UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE – ICBS

DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS-GRADUACÃO EM CIÊNCIAS BIOLÓGICAS-BIOQUÍMICA

A UTILIZAÇÃO DO ZEBRAFISH COMO MODELO PARA AVALIAR A INFLUÊNCIA DA EXPOSIÇÃO CRÔNICA AO ETANOL NOS SISTEMAS GLUTAMATÉRGICO, PURINÉRGICO E NÍVEIS DE BDNF

Eduardo Pacheco Rico

Porto Alegre, Fevereiro de 2011

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Eduardo Pacheco Rico

Prof. Dr. Diogo Onofre Gomes de Souza

Tese apresentada ao Program de Pós-Graduação em Ciências Biológicas: Bioquímica como pré-requisito parcial para a obtenção do título de doutor em ciências biológicas – bioquímica.

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"A coisa mais perfeita que podemos experimentar é o misterioso.

É a fonte de toda a arte e de toda a ciência verdadeira "

(Albert Einstein)

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Apresentação

A presente tese irá apresentar os resultados obtidos referente ao projeto que aborda o efeito do etanol nos sistema glutamatérgico, purinérgico e níveis de fator neurotrófico em cérebro de zebrafish. O projeto de pesquisa que engloba esta tese foi desenvolvido no Departamento de Bioquímica da UFRGS, Laboratório Neuroquímica e Psicofarmacologia da PUCRS, e Laboratório de Pesquisas em Câncer e do Laboratório Experimental de Hepatologia e Gastroenterologia, ambos do Centro de Pesquisas Experimentais do Hospital de Clínicas de do HCPA.

Sumário

I.1. Resumo

O zebrafish (Danio rerio) é uma espécie utilizada como modelo experimental em diversas áreas, tais como neurociências toxicologia. Seu genoma já está praticamente sequenciado e estudos demonstraram que muitos genes deste peixe são similares aos de mamíferos. Além disso, o zebrafish é um excelente modelo para estudar a função de diferentes sistemas de neurotransmissão. O consumo do etanol exerce diversas mudanças na coordenação motora, percepção sensorial e cognição promovendo um amplo espectro de alterações bioquímicas e fisiológicas nas células nervosas. Aqui, nós investigamos o efeito promovido pela exposição crônica de etanol nos sistemas purinérgico e glutamatérgico, e níveis de BDNF no SNC de zebrafish. Os transportadores de alta afinidade de aminoácidos (EAAT) regulam os níveis extracelulares de glutamato. Nós identificamos e descrevemos o padrão de expressão dos genes relacionados aos transportadores e as propriedades de captação de glutamato nas três importantes estruturas cerebrais de zebrafish (telencéfalo, tecto óptico e cerebelo). As pesquisas nos bancos de dados do seu genoma através de análise filogenética confirmaram a presença de diversos EAATs (*EAAT2, EAAT3*, três *EAAT1* parálogos e duas sequências parálogas para *EAAT5*). Também, a captação de glutamato dependente de sódio foi significativamente maior no tecto óptico, indicando diferenças funcionais entre as estruturas cerebrais. Os EAATs pertencem à família dos carreadores de soluto 1 (SLC1), que constitui os transportadores de alta afinidade de aminoácidos e transportadores de aminoácidos neutros. Recentemente, foi demonstrada uma análise filogenética e clonagem dos genes SLC1/EAAT identificando distintos membros desta família de transportadores. No sentido de unificar a designação dos genes SLC1/EAAT em zebrafish, foi proposta uma nomenclatura comum para estes grupos. O etanol promoveu uma diminuição significativa na captação de glutamato após 7 e 14 dias de exposição (30% e 54%, respectivamente), enquanto que após 28 dias, não foram observadas alterações. Na,K-ATPase, a enzima responsável pelo controle do gradiente iônico, não teve sua atividade alterada após todos os períodos de exposição testados. Além disso, os peixes expostos ao etanol durante 7 e 14 dias tiveram uma redução nos níveis de mRNA para SLC1A1/EAAT3. Entretanto, a expressão gênica do SLC1A8a,b/EAAT6a,b aumentou após todos os períodos testados, enquanto que SLC1A3a,b/EAAT1b aumentou somente após 28 dias. A prolongada exposição ao etanol não foi capaz de alterar as atividades da glutamina sintetase e glutaminase. Da mesma forma, etanol não alterou a hidrólise de ATP e GTP. Entretanto, foi verificada uma diminuição na hidrólise de ADP (46% e 34%) e GDP (48% e 36%) após 7 e 14 dias respectivamente. Após 7 e 14 dias de exposição ao etanol, também foi observada uma significativa alteração na hidrólise de AMP (48% e 36%), enquanto que a hidrólise de GTP foi inibida somente após 7 dias (46%). Os níveis de transcritos das nucleosídeo trifosfato difosfohidrolase (NTPDases) foram alteradas após 7, 14 e 28 dias. Em contraste, a expressão da *5'-nucleotidase* não foi alterada. A atividade da adenosina deaminase (ADA) na fração solúvel não foi modificada, mas uma redução da atividade na fração de membrana após 28 dias de exposição ao etanol (44%) foi observada. A análise da expressão gênica demonstrou que *ADA1* permaneceu inalterada, enquanto que os transcritos de ADAL, ADA2-2, ADA2-1, e sua isoforma truncada de "splicing" alternativo (ADA2-1/T) foram alteradas após a ação prolongada do etanol. Após 14 e 28 dias, etanol aumentou a expressão gênica do BDNF, mas não alterou os níveis de transcritos para trkB. A determinação da proteína BDNF através dos métodos de ELISA indicou um aumento de (51%), sendo confirmando imunohistoquímicamente. Estes resultados sugerem que a homeostase da função neurotrófica pode ser alterada pelo prolongado consumo de etanol. Esta tembém inclui uma revisão sobre o papel de diferentes neurotransmissores excitatórios e inibitórios em zebrafish, tais como, dopaminérgico, serotoninérgico, colinérgico, glutamatérgico, purinérgico, histaminérgico, nitrérgico, glicinérgico, gabaérgico, enfatizando aspectos farmacológicos e toxicológicos. Em suma, esta tese demonstra o efeito da exposição crônica ao etanol afeta o sistema glutamtérgico e purinérgico, expressão de BDNF em cérebro de zebrafish. O aumento do conhecimento global sobre os sistemas de neurotransmisão em zebrafish e o esclarecimento de efeitos farmacológicos e toxicológicos poderia contribuir para novas estratégias de pesquisa em ciências básicas e biomédicas.

Palavres chave: zebrafish; etanol; purinas; glutamato; neurotrofina.

I.2. Abstract

The zebrafish (Danio rerio) is a species used as experimental models in various fields such as neurosciences, toxicology. Its genome has already been sequenced and studies have shown that many genes are similar to those of mammals. Furthermore, zebrafish provides an excellent model to study the function of different neurotransmitter systems. The ethanol consumption exerts several changes in motor coordination, sensory perception and cognition, promoting a wide-spectrum of biochemical and physiological alterations on nervous cells. Here we investigated the effects promoted by chronic ethanol exposure on glutamatergic and purinergic systems, and BDNF levels in zebrafish CNS. High-affinity excitatory amino acid transporters (EAATs) regulate extracellular glutamate levels. We identified and described the expression profile of EAATs-related genes and the functional properties of glutamate uptake in three major brain structures from zebrafish (telencephalon, optic tectum and cerebellum). Searches on zebrafish genome databases and a phylogenetic analysis confirmed the presence of several EAAT-related genes (*EAAT2, EAAT3*, three *EAAT1* paralogs and two *EAAT5* sequences). Moreover, the glutamate uptake was significantly higher in optic tectum, which indicates functional differences within zebrafish brain structures. EAATs belong to the solute carrier family 1 (SLC1), that constitute high-affinity glutamate and neutral amino acid transporters. Recently, the phylogenetic analysis and cloning reporting of SLC1/EAAT genes from zebrafish identified distinct members of this transporter family. In order to unify the nomenclature of SLC1/EAAT genes in zebrafish, it was proposed a common nomenclature for these groups. The actions of ethanol on glutamate uptake showed a significant decrease in glutamate transport (30% and 54%) after 7 and 14 days of exposure, whereas after 28 days, no significant changes were detected. Na,K-ATPase, the enzyme responsible to generate ion gradients, did not alter after all exposure periods. Moreover, fish exposed to ethanol during 7 and 14 days exhibit a decrease of mRNA levels for SLC1A1/EAAT3. However, the gene expression of SLC1A8a,b/EAAT6a,b increased after all exposure periods, whereas SLC1A3a,b/EAAT1b increased only after 28 days. The prolonged ethanol exposure did not significantly change the glutamine synthetase and glutaminase activities. In the same way, ethanol did not alter the ATP and GTP hydrolysis. However, a decrease in ADP (46% and 34%) and GDP (48% and 36%) hydrolysis was verified after 7 and 14 days, respectively. After 7 and 14 days of ethanol exposure, a significant decrease in AMP hydrolysis (48% and 36%) was also observed, whereas GMP hydrolysis was inhibited only after 7 days (46%). Furthermore, nucleoside triphosphate diphosphohydrolase (*NTPDase)* transcript levels were altered after 7, 14, and 28 days. In contrast, *5'-nucleotidase* expression was not altered. Adenosine deaminase (ADA) activity from soluble fraction was not modified, but a decrease of ADA activity in membrane fraction after 28 days (44%) of ethanol exposure was observed. Gene expression analysis demonstrated that *ADA1* remained unaltered, whereas ADAL, ADA2-2, ADA2-1 transcripts, and its truncated alternative splice isoform (ADA2-1/T) were altered after prolonged ethanol exposure. After 14 and 28 days, ethanol increased the *BDNF* gene expression, but did not change the levels of trkB transcripts. The measurement of BDNF protein through ELISA kit anti-BDNF showed increased amounts after 28 days of exposure (51%), which was also confirmed by immunohistochemstry. These results suggest that the homeostasis of neurotrophic functions may be altered by prolonged ethanol consumption. Moreover, we present a review about the role of different excitatory and inhibitory neurotransmitters systems in zebrafish, such as dopaminergic, serotoninergic, cholinergic, glutamatergic, purinergic, histaminergic, nitrergic, glycinergic, and GABAergic systems, emphasizing pharmacological and toxicological aspects. In conclusion, this thesis demonstrates that chronic ethanol exposure affects the glutamatergic and purinergic systems, and BDNF expression in zebrafish brain. The significant increase in the global knowledge about the neurotransmitters systems in zebrafish and the elucidation of pharmacological and toxicological effects could lead to new strategies and appropriate priorities in research in order to support complementary insights on basic sciences and biomedical research.

Keywords: zebrafish; ethanol; purines; glutamate; neurotrofin.

I.3. Lista de Abreviaturas

A_2 – subtipo de receptor adenosinérgico facilitatório

A_1 – subtipo de receptor adenosinérgico inibitório

AC – adenilato ciclase

ADA – adenosina deaminase

ADH – álcool desidrogenase

ALDH – aldeído desidrogenase

AMPA – alfa-amino-3-hidróxi-metilisoxazol-propionato

AMPc- adenosina 3',5', monofosfato cíclico

BDNF- fator neurotrófico derivado do encéfalo

CD73 – proteína de superficie de linfócitos

CYP2E1 – citocromo P450

EAAT – transportador de aminoácidos excitatórios

ELISA – do inglês "Enzyme Linked Immunosorbent Assay"

ENT1 – transportador bidirectional de adenosina

ERK – do inglês "Extracellular signal-regulated kinases"

GABA – γ-acido aminobutírico

$GABA_A$ – receptor γ-acido aminobutírico

GLAST – do inglês "glutamate-aspartate transporter"

GLT-1 – do inglês "glutamate transporter".

GPI – glicosil-adenosina deaminase

G6PD – glicose-6-fosfato desidergenase

NAD^+ – nicotinamida adenina dinucleotídeo

MAPK – do inglês " Mitogen-activated protein kinase"

NTPDase – nucleosídeo trifosfato difosfoidrolase

SLC– carreador de solutos

LDH – lactato desidrogenase

mRNA – RNA mensageiro

Na+ /K+ - Bomba de sódio e potássio dependente de ATP

NMDA – N-metil-D-aspartato

P2 – receptor purinérgico para nucleotídeos

P1 – receptor purinérgico para nucleosídeos

P2X– receptor purinérgico ionotrópico

P2Y – receptor purinérgico metabotrópico

PKA – proteína cinase A, proteína cinase dependente de AMP cíclico

PKC – proteína cinase C, proteína cinase dependente de AMP cíclico

SNC – sistema nervoso central

trkB- receptor tirosina cinase do tipo B

I.4 Introdução

I.4.1 Zebrafish

O Zebrafish ou peixe-zebra (*Danio rerio*) é um pequeno teleósteo (3-4 cm) da família Cyprinidae, sendo uma espécie bastante conhecida pelo seu uso como ornamental. O pioneiro a estudar esta espécie foi George Streisinger que, no final da década de 60, aplicou as técnicas de análise mutacional para estudar o desenvolvimento embrionário do zebrafish (Grunwald & Eisen, 2002). Atualmente, este peixe é um modelo experimental consolidado em diversas áreas da ciência, tais como: genética e genômica, desenvolvimento, teratologia, comportamento, toxicologia e neurociências (Vascoto et al., 1997). Devido as favoráveis características que este peixe possui, o tornam um modelo experimental bastante atrativo em muitas áreas da Ciência, as quais podemos citar: pequeno custo e espaço requerido para manutenção, rápido desenvolvimento e ciclo biológico, grande prole, embriões translúcidos e suscetíveis à manipulação e microinjeção (Lele & Krone, 1996). O interesse pela espécie pode ser observado pelo número crescente de laboratórios que tem utilizado este teleósteo como um modelo experimental em suas pesquisas (Sprague et al., 2001). Foi criada uma rede de informações na web sobre o zebrafish (http://zfin.org), na qual laboratórios do mundo inteiro podem depositar informações sobre esta espécie (Sprague et al., 2003). Além do mais, existe um excelente, compreensivo e freqüentemente atualizado manual de manutenção e controle das condições em laboratórios sobre este teleósteo (Westerfield, 2000).

Nos últimos anos, houve um progresso considerável na genética e genômica do zebrafish. Em 2001, o Instituto Sanger iniciou o seqüenciamento do genoma total (Vogel, 2000; Stern & Zon, 2003), e o mitocondrial já está seqüenciado servindo de base para estudos filogenéticos (Broughton et al., 2001). Estudos do genoma do zebrafish pode servir

como um complemento funcional para o projeto genoma humano, o qual produz enormes quantidades de seqüências, mas carece de informações funcionais para a maioria dos genes identificados (Dooley & Zon, 2000). Além disso, grandes segmentos dos cromossomos do zebrafish estão em sintonia com os cromossomos humanos e de camondongo, e muitos genes apresentam um alto grau de similaridade, quando comparados em sua seqüência (Barbazuk et al., 2000).

O zebrafish se tornou o principal modelo experimental para o estudo do desenvolvimento de vertebrados (Anderson & Ingham, 2003). As características básicas de sua embriogênese são bem conhecidas, assim como o destino celular durante o seu desenvolvimento (Kimmel & Warga, 1988; Kimmel, 1989). Atualmente, um amplo espectro de estudos sobre o desenvolvimento de diversos sistemas, órgãos e patologias relacionadas são realizados nesta espécie (Dodd et al., 2000; Ackermann & Paw, 2003).Comparando-se as seqüências do genoma humano e o do zebrafish, muitos genes como os do ciclo celular, supressores tumorais e oncogenes se mostram conservados (Amatruda et al., 2002). Já foram observados muitos tipos de neoplasias no zebrafish, os quais são parecidos histologicamente e geneticamente com os humanos, o que mostra que a biologia do câncer é muito similar nestes organismos (Amatruda et al., 2002; Stern & Zon, 2003). Além disso, animais transgênicos podem ser gerados com alterações em genes específicos envolvidos no câncer (Long et al., 1997).

Recentemente, estudos avaliando características comportamentais do zebrafish foram desenvolvidos (Guo, 2004; Gerlai et al, 2000). A maioria dos trabalhos avaliou o efeito de pesticidas, drogas e xenobióticos na atividade comportamental desta espécie (Levin & Chen, 2004; Swain et al., 2004). Alguns estudos também observaram a

importância do comportamento inato e adquirido em modelos de agressão, sociabilidade e sua preferência por ambientes claros ou escuros (Serra et al., 1999).

Devido às vantagens de se usar o zebrafish como modelo experimental, o efeito agudo e crônico de diversas substâncias tóxicas pode ser avaliado facilmente. Devido ao pequeno espaço requerido por estes animais, uma quantidade menor de toxinas é empregada nos testes toxicológicos. Além disso, o efeito e a acumulação de diversas substâncias químicas vêm sendo testados no zebrafish desde o final dos anos 70 (Lele & Krone, 1996). Muitos compostos, tais como pesticidas, metais pesados, fenóis e misturas complexas de compostos já foram avaliados em diversos órgãos deste peixe, o que indica o crescente interesse nesta espécie como um modelo de toxicologia ambiental e bioindicação (Yamazaki et al., 2002).

Atualmente, muitos estudos são realizados nesta espécie para estudar as bases moleculares da neurobiologia, identificando genes envolvidos na formação de circuitos neuronais, no comportamento e nos mecanismos envolvidos na neuropatogênese (Vascotto et al., 1997; Guo, 2004). Muitos sistemas de neurotransmissão já foram identificados no zebrafish tais como: glutamatérgico (Edwards & Michel, 2002), colinérgico (Behra et al., 2002; Clemente et al., 2004), dopaminérgico (Boehmler al., 2004), serotoninérgico (Rink & Guo, 2004), histaminérgico (Kaslin & Panula, 2001), gabaérgico (Kim et al., 2004) e purinérgico (Kucenas et al., 2003; Rico et al., 2003; Senger et al., 2004). Estudos anteriores têm demonstrado alterações comportamentais no zebrafish após exposição ao etanol em diferentes linhagens (Dlugos e Rabin 2003), o que sugere que esta espécie é um excelente modelo para investigar os determinantes genéticos envolvidos na regulação das respostas ao etanol. A exposição aguda a diferentes concentrações de etanol já foi investigada, demonstrando que esta espécie é bastante adequada para estudos de neuroquímica, pois os

mecanismos de respostas comportamentais à intoxicação por etanol são bastante complexos (Gerlai et al., 2000).

I.4.2. Etanol

O consumo excessivo de álcool é um problema público em todo o mundo. Seu consumo abundante está associado com a ocorrência de muitas condições patológicas, como câncer, doenças hepáticas, danos cerebrais, entre outros. Após o consumo de álcool, muitos órgãos são capazes de metabolizar o etanol, mas a maior parte (mais de 90%) é metabolizada no fígado (Quertemont et al., 2005). Sua eliminação é através de uma cascata metabólica de degradação envolvendo múltiplas reações enzimáticas.

A principal via do metabolismo do álcool é através de duas reações enzimáticas que requerem nicotinamida adenina dinucleotídeo (NAD^+) como cofator. No primeiro passo, a enzima álcool desidrogenase (ADH) converte o etanol em acetaldeído, reduzindo o NAD^+ nesse processo. No segundo passo o acetaldeído é metabolizado a ácido acético (acetato) pela enzima aldeído desidrogenase (ALDH) reduzindo também NAD^+ (Swift, 2003). Em mamíferos distintas classes de ADH têm sido caracterizadas apresentando distintas características moleculares e cinéticas (Reimers, 2004). Além disso, duas classes de álcool desidrogenase foram caracterizadas em zebrafish e compartilham similaridade estrutural com as de mamíferos (Reimers, et al, 2004), mas apresentam características funcionais distintas.

Além da principal rota de degradação envolvendo ADH e ALDH, há duas menores vias oxidativas para a degradação do etanol em acetaldeído. Além disso, o citocromo P450 (CYP2E1) é responsável por uma pequena parte do total metabolizado e a catalase capaz de transformar etanol em acetaldeído a partir de um radical peróxido (Swift, 2003). Neste

contesto, acetaldeído e acetato vêm sendo investigados no sentido de esclarecer o envolvimento dos metabólitos da degradação do etanol, em respostas comportamentais e farmacológicas (Israel, et al., 1994; Quertemont, et al., 2005). Estudos têm demonstrado que o papel do acetaldeído em diversos efeitos neuroquímicos e farmacológicos promovidos pelo etanol. O acetaldeído, produto intermediário da ADH, é uma molécula altamente reativa que pode formar complexos com proteínas e outros componentes biológicos formando aductos.

A utilização desta espécie em pesquisas envolvendo drogas de abuso vem aumentando consideravelmente, principalmente no que se refere à intoxicação ao álcool. Estudos demonstram que o etanol causa alterações em zebrafish, tais como anormalidades craniofaciais, malformações cardíacas e prejuízos no seu desenvolvimento (Carvan III, 2004; Reimers, 2004; Bilotta, 2004). Isso faz com que esta espécie sirva como um importante modelo para biologia do câncer devido às diversas ações teratogências promovidas por este composto (Scalzo, 2004). Recentemente o zebrafish tem sido utilizado com sucesso em pesquisas que envolvam as mais diversas respostas comportamentais aos efeitos de drogas, dentre elas, o etanol. Foi demonstrado por Gerlai e colaboradores (2000) aterações em parâmetros como locomoção, aprendizado, agressão, interação social servindo de base para estudos genéticos, na qual linhagens de diferentes genótipos podem ser expostas ao etanol (Dlugos et al., 2003).

Trabalhos sugerem que a adenosina está envolvida nos efeitos comportamentais e neuronais induzidos pelo etanol (Dohrman et al., 1997). O etanol é uma droga amplamente consumida, exercendo uma variedade de ações no sistema nervoso central. Dentre os efeitos comportamentais induzidos, observa-se alterações na coordenação motora, na percepção sensorial e na cognição (Diana et al., 2003). Diversos estudos demonstraram que

o etanol altera a função neuronal por modificar rotas de transdução de sinais mediadas por hormônios e neurotransmissores. Estudos em cultura de células neurais demonstraram que o etanol, através da ativação dos receptores de adenosina do tipo A_2, estimula a sinalização mediada pela via da (cAMP/PKA) e a expressão gênica mediada pelo elemento regulator de AMPc e que este efeito é bloqueado pela subunidades beta e gama da proteína G inibitória (Arolfo et al., 2004). Foi demonstrado que os efeitos mediados pelo etanol no SNC está relacionado com alterações em diversos sistemas de neurotransmissão, dentre eles o sistema glutamtérgico (Esel, 2006).

I.4.3. Sinalização Glutamatérgica

A sinalização entre neurônios ocorre através de seus sinais químicos, mediada por neurotransmissores em um período de atividade curto na fenda sináptica. O término da resposta pode ser realizado através da metabolização por enzimas específicas, como ocorre para a acetilcolina, ATP e neuropeptídeos; ou também por retirada dos neurotransmissores da fenda sináptica por transportadores específicos, no caso de aminoácidos excitatórios (glutamato e aspartato), aminoácidos inibitórios (GABA e glicina) e monoaminas (Kandel et al, 1991).

Os aminoácidos glutamato e aspartato são os neurotransmissores excitatórios mais importantes do SNC e periférico de mamíferos, respectivamente. O glutamato desempenha um importante papel na manutenção da atividade do SNC e em fenômenos plásticos vinculados à aprendizagem e à memória (Meldrum, 2000). Outro aspecto de interesse é a participação do neurotransmissor glutamato na gênese de diversos quadros neurológicos como a epilepsia, encefalopatias isquêmicas, demência de Alzheimer e enfermidade de Huntington (Olney, 1990; Segovia et al., 2001).

A existência de uma grande variedade de receptores faz com que o neurotransmissor glutamato tenha uma ampla diversidade funcional. Os receptores glutamatérgicos são divididos em dois grupos: ionotrópicos e metabotrópicos (Gasic & Holmann, 1994). Os receptores ionotrópicos são uma família de canais iônicos e denominados de acordo com seu agonista sintético mais seletivo: NMDA, o qual ativa um canal de cálcio, AMPA e cainato, que permeiam a entrada de sódio e potássio. Os receptores metabotrópicos, acoplam-se a proteínas-G (proteínas ligantes de nucleotídeos da guanina) e modulam efetores intracelulares, como a fosfolipase C e adenilato ciclase.

Após sua liberação no espaço extracelular, não existem evidências quanto a efetiva metabolização do glutamato, ou seja, sua sinalização é finalizada quando este neurotransmissor é retirado da fenda sináptica por recaptação para o terminal pré-sináptico ou para as células gliais (Robinson, 1999). Quando captado por astrócitos, o glutamato pode ser metabolizado por diversas vias, na qual a formação de glutamina e a entrada no ciclo dos ácidos tricarboxílicos são quantitativamente mais importantes. A formação de glutamina é catalisada, de maneira dependente de ATP, pela glutamina sintetase, enzima presente em astrócitos, com pouca expressão em oligodendrócitos e ausente em neurônios. A glutamina pode ser liberada pelo astrócito e captada pelo neurônio, o qual a converterá a glutamato pela enzima glutaminase, localizada na mitocôndria, o glutamato formado pode ser então armazenado em vesículas para ser liberado (Anderson & Swanson, 2000). Os transportadores astrocitários são considerados como os principais responsáveis pela retirada do glutamato da fenda sináptica. Dois transportadores astrocitários já foram clonados e denominados de GLAST e GLT-1. Em humanos eles são designados como EAAT1 e EAAT2. GLT-1 e GLAST são amplamente distribuídos no cérebro. Os transportadores localizados nos neurônios também contribuem na manutenção das baixas concentrações

extracelulares de glutamato. O transportador neuronal de glutamato mais amplamente distribuído no cérebro é o EAAC1 (homólogo humano, EAAT3). Ele é encontrado em regiões não-sinápticas. Outros subtipos clonados incluem EAAT4, localizado em células de Purkinje e EAAT5, localizado em neurônios retinianos (Anderson et al., 2001).

Os EAAT localizados na membrana plasmática funcionam através do acoplamento a um gradiente eletroquímico formado pela Na+/K+ -ATPase, permitindo o transporte de aminoácidos ácidos contra seu gradiente de concentração. Os EAAT dependentes de sódio também dependem do potássio intracelular. Após a dissociação dos íons sódio e de glutamato, íons potássio presentes no citoplasma ligam-se ao transportador e o ativam. Em cada ciclo, pelo menos uma molécula de sódio é movida do espaço extracelular para o citoplasma e uma molécula de potássio é movida na direção oposta, sendo o transporte de glutamato acoplado à entrada de sódio (Robinson & Dowd, 1997).

Estudos realizados simultaneamente definiram o papel fisiológico do glutamato no SNC e também como mediador de eventos patológicos. O termo excitotoxicidade foi criado para definir a morte neuronal causada pela administração exógena de altas concentrações de glutamato ou compostos com ação agonística nos receptores glutamatérgicos (Olney, 1990). A exitotoxicidade glutamatérgica endógena está envolvida em danos neurológicos agudos como hipóxia, isquemia, traumatismo craniano e epilepsia (Meldrum, 2000) e em doenças neurodegenerativas crônicas como Alzheimer, Parkinson, Huntigton e Esclerose Amiotrófica Lateral (Segovia et al., 2001). A morte celular causada pela excitotoxicidade pode levar a subseqüentes liberações de glutamato que, em grande quantidade e através de processos não controlados levam a formação de uma "onda excitotóxica" atingindo as células neurais circundantes. O mecanismo principal relacionado a morte celular induzida por glutamato envolve o desequilíbrio iônico promovido pela entrada excessiva e

prolongada de cálcio, inicialmente por receptores ionotrópicos (resposta rápida e aguda) e posteriormente via modulação de canais de cálcio por receptores metabotrópicos (resposta tardia). O aumento de cálcio intracelular leva a ativação de uma série de cascatas enzimáticas que incluem, proteases, fosfolipases, óxido nítrico sintases e endonucleases que contribuem na morte celular (Meldrum, 2000).

I.4.4. Sinalização Purinérgica

O ATP foi descrito inicialmente como neurotransmissor através dos estudos que mostravam a sua liberação a partir de nervos sensoriais (Holton & Holton, 1954; Holton, 1959). Entretanto, sua ação como neurotransmissor só foi reconhecida pelos estudos realizados por Geoffrey Burnstock, que desenvolveu a hipótese purinérgica (Burnstock et al., 1970; Burnstock, 1972). O ATP pode ser armazenado e liberado para o meio extracelular juntamente com outros neurotransmissores, tais como: acetilcolina, glutamato, noradrenalina, serotonina e GABA (Burnstock, 1999; Burnstock, 2004] através de vesículas pré-sinápticas dependentes de cálcio (Phillis & Wu, 1981).

No sistema nervoso central e periférico, o ATP age como neurotransmissor excitatório e possivelmente como neuromodulador (Cunha, 2000; Salgado et al., 2000). O ATP extracelular pode influenciar a atividade sináptica ao interagir com receptores específicos. Os nucleotídeos e o nucleosídeo da adenina podem exercer seus efeitos através da ativação de receptores purinérgicos subdivididos em dois grandes grupos: P1 e P2. Os purinoreceptores do tipo P1 são mais eficientemente ativados por adenosina, enquanto que os purinoreceptores P2 são ativados por ATP (Ralevic & Bunrnstock, 1998). Os purinoceptores P2 e são divididos em duas subclasses, os receptores ionotrópicos P2X, que são canais iônicos dependentes de ligantes, e os receptores metabotrópicos P2Y, que são

acoplados a proteína G. Membros de ambos tipos de receptores são amplamente distribuídos no sistema nervoso central e periférico e estão envolvidos em uma miríade de funções (Barnard et al., 1997, Burnstock & Knigth, 2004).

A clonagem e caracterização molecular dos subtipos dos receptores P2X do zebrafish já foram realizadas(Diaz-Hernandez et al., 2002; Boué-Grabot et al., 2000; Egan et al., 2000; Norton et al., 2000). A análise da seqüência de nove genes sugere que cinco deles são ortólogos a genes dos receptores P2X de mamíferos, dois são parálogos e um ainda precisa ser devidamente classificado (Kucenas et al., 2003). Todas os subtipos de receptores P2X do zebrafish contêm resíduos altamente conservados, os quais são encontrados nas subunidades de mamíferos. Até o momento, na família de receptores P2Y foram identificados quatorze subtipos, mas somente oito são entidades moleculares que possuem respostas funcionais (Ralevic & Burnstock, 1998; Lazarowski et al., 2003. Illes & Ribeiro, 2004).

A análise da seqüência de nove genes sugere que cinco deles são ortólogos a genes dos receptores P2X de mamíferos, dois são parálogos e um ainda precisa ser devidamente classificado (Kucenas et al., 2003). Todos os subtipos de receptores P2X do zebrafish contêm resíduos altamente conservados, os quais são encontrados nas subunidades de mamíferos. Também existem evidências que os receptores P2Y estão envolvidos na transdução de sinal mediada por proteínas ligantes de GTP e proteínas cinases como a PKC, MAPK, ERK1/2 (Communi et al., 2000; Boeynaems et al., 2000).

A ação sinalizadora dos nucleotideos é terminada por uma cascata de enzimas localizadas na superfície celular. No caso da degradação extracelular do ATP, o produto final é o neuromodulador adenosina. Esta degradação pode inativar a sinalização mediada

pelo ATP através dos receptores P2 e aumentar a sinalização mediada pela adenosina através dos receptores P1 (Kato et al., 2004).

Além dos nucleotídeos de adenina, as ectonucleotidases podem atuar em outros nucleotídos, incluindo os derivados da guanina, hidrolisando GTP, GDP e GMP até guanosina. Por atuarem como moduladores de diversos processos tanto intracelulares quanto 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, uma vez que é capaz de promover a manutenção do tônus glutamatérgico fisiológico (Frizzo et al., 2005). Desta forma a presença de uma ecto-5´-nucleotidase é importante na regulação dos níveis extracelulares tanto de guanosina quanto de adenosina.

A adenosina é uma substância que pode ser formada nos espaços intracelular e extracelular. No meio extracelular esta purina se comporta como uma molécula sinalizadora, influenciando a transmissão sináptica e a atividade do sistema nervoso central (Ribeiro et al., 2003). Sua formação intracelular é devido a ação, principalmente, da enzima 5'-nucleotidase que hidrolisa AMP à adenosina e da ação de hidrólise do substrato S-adenosil-homocisteína. A adenosina gerada intracelularmente pode ser transportada ao espaço extracelular através de transportadores bidirecionais, por um mecanismo de difusão facilitada que regula os níveis intracelulares e extracelulares deste nucleosídeo (Fredholm et al., 2001). Entretanto, a adenosina não é considerada um neurotransmissor, pois não há indícios de que é armazenada em vesículas sinápticas (Brundege & Dunwiddie, 1997; Ribeiro et al., 2003).

A sinalização mediada por nucleotídeos extracelulares necessita de mecanismos eficientes para a inativação de seu sinal. Muitos trabalhos realizados evidenciaram a

presença de uma variedade de enzimas localizadas na superfície celular denominadas ectonucleotidases, que são capazes de hidrolisar, e assim, inativar a sinalização mediada por nucleotídeos (Zimmermann, 1994; 2001). Este grupo inclui a ecto-ATP difosfoidrolase (ecto-apirase, EC 3.6.1.5) e a ecto-ATPase (EC 3.6.1.3), pertencentes à família das NTPDases (nucleosídeo trifosfato difosfoidrolase), e a ecto-5'-nucleotidase (EC 3.1.3.5) (Battastini et al., 1991, 1995). A ecto-5'-nucleotidase, também conhecida como a proteína linfocitária CD73 hidrolisa nucleotídeos 5'-monofosfatatos púricos e pirimídicos ao respectivo nucleosídeo. Esta atividade enzimática é dependente de cátions divalentes, como cálcio e magnésio. A ecto-5'-nucleotidase é uma enzima ancorada a membrana plasmática por GPI, sendo que formas solúveis da enzima podem ser originadas mediante a ação de uma fosfolipase específica.

A hidrólise extracelular de ATP por essa via resulta na formação de ADP, AMP e adenosina, e o produto final das reações de hidrólise é o nucleosídeo, que pode agir sobre seus próprios receptores ou ser captado pela célula e participar na rota de salvação do metabolismo das purinas.

Muitos estudos demonstraram a presença de ectonucleotidases como a NTPDase (Sarkis & Saltó, 1991; Schetinger et al., 2001) e 5´-nucleotidase (Vogel et al., 1992; Volknandt, 1991) em teleósteos. Em zebrafish, estudos do nosso laboratório demonstraram a presença de uma NTPDase e uma ecto-5'-nucleotidase em membranas cerebrais (Rico et al., 2003; Senger et al., 2004).

A adenosina atua como um importante modulador sináptico no sistema nervoso central (Dunwiddie E Masino, 2001). Suas ações neuromodulatórias são exercidas através de um grupo de receptores P1, que estão divididos em quatro subtipos: A_1, A_{2A}, A_{2B} e A_3. Grande parte do conhecimento sobre a distribuição e funcionalidade destes receptores

corresponde aos receptores de baixa afinidade A_1 e A_{2A}. Os receptores A_1 e A_2 são acoplados a proteína G inibitória e estimulatória, respectivamente, porém recentes estudos evidenciam que estes receptores podem ser acoplados a outras proteínas G (Fredholm et al., 2001).

Estudos têm demonstrado que a adenosina formada a partir dos nucleotídeos da adenina age preferencialmente nos receptores A_{2A} e a adenosina liberada como tal age preferencialmente sobre os receptores A_1 (Sebastião E Ribeiro, 2000; Cunha et al., 2001). A presença de adenosina na fenda sináptica depende da sua liberação celular através de um sistema de transporte bidirecional ou da sua produção através do catabolismo extracelular do ATP, exercido pelas ectonucleotidases (Zimmermann, 2001).

I.4.5 BDNF

A família das neurotrofinas é caracterizada por proteínas de tamanho pequeno que são secretadas e modulam a função sináptica no sistema nervoso central e periférico durante o desenvolvimento e na fase adulta de vertebrados (Binder and Scharfman, 2004; Chao et al., 2006). Dentre suas funções estão a regulação da liberação do neurotransmissores e modulação de eventos de plasticidade sináptica de curta e longa duração (Todd et al., 2007). O BDNF está implicado na regulação da diferenciação e sobrevivência de populações específicas de neurônios na fase embrionária e também em diferentes funções no sistema nervoso adulto que inclui homeostase neuronal e processos relacionados à plasticidade do cérebro tais como: memória e aprendizado (Tyler et al., 2002; Yamada et al., 2002) e drogas de abuso (Bolanos e Nestler, 2004). A exposição ao etanol durante o desenvolvimento induz uma série de efeitos adversos que incluem déficits na migração dos neurônios, perda de células em diversas regiões do cérebro bem como uma

diminuição das conexões sinápticas estabelecidas (Guerri, 1998). Neste sentido, tem sido mostrado na literatura que a sinalização mediada pelas neurotrofinas durante o desenvolvimento é inibida pelo etanol. (Climent et al., 2002; Ohrtman et al., 2006). O gene que codifica para BDNF, os sete exons-5' não codificadores regulados por promotores distintos, e o padrão de expressão tecidual em diferentes estágios do desenvolvimento e órgãos de zebrafish adultos foram demonstrados (Huynh and Heinrich, 2001; Heinrich and Pagtakhan, 2004). O gene que codifica para o receptor trkB e o padrão de expressão em embrião e larva de zebrafish foi também descrito (Lum et al., 2001). Entretanto, embora a organização dos genes que codificam para BDNF e para o receptor trkB e seus padrões de expressão tecidual tenham sido descritos em zebrafish, nenhum estudo avaliando a correlação entre os níveis de expressão e a modulação de qualquer dos diferentes aspectos da transmissão sináptica foi realizado.

I.5 Objetivos

O objetivo principal deste estudo foi avaliar o efeito crônico do etanol nos sistemas glutamtérgico, purinérgico e função neurotrófica mediada pelo BDNF em cérebro de zebrafish.

Considerando que: (1) o zebrafish é um importante e consolidado modelo experimental em estudos neuroquímicos, (2) as sinalizações glutamatérgicas e purinérgicas, bem como neurotrofinas exercem um importante papel na sinalização no SNC e (3) receptores e enzimas, envolvidas nestes importantes sistemas de neurotransmissão, já foram descritos nesta espécie, a presente tese apresenta os seguintes objetivos específicos:

➢ Identificar e verificar a expressão dos genes pertencentes à família dos EAAT seguido da avaliação funcional do destes transportadores através da captação dependente de sódio em cérebro de zebrafish.

➢ Avaliar o efeito da exposição crônica ao etanol na captação de glutamato e expressão gênica de seus transportadores em cérebro de zebrafish.

➢ Estudar a influência da exposição crônica ao etanol na atividade e padrão de expressão das ectonucleotidases e adenosina deaminase em cérebro de zebrafish.

➢ Investigar possíveis alterações induzidas pelo etanol na expressão gênica e níveis de proteína do BDNF em cérebro de zebrafish.

II.1. Capítulo 1

Anélise funcional e de expressão dos transportadores de glutamato dependentes de sódio em cérebro de zebrafish. Artigo publicado no periódico *Brain Research Bulletin* (2010) 81, 517-523.

Research report

Expression and functional analysis of Na+-dependent glutamate transporters from zebrafish brain

Eduardo Pacheco Rico [a,b,*], Diogo Losch de Oliveira [a,b], Denis Broock Rosemberg [a,b],
Ben Hur Mussulini [a,b], Carla Denise Bonan [c,e], Renato Dutra Dias [c], Susana Wofchuk [a,b],
Diogo Onofre Souza [a,b], Maurício Reis Bogo [d,e,**]

[a] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde,
Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil
[b] Instituto Nacional em Excitotoxicidade e Neuroproteção (INCT-EN), 90035-003 Porto Alegre, RS, Brazil
[c] Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia, Faculdade de Biociências,
Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil
[d] Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências,
Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga 6681, 90619-900 Porto Alegre, RS, Brazil
[e] Instituto Nacional Translacional em Medicina (INCT-TN), 90035-003 Porto Alegre, RS, Brazil

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ABSTRACT

High-affinity excitatory amino acid transporters (EAATs) regulate extracellular glutamate levels. Zebrafish (*Danio rerio*) provides an excellent model to study the function of different neurotransmitter systems. Although the identification of the EAAT family is well established in the mammalian central nervous system (CNS), EAAT-related genes and their expression profile in zebrafish have not yet been reported. Here we identify and describe the expression profile of EAATs-related genes and functional properties of glutamate uptake in three major brain structures from zebrafish (telencephalon, optic tectum and cerebellum). Searches on zebrafish genome databases and a phylogenetic analysis confirmed the presence of several EAAT-related genes (EAAT2, EAAT3, three EAAT1 paralogs and two EAAT5 sequences). All sequences identified were expressed in the structures analyzed. EAAT2 and EAAT3 were the most prominent glutamate transporters expressed in all brain areas. A uniform expression was observed for EAAT1A, whereas higher EAAT1B transcript levels were detected in telencephalon. Lower amounts of EAAT1C transcripts were observed in cerebellum when compared to other structures. No EAAT4-related sequence was found in the zebrafish genome. The EAAT5A expression was similar to EAAT5B in the telencephalon, while EAAT5B was less expressed than EAAT5A in optic tectum and cerebellum. Moreover, the glutamate uptake was significantly higher in optic tectum, which indicates functional differences within zebrafish brain structures. Altogether, the study of glutamate uptake in zebrafish could be important to evaluate the modulation of glutamatergic signaling through pharmacological and toxicological studies.

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1. Introduction

Glutamate is the most widespread excitatory neurotransmitter in the mammalian CNS, being involved in many aspects of brain function such as learning and memory [48,26], development and ageing [50,7], and environmental adaptation [11]. However,

besides its essential roles in brain activity, the neurotransmitter glutamate may be potently toxic (excitotoxicity), when present in high concentrations in the synaptic cleft [11,64,29], and it has been shown that this excitotoxic effect is involved in various acute and chronic neurological disorders [32,33,53,58]. Thus, the clearance of extracellular glutamate, mainly mediated by sodium-dependent transport into astrocytes [1,52,64], is an essential parameter involved in the physiological/excitotoxic tonus of the glutamatergic system.

The EAATs represent a protein family that displays considerable homology (50–60% at the amino acid level) [6]. To date, five structurally distinct subtypes of excitatory amino acid transporters have been identified and characterized in the mammalian brain: EAAT1 [56], EAAT2 [40], EAAT3 [27], EAAT4 [18] and EAAT5 [4]. EAAT1 is primarily an astroglial transporter and the main transporter pro-

* Corresponding author at: Departamento de Bioquímica, ICBS, UFRGS, Rua Ramiro Barcelos 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brazil.
Tel.: +55 51 3316 5557; fax: +55 51 3316 5540.
** Corresponding author at: Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga 6681, CEP: 90619-900 Porto Alegre, RS, Brazil. Tel.: +55 51 3320 3500x4726; fax: +55 51 3320 3568.
E-mail addresses: eduprico@gmail.com (E.P. Rico), mbogo@pucrs.br (M.R. Bogo).

Table 1
PCR primer design.

EAAT member	Primers (5′–3′)	Tm (°C)	Cycles	PCR product (bp)	GenBank accession numbers	ZFIN ID (ZDB-GENE)
EAAT1A	TGTCACGTCACGAGCTGCGCTC (F) ACAAGAAAACAGTGGACCGCTCG (R)	57	30	321	mRNA BC063233 Protein AAH63233	030131-2159
EAAT1B	AGAAACCGCGGTCGCGCAGC (F) TGCCAATGAACACTGCGATGAAGG (R)	57	30	396	mRNA XM_679025 Protein XP_684117	–
EAAT1C	CGTGATCTTCACCGTGGCTGCTG (F) AGTTGACGTTCTCTGTCGCATTGACC (R)	57	35	497	mRNA NM_001109703 Protein NP_001103173	071004-45
EAAT2	GCTGTCTCGGAGGAGAACCTGGGCATTG (F) TCATCTCGATGTCGTCAGTCTTCCCGTG (R)	61	30	432	mRNA BC056751 Protein AAH56751	030131-7779
EAAT3	GGATGGAACTGCACTGTATGAAGCGGTG (F) GGACGATTCCAGCACCGTAGGCGTC (R)	61	30	287	mRNA NM_001002666 Protein NP_001002666	040718-414
EAAT5A	CGTTTGGCATTGTGTTTCTGGTGGCTG (F) TGAGCGATAAATATGGCCGCCACAG (R)	61	35	376	mRNA XM_678579 Protein XP_683671	–
EAAT5B	ATCGTCCTCACTTCAGTGGGTTTGC (F) GCAAAGGTTGTAACAGGCGGTGG (R)	57	35	330	mRNA XM_687808 Protein XP_692900	–
β-actin	GTCCCTGTACGCCTCTGGTCG (F) GCCGGACTCATCGTACTCCTG (R)	54	35	678	mRNA AAC13314 Protein AF057040	000329-1

tein present during CNS development [20]. EAAT2 is one of the two most abundant glutamate transporters in the adult CNS [30], and is an astroglial transporter expressed postnatally and responsible for up to 90% of all glutamate transport in adult tissue [45,61,63,31]. EAAT3, a neuronal glutamate transporter found at high densities on postsynaptic membranes, is present most notably in the hippocampus, cerebellum, and basal ganglia [21]. EAAT4 is a glutamate transporter largely limited to the Purkinje cells of the cerebellum [18,21], whereas EAAT5 is found primarily in the retina on photoreceptors and bipolar cells [4,43].

The zebrafish is a small freshwater teleost that has recently attained a pre-eminent position in biomedical research, being considered an important and emerging vertebrate model in many fields of biology including neuroscience [22]. This species exhibits genetic and anatomic conservation in relation to both mice and humans and a high degree of genetic homology [5,13]. Moreover, the zebrafish appears to be an attractive organism for high throughput screening applications, e.g., mutagenesis screening, forward genetics or drug discovery efforts applied to neurotoxicity tests [68,39].

Recently, the choice of this animal model to investigate some aspects of brain neurotransmission, including the glutamatergic system, has become more common. Studies have demonstrated the distribution and function of ionotropic glutamate receptors in the olfactory bulb [15,59], as well as embryonic expression of NMDA receptor subunit genes [10]. The glutamatergic modulator MK-801 was employed to examine behavioral parameters [57] as well as the role of glutamatergic receptors in learning and memory processes [36]. Furthermore, vesicular glutamate transport has been described in mutant zebrafish larvae, playing a key role in visual perception and behavior [54,37].

Although some parameters of the glutamatergic signaling in zebrafish have already been characterized, the expression and functional profile of glutamate transporters have not yet been reported. Theories about vertebrate neural and behavioral bases propose that brain evolution occurred in successive stages and that these bases have been conserved through phylogenesis. However, recent developmental, neuroanatomical and functional data indicate that the brain and behavioral evolution may have been more conservative than previously thought [47].

To provide new insights into the primary characteristics of the glutamatergic system in zebrafish, the aims of the present study were to identify the relative gene expression profiles of distinct members of the EAAT family and to carry out a preliminary investigation of some parameters of glutamate uptake in three brain structures in this species: the telencephalon, optic tectum, and cerebellum.

2. Experimental procedures

2.1. Materials

Reagents were purchased from Sigma Chemical CO (St. Louis, MO, USA). L-[³H]glutamate (specific activity 30 Ci mmol⁻¹) was purchased from Amersham International, UK. Platinum Taq DNA polymerase, TRIzol reagent, and SuperScript™ First-Strand III (Synthesis System for RT-PCR) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Animals

Adult wild-type zebrafish (*Danio rerio*) of both sexes (3–6 months-old) were obtained from a commercial supplier (Delphis, RS, Brazil). All fish were acclimated to their new environment for at least 2 weeks in a 50-l thermostated aquarium. The water was kept at $26 \pm 2\,°C$ under a 12-h light–dark controlled photoperiod and the animals were fed with commercial flake fish food twice a day. They were used according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, being healthy and free of any signs of disease. All procedures in the present study were approved by the Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS), protocol number 477/05-CEP.

2.3. Phylogenetic analysis and primers design

EAAT members were identified by NCBI Blast searches of GenBank, using the well-known *Homo sapiens* and *Rattus norvegicus* proteins as queries. The obtained sequences (supported by mRNA or EST data) were compared with the zebrafish protein database of the Zebrafish Information Network (ZFIN) (University of Oregon, Eugene, OR 97403-5274; World Wide Web URL: http://zfin.org) and the sequences were aligned using the ClustalX program [62]. A phylogenetic tree was constructed according to the Neighbor-Joining method [49] using proportional (*p*) distance with the MEGA 2.1 program [46]. In order to compare the zebrafish deduced amino acid sequences, an alignment was performed using ClustalX. 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 for the design of the primers avoided cross-amplification. The optimal conditions for primer annealing were determined (Table 1) and the β-actin primers were designed as described previously [9].

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

In order to obtain distinct brain structures from zebrafish, the animals were cryoanaesthetized and further euthanized by decapitation. Total RNA was isolated from telencephalon, optic tectum, and cerebellum using the TRIzol® reagent in accordance with the manufacturer's instructions. The purity of the RNA was spectrophotometrically determined 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 (Synthe-

sis System for RT-PCR) following the suppliers instructions. Each RNA sample was mixed with 1 µl of 50 µM Oligo (dt) and 1 µl annealing buffer (final volume of 8 µl) and then incubated in a thermal cycler at 65 °C for 5 min. The samples were immediately placed on ice for 1 min and 10 µl 2× First-Strand Reaction Mix and 2 µl SuperScript™ III/RNaseOUT™ Enzyme Mix were added. The products were mixed, incubated for 50 min at 50 °C and the reaction was terminated at 85 °C for 5 min. PCR reactions for different EAATs and β-actin genes were performed in a total volume of 20 µl, with 0.1 mM primers (Table 1), 0.2 mM dNTP, 2 mM MgCl$_2$ and 0.5 U Platinum Taq DNA polymerase. The following conditions were used for the PCR reactions: 1 min at 94 °C, 1 min at the annealing temperature (see Table 1), and 1 min at 72 °C for a number of cycles to ensure the linearity of transcript amplification. Postextension at 72 °C was performed 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 Kodak 1D Image Analysis Software after running all PCR products in a single gel.

2.5. Glutamate uptake

2.5.1. Tissue preparation

The animals were cryoanaesthetized and further euthanized for total brain excision. Telencephalon, optic tectum and cerebellum were dissected into Petri dishes humidified with Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl; 0.63 Na$_2$HPO$_4$; 4.17 NaHCO$_3$; 5.36 KCl; 0.44 KH$_2$PO$_4$; 1.26 CaCl$_2$; 0.41 MgSO$_4$; 0.49 MgCl$_2$ and 1.11 glucose, pH 7.2. Each structure was separated with the help of a magnifying glass and entirely transferred to paired 24-well culture plates containing 0.5 ml of HBSS. One plate from each pair was maintained at 37 °C and the other at 4 °C. The structures from the first plate were washed once with l ml of 37 °C HBSS and those of the second were washed with 1 ml of ice-cold HBSS containing N-methyl-D-glucamine (4 °C) instead of sodium chloride.

2.5.2. Uptake assay

Brain structures were preincubated at 37 °C for 15 min in 0.28 ml of HBSS. The uptake assay was carried out by adding 20 µl of a solution containing 0.33 µCi/ml L-[2,3–3 H]glutamate with unlabeled glutamate (to appropriate concentrations) at 37 °C. Incubations were stopped after 5 min (for telencephalon) and 7 min (for optic tectum and cerebellum) by washing out the glutamate remaining in the incubating medium followed by two washes with l ml ice-cold HBSS. The brain structures were immediately transferred to 0.5N NaOH and incubated overnight, resulting in a homogenate. Protein content was measured using aliquots of homogenate (10 µl) following the method described by Peterson [38]. Samples were taken for determination of the intracellular content of L-[2,3–3 H]glutamate by scintillation counting. Sodium-independent uptake was determined by using ice-cold (4 °C) HBSS containing N-methyl-D-glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the sodium-dependent uptake.

2.6. Statistical analysis

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

3. Results

3.1. Identification of zebrafish EAATs ortholog gene sequences and phylogenetic analysis

Proteins of *H. sapiens* (EAAT1: AAH37310; EAAT2: P43004; EAAT3: NP_004161; EAAT4: NP_005062; EAAT5: NP_006662) and *R. norvegicus* (EAAT1: NP062098; EAAT2: NP_058911; EAAT3: NP_037164; EAAT4: AAB72086; EAAT5: NP_001102443) were retrieved from GenBank and used as queries for the identification of EAAT-related ortholog sequences in zebrafish. Another nonmammalian organism, *Ambystoma tigrinum*, was added in order to compare the grouping of EAAT sequences since previous studies demonstrated the existence of different EAAT members in salamander [16,17].

The phylogenetic tree was constructed using the NeighborJoining method [49] and proportional (p) distance (Fig. 1). Five well-resolved terminal clades supported by high bootstrap values were identified. When both human and rat EAAT genes were used as queries, NCBI Blast searches of GenBank yielded seven complete EAAT-similar deduced amino acid sequences in
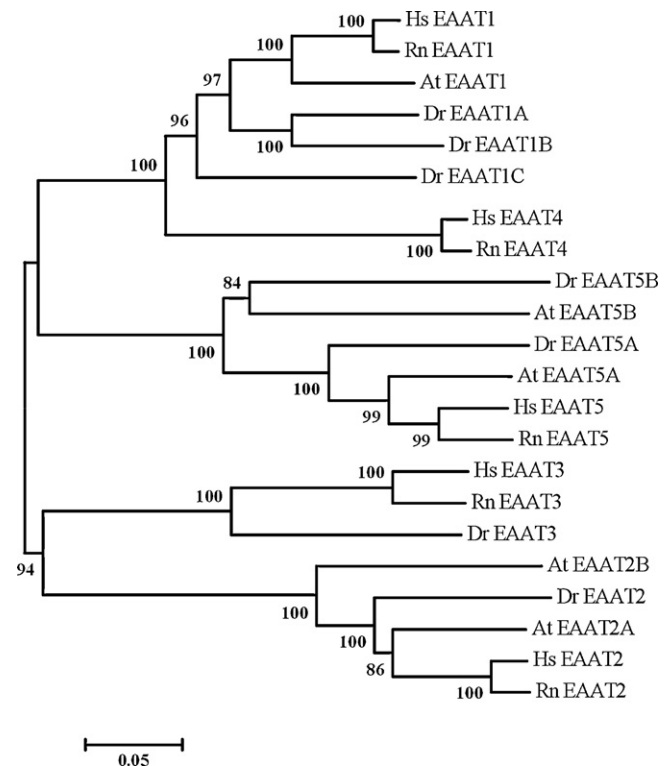

Fig. 1. Phylogenetic analysis of EAAT-related genes. The deduced amino acid sequences were aligned with the ClustalX program and the phylogenetic tree was constructed using the Neighbor-Joining method, proportional (p) distance with the MEGA 2.1 program. The phylogenetic tree grouped consistently *Danio rerio* (Dr), *Ambystoma tigrinum* (At), *Rattus norvegicus* (Rn) and *Homo sapiens* (Hs) EAATs ortholog sequences.

zebrafish (Fig. 1). In order to confirm the sequence identities and to obtain the information about the current data annotation, these sequences were compared with the zebrafish protein database at ZFIN. The AAH56751 and NP_001002666 sequences grouped consistently with both *H. sapiens* and *R. norvegicus* EAAT2 and EAAT3 clades, respectively, suggesting homologous function in zebrafish. The AAH63233, XP_684117 and NP_001103173 sequences (named EAAT1A, EAAT1B, and EAAT1C, respectively) must be paralogs since they were grouped together and formed the EAAT1 clade with *H. sapiens* and *R. norvegicus* sequences. The phylogenetic analysis did not enable any zebrafish sequence retrieved from the GenBank database to be grouped consistently with the EAAT4. Two other sequences, XP_683671 and XP_692900, also formed a clade with EAAT5 *H. sapiens* and *R. norvegicus* sequences. These sequences were named EAAT5A and EAAT5B, respectively. The sequences Dr XP_684117 (EAAT1B), Dr XP_683671 (EAAT5A) and Dr XP_692900 (EAAT5B) were classified by the GenBank database as predicted. Due to the high degree of similarity with the respective *H. sapiens* and *R. norvegicus* EAAT sequences and their consistent position on the phylogenetic tree, these paralog members were also analyzed.

3.2. Gene expression of EAAT members in brain structures

The relative expression profiles of EAAT-related genes were evaluated in different brain structures of zebrafish. RT-PCR experiments showed that transcripts for the seven glutamate transporters identified by phylogenetic analysis were present in all analyzed structures (Fig. 2). EAAT2 and EAAT3 mRNAs were detected at higher levels in all structures, compared with all the other mRNAs. Gene expression of the paralogs EAAT1A and EAAT1B was similar in telencephalon, whereas significantly higher levels of EAAT1A transcripts were detected in the optic tectum and cerebellum when

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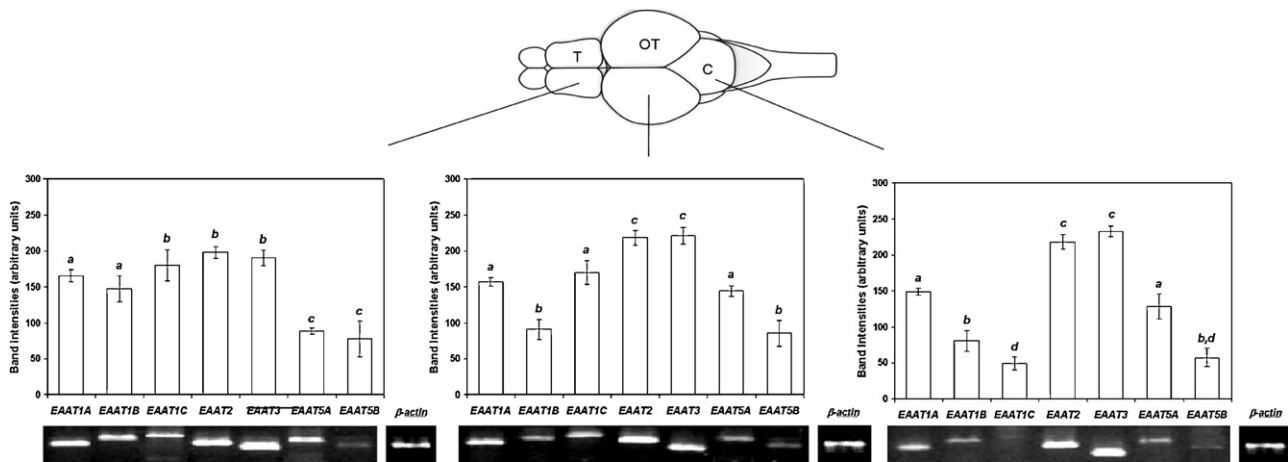
Fig. 2. Relative expression profile of EAAT genes in zebrafish brain. The amplifications resulted in a single product. The band intensities were measured by optic densitometry analysis for telencephalon (T), optic tectum (OT) and cerebellum (C) using the Kodak 1D Image Analysis Software. The results were expressed as mean ± S.E. of optical densitometry arbitrary units of four independent replicate RT-PCR experiments. Distinct letters mean statistically significant differences for EAAT genes expression in each structure ($p \leq 0.05$; one-way ANOVA followed by the Duncan post hoc test).
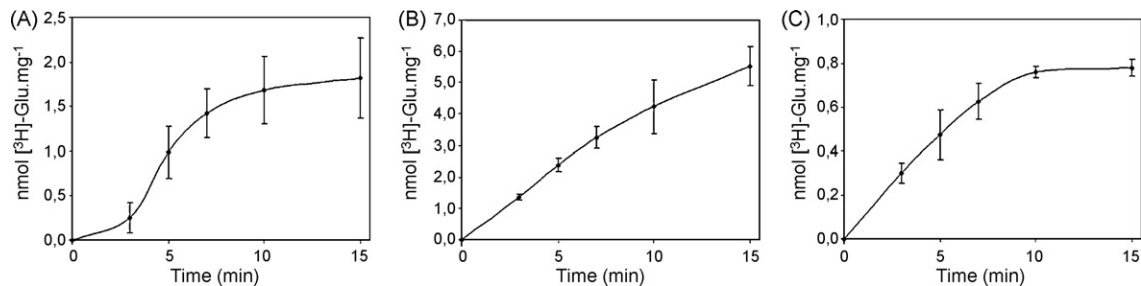
Fig. 3. Time course of Na$^+$-glutamate uptake in zebrafish telencephalon (A), optic tectum (B) and cerebellum (C). Brain structures were transferred to 24-well culture plates and preincubated at 37 °C for 15 min in 0.28 ml of HBSS. Uptake was assessed by adding 20 μl of a solution containing 0.33 μCi/ml L-[2,3–3 H]glutamate with 100 μM of unlabeled glutamate at 37 °C. The sodium-independent uptake was subtracted from the total uptake to obtain the sodium-dependent uptake. The data were expressed as mean ± S.E. of four different experiments, each in duplicate.

compared to EAAT1B. Concerning the EAAT1C, the results show the presence of similar mRNA levels in telencephalon and optic tectum and a lower amount in the cerebellum. The relative gene expression of EAAT5A was similar to that of EAAT5B in the telencephalon, while EAAT5B was significantly less expressed when compared to EAAT5A in optic tectum and cerebellum.

3.3. Glutamate uptake

Considering the identification of distinct EAAT mRNAs and their expression profiles in zebrafish brain, some preliminary parameters of glutamate transporter activity were investigated. In order to verify the influence of temperature on L-[3H]glutamate uptake, the assay was carried out in over a range from 21 to 45 °C (7 min of incubation, 100 μM of glutamate). Since the temperatures tested did not alter the glutamate uptake in all brain structures analyzed (data not shown), subsequent experiments were performed at 37 °C. Time courses for glutamate uptake in telencephalon, optic tectum and cerebellum were obtained over 3–15 min and these are represented in Fig. 3. A distinct glutamate uptake profile was observed in zebrafish brain structures. The uptake in telencephalon and cerebellum remained constant after 10 min incubation, whereas the glutamate uptake activity in optic tectum increased over the time period analyzed.

4. Discussion

In mammalian brain, it has been clearly demonstrated that glutamate transporter activity modulates the excito-

toxic/physiological tonus of the glutamatergic system, presenting a central role in brain function [11,33,64]. By way of comparison, therefore, here we investigated some EAAT parameters in zebrafish brain. In addition to the confirmation of EAAT-related sequences by a phylogenetic analysis, this is the first study to demonstrate the mRNA expression profile in zebrafish CNS of EAAT1, EAAT2, EAAT3 and EAAT5 members of the glutamate transporter family. Surprisingly, the EAAT4 glutamate transporter was not identified in zebrafish in this work. Despite this, it is clear that structures from zebrafish brain have the ability to transport glutamate in a Na$^+$-dependent fashion.

Searches in the GenBank database yielded seven EAAT-similar deduced amino acid sequences in zebrafish, which were consistently grouped to human, rat, and salamander EAAT orthologs, supporting the evolutionary conservation of each protein. For EAAT2 and EAAT3 only one related gene was found in the zebrafish genome. However, for EAAT1 three paralog members were found which were named EAAT1A, EAAT1B, and EAAT1C. For EAAT5 two paralog members were found which were named EAAT5A and EAAT5B. The presence of these paralog genes may be due to evolutionary genome duplication in the Teleostei infraclass [2,42], since previous studies have shown that teleosts tend to have expanded gene families as compared with mammals [19,41]. This gene family expansion could be caused by extra tandem duplication in the fish lineage, extensive loss of preexisting duplicates in the mammalian lineage or extra duplication of chromosomal segments, chromosomes, or the entire genome in the fish lineage [66]. Previous phylogenetic studies have argued against the idea that expanded families result from retention in the fish lin-

eage of a large number of duplicates that were present in the last common ancestor of zebrafish and humans [66]. Moreover, EAAT paralog genes have been found in organisms other than teleosts. Thus, five distinct glutamate transporter genes were identified expressed in the salamander retina: one EAAT1 subtype, two distinct EAAT2 subtypes (EAAT2A and EAAT2B), and two distinct members of the retina-specific subtype, EAAT5A and EAAT5B [16].

In this study, semiquantitative RT-PCR assays were performed to verify the mRNA expression of EAAT members of the glutamate transporter family in zebrafish brain structures. In mature mammalian brain, EAAT1 (GLAST) and EAAT2 (GLT-1) are expressed primarily in astrocytes and are responsible for up to 80% of glutamate removal from the synaptic cleft [45,11,24]. Here, we show that the orthologs EAAT1A, EAAT1B, EAAT1C, and EAAT2 genes are expressed and have a wide distribution in the zebrafish brain, as reflected in considerable levels of transcripts for these genes.

EAAT3 is a mammalian neuronal EAAT. Unlike the astrocytic glutamate transporters, it does not play a major role in clearing glutamate from the extracellular space [45,23,28], in spite of being an essential brain transporter. In the rat brain, the highest concentrations of EAAT3 are found in the hippocampus, cerebellum, and basal ganglia [55,11]. Here we show that there were high expression levels in several brain structures, as well as a wide distribution of transcripts for the EAAT3 gene. There is evidence pointing to the involvement of this transporter in the re-synthesis of GABA in presynaptic GABAergic neuronal terminals [51]. Moreover, it is far more effective at transporting cysteine than the astrocyte glutamate transporters [67,3] and cysteine is a substrate for the synthesis of glutathione, the main thiol antioxidant [14].

Electrophysiological studies of EAAT4 and EAAT5 have demonstrated a relatively large chloride conductance associated with transport activity. Here, we show that both EAAT5A and EAAT5B are expressed in zebrafish brain and their mRNAs were detectable in all the investigated structures, with the expression of EAAT5B being lower than that of EAAT5A. This result differs from data obtained in mammalian neuronal tissue [4], for which Northern blot assays showed a strong signal only in the retina. Furthermore, two clones of EAAT5 (sEAAT5A and sEAAT5B) have been isolated from salamander [16] and appear to be expressed both in Müller cells and in most neurons [16]. The significance of these findings still remains unclear, but it is possible to speculate that the functions of EAAT5 in fish and other vertebrates might be distinct.

Based upon the observed data for EAAT gene expression in zebrafish, we performed some preliminary experiments on the [^{3}H] glutamate uptake by the three brain structures and observed that all the structures presented glutamate uptake activity. It has been clearly demonstrated [8] that the equilibrium between the physiological/excitotoxic glutamatergic tonus is dependent on adequate activity in glutamate transporters. As there is no extracellular metabolism of glutamate, uptake of this amino acid is the unique mechanism responsible for maintaining extracellular concentrations below toxic levels in the long term, as well as for the control of glutamatergic function. Consequently, brain tissue needs a very efficient glutamate uptake system to protect itself against glutamate toxicity.

The presence of all the observed EAATs in all the structures investigated is intriguing, as compared with mammalian brain distribution. The area responsible for learning in zebrafish is the telencephalon, which is analogous to the hippocampus and amygdala in mammalian brain [44]. After induction of long-term potentiation (LTP), the activation of NMDA receptors and GLT-1 function play important roles through the regulation of extracellular levels of glutamate [35,60]. Electrophysiological evidence of NMDA receptor-mediated activity and synaptic plasticity, such as LTP, has already been reported in zebrafish brain [36]. Accord-

ingly, our results demonstrate the expression pattern of GLT-1 (EAAT2) in the telencephalon. Four EAAT subtypes are expressed in zebrafish brain and they may contribute to glutamate uptake in the optic tectum of zebrafish, which has been considered a useful vertebrate model in visual neuroscience. Given that the optic tectum is the area responsible for visual processing in the zebrafish brain [34], an interesting possibility is that the glutamate transporters may participate in visual functions in this species. Furthermore, the influence of central glutamatergic synapses on the acuity of visual perception in zebrafish has been demonstrated [54].

Cerebellar compartments in teleosts correspond to the mammalian vestibulocerebellar and non-vestibulocerebellar systems, participating in the control of balance and locomotion, respectively [65]. The Purkinje cells of the cerebellar molecular layer express EAAT4 in the CNS of adult rat [12] and human [25]. Considering that the EAAT4 gene has not been found in the zebrafish genome so far, other members of the EAAT family could play a role in the glutamatergic transmission involved in cerebellar function.

The different gene expression patterns of EAATs in zebrafish in comparison to those observed in human and rat reinforces the idea that the vertebrate neural and behavioral changes that support brain evolution occurred in successive stages and have been conserved through phylogenesis. In addition, knowledge of EAATs in this species sheds light upon their localization as well as functional differences in vertebrate glutamatergic signaling.

In summary, this report presents a phylogenetic analysis and the relative gene expression profiles of glutamate transporters in zebrafish, as well as preliminary data on glutamate uptake. The identification of EAAT-related genes involved in glutamate transport and the demonstration of uptake activity, together with the powerful genetic approaches that this organism offers could be important in determining neurotoxicity parameters and in providing a better understanding of the role of glutamatergic signaling in the neurobiology of this model species.

Conflict of interest

None.

References

[1] S.G. Amara, A.C. Fontana, Excitatory amino acid transporters: keeping up with glutamate, Neurochem. Int. 41 (2002) 313–318.
[2] A. Amores, A. Force, Y.L. Yan, L. Joly, C. Amemiya, A. Fritz, R.K. Ho, J. Langeland, V. Prince, Y.L. Wang, M. Westerfield, M. Ekker, J.H. Postlethwait, Zebrafish hox clusters and vertebrate genome evolution, Science 282 (1998) 1711–1714.
[3] K. Aoyama, M. Watabe, T. Toshio Nakaki, Regulation of neuronal glutathione synthesis, J. Pharmacol. Sci. 108 (2008) 227–238.
[4] J.L. Arriza, S. Eliasof, M.P. Kavanaugh, S.G. Amara, Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance, Proc. Natl. Acad. Sci. 94 (1997) 4155–4160.

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[5] 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.

[6] P.M. Beart, R.D. O'Shea, Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement, Br. J. Pharmacol. 150 (2007) 5–17.

[7] C. Beas-Zárate, M.Y. Sánchez-Ruíz, M.E. Ureña-Guerrero, A. Feria-Velasco, Effect of neonatal exposure to monosodium L-glutamate on regional GABA release during postnatal development, Neurochem. Int. 33 (1998) 217–232.

[8] A. Camacho, L. Massieu, Role of glutamate transporters in the clearance and release of glutamate during ischemia and its relation to neuronal death, Arch. Med. Res. 37 (2006) 11–18.

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

[10] J.A. Cox, S. Kucenas, M.M. Voigt, Molecular characterization and embryonic expression of the family of N-methyl-D-aspartate receptor subunit genes in the zebrafish, Dev. Dyn. 234 (2005) 756–766.

[11] N.C. Danbolt, Glutamate uptake, Prog. Neurobiol. 65 (2001) 1–105.

[12] Y. Dehnes, F.A. Chaudhry, K. Ullensvang, K.P. Lehre, J. Storm-Mathisen, N.C. Danbolt, The glutamate transporter EAAT4 in rat cerebellar Purkinje cells: a glutamate-gated chloride channel concentrated near the synapse in parts of the dendritic membrane facing astroglia, J. Neurosci. 18 (1998) 3606–3619.

[13] K. Dooley, L.I. Zon, Zebrafish: a model system for the study of human disease, Curr. Opin. Genet. Dev. 10 (2000) 252–256.

[14] R. Dringen, B. Pfeiffer, B. Hamprecht, Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione, J. Neurosci. 19 (1999) 562–569.

[15] J.G. Edwards, W.C. Michel, Pharmacological characterization of ionotropic glutamate receptors in the zebrafish olfactory bulb, Neuroscience 122 (2003) 1037–1047.

[16] S. Eliasof, J.L. Arriza, B.H. Leighton, S.G. Amara, M.P. Kavanaugh, Localization and function of five glutamate transporters cloned from the salamander retina, Vision Res. 38 (1998) 1443–1454.

[17] S. Eliasof, J.L. Arriza, B.H. Leighton, M.P. Kavanaugh, S.G. Amara, Excitatory amino acid transporters of the salamander retina: identification, localization, and function, J. Neurosci. 18 (1998) 698–712.

[18] W.A. Fairman, R.J. Vandenberg, J.L. Arriza, M.P. Kavanaugh, S.G. Amara, An excitatory amino-acid transporter with properties of a ligand-gated chloride channel, Nature 375 (1995) 599–603.

[19] A. Force, M. Lynch, F.B. Pickett, A. Amores, Y.L. Yan, J. Postlethwait, Preservation of duplicate genes by complementary, degenerative mutations, Genetics 151 (1999) 1531–1545.

[20] A. Furuta, L.J. Martin, C.L. Lin, M. Dykes-Hoberg, J.D. Rothstein, Cellular and synaptic localization of the neuronal glutamate transporters excitatory amino acid transporter 3 and 4, Neuroscience 81 (1997) 1031–1042.

[21] A. Furuta, J.S. Rothstein, L.J. Martin, Glutamate transporter protein subtypes are expressed differentially during rat CNS development, J. Neurosci. 17 (1997) 8363–8375.

[22] D.J. Grunwald, J.S. Eisen, Headwaters of the zebrafish – emergence of a new model vertebrate, Nat. Rev. Genet. 3 (2002) 717–724.

[23] T. Harada, C. Harada, K. Nakamura, H.M. Quah, A. Okumura, K. Namekata, T. Saeki, M. Aihara, H. Yoshida, A. Mitani, K. Tanaka, The potential role of glutamate transporters in the pathogenesis of normal tension glaucoma, J. Clin. Invest. 117 (2007) 1763–1770.

[24] J. Huguenard, Neurotransmitter supply and demand in epilepsy, Epilepsy Curr. 2 (2003) 61–63.

[25] Y.W. Inage, M. Itoh, K. Wada, S. Takashima, Expression of two glutamate transporters, GLAST and EAAT4, in the human cerebellum: their correlation in development and neonatal hypoxic-ischemic damage, J. Neuropathol. Exp. Neurol. 57 (1998) 554–562.

[26] I. Izquierdo, L.R. Bevilaqua, J.I. Rossato, J.S. Bonini, J.H. Medina, M. Cammarota, Different molecular cascades in different sites of the brain control memory consolidation, Trends Neurosci. 29 (2006) 496–505.

[27] Y. Kanai, M.A. Hediger, Primary structure and functional characterization of a high-affinity glutamate transporter, Nature 360 (1992) 467–471.

[28] F. Klaus, E.M. Gehring, A. Zürn, J. Laufer, R. Lindner, N. Strutz-Seebohm, J.M. Tavaré, J.D. Rothstein, C. Boehmer, M. Palmada, I. Gruner, U.E. Lang, G. Seebohm, F. Lang, Regulation of the Na(+)-coupled glutamate transporter EAAT3 by PIKfyve, Neurochem. Int. 54 (2009) 372–377.

[29] S. Lortet, B. Canolle, F. Masmejean, A. Nieoullon, Plasma membrane expression of the neuronal glutamate transporter EAAC1 is regulated by glial factors: evidence for different regulatory pathways associated with neuronal maturation, Neurochem. Int. 52 (2008) 1373–1382.

[30] L.T. Macnab, D.V. Pow, Expression of the exon 9-skipping form of EAAT2 in astrocytes of rats, Neuroscience 150 (2007) 705–711.

[31] J. Mallolas, O. Hurtado, M. Castellanos, M. Blanco, T. Sobrino, J. Serena, J. Vivancos, J. Castillo, I. Lizasoain, M.A. Moro, A.A. Dávalos, Polymorphism in the EAAT2 promoter is associated with higher glutamate concentrations and higher frequency of progressing stroke, J. Exp. Med. 203 (2006) 711–717.

[32] N.J. Maragakis, J.D. Rothstein, Glutamate transporters: animal models to neurologic disease, Neurobiol. Dis. 15 (2004) 461–473.

[33] N.J. Maragakis, J.D. Rothstein, Mechanisms of disease: astrocytes in neurodegenerative disease, Nat. Clin. Pract. Neurol. 2 (2006) 679–689.

[34] A.L. McDowell, L.J. Dixon, J.D. Houchins, J. Bilotta, Visual processing of the zebrafish optic tectum before and after optic nerve damage, Vis. Neurosci. 21 (2004) 97–106.

[35] R.C. Malenka, R.A. Nicoll, Long-term potentiation – a decade of progress? Science 285 (1999) 1870–1874.

[36] R.H. Nam, W. Kim, C.J. Lee, NMDA receptor-dependent long-term potentiation in the telencephalon of the zebrafish, Neurosci. Lett. 370 (2004) 248–251.

[37] N. Obholzer, S. Wolfson, J.G. Trapani, W. Mo, A. Nechiporuk, E. Busch-Nentwich, C. Seiler, S. Sidi, C. Sollner, R.N. Duncan, A. Boehland, T. Nicolson, Vesicular glutamate transporter 3 is required for synaptic transmission in zebrafish hair cells, J. Neurosci. 28 (2008) 2110–2118.

[38] G.L. Peterson, A simplification of the protein assay method of Lowry et al. which is more generally applicable, Anal. Biochem. 83 (1977) 346–356.

[39] R.T. Peterson, R. Nass, W.A. Boyd, J.H. Freedman, K. Dong, T. Narahashi, Use of non-mammalian alternative models for neurotoxicological study, Neurotoxicology 29 (2008) 546–555.

[40] G. Pines, N.C. Danbolt, M. Bjoras, Y. Zhang, A. Bendahan, L. Eide, H. Koepsell, J. Storm-Mathisen, E. Seeberg, B.I. Kanner, Cloning and expression of a rat brain L-glutamate transporter, Nature 360 (1992) 464–467.

[41] J. Postlethwait, A. Amores, A. Force, Y.L. Yan, The zebrafish genome, Methods Cell Biol. 60 (1999) 149–163.

[42] J.H. Postlethwait, Y.L. Yan, M.A. Gates, S. Horne, A. Amores, A. Brownlie, A. Donovan, E.S. Egan, A. Force, Z. Gong, C. Goutel, A. Fritz, R. Kelsh, E. Knapik, E. Liao, B. Paw, D. Ransom, A. Singer, M. Thomson, T.S. Abduljabbar, P. Yelick, D. Beier, J.S. Joly, D. Larhammar, F. Rosa, M. Westerfield, L.I. Zon, S.L. Johnson, W.S. Talbot, Vertebrate genome evolution and the zebrafish gene map, Nat. Genet. 18 (1998) 345–349.

[43] D.V. Pow, Amino acids and their transporters in the retina, Neurochem. Int. 38 (2001) 463–484.

[44] F. Rodríguez, J.C. López, J.P. Vargas, C. Broglio, Y. Gómez, C. Salas, Spatial memory and hippocampal pallium through vertebrate evolution: insights from reptiles and teleost fish, Brain Res. Bull. 57 (2002) 499–503.

[45] J.D. Rothstein, M. Dykes-Hoberg, C.A. Pardo, L.A. Bristol, L. Jin, R.W. Kuncl, Y. Kanai, M.A. Hediger, Y. Wang, J.P. Schielke, D.F. Welty, Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate, Neuron 16 (1996) 675–686.

[46] J.K. Ryu, J. Kim, S.H. Choi, Y.J. Oh, Y.B. Lee, S.U. Kim, B.K. Jin, ATP-induced in vivo neurotoxicity in the rat striatum via P2 receptors, Neuroreport 13 (2002) 1611–1615.

[47] C. Salas, C. Broglio, E. Durán, A. Gómez, F.M. Ocaña, F. Jiménez-Moya, F. Rodríguez, Neuropsychology of learning and memory in teleost fish, Zebrafish 3 (2006) 157–171.

[48] E. Salinska, A. Stafiej, Metabotropic glutamate receptors (mGluRs) are involved in early phase of memory formation: possible role of modulation of glutamate release, Neurochem. Int. 43 (2003) 469–474.

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

[50] G. Segovia, A. Del Arco, L. Prieto, F. Mora, Glutamate–glutamine cycle and aging in striatum of the awake rat: effects of a glutamate transporter blocker, Neurochem. Res. 26 (2001) 37–41.

[51] J.P. Sepkuty, A.S. Cohen, C. Eccles, A. Rafiq, K. Behar, R. Ganel, D.A. Coulter, J.D. Rothstein, A neuronal glutamate transporter contributes to neurotransmitter GABA synthesis and epilepsy, J. Neurosci. 22 (2002) 6372–6379.

[52] A.L. Sheldon, M.B. Robinson, The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention, Neurochem. Int. 51 (2007) 333–355.

[53] A.L. Sheldon, M.I. González, E.N. Krizman-Genda, B.T. Susarla, M.B. Robinson, Ubiquitination-mediated internalization and degradation of the astroglial glutamate transporter, GLT-1, Neurochem. Int. 53 (2008) 296–308.

[54] M.C. Smear, H.W. Tao, W. Staub, M.B. Orger, N.J. Gosse, Y. Liu, K. Takahashi, M.M. Poo, H. Baier, Vesicular glutamate transport at a central synapse limits the acuity of visual perception in zebrafish, Neuron 53 (2007) 65–77.

[55] S. Smith, J.A. Metcalfe, G. Elgar, Identification and analysis of two snail genes in the pufferfish (*Fugu rubripes*) and mapping of human SNA to 20q, Gene 247 (2000) 119–128.

[56] T. Storck, S. Schulte, K. Hofmann, W. Stoffel, Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain, Proc. Natl. Acad. Sci. 89 (1992) 10955–10959.

[57] H.A. Swain, C. Sigstad, F.M. Scalzo, Effects of dizocilpine (MK-801) on circling behavior, swimming activity, and place preference in zebrafish (*Danio rerio*), Neurotoxicol. Teratol. 26 (2004) 725–729.

[58] L. Struzynska, A glutamatergic component of lead toxicity in adult brain: the role of astrocytic glutamate transporters, Neurochem. Int. 55 (2009) 151–156.

[59] R. Tabor, R.W. Friedrich, Pharmacological analysis of ionotropic glutamate receptor function in neuronal circuits of the zebrafish olfactory bulb, PLoS ONE 3 (2008) 1416.

[60] K. Tanaka, Functions of glutamate transporters in the brain, Neurosci. Res. 37 (2000) 15–19.

[61] K. Tanaka, K. Watase, T. Manabe, K. Yamada, M. Watanabe, K. Takahashi, H. Iwama, T. Nishikawa, N. Ichihara, T. Kikuchi, S. Okuyama, N. Kawashima, S. Hori, M. Takimoto, K. Wada, Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1, Science 276 (1997) 1699–1702.

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

[63] S. Tilleux, S. Goursaud, E. Hermans, Selective up-regulation of GLT-1 in cultured astrocytes exposed to soluble mediators released by activated microglia, Neurochem. Int. 55 (2009) 35–40.

[64] A.V. Tzingounis, J.I. Wadiche, Glutamate transporters: confining runaway excitation by shaping synaptic transmission, Nat. Rev. Neurosci. 12 (2007) 935–947.

[65] K. Volkmann, S. Rieger, A. Babaryka, R.W. Koster, The zebrafish cerebellar rhombic lip is spatially patterned in producing granule cell populations of different functional compartments, Dev. Biol. 313 (2008) 167–180.

[66] I.G. Woods, P.D. Kelly, F. Chu, P. Ngo-Hazelett, Y.L. Yan, H. Huang, J.H. Postlethwait, W.S. Talbot, A comparative map of the zebrafish genome, Genome Res. 10 (2000) 1903–1914.

[67] N. Zerangue, M.P. Kavanaugh, Flux coupling in a neuronal glutamate transporter, Nature 383 (1996) 634–637.

[68] L.I. Zon, R.T. Peterson, In vivo drug discovery in the zebrafish, Nat. Rev. Drug Discov. 4 (2005) 35–44.
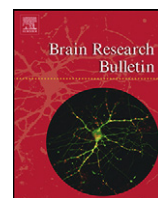
II.2. Capítulo 2

Nomenclatura dos transportadores de glutamato em zebrafish e outros organismos.

Artigo publicado no periódico *Brain Research Bulletin* (2010) 83, 297.

Letter to the Editor

Nomenclature of glutamate transporters in zebrafish and other vertebrates

Glutamate is the main excitatory neurotransmitter in the central nervous system. Its uptake is tightly regulated by a group of excitatory amino acid transporters (EAATs), that belong to the solute carrier family 1 (SLC1), that constitute high affinity glutamate and neutral amino acid transporters. Earlier names of these transporters (e.g. GLT-1, Glast) have been exchanged by a more coherent nomenclature, naming these genes EAATs and sometimes interchanged with the SLC1 nomenclature proposed by the HUGO gene nomenclature committee (http://www.genenames.org), which attempts to give meaningful names to every human gene family.

The recent cloning and reporting of SLC1/EAAT genes from zebrafish (*Danio rerio*) in this journal [4,5], exemplifies the confusion of nomenclature. The confusion partly originates from the realization that vertebrates have up to 7 subfamilies, while mammalian genomes (with the exception of monotremata) have lost 2 of these and hence only 5 copies are present in their genomes [3]. In order to clarify the nomenclature and the orthologous relationship of the cloned zebrafish genes, we propose to consolidate the nomenclature. As shown in Table 1, we propose to unify

[3] M. Gesemann, A. Lesslauer, C.M. Maurer, H.B. Schonthaler, Neuhauss SC, Phylogenetic analysis of the vertebrate excitatory/neutral amino acid transporter (SLC1/EAAT) family reveals lineage specific subfamilies, BMC Evol. Biol. 10 (2010) 117.
[4] M. Gesemann, C.M. Maurer, Neuhauss SC, Excitatory amino acid transporters in the zebrafish. Letter to "Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain" from Rico et al., Brain Res. Bull. (2010).
[5] E.P. Rico, D.L. de Oliveira, D.B. Rosemberg, B.H. Mussulini, C.D. Bonan, et al., Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain, Brain Res. Bull. 81 (2010) 517–523.

Stephan C.F. Neuhauss*
University of Zurich, Institute of Molecular Life Sciences, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Eduardo Pacheco Rico
Universidade Federal do Rio Grande do Sul, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Rua Ramiro Barcelos 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil

Matthias Gesemann**
University of Zurich, Institute of Molecular Life Sciences, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Table 1
Nomenclature of Glutamate Transporters.

Proposed name	Zebrafish	Other vertebrates
SLC1A1/EAAT3	*EAAT3* [4]; SLC1/EAAT3 [5,3]	EAAT3, EAAC1
SLC1A2a,b/EAAT2a,b	*EAAT2* [4]; SLC1A2a/EAAT2a, SLC1A2b/EAAT2b [5,3]	EAAT2, GLT-1
SLC1A3a,b/EAAT1a,b	*EAAT1a, EAAT1b* [4]; SLC1A3a/EAAT1a, SLC1A3b/EAAT1b [5,3]	EAAT1, GLAST, GLAST-1
SLC1A4	SLC1A4 [5,3]	SLC1A4
SLC1A5	SLC1A5 [5,3]	SLC1A5
SLC1A6/EAAT4	*EAAT1c* [4]; SLC1A6/EAAT4 [5,3]	EAAT4
SLC1A7a,b/EAAT5a,b	*EAAT5a* [4]; SLC1A7a/EAAT5a, SLC1A7b/EAAT5b [5,3]	EAAT5, EAAT5a [1,2]
SLC1A8a,b/EAAT6a,b	*EAAT5b* [4]; SLC1A8a/EAAT6a, SLC1A8b/EAAT6b [5,3]	EAAT5b [1,2]
SLC1A9/EAAT7	SLC1A9/EAAT7 [5,3]	EAAT2b [1,2]

the nomenclature in zebrafish and have also included proposed nomenclature for EAAT genes in the tiger salamander *Ambystoma tigrium*, which in earlier studies [1,2] was shown to have two EAAT2 and EAAT5 orthologs. In light of the recent phylogeny, we propose to rename AtEAAT2b and AtEAAT5b as AtEAAT6 and AtEAAT7, respectively, to reflect their true phylogenetic relationship. Proposing this new nomenclature for the SLC1/EAAT genes, we hope to avoid future confusion designating glutamate transporters isolated from zebrafish and other species.

References

[1] S. Eliasof, J.L. Arriza, B.H. Leighton, S.G. Amara, Kavanaugh MP, Localization and function of five glutamate transporters cloned from the salamander retina, Vision Res. 38 (1998) 1443–1454.
[2] S. Eliasof, J.L. Arriza, B.H. Leighton, M.P. Kavanaugh, S.G. Amara, Excitatory amino acid transporters of the salamander retina: identification, localization, and function, J. Neurosci. 18 (1998) 698–712.

* Corresponding author. Tel.: +41 44 6356040; fax: +41 44 6356897.

** Corresponding author. Tel.: +41 44 6356040; fax: +41 44 6356897.
E-mail addresses: stephan.neuhauss@imls.uzh.ch (S.C.F. Neuhauss), matthias.gesemann@imls.uzh.ch (M. Gesemann)

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II.3. Capítulo 3

Transportadores de glutamato são alterados após a exposição ao etanol em tecto óptico de zebrafish.

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Glutamate transporters are altered after chronic ethanol exposure in zebrafish optic tectum

Eduardo Pacheco Rico[a,b*], Andrei da Silveira Langoni[c], Diogo Lösch de Oliveira[a,b], Denis Broock Rosemberg[a,b], Ben Hur Mussulini[a,b], Kamila Cagliari Zenki[a,b], André Arigony Souto[e], Renato Dutra Dias[c,f], Maurício Reis Bogo[d,f], Susana Wofchuk[a,b], Carla Denise Bonan[c,f*], Diogo Onofre Souza[a,b]

[a] *Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil;*

[b] *Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção (INCT-EN), 90035-003, Porto Alegre, RS, Brazil;*

[c] *Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil;*

[d] *Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil;*

[e] *Faculdade de Química, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil;*

[f] *Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003, Porto Alegre, RS, Brazil.*

*Corresponding author:

Eduardo Pacheco Rico, Departamento de Bioquímica, ICBS, UFRGS. Rua Ramiro Barcelos 2600-Anexo. CEP: 90035-003. Porto Alegre, RS, Brazil. Tel.: +55 51 3316-5557; Fax: +55 51 3316-5540. e-mail: edurico@portoweb.com.br

Abstract

Alcoholism is a public health problem that leads to neurodegeneration, tremors, alcoholic psychosis, and delirium. Prolonged ethanol exposure affects both excitatory and inhibitory neurotransmissions systems. Glutamate is the major excitatory amino acid in the central nervous system that triggers ion channels or G protein-coupled receptors. Astrocytes control the glutamate-glutamine cycle in the brain by taking up glutamate through glutamate transporters and metabolizing it into glutamine via glutamine synthetase. In neurons, glutaminase is responsible to recycling glutamine in glutamate. Zebrafish is a biochemical and behavioral tool model to studies on alcoholism and alcohol-dependence. In this study, we evaluated the prolonged ethanol effect on glutamatergic system in optic tectum, an important structure that processes the motor function in zebrafish brain. Animals were exposed to 0.5% ethanol for 7, 14, and 28 days. After 7 and 14 days of ethanol exposure, a significant decrease in glutamate uptake (30% and 54%) was observed, whereas after 28 days, no significant changes were detected. Na,K-ATPase, the enzyme responsible to generate ion gradients, was not altered after all exposure periods. Moreover, fish exposed to ethanol during 7 and 14 days exhibit a decrease of mRNA transcripts for SLC1A1/EAAT3. However, the gene expression of SLC1A8a,b/EAAT6a,b increased after all ethanol exposure periods, whereas SLC1A3a,b/EAAT1b increased only after 28 days. Glutamine synthetase and glutaminase were not altered after prolonged ethanol exposure.

In conclusion, this study demonstrates that chronic ethanol exposure affect the glutamatergic system in zebrafish optic tectum, specifically the glutamate transport, an important mechanism that controls the extracellular glutamate levels.

Keywords: *glutamatergic system; ethanol; glutamate transporters; zebrafish.*

Abbreviations: CNS, Central nervous system; EAAT, excitatory amino acid transporters; PAG, phosphate-activated glutaminase; GS, glutamine synthetase.

Introduction

The prolonged alcohol consumption has been shown to cause aberrations in synaptic plasticity and related neuronal function. The neuro-adaptational changes induced by exposure to alcohol may be related to dysregulation of signaling systems, gene transcription, and protein expression at the cellular level (Pandey, 2004; Moonat et al., 2010). Glutamate is the most widespread and important excitatory neurotransmitter in the CNS and participates in plastic process such as neural development, learning and memory and neuronal networks establishment (Lhullier et al., 2004; Ozawa et al., 1998). However, overstimulation of the glutamatergic system may lead to excitotoxicity, a phenomenon involved in several CNS disorders (Nicolaidis et al., 2005).

The excess of extracellular glutamate is normally neutralized by high-affinity uptake mechanism executed by a family of glutamate transporter proteins. To date, five structurally distinct subtypes of Na^+-dependent excitatory amino acid transporters have been identified and characterized in the mammalian brain: EAAT1 (Storck et al., 1992; Takayasu et al., 2006) EAAT2 (Pines et al., 1992), EAAT3 (Kanai, 1992), EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997). EAAT1 is primarily an astroglial

transporter and the main transporter protein present during CNS development (Furuta et al., 1997). EAAT2 is one of the two most abundant glutamate transporters in the adult CNS (Macnab and Pow, 2007), and is an astroglial transporter expressed postnatally responsible for up to 90% of all glutamate transport in adult tissue (Rothstein et al., 1996; Tilleux et a., 2009). EAAT3, and EAAT4 are expressed in neurons (Furuta et al., 1997; Dehnes et al., 1998), whereas EAAT5 is present in glia and neurons from retina (Eliasof et al., 1998). After being taken up, glutamate is converted into glutamine exclusively in the cytoplasm of astrocytes by GS (EC 6.3.1.2) (Norenberg and Martinez-Hernandez, 1979; Holten and Gundersen, 2008). Glutamine subserves the metabolic demands of astrocytes, but is also exported to neurons where it serves both as oxidation substrate or precursor of amino acids neurotransmitters (Albrecht et al., 2000), and PAG (EC 3.5.1.2) is regarded as a pivotal enzyme in the production of transmitter glutamate (Kvamme et al., 2001). This mechanism dependent of transporters and enzymes involved into glutamate metabolism is known as glutamate-glutamine cycle.

Glutamatergic neurotransmission is involved in alcohol consumption. In mammalian CNS, chronic administration of ethanol alters the signal promoted by NMDA receptors, leading to an imbalance on intracellular Ca^{2+} in cells (Esel, 2006). Moreover, it has been demonstrated that ethanol affects the glutamate transporter system in different models (Smith, 1997; Melendez et al., 2005). However, to our knowledge, there are no studies about the effects promoted by ethanol in glutamatergic neurotransmission of zebrafish. Recently, our group reported the phylogenetic analysis and the relative gene expression profile of glutamate transporters, as well as preliminary data related to the glutamate uptake in zebrafish brain (Rico et al., 2010).

During the last ten years, adult zebrafish has served as an excellent model for studying the mechanisms associated to ethanol consumption through investigation of the neurobehavioral and neurochemical parameters such as: (i) locomotor activity, learning and memory, aggression, and social interaction (Gerlai et al., 2000; Scalzo and Levin, 2004); (ii) effects of drugs of abuse and addiction, including ethanol (Ninkovic and Bally-Cuif, 2006); (iii), investigation of genetic determinants involved in regulating the responses to ethanol of different zebrafish strains (Dlugos and Rabin, 2003; Zon and Peterson, 2005). (iiii) neurochemical studies about of neuronal signaling, as well as dopaminergic and serotoninergic (Chatterjee and Gerlai, 2009), cholinergic (Rico et al., 2007) and purinergic (Rico et al., 2008) systems. Furthermore, zebrafish share a genetic and anatomic conservation with mice and humans (Barbazuk et al., 2000). This species appears to be an attractive organism for high throughput screening applications, e.g., mutagenesis screening, forward genetics or drug discovery efforts applied to neurotoxicity tests (Bowman and Zon, 2010).

One of the most accessible brain regions of the zebrafish is the optic tectum. Analogous to the mammalian superior colliculus, the tectum is thought to process visual information from the environment for use in orienting and tracking movements such as those used in prey capture behavior (Ramdya et al., 2006; Gahtan et al., 2005). Glutamatergic system was described in neural circuit of this area, participating in the functions of tectal neurons (Kinoshita and Ito, 2006; Smear et al., 2007; Rico et al., 2010). Thus, considering the importance of glutamatergic system to comprehension of ethanol effects in CNS, the aim of this study was to evaluate the prolonged ethanol exposure on functional and transcriptional profile of glutamate transporters, Na,K-ATPase, GS, and PAG activities in adult zebrafish optic tectum.

2. Material and Methods

2.1. Zebrafish maintenance

Adult zebrafish of both sexes were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in a 50 L thermostated aquarium filled with continuously aerated unchlorinated water treated with Aquasafe® (Tetra, USA). The temperature was kept at 26 ± 2 °C under a 12-h light-dark controlled photoperiod, and the animals were fed twice a day until satiety with a commercial flake fish food (alcon BASIC®, Alcon, Brazil). The fish were used according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and the experiments were designed to minimize discomfort or suffering and also the number of fish used. The Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS) approved the protocol under license number 477/05—CEP.

2.2 Chemicals

Ethanol (C_2H_6O) was purchased from Merck (Darmstadt, Germany). Reagents were purchased from Sigma Chemical CO (St. Louis, MO). L-[3H]glutamate (specific activity 30 Ci $mmol^{-1}$) was purchased from Amersham International, UK. Platinum Taq DNA polymerase, Trizol reagent, and SuperScriptTM First-Strand III (Synthesis System for RT-PCR) were purchased from Invitrogen (Carlsbad, California, USA).

2.3. Ethanol exposure

Animals were introduced to the test aquariums (10 L) containing a solution of ethanol at 0.5% (v/v). The ethanol solution was replaced every two days, and the animals were maintained in the test aquarium for 7, 14, and 28 days. The chronic ethanol exposure has been based in previously studies, which was able to promote significant changes in locomotor activity and gene expression of zebrafish. (Gerlai et al., 2009; Pan et al., 2011). A preliminary ethanol assay by infrared analysis ensured that there were no alterations in ethanol concentration every 48 hours. Immediately after the exposure, the fish were cryoanesthesied and further decapitated for total brain isolation. The optic tectum was then removed and the experiments were subsequently performed.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

The expression of glutamate transporters was analyzed by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. Recently, the solute carriers (SLC) in zebrafish were identified (Gesemann et al., 2010; Rico et al., 2010). In this sense, a revision of the suggestive nomenclature was performed in order to establish a unification of among SLC and EAAT members (Neuhauss et al., 2010). To standardize the RNA extraction, all animals were euthanized at the same time of day (9:00−10:00 am). Total RNA from zebrafish optic tectum was isolated using the Trizol reagent (Invitrogen) in accordance with 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 Kit (Invitrogen, USA), following the supplier's instructions. The β-actin primers were used as described previously (Chen et

al, 2004). Primer sequences of EAAT-related genes were designed and RT-PCR conditions were chosen as described previously (Rico et al., 2010). The experimental conditions were optimized in order to determine the number of cycles that would allow product detection within the linear phase of band intensities analyzed. 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 employed 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.

2.5. Glutamate uptake

The optic tectum was removed and dissected into Petri dishes humidified with Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, pH 7.2. The structures were separated with the help of a magnifying glass and transferred to 24-well culture plates containing 0.5 mL of HBSS. One plate was maintained at 37° C and the other at 4° C. The structures from the first plate were washed once with 1.0 mL of 37° C HBSS and the second with 1.0 mL of ice-cold HBSS containing N-methyl-D-glucamine ($4°$ C) instead of sodium chloride. Brain structure was preincubated at $37°$ C for 15 min in 0.28 mL of HBSS. The uptake assay was carried out by adding 20 µL of a solution containing 0.33 µCi/mL L-[2, 3-3 H] glutamate with 100mM unlabeled glutamate at 37 °C during 7 min. The uptake was stopped with two ice-cold washes of 1.0 mL HBSS, immediately followed by the addition of 0.5 N NaOH. The samples were then kept overnight and aliquots of lysates were taken for determination of intracellular content of L-[2, 3-3 H]

glutamate by scintillation counting. The Na^+-independent uptake was determined by using an ice-cold (4° C) HBSS containing N-methyl-D-glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the Na^+-dependent uptake. Protein content was measured following the method described by Peterson (1977).

2.6. Glutamine synthetase assay

The enzyme assay was performed according to the method previously described (Petito et al., 1992). Briefly, optic tectum homogenate (75 µg protein) was added to 200 µL of reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole-HCl buffer (pH7,4); 10 2-mercaptoethanol; 50 hydroxylamine-HCl; 10 ATP and incubated for 45 min at 37 °C. The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): 370 ferric chloride, 670 HCl; 200 trichloroacetic acid. After centrifugation, the supernatant absorbance was measured at 530nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate, treated with ferric chloride reagent. Controls with the addition of the enzyme preparation after mixing with ferric chloride were used to correct non-enzymatic interference. Protein was measured by the Coomassie Blue method (Bradford, 1976) using bovine serum albumin as a standard. The linearity of absorbance towards time and protein concentration was previously determined. All enzyme assays were run in duplicate.

2.7. Glutaminase assay

The optic tectum was homogenized in 60 volumes (v/w) of chilled mannitol-sucrose-EDTA buffer MSTE (230mM-mannitol - 70mM-sucrose - 1mM-EDTA, pH7.4) in a motor driven Teflon-glass homogenizer. The samples were centrifuged at 600 x g for 10

min and the supernatant was kept at 4 ^{o}C. The resultant pellet was resuspended in MSTE buffer, and then centrifuged for 10 min at 600 x g. The resultant supernatant was added to supernatant obtained from first centrifugation and again centrifugated for 10 min at 8,000 x g. The final pellet was resuspended in Tris-HCl buffer (20 mM Tris, 140mM KCl, pH 7.4 frozen in liquid nitrogen and thawed for three times, and used in the enzyme assays. This freeze-thaw-wash procedure was used to ensure the lysis of the mitochondria preparation. All samples were maintained at 2–4 °C throughout preparation. After preparation of mitochondria, protein was measured by the method of Bradford (1976). Phosphate-activated glutaminase activity was measured either by following ammonia production or by measuring the glutamate formed by employing the glutamate dehydrogenase reaction (Ayoub et al., 1998). Glutaminase activity was determined as follows: mitochondrial solution (5 μg protein) was added to reaction medium containing 200 mM KH_2PO_4 (pH 7.4) in a final volume of 200 μL. The samples were preincubated for 10 min at 37 ^{o}C. The reaction was initiated by the addition of L-glutamine to a final concentration of 10 mM and stopped after 40 min by adding the samples on a 500 μL of phenol-nitroprusside reagent and measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported (Weisman et al., 1988). Controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used to correct non-enzymatic hydrolysis of glutamine. Samples were incubated at 37 °C for 15 min and the colorimetric assay was carried out at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity is expressed as nmol of NH_3 min^{-1} mg^{-1} of protein. All enzyme assays were run in duplicate.

2.8. Na,K-ATPase activity

For Na K-ATPase activity assay, the optic tectum were homogenized in 60 volumes (v/w) of chilled Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a Teflon-glass homogenizer. Brain membranes were prepared according described previously (Barnes et al., 1993). The reaction mixture for Na,K-ATPase activity assay contained (in mM): 5.0 $MgCl_2$, 80.0 NaCl, 20.0 KCl and 40.0 Tris–HCl with pH 7.4, in final volume of 200 μL. The brain fractions (3–5 μg protein) were preincubated for 10min at 37 °C. The reaction was initiated by addition of ATP and incubated during 5 min. The reaction was terminated by the addition of 200 μL 10% trichloroacetic acid and chilled on ice for 10 min. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na,K-ATPase activity was calculated by the difference between the two assays as previously described (Wyse et al., 1998). Released inorganic phosphate (Pi) was measured by the Colorimetric method (Chan et al., 1986). Specific enzyme activity was expressed as nanomoles of Pi released per minute per milligram of protein. All samples were run in triplicate.

2.9 Statistical analysis

All experiments were carried out in duplicate and means ± S.D. of at least three independent experiments are presented. Data were analyzed by one-way analysis of variance (ANOVA) and the post hoc analysis was performed through Duncan's multiple range test, considering a level of significance of 5%.

3. Results

The effect of chronic ethanol exposure (0.5% v/v) on the Na^+-dependent glutamate uptake in zebrafish optic tectum was evaluated after 7, 14, and 28 days. After 7 and 14 days of ethanol exposure, a significant decrease on glutamate uptake (30% and 54%, respectively) was observed (Fig.1A). However, 28 days of ethanol exposure did not modify the glutamate uptake. Considering that glutamate transporters are Na^+-dependent proteins that rely on sodium and potassium gradients generated principally by Na,K-ATPase, this enzyme activity was assessed after chronic ethanol exposure. The results showed that prolonged ethanol exposure did not alter Na,K-ATPase activity (Fig.1B).

To verify if chronic ethanol exposure modifies the transcriptional profile of glutamate transporters, we evaluated the gene expression pattern for EAAT-related genes (Fig.2). The results demonstrated a decrease on SLC1A1/EAAT3 transcript levels after 7 and 14 days of exposure. In contrast, mRNA transcript levels for SLC1A3a,b/EAAT1b increased after 28 days. Ethanol also produced an enhancement for SLC1A8a,b/EAAT6a,b after all exposure periods. No significant changes were detected for SLC1A3a,b/EAAT1a, SLC1A6/EAAT4, SLC1A2a,b/EAAT2a,b, and SLC1A7a,b/EAAT5a,b. Two important enzymes responsible to glutamate metabolism, PAG and GS were assessed in optic tectum of zebrafish. Preliminarily, incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. After chronic ethanol exposure, neither GS nor PAG did reveal significant changes in the enzyme activity (Table 1).

4. Discussion

Ethanol promotes several biochemical and physiological alterations on CNS, involving specific neurotransmitter systems (Esel, 2006). Among the multiple

neurotransmitters, glutamate is an excitatory neurotransmitter affected by ethanol intoxication (Diamond and Gordon, 1997). In the present study, we demonstrated that ethanol can alter the glutamate uptake and gene expression pattern of glutamate transporters in zebrafish optic tectum.

Considering the huge amounts of glutamate in the brain, besides its essential roles for the brain activity, the neurotransmissor glutamate may be potently toxic when present in high concentrations at synaptic cleft (Danbolt, 2001; Tzingounis and Wadiche, 2007; Lortet et al., 2008). Thus, the clearance of extracellular glutamate, mainly mediated by Na^+-dependent transport into astrocytes (Amara and Fontana, 2002; Sheldon and Robinson, 2007; Tzingounis and Wadiche, 2007), is an essential parameter involved in the physiologic/excitotoxic tonus of the glutamatergic system. Our results suggest that the inhibitory effect on Na^+-dependent glutamate observed after 7 and 14 days of ethanol exposure could induce an increase in the extracellular glutamate levels and a consequent hyperstimulation of glutamate receptors. Since it was not observed alterations on glutamate uptake after 28 days, we suggest that the functionality glutamate transport could be associated with the period of exposure.

The EAAT localized at the plasma membrane act by coupling an electrochemical gradient formed by Na,K-ATPase, allowing the transport of amino acids against their concentration gradient. The Na^+-dependent EAAT also depend on the intracellular potassium. In each cycle, at least one molecule of sodium is moved from the cytoplasm to the extracellular space and one molecule of potassium is moved in the opposite direction, with the glutamate transport coupled to sodium entry (Robinson and Dowd, 1997). In addition to the putative indirect reliance on Na,K-ATPase through ion gradients, previous studies has demonstrated the possibility of a more extensive link between glutamate

transporters and Na,K-ATPase (Rose et al., 2009). Our results showed that this enzyme was insensible after chronic ethanol exposure. Therefore, these results lead us to conclude that the inhibition of glutamate uptake after 7 and 14 days is independent of Na,K-ATPase activity.

Excitotoxic effects promoted by glutamate is involved in various acute and chronic neurological disorders (Maragakis and Rothstein, 2004; Maragakis and Rothstein, 2006; Struzynska, 2009). Regarding ethanol effects, it was documented that chronic exposure up-regulates the expression of NMDA-receptors number and function caused by its physiological response in cortical neurons (Chandler et al., 1993). Nonetheless, ethanol has also been reported to interact with glutamate uptake in different models. Our results suggest that the inhibitory effect on glutamate uptake observed after 7 and 14 days of ethanol exposure could impair the glutamatergic signaling in zebrafish optic tectum. These functional alterations in glutamatergic function could play a role in some neurotoxicological characteristics promoted by prolonged ethanol exposure.

EAATs belong to the solute carrier family 1 (SLC1), that constitute high affinity glutamate and neutral amino acid transporters. Recently, the phylogenetic analysis and cloning reporting of SLC1/EAAT genes from zebrafish identified distinct members of this transporter family (Rico, et al., 2010; Gesemann et al., 2010). In order to unify the nomenclature of SLC1/EAAT genes in zebrafish, we proposed a common nomenclature for these groups (Neuhauss et al., 2010). To verify if ethanol could modify EAATs at transcriptional level, the analysis of gene expression pattern of EAAT-related genes was conducted. The lower SLC1A1/EAAT3 gene expression detected could play a role, at least in part, in the alterations on glutamate uptake promoted by chronic ethanol exposure. On the other hand, the increases of SLC1A3a,b/EAAT1b and SLC1A8a,b/EAAT6a,b

demonstrate that each member of glutamate transporters gene expression show a characteristic response profile to ethanol. Chronic ethanol exposure promotes changes in the expression of a number of genes belonging to diverse functional groups (Liu et al., 2004; Mayfield et al., 2002). Moreover, there is growing evidence that alcoholism results from the interaction of genetic and environmental factors influencing both its expression and its course (Pinto and Ansseau, 2009). Recently, it was performed a global gene expression analysis in brain of zebrafish exposed chronically to ethanol in order to identify molecular putative targets (Pan et al., 2011). Besides, many SLC were verified as well as zinc, nucleoside, glucose, sodium/sulphate and taurine, but not glutamate transporters. Thus, our findings demonstrating the influence of ethanol exposure on the transcriptional profile of EAAT-related genes could be important to contribute in this way.

The glutamate-glutamine cycle has been the dominant paradigm for understanding the coordinated, compartmentalized activities of GS and PAG in support of functional glutamate trafficking *in vivo* (Maciejewski and Rothman, 2008). The coupling of GS and PAG is essential for glutamine homeostasis and neurotransmitter generation and recycling (Schousboe, 2003). To date, the activity of these enzymes has not previously been demonstrated in brain of adult zebrafish. This study is the first to access these important pathways that participate on glutamate glutamine cycle in zebrafish. The results showed that after chronic ethanol exposure, GS and PAG activity did not change, suggesting that ethanol promotes changes in the transport system of glutamate, but not in the enzymes involved in its metabolism.

5. Conclusions

These findings demonstrate for the first time the effects promoted by ethanol on parameters of glutamatergic system in zebrafish optic tectum. The changes induced by chronic ethanol exposure on functional and transcriptional profile of Na^+-dependent glutamate transporters suggest that the glutamatergic neurotransmission is an interesting target for potential pharmacological studies using zebrafish as a complementary vertebrate model. Our results also help to clarify the importance of neurochemical effects on glutamatergic tonus associated to alcohol consumption.

Conflict of interest

There are no competing interests.

Acknowledgements

References

Albrecht, J., Dolińska, M., Hilgier, W., Lipkowski, A.W., Nowacki, J. 2000. Modulation of glutamine uptake and phosphate-activated glutaminase activity in rat brain

mitochondria by amino acids and their synthetic analogues. Neurochem. Int. 36, 341-7.

Amara, S.G., Fontana, A.C. 2002. Excitatory amino acid transporters: keeping up with glutamate. Neurochem. Int. 41, 313-318.

Arriza, J.L., Eliasof, S., Kavanaugh, M.P., Amara, S.G. 1997. Excitatory amino acid transporter5, a retinal glutamate transporter coupled to a chloride conductance, Proc. Natl. Acad. Sci. 94, 4155–4160.

Ayoub, G.S., Grutsis, S., Simko, H. 1998. Imaging of endogenous neurotransmitter release. J. Neurosci. Methods. 81, 113-9.

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, 1351-1358.

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.

Bowman, T.V., Zon, L.I. 2010. Swimming into the future of drug discovery: in vivo chemical screens in zebrafish. ACS Chem Biol. 19, 159-61.

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.

Chan, K.M., Delfer, D., Junger K.D. 1986. A direct colorimetric assay for Ca2+-stimulated ATPase activity, Anal Biochem 157, 375–380.

Chandler, L.J., Newsom, H., Sumners, C., Crews, F. 1993. Chronic ethanol exposure potentiates NMDA excitotoxicity in cerebral cortical neurons. J. Neurochem. 60, 1578-81.

Chatterjee, D., Gerlai, R. 2009 High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. Behav. Brain. Res. 8, 200, 208-13.

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.

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

Dehnes, Y., Chaudhry, F.A., Ullensvang, K., Lehre, K.P., Storm-Mathisen, J., Danbolt, N.C. 1998. The glutamate transporter EAAT4 in rat cerebellar Purkinje cells: a glutamate-gated chloride channel concentrated near the synapse in parts of the dendritic membrane facing astroglia. J. Neurosci. 18, 3606–3619.

Diamond, I., Gordon, A.S. 1997. Cellular and molecular neuroscience of alcoholism. Physiol Rev. 77, 1-20.

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

Eliasof, S., Arriza, J.L., Leighton, B.H., Kavanaugh, M.P., Amara, S.G. 1998. Excitatory amino acid transporters of the salamander retina: identification, localization, and function. J Neurosci. 18, 698 –712.

Eşel, E. 2006. Neurobiology of alcohol withdrawal inhibitory and excitatory neurotransmitters. Turk Psikiyatri Derg. 17, 129-37.

Fairman, W.A., Vandenberg, R.J., Arriza, J.L., Kavanaugh, M.P., Amara, S.G. 1995. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. 375, 599–603.

Furuta, A., Martin, L.J., Lin, C.L., Dykes-Hoberg, M., Rothstein, J.D. 1997. Cellular and synaptic localization of the neuronal glutamate transporters excitatory amino acid transporter 3 and 4, Neuroscience. 81, 1031–1042.

Gahtan, E., Tanger, P., Baier, H. 2005. Visual prey capture in larval zebrafish is controlled by identified reticulospinal neurons downstream of the tectum. J. Neurosci 25, 9294–303.

Gerlai, R., Chatterjee, D., Pereira, T., Sawashima, T., Krishnannair, R. 2009. Acute and chronic alcohol dose: population differences in behavior and neurochemistry of zebrafish. Genes Brain Behav. 8, 586-99.

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.

Gesemann, M., Lesslauer, A., Maurer, C.M., Schönthaler, H.B., Neuhauss, S.C. 2010. Phylogenetic analysis of the vertebrate excitatory/neutral amino acid transporter (SLC1/EAAT) family reveals lineage specific subfamilies. BMC Evol. Biol.10, 117.

Holten, A.T., Gundersen, V. 2008. Glutamine as a precursor for transmitter glutamate, aspartate and GABA in the cerebellum: a role for phosphate-activated glutaminase. J. Neurochem. 104, 1032-42.

Kanai, Y., Hediger, M.A. 1992. Primary structure and functional characterization of a high-affinity glutamate transporter. 360, 467–471.

Kinoshita, M., Ito, E., 2006. Roles of periventricular neurons in retinotectal transmission in the optic tectum. Prog. Neurobiol. 79, 112–121.

Kvamme, E., Torgner, I.A., Roberg, B. 2001. Kinetics and localization of brain phosphate activated glutaminase. J Neurosci. Res. 66, 951-8.

Lhullier, F.L., Nicolaidis, R., Riera, N.G., Cipriani, F., Junqueira, D., Dahm, K.C., Brusque, A.M., Souza, D.O. 2004. Dehydroepiandrosterone increases synaptosomal glutamate release and improves the performance in inhibitory avoidance task. Pharmacol. Biochem. Behav. 77, 601-6.

Liu, J., Lewohl, J.M. Dodd, P.R. Randall, P.K. Harris, R.A. Mayfield, R.D. Gene expression profiling of individual cases reveals consistent transcriptional changes in alcoholic human brain, J. Neurochem. 90, 1050-1058.

Lortet, S., Canolle, B., Masmejean, F., Nieoullon, A. 2008. Plasma membrane expression of the neuronal glutamate transporter EAAC1 is regulated by glial factors: evidence for different regulatory pathways associated with neuronal maturation. Neurochem. Int. 52, 1373-1382.

Maciejewski, P.K., Rothman, D.L. 2008. Proposed Cycles for Functional Glutamate Trafficking in Synaptic Neurotransmission Neurochem Int. 52, 809–825.

Macnab, L.T., Pow, D.V. 2007. Expression of the exon 9-skipping form of EAAT2 in atrocytes of rats, Neuroscience. 150, 705–711.

Maragakis, N.J., Rothstein, J.D. 2004. Glutamate transporters: animal models to neurologic disease. Neurobiol. Dis. 15, 461-473.

Maragakis, N.J., Rothstein, J.D., 2006. Mechanisms of Disease: astrocytes in neurodegenerative disease. Nat. Clin. Pract. Neurol. 2, 679-689.

Mayfield, R.D., Lewohl, J.M. Dodd, P.R. Herlihy, A. Liu, J. Harris, R.A. 2002. Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. J. Neurochem. 81, 802-813.

Melendez, R.I., Hicks, M.P., Cagle, S.S., Kalivas, P.W. 2005. Ethanol exposure decreases glutamate uptake in the nucleus accumbens. Alcohol Clin. Exp. Res. 29, 326-33.

Moonat, S., Starkman B.G., Sakharkar A., Pandey S.C. 2010. Neuroscience of alcoholism: molecular and cellular mechanisms. Cell. Mol. Life Sci. 67, 73-88.

Neuhauss, S.C., Rico, E.P., Gesemann, M. Nomenclature of glutamate transporters in zebrafish and other vertebrates. Brain Res. Bull. 83, 297.

Nicolaidis, R., Bruno, A.N., Sarkis, J.J., Souza, D.O. 2005. Increase of adenine nucleotide hydrolysis in rat hippocampal slices after seizures induced by quinolinic acid. Neurocheml. Res. 30, 385-90.

Ninkovic, J., Bally-Cuif, L. 2006. The zebrafish as a model system for assessing the reinforcing properties of drugs of abuse. Methods. 39, 262-74.

Norenberg, M.D., Martinez-Hernandez, A. 1979. Fine structural localization of glutamine synthetase in astrocytes of rat brain. Brain Res. 161, 303-10.

Ozawa, S., Kamiya, H., Tsuzuki, K. 1998. Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol. 54, 581-618.

Pan, Y., Kaiguo, M., Razak, Z., Westwood, J.T., Gerlai, R. 2011. Chronic alcohol exposure induced gene expression changes in the zebrafish brain. Behav. Brain. Res. 216, 66-76.

Pandey, S.C. 2004. The gene transcription factor cyclic AMP-responsive element binding protein: role in positive and negative affective states of alcohol addiction. Pharmacol. Ther. 104, 47-58.

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83, 346-56.

Petito, C.K., Chung, M.C., Verkhovsky, L.M., Cooper, A.J. 1992. Brain glutamine synthetase increases following cerebral ischemia in the rat. Brain Res. 569, 275-80.

Pines, G., Danbolt, N.C., Bjoras, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E., Kanner, B.I. 1992. Cloning and expression of a rat brain l-glutamate transporter. Nature 360, 464–467.

Pinto, E., Ansseau M. 2009. Genetic factors of alcohol-dependence. Encephale. 35, 2009, 461-469.

Ramdya, P., Reiter, B., Engert, F. 2006. Reverse correlation of rapid calcium signals in the zebrafish optic tectum in vivo. J. Neurosci. Methods. 157, 230-7.

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. 2009. Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain. Brain Res. Bull. 81, 517-23.

Rico, E.P., de Oliveira, D.L., Rosemberg, D.B., Mussulini, B.H., Bonan, C.D., Dias, R.D., Wofchuk, S., Souza, D.O. 2010. Bogo MR.Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain. Brain Res. Bull. 81, 517-23.

Rico, E.P., de Oliveira, D.L., Rosemberg, D.B., Mussulini, B.H., Bonan, C.D., Dias, R.D., Wofchuk, S., Souza, D.O., Bogo, M.R. 2010. Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain. Brain Res. Bull. 81, 517-23.

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, 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, 290-6.

Robinson, M.B., Dowd, L.A. 1997. Heterogeneity and functional properties of subtypes of sodium-dependent glutamate transporters in the mammalian central nervous system. In: Adv. Pharmacol. 37, 69-113.

Rose, E.M., Koo, J.C., Antflick, J.E., Ahmed, S.M., Angers, S., Hampson, D.R. 2009. Glutamate transporter coupling to Na,K-ATPase. J. Neurosci. 29, 8143-55.

Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M.A., Wang, Y., Schielke, J.P., Welty, D.F. 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate, Neuron. 16, 675–686.

Scalzo, F.M., Levin, E.D., 2004. The use of zebrafish (Danio rerio) as a model system in neurobehavioral toxicology. Neurotoxicol. Teratol. 26, 707–708.

Schousboe, A. 2003. Role of astrocytes in themaintenance andmodulation of glutamatergic and GABAergic neurotransmission. Neurochem. Res. 28, 347–52.

Sheldon, A.L., Robinson, M.B. 2007. The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem. Int. 51, 333-355.

Smear, M.C., Tao, H.W., Staub, W., Orger, M.B., Gosse, N.J., Liu, Y., Takahashi, K., Poo, M.M., Baier, H. 2007. Vesicular glutamate transport at a central synapse limits the acuity of visual perception in zebrafish. Neuron. 53, 65-77.

Smith, T.L. 1997. Regulation of glutamate uptake in astrocytes continuously exposed to ethanol. Life Sci. 61, 2499-505.

Storck, T., Schulte, S., Hofmann, Stoffel, K. 1992. Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain. Proc. Natl. Acad. Sci. 89, 10955–10959.

Struzynska, L. 2009. A glutamatergic component of lead toxicity in adult brain: The role of astrocytic glutamate transporters. Neurochem. Int. 55, 151–156.

Takayasu, Y., Iino, M., Shimamoto, K., Tanaka, K., Ozawa, S. 2006. Glial glutamate transporters maintain one-to-one relationship at the climbing fiber-Purkinje cell synapse by preventing glutamate spillover. J Neurosci. 14, 6563-72.

Tilleux, S., Goursaud, S., Hermans, E., 2009. Selective up-regulation of GLT-1 in cultured astrocytes exposed to soluble mediators released by activated microglia, Neurochem. Int. 55, 35–40.

Tzingounis, A.V., Wadiche, J.I. 2007. Glutamate transporters: confining runaway excitation by shaping synaptic transmission. Nat. Rev. Neurosci. 12, 935-947.

Weisman, M.I., Caiolfa, V.R., Parola, A.H., 1988. Adenosine deaminase-complexing protein from bovine kidney. Isolation of two distinct subunits. J. Biol. Chem. 263, 5266–5270.

Wyse, A.T.S., Brusque, A.M., Silva, C.Q., Streck, E.L., Wajner M., Wannmacher, C.M.D. 1998. Inhibition of Na+, K+-ATPase from rat brain cortex by propionic acid, Neuroreport 9, 1719–1721.

Zon, L.I., Peterson, R.T., 2005. In vivo drug discovery in the zebrafish. Nat. Rev. Drug Discov. 4, 35–44.

Legend for Figures

Fig. 1: Effect of chronic ethanol exposure on Na^+-dependent glutamate uptake (A) and Na,K-ATPase activity (B) in zebrafish optic tectum. The data were expressed as means ± S.D. of different experiments ($n = 8$), each in duplicate. Data were analyzed by ANOVA followed by Duncan's post hoc test considering $P \leq 0.05$ as statistical significance. * Significantly different from control group.

Fig. 2: Transcriptional profile of EAAT-related genes in zebrafish optic tectum after chronic ethanol exposure. Band intensities were measured by optical densitometry (arbitrary units) for glutamate transporters compared to β-actin using the freeware ImageJ 1.37 for Windows. The results were expressed as means ± S.D. of four independent RT-PCR experiments performed in duplicate. Data were analyzed by ANOVA followed by Duncan's post hoc test considering $P \leq 0.05$ as statistical significance. * Significantly different from control.
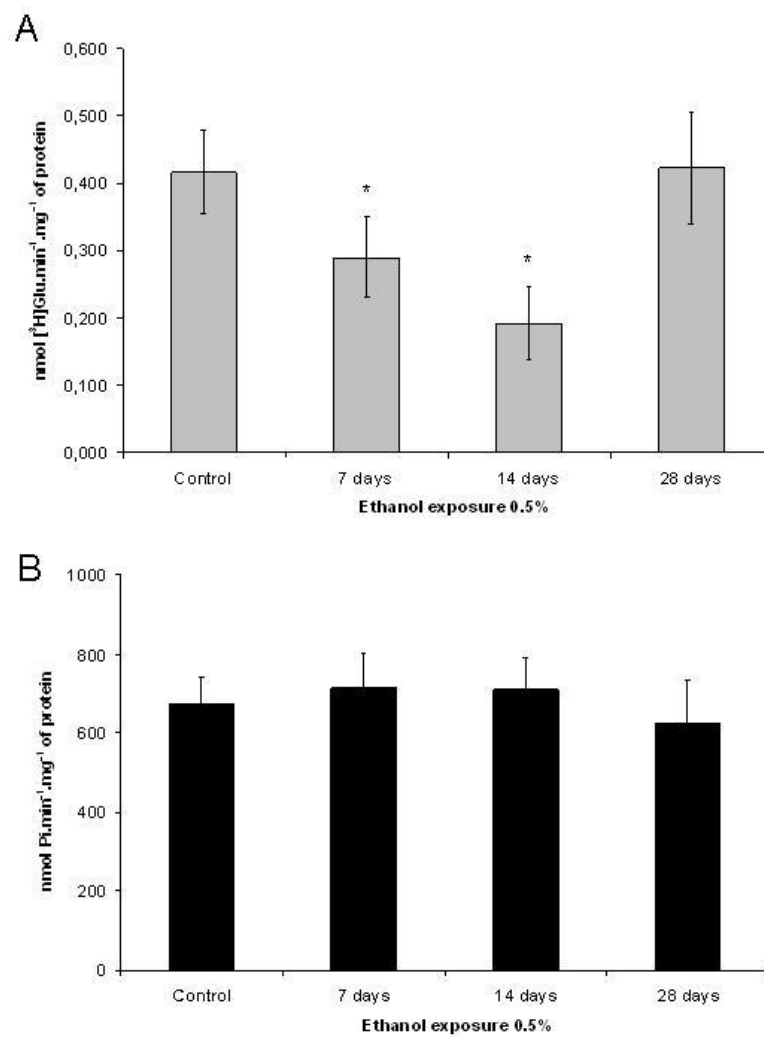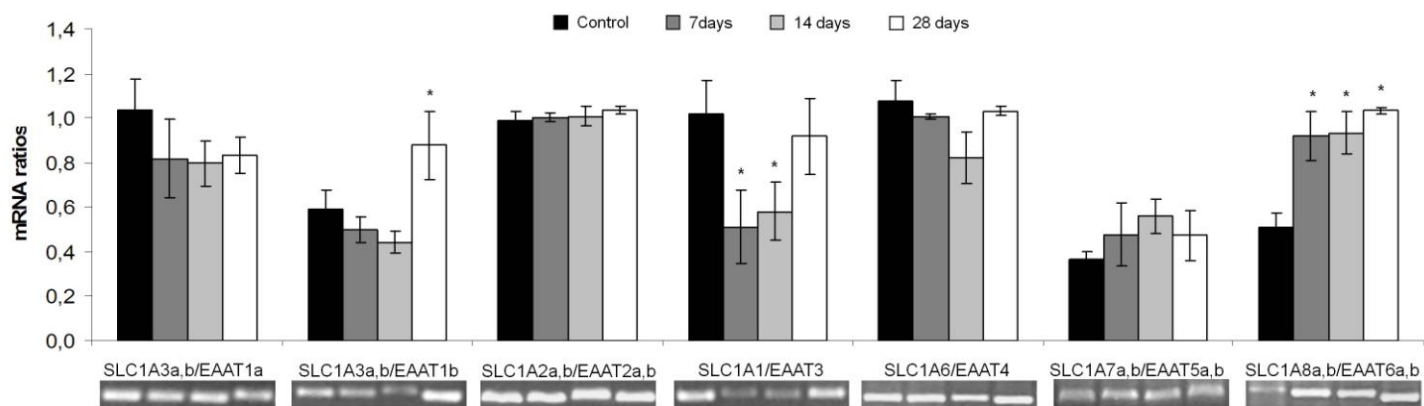
Figures:

Figure 1

Figure 2

Table 1: Effect of chronic ethanol exposure on GS and PAG activities in zebrafish brain.

Enzymes	Control	7 days	14 days	28 days
GS	155.21 ± 25.1	137.3 ± 26.7	122.7 ± 17.3	161.7 ± 15.8
PAG	51.8 ± 10.1	45.6 ± 7.3	42.8 ± 10.1	48.1 ± 11.3

The specific activities (means ± S.D.) of GS and PAG were expressed as γ-glutamylhydroxamate. $min^{-1}.mg^{-1}$ of protein and nmol of NH3 $.min^{-1}$ $.mg^{-1}$ of protein, respectively.

II.4. Capítulo 4

Tratamento crônico com etanol altera a hidrólise dos nucleotídeos das purinas e o padrão de expressão gênica em cérebro de zebrafish.

Artigo submetido ao periódico *Neurotoxicology*.

Chronic ethanol treatment alters purine nucleotide hydrolysis and nucleotidase gene expression pattern in zebrafish brain

Eduardo Pacheco Rico[a,b*], Denis Broock Rosemberg[a,b], Andrei da Silveira Langoni[c], André Arigony Souto[e], Renato Dutra Dias[c,f], Maurício Reis Bogo[d,f], Carla Denise Bonan[c,f*], Diogo Onofre Souza[a,b]

[a]*Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil;* [b]*Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção (INCT-EN), 90035-003, Porto Alegre, RS, Brazil;* [c]*Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia;* [d]*Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular,* [e]*Faculdade de Química,Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil;* [f]*Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003, Porto Alegre, RS, Brazil.*

*Corresponding authors:

Eduardo Pacheco Rico, Departamento de Bioquímica, ICBS, UFRGS. Rua Ramiro Barcelos 2600-Anexo. CEP: 90035-003. Porto Alegre, RS, Brazil. Tel.: +55 51 3316-5557; Fax: +55 51 3316-5540. e-mail: edurico@portoweb.com.br

Carla Denise Bonan, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil. Tel.: +55 51 3353-4158; Fax: +55 51 3320 3568. e-mail: cbonan@pucrs.br

Abstract

Ethanol is a widely consumed drug that acts on the central nervous system (CNS), modifying several signal transduction pathways activated by hormones and neurotransmitters. The zebrafish is an experimental model for the study of human diseases and the use of this species in biochemical and behavioral studies on alcoholism and alcohol-dependence has increased recently. However, there are no data concerning the effects of chronic ethanol exposure on the purinergic system, where extracellular nucleotides act as signaling molecules. Purinergic signaling is controlled by a group of enzymes named ectonucleotidases, which include NTPDases and ecto-5'-nucleotidase already characterized in zebrafish brain. The aim of this study was to evaluate nucleotide hydrolysis by NTPDases and ecto-5'-nucleotidase after long-term ethanol exposure. Additionally, the gene expression patterns of NTPDases1-3 and 5'-nucleotidase were determined. Animals were exposed to 0.5% ethanol for 7, 14, and 28 days. There were no significant changes in ATP and GTP hydrolysis after all treatments. However, a decrease in ADP (46% and 34%) and GDP (48% and 36%) hydrolysis was verified after 7 and 14 days, respectively. After 7 and 14 days of ethanol exposure, a significant decrease in AMP hydrolysis (48% and 36%) was also observed, whereas GMP hydrolysis was inhibited only after 7 days (46%). NTPDase2_mv and NTPDase3 mRNA transcript levels decreased after 7 and 14 days, respectively. In contrast, ethanol increased NTPDase1, NTPDase2_mq and NTPDase3 transcript levels after 28 days of exposure. NTPDase2_mg and 5'- nucleotidase gene expression was not altered. The ectonucleotidase pathway may be a target of chronic ethanol toxicity. Therefore, regulation of the purinergic system could play a key role in the neurochemical mechanisms underlying the effects of ethanol on the CNS.

Keywords: NTPDase; 5'- nucleotidase; ethanol; ectonucleotidases; zebrafish.

1. Introduction

Alcohol abuse and alcoholism involve interactions among a number of neural mechanisms, including acute sensitivity to alcohol, development of tolerance, and dependence. The psychotropic effects of alcohol are mediated by complex interactions with ion channel systems and neurotransmitters, e.g. GABA, glutamate, dopamine, and noradrenaline (Esel, 2006), leading to the typical behavioral effects on motor coordination, sensory perception and cognitive performance (Fleming et al., 2001). Ethanol is able to disrupt purinergic signaling by inducing changes in ATP-activated P2X receptor function (Franke and Illes, 2006) and also by increasing the extracellular adenosine levels (Mailliard and Diamond, 2004).

ATP is a well-known co-transmitter together with classical transmitters in most nerves in the peripheral and CNS, although concentrations vary according to the tissue and species and in different developmental and pathophysiological circumstances (Burnstock, 2009a; Zimmermann, 2008). The many effects of ATP as a neurotransmitter are mediated by a series of nucleotide-selective receptors collectively named purinoceptors: P2 receptors (sensitive to ATP and ADP), and P1 receptors (sensitive to adenosine) (Burnstock, 2009b). P2 receptors are divided in two main families known as P2X and P2Y, which are ligand-gated ion channels and G protein-coupled receptors, respectively (Greig et al., 2008). Seven subtypes of P2X receptors (Gever et al., 2006) and eight subtypes of P2Y receptors have been recognized so far (Burnstock, 2006). Adenosine can mediate different cellular functions by operating G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3), which can inhibit (A_1 and A_3) or facilitate (A_{2A} and A_{2B}) neuronal communication (Fredholm et al., 2001).

After release, 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 (Abbracchio et al., 2009). Thus, ectonucleotidases not only control the lifetime of nucleotide ligands but also produce ligands for additional P2 receptors and nucleosides by degrading or interconverting the originally released ligands (Zimmermann, 2006a). The ectonucleotidases include the ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase phosphodiesterases (E-NPPs), alkaline phosphatases and ecto-5´-nucleotidase (Yegutkin, 2008; Zimmermann, 2006a). Individual enzymes differ in substrate specificity and product formation. E-NTPDases and E-NPPs hydrolyze ATP and ADP to AMP, which is further hydrolyzed to adenosine by ecto-5'-nucleotidase. Alkaline phosphatases equally hydrolyze nucleoside tri, di and monophosphates. Four of the NTPDases are typical cell surface-located enzymes with their active site facing extracellularly (NTPDase1, 2, 3, 8). By contrast, NTPDases5 and 6 exhibit an intracellular localization and undergo secretion after heterologous expression. Meanwhile, NTPDases4 and 7 are located entirely intracellularly, facing the lumen of cytoplasmic organelles (Robson et al., 2006). ATP:ADP hydrolysis ratios for NTPDase1, 2, 3, and 8 are ~1-1.5:1, 10-40:1, 3-4:1 and 2:1, respectively (Kukulski et al., 2005; Yegutkin, 2008; Zimmermann, 2000).

Biochemical, molecular, and immunohistochemical studies have already described the ectonucleotidases in zebrafish (Rico et al., 2003; Ricatti et al., 2009; Senger et al., 2004). Bioinformatic and molecular studies reported and identified phylogenetically one ortholog gene for NTPDase1 (Senger et al., 2006), three paralog genes for NTPDase2 (Rico et al., 2006) and one gene for NTPDase3 (Appelbaum et al., 2007). Furthermore, the

cloning and characterization of two P2X receptor subunits from zebrafish have been described (Boué-Grabot et al., 2000; Kucenas et al., 2003). Recently, two zebrafish A_{2A} and one A_{2B} genes were identified in developing embryos, and their expression was demonstrated in the CNS (Boehmler et al., 2009).

As a model for use in neuroscience the zebrafish possesses numerous advantages including presenting a good balance between the simplicity and complexity of its organs and systems. This teleost is an excellent model organism for use in forward genetics, has a relatively short generation time, and its progeny numbers facilitate large scale screens. Reports have described the use of zebrafish to study the reinforcing effects of drugs of abuse, such as morphine (Bretaud et al., 2007), cocaine (Darland and Dowling, 2001), and ethanol (Ninkovic and Bally-Cuif, 2006), while the effects of acute ethanol treatment have been shown on zebrafish swimming behavior (Gerlai et al., 2000). Furthermore, the zebrafish has been adopted as a genetic system for large-scale screening (Lockwood et al., 2004), and for the identification of genes that regulate the sensitivity and resistance to alcohol (Carvan et al., 2004). The adaptation of adult zebrafish after chronic exposure to ethanol has been demonstrated, such that tolerance to the acute effects of the drug develops (Dlugos and Rabin, 2003; Gerlai et al., 2006).

Behavioral parameters have been studied in the range of 0.25 to 1%, indicating a U-shaped dose response curve. These findings suggested that alcohol has a facilitatory effect at lower and inhibitory effect at higher doses (Gerlai et al., 2000). Recently, it was demonstrated that different adult zebrafish strains were exposed to 0.5% ethanol for a period of 10 weeks to analyze swimming behavior (Dlugos et al., 2010). Furthermore, our group demonstrated changes in NTPDase activities and expression and observed that 0.5%

was the concentration that promoted the strongest inhibition on NTPDase activities after of acute ethanol exposure in zebrafish brain membranes (Rico et al., 2006).

The aim of this study was to evaluate changes in adenine and guanine nucleotide hydrolysis promoted by ectonucleotidases in zebrafish brain after long-term ethanol treatment, as well as to investigate the gene expression pattern of NTPDases (1, 2, and 3) and 5´-nucleotidase in these animals.

2. Material and Methods

2.1 Zebrafish maintenance

Adult zebrafish of both sexes were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in a 50 L thermostated aquarium filled with continuously aerated unchlorinated water with Aquasafe® (Tetra). The temperature was kept at 26 ± 2 °C under a 12-h light-dark controlled photoperiod, and the animals were fed twice a day until satiety with a commercial flake fish food (alcon BASIC®, Alcon, Brazil). The fish were used according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and the experiments were designed to minimize discomfort or suffering and also the number of fish used. The Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS) approved the protocol under license number 477/05—CEP.

2.2 Chemicals

Ethanol (C_2H_6O) was purchased from Merck (Darmstadt, Germany). Trizma base, malachite green, ammonium molybdate, polyvinyl alcohol, EDTA, EGTA, sodium citrate,

Coomassie Blue G, bovine serum albumin, calcium chloride, magnesium chloride, and nucleotides (ATP, GTP, ADP, GDP, AMP, and GMP) were purchased from Sigma (USA). All other reagents used were of analytical grade.

2.3 Ethanol treatment

For *in vivo* treatments, animals were introduced to the test aquariums (10 L) containing a solution of ethanol at 0.5% (v/v). The ethanol solution was replaced every two days, and the animals were maintained in the test aquarium for 7, 14, and 28 days. A preliminary ethanol assay by infrared analysis ensured that there was no alteration in ethanol concentration every 48 hours. Immediately after the exposure, the fish were euthanized and membrane preparations were obtained.

2.4 Membrane preparation

Brain membranes were prepared as described previously (Barnes et al., 1993). Zebrafish were cryoanaesthetized, then euthanized. Their brains were removed by dissection and briefly homogenized in 60 volumes (v/w) of chilled Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor driven Teflon-glass homogenizer. The samples were centrifuged at 1,000 x g for 10 min and the pellet was discarded. The supernatant was then centrifuged for 25 min at 40,000 x g. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris-citrate buffer, and centrifuged for 20 min at 40,000 x g. This freeze-thaw-wash procedure was used to ensure the lysis of the brain membranes. The final pellet was resuspended and used in the enzyme assays. All samples were maintained at 2–4 °C throughout preparation.

2.5 Nucleotide hydrolysis assay

The conditions for the NTPDase and 5′-nucleotidase assays have been described previously (Rico et al., 2003; Senger et al., 2004). Briefly, zebrafish brain membranes (3–10 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for NTPDase activity) or 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 and the reaction was initiated by the addition of substrate (ATP, ADP, AMP, GTP, GDP or GMP) to a final concentration of 1 mM. The reaction was stopped after 30 min by the addition of trichloroacetic acid in a final concentration of 5% and the samples were chilled on ice for 10 min. The inorganic phosphate (Pi) release was determined by adding 1 ml of a mixture containing 2.3% polyvinyl alcohol, 5.7% ammonium molybdate and 0.08% malachite green (Chan et al., 1986). Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct for nonenzymatic hydrolysis of the substrates. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity was expressed as nanomoles of Pi released per minute per milligram of protein. All enzyme assays were run in triplicate.

2.6 Protein determination

Protein was measured using Coomassie Blue as color reagent (Bradford, 1976) and bovine serum albumin as a standard.

2.7 RT-PCR experiments

The expressions of NTPDase 1, 2, 3, and 5′-nucleotidase were analyzed by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. The optimal conditions for primer annealing for NTPDase1, different NTPDases2 and 5′-nucleotidase were determined from information on GenBank and data previously published in the literature (Rico et al., 2008; Senger et al., 2006). RT-PCR conditions for NTPDase3 were optimized in order to determine the number of cycles that would allow product detection within the linear phase of mRNA transcripts amplification. The β-actin primers were designed as described previously (Chen et al., 2004) (see Table 1).

After chronic ethanol treatments, zebrafish brains was isolated for total RNA extraction using the TRIzol® reagent (Invitrogen) in accordance with the manufacturer's instructions. RNA was quantified by spectrophotometry and all samples were adjusted to 160 ng/µl. cDNA species were synthesized with the SuperScript™ First-Strand (Synthesis System for RT-PCR) Invitrogen Kit® following the suppliers' instructions. PCR reactions for different NTPDase2, NTPDase3, 5´-nucleotidase and β-actin genes were performed in a total volume of 20 µl, containing 0.1 µM primers (Table 1), 0.2 µM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA Polymerase® (Invitrogen). The PCR conditions for NTPDase1 were similar to those described above, except that 1.5 mM $MgCl_2$ was employed. The following conditions were used for the PCR reactions: 1 min at 94 °C, 1 min at the annealing temperature (Table 1), and 1 min at 72 °C for 35 cycles. Post-extension at 72 °C was performed for 10 min. For each set of PCR reactions a negative control was included. PCR products were analyzed on a 1% agarose gel containing GelRed® (Biotium) 10x, and visualized with ultraviolet light. The Low DNA Mass Ladder® (Invitrogen) was used as a molecular marker and PCR products were normalized by employing β-actin as a constitutive gene. The band intensities were measured by optical densitometry using the

freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the abundance of each mRNA compared to β-actin. Each experiment was repeated four times using RNA isolated from independent extractions. The expression analysis was performed in triplicate and representative data are shown.

2.8 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), being expressed as means ± S.D. Post-hoc analysis was performed through Duncan's multiple range test, considering a level of significance of 5%.

3. Results

In this study, we verified the effects of chronic ethanol treatment on ectonucleotidases (NTPDase and 5'-nucleotidase), responsible for regulating the extracellular concentrations of purine and pyrimidine nucleotides. To evaluate the *in vivo* effect of this alcohol on ectonucleotidase activities and gene expression patterns, animals were exposed to ethanol at a concentration of 0.5% (v/v) for 7, 14, and 28 days. ATP hydrolysis did not present significant changes after any of the time periods of ethanol exposure tested, whereas there was a significant decrease in ADP hydrolysis at 7 and 14 days 46%; p=0.001033 and 34%; p=0.004095, respectively (Fig. 1A). Similarly, GTP hydrolysis was not altered after these periods of exposure whereas there was an inhibition of GDP hydrolysis at 7 and 14 days 48%; p=0.000255 and 36%; p=0.00339, respectively) (Fig. 1B). Since the effects of ethanol could be a consequence of transcriptional control, RT-PCR analyses of nucleotidase gene expression were performed. The β-actin expression was normalized to allow for comparison in different experimental conditions. Chronic

exposure to ethanol for 28 days produced an increase in NTPDase1 (46%; $p=0.021523$) (Fig. 2A), NTPDase2_mq (47%; $p=0.027847$) (Fig. 2B), and NTPDase3 (45%; $p=0.013578$) (Fig. 2C) mRNA transcript levels. In contrast, NTPDase2_mv (24%; $p=0.022731$) and NTPDase3 (23%; $p=0.013578$) mRNA transcript levels exhibited a decrease at 7 and 14 days (Fig. 2B and C), respectively. Different periods of chronic ethanol exposure did not alter NTPDase2_mg gene expression (Fig. 2B). After 7 and 14 days of ethanol exposure we observed a significant decrease in AMP hydrolysis 48%; $p=0.001689$ and 36%; $p=0.00339$, respectively, whereas GMP hydrolysis was inhibited only after 7 days of treatment (46%; $p=0.000255$). Nevertheless, the RT-PCR analysis showed that 7-, 14-, and 28-day exposure to ethanol did not significantly alter 5′-nucleotidase gene expression.

4. Discussion

There is increasing awareness that purines and pyrimidines play important long-term roles in cell proliferation and growth (Burnstock, 2006), induction of apoptosis, and anticancer activity (White and Burnstock, 2006). In this study, we found that long-term ethanol exposure promoted significant changes in ectonucleotidase activities and gene expression demonstrating that this alcohol may be able to induce functional and transcriptional modulation of NTPDases and 5´-nucleotidase from zebrafish brain. Our results show that after 7 and 14 days of ethanol exposure ADP and GDP hydrolysis were significantly decreased, whereas there were no significant changes in ATP and GTP hydrolysis. Although these results seem controversial, previous study had already demonstrated that zebrafish present distinct NTPDase members that have a different expression profile within tissues (Rosemberg et al., 2010). Therefore, the differential

inhibitory effect observed for triphosphate and diphosphate nucleotide hydrolysis could be a consequence of the presence and different functionality of distinct NTPDase proteins in zebrafish CNS. The nucleosides adenosine and guanosine can be released *per se* or generated from nucleotides (ATP, ADP, AMP, GTP, GDP, GMP) that are metabolized by ectonucleotidases (Oses et al, 2007). Our results also showed that AMP hydrolysis in zebrafish brain membranes was decreased after 7- and 14- day ethanol exposure, while GMP hydrolysis was also decreased although only after 7 days. These effects suggest that prolonged ethanol treatment can modulate the activity of ecto-5´-nucleotidase, the rate limiting enzyme for extracellular adenosine and guanosine production. Adenosine and guanosine have been implicated in several extracellular roles, such as in protecting neurons against excitotoxic damage through different mechanisms (Oleskovicz et al., 2008; Cunha, 2005). It is also known that ethanol inhibits purine reuptake through the type I equilibrative nucleoside transporter (ENTI) (Choi et al., 2004; Newton and Messing, 2006). Therefore, the inhibitory influence exerted by ethanol on ectonucleotidases could be a compensatory mechanism to avoid a significant increase of adenosine levels, which could lead to the desensitization of adenosine receptors (Kiselevski et al., 2003). Here we demonstrated the inhibitory effect on NTPDase and 5´-nucleotidase activities in zebrafish brain after 7 and 14 days, but not 28 days. These alterations on nucleotide hydrolysis could be important to explain the functional action of ethanol and its tolerance over time on purinergic neurotransmission of zebrafish brain. Adenosine is a neuromodulator responsible to control the release of several neurotransmitters, including acetylcholine, serotonin, norepinephrine, dopamine, GABA, and glutamate (Dohrman et al., 1997). Furthermore, ectonucleotidases have an important regulatory mechanism that control external concentration of nucleotides and hence regulate P2-mediated signaling. Once the prolonged action of alcohol and their

tolerance about behavioral parameters have been studied in zebrafish, our findings could elucidate the importance of the purinergic system in chronic alcohol abuse.

Experimental evidence has shown that ethanol exerts its pharmacological effects by modulating the function of many membrane components, such as those linked to intracellular signal transduction pathways (Nagy, 2004). It has also been suggested that the lipid composition and the degree of ethanol influence on the physicochemical structure of the membrane may play a role in the modulation of membrane protein functions (Carrasco et al., 2007). A feature of the NTPDase family is that these proteins are firmly anchored to membranes (Zimmermann, 2006b), whereas 5´-nucleotidase is linked to the plasma membrane by a glycosylphosphatidylinositol anchor (Bianchi and Spychala, 2003). Thus, we can not exclude the possibility that the observed inhibition of NTPDase and 5´-nucleotidase could be due to an effect of ethanol on conformational protein structure, inducing functional alterations in these enzymes. Chronic and heavy alcohol abuse is marked by a number of biochemical and physiological changes in the CNS, such as (i) changes in intracellular signaling cascades including those containing cyclic adenosine 3´, 5´-monophosphate (cAMP)- dependent protein kinase A (PKA), protein kinase C (PKC), tyrosine kinase and phospholipase D (Newton and Messing, 2006); (ii) neuronal responses through the release of several hormones and neurotransmitters (Mailliard and Diamond, 2004); and (iii) enhancement of oxidative stress and lipid peroxidation through induction of free radical formation (Sun and Sun, 2001). The results of *in vivo* experiments suggest that ethanol could modulate ectonucleotidase activities indirectly, probably by affecting signal transduction pathways. In accordance with this hypothesis, it has already been reported that ethanol does not act directly on ectonucleotidase activities in zebrafish brain (Rico et al., 2008). Furthermore, the observed divergence between *in vitro* and *in vivo* effects on

nucleotide hydrolysis reinforces the idea that ethanol does not act directly on ectonucleotidase activities (Rico et al., 2008). Moreover, when considering the direct/indirect nature of its effects it is important to note that ethanol can be metabolized to acetaldehyde forming acetaldehyde adducts, which may be associated with brain and other organ damage (Niemela, 2001; Nakamura et al., 2003) or affect the activity of different neurotransmitter systems nonselectively (Vengeliene et al., 2008).

Although ethanol affects various biochemical processes such as neurotransmitter release, enzyme function, and ion channel kinetics, the specific molecular sites to which ethanol molecules bind to produce these myriad effects are not completely known (Harris et al., 2008). The sensitivity of the CNS to chronic alcohol administration leads to adaptive changes that are manifested as tolerance and physical dependence. The neuronal adaptations underlying these behavioral responses to ethanol exposure involve molecular mechanisms that are affected both directly and indirectly by ethanol (Lovinger and Crabbe, 2005). However, in the present study it was not possible to detect changes in nucleotide hydrolysis when zebrafish were exposed to ethanol for 28 days, while some effects were observed at 7 and 14 days.

Genomic studies have identified changes in the expression of a number of genes belonging to diverse functional groups after chronic ethanol exposure (Liu et al., 2004; Mayfield et al., 2002). In order to verify whether the NTPDase and 5´-nucleotidase genes were modulated when zebrafish were chronically exposed to ethanol, we performed RT-PCR experiments after 7, 14 and 28 days of treatment. We observed that each gene displays a specific profile of response according to the time of treatment. Interestingly, NTPDase1, NTPDase2_mq and NTPDase3 mRNA levels were significantly increased after 28 days of treatment, suggesting that the absence of effect on nucleotide hydrolysis observed with this

treatment is not directly related to a higher gene expression. Studies have shown that the most pronounced and consistent changes induced by ethanol were observed in gene families encoding mitochondrial proteins, as well as proteins involved in signal transduction and synaptic transmission (Damodaran et al., 2006). The identification of ethanol-sensitive genes is important for a complete understanding of its molecular effects (Liu et al., 2004; Mayfield et al., 2002; Sokolov et al., 2003).

Theories about vertebrate neural and behavioral basis sustain that brain evolution occurred in successive stages and have been conserved through phylogenesis. However, recent developmental, neuroanatomical and functional data indicate that the brain and behavioral evolution may have been more conservative than previously though (Salas et al., 2006). Furthermore, zebrafish has shown genetic and anatomic conservation with both mice and humans and a high degree of genetic homology Barbazuk et al., 2000; Dooley and Zon, 2000). Therefore, these alterations observed in purinergic system after chronic ethanol exposure could be important to clarify the basis of neurotransmission system since zebrafish appears to be an attractive organism for high throughput screening applications as well as mutagenesis screening, forward genetics or drug discovery efforts applied to neurotoxicity tests (Zon and Peterson, 2005; Peterson et al., 2008).

5. Conclusion

We demonstrated that prolonged ethanol exposure promotes changes in activity and gene expression in the enzyme pathway responsible for controlling extracellular nucleotide levels and, consequently, purinergic signaling. It is important to emphasize that the current report provides implications for future studies in relation to modeling the underlying mechanisms related to the alcohol-mediated responses and also to its potential toxicological

actions and tolerance in adult zebrafish. These results reinforce the idea that the zebrafish is an excellent animal model to investigate neurochemical and molecular mechanisms involved in regulating responses to ethanol.

Conflict of interest

There are no competing interests.

Acknowledgements

References

Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signalling in the nervous system: an overview. Trends Neurosci 2009;32:19-29.

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

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

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

Bianchi V, Spychala J. Mammalian 5'-nucleotidases. J Biol Chem 2003;278: 46195-98.

Boehmler W, Petko J, Woll M, Frey C, Thisse B, Thisse C, Canfield VA, Levenson R. Identification of zebrafish A2 adenosine receptors and expression in developing embryos. Gene Expr Patterns 2009;9:144-51.

Boué-Grabot E, Akimenko MA, Séguéla P. Unique functional properties of a sensory neuronal P2X ATP-gated channel from zebrafish. J Neurochem 2000;75: 1600-07.

Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:218–54.

Bretaud S, Li Q, Lockwood BL, Kobayashi K, Lin E, Guo S. A choice behavior for morphine reveals experience-dependent drug preference and underlying neural substrates in developing larval zebrafish. Neuroscience 2007;146:1109-16.

Burnstock G. Pathophysiology and therapeutic potential of purinergic signaling. Pharmacol Rev 2006;58:58–86.

Burnstock G. Purinergic cotransmission. Exp Physiol 2009a;94:20-4.

Burnstock G. Purinergic signalling: past, present and future. Braz J Med Biol Res 2009b;42:3-8.

Burnstock G. Purinergic signaling. Br J Pharmacol 2006;147:172-81.

Carrasco MP, Jiménez-López JM, Segovia JL, Marco C. Effects of ethanol on the remodeling of neutral lipids and phospholipids in brain mitochondria and microsomes. Neurochem Int 2007;50:858-65.

Carvan 3rd MJ, Loucks E, Weber DN, Williams FE. Ethanol effects on the developing zebrafish: neurobehavior and skeletal morphogenesis. Neurotoxicol Teratol 2004;26:757-68.

Chan K, Delfert D, Junguer KD. A direct colorimetric assay for Ca+2-ATPase activity. Anal Biochem 1986;157:375–80.

Chen WY, John JAC, Lin CH, Lin HF, Wu SC, Lin CH, Chang CY. Expression of metallothionein gene during embryonic and early larval development in zebrafish. Aquat Toxicol 2004;69:215–27.

Choi DS, Cascini MG, Mailliard W, Young H, Paredes P, McMahon T, Diamond I, Bonci A, Messing RO. The type 1 equilibrative nucleoside transporter regulates ethanol intoxication and preference. Nat Neurosci 2004;7:855–61.

Cunha RA. Neuroprotection by adenosine in the brain: From A1 receptor activation to A_{2A} receptor blockade. Purinergic Signal 2005;1:111-34.

Damodaran S, Dlugos CA, Wood TD, Rabin RA. Effects of chronic ethanol administration on brain protein levels: a proteomic investigation using 2-D DIGE system. Eur J Pharmacol 2006;547:75-82.

Darland T, Dowling JE. Behavioral screening for cocaine sensitivity in mutagenized zebrafish. Proc Natl Acad Sci U S A 2001;98:11691-6.

Dlugos CA, Rabin RA. Ethanol effects on three strains of zebrafish: model system for genetic investigations. Pharmacol Biochem Behav 2003;74:471-80.

Dohrman DP, Diamond I, Gordon AS. The role of the neuromodulator adenosine in alcohol's actions. Alcohol Health Res World. 1997; 21, 136-143.

Dooley K, Zon LI. Zebrafish: a model system for the study of human disease. Curr. Opin. Genet. Dev. 2000; 10:252-256.

Esel E. Neurobiology of alcohol withdrawal inhibitory and excitatory neurotransmitters. Turk Psikiyatri Derg 2006;17:129-37.

Fleming M, Mihic SJ, Harris RA. Ethanol. In: (Hardman, J.G., Limbird, L.E. Eds.), The Pharmacological Basis of Therapeutics Tenth ed. New York: McGraw-Hill, pp. 429–45; 2001.

Franke H, Illes P. Involvement of P2 receptors in the growth and survival of neurons in the CNS. Pharmacol Ther 2006;109:297-324.

Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology: XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 2001;53:527–52.

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, Lee V, Blaser R. Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (*Danio rerio*). Pharmacol Biochem Behav 2006; 85:752-61.

Gever J, Cockayne DA, Dillon MP. Pharmacology of P2X channels. Pflugers Arch 2006;452:513–37.

Greig AV, Linge C, Burnstock G. Purinergic receptors are part of a signalling system for proliferation and differentiation in distinct cell lineages in human anagen hair follicles. Purinergic Signal 2008;4:331-8.

Harris RA, Trudell JR, Mihic SJ. Ethanol's molecular targets. Sci Signal 2008;1: re7.

Kiselevski Y, Oganesian N, Zimatkin S, Szutowicz A, Angielski S, Niezabitowski P, Uracz W, Gryglewski RJ. Acetate metabolism in brain mechanisms of adaptation to ethanol. Med Sci Monit 2003;9:178–82.

Kucenas S, Li Z, Cox JA, Egan TM, Voigt MM. Molecular characterization of the zebrafish P2X receptor subunit gene family. Neuroscience 2003;121:935-45.

Kukulski F, Lévesque SA, Lavoie EG, Lecka J, Bigonnesse F, Knowles AF, Robson SC, Kirley TL, Sévigny J. Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. Purinergic Signal 2005;1:193-204.

Liu J, Lewohl JM, Dodd PR, Randall PK, Harris RA, Mayfield RD. Gene expression profiling of individual cases reveals consistent transcriptional changes in alcoholic human brain. J Neurochem 2004;90:1050-8.

Lockwood B, Bjerke S, Kobayashi K, Guo S. Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening. Pharmacol Biochem Behav 2004;77:647-54.

Lovinger DM, Crabbe JC. Laboratory models of alcoholism: treatment target identification and insight into mechanisms. Nat Neurosci 2005;8:1471-80.

Mailliard WS, Diamond I. Recent advances in the neurobiology of alcoholism: the role of adenosine. Pharmacol Ther 2004;101:39-46.

Mayfield RD, Lewohl JM, Dodd PR, Herlihy A, Liu J, Harris RA. Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. J Neurochem 2002;81:802-13.

Nagy LE. Molecular aspects of alcohol metabolism: transcription factors involved in early ethanol-induced liver injury. Annu Rev Nutr 2004;24:55-78.

Newton PM, Messing RO. Intracellular signaling pathways that regulate behavioral responses to ethanol. Pharmacol Ther 2006;109:227–37.

Nakamura K, Iwahashi K, Furukawa A, Ameno K, Kinoshita H, Ijiri I, Sekine Y, Suzuki I, Iwata Y, Minabe Y, Mori N. Acetaldehyde adducts in the brain of alcoholics. Arch Toxicol 2003;77:591-3.

Niemela O. Distribution of ethanol-induced protein adducts in vivo: relationship to tissue injury. Free Radic Biol Med 2001;31:1533-8.

Ninkovic J, Bally-Cuif L. The zebrafish as a model system for assessing the reinforcing properties of drugs of abuse. Methods 2006;39:262-74.

Oleskovicz SP, Martins WC, Leal RB, Tasca CI. Mechanism of guanosine-induced neuroprotection in rat hippocampal slices submitted to oxygen-glucose deprivation. Neurochem Int 2008;52:411-8.

Oses JP, Viola GG, de Paula Cognato G, Júnior VH, Hansel G, Böhmer AE, Leke R, Bruno AN, Bonan CD, Bogo MR, Portela LV, Souza DO, Sarkis JJ. Pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid. Epilepsy Res 2007;75:104-11.

Peterson RT, Nass R, Boyd WA, Freedman JH, Dong K, Narahashi T. Use of non-mammalian alternative models for neurotoxicological study. Neurotoxicology, 2008; 29:546-555.

Ricatti MJ, Alfie LD, Lavoie EG, Sévigny J, Schwarzbaum PJ, Faillace MP. Immunocytochemical localization of NTPDases1 and 2 in the neural retina of mouse and zebrafish. Synapse 2009;63:291-307.

Rico EP, Rosemberg DB, Senger MR, de B. Arizi M, Bernardi GF, Dias RD, Bogo MR, Bonan CD. Methanol alters ectonucleotidases and acetylcholinesterase in zebrafish brain. Neurotoxicol Teratol 2006;28:489-96.

Rico EP, Rosemberg DB, Senger MR, de Bem Arizi M, Dias RD, Souto AA, Bogo MR, Bonan CD. Ethanol and acetaldehyde alter NTPDase and 5'-nucleotidase from zebrafish brain membranes. Neurochem Int 2008;52:290-6.

Rico EP, Senger MR, da G. Fauth M, Dias RD, Bogo MR, Bonan CD ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*). Life Sci 2003;73: 2071-82.

Robson SC, Sevigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signal 2006;2:409–30.

Rosemberg DB, Rico EP, Langoni AS, Spinelli JT, Pereira TC, Dias RD, Souza DO, Bonan CD, Bogo MR. NTPDase family in zebrafish: Nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart. Comp Biochem Physiol B Biochem Mol Biol 2010; 3:230-240.

Salas C, Broglio C, Durán E, Gómez A, Ocaña FM, Jiménez-Moya F, Rodríguez F. Neuropsychology of learning and memory in teleost fish. Zebrafish. 2006;3: 157-171.

Senger MR, Rico EP, de Bem Arizi M, Frazzon AP, Dias RD, Bogo MR, Bonan CD. Exposure to Hg2+ and Pb2+ changes NTPDase and ecto-5'-nucleotidase activities in central nervous system of zebrafish (*Danio rerio*). Toxicology 2006;226: 229-37.

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;139:203-7.

Sokolov BP, Jiang L, Trivedi NS, Aston C. Transcription profiling reveals mitochondrial, ubiquitin and signaling systems abnormalities in postmortem brains from subjects with a history of alcohol abuse or dependence. Neurosci Res 2003;72: 756-67.

Sun AY, Sun GY. Ethanol and oxidative mechanisms in the brain. J Biomed Sci 2001;8:37–43.

Vengeliene V, Bilbao A, Molander A, Spanagel R Neuropharmacology of alcohol addiction. Br J Pharmacol 2008;154:299-315.

White N, Burnstock G. P2 receptors and cancer. Trends Pharmacol Sci 2006;27: 211-7.

Yegutkin GG. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochim Biophys Acta 2008;1783:673-94.

Zimmermann H. ATP and acetylcholine, equal brethren. Neurochem Int 2008;52: 634-48.

Zimmermann H. Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch Pharmacol 2000;326:299-309.

Zimmermann H. Ectonucleotidases in the nervous system. Novartis Found Symp 2006a;276:113-28.

Zimmermann H. Nucleotide signaling in nervous system development. Pflugers Arch 2006b;452:573-88.

Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. Nat. Rev. Drug Discov. 2005; 4: 35-44.

Legend of Figures

Fig.1: Effect of different time exposures to ethanol on ATP and ADP (A) and GTP and GDP (B) hydrolysis in zebrafish brain membranes. Bars represent the mean±S.D. of at least five different experiments. Control specific activities for ATP, ADP, GTP and GDP

hydrolysis were 536.44±52.22, 182.05±29.96, 158.80±29.67 and 35.93±6.23 nmol Pi min^{-1}.mg^{-1} of protein, respectively. Data were analyzed by ANOVA followed by Duncan's post hoc test (P≤0.05, when compared to control group). *significantly different from control.

Fig. 2: Gene expression patterns in zebrafish brain after treatment with ethanol. The band intensities were measured by optical densitometry for NTPDase1 (A) NTPDase2_mg, NTPDase2_mq, NTPDase2_mv (B), and NTPDase3 (C) using the freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the abundance of each mRNA compared to β-actin. The results were expressed as mean±S.D. of optical densitometry arbitrary units of four independent replicate RT-PCR experiments. Data were analyzed by ANOVA followed by Duncan's post hoc test (P≤0.05, when compared to control group). *significantly different from control.

Fig. 3: Effect of different time exposures to ethanol on AMP and GMP (A) hydrolysis in zebrafish brain membranes. Bars represent the mean±S.D. of at least five different experiments. Control specific activities for AMP and GMP hydrolysis were 36.76±9.06 and 27.26±3.36 nmol Pi min^{-1}.mg^{-1} of protein, respectively. (B) represents the gene expression patterns of 5´-nucleotidase after treatment with ethanol in zebrafish brain. Data were analyzed by ANOVA followed by Duncan's post hoc test (P≤0.05, when compared to control group). *significantly different from control.
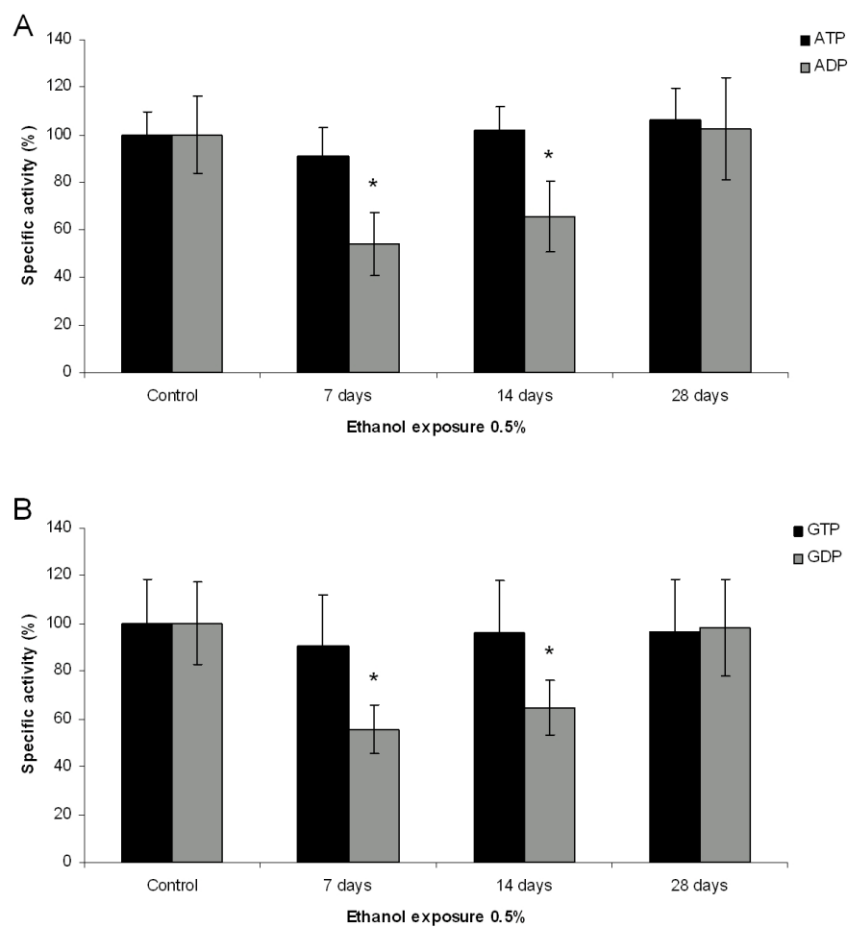
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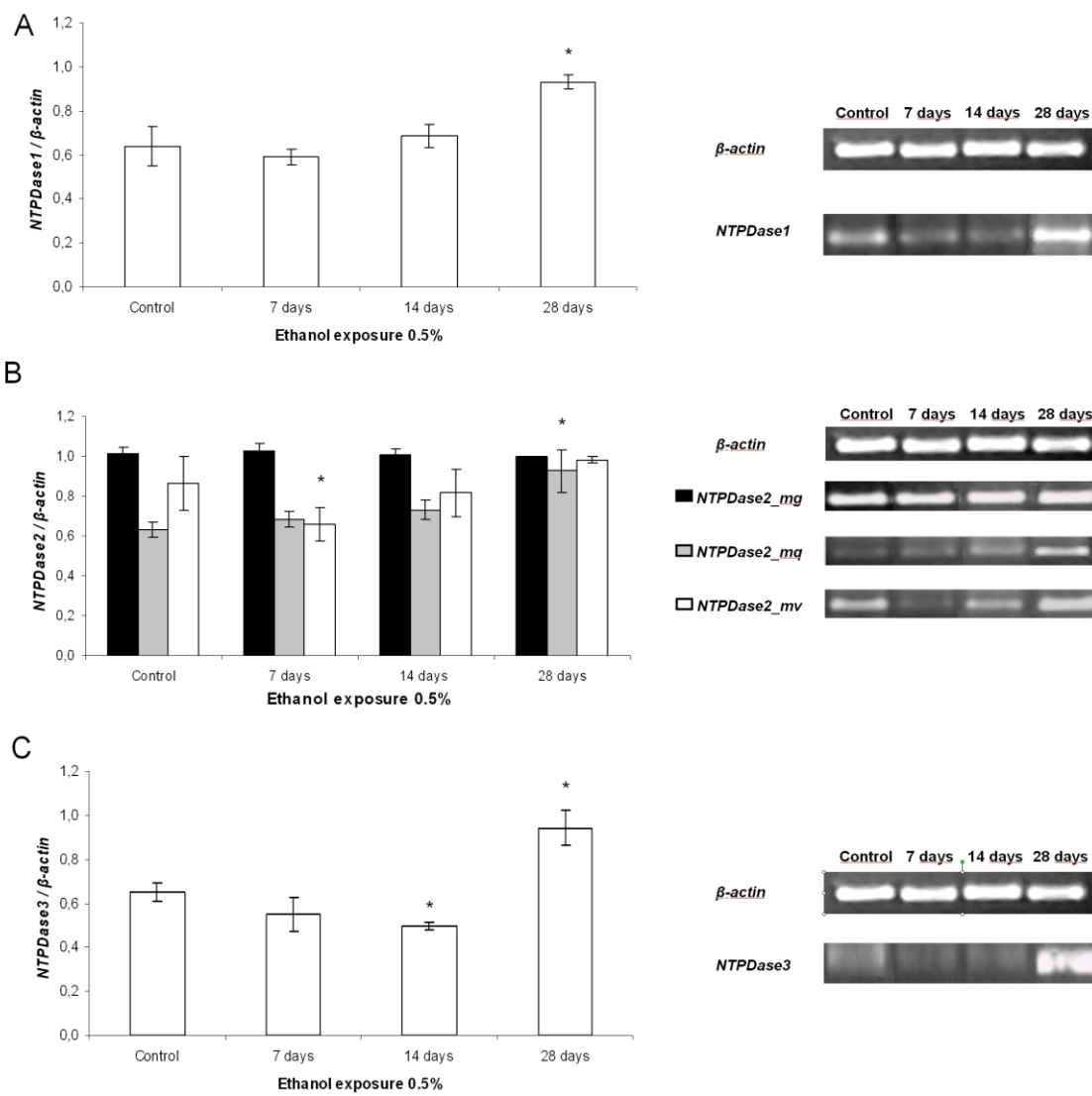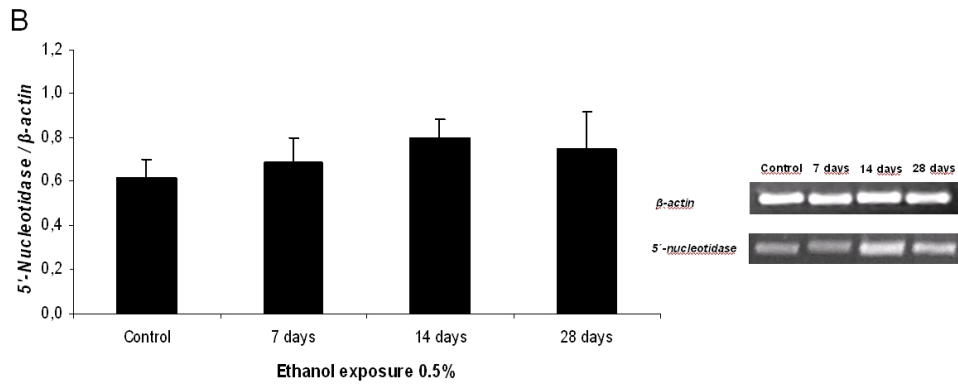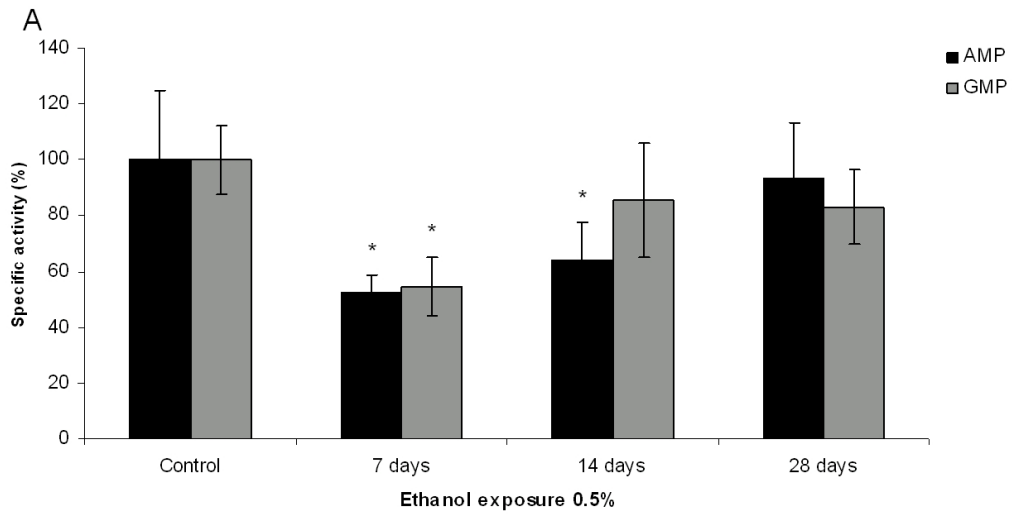
Figure 1

Figure 2

Figure 3

Table 1 : PCR primer design

Enzymes	Sequences (5'-3')	Annealing temperature (ºC)	PCR product (i)	GenBank Accession number	ZFIN ID (ZDB-GENE)
NTPDase1	CCCATGGCACAGGCCGGTTG (forward)				
	GCAGTCTCATGCCAGCCGTG (reverse)	54	380	AAH78240	040801-58
NTPDase2_mg*	GGAAGTGTTTGACTCGCCTTGCACG (forward)				
	CAGGACACAAGCCCTTCCGGATC (reverse)	64	554	XP_697600	---
NTPDase2_mq*	CCAGCGGATTTAGAGCACGCTG (forward)				
	GAAGAACGGCGGCACGCCAC (reverse)	64	313	XP_687722	040724-67
NTPDase2_mv*	GCTCATTTAGAGGACGCTGCTCGTG (forward)				
	GCAACGTTTTCGGCAGGCAGC (reverse)	64	263	AAH78419	040724-187
NTPDase3	TACTTTCTTTGGACAGAGCAACCCTG (forward)				
	AAGCATATAGCCCAGGGACCAGG (reverse)	62	424	ABR15509	030131-6186
5´-nucleotidase	ACCTCCGAGGAGTGTCGCTTTCG (forward)				
	CCTTGTTGGGGACCAGCGGTTC (reverse)	54	433	NP_957226	040426-1261
β-actin	GTCCCTGTACGCCTCTGGTCG (forward)				
	GCCGGACTCATCGTACTCCTG (reverse)	54	678	AAC13314	000329-1

* Correspond to the two first amino acids residues of the protein sequence.

II.5. Capítulo 5

Atividade e padrão de expressão gênica da adenosina deaminase após a exposição crônica ao etanol em cérebro de zebrafish. Artigo submetido ao periódico *Alcohol*.

Adenosine deaminase activity and gene expression patterns are altered after chronic ethanol exposure in zebrafish brain

Eduardo Pacheco Rico[a,b*], Andrei da Silveira Langoni[c], Denis Broock Rosemberg[a,b], André Arigony Souto[e], Renato Dutra Dias[c,f], Maurício Reis Bogo[d,f], Carla Denise Bonan[c,f*], Diogo Onofre Souza[a,b]

[a]*Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil;* [b]*Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção (INCT-EM), 90035-003, Porto Alegre, RS, Brazil;* [c]*Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia;* [d]*Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular,* [e]*Faculdade de Química, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil;* [f]*Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003, Porto Alegre, RS, Brazil.*

*Corresponding author:

Eduardo Pacheco Rico, Departamento de Bioquímica, ICBS, UFRGS. Rua Ramiro Barcelos 2600-Anexo. CEP: 90035-003. Porto Alegre, RS, Brazil. Tel.: +55 51 3316-5557; Fax: +55 51 3316-5540. E-mail: edurico@portoweb.com.br

Abstract

Ethanol alters the homeostasis between excitatory and inhibitory neurotransmitters and its intoxication reveals adenosine as responsible to modify several responses including signal transduction. Zebrafish has been recently investigated for knowledge the prolonged effect of ethanol on behavioral and biochemical parameters. The aim of this study was to evaluate the soluble and membrane adenosine deaminase activities and gene expression in zebrafish brain. Animals were exposed to 0.5% ethanol for 7, 14, and 28 days. There were no significant changes in ADA activity from soluble fraction after all treatments. However, we verified a decrease of ADA activity in membrane fraction after 28 days (44%) of ethanol exposure. *ADA1* was not altered whereas mRNA transcript levels for *ADAL* presented an increase after 28 days of ethanol exposure (34%). *ADA2-1* showed a decrease (26%) followed by an increase (17%) of transcripts after 14 and 28 days of ethanol exposure, respectively. However, *ADA2-1* truncated alternative splice ignalin *(ADA2-1/T)* demonstrated a reduction after 28 days (20%). *ADA2-2* was decreased (22%) followed by an increase (109%) of transcripts after 14 and 18 days of ethanol exposure, respectively. Altogether, the purine catabolism promoted by ADA may be an important target of the chronic toxicity induced for ethanol.

Keywords: adenosine deaminase; ethanol; chronic; zebrafish.

Abbreviations: ADA: adenosine deaminase; CNS: central nervous system; GABA gamma-aminobutyric acid.

Introduction

Chronic and excessive ethanol consumption is associated with various biochemical and physiological changes in CNS. Some of these changes are pertaining to alteration of specific neurotransmitter systems (Chandler et al., 1997) and signaling pathways (Hoek and Kholodenko, 1998). Besides GABA, glutamate, dopamine, and noradrenaline, ethanol acts on purinergic signaling changing P2X receptor function (Franke and Illes, 2006) and also the adenosine levels (Mailliard and Diamond, 2004). The purinergic signaling involves the important roles of nucleotides and nucleosides in CNS. After released, ATP is catabolized to adenosine via ectonucleotidase pathway, such as nucleotide pyrophosphatase/phosphodiesterases (NPP), nucleoside triphosphate diphosphohydrolases (NTPDases), and 5′-nucleotidase, or it can be released from any cell when the intracellular concentration rises (Fredholm, 2002; Yegutkin, 2008). Extracellular adenosine acts as a neuromodulator in the CNS (Ralevic and Burnstock, 1998; Burnstock, 2006) and can mediate different cellular functions by operating G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3), which can inhibit (A_1 and A_3) or facilitate (A_{2A} and A_{2B}) neuronal communication (Burnstock, 2007.

Adenosine deaminase (ADA, EC 3.5.4.4) is an enzyme in the purine catabolic pathway that catalyses the conversion of adenosine and deoxyadenosine into inosine and deoxyinosine respectively. This enzyme can also be considered as an ecto-enzyme since it is found on the surface membranes of many cells (Franco et al., 1997; Kanbak et al., 2008). Adenosine is involved in several acute and chronic effects of ethanol (Dar et al., 1983; Newton and Messing, 2006). In mammalian brains, adenosine deaminase activity is located mainly in the cytosol, but the presence of ecto-adenosine deaminase has been also

established on the surface of synaptosomes and neurons by activity assays and immunohistochemistry (Richardson et al., 1987; Ruiz et al., 2000).

Since the introduction of zebrafish into the laboratory, many milestones have been achieved that firmly establish this specie as a prominent genetic model organism for biology and medicine. Many properties make zebrafish an organism of easy maintenance which provides advantages to understand the molecular and cellular mechanisms of behavior and behavioral disorders. Forward genetic studies in zebrafish (Dooley and Zon, 2000; Shin and Fishman, 2002) represent an important complementary approach to uncover novel molecular mechanisms underlying behavioral sensitivity to ethanol. While high-throughput behavioral assays for ethanol sensitivity have been established (Lockwood et al., 2004), it has been difficult to recover behavioral mutants or underlying molecular lesions.

Studies from our laboratory demonstrated the presence of NTPDase (Rico et al., 2003), 5′-nucleotidase (Senger et al., 2004), and adenosine deaminase (Rosemberg et al., 2008) activities in zebrafish brain. Besides, we have shown that exposure to ethanol changes NTPDase and ecto-5′-nucleotidase activities in the CNS of this animal model (Rico et al., 2008). Our group also reported the differential expression pattern of ADA-related genes in zebrafish tissues, including brain, confirming that these genes (*ADA1, ADAL, ADA2-1,* and *ADA2-2*) are present in this species (Rosemberg et al., 2007a). Recently, two zebrafish A_{2A} and one A_{2B} genes were identified in developing embryos, and their expression was demonstrated in the CNS (Boehmler et al., 2009).

Therefore, considering that adenosine levels can be associated with neurological effects promoted by ethanol and that ADA is responsible for controlling adenosine levels, in the present study we investigated the influence of chronic ethanol exposure on ADA

activity in zebrafish brain. Additionally, the gene expression patterns of ADA-related genes were determined in these animals.

Material and Methods

Zebrafish maintenance

Adult zebrafish of both sexes were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in a 50 L thermostated aquarium filled with continuously aerated unchlorinated water with Aquasafe® (Tetra). The temperature was kept at 26 ± 2 °C under a 14/10-h light-dark controlled photoperiod, and the animals were fed with commercial fish pellet twice a day. The fish were used in accordance to the National Institutes of Health Guide for Care and Use of Laboratory Animals and the experiments were designed to minimize discomfort or suffering and also the number of fish used.

Chemicals

Ethanol (C_2H_6O; CAS number 64-17-5) was purchased from Merck (Darmstadt, Germany). Adenosine, EGTA, EDTA, Coomassie Blue G, and bovine serum albumin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phenol and sodium nitroprusside were purchased from Merck (Darmstadt, Germany). All reagents used were of high analytical grade.

Ethanol treatment

For *in vivo* treatments, animals were introduced to the test aquariums (10 L) containing a solution of ethanol at 0.5% (v/v). The ethanol solution was replaced every two days, and the animals were maintained in the test aquarium for 7, 14, and 28 days. A preliminary ethanol assay by infrared analysis ensured that there was no alteration in

ethanol concentration every 48 hours. Immediately after the exposure, the fish were euthanized and brain membranes were prepared.

Preparation of soluble and membrane fractions

Zebrafish were cryoanesthetized and immediately euthanized by decapitation. Whole brains were initially homogenized in 20 volumes (v/w) of chilled phosphate buffered saline (PBS) 2 mM EDTA, 2 mM EGTA, pH 7.4, in a glass-Teflon homogenizer in order to obtain both cellular fractions. Brain membranes were prepared according to a method described previously (Barnes et al., 1993) with minor modifications. The homogenate was centrifuged at $800 \times g$ for 10 min and the pellet was discarded. After removing the nuclear and cell debris, the supernatant was centrifuged for 25 min at $40\,000 \times g$. The resulting supernatant and pellet corresponded to the soluble and membrane fractions, respectively. The supernatant was collected and kept on ice for enzyme assays. The pellet was frozen in liquid nitrogen, thawed, resuspended in PBS once and centrifuged for 20 min at $40\,000 \times g$. This freeze–thaw-wash procedure was used to ensure the lysis of the brain membranes vesicles. The final pellet was resuspended and used for biochemical assays. The material was maintained at 2–4 °C throughout preparation.

Determination of adenosine deaminase activity

Adenosine deaminase activity was determined using a Berthelot reaction as previously reported (Weisman et al., 1988; Rosemberg et al., 2008). The brain fractions (5–10 μg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for the assays with soluble and membrane fractions, respectively, in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C, and the reaction was initiated by the addition of adenosine to a final concentration of 1.5 mM. The reaction was stopped by the addition of

500 μL of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL) after incubation for 75 min (soluble fraction) or 120 min (membrane fraction). Controls with the addition of the enzyme preparation after mixing with the phenol-nitroprusside reagent were used to correct for non-enzymatic hydrolysis of substrates. The reaction mixtures were immediately added to 500 μL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed. Samples were incubated at 37 °C for 15 min and the ammonia produced was quantified by a colorimetric assay at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity was expressed as nmol of NH_3 min^{-1} mg $protein^{-1}$.

Protein determination

Protein was measured using Coomassie Blue as the color reagent (Bradford, 1976). A protein curve was prepared using bovine serum albumin as standard (Stoscheck, 1990).

Reverse transcription-polymerase chain reaction (RT-PCR)

The expression of ADA-related genes *ADA1, ADA2-1, ADA2-2*, and *ADAL* was analyzed by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. To standardize the RNA extraction, all animals were euthanized at the same time of day (9:00–10:00 am). Total RNA from zebrafish brain was isolated using the Trizol reagent (Invitrogen) in accordance with 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 Kit (Invitrogen, USA), following the supplier's instructions. The *β-actin* primers were used as described

previously (Chen et al, 2004). Primer sequences of ADA-related genes were designed and RT-PCR conditions were chosen as described previously (Rosemberg et al., 2007b). The experimental conditions were optimized in order to determine the number of cycles that would allow product detection within the linear phase of band intensities analyzed. 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 employed 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.

Statistical analysis

All experiments were carried out in duplicate and means ± S.D. of at least three independent experiments are presented. Data were analyzed by one-way analysis of variance (ANOVA) and the post hoc Tukey's test was employed where results achieved significance. *P*-Values ≤0.05 were considered as significant.

Results

The results obtained for ADA activity after chronic ethanol exposure in zebrafish brain are presented in Figure 1. As can be observed, in soluble fractions, ADA activity of zebrafish exposed for 7, 14 and 28 days did not significantly change as compared to the control group (Fig.1A). However, in membrane fractions, after 28 days of ethanol exposure it was possible to observe a decrease of ADA activity (44%), while 7 and 14 days of ethanol exposure did not promote significant changes in ADA activity (Fig.1B).

In order to verify whether the ADA-related genes could be modulated when zebrafish were exposed to chronic ethanol, we have performed semi-quantitative RT-PCR

experiments after 7, 14 and 28 days. Ethanol exposure modified the gene expression pattern of ADA-related genes in zebrafish brain (Fig. 2). *ADA1* transcripts were not altered in all times of ethanol exposure in zebrafish brain (Fig. 2A). However, *ADAL* presented an increase in the level of transcripts after 28 days of ethanol exposure (34%), while that 7 and 14 days did not induce significant changes (Fig. 2B). Considering the member related to ADA2-1, it was previously identified an *ADA2-1* truncated alternative splice   ignalin *(ADA2-1/T)*, which was expressed at different intensities (Rosemberg et al., 2007b). There were not alterations of *ADA2-1T* after ethanol exposure for 7 and 14 days, while transcript levels demonstrated a reduction after 28 days (20%). Interestingly, *ADA2-1* showed a decrease (26%) of transcripts followed by an increase of transcripts (17%) after 14 and 28 days of ethanol exposure, respectively (Fig.2C). Similarly to *ADA2-1*, *ADA2-2* demonstrated a decrease (22%) of transcripts followed by a strong increase of transcripts (109%) after 14 and 28 days of ethanol exposure, respectively (Fig2D).

Discussion

The chronic ethanol consumption is associated with neurochemical changes in the CNS. Ethanol interferes with the function of adenosinergic system, and may mediate some effects of ethanol, such as intoxication, motor coordination and sedation (Dohrman et al., 1997). Furthermore, studies have shown that extracellular concentrations of adenosine may also be regulated by ecto-ADA activity (Franco et al., 1998; Romanowska et al., 2007). In this study, we found an inhibition of ADA activitiy in membrane fractions of zebrafish brain exposed to 28 days to ethanol exposure. Differently, we did not observe significant alterations in soluble fractions. Ethanol has an aliphatic moiety and provides a lipophilic group that can interact with non-polar domains of macromolecules. This signal-chemical

property governs the forces of interaction of ethanol with biological substrates (Fadda and Rossetti, 1998). Although ethanol can disturb the natural thermal balance that maintains membrane architecture and can alter membrane microdomains that determine protein-membrane and protein-ligand interactions (Wang et al., 1993). Furthermore, studies demonstrate points to a specificity of action of ethanol directly on membrane proteins producing on formational changes that alter their function (Dickinson et al., 1993; Li et al., 1994; Lovinger, 1997).

Ethanol can affect these systems directly, and/or the interactions among these systems become important in the actions promoted by ethanol. It was demonstrated that acetaldehyde and acetate play a key role in the brain mediating some actions of ethanol (Israel et al., 1994; Deitrich, 2004). Furthermore, it is well established that acetaldehyde mediates the toxic effects of ethanol, and studies were aimed at unraveling its effects in pathological conditions (Quertemont et al, 2005). According to this knowledge, we may not discard the involvement of these metabolites in promoting alterations on ADA activity after chronic ethanol exposure.

Ethanol stimulates cAMP signaling through at least two mechanisms. The first mechanism has been demonstrated through the inhibition of the type I equilibrative nucleoside transporter (ENT 1), which leads to accumulation of extracellular adenosine (Choi et al, 2004). The second involves metabolism of ethanol by liver generating acetate, which is converted to Acetyl-CoA, a process that requires ATP and yields AMP (Carmichael et al., 2001). This AMP is converted to adenosine by the 5´-nucleotidase (Bianchi et al., 2003), leading to an increase of adenosine levels. In this study we demonstrated an inhibition on ADA activity from membrane fractions after 28 days of ethanol exposure. Since it has been demonstrated the presence of ADA activity in the brain

membranes suggesting the existence of an ecto-ADA in zebrafish, ADA activity could be another important pathway to study the control of extracellular adenosine levels.

Ethanol has been proposed to stimulate adenosine A_{2A} receptors by inhibition of (ENT1) in culture dNG108-15 cells (Nagy et al., 1990). Since A_{2A} receptors are coupled to G protein coupled receptors (G_S-coupled), this increases levels of intracellular cAMP and stimulates protein kinase A (PKA) (Mailliard and Diamond, 2004). This activation of PKA permits to phosphorylate substrates. Therefore, we suggest that the inhibition observed adenosine deamination after 28 days of ethanol exposure from membrane fractions could be related, at least in part, to the modulation of this intracellular pathway.

Precisely, genetics is a promising way benefiting from many advances in genetic epidemiology, cellular and molecular biology, neuroimaging and pharmacology. In parallel with a better understanding of the neurobiology of addictions and associated behaviors, these techniques led to the identification of brain mechanisms in which a genetic variation may influence the individual vulnerability towards alcohol dependence. Moreover, there is growing evidence that alcoholism results from the interaction of genetic and environmental factors influencing both its expression and its course. (Pinto and Ansseau. 2009). Studies have previously demonstrated that chronic ethanol exposure promoted changes in the expression of a number of genes belonging to diverse functional groups (Liu et al., 2004; Mayfield et al., 2002). In order to verify whether chronic ethanol exposure could affect ADA-releated genes of zebrafish at transcriptional level, we performed RT-PCR analysis. We observed that each gene displays a specific profile of response according to the time of ethanol exposure. Considering these differences observed, we suggest that each gene could be modulated by independent mechanisms. Thus, the effects observed in all ADA-related genes, could represent the scenario of expression of members of the ADA family for each

period of exposure. However, the mechanisms involved in this modulatory effect of ethanol on ADA transcripts from zebrafish brain still require further investigations.

Taken together, this study demonstrated that ethanol exposure chronically inhibited ADA activity in zebrafish brain, suggesting that adenosine/inosine levels could be altered by this alcohol. Besides, we showed that ethanol induces alterations of ADA-related genes expression profile. Our results could help to clarify the importance of neurochemical effects on control of adenosine levels associated to ethanol consumption.

Conflict of interest

There are no competing interests.

References

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.

Bianchi, V., Spychala, J. (2003). Mammalian 5´-nucleotidases. J. Biol. Chem. *278*, 46195–46198.

Boehmler, W., Petko, J., Woll, M., Frey, C., Thisse, B., Thisse, C., Canfield, V.A., Levenson, R. (2009). Identification of zebrafish A2 adenosine receptors and expression in developing embryos. Gene Expr. Patterns. *9*, 144-151.

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. (2006). Purinergic signalling- an overview. Novartis Found. Symp. *276*, 26-48.

Burnstock, G. (2007). Purine and pyrimidine receptors. Cell. Mol. Life. Sci. *64*, 1471–1483.

Carmichael, F.J., Israel, Y., Crawford, M., Minhas, K., Saldivia, V., Sandrin, S., Campisi, P., Orrego, H. (1991). Central nervous system effects of acetate: contribution to the central effects of ethanol. J. Pharmacol. Exp. Ther. *259*, 403-408.

Choi, D.S., Cascini, M.G., Mailliard, W., Young, H., Paredes, P., McMahon, T., Diamond, I., Bonci, A., Messing, R.O. (2004). The type 1 equilibrative nucleoside transporter regulates ethanol intoxication and preference. Nat. Neurosci. *7*, 855–861.

Chandler, L.J., Sutton, G., Norwood, D., Sumners, C., Crews, F.T. (1997). Chronic ethanol increases N-methyl-D-aspartate-stimulated nitric oxide formation but not receptor density in cultured cortical neurons. Mol. Pharmacol. *51*, 733-40.

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

Dar, M.S., Mustafa, S.J., Wooles, W.R. (1983). Possible role of adenosine in the CNS effects of ethanol. Life Sci. *33*, 1363-1374.

Deitrich, R.A. (2004). Acetaldehyde : deja vu du jour. J. Stud. Alcohol. *65*, 557–572.

Dickinson, R., Franks, N.P., Lieb, W.R. (1993). Thermodynamics of anesthetic/protein interaction. Temperature studies on firefly luciferase. Biophys. J. *64*, 1254-1271.

Dohrman, D.P., Diamond, I., Gordon, A.S. (1997). The role of the neuromodulator adenosine in alcohol's actions. Alcohol Health Res World. *21*, 136-143.

Dooley, K., Zon, L.I. (2000). Zebrafish: a model system for the study of human disease. Curr. Opin. Genet. Dev. *10*, 252–256.

Fadda, F., Rossetti, Z.L. (1998). Chronic ethanol consumption: from neuroadaptation to neurodegeneration. Prog. Neurobiol. *56*. 385-431.

Franco, R., Casadó, V., Ciruela, F., Saura, C., Mallol, J., Canela, E.I., Lluis, C. (1997). Cell surface adenosine deaminase: much more than an ectoenzyme, Prog. Neurobiol. *52*, 283-294.

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

Franke, H., Illes, P. (2006). Involvement of P2 receptors in the growth and survival of neurons in the CNS. Pharmacol. Ther. *109*, 297-324.

Fredholm, B.B. (2002). Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ. *14*, 1315–1323.

Hoek, J.B., Kholodenko, B.N. (1998). The intracellular signaling network as a target for ethanol. Alcohol Clin. Exp. Res. *22*, 224S-230S.

Israel, Y., Orrego, H., Carmichael, F.J. (1994). Acetate-mediated effects of ethanol, Alcohol Clin. Exp. Res. *18*, 144–148.

Kanbak, G., Arslan, O.C., Dokumacioglu, A., Kartkaya, K., Inal, M.E. (2008). Effects of chronic ethanol consumption on brain synaptosomes and protective role of betaine. Neurochem. Res. *33*, 539-544.

Li, C., Peoples, R.W., Weight, F.F. (1994). Alcohol action on a neuronal membrane receptor: evidence for a direct interaction with the receptor protein. Proc. Natl. Acad. Sci. U.S.A. *91*, 8200-8204.

Liu, J., Lewohl, J.M., Dodd, P.R., Randall, P.K., Harris, R.A., Mayfield, R.D. (2004). Gene expression profiling of individual cases reveals consistent transcriptional changes in alcoholic human brain. J. Neurochem. *90*, 1050-1058.

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

Lovinger, D.M. (1997). Alcohols and neurotransmitter gated ion channels: past, present and future. Naunyn-Schmiedeberg's Arch. Pharmacol. *356*, 267-282.

Mailliard, W.S., Diamond, I. (2004). Recent advances in the neurobiology of alcoholism: the role of adenosine. Pharmacol Ther. *101*, 39-46.

Mayfield, R.D., Lewohl, J.M., Dodd, P.R., Herlihy, A., Liu, J., Harris, R.A. (2002). Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. J. Neurochem. *81*, 802-813.

Nagy, L.E., Diamond, I., Casso, D.J., Franklin, C., Gordon, A.S. (1990). Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. J. Biol. Chem. *265*, 1946-1951.

Newton, P.M., Messing, R.O. (2006). Intracellular signaling pathways that regulate behavioral responses to ethanol. Pharmacol. Ther. *109*, 227-237.

Pinto, E., Ansseau, M. (2009). Genetic factors of alcohol-dependence. Encephale. *35*, 461-469.

Quertemont, E., Tambour, S., Tirelli, E. (2005). The role of acetaldehyde in the neurobehavioral effects of ethanol: a comprehensive review of animal studies. Prog. Neurobiol. *75*, 247–274.

Ralevic, V., Burnstock, G. (1998). Receptors for purines and pyrimidines. Pharmacol. Rev. *50*, 413-492.

Richardson, P.J., Brown, S.J. Bailyes, E.M. Luzio, J.P. (1987). Ectoenzymes control adenosine modulation of immunoisolated cholinergic synapses. Nature. *327*, 232–234.

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.

Rico, E.P., Senger, M.R., Fauth, M. da 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.

Romanowska, M., Ostrowska, M., Komoszyński, M.A. (2007). Adenosine ecto-deaminase (ecto-ADA) from porcine cerebral cortex synaptic membrane. Brain Res. *1156*, 1–8.

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*, 96-101.

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*, 1526-1534.

Rosemberg, D.B., Rico, E.P., Senger, M.R., Arizi, M.B., Dias, R.D., Bogo, M.R. (2007b). Acute and subchronic copper treatments alter extracellular nucleotide hydrolysis in zebrafish brain membranes. Toxicology *236*, 132–139.

Ruiz, M.A., Escriche, M., Lluis, C., Franco, R., Martin, M., Andres, A., Ros, M. (2000). Adenosine A1 receptor in cultured neurons from rat cerebral cortex: colocalization with adenosine deaminase. J. Neurochem. *75*, 656–664.

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.

Shin, J.T., Fishman, M.C. (2002). From zebrafish to human: modular medical models. Annu. Rev. Genomics. Hum. Genet. *3*, 311–340.

Stoscheck, C.M. (1990). Quantitation of protein. Methods Enzymol. *182*, 50–68.

Wang, D.C., Taraschi, T.F., Rubin, E., Janes, N. (1993). Configurational entropy is the driving force of ethanol action on membrane architecture. Biochim. Biophys. Acta. *1145*, 141- 148.

Weisman, M.I., Caiolfa, V.R., Parola, A.H. (1988). Adenosine deaminase-complexing protein from bovine kidney. Isolation of two distinct subunits. J. Biol. Chem. *263*, 5266–5270.

Yegutkin, G. (2008). Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochim. Biophys. Acta. *1783*, 673–694.

Figures

Fig.1: Effect of different time exposures to ethanol on adenosine hydrolysis by soluble (A) and membrane (B) preparations from zebrafish brain. The enzyme activity was determined as described in Material and methods. Bars represent the mean ± S.D. of at least five different experiments. Specific activities were expressed as nmol NH_3 $min^{-1}.mg^{-1}$ of protein, respectively. Data were analyzed by ANOVA followed by Duncan's post hoc test ($P \leq 0.05$, when compared to control group). *significantly different from control.

Fig. 2: Gene expression patterns in zebrafish brain after treatment with ethanol. The band intensities were measured by optical densitometry for *ADA1* (A), *ADAL* (B), *ADA2-1* (C), and *ADA2-2* (D) using the freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the abundance of each mRNA compared to β-actin. The results were expressed as mean ± S.D. of optical densitometry arbitrary units of four independent replicate RT-PCR experiments. Data were analyzed by ANOVA followed by Duncan's post hoc test ($P \leq 0.05$, when compared to control group). *significantly different from control.
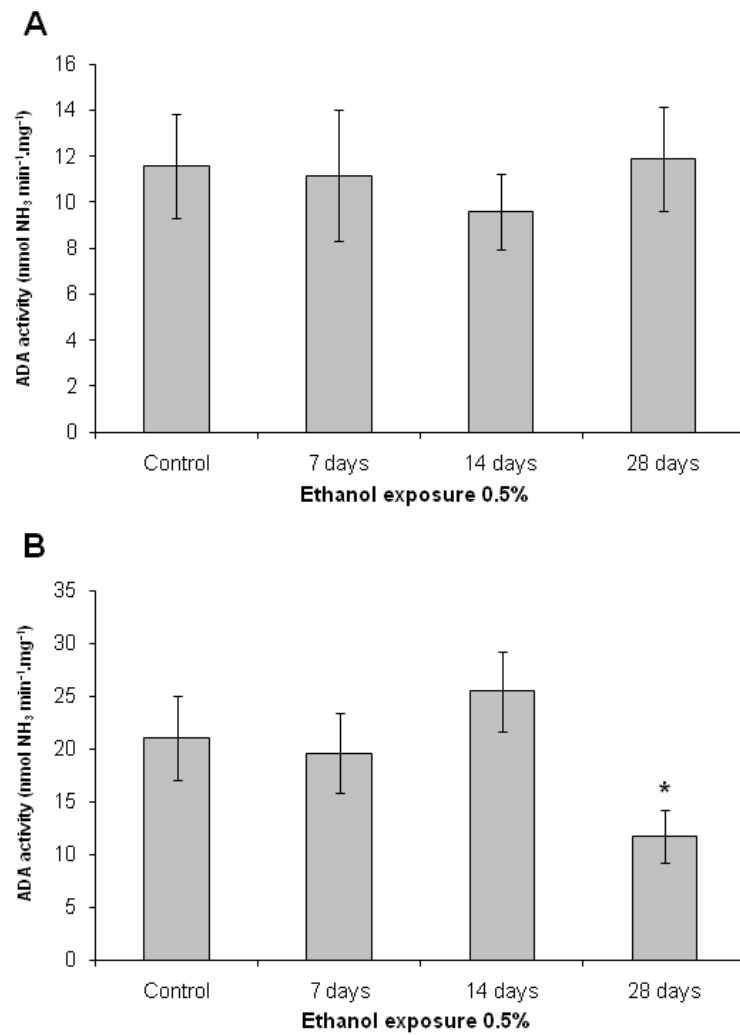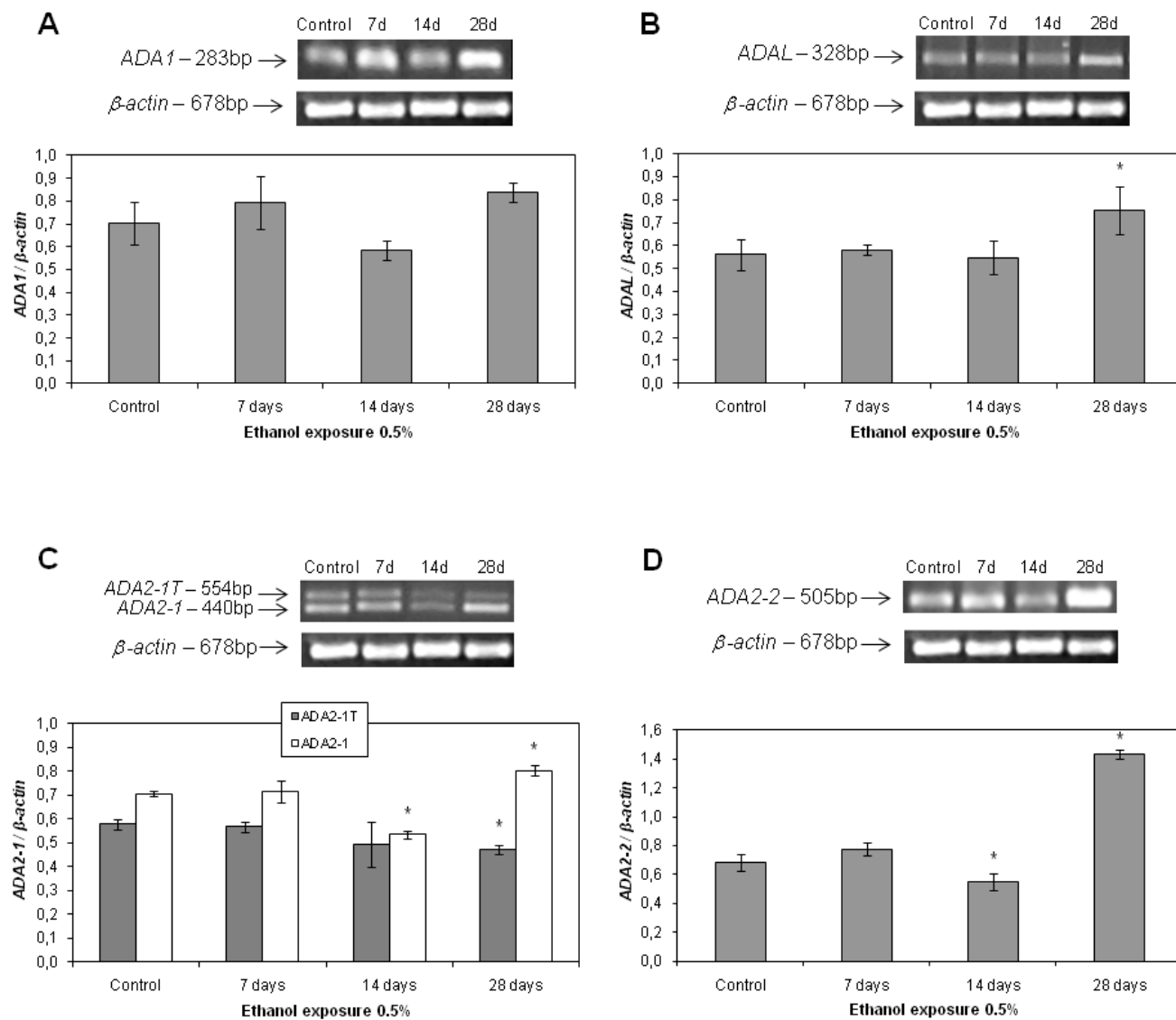
Figures:

Figure 1

Figure 2

II.6. Capítulo 6

Exposição crônica ao etanol aumenta os níveis de BDNF em cérebro de zebrafish.

Artigo submetido ao periódico *Archives of Biochemstry and Biophysics*.

Chronic ethanol exposure increases BDNF levels in zebrafish brain

Eduardo Pacheco Rico[a,b*], Denis Broock Rosemberg[a,b], Caroline Brunetto de Farias[g], Pedro Guilherme Shefer[h], Jonathan Tesch Spinelli[d], Talita Carneiro Brandão Pereira[d], André Arigony Souto[e], Carla Denise Bonan[c,f], Temis Reverbel da Silveira[h], Rafael Roesler[g], Maurício Reis Bogo[c,f*], Diogo Onofre Souza[a,b]

[a] *Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil;*

[b] *Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção (INCT-EM), 90035-003, Porto Alegre, RS, Brazil;*

[c] *Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia; Departamento de Biologia Celular e Molecular; Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil;*

[d] *Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular; Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil;*

[e] *Faculdade de Química, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil;*

[f] *Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003, Porto Alegre, RS, Brazil.*

[g]*Cancer Research Laboratory, Academic Hospital Research Center, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.*

[h] *Experimental Hepatology Laboratory, Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil.*

* Addresses for correspondence:

Eduardo Pacheco Rico, Departamento de Bioquímica, ICBS, UFRGS. Rua Ramiro Barcelos 2600 anexo. CEP: 90035-003. Porto Alegre, RS, BRAZIL. Tel: +55 51 3316-5557. Fax: +55 51 3316-5540. E-mail: edurico@portoweb.com.br;

Maurício Reis Bogo, Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681. CEP: 90619-900. Porto Alegre, RS, Brazil. Tel: +55 51 3320 3500x4726. Fax: +55 51 3320 3568. E-mail: mbogo@pucrs.br

Abstract

Alcohol consumption is one of the biggest and costliest diseases whose mechanisms are not fully understood. Most research has focused on ethanol modulation of cell surface signaling involving neurotransmitters, modulators and trophic signaling. Brain-derived neurotrophic factor (BDNF) is a widely expressed neurotrophin that modulates both short and long-term synaptic plasticity. However, there are no studies about the effect of chronic consumption of ethanol on the BDNF levels in the brain of zebrafish model. In the present study, we examined the effects of 7, 14 and 28 days of ethanol exposure in mRNA expression and protein levels of BDNF and its high affinity receptor, trkB, in adult zebrafish brain. After 14 and 28 days, ethanol increased BDNF mRNA levels, whereas gene expression of trkB was not altered. The measurement of BDNF protein through ELISA kit anti-BDNF showed an increase after 28 days of exposure (51%), which was also confirmed by immunohistochemstry. Altogether, our results demonstrate that ethanol increases BDNF gene and protein expression in zebrafish brain, suggesting that the homeostasis of neurotrophic functions may be altered by prolonged ethanol consumption.

Keywords: *neurotrophins; BDNF; ethanol; chronic; zebrafish.*

Abbreviations: BDNF: brain-derived neurotrophic factor; CNS: central nervous system; CREB: cAMP-responsive element binding; GABA: gamma-aminobutyric acid; trkB: high-affinity tyrosine kinase receptor.

Introduction

Alcohol abuse and alcoholism are significant health issues throughout the world. Despite the scale of the problem, an important approach to rational pharmacological treatments is to understand the mechanisms by which ethanol alters nervous system function. Various insults, such as promoted by alcohol, can induce marked changes in the level of gene expression of neurotrophins, ultimately leading to functional alterations in the CNS [1,2].

Neurotrophin signaling regulates cell survival, proliferation, the fate of neural precursors, axon and dendrite growth and patterning, and the expression and activity of functionally important proteins, such as ion channels and neurotransmitter receptors [3]. BDNF is a member of a family of neurotrophic factors which includes nerve growth factor, neurotrophin-3, neurotrophin-4/5, neurotrophin-6 and neurotrophin-7 [4,5]. BDNF acts mainly through trkB receptor [1,6,7] and it is involved in the survival of neurons and participates in neuroprotection against various insults [2]. Besides, BDNF has also been shown to elicit rapid action potentials influencing neuronal excitability. It has been demonstrated a putative role in activity-dependent synaptic plasticity events like long-term potentiation, learning tasks and memory [8].

Zebrafish is a small freshwater teleost widely used in genetic and biochemical studies that combines the characteristics of a vertebrate with the scalability of an invertebrate [9,10]. The knowledge accrued from the "Zebrafish Genome Project" together with the ability to rapidly absorb (assimilate) chemicals from the water, make zebrafish an attractive model for toxicological studies and evaluation of human diseases phenotypes [9,11]. Furthermore, it has been shown that zebrafish genes are highly conserved, since its genome presents similarities with the human genome [12]. Concerning the BDNF signaling, the cloning and structural analysis of the zebrafish BDNF gene demonstrated that its intron/exon organization is similar

to mammalian BDNF gene [13,14]. Furthermore, the gene which encodes the neurotrophin receptor trkB is expressed throughout embryonic development in the zebrafish [15], and the neurotrophin signaling has been increasingly studied to comprehension of its physiological importance in zebrafish brain.

In the recent years, an exponential increase in the number of publications in field of animal behavioral neuroscience addressing to alcohol alterations were investigated as such as aggression [16], anxiety [17], memory [18], social preference [19], and addiction [20] by using zebrafish. Besides, neurochemical investigations on cholinergic [21], purinergic [22, 23], glutamatergic [24] and dopaminergic [25] neurotransmitter systems were established. However, to our knowledge, this is the first study to evaluate the correlation of BDNF signaling with chronic ethanol exposure in this model so far. Therefore, considering that neurotrophic factors could be a target of ethanol, we designed the present study to investigate the relative changes on BDNF levels and trkB receptor in zebrafish brain at different periods on ethanol exposure.

2. Materials and methods

2.1. Zebrafish maintenance

Adult zebrafish of both sexes were obtained from commercial supplier (Red Fish, RS, Brazil) and acclimated for at least 2 weeks in a 50 L aquarium, being fed twice daily. The fish were kept between 25-28 °C under a 12-h light–dark photoperiod. The use of animals was in accordance to according to the National Institute of Health Guide for Care and Use of Laboratory Animals and the experiments were designed to minimize discomfort or suffering to

the animals, as well the number used. The Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) approved the protocol under the number 477/05—CEP.

2.2. Ethanol treatment

For *in vivo* treatments, animals were introduced to the test aquariums (10 L) containing a solution of ethanol purchased Merck (Darmstadt, Germany) at 0.5% (v/v). The ethanol solution was replaced every two days, and the animals were maintained in the test aquarium for 7, 14, and 28 days. A preliminary ethanol assay by infrared analysis ensured that there was no alteration in ethanol concentration every 48 hours. The chronic ethanol exposure was based in previous studies, which induced significant changes in locomotor activity and gene expression of zebrafish [26,27]. Immediately after the exposure, the fish were chryoanesthesied and further euthanized by decapitation. The brains were excised and the subsequent experiments were performed.

2.3 RT-PCR experiments

Total RNA was isolated from zebrafish brain using Trizol reagent purchased from Invitrogen (Carlsbad, California, USA) in accordance with manufacturer instructions. RNA was quantified by spectrophotometry and all samples were adjusted to 160 ng/µL. cDNA species were synthesized with SuperScriptTM First-Strand Kit (Invitrogen) following the suppliers. Each RNA sample was mixed with 1 µL of 50 M Oligo (dt) and 1 µL Annealing Buffer (final volume of 8 µL), being incubated in a thermal cycler at 65 °C for 5 min. Immediately, the samples were placed on ice for 1 min and 10 µL 2X First- Strand Reaction Mix and 2 µL SuperScript™ III/RNaseOUT™ Enzyme Mix were added. The products were mixed, incubated by 50 min at 50 °C and the reaction was terminated at 85 °C for 5 min. The primer

sequences 5´-GCTTGAGGTGGAAGGGGAAGCGAC-3´ (forward) and 5´-CCCGCCGTGCGGGGTCCGAG-3´ (reverse) for *BDNF* were designed using the program Oligos 9.6. The primer specificity was checked by comparing each primer with the zebrafish genome to confirm that it would recognize only its specific target sequence and by nucleotide sequencing, which confirmed that the strategy adopted to design the primers avoided cross-amplification. The *trkB* 5´- GGTTTATTAGACGAGCAACCC-3´ (forward) and GGCAGCGATTCCCACGAC-3´ (reverse) primers were designed according [15]. The *β-actin* primers forward (5´-GTCCCTGTACGCCTCTGGTCG-3´) and reverse (5´-GCCGGACTCATCGTACTCCTG-3´) were used as described previously [28].

PCR reactions for *BDNF* and *trkB* and genes were performed in a total volume of 20 μL, 0.1 mM primers, 0.2 mM dNTP, 2 mM $MgCl_2$ and 0.5 U Platinum Taq DNA polymerase (Invitrogen). The following conditions were used for the PCR reactions: desnaturation at 94 ^{o}C for 1 min, followed for 1 min at 54°C (*β-actin*) and 58 ^{o}C (*BDNF* and *trkB*) for annealing temperature for 35 (*β-actin* and *trkB*) and 40 (*BDNF*) cycles and 1 min at 72 ^{o}C for extension. The number of cycles was chosen in order to ensure the linearity of band intensities analyzed. Post-extension at 72 ^{o}C was performed for 10 min. PCR products were analyzed on 1% agarose gel, containing GelRed® 10x and visualized with ultraviolet light. The Invitrogen 1 Kb ladder was used as molecular marker and normalization was performed employing *β-actin* as a constitutive gene. The band intensities were measured by optic densitometry analysis and the relative expression was established for each gene using the Image J 1.37 for Windows software.

2.4. BDNF protein measurement by ELISA immunoreaction

The measurement of BDNF levels in zebrafish brain was performed as previously described [29]. Immediately after the exposures, zebrafish brains were dissected and stored at $-80\ °C$ for biochemical analyses. BDNF levels were measured by anti-BDNF sandwich-ELISA, according to the manufacturer instructions (Chemicon International Inc., Temecula, CA, USA). Briefly, zebrafish brains were homogenized in phosphate-buffered solution with 1 mM phenylmethylsulfonyl fluoride and 1 mM ethyleneglycoltetraacetic acid. Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in sample diluent and standard curve ranged from 7.8 to 500 pg/mL of BNDF. The plates were then washed four times with wash buffer and a monoclonal anti-BNDF rabbit antibody diluted 1:1000 in sample diluent was added to each well and incubated for 3 h at room temperature. After washing, a peroxidase-conjugated anti-rabbit antibody (horseradish peroxidase enzyme; diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After addition of streptavidin enzyme, substrate (3,3′,5,5′- tetramethylbenzidine) and stop solution, the amount of BDNF was determined by absorbance in 450 nm. The standard curve demonstrates a direct relationship between optical density and BDNF concentration. BDNF was expressed as pg of BDNF per mL of serum obtained from brain homogenate. Total protein was measured by Coomassie Blue method [30] using bovine serum albumin as a standard.

2.5. Immunohistochemical analysis of BDNF expression

Immunohistochemical analysis was used to access the BDNF expression in zebrafish brain. The primary antibody used was a mouse monoclonal antibody raised against an extracellular domain of the human trkB receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Zebrafish brains were maintained in 5 mL of 10% paraformaldehyde and embedded into paraffin wax. Four-micrometerthick sections were mounted on organosilane-coated slides and

dried overnight at 37 $^{\circ}$C. Sections were deparaffinized in stove, rehydrated in graded alcohols, and washed with distilled water. The procedure to antigenic recuperation was performed in the microwave, the inactivation of the endogenous peroxidase through immersion in hydrogen peroxide, and blocking cross-reaction with normal serum. The primary antibody diluted in solution (1:50) was incubated for 12 h at 4 $^{\circ}$C, followed by an application of the streptavidin–biotin–peroxidase complex (LSAB, Dako) and the revelation with diaminobenzidine tetrahidroclore (Kit DAB, Dako). Cell nuclei were lightly counterstained with hematoxylin–eosin as a control [31,32].

2.6. Statistical analysis

Data were expressed as means ± S.D. and analyzed using one-way ANOVA for multiple group comparison followed by *post hoc* analysis carried out by Duncan multiple range tests, considering $P \leq 0.05$ as significant.

3. Results

The effect of chronic ethanol exposure (7 to 28 days) on BDNF and trkB levels in zebrafish brain was evaluated. RT-PCR analyses of gene expression for *BDNF* and *trkB* genes were performed. The *β-actin* expression was normalized in order to allow the comparison in different experimental conditions. The results showed that the mRNA levels for *trkB* did not present significant changes after any of the time periods of ethanol exposure tested (Fig. 1B). However, chronic ethanol exposure produced a significant increase in BDNF transcript levels at 14 and 28 days (around 39% and 56%, respectively) as compared to control group (Fig. 1A). The measurement of BDNF protein levels using ELISA kit anti-BDNF was also performed. We observed a significant increase of BDNF immunoreaction (51%) after 28 days of ethanol

exposure (Fig. 2A). Moreover, when the tissues were immunostained with BDNF antibody, the intensity showed by the control group was similar to the 7, and 14 days-exposed groups in optic tectum. Nevertheless, this structure had a substantial increase of anti-BDNF following the ethanol exposure for 28 days (Fig. 2B).

4. Discussion

Brain neurotrophins, especially BDNF, are known to play crucial roles in synaptic plasticity in the CNS during development and adulthood [33]. Recent biochemical and genetic data implicate BDNF and its associated signaling intermediates with chronic ethanol exposure and addiction [34,35]. As the long-term ethanol treatment models can alter neurotrophin expression, in this study we verified whether chronic ethanol exposure alters the gene and protein expression of BDNF and trkB in zebrafish brain. Our results showed that after 7 days of ethanol exposure, there were no significant changes in the both mRNA and BDNF protein levels. In contrast, increased levels of transcripts were detected after 14 and 28 days, but only after 28 days we detected an increase on BDNF protein amounts.

It has been described that alcohol intoxication induces a defect in global protein synthetic rates that is associated to impaired translation of mRNA at the level of peptide-chain initiation. Decreased translational efficiency may result from inhibition of peptide-chain initiation and/or elongation/termination. Relative rates of peptide-chain initiation and elongation can be assessed by the measurement of protein synthetic rates coupled with analysis of the distribution of ribosomal subunits between free subunits and polysomes. The amount of RNA in free ribosomal subunits reflects the balance between the rates of peptide-chain initiation and elongation/termination [36]. It is known that alcohol causes disaggregation of polysomes into free ribosomes [37-39]. Ethanol exposure for 14 days showed a significant increase in the

expression of mRNA for *BDNF* in zebrafish brain. However, ELISA immunoquantification and immunohistochemical data did not demonstrate significant difference. Therefore, these results suggest that during this exposure period, it is possible that the translational machinery could be altered. Studies have shown an increased BDNF expression after chronic ethanol consumption, suggesting that it may chronically modify neuronal functions through alterations in growth factors and its receptors [40,41]. Ethanol is able to modulate CREB, leading to a change in expression of various CREB target genes. The CREB-related signaling pathway influences upstream and downstream mediators, revealing a complex signaling network that is often usurped by ethanol addiction [42]. Thus, the increase of BDNF transcripts and protein levels after the longest period of ethanol exposure could be associated to ethanol effects on CREB in zebrafish brain.

The neuroprotective role of BDNF against several insults, such as hypoxia-ischemia, hypoglycemia, and ethanol exposure has been demonstrated [43-45]. In our findings, we observed an increased BDNF expression after the prolonged ethanol exposure. In this sense, it is possible to suggest that the increase in BDNF levels could be associated with its possible neurotrophic function, as well as, development, survival and plasticity of brain. Therefore, the increased BDNF levels observed in the current study could reflect a compensatory response against the neurotoxic effects promoted by prolonged ethanol exposure.

Since we found that the expression level of BDNF was elevated in the zebrafish brain after the longest ethanol exposure, and considering that BDNF alters gene expression [46,47], we determined whether chronic ethanol exposure changes trkB gene expression. The results showed that ethanol did no significant alterations on trkB mRNA expression during 7-to 28 days of ethanol exposure in zebrafish brain. The lack of changes observed in trkB expression is consistent with recent findings showing that ethanol did not affect trkB levels in cortical

neuronal cultures and *in vitro* [48] and trkB mRNA in rat hippocampus *in vivo* [49]. Meanwhile, evidence in literature report that ethanol causes changes in the expression of trkB in different adult models [50,51] and these studies point to the necessity to better understand the role of neurotrophins in the alcoholism.

The psychotropic effects of ethanol are mediated by complex neurotransmitter systems, including the GABAergic signaling. Ethanol act in the GABAergic system mainly through its agonistic effect on GABA$_A$ receptors, which is connected to a chloride channel and regulates the passage of chloride into the cells [52]. BDNF has been proposed to play a critical role in regulating fast synaptic inhibition through GABAergic synaptic transmission. Furthermore, BDNF induces changes in synaptic inhibition by modulating GABA$_A$ receptor phosphorylation and cell-surface stability, suggesting the importance of this neurotrophin signaling in synaptogenesis of GABAergic contacts [53]. Therefore, considering the effect of alcohol on GABAergic system and the importance of BDNF in the formation and functional maturation of inhibitory synapses, our results suggest that the increased expression of BDNF in zebrafish brain could be a dependent response to ethanol effects by modulation of the GABAergic synapse.

Several genes predisposing to chronic alcohol consumption have been described and progress is slowly being made towards integrating these polymorphisms with behavioral phenotypes. Neurotrophins bind to specific Trk receptors activating, among others, phospholipase C, Ras-MAP kinase, and phosphatidylinositol 3 kinase cell signaling pathways [54,55]. These changes in neurochemical functions lead to alterations in the basis for behavioral pattern as learning, memory and, anxiety [56]. The behavioral profiles such as anxiety, memory, social preference, and addiction [17-20] have been studied in zebrafish. Considering that the chronic ethanol exposure promotes changes in the levels of BDNF in zebrafish brain, this neurotrophin could

be involved, at least in part, in the behavioral responses. Since adult zebrafish has been widely used for studying several behavioral paradigms, we understand that this study provides new approaches regarding the effects of ethanol in the CNS of this species. In this sense, we suggest that BDNF through Trk receptor could be involved to mediate intracellular routes induced by ethanol. Altogether, the observed changes in BDNF levels suggest the importance of the prolonged ethanol effect in modulating signaling pathways related to this neurotrophin in CNS.

Acknowledgements

References

[1] O. Lindvall, Z. Kokaia, J. Bengzon, E. Elmér, M. Kokaia, Neurotrophins and brain insults. Trends Neurosci. 17 (1994) 490-496.

[2] H. Okamoto, T. Miki, K.Y, Lee, T. Yokoyama, H. Kuma, H. Gu, H.P. Li, Y. Matsumoto, I. Yamaoka, K. Fusumada, T. Imagawa, Z.W. Wang, Y. Nakamura, Y. Takeuchi, Effects of chronic ethanol administration on the expression levels of neurotrophic factors in the rat hippocampus. Okajimas Folia Anat. Jpn. 83 (2006) 1-6.

[3] E.J. Huang, L.F. Reichardt, Trk Receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. 72 (2003) 609–642.

[4] T. Aid, A. Kazantseva, M. Piirsoo, K. Palm, T. Timmusk, Mouse and rat BDNF gene structure and expression revisited. J. Neurosci Res. 85 (2007) 525-535.

[5] T. Miki, H. Kuma, T. Yokoyama, K. Sumitani, Y. Matsumoto, T. Kusaka, K. Warita, Z.Y. Wang, N. Hosomi, T. Imagawa, K. S Bedi, S. Itoh, Y. Nakamura, Y. Takeuchi, Early postnatal ethanol exposure induces fluctuation in the expression of BDNF mRNA in the developing rat hippocampus. Acta Neurobiol. Exp. (Wars). 68 (2008) 484-493.

[6] S.P. Squinto, T.N. Stitt, T.H. Aldrich, S. Davis, S.M. Bianco, C. Radziejewski, D.J. Glass, P. Masiakowski, M.E. Furth, D.M. Valenzuela, trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell. 65 (1991) 885–893.

[7] T. Timmusk, K. Palm, M. Metsis, T. Reintam, V. Paalme, M. Saarma, H. Persson, Multiple promoters direct tissue-specific expression of the rat BDNF gene. Neuron. 10 (1993) 475-489.

[8] A. Nair, V.A. Vaidya, Cyclic AMP response element binding protein and brain-derived neurotrophic factor: molecules that modulate our mind? J. Biosci. 31 (2006) 423-434.

[9] P. Goldsmith, Zebrafish as a pharmacological tool: the how, why and when. Curr. Opin. Pharmacol. 4 (2004) 504–512.

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

[11] A.J. Hill, H. Teraoka, W. Heideman, R.E. Peterson, Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci. 86 (2005) 6-19.

[12] W.B. Barbazuk, I. Korf, C. Kadavi, J. Heyen, S. Tate, E. Wun, J.A. Bedell, J.D. McPherson, S.J. Johnson, The syntenic relationship of the zebrafish and human genomes. Genome Res. 10 (2000) 1351–1358.

[13] G. Huynh, G. Heinrich, Brain-derived neurotrophic factor gene organization and transcription in the zebrafish embryo. Int. J. Dev. Neurosci. 19 (2001) 663-73.

[14] M. Hashimoto, G. Heinrich, Brain-derived neurotrophic factor gene expression in the developing zebrafish. Int. J. Dev. Neurosci. 15 (1997) 983-997.

[15] T. Lum, G. Huynh, G. Heinrich, Brain-derived neurotrophic factor and trkB tyrosine kinase receptor gene expression in zebrafish embryo and larva. Int. J. Dev. Neurosci. 19 (2001) 569-587.

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

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

[18] T. Darland, J.E. Dowling, Behavioral screening for cocaine sensitivity in mutagenized zebrafish. Proc. Natl. Acad. Sci. U S A. 98 (2001) 11691-11696.

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

[20] J. Ninkovic, L. Bally-Cuif, The zebrafish as a model system for assessing the reinforcing properties of drugs of abuse. Methods. 39 (2006) 262-274.

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

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

[23] 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.

[24] E.P. Rico, D.L. de Oliveira, D.B. Rosemberg B.H. Mussulini, C.D. Bonan, R.D. Dias RD, S. Wofchuk, D.O. Souza, M.R. Bogo, Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain. Brain Res. Bull. 81 (2010) 517-523.

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

[26] R. Gerlai, D. Chatterjee, T. Pereira, T. Sawashima, R. Krishnannair. Acute and chronic alcohol dose: population differences in behavior and neurochemistry of zebrafish. Genes Brain Behav. 8 (2009) 586-599.

[27] Y. Pan, M. Kaiguo, Z. Razak, J.T. Westwood, R. Gerlai. Chronic alcohol exposure induced gene expression changes in the zebrafish brain. Behav Brain Res. 216 (2011) 66-76.

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

[29] B.N. Frey, A.C. Andreazza, K.M. Ceresér, M.R. Martins, S.S. Valvassori, G.Z. Réus, J. Quevedom F. Kapczinski, Effects of mood stabilizers on hippocampus BDNF levels in an animal model of mania. Life Sci. 79 (2006) 281-286.

[30] M.M Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72 (1976) 218–254.

[31] C.B. Farias, R.G. Lima, L.O. Lima, D.G. Flores, L. Meurer, A.L. Brunetto, G. Schwartsmann, R. Roesler, Stimulation of proliferation of U138-MG glioblastoma cells by gastrin-releasing peptide in combination with agents that enhance cAMP signaling. Oncology, 75 (2008) 27–31.

[32] D.G. Flores, C.B. de Farias, J. Leites, M.S. de Oliveira, R.C. Lima, A.S. Tamajusuku, L.P. Di Leone, L. Meurer, A.L. Brunetto, G. Schwartsmann, G. Lenz, R. Roesler, Gastrin-releasing peptide receptors regulate proliferation of C6 glioma cells through a phosphatidyli-nositol3-kinase-dependent mechanism. Curr. Neurovasc. Res. 5 (2008) 99–105.

[33] V. Lessmann, T. Brigadski, Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. Neurosci. Res. 65 (2009) 11-22.

[34] C.A. Bolaños, E.J. Nestler, Neurotrophic mechanisms in drug addiction. Neuromolecular Med. 5 (2004) 69-83.

[35] M.I. Davis, Ethanol-BDNF interactions: still more questions than answers. Pharmacol Ther. 118 (2008) 36-57.

[36] A.M. Karinch, J.H. Martin, T.C. Vary, Acute and chronic ethanol consumption differentially impact pathways limiting hepatic protein synthesis. Am. J. Physiol. Endocrinol. Metab. 295 (2008) E3-9.

[37] C.H. Lang, D. Wu, R.A. Frost, L.S. Jefferson, T.C. Vary, S.R. Kimball, Chronic alcohol feeding impairs hepatic translation initiation by modulating eIF2 and eIF4E. Am. J. Physiol. 277 (1999) E805-814.

[38] M.A. Rothschild, M. Oratz, J. Morland, S.S. Schreiber, A. Burks, B. Martin, Effects of ethanol on protein synthesis and secretion. Pharmacol. Biochem. Behav. 13 (1980) 31-36.

[39] K.M. Huber, M.S. Kayser, M.F. Bear, Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. Science. 288 (2000) 1254-1257.

[40] N.N. McGough, D.Y. He, M.L. Logrip, J. Jeanblanc, K. Phamluong, K., Luong, V. Kharazia, P.H. Janak, D. Ron, RACK1 and brain-derived neurotrophic factor: a homeostatic pathway that regulates alcohol addiction. J. Neurosci. 24 (2004) 10542-10552.

[41] M.W. Miller, Repeated episodic exposure to ethanol affects neurotrophin content in the forebrain of the mature rat. Exp. Neurol. 189 (2004) 173-181.

[42] S. Moonat, B.G. Starkman, A. Sakharkar, S.C. Pandey, Neuroscience of alcoholism: molecular and cellular mechanisms. Cell Mol. Life Sci. 67 (2009) 73-88.

[43] J.J. Mitchell, M. Paiva, D.W. Walker, M.B. Heaton, BDNF and NGF afford in vitro neuroprotection against ethanol combined with acute ischemia and chronic hypoglycemia. Dev. Neurosci. 21 (1999) 68-75.

[44] B.H. Han, D.M. Holtzman, BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. J. Neurosci. 20 (2000) 5775-5781.

[45] M.B. Heaton, D.S. Kim, M. Paiva, Neurotrophic factor protection against ethanol toxicity in rat cerebellar granule cell cultures requires phosphatidylinositol 3-kinase activation. Neurosci, Lett. 291 (2000) 121−125.

[46] S.D. Croll, S.J. Wiegand, K.D. Anderson, R.M. Lindsay, H. Nawa, Regulation of neuropeptides in adult rat forebrain by the neurotrophins BDNF and NGF. Eur. J. Neurosci. 6 (1994) 1343-1353.

[47] O. Guillin, N. Griffon, E. Bezard, L. Leriche, J. Diaz, C. Gross, P. Sokoloff, Brain-derived neurotrophic factor controls dopamine D3 receptor expression: therapeutic implications in Parkinson's disease. Eur. J. Pharmacol. 480 (2003) 89-95.

[48] G.K. Seabold, J. Luo, M.W. Miller, Effect of ethanol on neurotrophin-mediated cell survival and receptor expression in cultures of cortical neurons. Brain Res. Dev. Brain Res. 108 (1998) 139-145.

[49] L. Zhang, H.S. Dhillon, S. Barron, R.R. Hicks, R.M. Prasad, K.B. Seroogy, Effects of chronic ethanol administration on expression of BDNF and trkB mRNAs in rat hippocampus after experimental brain injury. Brain Res. Mol. Brain Res. 79 (2000) 174-179.

[50] J.K. Baek, M.B. Heaton, D.W. Walker, Up-regulation of high-affinity neurotrophin receptor, trk B-like protein on western blots of rat cortex after chronic ethanol treatment. Brain Res. Mol. Brain Res. 40 (1996) 161-164.

[51] L. Tapia-Arancibia, F. Rage, L. Givalois, P. Dingeon, S. Arancibia, F. Beaugé, Effects of alcohol on brain-derived neurotrophic factor mRNA expression in discrete regions of the rat hippocampus and hypothalamus. J Neurosci. Res. 63 (2001) 200-208.

[52] E. Esel, Neurobiology of alcohol withdrawal inhibitory and excitatory neurotransmitters. Turk. Psikiyatri. Derg. 17 (2006) 129-137.

[53] N.J. Jovanovic, P. Thomas, J.T. Kittler, T.G. Smart, S.J. Moss, Brain-derived neurotrophic factor modulates fast synaptic inhibition by regulating GABA(A) receptor phosphorylation, activity, and cell-surface stability. J. Neurosci. 24 (2004) 522-530.

[54] M.V. Chao, Neurotrophins and their receptors: a convergance poit for many signalling pathways. Nat. Rev. Neurosci. 4 (2003) 299-309.

[55] A.K. McAllister, L.C. Katz, D.C. Lo, Neurotrophins and synaptic plasticity. Annu. Rev. Neurosci. 22 (1999) 295-318.

[56] R.H. Lipsky, A.M. Marini, Brain-derived neurotrophic factor in neuronal survival and behavior-related plasticity. Ann. N. Y. Acad. Sci. 1122 (2007) 130-143.

Figure Legends

Fig. 1: Gene expression patterns in zebrafish brain after treatment with ethanol. The band intensities were measured by optical densitometry for *BDNF* (A) and *trkB* (B) using the freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the abundance of each mRNA compared to *β-actin*. The results were expressed as means ± S.D. of optical densitometry (arbitrary units) of four independent replicate RT-PCR experiments. Data were analyzed by ANOVA followed by Duncan's post hoc test ($P \leq 0.05$, as compared to control group). * Significantly different from control.

Fig. 2: Effects of chronic ethanol exposure (7, 14 and 28 days) on BDNF protein levels in zebrafish brain. (A) Quantification of BDNF protein amounts by ELISA assay. Bars represent means ± S.D. of at least five different experiments, each in duplicate. Data were analyzed by ANOVA followed by Duncan's post hoc test ($P \leq 0.05$, as compared to control group). * Significantly different from control. (B) BDNF immunohistochemstry for control group and ethanol exposure for 7, 14, and 28 days. The primary antibody diluted in solution (1:50) was incubated for 12 h at 4 $^{\circ}$C, followed by an application of the streptavidin–biotin–peroxidase complex and the revelation with diaminobenzidine tetrahidroclore. Cell nuclei were lightly counterstained with hematoxylin–eosin as a control.

Figures:

A

B
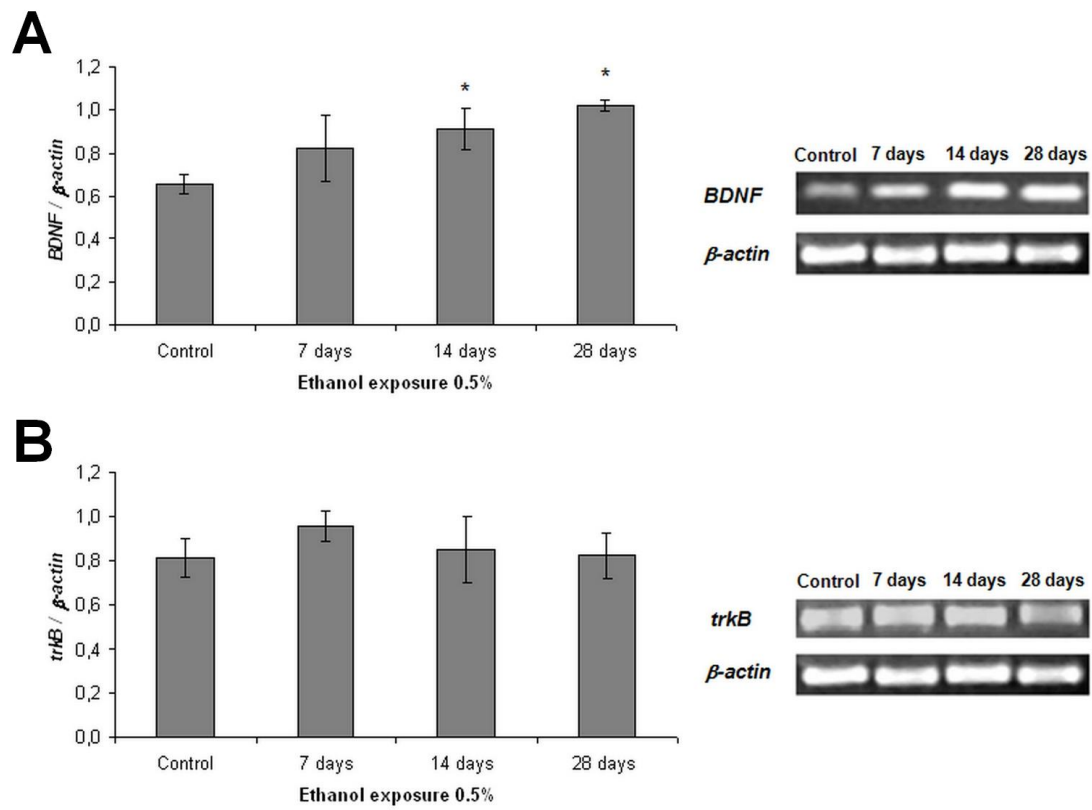
Figure 1

A
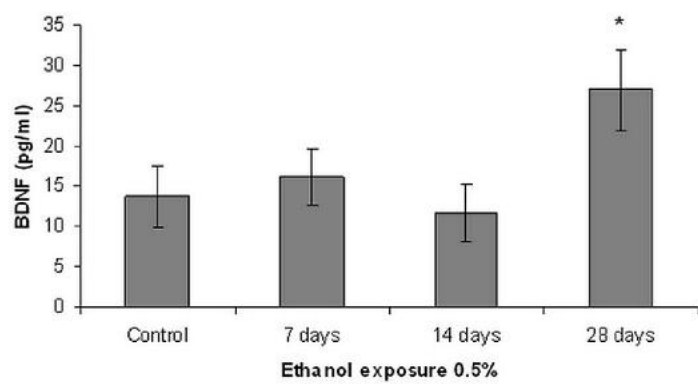
B
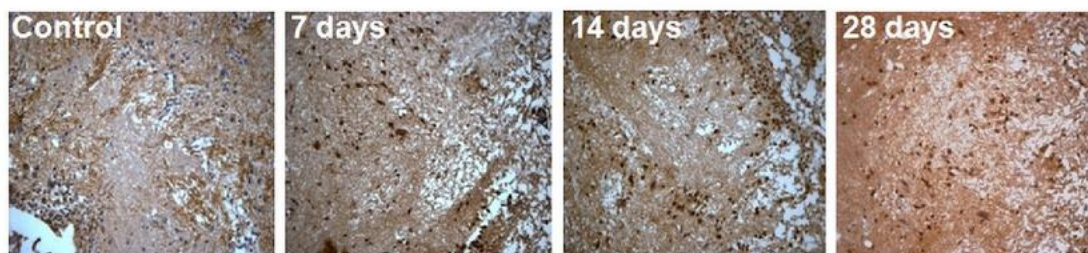
Figure 2

II.7. Capítulo 7

Sistemas de neurotransmissão em zebrafish como potencial alvo farmacológico e toxicológico. Artigo submetido ao periódico *Neurotoxicology and Teratology*.

Neurotransmitter systems in zebrafish as potential pharmacological and toxicological targets

Rico, E.P.[a,b]; Rosemberg, D.B.[a,b]; Seibt, K.J.[c,d], Capiotti, K.M. [c,d], Da Silva, R.S. [c,d], Bonan C.D [c,d,*]

[a] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil;

[b] Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção (INCT-EN), 90035-003, Porto Alegre, RS, Brazil;

[c] Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia; Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil

[d] Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003, Porto Alegre, RS, Brazil.

Corresponding author:

Bonan, C.D.,Laboratório de Neuroquímica e Psicofarmacologia Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul; Avenida Ipiranga, 6681, 90619-900. Porto Alegre, RS, Brazil. Tel.: +55 51 3353 4158; fax: +55 51 3320 3568. cbonan@pucrs.br

Abstract

The recent advances in neurobiology have emphasized the study of the brain structure and function and its relation with numerous pathological and toxicological events. Neurotransmitters are chemicals substances that are used to relay, amplify, and modulate electrical signals between neurons and/or another cell. Their signaling mediates rapid intercellular communication by interacting with cell surface receptors, often activating second messenger systems and regulating the activity of ion channels. Changes in functional balance of neurotransmitters have been implicated in the failure of cell function. In addition, abnormalities in the production or functioning of neurotransmitters are induced by several toxicological compounds, many of them found in the environment. Zebrafish has been increasingly used as an animal model for biomedical research, mainly due to its genetic tractability and ease of maintenance. These features make this species a versatile tool into pre-clinical drug discovery and toxicological investigations. Here we present a review about the role of different excitatory and inhibitory neurotransmitters systems in zebrafish such as dopaminergic, serotoninergic, cholinergic, glutamatergic, purinergic, histaminergic, nitrergic, glycinergic, and GABAergic systems, emphasizing pharmacological and toxicological aspects. The significant increase in the global knowledge about the neurotransmitters systems in zebrafish and the elucidation of pharmacological and toxicological effects could lead to new strategies and appropriate priorities in research in order to offer support to insights on biomedical and environmental research.

1. Introduction

Neurotransmitters are chemical messengers that lead, amplify, and modulate signals between neurons and other cells in the body. In most cases, they are released from the axon terminal after an action potential from synapse and exert their effects by binding to specific receptors on the neuronal postsynaptic membrane. The neuronal activity of a neuron depends on the balance between the number of excitatory and inhibitory processes affecting it, which may occur individually or simultaneously (Prange et al., 2004). The consequences of the neurotransmitter-mediated signaling can influence the regulation of metabolic manifestations directly by central nervous system (CNS) function or through the hypothalamic-pituitary-end organ axis. Considering that brain disorders are associated with abnormal production or functioning of neurotransmitters, experimental approaches involving proteins that participate of neurotransmission (transporters, receptors, and enzymes) have been characterized. These assays include the analysis of different aspects, such as localization, function, and pharmacological properties (Raiteri, 2006). From this paradigm, there is a growing interest in the utilization of biological models to investigate the basis of neurotransmission. Although many researchers study these parameters using distinct cell cultures, the use of the whole organism is a tempting strategy that allows the screening of processes that are not easily replicated in vitro, such as organ development. Furthermore, drug metabolism is an important factor in the conservation of drug activity across species. Since in vitro studies provide speed and efficiency to screen a larger number of compounds, whole organisms offer several advantages over cell lines for the increasing of chemical genetic screens, providing information on tissue specificity, toxicity, and accounting for bioavailability. This *in vivo* assessment represents an important role in order to complement *in vitro* assays beyond support tests in other more complex organisms.

During the last years, zebrafish is becoming a consolidated model system 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 zebrafish genome shares similarities with the human genome (Barbazuk et al., 2000). The molecular basis of neurobiology has been also studied in this species, identifying genes involved in the formation of neural circuitry, behavior and mechanisms related to neuropathogenesis. Considering the advantages of zebrafish as experimental model, the acute and chronic effect of several compounds may be easily evaluated. Furthermore, considering the small space required by this specie and features of the embryos, a lesser amount of drugs may be used in toxicological and pharmacological assays and changes throughout development may be observed. The characterization of alternative animal models, which permit embriologic and molecular screenings, contributes to a better knowledge about neurochemical mechanisms as well as helps in the development and screening of new drugs. Zebrafish has been used to screening a large library of compounds and small molecules that disrupt a phenotype in a biological assay analogous to traditional genetic screens. Besides, zebrafish system has been applied as an emergent tool in the general aspects of omics approaches for analyses of transcriptome, proteome, and metabolome (Sukardi et al., 2010).

Excitatory and inhibitory neurotransmitters are involved in several events in CNS. Many neurodegenerative diseases are correlated with the brain cells dysfunction, and, consequently, changes in signaling systems. One focus of therapeutic approach in neurodegenerative diseases has been replacement strategies of neurotransmitters, such as levodopa (a dopamine precursor) for Parkinson's disease, or memantine (a glutamate

receptor [N-methyl-d-aspartic acid] antagonist), for the treatment of Alzheimer's disease (Maragakis and Rothstein, 2004). This focus can be strategically incorporated into chemical screening, and injury models using this species.

Neurotoxicity occurs when the exposure to toxicants alters the normal activity of the nervous system, including neural transmission, connection and survival. The neuronal toxicity can result from exposure to drugs used for chemotherapy, radiation treatment, and organ transplantation as well as from food additives and environmental toxicants (Parng et al., 2007). The ability to examine the entire nervous system and to visually evaluated the brain in early stages of development makes zebrafish an exceptional model to neurotoxicity assessments. In order to understand the biological basis of cumulative effect of pollutants and other chemicals, the investigation of environmental toxicants (e.g. organic compounds and heavy metals) on zebrafish neurotransmission has been increased. In this regard, this species has helped to understand the neurochemistry and molecular aspects, providing interesting and emergent tools for future studies and applications. The technology advancements and the knowledge of the zebrafish genome allowed the improvement and development of sophisticated strategies as well as mutant individuals, genetic and tissue manipulation, genetic morpholinos and microarray technology, and new bioassays for toxic and therapeutic endpoints (Hill et al., 2005). These entire advances associated to the simplicity for evaluating the morphological, biochemical, and physiological information at all stages of early development, in juveniles, and adults of both sexes make this species ideal to identify adverse effects of chemical exposure. The current review will focus the role of different excitatory and inhibitory neurotransmitters systems in zebrafish such as dopaminergic, serotoninergic, cholinergic, glutamatergic, purinergic, histaminergic, nitric

oxide synthase, glycinergic, and GABAergic systems (Figure 1), emphasizing their importance and potential application at pharmacological and toxicological studies.

2. Dopamine and serotonine

The aminergic neurotransmitters, such as dopamine and serotonin, are mediators of several important brain functions. Abnormalities in their levels have been implicated in distinct CNS diseases in humans (Belmaker, 2008; Murray et al., 2008). Although dopaminergic neurons account for less than 1% of the total neuronal population of the brain, they have an important effect on brain physiology. Dopamine regulates locomotion, cognition, emotion, and reward (Goldman-Rakic 1998; Schultz, 2002). The effects promoted by dopamine are mediated by a group of G-protein-coupled receptors. In mammals there are five dopamine receptor (DR) subtypes that are grouped into two families based on pharmacological profiles and sequence similarities. 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 plays fundamental roles during development and plasticity of the vertebrate CNS. The serotonergic neurons in mammalian CNS are mainly 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 central nervous system (Cote et al., 2007; Fricker et al., 2005; Gaspar et al., 2003). In agreement with these multiple activities, the dysfunction of serotonergic neurons, during development or even

adulthood, has been implicated in several psychiatric diseases, including depression, addiction to drugs, and schizophrenia (Lucki, 1998; Sallinen et al., 2009).

Zebrafish domaninergic and serotonergic systems share similarities to the respective mammalian systems, and thus this species is a feasible model to the evaluation of 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 promoted by alcohol on adult brain function (Gerlai et al. 2000). Alcoholism and alcohol abuse are known to affect aminergic neurons and 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 as well as 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 to its computer animated image were enhanced or impaired after acute exposure to intermediate or high doses alcohol, respectively. In addition, Chatterjee and Gerlai (2009) showed that there are significant changes in levels of serotonin and dopamine and its metabolites in zebrafish after alcohol treatment (Chatterjee & Gerlai, 2009).

Dopaminergic deficiency in the zebrafish brain has been already induced by systemic administration of two catecholaminergic neurotoxins, 6-hydroxydopamine (6-OHDA) and 1-methyl- 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The levels of dopamine and noradrenaline decreased significantly after the injection of MPTP and 6-OHDA. Both drugs produced significant alterations in the swimming behaviour of zebrafish. The general locomotor activity (distance moved and velocity) was markedly

decreased and the fish displayed altered the swimming pattern (Anichtchik et al., 2004). A recent report demonstrated that nervous system development is disrupted in developing zebrafish exposed to monovalent silver ion (Ag^+) (Powers et al., 2010). This study shown that Ag^+ is a developmental neurotoxicant that causes persistent neurobehavioral effects, reinforcing health concerns about Ag^+ released from silver nanoparticles. Early developmental exposure to Ag^+ elevated DA and 5HT turnover in adult zebrafish. These changes cannot be explained by alterations in basal levels, and thus are indicative of increased presynaptic activity for these two neurotransmitters. The novel finding that developmental Ag^+ exposure produces lasting behavioral changes in association with altered DA and 5HT synaptic function, highlights the potential impact that elevations in Ag^+ exposure could have in human and environmental populations (Powers et al., 2010).

Giacomini and collaborators (2006) investigated the effects of antipsychotics on larval zebrafish. The antipsychotics haloperidol and fluphenazine produced hypoactivity associated to erratic swimming bouts, which are recovered by coadministration with the dopamine precursor levodopa (Giacomini et al., 2006). Clozapine, an atypical antipsychotic, also induced hypoactivity, an effect that is prevented by the D4 receptor selective agonist ABT-724 but not by quinpirole, a D2/D3 agonist, that produces hyperactivity on zebrafish larvae (Boehmler, 2007). The acute administration of fluoxetine, a selective serotonin reuptake inhibitor, produces a hyperlocomotor effect accompanied by diminished expression of SERT and 5-HT1A receptor in the spinal cord, but not in zebrafish larvae brain (Airhart et al., 2007). Others researchers reported that the hallucinogenic drug lysergic acid diethylamide (LSD), a nonselective serotonin receptor agonist, produces a pattern of disorganized exploration in diverse models, including the novel tank diving test and the scototaxis test (Grossman et al., 2010).

3. Acetylcholine

Acetylcholine (ACh) is a classical signaling molecule that elicits several actions on living systems. Besides its role at neuromuscular junctions, ACh acts as an important neurotransmitter in the CNS (Panula et al., 2010). After being released by electrical stimuli, ACh activates two classes of receptors (AChRs): the ionotropic nicotinic ACh receptors (nAChRs) and G-protein-coupled muscarinic AChRs (mAChRs). While the muscarinic AChRs may be involved in neurotransmission, neuromodulation (Brown, 2010) and also in olfactory mechanisms (Durand et al., 1998), the nicotinic AChR activation 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-tuning regulation of ACh-mediated signaling is performed by acetylcholinesterase (AChE, EC 3.1.1.7) activity. This enzyme is a serine hydrolase related to type B carboxylesterases family, which cleaves ACh into choline and acetate, terminating cholinergic transmission. The synthesis *de novo* of ACh is dependent of choline acetyltransferase (ChAT, EC 2.3.1.6) activity, which catalyzes the reaction of acetate and choline at pre-synaptic neurons (Jamal et al., 2009).

The identification of cholinergic neurons in 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 the distinct methodological approach used, the anatomical identification of ChAT immunoreactive neurons differs among these studies. Although Mueller et al. (2004) detected a significant staining only in the lateral nucleus of the ventral telencephalic area, Kaslin et al. (2004) observed the presence of ChAT immunocontent in the central, dorsal and subcommissural nuclei of the ventral

telencephalic area of adult animals. In diencephalon, the preoptic area, dorsal thalamus, pretectal nuclei and hypothalamus displayed distinct groups of ChAT positive staining. Prominent groups were also detected in the mesencephalon, where the optic tectum (OT) and tegmentum showed immunoreactive cells (Clemente et al., 2004; Kaslin et al., 2004; Mueller et al., 2004). The main developmental pattern of ChAT positive neurons were described for zebrafish (Arenzana et al., 2005). In this study, it was demonstrated that, at 60 hpf, the tegmental ChAT positive neurons may be identified as parts of the oculomotor, trochlear and rostral tegmental nuclei, whereas the tectal cholinergic neurons develop only at 5 dpf.

Regarding ACh receptors, the muscarinic AChRs have already been characterized in zebrafish brain by radioligand binding techniques. Similar to rodents, this species might be a useful model organism for evaluating the role of cholinergic systems in learning, memory and behavior (Williams and Messer, 2004). Moreover, the M(2) muscarinic receptor has been described to play a role in the initiation of hypoxic bradycardia in larval zebrafish, since severe hypoxia levels significant increased the relative mRNA expression of M(2) and the cardiac type beta-adrenergic receptors (beta1AR, beta2aAR, and beta2bAR) at 4 dpf (Steele et al., 2009). The actions of nicotine in memory and behavioral tasks have already been reported in adult zebrafish (Levin and Chen, 2004; Levin et al., 2007). It has been demonstrated that low nicotine doses significantly improved fish memory, whereas higher nicotine doses have diminished effect and induced an impairment of memory (Levin and Chen, 2004). Additionally, a potential anxiolytic effect of nicotine in zebrafish has been suggested by evaluation of the vertical swimming in the novel tank paradigm (Levin et al., 2007) and both nicotinic alpha7 and alpha4beta2 receptors could be involved in this response (Bencan and Levin, 2008). Considering the effects of organophosphate pesticides,

a recent study showed that zebrafish is a sensitive model of persisting neurobehavioral effects of developmental chlorpyrifos exposure and it was suggested a putative role of AChR in triggering these effects (Eddins et al., 2010).

There is evidence suggesting the importance of AChE activity and expression in regulating zebrafish brain functions. The early expression of AChE in diverse cell types suggests that it may have a developmental role and thus, could be a putative target for neurotoxicity in zebrafish (Hanneman and Westerfield, 1989). In this regard, the effects promoted by metals (Senger et al., 2006a 2006b; Richetti et al., 2010), 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 had already been studied in zebrafish brain. A recent report also demonstrated that the alterations in AChE activity induced by ethanol could be related to changes on oxidative stress parameters, which were prevented by the pretreatment with taurine (Rosemberg et al., 2010a). Based on these data, the evaluation of cholinergic system parameters emerges as a tempting strategy to assess neurochemical, behavioral, and toxicological phenotypes in both larval and adult zebrafish.

4. Glutamate

Glutamate is the main excitatory neurotransmitter in the vertebrate CNS. It is involved in a great number of brain functions (Ozawa et al., 1998; Anderson and Swanson, 2000; Danbolt, 2001), such as memory and learning (Izquierdo and Medina, 1997), development and ageing (Segovia et al., 2001) and adaptation to the environment (Danbolt, 2001; Mattson et al.,2002). However, the glutamate concentration profile at the synaptic cleft is variable and it may also act as an excitotoxin when its receptors are over-stimulated (Anderson and Swanson, 2000; Danbolt, 2001; Maragakis and Rothstein, 2004). The

maintenance of extracellular glutamate concentrations below neurotoxic levels is an essential role of glial cells and this is achieved through high affinity sodium-dependent glutamate transporters present mainly in astrocytes (Danbolt, 2001; Chen and Swnason, 2003). Glutamate toxicity has been related to neuronal death in ischemia and trauma (Choi, 1988; Ikonomidou et al., 1989), and several neurodegenerative disorders such as Huntingdon's and Alzheimer's diseases (Brewer, 2000; Danbolt, 2001; Ingram et al., 2001; Maragakis and Rothstein, 2001, 2004; Segovia et al., 2001).

Studies aimed at identifying and understanding the basis of glutamatergic signaling has emerged. The 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 excitatory amino acid transporters have been identified and characterized in the mammalian brain. The presence of EAAT-related sequences by phylogenetic analysis and the mRNA expression profile in zebrafish CNS have been described recently (Rico et al., 2010). Besides, evolutionary history of EAATs was also analyzed and these members were included on the solute carrier 1 (SLC1) gene family (Gesemann et al., 2010, Neuhauss et al., 2010). Once identified these EAAT-related genes, some preliminary parameters of glutamate transporter activity were investigated through sodium-dependent glutamate uptake in distinct zebrafish brain structures (Rico et al., 2010).

Hair cells are the sensory receptors for auditory and vestibular system in zebrafish. They detect sound, movement and transmit this information specialized through ribbon synapses, which coordinates synaptic vesicles. It was demonstrated that zebrafish mutant for vesicular glutamate transporter 3 (*vglut3*), hair cells presented a decrease in the number of ribbon-associated synaptic vesicles, indicating for this glutamate transporter for synaptic transmission (Obholzer et al., 2008). This family of proteins mediates the glutamate uptake

by synaptic vesicles, being necessary for glutamatergic transmission in retina. Other researchers reported that 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 primarily divided into 2 main categories: metabotropic and ionotropic receptors. Metabotropic receptors trigger intracellular secondary messengers through G-proteins. On the other hand, ionotropic receptors are ligand-gated ions channel managing rapid changes in sodium, calcium, and potassium conductances. Subtypes of ionotropic receptors are N-methyl-D-aspartate (NMDA), AMPA and kainate (KA). The molecular characterization and embryonic expression of the family of NMDA receptor subunit genes has 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 hippocampus and amygdala of mammalian brain (Portavella et al., 2002; Rodríguez et al., 2002). Considering that long-term potentiation (LTP) is the representative synaptic modification underlying the process of learning and memory, Nam and collaborators (2004) demonstrated the NMDA receptor-dependent long-term potentiation in the telencephalon of the zebrafish. In this context, a simple protocol of inhibitory avoidance task in adult zebrafish demonstrated that the resulting memory is robust, long-lasting and sensitive to NMDA-receptor antagonist MK-801 given in 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. Variations in the subunit composition of each of the three iGluR sub-types further contribute to unique cellular responses elicited by glutamate (Nakanishi et al., 1994). Edwards and Michel (2003)

demonstrated the pharmacological characterization of ionotropic glutamatergic receptors in olfactory bulb. This group advanced the understanding of the glutamatergic system in the teleosts by characterizing the distribution of functional NMDA and KA-stimulated neurons. Furthermore, Tabor and Friedrich (2008) pharmacologically investigated the ionotropic glutamate receptor function in neuronal circuits of the zebrafish olfactory bulb.

There are also studies evaluating the glutamatergic signaling beyond CNS. There is growing interest in to understand the the role of signaling molecules in visual function in zebrafish retina. Previous studies on 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). Besides the visual system, the importance of glutamate receptors was demonstrated in the peripheral nervous system. Activation of ionotropic glutamate receptors on peripheral axons of primary motoneurons mediates transmitter release at the zebrafish neuromuscular junction (Todd et al., 2004).

5. Purine nucleotides and nucleosides

Nucleosides and nucleotides play their actions through activation of specific membrane receptors named purinoceptors, which are divided in two purinergic receptor families named P1 and P2 (Burnstock, 1978). Purinergic receptors were divided on the basis in the response to specific agonists and molecular cloning (Burnstock and Kennedy, 1985). Extracellular nucleotides exert their effects through two major receptor subfamilies: P2X, which are ligand-gated ion channels comprising a family of seven receptors, and P2Y, a group of eight G-protein coupled receptors (Khak et al., 2001; Abbrachio 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, considered a short-term effect, is mediated by P2X receptors that bind mainly ATP, whereas long-term effects, such as cytotoxicity, cell proliferation, differentiation and migration, occur mainly through P2Y receptors that bind both purine and pyrimidine nucleotides (Agresti et al., 2005; Fields and Burnstock, 2006). Adenosine is the endogenous agonist of P1 receptors family, which is composed by four subtypes of G-protein-coupled receptors named A_1, A_{2A}, A_{2B} and A_3 (Burnstock, 1978). A P2X subunit cloned from the zebrafish that is an orthologue of the mammalian P2X(3) subunit has been described (Egan et al., 2000). The zebrafish P2X(3) subunit mRNA is exclusively expressed at high levels in trigeminal neurons and Rohon-Beard cells during embryonic development (Boue-Gabrot et al., 2000; Norton et al., 2000). Recently, it has been shown that the *p2rx3.1* function in ectodermal cells is involved in purinergic signaling essential for proper craniofacial development and sensory circuit formation in the embryonic and larval zebrafish (Kucenas et al., 2009). The cloning and characterization of zebrafish P2X(4) and P2X(5) subunits were also performed (Diaz-Hernandez et al., 2002) and a more complete analysis of P2X family identified nine genes, which 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 subsets of Rohon-Beard neurons. In contrast to mammals, *p2rx2* was not expressed in hypocretin cells (Appelbaum et al., 2007). Previous studies also provided evidence for the presence of a P2Y1 receptor in the zebrafish thrombocytes (Gregory and Jagadeeswaran, 2002). Regarding to P1 receptors, two zebrafish A2A (*adora2a.1 and adora2a.2*) genes and one A_2B (*adora2b*) adenosine receptor gene were identified in the CNS of developing embryos. In addition, caffeine, an A_2A adenosine receptor antagonist, is neuroprotective against the adverse effects of MPTP in zebrafish

embryos, suggesting that these receptors in zebrafish may serve as useful targets for testing novel therapeutic strategies for the treatment of Parkinson disease (Boehmler et al., 2009).

Considering their importance in cell signaling, the concentration of extracellular nucleotides is tightly regulated by a variety of cell surface-located enzymes named 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 (ecto-nucleotide pyrophosphate/phosphodiesterases), alkaline phosphatases and ecto-5'-nucleotidase (Robson et al., 2006; Schetinger et al., 2007; Yegutkin, 2008). In addition 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 to nucleotide and adenosine receptors (Bonan et al., 2001; Cunha, 2001). 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 relative *entpd1-6* and *entpd8* expression profile was observed in brain, liver, and heart of zebrafish (Rosemberg et al., 2010b) and studies also showed that *entpd3* was expressed with *p2rx8* in hypothalamic region (Appelbaum et al, 2007) In zebrafish retina, NTPDases1 and 2 appear to be expressed within the germinal margin that contains proliferative and differentiating cells (Ricatti et al., 2009). Other group of enzymes involved in the control of purinergic signaling is adenosine deaminase, which is responsible for cleaving the neuromodulator

adenosine to inosine. Two members of ADA subfamilies, known as ADA1 and ADA2, were described and evidence demonstrated another similar protein group named ADAL (adenosine deaminase "like"). The existence of different ADA-related genes, their distinct expression pattern and a truncated *ADA2-1* isoform suggest a high degree of complexity in zebrafish adenosinergic system (Rosemberg et al., 2007a). The kinetic properties of these enzymes in membranes and soluble fraction of zebrafish brain were determined, indicating that the presence of ADA activity is important to regulate the adenosine/inosine levels in the CNS of this species (Rosemberg et a.,l 2008).

Several studies have demonstrated that these enzymes may be a target of neurotoxic effects induced by pesticides, alcohols, and metals. Exposure to carbofuran and malathion during 7 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 the modulation of extracellular nucleotide and nucleoside levels. Methanol or ethanol exposures, during 1 hour, decreased NTPDase activity and NTPDase1 and three isoforms of NTPDase2 mRNA transcript levels in zebrafish brain. However, no significant alterations on ecto-5'-nucleotidase activity was verified after both exposures to methanol or ethanol (Rico et al., 2006; 2008). Exposure to lead and mercury, during 24 h, 96 h and 30 days caused differential inhibitory effects on ATP, ADP and AMP hydrolysis whereas there are no significant changes in the expression of NTPDase1 and 5'-nucleotidase, following 30 days of exposure to both metals (Senger et al., 2006a). Soluble ADA activity was also decreased after both acute (24 hours) and subchronic (96 hours) exposures to mercury whereas in brain membranes the enzyme activity was inhibited only after subchronic exposure. Semiquantitative RT-PCR analysis showed that mercury chloride did not alter ADA gene expression (Senger et al., 2010).

Acute copper treatment during 24 hours altered ATP hydrolysis; however, subchronic treatment (during 96 hours) inhibited 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 exposures during 24 and 96 h whereas subchronic copper treatment also reduced the NTPDase2_mq and ecto-5'-nucleotidase expression (Rosemberg et al., 2007b). The co-existence of several enzymes in 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 in the nucleotide levels, may play a modulatory role during the evolution of neurotoxicity promoted by metals, pesticides, and organic compounds. Thus, identifying changes induced by metals and the exact mechanisms by which these enzymes regulate local nucleotide and nucleoside concentrations could be an important strategy to better understand their role as a potential target for several neurotoxins.

6. Histamine

The histaminergic system (HS) is involved in regulatory mechanisms in the brain, including alertness and sleep, physiological and behavioral processes, circadian rhythm, locomotor activity, and feeding/drinking (Schwartz, 1991). The histidine descarboxylase enzyme is responsible by histamine (HA) synthesis; however, histaminergic innervations and the molecular cloning and expression of L-histidine descarboxylase in zebrafish brain were recently described (Ericksson et al., 1998). The zebrafish histaminergic system resembles that of other vertebrates, with HA-immunoreactive fibers of different densities in all brain areas except in the cerebellum. In zebrafish embryos, the first histamine-immunoreactive neurons emerge in the ventral hypothalamus at about 85 hours post-

fertilization and at 90 hours immunoreactive fibers terminated in the dorsal telencephalon. The HA content in the adult zebrafish brain has a circadian variation with decreased concentrations during the light period, similarly to those observed in rodents (Mochizuki et al., 1992). The HS appears during the period when the larva starts to actively search for prey, thus the proposed regulator role of HS can be well defined in this context. The HA-immunoreactive neurons, which appear first in the larva likely belong to the same population of adult neurons, since they are located in the developing ventral hypothalamus and also innervate the rostrodorsal telencephalon, which is a major projection area in the adults. This area is considered in zebrafish as corresponding to the mammalian amygdala and the hippocampus, which is densely innervated by histaminergic fibres (Ericksson et al., 1998; Peitsaro et al., 2003).

The HS can have a similar alerting role in the zebrafish OT. Beyond OT, more two other regions which have been described as receiving much of its input from the auditory, lateral line, and visual system. The dorsal telencephalon and the torus semicircularis also receive a dense histaminergic innervation (Northcutt, 1981; Meek, 1990). The concentrations of the HA in zebrafish brain of are in the same range, although slightly lower, when compared to the values from higher vertebrates (Yamatodani et al., 1991).

The HA acts through ate least four types of characterized G-protein-coupled receptor in mammals: histamine H_1, H_2, H_3, and H_4 receptor (Liu et al., 2001). In the zebrafish brain histamine H_3-like receptor binding and H_3-related G-protein have been described (Peitsaro, et al., 2000). All three zebrafish HA receptor were expressed in the brain, suggesting an involvement in CNS regulation. The H_1 receptor is expressed in intestine, liver, and spleen whereas expression H_2 receptor was found in periphery in gills, heart and spleen. All three receptors were also expressed very early during development,

suggesting regulatory roles in the period. Binding sites for the H_2 and H_3- ligands are identified in the zebrafish brain in the corresponding mammalian area (Peitsaro et al., 2007).

Data concerning the zebrafish HS indicates that HA is mostly present in the CNS (Ericksson et al., 1998) and manipulation of HA levels alters behavior.This fact demonstrates that the HA content from zebrafish brain can be reduced by α-fluoromethylhistidine, and this decrease is associated with changes in the exploratory behavior and in T-maze performance. These changes might be due to reduced anxiety and some memory-related mechanisms after HA depletion (Peitsaro et al, 2003). As demonstrated by Renier (2007), that histaminergic H_1 antagonists produce a concentration-dependent reduction in immobility, with the higher concentrations producing a complete state of unresponsive immobility similar to general anesthesia.

7. Nitric Oxide

Nitric oxide (NO) is formed by endogenous NO synthase (NOS) and is involved in various normal and pathophysiological, and developmental events, suggesting a participation in plasticity processes (Mize et al., 1998; Moncada et al., 1998). NO is a free radical molecule that is formed in biological tissues from L-arginine by three major nitric oxide synthase isoforms (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), 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 both embryonic and post-embryonic life stages (Fritsche et al., 2000). Holmqvist et al. (2000) demonstrated that neuronal NOS isoform (nNOS) mRNA-expressing populations are closely associated with the proliferation zones that generate new cells throughout life,

which are ventricular regions of the telencephalon, diencephalon, and mesencephalon. The expression of NOS in zebrafish embryos was detected at 16 hours post-fertilization in the hypothalamus and, during 3 days post-fertilization, was present in discrete CNS locations (Pool et al., 2003).

8. Glycine and GABA

The correct development of spinal cord implies in normal control of movements and integration of signals from periphery. In order to promotes this integration several types of neurons and neurotransmitter systems work in a harmonized way. A balance between both excitatory (glutamate) and inhibitory (glycine and GABA) neurotransmitters are involved in this issue. Glycine receptors (GlyRs) and GABA receptors are members of the ligand-gated chloride channel family. As one of the predominant inhibitory neurotransmitter in vertebrate brain stem and spinal cord, glycine is also critically important for the regulation of interneuron differentiation during development of 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 established soon after patterning of the hindbrain and spinal cord (Saint-Amant and Drapeau, 2000). Two types of postsynaptic glycinergic receptors with different subconductance and sensibility to picrotoxin were pharmacologically identified on the Mauthner cells of zebrafish larvae (52hpf) (Legender, 1997). These types of postsynaptic receptors have been pointed as the determinant receptors to control the synaptic events on the Mauthner cells (Legender, 1998). Mutants defective in glycinergic synaptic transmission due to a lack of synaptic aggregation of GlyRs exhibit simultaneous motor neurons activation on both sides resulting in bilateral

contraction of axial muscles (Hirata et al., 2010). The distribution of GABA-containing neurons appears in zebrafish olfactory bulb (OB), telencephalon, tectum stratum, and in the hypothalamus (Kim et al., 2004). GABA receptors in zebrafish brain are present at the molecular layer, Purkinje cells and groups of presumed Golgi cells in the granular layer, both in the cerebellar corpus and valve (Delgado and Schmachtenberg, 2008). Glycine and GABA can activate homomeric GlyR channels with similar single-channel conductances but different kinetics in zebrafish (Fucile et al., 1999). The glycine receptor alpha subunits from zebrafish have already been characterized and showed that the predicted amino acid sequences were 86%, 81% and 77% identical to mammalian isoforms alpha1, alpha3 and alpha2, respectively (David-Watine et al., 1999). Moreover, the zebrafish glycine receptor demonstrated unexpectedly high sensitivity to taurine, being also activated by GABA and antagonized by nanomolar concentrations of strychnine (Imboden et al., 2001). These results were consistent with physiological findings in other fish (e.g. lamprey and goldfish), suggesting that glycine receptor from teleosts displays a lower selectivity to neurotransmitters than that reported for glycine mammalian receptors (David-Watine et al., 1999).

The glycinergic and GABAergic inhibitory control of motor neurons, the balance between excitatory and inhibitory synapses into interneurons and motor neurons underlies normal functioning of locomotor circuits that produce rhythmic motor output (Grillner et al., 1995; Hultborn and Nielsen, 2007). Since neurological effects can be detected by movement disorders, these systems appear as good choices to neurotoxicological assessment, even though few studies still contribute to this issue. In fact, a study using a phenylpyrazole insecticide (fipronil) on zebrafish embryos showed that although this insecticide acts as an inhibitor of GABA receptors, in zebrafish it may inhibit a structurally

related GlyR subtype expressed during development of spinal locomotor pathways. This inhibitory action on GlyR had no effect on the morphology of zebrafish embryos until 30 hours post-fertilization, but after this period, embryos began to show reduced body length, notochord degeneration, abnormal axial muscle morphology, and consequently locomotor defects (Stehr et al., 2006).

Baraban et al. (2005) reported that the exposure to the common convulsant agent, pentylenetetrazole, induced changes in zebrafish behavior, neural activity and significantly increased the mRNA expression of c-fos. 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 the present review, we highlight the different neurotransmitter systems described in zebrafish and their pharmacological and toxicological implications. These advances reinforce the benefits that zebrafish offers and the efforts that researchers have done in order to understand the signaling functions promoted by neurotransmitter systems in this species. In addition, it is important to reinforce the idea that this vertebrate model is an interesting tool to perform preclinical assays in large scale before pharmacological validation in rodent models and to study characteristics of aquatic toxicity and drug abuse (Figure 2). Therefore, zebrafish becomes an attractive and potential organism for several screening strategies used in drug discovery and neurotoxicity assays.

Acknowledgments

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA. 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, Volonté C, Aloisi F, Visentin S. Metabotropic P2 receptor activation regulates oligodendrocyte progenitor migration and development. Glia. 2005; 15;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, W. K., Cooper, C. E. and Knowles, R.G. Nitric oxide synthases: structure, function and inhibition. Biochem. J. Vol. 357, pp. 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, 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 Jan; 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, Bedell JA, McPherson JD, Johnson SL. The syntenic relationship of the zebrafish and human genomes, Genome Res. 2000; 10:1351–1358.

Belmaker 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;6:155–166.

Boehmler W, Petko J, Woll M, Frey C, Thisse B, Thisse C, Canfield VA, Levenson R. 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 Feb 19;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, 433-440.

Burnstock G. Purinergic signalling in neuron-glia interactions. Nat Rev Neurosci. 2006; 7(6):423-436.

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): 208–213.

Chen Y, Swnason RA. Astrocytes and brain injury. J. Cerebr.Blood F.Met 2003; 23:137–149.

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, Arévalo R. Cholinergic elements in the zebrafish central nervous system: Histochemical and immunohistochemical analysis. J Comp Neurol. 2004; 474(1): 75-107.

Cofiel LPV and Matiolli R. L-histidine enhances learning in stressed zebrafish. Braz J Med Biol Res 2009; 42:128-134.

Connaughton VP, Behar TN, Liu WLS, Massey SC. Immunocytochemical localization of excitatory and inhibitory neurotransmitters in the zebrafish retina. Vis. Neurosci 1999; 16:483–490.

Cote F, Fligny C, Bayard E, Launay JM, Gershon MD, Mallet J, Vodjdani G. Maternal serotonin is crucial for murine embryonic development. Proc Natl Acad Sci U S A 2007; 104: 329–334.

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.

Cunha RA. Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals. Neurochem Res 2001;26: 979-991.

Danbolt NC. Glutamate uptake. Prog. Neurobiol 2001; 65:1–105.

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

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 M, 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 JS 2nd, Imai M, Edelberg JM, Rayburn H, Lech M, Beeler DL, Csizmadia E, Wagner DD, Robson SC, Rosenberg RD. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med. 1999; 5(9):1010-7.

Ericksson 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. and Peitser, B. Nitric oxide and vascular reactivity in development zebrafish, Danio rerio. Am. J. Physiol. Reg. Int. Comp. Physiol. Vol. 279, pp. 2200-2207.

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): 369-383.

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, 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 Aug;8(6):586-99.

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; 67:773–782.

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; 28:245–250.

Goldman-Rakic, P. S. (1998) The cortical dopamine system: role in memory and cognition. Adv Pharmacol 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, Orlovsky GN, Wallen P. Neural networks that coordinate locomotion and body orientation in lamprey. Trends Neurosci 1995; 18:270–279.

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; 214:277–284.

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, Fjose A Seo HC. Identification and distribution of nitric oxide synthase in the brain the zebrafish. Neuroscience Letters. 2000; 292:119-122.

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; 189:111–121.

Ivetac I, Becanovic J, Krishnapillai V. Zebrafish: genetic tools and genomics, Asia–Pacific J. Mol. Biol. Biotechnol. 2000; 8:1–11.

Ikonomidou C, Price MT, Mosinger JL, Frierdich G, Labruyere J, Salles KS, Olney JW. 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.

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

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, Kawamoto T, Kitagawa K, Nakayama K, Ijiri I, Kinoshita H. Cholinergic alterations following alcohol exposure in the frontal cortex of Aldh2-deficient mice models. Brain Res. 2009; 1295: 37-43.

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, Voigt M, Humphrey PP. 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, 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.

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.

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):2856–2870.

Legendre P. Pharmacological Evidence for Two Types of postsynaptic Glycinergic Receptors on the Mauthner Cell of 52-h-Old Zebrafish Larvae. J Neurophysiol 1997; 77:2400-2415.

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

Levin ED, Bencan Z, Cerutti DT. Anxiolytic effects of nicotine in zebrafish. Physiol Behav. 2007; 90(1): 54-8.

Levin ED, Chen E. Nicotinic involvement in memory function in zebrafish. Neurotoxicol Teratol. 2004; 26(6): 731-5.

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, N.J., Rothstein, J.D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiol. Dis. 2004; 15:461–473.

Mattson MP, Chan SL, Duan W. Modification of brain ageing and neurodegenerative disorders by genes, diet, and behavior. Physiol. Rev. 2002; 82:637–672.

McDearmid JR, Liao M, Drapeau P. Glycine receptors regulate interneuron differentiation during spinal network development. Proc Natl Acad Sci U S A. 2006; 103(25):9679-84.

Meek J. Tectal morphology: connections, neurons and synapses. (1990) In Douglas, R.H. & Djamgoz, M. B. A. (eds), The visual system of Fish. Chapman and Hall, London, pp. 239-277.

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. Dopamine receptors: from structure to function. Physiol Rev 1998; 78, 189–225.

Mize R, Dawson T, Dawson V and Friedlander M. Nitric oxide in brain development, plasticity, and disease. Progress in Brain Research, Vol 118, Elsevier, Amsterdam, 1998.

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):391–394.

Moncada S, Nistico G, Bagetta G, Higgs E. Nitric oxide and the cell proliferation, differentiation and death, Portland Press, UK, 1998.

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 Suppl 2008;3:S129–134.

Nakanishi S, Masu M, Bessho Y, Nakajima Y, Hayashi Y, Shigemoto R; Molecular diversity of glutamate receptors and their physiological functions. Experientia Supplements 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. (1981) In: Davis, R. E. & Northcutt, R.G. Fish Neurobiology. 2: Higher Brain Areas and Functions. University of Michigan Press, Ann. Arbor, pp. 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.

Obholzer N, Wolfson S, Trapani JG, Mo W, Nechiporuk A, Busch-Nentwich E, Seiler C, Sidi S, Söllner C, Duncan RN, Boehland A, Nicolson T. 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, Chen YC, Priyadarshini M, Kudo H, Semenova S, Sundvik M, Sallinen V. The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. Neurobiol Dis. 2010; 40(1): 46-57.

Panula P, Sallinen V, Sundvik M, Kolehmainen J, Torkko V, Tiittula A, Moshnyakov M, Podlasz P. Modulatory neurotransmitter systems and behavior: towards zebrafish models of neurodegenerative diseases. Zebrafish. 2006; 3(2):235-47.

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.

Peitsaro N, Kaslin J, Anitchick O, V, Panula P. Modulation of histaminergic system and behaviour by alpha-fluorothylhistidine in zebrafish. J. Neurochem 2003; 86:432-41.

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, 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; 205-214-73.

Pool KL, Richardson M., Lan SC., Khoo HE, Korzh V. Expression pattern of neuronal nitric oxide synthase in embryonic zebrafish. Gene Expression Patterns, 2007; 3(4):463-466.

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. 2010; Oct 28. Article in press.

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 Jun;58(2):162-93.

Renier C, Faraco JH, Bourgin P, Motley T, Bonaventure P, Rosa F, Mignot E. Genomic and functional conservation of sedative-hypnotic targets in the zebrafish. Pharmacogenet Genomics. 2007; 17(4):237-53.

Ricatti MJ, Alfie LD, Lavoie EG, Sévigny J, Schwarzbaum PJ, Faillace MP. Immunocytochemical 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. 2010; doi:10.1016/j.neuro.2010.11.001

Rico EP, Rosemberg DB, Dias RD, Bogo MR, Bonan CD. Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicol Lett. 2007; 174(1-3): 25-30.

Rico EP, Rosemberg DB, Senger MR, Arizi Mde B, Bernardi GF, Dias RD, Bogo MR, Bonan CD. Methanol alters ecto-nucleotidases and acetylcholinesterase in zebrafish brain. Neurotoxicol Teratol. 2006; 28(4): 489-96.

Rico EP, Rosemberg DB, Senger MR, de Bem Arizi M, Dias RD, Souto AA, Bogo MR, Bonan CD. Ethanol and acetaldehyde alter NTPDase and 5'-nucleotidase fromzebrafish brain membranes. Neurochem Int. 2008; 52(1-2):290-6.

Rico EP, Senger MR, Fauth Mda G, Dias RD, Bogo MR, Bonan CD. ATP and ADP hydrolysis in brain membranes of zebrafish (Danio rerio). Life Sci. 2003 Sep 5; 73(16):2071-82.

Rico EP, de Oliveira DL, Rosemberg DB, Mussulini BH, Bonan CD, Dias RD, Wofchuk S, Souza DO, Bogo MR. 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, 409–430.

Rodd-Henricks ZA, McKinzie DL, Edmundson VE, Dagon CL, Murphy JM, McBride WJ, Lumeng L, Li TK. 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, da Rocha RF, Rico EP, Zanotto-Filho A, Dias RD, Bogo MR, Bonan CD, Moreira JC, Klamt F, Souza DO. 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, Guidoti MR, Dias RD, Souza DO, Bonan CD, Bogo MR. 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, Langoni AS, Spinelli JT, Pereira TC, Dias RD, Souza DO, Bonan CD, Bogo MR. NTPDase family in zebrafish: Nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart. Comp Biochem Physiol B Biochem Mol Biol. 2010; 155(3):230-40.

Rosemberg DB, Rico EP, Senger MR, Arizi Mde B, Dias RD, Bogo MR, Bonan CD. 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, Souza DO. Kinetic characterization of adenosine deaminase activity in zebrafish (Danio rerio) brain. Comp Biochem Physiol B Biochem Mol Biol. 2008; 151(1):96-101.

Saint-Amant L, Drapeau P. Motoneuron activity patterns related to the earliest behavior of the zebrafish embryo. J Neurosci 2000; 20:3964–3972.

Sallinen V, Sundvik M, Renila I, Peitsaro N, Khrustalyov D, Anichtchik O, Toleikyte G, Kaslin J, Panula P. 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, 1-5 l.

Seibt KJ, Oliveira Rda 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.

Segovia G, Porras A, Del Arco A, Mora F. Glutamatergic neurotransmission in aging: a critical perspective. Mech Ageing Dev. 2001; Jan;122(1):1-29.

Senger MR, Rico EP, de Bem Arizi M, Frazzon AP, Dias RD, Bogo MR, Bonan CD. 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, 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, Bonan CD. Carbofuran and malathion inhibit nucleotide hydrolysis in zebrafish (Danio rerio) brain membranes. Toxicology. 2005; 212(2-3):107-15.

Senger MR, Rosemberg DB, Rico EP, de Bem Arizi M, Dias RD, Bogo MR, Bonan CD. 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, Bogo MR, Bonan CD. 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, Takahashi K, Poo MM, Baier H. 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.

Stehr CM, Linbo TL, Incardona JP, Scholz NL. The Developmental Neurotoxicity of Fipronil: Notochord Degeneration and Locomotor Defects in Zebrafish Embryos and Larvae. Toxicol Sci 2006; 92(1): 270–278.

Sukardi H, Ung CY, Gong Z, Lam SH. Incorporating zebrafish omics into chemical biology and toxicology. Zebrafish. 2010; 7(1):41-52.

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, McBride WJ. 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.

Vascotto SG, Beckham Y, Kelly GM. The zebrafish's swim to fame as an experimental model in biology, Biochem. Cell. Biol. 1997; 75: 479–485.

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 WS Jr. 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. (1991). Structure and functions of the histaminergic neuron system. In Histamine and Histamine Agonists. Springer, Heidelberg, pp. 243-283.

Yegutkin GG. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochim Biophys Acta 2008; 1783(5):673-694.

Zimmermann H. Ectonucleotidases: some developments and a note on nomenclature. Drug Dev Res. 2001; 52: 44-56.

Figure Legends

Figure.1: Hallmarks of research about neurotransmission in zebrafish. The timeline shows important studies related to players (enzymes, receptors, transporters) involved in neurotransmitter systems described in this species in the last 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; 5HT, serotonin.

Figure 2: The zebrafish model offers the potential to evaluate the effects promoted by several compounds. The figure shows some emergent approaches and perspectives to study the cellular, morphological, physiological, and behavioral aspects using both larvae and adult zebrafish. The strategies described could be used as interesting tools for testing a potential neuroprotective action of distinct compounds in a fast and large-scale manner.

Figures

Na⁺-dependent Glu transporters in brain (Rico et al., 2010)

5HT and DA levels after alcohol treatment (Chatterjee and Gerlai, 2009)

GABA receptors in the cerebellar corpus and (Delgado and Schmachtenberg, 2008)

Different ADA members and kinetic properties (Rosemberg et al., 2007)

DA and 5HT systems have similarities to mammals (Panula et al., 2006)

The developmental pattern of ChAT positive neurons (Arenzana et al., 2005)

Characterization of brain ecto-5'-nucleotidase (Senger et al., 2004)

Modulation of HA system and behavior by α-fluoromethylhistidine (Peitsaro et al., 2004).

Localization of cholinergic elements in the CNS (Clemente et al., 2004)

Distribution of GABA-containing neurons (Kim et al., 2004)

ATP and ADP hydrolysis in brain membranes (Rico et al., 2003)

Expression of NOS in CNS (Pool, et al., 2003)

Characterization of iGluR in olfactory bulb (Edwards and Michel, 2003)

Purinergic P2X(3) subunit mRNA is expressed during embryonic development (Boue-Gabrot et al., 2000; Egan et al., 2000; Norton et al., 2000)

Gly and GABA activate homomeric GlyR channels with similar single-channel conductances but different kinetics (Fucile et al., 1999)

Development of HA neurons and expression of histidine decarboxylase mRNA in the brain (Eriksson et al., 1998)

Pharmacological identification of Gly receptors on the Mauthner cells (Legender, 1997)

2010

2005

2000

1995

Figure 1

Figure 2

III.1 Discussão

Os resultados apresentados neste estudo demonstram o efeito da exposição crônica (7, 14 e 28 dias) ao etanol sobre parâmetros dos sistemas glutamatérgico, purinérgico, bem como sua influência sobre a expressão de BDNF em cérebro de zebrafish. Com o propósito de caracterizar os membros relacionados aos EAATs nesta espécie, no primeiro capítulo foi realizada um busca no seu genoma através de uma análise filogenética, na qual foram identificados diferentes genes relacionados á família dos transportadores de glutamato dependentes de sódio. Utilizando as sequências gênicas deduzidas de outros organismos como parâmetro de busca, foram encontrados ortólogos de EAAT$_{(1-5)}$ em zebrafish, os quais agruparam com elevado valor de suporte aos respectivos transportadores de outras espécies.

Considerando os cinco membros de EAAT conhecidos, a estratégia utilizada possibilitou encontrar sete genes relacionados a esta família de transportadores. Para os genes relacionados aos membros EAAT1 e EAAT5, foram identificados mais de um parálogo. Este fato poderia ser atribuído a um evento de duplicação genômica ocorrido na infraclasse dos teleósteos (Amores et al., 1998; Postlethwait et al., 1998), uma vez que foi demonstrado que os mesmos tendem a apresentar distintas cópias para uma mesma família gênica em comparação aos mamíferos (Force et al., 1999; Postlethwait et al., 1999). Embora muitos genes tenham sido perdidos evolutivamente, algumas sequências gênicas se apresentam duplicadas e com um elevado grau de homologia com os genes de mamíferos.

A análise transcricional demonstrou um perfil diferencial do padrão de expressão dos EAATs em telencéfalo, tecto óptico e cerebelo de zebrafish, sugerindo que os diversos membros previamente identificados poderiam exercer um papel na captação de glutamato nestas estruturas cerebrais. Desta forma, parâmetros referentes à captação de glutamato dependente de sódio foram verificados e um distinto perfil de captação foi observado

através das variáveis tempo e temperatura. Sabe-se que o controle dos níveis extracelulares de glutamato exercido pelos transportadores dependentes de sódio é extremamente importante para a manutenção do tônus glutamatérgico sináptico (Camacho e Massieu, 2006). Portanto, esta primeira demonstração funcional do transporte de glutamato dependente de sódio em cérebro de zebrafish indica seu papel no sentido de estabelecer o controle da sinalização glutamatérgica nesta espécie.

Recentemente, foi realizada uma análise filogenética relacionada à família de proteínas carreadoras de solutos conhecida como "solute carrier family 1 (SLC1)" (Gesemann et al., 2010). Considerando que os EAATs estão incluídos na família SLC1, no segundo capítulo foi apresentado o estabelecimento de uma nomenclatura proposta por HUGO Gene Nomenclature Committee no sentido de definir uma designação comum a todos estes transportadores (Neuhauss et al., 2010). A disfunção do transporte de glutamato em SNC é uma característica que ocorre em diversas situações patológicas, tais como morte neuronal, isquemia, hipóxia e trauma (Choi, 1988; Ikonomidou et al., 1989; Danbolt, 2001). O acúmulo de glutamato na fenda sináptica acima dos níveis fisiológicos promove um aumento na estimulação de seus receptores, gerando o fenômeno conhecido como excitotoxicidade (Nicolaidis et al., 2005).

Diversos estudos neuroquímicos têm surgido no sentido de elucidar as ações do álcool nos sistemas de neurotransmissão (Rico et al., 2007; Rico et al., 2008; Heinz et al., 2009; Silberman et al., 2009; Stuber et al., 2010). Alterações na sinalização glutamatérgica parecem desempenhar um papel chave no processo neurodegenerativo induzido pelo consumo excessivo de álcool. Esel (2006) demonstrou que a administração crônica de etanol promove modificações na sinalização em receptores NMDA, causando um desequilíbrio nos níveis intracelulares de Ca^{2+}. Além disso, o etanol é capaz de afetar o

sistema de transporte de glutamato em diferentes modelos experimentais, sugerindo que múltiplos fatores poderiam estar envolvidos nos processos de abuso de álcool, tolerância e dependência (Smith, 1997; Melendez et al., 2005).

A busca de modelos animais alternativos que possibilitam avaliar parâmetros bioquímicos, moleculares e toxicológicos tem contribuído para compreender mecanismos biológicos associados, bem como ajudar no desenvolvimento e o "screening" de compostos. Na última década, a aplicação do zebrafish em estudos neuroquímicos e comportamentais relacionados aos efeitos promovidos pelo álcool está possibilitando um avanço no conhecimento dos mecanismos envolvidos no abuso de álcool (Dlugos & Rabin, 2003; Lockwood et al., 2004; Maximino et al., 2011). Devido à presença de um amplo e complexo repertório comportamental, o zebrafish adulto está consolidado como um atrativo modelo capaz de mimetizar os efeitos relacionados à intoxicação por etanol verificados em roedores, tais como a resposta bifásica na atividade locomotora (Gerlai et al., 2000). Em virtude do surgimento de evidências relacionadas ao sistema glutamatérgico no SNC do zebrafish e do crescente uso desta espécie em investigações envolvendo a ação prolongada do etanol, no terceiro capítulo estudamos o efeito crônico do etanol nos parâmetros relacionados ao transporte de glutamato previamente caracterizado no primeiro capítulo. A exposição ao etanol foi capaz de promover alterações na captação de glutamato dependente de sódio, bem como no padrão de expressão gênica dos transportadores de glutamato. Esses resultados sugerem que o efeito inibitório observado poderia provocar um desbalanço nos níveis de glutamato na fenda sináptica de forma que a sinalização glutamatérgica poderia estar mediando os efeitos neurotóxicos promovidos pelo etanol.

O ATP é um cotransmissor liberado com clássicos neurotransmissores na maioria das sinapses do SNC, incluindo o glutamato (Burnstock, 2004). As ectonucleotidases são

enzimas capazes de hidrolisar, e assim, controlar os níveis extracelulares de nucleotídeos para os receptores P2 quanto de nucleosídeos para os receptores P1 (Bonan et al., 2001; Sevigny et al., 2002). Além do mais, foi demonstrado o efeito agudo do etanol nas ectonucleotidases em membranas cerebrais de zebrafish (Rico et al., 2008). Desse modo, no quarto capítulo foi verificado se a exposição crônica ao etanol poderia alterar as ectonucleotidases tanto em nível molecular quanto cinético nesta espécie. Após 7 e 14 dias de exposição, foram observadas alterações na atividade das NTPDases. Uma vez que estas enzimas contribuem para a manutenção dos efeitos fisiológicos dos nucleotídeos extracelulares, a influência desta cascata enzimática envolvida no controle destes nucleotídeos tem sido proposta em diversas situações fisiológicas (Agteresch et al., 1999). Assim, nossos resultados sugerem que o efeito inibitório observado na hidrólise de ATP e ADP após a exposição crônica ao etanol poderia induzir um aumento nos níveis extracelulates de ATP e, consequentemente, promover diversos processos relacionados com a excitabilidade cerebral. Além do efeito do etanol no catabolismo das purinas, também tem surgido evidências relacionadas ao seu consumo em torno dos receptores purinérgicos. Um recente estudo utilizando uma abordagem genômica/proteômica identificou que o gene para o receptor P2X4 (*p2rx4*) é um potencial candidato para a predisposição a consumo de etanol (Tabakoff et al. 2009).

A adenosina, produto final da via das ectonucleotidases, atua como um importante modulador sináptico no SNC (Dunwiddie & Masino, 2001). Este nucleosídeo exerce seus efeitos em receptores inibitórios (A_1 e A_3) e facilitatórios (A_{2A} e A_{2B}) (Cunha, 2001). A sinalização mediada pela adenosina pode ser regulada através de transportadores, seguida por fosforilação à AMP pela adenosina cinase ou desaminada à inosina através da ação da ADA intracelular. Entretanto, estudos demonstraram a presença de uma ecto-ADA, a qual

está ancorada à membrana plasmática, controlando os níveis de adenosina extracelular para seus respectivos receptores P1(Franco et al., 1998). Estudos realizados por Choi et al. (2004) demonstraram que o etanol promove uma inibição no transportador bidirecional de adenosina (ENT1), levando a um acúmulo de adenosina no espaço extracelular em cultura de astrócitos. Com a finalidade de verificar se o etanol poderia alterar outros parâmetros da sinalização adenosinérgica, no quinto capítulo, foi avaliada a ação promovida pela exposição crônica ao etanol na atividade de desaminação de adenosina em frações solúvel (citosólica) e de membrana cerebrais em zebrafish. Embora não tenham sido observadas alterações na atividade da ADA citosólica, ocorreu uma significativa inibição da ecto-ADA após 28 dias de exposição. Foi demonstrado que esta enzima está co-localizada com receptores A_1 e A_{2B} em células neuronais e gliais, sugerindo um envolvimento na regulação da sinalização adenosinérgica (Franco et al., 1998; Herrera et al., 2001). Este resultado permite sugerir que através da redução da atividade da ecto-ADA promovida pelo etanol, um possível acúmulo dos níveis extracelulares de adenosina poderia modificar a sinalização mediada pelo nucleosídeo em cérebro de zebrafish.

A relação entre o sistema adenosinérgico e fatores neurotróficos tem sido estabelecida. Embora o mecanismo ainda não esteja completamente elucidado, sabe-se que sinalização via receptores A_{2A} modula a função, transcrição e liberação de BDNF (Patterson et al., 2001; Potenza et al., 2007). Desta forma, o sexto capítulo teve como objetivo verificar o efeito crônico do etanol na expressão de BDNF e de seu receptor trkB em cérebro de zebrafish. Neste estudo foi encontrado um aumento na expressão gênica do *BDNF* após 14 e 28 dias de exposição. Interessantemente, somente após 28 dias foi detectado um aumento significativo nos níveis de proteína para BDNF.

A relação entre os resultados encontrados referentes às sinalizações glutamatérgica e purinérgica com os níveis de BDNF poderia ser interpretada de formas distintas. As reduções tanto na captação de glutamato dependente de sódio quanto na atividade das ectonucleotidases após 7 e 14 dias de exposição poderiam promover uma resposta biológica no sentido de conduzir a um aumento na expressão de *BDNF* após 14 e 28 dias. Sendo assim, a disfunção glutamatérgica e purinérgica poderia ser uma causa para o posterior aumento dos níveis de BDNF. Também foram observadas diminuições na atividade da ecto-ADA e um retorno da hidrólise de nucleotídeos aos valores basais após 28 dias de exposição, o que poderia elevar os níveis de adenosina extracelular. Cunha (2008) demonstrou que a sinalização mediada pelos receptores A_{2A} ocorre, preferencialmente, através da união da adenosina formada pela cascata das ectonucleotidases, enquanto que os receptores A_1 seriam predominantemente ativados pela adenosina transportada do meio intracelular para a fenda sináptica. Uma vez que a ativação de receptores A_{2A} leva a uma ativação da AC, ocorre um aumento na geração de AMPc. Este aumento do AMPc promoveria a dissociação da subunidade regulatória da PKA para subunidade catalítica permitindo a fosforilação de outros alvos intracelulares, tais como o CREB (Newton & Messing, 2006). A via de sinalização do CREB é capaz de influenciar diversos mediadores celulares, originando uma complexa rede de sinalização a qual é afetada pelo etanol. (Moonat et al. 2009). Assim, o aumento dos níveis de transcrito e proteína após o período mais longo de exposição ao etanol (28 dias) poderia também estar associado aos efeitos do etanol na sinalização mediada por CREB em cérebro de zebrafish.

O sistema de neurotrofinas é capaz de regular sobrevivência celular, proliferação, destino de precursores neurais, crescimento de dentritos e axônios e a funcionalidade da expressão e atividade de importantes proteínas tais como canais iônicos, e receptores de

neurotransmissores, dentre eles os associados à sinalização glutamatérgica (Nestler, 2001; Huang & Reichardt, 2003; Kalivas, 2009). Considerando a importância da função neurotrófica, é possível sugerir que o aumento na expressão de BDNF poderia ser responsável por promover novamente um equilíbrio no tônus glutamatérgico e purinérgico.

O etanol atua no sistema gabaérgico através da ativação dos receptores $GABA_A$ (Santhakumar et al., 2007). Os efeitos inibitórios promovidos pelo consumo crônico do etanol induzem neuroadaptação, estimulando a regulação via sistema glutamatérgico e contrabalançando a ação inibitória do etanol (De Witte, 2004). O etanol foi capaz de promover reduções tanto na captação de glutamato bem como nas atividades das NTPDases após 7 e 14 dias. Contudo, não foram observadas alterações após 28 dias. Desta forma, podemos sugerir que em períodos mais prolongados de exposição ao etanol, ocorreria algum evento adaptativo, no qual estes parâmetros não são afetados.

Os resultados apresentados nesta tese apontam que o etanol é capaz de modular diferentes sistemas de neurotransmissão em zebrafish. Considerando que este modelo é capaz de absorver diretamente compostos adicionados na água, o zebrafish oferece vantagens para estudos que visam uma melhor compreensão das bases neuroquímicas envolvidas em respostas comportamentais e toxicológicas. Assim, o sétimo capítulo propõe uma revisão abordando a relevância deste vertebrado para estudos relacionados a aspectos farmacológicos e toxicológicos. O estudo relacionou a importância de distintos sistemas de neurotransmissão excitatório e inibitório em zebrafish, tais como dopaminérgico, serotoninérgico, colinérgico, glutamatérgico, purinérgico, histaminérgico, nitrérgico, glicinérgico e gabaérgico. A semelhança genômica e funcional dos sistemas de neurotransmissão do zebrafish comparado aos de mamíferos, faz desta espécie um potencial modelo para estudos em larga escala como uma versátil ferramenta pré-clínica para a busca

de novas drogas, bem como para investigações toxicológicas. O significativo aumento do conhecimento em torno dos sistemas de neurotransmissão em zebrafish e a elucidação dos efeitos farmacológicos e toxicológicos poderiam levar a novas estratégias e apropriadas prioridades na pesquisa no sentido de oferecer suporte a descobertas nas áreas ambiental e biomédica.

III.2 Conclusão final

Mesmo que os mecanismos responsáveis de causa e tolerância dos efeitos do etanol sobre os parâmetros neuroquímicos abordados nesta tese ainda não estejam bem esclarecidos, é importante ressaltar que as respostas observadas poderiam estar relacionadas com uma ação promovida pelo álcool no SNC dependente do tempo de exposição. Com os resultados apresentados nesta Tese de Doutorado, podemos concluir que os sistemas purinérgico e glutamatérgico, bem como a expresão de fator neurotrófico são alterados pelo etanol em cérebro em cérebro de zebrafish. Portanto, nosso trabalho contribui para um melhor esclarecimento sobre a farmacologia deste álcool e o papel das destas sinaçizações estudadas nas respostas promovidas após a exposição crônica do etanol no SNC de zebrafish.

Referências Bibliográficas

Ackermann, G. E., Paw, B. H. 2003. Zebrafish: a genetic model for vertebrate organogenesis and human disorders. Front. Biosci. 8, 1227-1253.

aging: a critical perspective. Mechan. Aging Develop. 122, 1-29.

Agteresch, H.J., Dagnelie, P.C., van den Berg, J.W., Wilson, J.H. 1999. Adenosine triphosphate: established and potential clinical applications. Drugs 58, 211–232.

Amatruda, J. F., Shepard, J. L., Stern, H. M., Zon, L. I. 2002, Zebrafish as a cancer model system. Cancer Cell 1, 229-231.

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.

Anderson, C. M., Bridges, R. J., Chamberlin, A. R., Shimamoto, K., Yasuda-Kamatami, Y., Swanson, R. A., 2001., Differing effects of substrate and non-substrate transport inhibitors on glutamate uptake reversal. J. Neurochem. 79, 1207-1216.

Anderson, C. M., Swanson, R. A., 2000., Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia. 32, 1–14.

Anderson, K. V., Ingham, P. W. 2003. The transformation of the model organism: a decade of developmental genetics. Nat. Genet. 33, 285-293.

Arolfo M.P., Yao L., Gordon A.S., Diamond I., Janak P.H. 2004 Ethanol operant self-administration in rats is regulated by adenosine A2 receptors, Alcohol Clin. Exp.Res. 28, 1308-1316.

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 humam genomes. Gen. Res. 10, 1351-1358.

Barnard, E. A., Simon, J., Webb, T. E. 1997. Nucleotide receptors in the nervous system: an abundant component using diverse transductional mechanisms. Mol. Neurobiol. 15, 103-129.

Battastini, A.M., da Rocha J.B., Barcellos C.K., Dias, R.D., Sarkis, J.J. 1991. Characterization of an ATP diphosphohydrolase (EC 3.6.1.5) in synaptosomes from cerebral cortex of adult rats. Neurochem. Res. 16, 1303-1310.

Battastini, A.M., Oliveira, E.M.; Moreira, C.M.; Bonan, C.D.; Sarkis, J.J.; Dias, R.D. 1995. Solubilization and characterization of an ATP diphosphohydrolase (EC 3.6.1.5) from rat brain synaptic plasma membranes. Biochem. Mol. Biol. Int. 37, 209-219.

Behra, M., Cousin. X., Bertrand, C., Vonesch, J. L., Biellmann, D., Chatonnet, A., Strahle, U. 2002. Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo. Nat. Neurosci. 5, 111-118.

Bilotta, J., Saszik, S., Givin, C.M., Hardesty, H.R., Sutherland S.E. 2002. Effects of embryonic exposure to ethanol on zebrafish visual function, Neurotox. and Teratol. 24, 759-766.

Binder, D.K., Scharfman, H.E. 2004. Brain-derived neurotrophic factor. Growth Factors. 22, 123-31.

Boehmler, W., Obrecht-Pflumio, S., Canfield, V., Thisse, C., Thisse, B., Levenson, R. 2004. Evolution and expression of D2 and D3 dopamine receptor genes in zebrafish. Dev. Dyn. 230 , 481-493.

Boeynaems, J. M., Communi, D., Savi, P., Herbert, J. M. 2000. P2Y receptors: in the middle of the road. TIPS 21, meeting report.

Bolaños, C.A., Nestler, E.J. 2004. Neurotrophic mechanisms in drug addiction. Neuromolecular Med. 5, 69-83.

Bonan, C.D., Schetinger, M.R.C., Battastini, A.M.O., Sarkis, J.J.F. 2001. Ectonucleotidase and synaptic plasticity: implications in physiological and pathological condictions. Drug. Dev. Res. 52, 57-65.

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

Broughton, R. E., Milan, J. E., Roe, B. A. 2001. The complete sequence of the zebrafish (*Danio rerio*) mitochondrial genome and evolutionary patterns in vertebrate mitochondrial DNA. Genome Res. 11, 1958-1967.

Brundege, J. M., Dunwiddie, T. V. 1997. Role of adenosine as a modulator of synaptic activity in the central nervous system. Ad. Pharmacol. 39, 353-391.

Burnstock G. 2004. Cotransmission, Curr. Opin. Pharmacol. 4, 47-52.

Burnstock, G. 1972. Purinergic nerves. Pharmacol. Rev. 24, 509-581.

Burnstock, G. 1999. Purinergic cotransmission. Brain Res. Bull. 50, 355-357.

Burnstock, G. 2004. Cotransmission, Curr. Opin. Pharmacol. 4, 47-52.

Burnstock, G., Campbell, G., Satchell, D. G., Smythe, A. 1970. Evidence that adenosine triphosphate or a related nucleotide is the transmittersubstance released by non-adrenergic inhibitory nerves in the gut. Br. J. Pharmacol. 40, 668-688.

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

Camacho, A.,Massieu, L. 2006. Roleof glutamate transporters in the clearance and

Carvalho, A.L., Caldeira, M.V., Santos, S.D., Duarte, C.B. 2008. Role of the brain-derived neurotrophic factor at glutamatergic synapses. Br J Pharmacol. 153, 1:S310-24.

Carvan 3rd, M.J., Loucks, E., Weber, D.N., Williams, F.E., 2004. Ethanol effects on the developing zebrafish: neurobehavior and skeletal morphogenesis. Neurotoxicol. Teratol. 26, 757-768.

Chao, M.V., Rajagopal, R., Lee, F.S. 2006. Neurotrophin signalling in health and disease. Clin. Sci. (Lond). 110, 167-73.

Choi DS, Cascini MG, Mailliard W, Young H, Paredes P, McMahon T, Diamond I, Bonci A, Messing RO. 2004. The type 1 equilibrative nucleoside transporter regulates ethanol intoxication and preference. 7, 855-61.

Choi, D.W. 1988. Glutamate neurotoxicity and disease of nervous system. Neuron 1, 623-34.

Clemente, D., Porteros, A., Weruaga, E., Alonso, J.R., Arenzana, F.J., Aijon, J., Arevalo, R. 2004. Cholinergic elements in the zebrafish central nervous system: Histochemical and immunohistochemical analysis, J. Comp. Neur. 474, 75-107.

Climent, E., Pascual, M., Renau-Piqueras, J., Guerri, C. 2002. Ethanol exposure enhances cell death in the developing cerebral cortex: role of brain-derived neurotrophic factor and its signaling pathways. J. Neurosci. Res. 68, 213-25.

Communi, D., Janssens, R., Suarez-Huerta, N., Robaye, B., Boeynaems, J. M. 2000. Advances in signalling by extracellular nucleotides: the role and transduction mechanisms of P2Y receptors. Cell. Sig. 12, 351-360.

Cunha, R. A. 2001. Adenosine as a neuromodulator and as a homeostatic regulator in thenervous system: different roles, different sources and different receptors. Neurochem. Int. 38, 107-125.

Cunha, R. A., Ribeiro, J. A. 2000. ATP as a presynaptic modulator. Life Sci. 68, 119-137.

Cunha, R.A. 2008. Different cellular sources and different roles of adenosine: A1 receptor-mediated inhibition through astrocytic-driven volume transmission and synapse-restricted A2A receptor-mediated facilitation of plasticity. Neurochem Int. 52, 65-72.

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

De Witte P. Imbalance between neuroexcitatory and neuroinhibitory amino acids causes craving for ethanol. Addict Behav. 2004 Sep;29(7):1325-39.

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, 354-361.

Diaz-Hernandez, M., Cox, J. A., Migita, K., Haines, W., Egan, T. M., Voigt, M. M. 2002. Cloning and characterization of two novel zebrafish P2X receptor subunits. Biochem. Biophys. Res. Com. 295 (4), 849-853.

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

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

Dodd, A., Curtis, P. M., Williams, L. C., Love, D. A. 2000. Zebrafish: bridging the gap between development and disease. Hum. Mol. Genet. 9 (16), 2443-2449.

Dohrman, D.P., Diamond, I., Gordon, A.S., 1997. The role of the neuromodulator adenosine in alcohol's actions, Alcohol Health Res. World, 21, 136-143.

Dooley, K., Zon, L. I. 2000. Zebrafish: a model system for the study of human disease. Curr. Op. Genet. Dev. 10, 252-256.

Dunwiddie, T.V.; Masino, S.A. 2001.The role and regulation of adenosine in the central nervous system. Annu. Rev. Neurosci. 24 31-55.

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

Egan, T. M., Cox, J. A., Voigt, M. M. 2000. Molecular cloning and functional characterization of the zebrafish ATP-gated ionotropic receptor P2X3 subunit. FEBS Lett. 475, 287-290.

Esel, E. 2006. Neurobiology of alcohol withdrawal inhibitory and excitatory neurotransmitters. Turk Psikiyatri Derg. 17, 129-37.

Esel, E. 2006. Neurobiology of alcohol withdrawal inhibitory and excitatory neurotransmitters. Turk Psikiyatri Derg. 17, 129-37.

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, 1531-1545.

Franco, R., Mallol, J., Casado, V., Lluis, C., Canela, E.I., Saura, C., Blanco, J., Ciruela, F. 1998. Ecto-adenosine deaminase: an ecto-enzyme and a co-stimulatory protein acting on a variety of cell surface receptors. Drug Develop. Res. 45, 261–68.

Fredholm, B. B., Lizerman, A. P., Jacobson, K. A., Klotz, K. N., Linden, J. 2001. International union of pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol. Rev. 53 (4), 527-552

Frizzo, M.E., Schwalm, F.D., Frizzo, J.K., Soares, F.A., Souza, D.O. 2005. Guanosine enhances glutamate transport capacity in brain cortical slices. Cell Mol Neurobiol. 25, 913-21.

Gasic, G. P., Hollman, M., 1994., Molecular neurobiology of glutamate receptors. Annu. Rev. Physiol. 54: 507-536.

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.

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, 773-82.

Gesemann, M., Maurer, C.M., Neuhauss, S.C. 2010. Excitatory amino acid transporters in the zebrafish: Letter to "Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain" from Rico et al. Brain Res Bull. 83, 202-6.

Grunwald, D. J., Eisen, J. S. 2002. Headwaters of the zebrafish – emergence of a new model vertebrate. Nat. Rev. Genet. 3, 717-724.

Guerri, C. 1998. Neuroanatomical and neurophysiological mechanisms involved in central nervous system dysfunctions induced by prenatal alcohol exposure. Alcohol Clin. Exp. Res. 22, 304-12.

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

Heinrich, G., Pagtakhan, C.J. 2004. Both 5' and 3' flanks regulate Zebrafish brain-derived neurotrophic factor gene expression. BMC Neurosci. 5, 19.

Heinz, A., Beck, A., Wrase, J., Mohr, J., Obermayer, K., Gallinat, J., Puls, I., 2009. Neurotransmitter systems in alcohol dependence. Pharmacopsychiatry. 42 Suppl 1:S95-S101.

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, 127–134.

Holton, F.A.; Holton, P. 1954. The capillary dilator substances in dry powders of spinal roots; a possible role of adenosine triphosphate in chemical transmission from nerve endings. J. Physiol. 126, 124-140.

Holton, P. 1959. The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. J. Physiol. 145, 494-504.

Huang, E.J., Reichardt, L.F. 2003. Trk Receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. 72, 609–642.

Huynh, G., Heinrich, G. 2001. Brain-derived neurotrophic factor gene organization and transcription in the zebrafish embryo. Int. J. Dev. Neurosci. 19, 663-73.

Ikonomidou, C., Price, M.T., Mosinger, J.L., Frierdich, G., Labruyere, J., Salles, K.S., Olney, J.W. 1989. Hypobaric-ischemic conditions produce glutamate-like cytopathology in infant rat brain. J Neurosci. 9, 1693-700.

Illes, P., Ribeiro, J. A. 2004. Molecular physiology of P2 receptors in the central nervous system. Eur. J. Pharmacol. 483, 5-17.

Israel, Y., Orrego, H., Carmichael, F.J. 1994. Acetate-mediated effects of ethanol, Alcohol Clin. Exp. Res. 18, 144-148.

Kalivas, P.W. 2009. The glutamate homeostasis hypothesis of addiction. Nat Rev Neurosci.10, 561-72.

Kandel, E., Schwartz, J., Jessel,. 1991., T. Principles of neural science. Elsevier, 1991.

Kaslin, J., Panula, P. 2001. Comparative anatomy of the histaminergic and other aminergic system in zebrafish (*Danio rerio*). J. Comp. Neurol. 440, 342-377.

Kato, F., Kawamura, M., Shigetomi, E., Tanaka, J., Inoue, K. 2004. ATP- and adenosine-mediated signaling in the central nervous system: synaptic purinoceptors: the stage for ATP to play its "dual-role". J. Pharmacol. Sci. 94, 107-111.

Kimmel, C. 1989. Genetics and early development of zebrafish. Trends Genet. 5, 283-288

Kimmel, C., Warga, R. 1988. Cell lineage and developmental potential of cells in the zebrafish embryos. Trends Genet. 5, 68-74.

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

Lazarowski, E. R., Boucher, R. C., Harden, T. K. 2003. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y- receptor activating molecules. Mol. Pharmacol. 64 , 785-795.

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

Levin, E. D., Chen, E. 2004. Nicotinic involvement in memory function in zebrafish. Neurobiol. Teratol. 26, 731-735.

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, 647-54.

Long, Q., Meng, A., Wang, H., Jessen, J. R., Farrell, M. J., Lin, S. 1997. GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene.Development. 124 (20), 4105-4111.

Lum, T., Huynh, G., Heinrich, G. 2001. Brain-derived neurotrophic factor and TrkB tyrosine kinase receptor gene expression in zebrafish embryo and larva. Int. J. Dev. Neurosci. 19, 569-87.

Mattson, M.P. 2008. Glutamate and neurotrophic factors in neuronal plasticity and disease. Ann N Y Acad Sci. 1144, 97-112.

Maximino, C., da Silva, A.W., Gouveia, A. Jr., Herculano, A.M. 2011. Pharmacological analysis of zebrafish (Danio rerio) scototaxis. Prog Neuropsychopharmacol Biol Psychiatry. Artigo in press.

Med. Res. 37, 11–18.

Meldrum, B.S., 2000., Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J. Nutrit. 130, 1007S-1015S.

Melendez, R.I., Hicks, M.P., Cagle, S.S., Kalivas, P.W. 2005. Ethanol exposure decreases glutamate uptake in the nucleus accumbens. Alcohol Clin. Exp. Res. 29, 326-33.

Moonat, S., Starkman, B.G., Sakharkar, A., Pandey, S.C. 2009. Neuroscience of alcoholism: molecular and cellular mechanisms. Cell Mol. Life Sci. 67, 73-88.

Nestler, E.J. 2001. Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci. 2, 119-28.

Neuhauss S.C., Rico E.P., Gesemann M. 2010. Nomenclature of glutamate transporters in zebrafish and other vertebrates. Brain Res Bull. 83, 297.

Newton, P.M., Messing, R.O. 2005. Intracellular signaling pathways that regulate behavioral responses to ethanol. Pharmacol Ther. 109, 227-37.

Nicolaidis, R., Bruno, A.N., Sarkis, J.J., Souza, D.O. 2005. Increase of adenine nucleotide hydrolysis in rat hippocampal slices after seizures induced by quinolinic acid. Neurocheml. Res. 30, 385-90.

Norton, W. H., Rohr, K. B., Burnstock, G. 2000. Embryonic expression of a P2X3 receptor encoding gene in zebrafish. Mech. Dev. 9, 149-152.

Ohrtman, J.D., Stancik, E.K., Lovinger, D.M., Davis, M.I. 2006. Ethanol inhibits brain-derived neurotrophic factor stimulation of extracellular signal-regulated/mitogen-activated protein kinase in cerebellar granule cells. Alcohol. 39, 29-37.

Olney, J. W., 1990., Excitotoxic amino acids and neuropsychiatric disorders. Ann. Rev.

Ortega, F., Pérez-Sem, R., Morente, V., Delicado, E.G., Miras-Portugal, M.T. 2010 P2X7, NMDA and BDNF receptors converge on GSK3 phosphorylation and cooperate to promote survival in cerebellar granule neurons. Cell Mol Life Sci. 67, 1723-33.

Patterson, S.L., Pittenger, C., Morozov, A., Martin, K.C., Scanlin, H., Drake, C., Kandel, E.R. 2001. Some forms of cAMP-mediated long-lasting potentiation are associated with release of BDNF and nuclear translocation of phospho-MAP kinase. Neuron. 32, 123-40. Pharmacol. Toxicol. 30, 47-71.

Phillis, J.W. & Wu, P.H. 1982. The effect of various centrally active drugs on adenosine uptake by the central nervous system. Comp. Biochem. Physiol. C. 72, 179-87.

Postlethwait, J., Amores, A., Force, A., Yan, Y.L. 1999. The zebrafish genome. Methods Cell Biol. 60, 149-163.

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, 345-349.

Potenza, R.L., Tebano, M.T., Martire, A., Domenici, M.R., Pepponi, R., Armida, M., Pèzzola, A., Minghetti, L., Popoli, P. 2007. Adenosine A_{2A} receptors modulate BDNF both in normal conditions and in experimental models of Huntington's disease. Purinergic Signal. 3, 333–338.

Quertemont, E., Tambour, S., Tirelli, E. 2005. The role of acetaldehyde in the neurobehavioral effects of ethanol: a comprehensive review of animal studies. Prog. Neurobiol. 75, 247-274.

Ralevic, V., Burnstock, G. 1998. Receptors for purines and pyrimidines. Pharmacol. Rev. 50, 413-492.

Reimers, M.J., Flockton, A.R., Tanguay, R.L. 2004. Ethanol- and acetaldehyde-mediated developmental toxicity in zebrafish. Neurotoxicol. Teratol. 26, 769-781.

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, 38303-38312.

Release of glutamate during ischemia and its relation to neuronal death, Arch.

Ribeiro, J., A., Sebastião, A. M., Mendonça, A. 2003. Participation of adenosine receptors in neuroprotection. Drug. News Pers. 16 , 80-86.

Rico, E. P., Senger, M. R., Fauth, M. 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., Dias, R.D., Bogo, M.R., Bonan, C.D. 2007. Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicol Lett. 174, 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, 290-6.

Rink, E., Guo, S. 2004. The too few mutant selectively affects subgroups of monoaminergic neurons in the zebrafish forebrain. Neurosci. 127, 147-154.

Robinson, M. B., 1999., The family of sodium-dependent glutamate transporters: a focus on the GLT-1/EAAT2 subtype. Neurochem. Int. 33, 479-491.

Robinson, M. B., Dowd, L. A., 1997., Heterogeneity and functional properties of subtypes of sodium-dependent glutamate transporters in the mammalian central nervous system. In: Adv. Pharmacol. 37, 69-113.

Salgado, A. I., Cunha, R. A., Ribeiro, J. A. 2000. Facilitation by P2 receptor activation of acetylcholine release from rat motor nerve terminals: interaction with presynaptic nicotinic receptors. Brain Res. 877, 245-250.

Santhakumar V, Wallner M, Otis TS. Ethanol acts directly on extrasynaptic subtypes of GABAA receptors to increase tonic inhibition. Alcohol. 2007 May;41(3):211-21.

Sarkis, J. J. F., Saltó, C. 1991. Characterization of a synaptosomal ATP diphosphohydrolase from the eletric organ of Torpedo marmorata. Brain Res. Bull. 26, 871-876.

Scalzo, F.M, Levin, E.D. 2004. The use of zebrafish (*Danio rerio*) as a model system in neurobehavioral toxicology. Neurotoxicol. Teratol. 26, 707-708.

Schetinger, M. R. C., Vieira, V. L. P., Morsch, V. M., Balz, D. 2001. ATP and ADP hydrolysis in fish, chicken and rat synaptosomes. Comp. Biochem. Physiol. 128B, 731-741.

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. 2007 116, 401-16.

Sebastião, A.M.; Ribeiro, J.A., 2000. *Trends Pharmacol Sci.* 21(9),341-6.

Segovia, G., Porras, A., Del Arco, A., Mora, F., 2001., Glutamatergic transmission in

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. 139B, 203-207.

Serra, E. L., Medalha, C. C. Mattioli, R. 1999. Natural preference of zebrafish (Danio rerio) for a dark environment. Braz. J. Med. Biol. Res. 32, 1551-1553.

Sevigny, J., Sundberg, C., Braun, N., Guckelberger, O., Csizmadia, E., Qawi, I., Imai, M., Zimmermann, H., Robson, S.C. 2002. Differential catalytic properties and vascular topography of murine nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) and NTPDase2 have implications for thromboregulation Blood. Apr. 99, 2801-2809.

Silberman, Y., Bajo, M., Chappell, A.M., Christian, D.T., Cruz, M., Diaz, M.R., Kash, T., Lack, A.K., Messing, R.O., Siggins, G.R., Winder, D., Roberto, M., McCool, B.A., Weiner, J.L. 2009. Neurobiological mechanisms contributing to alcohol-stress-anxiety interactions. Alcohol. 43, 509-19.

Smith, T.L. 1997. Regulation of glutamate uptake in astrocytes continuously exposed to ethanol. Life Sci. 61, 2499-505.

Sprague J, Doerry E, Douglas S, Westerfield M. 2001. The Zebrafish Information Network (ZFIN): a resource for genetic, genomic and developmental research. Nucleic Acids Res. 29 (1), 87-90.

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 Acid Res. 31, 241-243.

Stern, H. M., Zon, L. I. 2003. Cancer genetics and drug discovery in the zebrafish. Nature Rev. Cancer 3, 1-7.

Stuber, G.D., Hopf, F.W., Tye, K.M., Chen, B.T., Bonci, A. 2010. Neuroplastic alterations in the limbic system following cocaine or alcohol exposure. Curr Top Behav Neurosci. 3, 3-27.

Swain, H. A., Sigstad, C., Scalzo, F. M. 2004. Effects of dizocilpine (MK-801) on circling behavior, swimming activity, and place preference in zebrafish (Danio rerio). Neurotoxicol. Teratol. 26, 725-729.

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

Tabakoff, B., Saba, L., Printz, M., Flodman, P., Hodgkinson, C., Goldman, D., Koob, G., Richardson, H.N., Kechris, K., Bell, R.L., Hübner, N., Heinig, M., Pravenec, M., Mangion, J., Legault, L., Dongier, M., Conigrave, K.M., Whitfield, J.B., Saunders, J., Grant, B., Hoffman, P.L. 2009. Genetical genomic determinants of alcohol consumption in rats and humans. BMC Biol. 27, 7-70.

Todd, K.J., Auld, D.S., Robitaille, R. 2007. Neurotrophins modulate neuron-glia interactions at a vertebrate synapse. Eur. J. Neurosci. 25, 1287-96.

Tyler, W.J., Zhang, X.L., Hartman, K., Winterer, J., Muller, W., Stanton, P.K., Pozzo-Miller, L. 2006. BDNF increases release probability and the size of a rapidly recycling vesicle pool within rat hippocampal excitatory synapses. J. Physiol. 574, 787-803.

Ulmann, L., Hatcher, J.P., Hughes, J.P., Chaumont, S., Green, P.J., Conquet, F., Buell, G.N., Reeve, A.J., Chessell, I.P., Rassendren, F. 2008. Up-regulation of P2X4 receptors in spinal microglia after peripheral nerve injury mediates BDNF release and neuropathic pain. J Neurosci. 28, 11263-8.

Vascotto, S. G., Beckham, Y., Kelly, G.M. 1997. The zebrafish swim to fame as an experimental model in biology. Biochem. Cell. Biol. 75, 479-485.

Vogel, M., Kowalewski, H., Zimmermann, H., Hooper, N. M., Turner, A. J. 1992. Soluble low-Km 5'-nucleotidase from electric-ray (*Torpedo marmorata*) electric organ and bovine cerebral cortex is derived from the glycosyl-phosphatidylinositol-anchored ectoenzyme by phospholipase C cleavage. Biochem. J. 284, 621-624.

Volknandt, W., Vogel, M., Pevsner, J., Misumi, Y., Ikehara, Y., Zimmermann, H. 5'-nucleotidase from the electric ray electric lobe. Primary structure relation to mammalian and procariotic enzymes. Eur J. Biochem. 202, 855-861.

Westerfield, M. 2000. The zebrafish Book: A guide for the laboratory use of zebrafish (*Danio rerio*) (4th ed.). Eugene.OR: University of oregon Press.

Yamada, K., Mizuno, M., Nabeshima, T. 2002. Role for brain-derived neurotrophic factor in learning and memory. Life Sci. 70, 735-44.

Yamazaki, K., Teraoka, H., Dong, W., Stegeman, J. J., Hiraga. T. 2002. cDNA cloning and Expressions of cytocrome P450 1A in zebrafish embrios. J. Vet. Med. Sci. 64, 829-833.

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

Zimmermann, H. 1994. Signalling via ATP in the nervous system. Trend. Neurosc. 17, 420-426.

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