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**CARACTERIZAÇÃO COMPORTAMENTAL DO MODELO DE CONVULSÕES
INDUZIDAS POR PENTILENOTETRAZOL EM *ZEBRAFISH* ADULTO**

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# CARACTERIZAÇÃO COMPORTAMENTAL DO MODELO DE CONVULSÕES INDUZIDAS POR PENTILENOTETRAZOL EM *ZEBRAFISH* ADULTO

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

Orientador: Diogo Lösch de Oliveira

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*"O ontem é história, o amanhã é um mistério, mas o hoje é uma dádiva. É por isso que se chama presente."*

**Oogway**

**Aos Antepassados**

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# SUMÁRIO

**Parte I.**
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# RESUMO

O pentilenotetrazol (PTZ) é um agente convulsivo amplamente utilizado em modelos animais para investigações envolvendo crises convulsivas. Embora haja um crescente número de estudos envolvendo zebrafish adulto e convulsão, não há até o momento uma caracterização comportamental detalhada do modelo de indução de crises por PTZ neste animal. Portanto, o objetivo deste estudo foi realizar uma caracterização detalhada das manifestações comportamentais no modelo de convulsão induzida por PTZ em zebrafish adulto. Grupos de 12 animais foram submetidos, por imersão, a distintas diferentes concentrações de PTZ (5, 7.5, 10 e 15 mM). O comportamento convulsivo foi observado durante 20 minutos. Os animais apresentaram os seguintes escores comportamentais: (0) nados curtos; (1) aumento na atividade natatória e na abertura opercular; (2) movimentos erráticos e acelerados; (3) movimentos circulares; (4) convulsão clônica; (5) convulsão tônica; (6) morte. Os animais expostos a diferentes distintas concentrações de PTZ apresentaram diferentes distintos perfis convulsivos, intensidades de convulsão e latência para chegar aos escores. Apenas os animais imersos na concentração de 15 mM apresentaram maior tempo para retornar ao comportamento normal após a exposição ao PTZ. A mortalidade foi de 33% e 50% para as concentrações de 10 mM e 15 mM, respectivamente. No intuito de avaliar abordagem perfil comportamental frente ao pré-tratamento de um fármaco anticonvulsivo, 12 animais foram expostos a diazepam (DZP) por 40 min e após expostos ao PTZ (10 mM). O tratamento com DZP atenuou a severidade da convulsão, mas não o tempo necessário para retornar ao comportamento normal. Levando em consideração que este modelo baseia-se na imersão direta do animal a uma solução de PTZ, avaliamos também as concentrações cerebrais deste agente convulsivo. Além disso, investigamos também uma possível correlação entre a concentração cerebral de PTZ, o tempo e a concentração de exposição. Os resultados decorrentes da exposição dos animais a 5 e 10 mM de PTZ, apontam para uma correlação tempo e concentração dependentes. Os resultados deste trabalho demonstram uma caracterização comportamental detalhada ao longo do tempo de exposição ao PTZ, além de avaliar pela primeira vez a intensidade de convulsão, tempo de retorno ao comportamento normal e mortalidade dos animais. Além disso, pela primeira vez foram determinadas as concentrações cerebrais deste composto neste modelo de convulsão em zebrafish adulto. Por fim, espera-se que tais ferramentas comportamentais e de quantificação cerebral deste composto possam ser úteis em futuras investigações translacionais e estudos que tenham como objetivo melhor compreender esta desordem.

## ABSTRACT

Pentylenetetrazole (PTZ) is a common convulsant agent used in animal models to investigate the mechanisms of seizures. Although adult zebrafish have been recently used to study epileptic seizures, a thorough characterization of the PTZ-induced seizures in this animal model is missing. The goal of this study was to perform a detailed temporal behavior profile characterization of PTZ-induced seizure in adult zebrafish. The behavioral profile during 20 min of PTZ immersion (5, 7.5, 10, and 15 mM) was characterized by stages defined as scores: (0) short swim, (1) increased swimming activity and high frequency of opercular movement, (2) erratic movements, (3) circular movements, (4) clonic seizure-like behavior, (5) fall to the bottom of the tank and tonic seizure-like behavior, (6) death. Animals exposed to distinct PTZ concentrations presented different seizure profiles, intensities and latencies to reach all scores. Only animals immersed into 15 mM PTZ showed an increased time to return to the normal behavior (score 0), after exposure. Total mortality rate at 10 and 15 mM were 33% and 50%, respectively. Considering all behavioral parameters, 5, 7.5, 10, and 15 mM PTZ, induced seizures with low, intermediate, and high severity, respectively. Pretreatment with diazepam (DZP) significantly attenuated seizure severity. Finally, the brain PTZ levels in adult zebrafish immersed into the chemoconvulsant solution at 5 and 10 mM were comparable to those described for the rodent model, with a peak after a 20-min of exposure. The PTZ brain levels observed after 2.5-min PTZ exposure and after 60-min removal from exposure were similar. Altogether, our results showed a detailed temporal behavioral characterization of a PTZ epileptic seizure model in adult zebrafish. These behavioral analyses and the simple method for PTZ quantification could be considered as important tools for future investigations and translational researches.

# ABREVIAÇÕES

*c-Fos* – Proto-oncogêne de fatores de transcrição

DAE(s) – Drogas anti-epilépticas

GABA – Ácido gama-aminobutírico

$GABA_A$ – Receptor tipo A do neurotransmissor Ácido gama-aminobutírico

$GABA_B$ – Receptor tipo B do neurotransmissor Ácido gama-aminobutírico

$GABA_C$ – Receptor tipo C do neurotransmissor Ácido gama-aminobutírico

I.P. - Intraperitonial

PTZ – Pentilenotetrazol

S.C. - Subcutâneo

SNC – Sistema Nervoso Central

## INTRODUÇÃO

### 1. EPILEPSIAS

O termo epilepsia engloba um conjunto de distúrbios que acometem o sistema nervoso central (SNC), os quais apresentam crises epilépticas recorrentes caracterizadas por descargas transitórias, excessivas e hipersincrônicas das células nervosas (Fisher et al., 2005). A atividade neuronal anormal durante as crises epilépticas pode acometer uma determinada região do SNC (convulsão focal) ou ocorrer de forma generalizada, afetando ambos hemisférios cerebrais. Dependendo da região de origem da crise convulsiva, o paciente pode apresentar manifestações visuais, olfativas, auditivas, mnemônicas, motoras, etc. Por essa vasta gama de possibilidades autores descrevem esta desordem como "epilepsias" (Bell et al., 2011; Caplan et al., 2008; Kavros et al., 2008).

Estima-se que aproximadamente 3% da população mundial apresenta epilepsias, sendo que cerca de 80% encontram-se em países com baixo índice de desenvolvimento humano (Newton e Garcia, 2012). Para o diagnóstico das epilepsias, considera-se: (i) histórico de ocorrência de pelo menos uma crise convulsiva; (ii) alterações cerebrais fisiológicas e anatômicas persistentes, predispondo o paciente a futuras crises convulsivas; e (iii) condições associadas como: alterações psicológicas, cognitivas, neurobiológicas, sociais, aumento de agressividade, ansiedade, déficit de atenção, confusão mental, depressão, déficit de memória, entre outros (Fisher et al., 2005; Lin et al., 2012).

Crises convulsivas são sinais transitórios da ativação excessiva do SNC. Segundo Gowers (1881), esta hiperativação resulta da potenciação dos

mecanismos excitatórios, ou da falha dos mecanismos inibitórios do SNC. A convulsão é a principal manifestação clinica que caracteriza as epilepsias (Baraban, 2007; Lin et al., 2012). Para tanto, busca-se modelos animais desta desordem visando o estudo dos mecanismos neuroquímicos e eletrofisiológicos envolvidos na gênese e na manutenção das crises epilépticas, bem como o desenvolvimento de fármacos que previnam tais manifestação.

## 1.1. Modelos animais

Durante o último século, diversos modelos animais foram desenvolvidos na tentativa de mimetizar esta desordem e com o intuito de aprofundar o conhecimento sobre as epilepsias. Apesar do frequente aparecimento de diferentes modelos experimentais, geralmente cada modelo está restrito a algumas das características desta ou daquela forma de epilepsia, sendo improvável estudar esta desordem em um único modelo animal (Loscher, 2011). Neste sentido, a caracterização de múltiplos modelos experimentais de epilepsias ou de crises convulsivas permite uma compreensão mais ampla dos fenômenos relacionados com esta desordem (Coppola and Moshe, 2012).

Inicialmente, os modelos foram classificados em genéticos e não genéticos (Loscher, 1984). O primeiro consiste na geração de crises espontâneas geralmente relacionadas a alterações na formação e/ou no desenvolvimento do sistema nervoso (Loscher, 1984). Tal abordagem permite estudar a desordem de forma crônica, tendo em vista que ao decorrer do desenvolvimento do animal ocorreram crises convulsivas recorrentes. Por outro lado, esta mesma abordagem torna difícil o estudo da crise convulsiva em si, já que é complicado estipular um controle do início da manifestação. O segundo consiste na submissão do animal a estímulos químicos ou elétricos, os quais culminam em crises convulsivas (Stewart et al., 2012). Este tipo de abordagem

permite precisar o momento exato do início da manifestação, tendo em vista que será resultado da aplicação pontual de um fármaco ou estímulo elétrico. Dependendo do fármaco testado, pode ainda haver a subdivisão em modelos agudos ou crônicos. Dentre os modelos crônicos destacam-se o modelo de LiCl-pilocarpina (Tang et al., 2011) e o modelo do ácido caínico (Bloss e Hunter, 2010). Após a aplicação dos fármacos, os animais apresentam uma crise prolongada, denominada de status epilepticus, o qual é seguido pelo surgimento de crises recorrentes. Para os modelos agudos, encontra-se em destaque o modelo do pentilenotetrazol (PTZ), o qual induz uma crise convulsiva rápida com baixa mortalidade (Rubio et al., 2010). Ressalta-se que utilizando compostos indutores de crises agudas, pode-se ainda gerar crises crônicas através do método de abrasamento ("*Kindling*") (Takechi et al., 2012).

Essa diversidade de modelos animais permitem a pesquisadores estudarem desde alterações genéticas que levam a crises, como por exemplo mutações em canais de K+ (Robbins e Tempel, 2012), até crises convulsivas pontuais que indivíduos possam vir a ter durante a vida decorrente de períodos de hipóxia-isquemia (Burns et al., 2012), glioma (Buckingham e Robel, 2013), episódios de febre (Byler et al., 2013), etc. A crise convulsiva é um sintoma não só da epilepsia, mas de diferentes patologias tais como, a Doença de Alzheimer (Pandis and Scarmeas, 2012),a Doença de Parkinson (Boison et al., 2010), o Diabetes mellitus (Verrotti et al., 2012), entre outras. Os modelos agudos de convulsão, permitem estudar pontualmente este sintoma e realizarem triagens de fármacos que inibam especificamente a crise convulsiva.

## 1.2. Pentilenotetrazol

O pentilenotetrazol é um agente convulsivo capaz de gerar tanto crises agudas, quanto crônicas (método do abrasamento) (Takechi et al., 2012). Este

é um dos compostos mais utilizados nos últimos 60 anos com o propósito de desenvolvimento de drogas anti-epilépeticas (DAE) (Loscher, 2011).

Após uma administração subcutânea (s.c.) ou intraperitoneal (i.p.) de PTZ em roedores, os animais apresentam crises convulsivas tônico-clônicas com duração de 5 min (Brito et al., 2006). A ação convulsiva do PTZ deve-se ao seu antagonismo não competitivo sobre os receptores $GABA_A$, diminuindo assim a ação inibitória do GABA no SNC (Ramanjaneyulu e Ticku, 1984).

O GABA é o principal neurotransmissor inibitório do sistema nervoso central (Rowley et al., 2012). Ao ser liberado pelo neurônio pré-sinaptico, este neurotransmissor atua em receptores ionotrópicos ($GABA_A$ e $GABA_C$) (Laurie et al., 1992; Perfilova e Tiurenkov, 2011) ou metabotrópicos ($GABA_B$) (Bowery, 1997), localizados na pré- e na pós-sinapse. O GABA ao ligar-se no receptor $GABA_A$ na pós-sinapse promove a abertura do canal iônico do receptor, permitindo o influxo de íons $Cl^-$ e o efluxo de íons $K^+$ do neurônio alvo, resultando na sua hiperpolarização (Sieghart e Sperk, 2002). À medida que o PTZ atinge o sistema nervoso central, este se liga aos receptores $GABA_A$ impedindo a ação do GABA, consequentemente bloqueando o influxo de íons $Cl^-$ e o efluxo de íons $K^+$ do neurônio (Ramanjaneyulu e Ticku, 1984). Devido a sua ação antagonista sobre os receptores $GABA_A$, o PTZ provoca um desequilíbrio no balanço excitação/inibição no SNC, resultando na hiperexcitabilidade neuronal e gerando uma crise epiléptica generalizada (Ferando e Mody, 2012).

Estima-se que 97% dos animais injetados com a concentração de 60 mg/Kg apresentem as seguintes manifestações (Loscher, 2011): score 1 – clonismos faciais; score 2 – movimentação de flexão e extenção da cabeça; score 3 – clonismos de patas; score 4 – respostas de orientação, onde o animal

permanece de pé apenas sobre as patas traseiras ("*rearing*") seguido de clonismos de patas e queda; e score 5 – crises tônico/clônicas generalizadas (Bao et al., 2011; Rauca et al., 2004). A latência média para a manifestação completa das crises é de aproximadamente 15 min após a injeção (Brito et al., 2006). Decorrente da alta reprodutibilidade do modelo de PTZ e fácil observação da crise (visual), pesquisadores utilizam-no para testes de compostos com potencial (Shorvon, 2009a; Shorvon, 2009b).

## 1.3. Refratariedade

Apesar do avanço no tratamento das epilepsias e o desenvolvimento de inúmeros protocolos pré-clínicos nas últimas décadas, persiste a busca por fármacos que apresentem uma melhor eficácia e tolerabilidade (Loscher e Schmidt, 2011). A utilização de modelos animais de epilepsia ainda é uma estratégia de primeira linha para a descoberta de novas drogas anti-epilépticas.

Atualmente, estima-se que 30% dos pacientes epilépticos sejam refratários aos tratamentos farmacológicos disponíveis (Kwan et al., 2010; Loscher e Schmidt, 2011). Segundo Loscher, (2011), essa alta porcentagem de refratariedade pode estar relacionada ao uso de apenas alguns modelos animais para triagem de novas DAEs, os quais focam-se principalmente no uso de roedores. Essa restrição resultaria em fármacos redundantes, com respostas similares. Portanto, é de grande valia a caracterização e implementação de novos modelos animais, que possibilitem novos achados e, além disso, facilitem e diminuam o custo das triagens farmacológicas.

## 2. *Zebrafish*

O *zebrafish* (*Danio rerio*), conhecido popularmente por peixe-zebra ou "paulistinha", é um teleósteo de 2-4 cm pertencente à família Cyprinidae, o qual foi utilizado pela primeira vez para pesquisas científicas por George Streisinger

no Instituto de Tecnologia da Califórnia (EUA) (Grunwald e Eisen, 2002). Devido ao seu ovo ser translucido e possuir um rápido desenvolvimento (2 meses), adquiriu aplicabilidade em estudos relacionados à biologia do desenvolvimento e embriogênese (Lele and Krone, 1996; Stern e Zon, 2003).

Além disso, outras características tornaram esta espécie extremamente atrativa quando comparado à outros modelos animais, tais como mosca-das-frutas (*Drosophila melanogaster*), rato (*Rattus norvegicus*) e camundongo (*Mus musculus*). Dentre as vantagens apresentadas, destacam-se as seguintes: pequeno espaço requerido para a manutenção; baixo custo; e praticidade para triagens em larga escala (Lieschke e Currie, 2007).

Houve progresso considerável no estudo da genética e da genômica do *zebrafish* na última década. Em 2001, o Instituto Sanger começou o sequenciamento do genoma dessa espécie e identificação de regiões codificadoras (Stern e Zon, 2003; Vogel, 2000). O *zebrafish* possui diversos genes evolutivamente conservados e apresenta um alto grau de homologia com genes de mamíferos (Barbazuk et al., 2000; Lieschke e Currie, 2007). Devido ao crescimento no número de estudos utilizando esse vertebrado, foi criada em 1994 uma rede de informações denominada ZFIN (http://zfin.org), na qual laboratórios de diversos países podem depositar informações acerca da genética, histologia, anatomia, fisiologia e bioquímica dessa espécie. Adicionalmente, o ZFIN disponibiliza também um manual de criação e manutenção em laboratório do *zebrafish* (Sprague et al., 2003).

Desde os anos 60 e principalmente na última década, o *zebrafish* tem franca expansão nas áreas da bioquímica (Rico et al., 2011; Taylor et al., 2004), das neurociências (Edwards e Michel, 2002), da farmacologia (Goldsmith, 2004) e da biologia do comportamento (Blaser e Rosemberg, 2012;

Egan et al., 2009; Gerlai, 2003; Guo, 2004; Rosemberg et al., 2012). Por apresentar tamanho pequeno e fácil absorção de compostos diluídos em água, a quantidade em gramas das moléculas teste passa a ser uma fração reduzida daquela necessária para roedores (Goldsmith, 2004). Isto facilita e reduz custos dos processos de triagem em larga escala de possíveis compostos terapêuticos (Rico et al., 2011; Stern e Zon, 2003). Outro ponto, é a utilização deste modelo animal como primeiro passo para estudar achados *in vitro,* o que permite detalhar complexas interações exercidas por diferentes compostos, assim como a relação dos mecanismos envolvidos no efeito promovido por moléculas distintas em sistemas biológicos (Rico et al., 2011). A utilização do *zebrafish* como modelo animal em pesquisas relacionadas a doenças humanas já foi previamente descrita (Hammes et al., 2012; Hortopan et al., 2010a).

A necessidade de desenvolvimento de novas DAEs que venham a diminuir a porcentagem de pacientes epilépticos refratários demanda um desafio na triagem de novos compostos anticonvulsivos e/ou antiepilépticos. Isto enfatiza a necessidade do desenvolvimento de novos modelos animais de convulsão e epilepsia (Loscher, 2011). Como os modelos de roedores apresentam um alto custo de manutenção ou difícil manipulação genética, organismos inferiores emergem como espécies úteis para a triagem inicial de fármacos ou mutações genéticas relacionadas com epilepsia (Berghmans et al., 2007). Apesar de nos últimos anos invertebrados (*Caenorhabditis elegans* e a *Drosophila melanogaster*) fornecerem achados sobre epilepsia, a ausência de um sistema nervoso complexo limita sua aplicabilidade (Baraban, 2007).

Desta forma, o *zebrafish* emerge como um potencial modelo animal para o estudo das epilepsias. Além de ser um vertebrado, apresenta homologia fisiológica e similaridade dos constituintes da barreira hematoencefalica ao se

comparar com humanos (Barbazuk et al., 2000; Eliceiri et al., 2011; Jeong et al., 2008; Rico et al., 2010). Outro ponto a ser considerado é a disponibilidade de utilizar este animal em uma abordagem ontogenética desta desordem.

## 2.1. *Zebrafish* larva e epilepsia

O ano de 2005 marca o início da aplicabilidade deste modelo animal para estudos envolvendo epilepsia. Baraban et al., (2005) publicaram um trabalho pioneiro no qual submeteram larvas de *zebrafish* a diferentes concentrações de pentilenotetrazol. O resultado foi visto como uma manifestação convulsiva tonico-clônica. Além disso, os autores observaram também uma elevada atividade eletroencefalográfica epileptiforme no SNC das larvas bem como o aumento na expressão de sinalizadores de atividade neuronal (c-*fos*). Após este estudo, Berghmans et al., (2007) demonstraram a primeira aplicabilidade deste modelo em triagens de compostos anti-convulsivos. Utilizando o padrão convulsivo descrito por Baraban et al., (2005) e o baixo espaço ocupado pelas larvas, este grupo utilizou uma placa de 96 poços, colocando um animal por poço e adicionando PTZ, ou PTZ+composto a ser testado, realizando uma vasta triagem de compostos classicamente aplicados a humanos. Tão importante quanto à aplicabilidade do modelo foram os achados frente às respostas a DAEs clássicos. O modelo de roedor induzido por PTZ responde limitadamente a DAEs que atuam via sistema GABAérgico (Loscher, 1984). Contudo neste estudo, além desta mesma resposta vista no modelo de roedor, fármacos que atuam por outras vias, como por exemplo, a glutamatérgica, também apresentaram resposta anticonvulsiva. Este achado aponta para a possibilidade de realizar uma ampla triagem farmacológica com um único modelo de convulsão, diferentemente do observado em roedores. Outro ponto debatido pelos pesquisadores foi o fato de larvas de *zebrafish*

frente a DAEs apresentarem sedação, gerando a dúvida se o fármaco inibiu a convulsão a nível de SNC, ou apenas tornou o animal imóvel. Afrikanova et al., (2013), dando continuidade a este trabalho, demonstraram por intermédio de técnicas eletrofisiológicas que mesmo com potencial efeito sedativo os DAEs testados impediram a convulsão a nível de SNC.

Além disso, desenvolveu-se larvas de *zebrafish* modificadas geneticamente que apresentaram crises recorrentes e refratárias (Baraban et al., 2007; Bassuk et al., 2008; DiBella et al., 2009; Hortopan et al., 2010a; Hortopan et al., 2010b; Teng et al., 2010; Teng et al., 2011).

## 2.2. *Zebrafish* adulto e epilepsia

Apesar dos grandes avanços disponíveis na literatura em relação à utilização das larvas de *zebrafish* para estudar epilepsias, este modelo apresenta algumas limitações devido ao estágio de desenvolvimento dos animais. A epilepsia engloba uma série de modificações neuroquímicas (Rico et al., 2011) e comportamentais (Blaser and Rosemberg, 2012; Cachat et al., 2011; Lee et al., 2010; Rosemberg et al., 2011; Rosemberg et al., 2012), cujos protocolos experimentais de estudo não apresentam caracterização definida para larvas de *zebrafish*. No que diz respeito ao peixe adulto, seu SNC é mais desenvolvido sendo possível  avaliar parâmetros de funcionalidade dos sistemas colinérgico (Rico et al., 2007), dopaminérgico e serotoninérgico (Chatterjee e Gerlai, 2009), purinérgico (Rosemberg et al., 2008), e glutamatérgico (Rico et al., 2010).. Além disso, existem ferramentas disponíveis para avaliar ansiedade (Blaser e Rosemberg, 2012), agressividade (Oliveira et al., 2011), perfil exploratório (Rosemberg et al., 2011) e memória (Lee et al., 2010). Tais características encontram-se alteradas em pacientes epilépticos.

Decorrente destas características Lee et al., (2010), observaram pela primeira vez não só os efeitos da imersão do animal adulto em PTZ, mas também o impacto da convulsão sobre a memória. Com isso, o *zebrafish* deixaria de ser um modelo apenas para estudar efeitos de fármacos no limiar de convulsão, mas poderia também ser utilizado para triagens de fármacos que atenuariam outros sintomas apresentados pelos pacientes epilépticos.

No mesmo ano, Wong et al., (2010), publicou um trabalho medindo alterações nos níveis de cortisol em peixes convulsionados e alterações comportamentais motoras após a exposição ao PTZ. Diferentemente da larva, os autores observaram que as manifestações convulsivas eram muito mais complexas em animais adultos. A larva apresenta movimentos acelerados (score 1), movimentos circulares (score 2) e movimentos repetitivos seguidos de queda e imobilidade (score 3). Já o adulto apresentou, aumento na frequência de abertura opercular, elevação na atividade natatória, movimentos circulares, movimentos repetitivos, queda ao fundo do aquário e rígida extensão do corpo. Contudo, diferente da larva, não havia demonstração eletroneurofisiológica, tão pouco estudos da expressão de *c-fos* no SNC.

Decorrente disto, Pineda et al., (2011) demonstraram a atividade eletrofisiológica de animais adultos expostos a diferentes tempos e concentrações de PTZ. Neste mesmo ano, Siebel et al., (2011) demonstraram que este animal poderia ser utilizado para estudar alterações neuroquímicas decorrentes das convulsões ao avaliar alterações do sistema purinérgico em animais expostos a diferentes concentrações de PTZ. Já em 2012, Stewart et al., mostraram um aumento na expressão de *c-fos* a nível de SNC de *zebrafish* adultos imersos em PTZ. Contudo, diferente do trabalho de Baraban et. al., (2005), não havia ainda nenhuma definição de estágios de convulsão.

Tal proposta foi vista no modelo de convulsão induzido por ácido caínico em *zebrafish* adulto realizado por Alfaro et al., (2011). Neste trabalho, os autores identificaram estágios convulsivos e os classificaram na forma de uma escala de escores ao longo do tempo. Este achado facilita o entendimento da convulsão, vide o grande sucesso do trabalho de Racine, (1972).

No ano de 2012, Demond et al. publicou um estudo unindo esses achados, propondo um sistema de escore básico para estudar convulsão em *zebrafish*. Apesar da boa perspectiva apresentada pelo trabalho, alguns pontos ainda ficaram sem elucidação, tendo em vista que a escala proposta foi baseada em diferentes agentes pró-convulsivantes com concentrações únicas, diferente do proposto por Baraban et al., (2005) e Alfaro et al., (2011).

Cabe enfatizar que dentre as questões não levantadas nos estudos citados anteriormente destacam-se: (I) O sistema de escores proposto por Desmond et al., (2012) enquadra-se neste modelo?; (II) Qual a sequência de manifestações ao longo do tempo após a exposição ao PTZ?; (III) Qual o comparativo com larvas de *zebrafish*?; (IV) Como se caracteriza o perfil convulsivo frente a diferentes concentrações de PTZ?; (V) Qual a latência para os animais atingirem a convulsão tonico-clonica?; (VI) Qual a estimativa de tempo para o animal se recuperar das crises convulsivas após a retirada da solução de PTZ?; (VII) Tendo em vista que este é um modelo de imersão do animal em uma solução de PTZ, a presença deste fármaco no SNC do animal depende do tempo e da concentração de exposição? (VIII) Qual a taxa de mortalidade para este modelo?

**3.OBJETIVO**

**3.1.Objetivo geral**

No intuito de responder tais perguntas, o objetivo deste trabalho foi detalhar a crise convulsiva induzida por PTZ em *zebrafish* adulto através de uma análise temporal das manifestações comportamentais convulsivas. Além disso, determinar as concentrações de PTZ no sistema nervoso central do zebrafish adulto.

**3.2.Objetivos específicos**

- Definir os scores comportamentais de convulsão neste modelo.

- Avaliar a progressão dos escores durante o tempo de exposição a diferentes concentrações de PTZ.

- Avaliar a latência para a convulsão tônico-clônica.

- Identificar o tempo que os animais levam para retornar ao comportamento basal após a retirada do PTZ

- Quantificar a mortalidade dos animais expostos as diferentes concentrações de PTZ até 7 dias após a exposição.

- Quantificar a concentração de PTZ no SNC do *zebrafish*, durante e após a exposição às soluções de 5 mM e 10 mM de PTZ.

**Parte II.**
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**RESULTADOS**

**CAPÍTULO I**

**Seizures induced by pentylenetetrazole in the adult zebrafish: a detailed behavioral characterization.**

Ben Hur M. Mussulini[a,*], Carlos E. Leite[b], Kamila C. Zenki[a], Luana Moro[a], Suelen Baggio[a], Eduardo P. Rico[a], Denis B. Rosemberg[a], Renato D. Dias[a], Tadeu M. Souza[a], Maria E. Calcagnotto[a], Maria M. Campos[b], Ana M. Battastini[a], Diogo L. de Oliveira[a,*].

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# Seizures Induced by Pentylenetetrazole in the Adult Zebrafish: A Detailed Behavioral Characterization

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## Abstract

Pentylenetetrazole (PTZ) is a common convulsant agent used in animal models to investigate the mechanisms of seizures. Although adult zebrafish have been recently used to study epileptic seizures, a thorough characterization of the PTZ-induced seizures in this animal model is missing. The goal of this study was to perform a detailed temporal behavior profile characterization of PTZ-induced seizure in adult zebrafish. The behavioral profile during 20 min of PTZ immersion (5, 7.5, 10, and 15 mM) was characterized by stages defined as scores: (0) short swim, (1) increased swimming activity and high frequency of opercular movement, (2) erratic movements, (3) circular movements, (4) clonic seizure-like behavior, (5) fall to the bottom of the tank and tonic seizure-like behavior, (6) death. Animals exposed to distinct PTZ concentrations presented different seizure profiles, intensities and latencies to reach all scores. Only animals immersed into 15 mM PTZ showed an increased time to return to the normal behavior (score 0), after exposure. Total mortality rate at 10 and 15 mM were 33% and 50%, respectively. Considering all behavioral parameters, 5, 7.5, 10, and 15 mM PTZ, induced seizures with low, intermediate, and high severity, respectively. Pretreatment with diazepam (DZP) significantly attenuated seizure severity. Finally, the brain PTZ levels in adult zebrafish immersed into the chemoconvulsant solution at 5 and 10 mM were comparable to those described for the rodent model, with a peak after a 20-min of exposure. The PTZ brain levels observed after 2.5-min PTZ exposure and after 60-min removal from exposure were similar. Altogether, our results showed a detailed temporal behavioral characterization of a PTZ epileptic seizure model in adult zebrafish. These behavioral analyses and the simple method for PTZ quantification could be considered as important tools for future investigations and translational research.

## Introduction

Epilepsy is a neurological disorder characterized by recurrent spontaneous epileptic seizures associated with distinct neurobiological and behavioral alterations [1]. One of the methods used to investigate epileptic seizures in experimental models consists on the analysis of the behavioral profile through a seizure stage-score classification [2]. This characterization is well established in rodents for seizures induced by electrical kindling Racine et al. [3] and for chemoconvulsant drugs, such as kainate (KA), pilocarpine and pentylenetetrazole (PTZ) [4]. Exposure to PTZ induces a concentration-dependent sequence of stereotyped behavioral changes that starts with orofacial movements and culminates in clonus-like seizures in rodent models. This seizure model has been widely used in the past 6 decades for discovery and development of several antiepileptic drugs (AED), such as benzodiazepines, valproate, gabapentine, etc [5,6,7]. Despite the advances in new AED discovery, 30% of epileptic patients still suffer with refractory

epilepsy [8,9]. Löscher et al. [5], in a critical review about the current animal models of seizure and epilepsy employed to discovery and development of new AED, pointed out that this high refractoriness could be a result from using always the same pro-convulsant focusing in rodent models.

In this context, the zebrafish (*Danio rerio*) emerges as a new animal model to evaluate the effects of classical pro-convulsant drugs in order to develop and characterize new AED [10–14]. This species exhibits several anatomic similarities and a high genetic homology with mice and humans [15,16]. Moreover, zebrafish presents a tight junction-based blood–brain barrier similar to higher vertebrates, with substantial macromolecule permeability, which makes this model an attractive organism for high throughput screening applications and AED discovery [17,18]. Furthermore, this model offers a potential non-discriminatory screening for AED [13], in contrast to that for rodents where usually seizures induced by PTZ were used to identify anticonvulsants agents that acts mainly through GABA [19]. All

these aspects contributes to an increased number of investigations involving chemoconvulsant induced-seizures in zebrafish, and epilepsy research through genetic models that are either susceptible or resistant to seizures, and mutations associated with known human epilepsy syndromes [20–29]. These investigations are based mostly in Baraban et al. [20] study, which showed that zebrafish larvae exposed to PTZ displays complex and stereotypical patterns of seizure behavior sequence, ictal- and interictal-like electrical activity in immobilized animal, and *c-fos* expression in brain regions. However, the use of adult zebrafish, which shows a broader behavioral repertoire [30–33], with a fully developed central nervous system (CNS), when compared to zebrafish larvae [34,35], could improve the currently protocols for new AED research and to study the mechanisms underlying seizures.

Although electrophysiology pattern of PTZ induced seizure activity [31], *c-fos* expression [32], and some behavioral analyses have already been demonstrated in adult zebrafish exposed to PTZ [22,36,37], questions regarding the behavioral profile of the seizure pattern remain unanswered, such as: (I) Is the score system proposed by Desmond et al. [35] for studying seizure in adult zebrafish suitable for all behavior seizure manifestations in PTZ model?; (II) What is the sequence of behaviors during the exposure to PTZ?; (III) Are these manifestations similar to larvae?; (IV) Does the seizure behavior profile change with alterations of drug concentration?; (V) What is the latency to reach the clonus-like behavior in distinct concentrations of PTZ exposure?; (VI) How long does animals take to return to a normal behavior after PTZ exposure?; (VII) Does the PTZ levels in the fish brain depends on the concentration and time of exposure?; (VIII) What is the mortality rate of fish exposed to PTZ at distinct concentrations?

In order to answer these questions, we performed a detailed behavioral seizure analysis of PTZ-induced seizures in adult zebrafish. Additionally, we performed a quantification of brain PTZ levels during and after exposure to this chemoconvulsant drug. All of these analysis were based on the pioneer work performed by Alfaro et al. [21] where they characterized a detailed seizure scale for kainic acid-induced epileptic seizures in adult zebrafish.

## Materials and Methods

### Ethics Statement

All procedures with animal subjects have been approved by the Ethic Committee for Use of Animals - CEUA from Universidade Federal do Rio Grande do Sul (protocol number 22214).

### Reagents

Pentylenetetrazole (PTZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Diazepam (DZP) was purchased from União Química Nacional S/A (Pouso Alegre, MG, Brazil). Acetonitrile and methanol were purchased from Merck® (Darmstadt, Hessen, Germany). Pure Ultra pure water was obtained from a Millipore Corporate® Milli-Q water system (Billerica, Massachusetts, USA). All HPLC components and software ChemStation were from Agilent Technologies® Inc. Santa Clara, California, USA.

### Animals

Adult zebrafish (*Danio rerio*; 4 to 6 months-old, ±50:50 male:female ratio) of heterogeneous wild-type stock (standard short-fin phenotype) were obtained from a local commercial supplier (Delphis, RS, Brazil). Animals were carefully weighted and measured in order to select the ones with similar weight and size (35±2 mg and 2±0.15 cm, respectively) to avoid putative

variations of drugs pharmacodynamic and pharmacokinetic. Fish were housed in 50-L aquariums (80-100 fish per aquarium) for at least 2 weeks prior to the experiments in order to acclimatize to the animal facility. All tanks were filled with non-chlorinated water previously treated with 132 μL/L AquaSafe® (Tetra, VA, USA) and kept under mechanical and chemical filtration at a targeted temperature of 26±2°C and water pH at 7.0 to 8.0 (system water). The room illumination was provided by ceiling-mounted fluorescent lamps on a 14/10 light/dark photo period cycle (lights on at 7:00 am). Animals were fed twice a day with a commercial flake fish food (Alcon BASIC®, Alcon, Brazil). All animals used in this study were experimentally naive, healthy and free of any signs of disease. They were maintained according to the National Institute of Health Guide for Care and Use of Laboratory Animals (2011).

### Treatments and Seizure Behavioral Characterization

To induce experimental epileptic seizures, animals (*n* = 12 in each group) were individually exposed to 5, 7.5, 10, and 15 mM PTZ, readily dissolved in water. All PTZ concentrations and the time of exposure were based on previous reports in order to induce clonus-like seizure responses [20,22,36,38,39]. The control group was exposed to system water only. In order to investigate the effect of a classical AED on PTZ-induced seizures in zebrafish, a GABA$_A$ positive allosteric modulator diazepam (DZP) was used. Two groups of animals were exposed to 75 μM DZP for 40 min in a beaker containing a 0.5-L solution. Afterwards, the animals were rapidly transferred to a beaker containing system water to remove the excess of DZP. One group was further transfer to a tank with system water to investigate the DZP sedative effect (DZP control group) and another group to a similar tank containing 10 mM PTZ solution (DZP/10 mM PTZ group). All conditions for DZP experiments were previously set up by our group (data not shown).

The detailed behavioral seizure profile characterization was performed during the same time frame each day (from 10:00 am to 4:00 pm). The apparatus consisted of a tank (20 cm width×13 cm height×7 cm length) filled with 1.5 L of PTZ solution or system water. In order to keep the same experimental conditions, animals were randomly handled from their home tanks and individually transferred to beakers filled only with system water for the same period of DZP pretreatment (40 min). Animals were carefully placed individually in the tanks and their behavioral seizure activity was recorded for a single session of 20 min. At each experiment a fish was placed individually into the determined treatment solution, which was not used in subsequent experiments. Assays were performed at three independent days with 4 animals in each group per day. All experimental procedures were performed on a silent room.

A webcam (Microsoft® LifeCam 1.1 with Auto-Focus) was placed 30 cm from the testing tank to ensure good video recording and to monitor the location and swimming activity of the fish. All tank walls were coated with white background cover, in order to avoid the reflex of the animal in the walls and in the tank bottom, and to ensure a uniform background for the video analysis. To boost the contrast between the background and zebrafish, two 60-watts light bulbs were placed 50 cm behind the tanks. All behavioral data were evaluated by two trained observers in a blinded fashion (inter-rater reliability ≥0.92). All necessary precautions were taken to ensure representative behavioral results and also to avoid handling stress. Throughout the experiments, the fish were gently transferred between home tanks, beakers, and experimental apparatus. All fish were handled and tested at similar way and the behaviors were recorded in the same room, which kept the manipulation, water quality, and illumination uniform and constant between trials.

## Epileptic Seizure Stage Score

The fish ($n = 6$) were immersed into a PTZ solution (5–15 mM) and monitored for 60 min to evaluate epileptic seizures-related behavior. To characterize each stage, behavioral manifestations were evaluated according to the literature [22,35,37] and the sequence of behavioral manifestations was described using a range from lower to higher concentration of PTZ tested in our study. It is important to emphasize that random alterations in behavioral induced by PTZ (e.g., jumping) were not considered. For each stage, we assigned a specific score, described in Table 1 (see Video S1).

## Washout Period and Survival Assessments

After PTZ exposure, we transferred the fish to an intermediary beaker containing system water to eliminate all the residual PTZ in contact with the animal. Immediately after, we than transferred the fish to another beaker with a new clean system water to evaluate the washout period. During the washout of PTZ, groups were observed for more three hours, to determine the latency to return to score 0. We considered that the animal fully returned to the basal behavior when it reached the score 0 and remained in this score until the end of the 3 hours. In order to access the survival rate, the fish were individually transferred to 1 L recipient, filled with system water. The water was renewed every 24 h and the survival was assessed (the total time of this protocol was 168 h). The Figure 1 illustrates the protocol used in this study.

## Determination of PTZ Levels in the Zebrafish Brain

To quantify the PTZ levels that reached the zebrafish brain, we performed experiments using high-performance liquid chromatography equipped with an isocratic pump, diode array detector (DAD), degasser and manual injection system. Chromatographic separations were performed using a reverse-phase column (250 mm×4 mm, 5 mm LiChrospher® 100 RP-18). The column was protected by a guard column (4×4 mm, 5 mm LiChrospher® 100 RP-18) and maintained at a temperature $22 \pm 2°C$. The mobile phase was a mixture of phosphate buffer 10 mM pH 6.9: methanol: acetonitrile (60:35:5, v/v/v). The flow rate of 0.8 mL/min was maintained isocratically, the DAD was set at 202 nm and the total run time was 6 minutes.

The PTZ determination was based on the method previously described by Soto-Otero et al. [40]. Each independent experiment was performed using a pool of three whole brains of animals from the PTZ groups (5 mM and 10 mM) after the exposure of 2.5 min, 20 min, and after 60 min PTZ washout. Briefly, the animals underwent cryoanesthesia and euthanized by decapitation. The cranial skulls were excised; the brains were removed and rapidly homogenized in 1 mL of cold PBS using a glass–Teflon homogenizer in ice. The samples ($n = 4$ per group) were centrifuged at 13.500 $g$ for 5 min at $4°C$ in 1.5 mL tubes, and the supernatants were collected for PTZ analysis. Nine hundred microliters of supernatant was treated with 5.0 mL of dichloromethane. The tube was mixed for 30 s, an excess of ammonium sulfate was added with calibrated spatula and the tube contents were mixed again for 30 s. The samples were centrifuged at 800 $g$, the organic phase was separated and totally dried with nitrogen at room temperature. Following, the dried samples were reconstituted with 100 µL of the mobile phase and 20 µL was injected into HPLC. Standard curve measures (1–75 µg/mL) can be accessed in Figure S1.

## Statistics

Non-parametric data of seizure scores were expressed as median $\pm$ interquartile range. Scatter plots were designed to enable the analysis of variance across time performed by Friedman test followed by Dunn's Multiple Comparison test as post hoc. Cumulative frequency was determined using the percentage of animal that reached each score across time for the respective treatment tested. The area under the curve (AUC), latency, and washout period were represented as mean $\pm$ S.E.M and analyzed by the one-way ANOVA followed by the Bonferroni's test as post hoc. Student's $t$ test was used to compare the DZP/10 mM PTZ and 10 mM PTZ groups. The PTZ quantification in brain were expressed as mean $\pm$ S.E.M and analyzed by two-way ANOVA followed by Bonferroni's test post-hoc. The survival time was compared among groups using the log-rank test of trend. In all analyses, the significance level was taken as $p \leq 0.05$.

## Results

The behavioral analysis shown that the control group exhibited spontaneous usual swimming movements consisted by repeated short swims (data not shown). On the other hand, animals immersed into PTZ solution presented behavioral epileptic seizures, classified in different scores as shown in Table 1. Figure 2A depicts the temporal behavioral profile of animals exposed to different PTZ concentrations. There was a rapid score progression in the first 5-min period for 10 and 15 mM PTZ (Friedman test, $p < 0.0001$; Dunn's Multiple Comparison test, $p < 0.05$). Therefore, in order to perform an analysis across time,

**Table 1.** Score phenotype of the PTZ seizure model in adult zebrafish.

| SCORE | Behavior phenotype |
|---|---|
| 0 | Short swim mainly in the bottom of the tank. |
| 1 | Increased swimming activity and high frequency of opercular movement. |
| 2 | Burst swimming, left and right movements, and erratic movements. |
| 3 | Circular movements. |
| 4 | Clonic seizure-like behavior (abnormal whole-body rhythmic muscular contraction). |
| 5 | Fall to the bottom of the tank, tonic seizure-like behavior (sinking to the bottom of the tank, loss of body posture, and principally by rigid extension of the body). |
| 6 | Death. |

All behavior phenotypes present in video S1 defined by scores.
doi:10.1371/journal.pone.0054515.t001
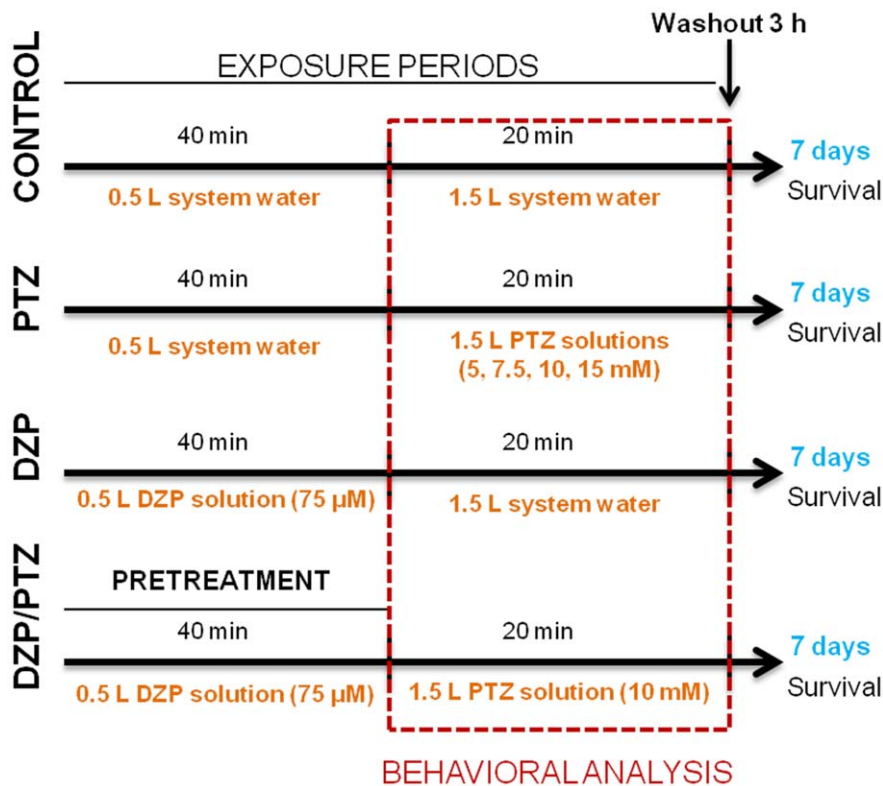
**Figure 1. Experimental protocol schematic representation.** The methodological approach used to evaluate the epileptic seizure-like behavior (experimental conditions, exposure periods, washout period, and survival evaluation after behavioral tests).
doi:10.1371/journal.pone.0054515.g001

the first 5 min and the remaining 15 min were divided into 30 s and 150 s intervals, respectively. Animals immersed into the higher concentrations (10 and 15 mM) reached scores 4 and 5 faster than animals exposed to lower concentrations (5 and 7.5 mM) of PTZ. Animals immersed into 5 mM PTZ showed repetitive low scores (1 and 2) during the first 5-min and only presented scores 4 and 5 in the last 15-min period. Animals exposed to 7.5 mM PTZ presented an intermediate profile between 5 mM and higher concentrations, alternating from scores 3 to 5, starting at 210 to 1200 s (Friedman test, $p<0.0001$; Dunn's Multiple Comparison test, $p<0.05$).

The time necessary to reach score 5 were: 1050 s for 5 mM; 600 s for 7.5 mM; 240 s for 10 mM; and 180 s for 15 mM. Furthermore, the animals immersed into 10 and 15 mM PTZ showed a rapid change from scores 0–2 to score 4 (16.66 and 58.33%, respectively) (Figure 2B). The score 5 was not observed in all animals treated with lower concentrations (only 83.33% and 75% of animals reached this score at 7.5 mM and 5 mM, respectively). Scatter plot representations were performed in order to see each animal behavioral seizure profile across time described in the score curves (Figure S2).

The analysis of the score curves (Figure 2A) suggests that there are three different moments for the PTZ-induced seizures in zebrafish. In the first moment (0 to 150 s), higher PTZ concentrations induced seizures with score 4; in the second (150 to 300 s), PTZ at 7.5 mM induced seizures with scores 3–4; and in the third (300 to 1200 s), all PTZ concentrations induced seizures with scores 3–5 (Figure S3A). In order to evaluate the seizure intensity across time in these three moments, we measured the area under score curve for each animal for each PTZ concentration (Figure 2C). In the first interval (0–150 s), animals from

15 mM PTZ group presented higher seizure intensity than 10 mM PTZ and both concentrations displayed higher seizure intensity when compared to 5 and 7.5 mM (one-way ANOVA, $F[4,59]=44.56$, $p<0.0001$; Bonferroni test, $p<0.05$). In the second interval (150–300 s), the seizure intensity was lower in 5 mM PTZ group when compared with other groups (one-way ANOVA, $F[4,59]=28.12$, $p<0.0001$; Bonferroni test $p<0.05$). In the last interval (300–1200 s), animals from 15 mM PTZ group showed a higher seizure intensity when compared to 5 and 7.5 mM PTZ (one-way ANOVA, $F[4,59]=20.10$, $p<0.0001$; Bonferroni test, $p<0.05$). Figure S3B shows the seizure intensity during the total time of observation (1200 s).

Animals immersed into 15 mM PTZ solution showed lower latency to the first episode of seizure score 4 when compared to animals immersed into 5 and 7.5 mM PTZ. In addition, animals exposed to 5 mM PTZ presented higher latency to the score 4 when compared to all other concentrations (one-way ANOVA, $F[4,59]=49.43$, $p<0.0001$; Bonferroni test, $p<0.05$), (Figure 2D). In the washout period only 15 mM PTZ group required a longer time to return to score 0, when compared to other concentration groups (one-way ANOVA; $F[4,59]=21.16$, $p<0.0001$; post-hoc, $p<0.05$) (Figure 3).

As shown in Figure 4, higher PTZ levels in the brain were found in animals exposed to 10 mM PTZ when compared with 5 mM PTZ (two-way ANOVA, concentration effect, $F[1,12]=152.4$, $p<0.0001$). Additionally, there was a peak of PTZ in the brain after 20-min of exposure. Similar levels were observed after a 2.5-min exposure and after a 60-min removal from the PTZ solutions (two-way ANOVA, time effect, $F[2,12]=176.9$, $p<0.0001$). There was a positive interaction between exposure time and PTZ
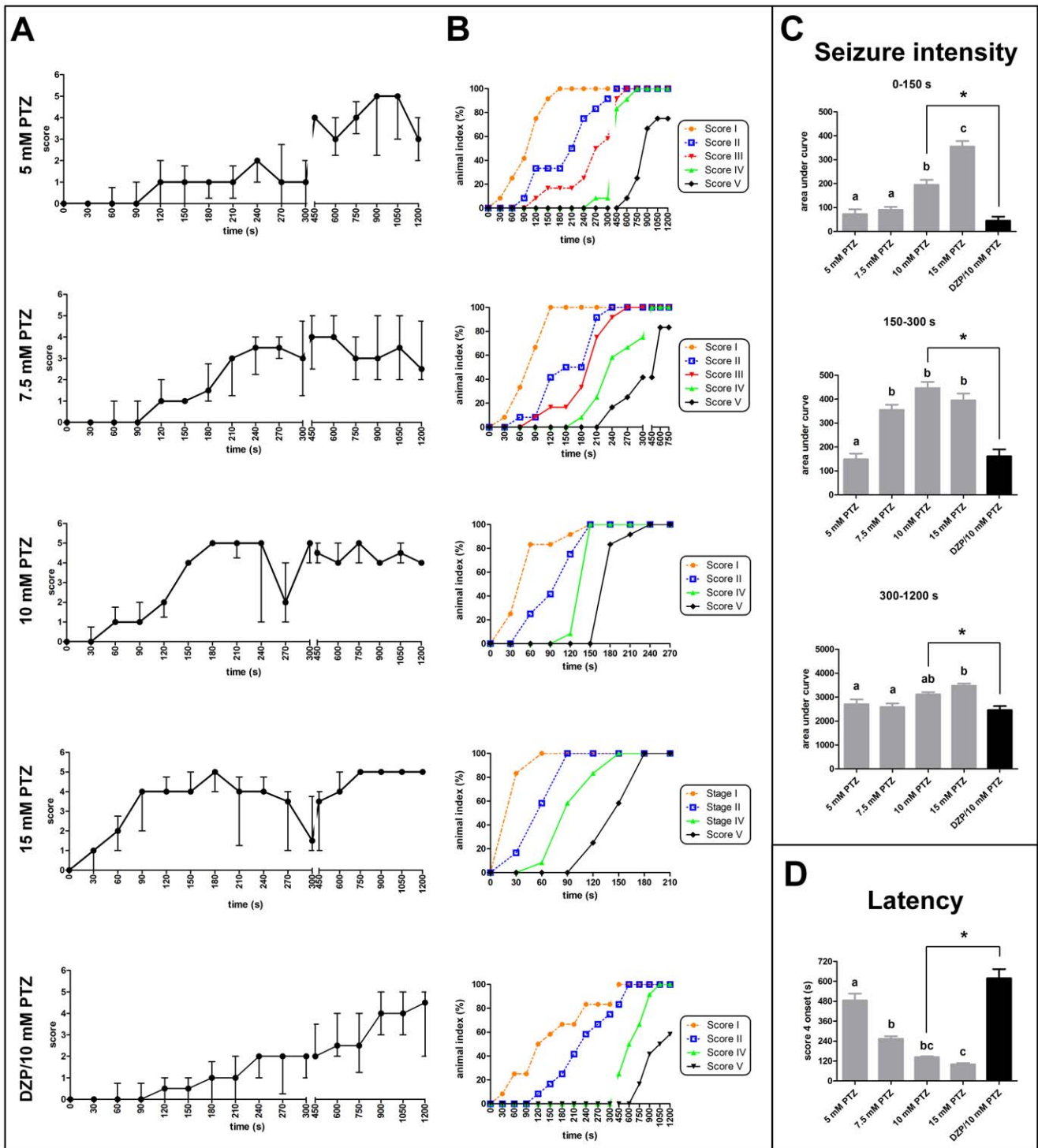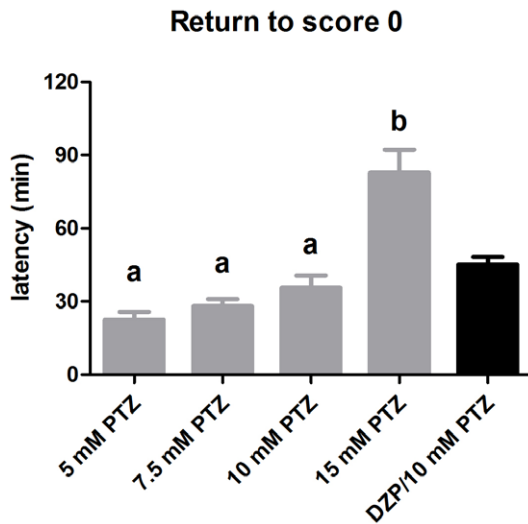
**Figure 2. Behavioral profile of PTZ-induced seizures in adult zebrafish.** The main characteristic seizure behavior induced by 5–15 mM PTZ and DZP/10 mM PTZ treatments during 20 min ($n = 12$). (A) Seizure score (only the highest score reached was consider in each interval) and cumulative frequency (B). Data are represented as median ± interquartile range and as the animal index (%) that reached the scores across time, respectively. (C) Seizure intensity during distinct moment tests (0–150, 150–300, and 300–1200 s) evaluated by the area under curve observed for each treatment. (D) Latency to score 4 onset. Data from seizure intensity and latency are represented as mean ± S.E.M and analyzed by one-way ANOVA followed by Bonferroni's test as post-hoc. Distinct letters indicate statistical differences among PTZ-treated groups (gray bars). The DZP/10 mM PTZ is represented as black bars and compared to 10 mM PTZ group by the Student's t test. *indicates significant difference between groups.
doi:10.1371/journal.pone.0054515.g002

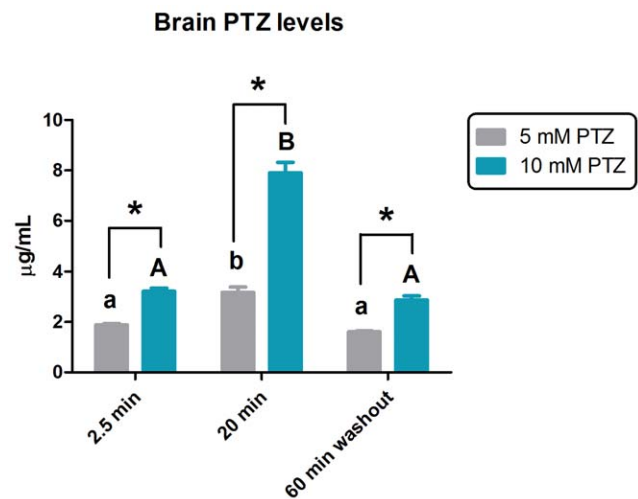concentration (two-way ANOVA, time X concentration effect: $F_{[2,12]} = 52.41$, $p < 0.0001$; Bonferroni test, $p < 0,01$).

After the DZP pretreatment, fish remained immobilized during the initial 7 min of observation. Afterwards, fish began
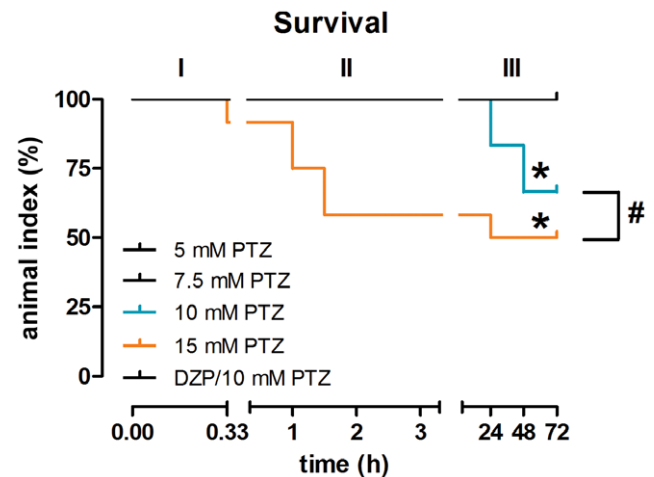
## Return to score 0



**Figure 3. Latency to score 0 during the washout period.** Data are represented as mean ± S.E.M and analyzed by one-way ANOVA followed by Bonferroni's test as post-hoc. Distinct letters indicate statistical differences among PTZ-treated groups (gray bars). The DZP/10 mM PTZ is represented as black bars and compared to 10 mM PTZ group by Student's $t$ test with no statistical difference.
doi:10.1371/journal.pone.0054515.g003

to present short movements in the bottom of the tank and, after 15 min the motor activity was recovered, being similar to the control group. Two out of 12 animals immersed into DZP died during observation (data not shown). Zebrafish from DZP/10 mM PTZ group presented different temporal seizure profile compared with 10 mM PTZ group (Figure 2A). In 5 min, all animals exposed to 10 mM PTZ reached scores 4 and 5. However, 66% of animals exposed to DZP/10 mM PTZ presented scores 1 and 2, 16% reached score 3 and 16% exhibited score 0. In the last 15 min, animals exposed to 10 mM PTZ presented an alternation between scores 4–5. On the other hand, fish pretreated with DZP presented scores from 2 to 5 (Friedman test, $p<0.05$; Dunn's Multiple Comparison test, $p<0.05$). As the cumulative curve shows, 16.66% of animals exposed to 10 mM PTZ and 41.66% of animals exposed to DZP/10 mM, did not present score 3 prior to the first score 4. The time required to reach score 1–5 is shown in the Figure 2B. Score 5 was observed in 58.33% of the animals pretreated with DZP, and exhibited lower seizure intensity during the entire observation (Student's $t$ test, $p<0.0001$), (Figure 2C and S3). The DZP/10 mM PTZ group presented longer latency than the 10 mM PTZ group to reach the score 4 (Student's $t$ test, $p<0.0001$), (Figure 2D). Concerning the washout period (Figure 3), the time to return to score 0 was similar for animals treated with DZP/10 mM PTZ and 10 mM PTZ.

Only animals under the highest concentration (15 mM) died (score 6) during exposure time and washout period. In the entire experiment, mortality rate was 33.33% and 50% at 10 mM and 15 mM, respectively. Log-rank test for trend indicates a different survival profile between these treatments ($X^2[1] = 16.20$, $p<0.0001$), and Hazard Ratio assumes that the mortality rate occurred 2.25 faster at 15 mM than at 10 mM PTZ. All other treatments, including the DZP pretreatment, had 100% of survival (Figure 5). After 72 h, survival was stable for all treatments (data not shown).

## Brain PTZ levels



**Figure 4. Quantification of the PTZ levels in the brain.** The concentration of PTZ in the brain was determined as μg PTZ/mL sample at 5 mM (gray bars) with values of 1.885, 3.175, 1.612, and 10 mM (blue bars) with values of 3.222, 7.905, and 2.870, after 2.5 and 20-min of PTZ exposure, and after 60-min washout. Data are expressed as mean ± S.E.M and analyzed by two-way ANOVA followed by Bonferroni's test as post-hoc. Distinct letters indicate statistical difference within the same group at different periods, (lower case letter for 5 mM and capital letters for 10 mM) whereas the asterisks (*) indicates significant difference between both PTZ groups for each time.
doi:10.1371/journal.pone.0054515.g004



**Figure 5. Survival evaluation.** Kaplan–Meier plot representing the animal index (%) that survived in 3 distinct periods: I – PTZ exposure; II – Washout period; III – survival evaluation in each 24 h after behavioral experiment procedures. Data are analyzed using the log-rank test for trend to compare groups. *Indicate significant differences from 100% survival, whereas the symbol (#) represents statistical difference profile between 10 and 15 mM PTZ groups.
doi:10.1371/journal.pone.0054515.g005

## Discussion

In the current study, we described a detailed behavioral characterization of the adult zebrafish epileptic seizure model induced by PTZ. In the past decade, Baraban et al. [20] demonstrated that PTZ elicits seizures in zebrafish larvae. More recently, several studies have extended this model to adult

zebrafish, using three main analyses: electrophysiological evaluation in immobilized animals [39], *c-fos* expression in the CNS [37], and behavioral endpoint parameters [22]. However, a detailed description of the behavioral sequence profile of PTZ-induced seizures in free-swimming zebrafish has not been performed. To our knowledge, this is the first study to describe a temporal behavioral characterization of PTZ-induced seizures and to measure PTZ levels in the brain of adult zebrafish. Both parameters may contribute to make this model useful not only for new AED discovery, but also to elucidate the molecular, neurochemical, and cellular mechanisms underlying seizures.

Among the different methodological approaches described in the literature to define seizure stage-scores, we decided to use a wide range of PTZ concentrations, similarly to the method purposed for larvae zebrafish PTZ model [20] and adult zebrafish KA model [21]. This approach of using different concentrations of PTZ was probably the main reason why distinct scores of seizures were different from what was previously suggested by Desmond et al. [35]. In his study, the authors presented a score system based mostly on one concentration of pro-convulsant drugs exposure (11 mM PTZ, 250 mg/L caffeine, and 0.17 mM picrotoxin). Likewise, we found that exposing the zebrafish to high PTZ concentration, such as 10 mM, our seizure score 3 was skipped, jumping directly from score 2 to 4, in some animals. Similar results can be verified in rodent models when they are injected with high doses of PTZ [40]. Moreover, Desmond et al. [35] define the initial score as freezing and hyperventilation (score 1). At any point the authors described circular behavior and/or whirlpool-like swimming as an independent manifestation from clonic-like seizure. On the other hand, like Baraban et al. (2005), we defined the initial increased activity with hyperventilation as score 1. To better clarify each seizure stage, we divided the scores 3 and 4 from Desmond et al. [35] (circular and/or spiral swimming, rapid movements from left to right, erratic movements, abnormal spasm-like muscular contractions, rapid whole-body clonic-like convulsions and tonic seizures with rigid extension of the body, loss of body posture, sinking to the bottom of the tank, spasms for several minutes, respectively), into score 3 (circular and/or spiral swimming), score 4 (clonic seizure-like behavior), and score 5 (fall to the bottom of the tank, loss of body posture, and tonic seizure-like behavior) (Table 1). This distinction is crucial since each of these stages were reached in a clearly independent time-period, when zebrafish were exposed to the lowest PTZ concentration tested (5 mM). Additionally, not every animal reached the score 5 with 5 mM and 7.5 mM PTZ, showing that clonic-like seizure behavior occurs independently from tonic-like seizure behavior. Since the score 3 in zebrafish larvae exposed to PTZ [20] is defined as a clonus-like convulsion leading to loss of body posture for 1–3 s, we could not use this score for the adult animal. All these manifestations in larvae occur almost at the same time probably because to its immature CNS. What is define as a immobility in larvae can be interpreted as a rigid and extended body posture (tonic-like seizure) that occur independently from clonus-like convulsion behavior in adult zebrafish. Our data also shows that animals rapidly assume left-to-right and erratic movements in the beginning of the behavioral manifestations (score 2) and prior to the circular movements (score 3). However, it is important to emphasize that the animals return to lower scores during PTZ exposure, even after reaching score 5.

Temporal profiles of seizure behavioral manifestation induced by PTZ present a different sequence of score from KA model [21]. The KA score 3, whirlpool-like swimming, corresponds to our score 1; and the score 5, rapid whole-body clonus-like convulsions, correspond to our score 4. This difference may suggest that it is not possible to apply a single stage-score behavior characterization for distinct chemoconvulsant. Nevertheless, animals that received the highest dose of KA or highest concentration of PTZ evolved directly from score 2 to 4, showing that a temporal behavioral profile occurs in different epileptic seizures models.

Seizure intensity was quantified as the area under curve (Figure 2C) in order to measure how fast the animals reach scores 4 and 5 and remain at these scores. Our results show that this parameter increased with PTZ concentration exposures.

In rodent models, the latency to clonic seizure-like behavior is used in screening studies of new AED discovery [41,42]. However, our results indicate that the analyses of several parameters, rather than only one, could provide a better and reliable instrument that could be used for screening new drugs. Animals exposed to 7.5 mM PTZ or 10 mM PTZ presented similar latency to reach to score 4, but different seizure intensities (Figures 2D and 2C). Additionally, the seizure score curve and the cumulative curve of animal exposed to 7.5 mM PTZ were variable, but the total seizure intensity was similar to 5 mM and different from 10 mM (Figures 2B and 2A). Therefore, these four above-mentioned parameters (score curve, cumulative curve, intensity and latency) would be the foremost method to interpret behavior in different seizure models.

Despite the described well absorption of PTZ by zebrafish, formal HPLC measurements of PTZ in the brain have never been reported for this model [13]. In our study, PTZ brain levels denote a concentration exposure-dependence, presenting a positive interaction between concentration and time of immersion. Additionally, the PTZ brain level of 3.2 μg/mL (1 mL = 3-brain pool) appears to be closely correlated with the first score 5 plateau (Figure 2A). Importantly, our quantification profile was similar to rodent models where a peak of PTZ concentration in the brain is followed by its decreased to the initial levels without reaching zero concentration after 1 h of exposure [40,43].

Another aspect to be considered is the recovery time after PTZ exposure. Our focus was not to elucidate a post-ictal period from an electrophysiological point of view, but to characterize only the behavioral phenotype of seizures induced by PTZ. We measured the time to return to score 0 and to remain at this score phenotype for 3 h after to remove from the PTZ solution. Our results showed that animals exposed to 5–10 mM exhibited similar time to return to score 0 and similar brain levels of PTZ at 2.5 min post-exposure and after a 60-min washout, even though they presented a completely different epileptic seizure profile. Rodent models show important neurochemical changes during the ictal and post-ictal periods induced by PTZ. Those changes could be related to a post-ictal depression after seizure clonus-like behavior, a period when the CNS remains refractory to further seizure activity [43,44,45]. This could explain why, even with detectable levels of PTZ in the brain, the fish returned to the normal behavior. Further studies should be performed to explain why the behavior returns to score 0 at a similar time for all PTZ concentration, except for 15 mM.

Distinctly from seizure intensity, we defined seizure severity as the sum of seizure intensity, latency to return to score 0, and mortality rate. We observed that the severity of PTZ-induced seizure at 15 mM is higher than 10 mM, despite of similar intensity profiles. The higher severity of seizure induced by 15 mM PTZ is associated with longer latency to return to score 0 and mortality rate of 50%. PTZ at 10 mM induced seizures with an intermediary severity, with mortality rate of 33%. On the other hand, 5 and 7.5 mM PTZ induced seizures with lower severity and no changes in total intensity, latency to return to score 0 or mortality rate. Although 15 mM PTZ appears to be a good concentration to study seizure severity, the increase in mortality

rate is an important limiting factor in epileptic seizure models [44]. However, in the work published by Pineda et al. [39], where fish were exposed to 15 mM PTZ for only three minutes, the authors mentioned that the mortality rate was zero. Moreover, they also showed that during a continuous exposure to 15 mM PTZ for 90 min, the neuronal activity increased up to 35 min, and only at 90 min of exposure the recording amplitude reversed approximately to the pattern of flat-line EEG, indicative of brain death.

Besides the article mentioned above, the first publication of adult zebrafish and PTZ used 10 mM PTZ and exposure time of 10 min repeated 3 times (total 30 min) [36]. Following this publication, Wong et al. [22] tested 11 mM PTZ for 20 min; Siebel et al. [38] used distinct concentrations (2.5 mM, 5 mM and 15 mM) during 20 min; and Stewart et al. [37] exposed the fish to 11 mM PTZ solution for 20 min. However, no data regarding mortality rate were addressed by any of these publications. Here we show that the amount of PTZ brain levels depend on the exposure time to this drug. The increase in severity for 15 mM PTZ may be attributed to the longer exposure time-period. So, we decided to use 20 min of PTZ exposure and the range of 5, 7.5, 10 and 15 mM PTZ based mostly on these previous literature, aiming to perform a detailed seizure behavior characterization for future studies of this model. As our data showed similar low severity for 5 and 7.5 mM, smaller concentrations (2.5 and 3.75 mM) were not used. This approach of using 20 min and a low concentration of exposure, could be interesting to neurochemical mapping the seizure, since the dopaminergic and serotoninergic [46], cholinergic [47], purinergic [48], and glutamatergic [16] systems have been already described in adult zebrafish.

Due to the great variability of the seizure induced by 7.5 mM PTZ, confirmed by the scatter plot (Figure S2) and the mortality rate of 15 mM, we chose the concentrations of 5 and 10 mM to quantify the levels of PTZ in the brain. Since the mortality rate of 10 mM PTZ induced-seizure were similar to 6 mg/kg KA injection [14] and lower than 15 mM PTZ in adult zebrafish, we decided to use 10 mM PTZ concentration to test all parameters of seizure severity, such as, score curve profile, and latency to reach score 4. These parameters were also tested, when fish are pretreated with a classical anticonvulsant, DZP, to test this behavioral tools for future use in AED research. We observed a decrease in seizure severity in the pretreatment with DZP when compared with 10 mM PTZ treatment alone. The difference in seizure severity for pretreatment with DZP and for all PTZ exposure concentrations could be mathematically demonstrated by the area under the curve analysis, providing a very suitable analysis tool for future AED research. Although the latency to return to score 0 was not affected, it is important to mention that DZP pretreatment presented zero mortality rate.

It is important to point out that the large volume of compounds used in this study (what could be seen as a disadvantage when compared to rodent models), is direct correlated to our main objective. To perform a behavior characterization, a large space is necessary not only for the animals display any behavior alteration, but also to be easier for the observer to detect such alterations. Nevertheless, based on our work, future researchers will be able to have a detailed temporal behavioral repertoire in a smaller volume of PTZ (0.3 L should be enough to perform the same analysis). Considering this small volume or even the intra-peritoneal injection of drugs in adult zebrafish (e.g. MK-801 and DNQX [21]), the amount of AED to be used in future studies may be insignificant when compared to rodent models. Furthermore, the maintenance of zebrafish to investigate the effects of possible anticonvulsant drugs, and mechanisms underlying seizures in future experiments (e.g., behavioral tasks, neurochemical changes,

neuronal reorganization and activity) makes zebrafish a lot cheaper and faster than rodent models.

To date, electrophysiology recording in freely swimming adult zebrafish is still a challenge. Despite the studies performed by Pineda et al. [39,49], the actual EEG methodology is restricted to anesthetized and immobilized animal, making it impossible to correlate the electrophysiological data with the behavior manifestation. We hope that, as soon as new technical approaches emerge to record neuronal activity in freely swimming animal, our behavioral characterization can be used as a background for mapping neuronal activity during the different seizure stages based on the scores proposed by this study.

## Conclusion

In summary, our results described a detailed temporal characterization of the stage-score manifestations of adult zebrafish exposed to distinct PTZ concentrations. We thoroughly described important parameters, such as score curve profile, cumulative score frequency, seizure intensity, latency to score 4 onset, scatter plot score curve, latency to return to score 0, mortality rate and seizure severity. Furthermore, we showed, for the first time, that PTZ brain levels depend on PTZ concentration and exposure time, exhibiting similar profile to rodent models of PTZ injection. Therefore, the behavioral analyses and the simple method for PTZ quantification described here could be considered as important tools for future investigations and translational researches.

## Supporting Information

**Figure S1   PTZ standard curve.** The figure shows the: A) HPLC chromatogram of PTZ detection for different concentrations (1–75 µg/mL); B) linear correlation plot of area for each PTZ concentration.
(TIFF)

**Figure S2   Scatter plot score curve for the experimental groups.** The figure depicts the higher score reached by each animal from 5–15 mM PTZ and DZP/10 mM PTZ groups during the observation time. Each symbol corresponds to its respective animal at each group ($n = 12$). Each symbol represents the profile of a single animal during each interval analyzed and the animal is limited to only one treatment.
(TIFF)

**Figure S3   Comparative score curves.** (A) Overlap of all treatment curves to clarify the 3 moments in the score curves. (B) Seizure intensity for total observation time. Data are represented as mean $\pm$ S.E.M and analyzed by one-way ANOVA followed by Bonferroni's test as post-hoc. Distinct letters indicate statistical difference between PTZ-treated groups (gray bars). The DZP/10 mM PTZ is represented as black bar and compared to 10 mM PTZ group by Student's $t$ test. The asterisks (*) indicates significant difference between both groups.
(TIFF)

**Video S1   Epileptic seizure stage-score induced by PTZ in adult zebrafish.** The video demonstrates the representative behavioral scores (0–6) exhibited by zebrafish exposed to 10 mM PTZ.
(AVI)

## Author Contributions

Conceived and designed the experiments: BHM CEL EPR DBR DLO. Performed the experiments: BHM CEL KCZ LM SB EPR. Analyzed the data: BHM CEL EPR DBR TMS DLO. Contributed reagents/materials/ analysis tools: RDD MMC AMB DLO. Wrote the paper: BHM CEL EPR DBR RDD TMS MEC DLO.

# References

1. Fisher RS, van Emde Boas W, Blume W, Elger C, Genton P, et al. (2005) Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia 46: 470–472.
2. Sarkisian MR (2001) Overview of the Current Animal Models for Human Seizure and Epileptic Disorders. Epilepsy Behav 2: 201–216.
3. Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol 32: 281–294.
4. Morimoto K, Fahnestock M, Racine RJ (2004) Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog Neurobiol 73: 1–60.
5. Loscher W (2011) Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. Seizure 20: 359–368.
6. Shorvon SD (2009) Drug treatment of epilepsy in the century of the ILAE: the second 50 years, 1959–2009. Epilepsia 50 Suppl 3: 93–130.
7. Shorvon SD (2009) Drug treatment of epilepsy in the century of the ILAE: the first 50 years, 1909–1958. Epilepsia 50 Suppl 3: 69–92.
8. Loscher W, Schmidt D (2011) Modern antiepileptic drug development has failed to deliver: ways out of the current dilemma. Epilepsia 52: 657–678.
9. Kwan P, Arzimanoglou A, Berg AT, Brodie MJ, Allen Hauser W, et al. (2010) Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. Epilepsia 51: 1069–1077.
10. Penberthy WT, Shafizadeh E, Lin S (2002) The zebrafish as a model for human disease. Front Biosci 7: d1439–1453.
11. Kari G, Rodeck U, Dicker AP (2007) Zebrafish: an emerging model system for human disease and drug discovery. Clin Pharmacol Ther 82: 70–80.
12. Ingham PW (2009) The power of the zebrafish for disease analysis. Hum Mol Genet 18: R107–112.
13. Berghmans S, Hunt J, Roach A, Goldsmith P (2007) Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy Res 75: 18–28.
14. Sierra S, Alfaro JM, Sanchez S, Burgos JS (2012) Administration of docosahexaenoic acid before birth and until aging decreases kainate-induced seizures in adult zebrafish. Brain Res Bull 88: 467–470.
15. Barbazuk WB, Korf I, Kadavi C, Heyen J, Tate S, et al. (2000) The syntenic relationship of the zebrafish and human genomes. Genome Res 10: 1351–1358.
16. Rico EP, de Oliveira DL, Rosemberg DB, Mussulini BH, Bonan CD, et al. (2010) Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain. Brain Res Bull 81: 517–523.
17. Eliceiri BP, Gonzalez AM, Baird A (2011) Zebrafish model of the blood-brain barrier: morphological and permeability studies. Methods Mol Biol 686: 371–378.
18. Jeong JY, Kwon HB, Ahn JC, Kang D, Kwon SH, et al. (2008) Functional and developmental analysis of the blood-brain barrier in zebrafish. Brain Res Bull 75: 619–628.
19. Loscher W (1984) Genetic animal models of epilepsy as a unique resource for the evaluation of anticonvulsant drugs. A review. Methods Find Exp Clin Pharmacol 6: 531–547.
20. Baraban SC, Taylor MR, Castro PA, Baier H (2005) Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience 131: 759–768.
21. Alfaro JM, Ripoll-Gomez J, Burgos JS (2011) Kainate administered to adult zebrafish causes seizures similar to those in rodent models. Eur J Neurosci 33: 1252–1255.
22. Wong K, Stewart A, Gilder T, Wu N, Frank K, et al. (2010) Modeling seizure-related behavioral and endocrine phenotypes in adult zebrafish. Brain Res 1348: 209–215.
23. Hortopan GA, Dinday MT, Baraban SC (2010) Spontaneous seizures and altered gene expression in GABA signaling pathways in a mind bomb mutant zebrafish. J Neurosci 30: 13718–13728.
24. Hortopan GA, Dinday MT, Baraban SC (2010) Zebrafish as a model for studying genetic aspects of epilepsy. Dis Model Mech 3: 144–148.
25. Teng Y, Xie X, Walker S, Saxena M, Kozlowski DJ, et al. (2011) Loss of zebrafish lgi1b leads to hydrocephalus and sensitization to pentylenetetrazol induced seizure-like behavior. PLoS One 6: e24596.
26. Baraban SC, Dinday MT, Castro PA, Chege S, Guyenet S, et al. (2007) A large-scale mutagenesis screen to identify seizure-resistant zebrafish. Epilepsia 48: 1151–1157.
27. Bassuk AG, Wallace RH, Buhr A, Buller AR, Afawi Z, et al. (2008) A homozygous mutation in human PRICKLE1 causes an autosomal-recessive progressive myoclonus epilepsy-ataxia syndrome. Am J Hum Genet 83: 572–581.
28. DiBella LM, Park A, Sun Z (2009) Zebrafish Tsc1 reveals functional interactions between the cilium and the TOR pathway. Hum Mol Genet 18: 595–606.
29. Teng Y, Xie X, Walker S, Rempala G, Kozlowski DJ, et al. (2010) Knockdown of zebrafish Lgi1a results in abnormal development, brain defects and a seizure-like behavioral phenotype. Hum Mol Genet 19: 4409–4420.
30. Rosemberg DB, Rico EP, Mussulini BH, Piato AL, Calcagnotto ME, et al. (2011) Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments. PLoS One 6: e19397.
31. Blaser RE, Rosemberg DB (2012) Measures of anxiety in zebrafish (Danio rerio): dissociation of black/white preference and novel tank test. PLoS One 7: e36931.
32. Rosemberg DB, Braga MM, Rico EP, Loss CM, Cordova SD, et al. (2012) Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol. Neuropharmacology 63: 613–623.
33. Cachat J, Stewart A, Utterback E, Hart P, Gaikwad S, et al. (2011) Three-dimensional neurophenotyping of adult zebrafish behavior. PLoS One 6: e17597.
34. Muller UK, van Leeuwen JL (2004) Swimming of larval zebrafish: ontogeny of body waves and implications for locomotory development. J Exp Biol 207: 853–868.
35. Desmond D, Kyzar E, Gaikwad S, Green J, Riehl R, et al. (2012) Assessing epilepsy-related behavioral phenotypes in adult zebrafish.; Press H, editor. New York.
36. Lee Y, Kim D, Kim YH, Lee H, Lee CJ (2010) Improvement of pentylenetetrazol-induced learning deficits by valproic acid in the adult zebrafish. Eur J Pharmacol 643: 225–231.
37. Stewart AM, Desmond D, Kyzar E, Gaikwad S, Roth A, et al. (2012) Perspectives of zebrafish models of epilepsy: what, how and where next? Brain Res Bull 87: 135–143.
38. Siebel AM, Piato AL, Capiotti KM, Seibt KJ, Bogo MR, et al. (2011) PTZ-induced seizures inhibit adenosine deamination in adult zebrafish brain membranes. Brain Res Bull 86: 385–389.
39. Pineda R, Beattie CE, Hall CW (2011) Recording the adult zebrafish cerebral field potential during pentylenetetrazole seizures. J Neurosci Methods 200: 20–28.
40. Soto-Otero R, Mendez-Alvarez E, Sierra-Paredes G, Galan-Valiente J, Aguilar-Veiga E, et al. (1987) High-performance liquid chromatographic procedure for quantitative determination of pentylenetetrazol in serum and discrete areas of rat brain. Anal Biochem 165: 331–336.
41. Vermoesen K, Serruys AS, Loyens E, Afrikanova T, Massie A, et al. (2011) Assessment of the convulsant liability of antidepressants using zebrafish and mouse seizure models. Epilepsy Behav 22: 450–460.
42. Bachiega JC, Blanco MM, Perez-Mendes P, Cinini SM, Covolan L, et al. (2008) Behavioral characterization of pentylenetetrazol-induced seizures in the marmoset. Epilepsy Behav 13: 70–76.
43. Ramzan IM, Levy G (1985) Kinetics of drug action in disease states. XIV. Effect of infusion rate on pentylenetetrazol concentrations in serum, brain and cerebrospinal fluid of rats at onset of convulsions. J Pharmacol Exp Ther 234: 624–628.
44. Goodman LS, Grewal MS, Brown WC, Swinyard EA (1953) Comparison of maximal seizures evoked by pentylenetetrazol (metrazol) and electroshock in mice, and their modification by anticonvulsants. J Pharmacol Exp Ther 108: 168–176.
45. Yonekawa WD, Kupferberg HJ, Woodbury DM (1980) Relationship between pentylenetetrazol-induced seizures and brain pentylenetetrazol levels in mice. J Pharmacol Exp Ther 214: 589–593.
46. Chatterjee D, Gerlai R (2009) High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. Behav Brain Res 200: 208–213.
47. Rico EP, Rosemberg DB, Dias RD, Bogo MR, Bonan CD (2007) Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicol Lett 174: 25–30.
48. Rosemberg DB, Rico EP, Senger MR, Dias RD, Bogo MR, et al. (2008) Kinetic characterization of adenosine deaminase activity in zebrafish (Danio rerio) brain. Comp Biochem Physiol B Biochem Mol Biol 151: 96–101.
49. Pineda R, Beattie CE, Hall CW (2012) Closed-loop neural stimulation for pentylenetetrazole-induced seizures in zebrafish. Dis Model Mech.

**Figura S1.**

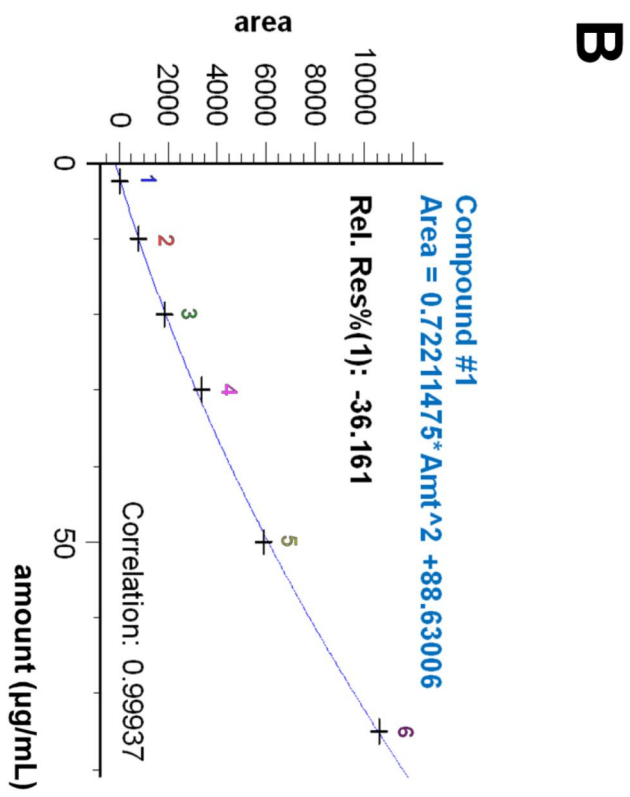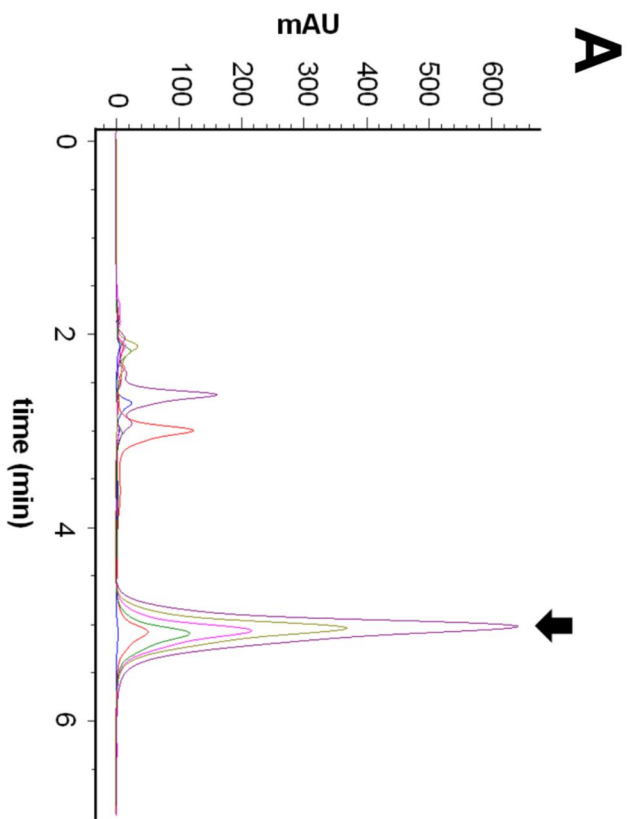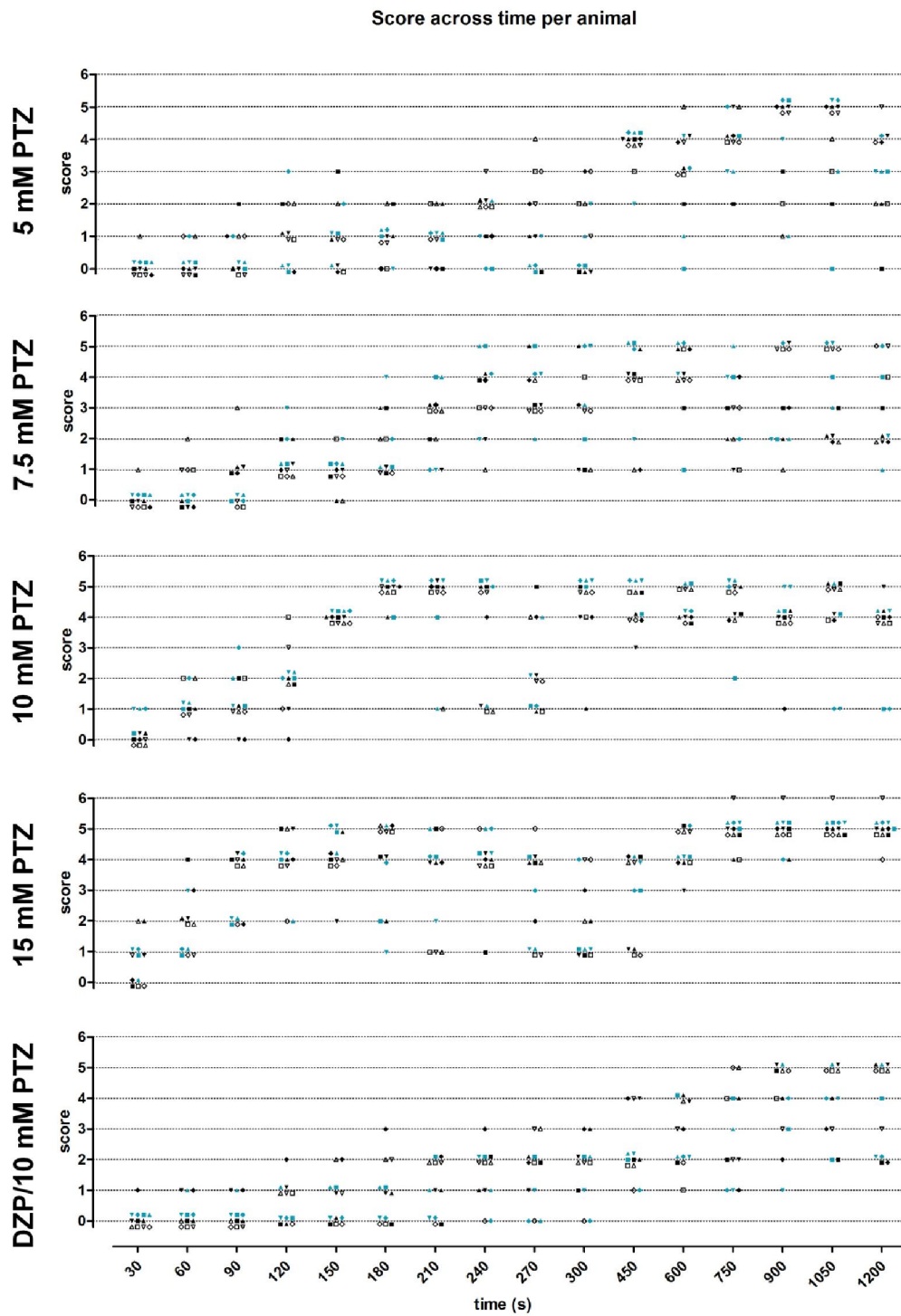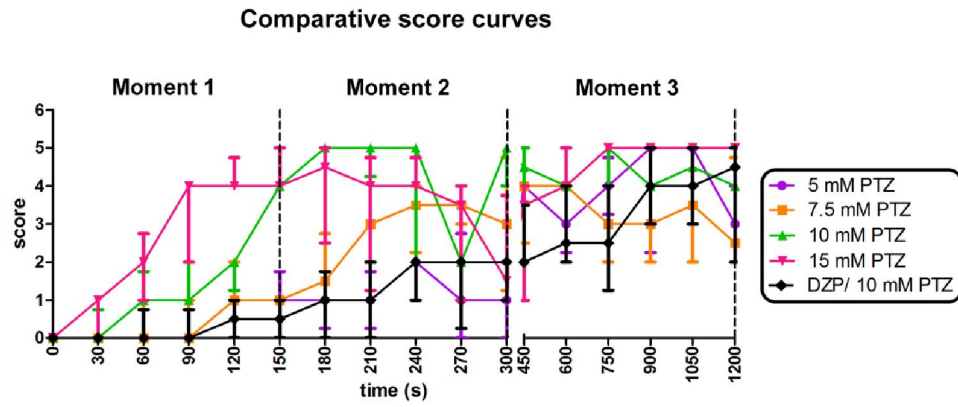**Figura S2.**

Score across time per animal

**Figura S3.**

**A**



**Comparative score curves**

**B**



**Seizure intensity**

**Vídeo S1.** Online:

http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.005451
5

**Parte III.**
_____
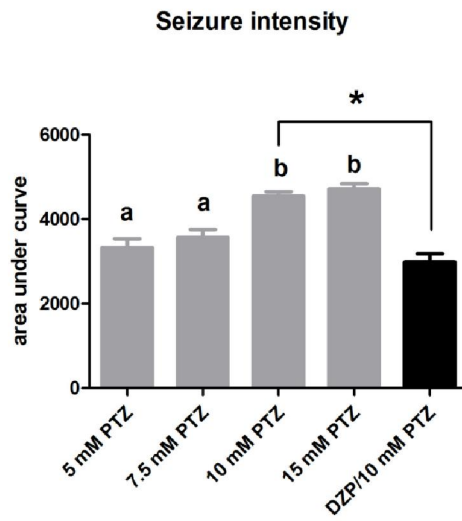
**DISCUSSÃO**

O presente estudo descreve uma caracterização detalhada do comportamento convulsivo do *zebrafish* ao ser imerso em pentilenotetrazol. Além disso, foi determinada a concentração de PTZ no SNC do *zebrafish*.

No intuito de realizar uma caracterização comportamental convulsiva do *zebrafish* adulto imerso em PTZ, optou-se pela estratégia utilizada por Baraban et al., (2005), e Alfaro et al., (2011). Nestes dois trabalhos foi utilizado um gradiente de concentrações do agente quimioconvulsivo e, consequentemente, a interpretação dos scores não foi similar ao estudo de Desmond et al., (2012).

**Tabela 1** – Scores de convulsão de *zebrafish* já descritos na literatura:

| Autor: | Desmond et al. 2012 | Baraban et al. 2005 | Alfaro et al. 2011 | Mussulini et al. 2013 |
|---|---|---|---|---|
| **Score 1** | Initial freezing with hyperventilation | Initially fish were observed to dramatically increase their swim activity | Immobility and hyperventilation of the animal. | Increased swimming activity and high frequency of opercular movement. |
| **Score 2** | Hyperlocomotion | Rapid "whirlpool-like" circling swim | Whirlpool-like swimming behaviour | Burst swimming, left and right movements, and erratic movements. |
| **Score 3** | Clonic seizures | Clonus-like convulsions leading to a loss of posture, e.g. fish falls to one side and remains immobile for 1–3 s | Rapid movements from right to left. | Circular movements. |
| **Score 4** | Tonic seizures | | Abnormal and spasmodic muscular contractions. | Clonic seizure-like behavior. |
| **Score 5** | Death | | Rapid whole-body clonus-like convulsions. | Fall to the bottom of the tank Tonic seizure-like behavior |
| **Score 6** | | | Sinking to the bottom of the tank and spasms for several minutes. | Death. |
| **Score 7** | | | Death. | |

Conforme visto na tabela 1 e no **Vídeo S1**, além dos 5 scores descritos por Desmond et. al., (2012), no presente estudo os animais apresentaram movimentos circulares após a hiperlocomoção e anteriormente a convulsão clônica. Isto pode estar relacionado ao fato de Desmond et al., (2012), terem baseado seu sistema de escores em apenas uma concentração de PTZ (11 mM). No presente estudo, na concentração de 10 mM de PTZ percebe-se que nem todos os animais apresentaram movimentos circulares, passando do escore 2 para o 4 (16.66%). A porcentagem de animais que apresentaram movimentos circulares quando imersos em 15 mM de PTZ é ainda menor (41.66%), ressaltando desta maneira a importância de se utilizar um gradiente de concentração ao caracterizar escores de convulsão de compostos pró-convulsivos. Além disso, os animais apresentaram, inicialmente, aumento da atividade locomotora e da frequência da abertura opercular, e não um período de imobilidade. Esta imobilidade é vista apenas no modelo de ácido caínico, diferentemente do modelo de PTZ em larva, o qual denota um aumento de atividade inicial Baraban et. al., (2005). Outro ponto a ser levado em consideração é a sequência diferente de manifestações ao se comparar com o modelo de ácido caínico e PTZ em *zebrafish* adulto. Embora tenha sido pioneiro o trabalho de Desmond et. al., (2012), ao propor uma tabela de escores genérica como base para estudos de modelos de convulsão em *zebrafish*, o que pode ser observado posteriormente é que essa tabela não pode ser aplicada nos modelos de convulsão de *zebrafish* adulto (nomeadamente PTZ e ácido caínico). Portanto, emerge a necessidade de detalhar o comportamento do zebrafish frente a imersão de possíveis compostos pró-convulsivos, (Afrikanova et al., 2013).

As limitações também aparecem ao se comparar os escores de *zebrafish* para larva e adulto, pois percebe-se diferenças marcantes no comportamento geral. Por exemplo, a larva apresenta apenas 3 escores de convulsão e manifestações clônicas e tônicas ocorrendo praticamente ao mesmo tempo. Já no adulto, temos 5 escores convulsivos (6º escore é a morte do animal) e neste caso, como pode ser visto pelos resultados obtidos dos grupos 5 mM e 7.5 mM (**Figura 2B** do artigo)**,** tais manifestações em zebrafish adulto ocorrem em tempos de exposição diferentes. Isto está provavelmente relacionado ao fato do animal adulto possuir um SNC mais desenvolvido em relação a larva ( Desmond et al., 2012; Muller and van Leeuwen, 2004).

Conforme visto na **Figura 2** do artigo o perfil comportamental dos animais é diferente para cada concentração de exposição ao PTZ. No intuito de melhor compreender essa diferença e até mesmo para uma aplicabilidade estatística mais profunda, desenvolveu-se um método para quantificar a intensidade da crise convulsiva. Considerando que a intensidade está relacionada com a velocidade que um animal chega ao score 4 e 5, e o quanto ele se mantem nesses escores, ao avaliarmos a área sobre a curva na **Figura 2A** de cada animal, haverá um valor matemático maior para animais expostos a 15 mM de PTZ. Por outro lado, os animais que demoraram a apresentar escores 4 e 5 e que retornaram aos escores iniciais, tais como aqueles imersos em 5 mM de PTZ, apresentaram um valor matemático inferior.

Ao sobrepor as curvas presentes na **Figura 2A** (**Figura S3**), e comparar os peixes imersos em todas as concentrações de exposição, foi possível visualizar três momentos com perfis distintos de convulsão. Com essas observações calculou-se as respectivas intensidades para cada animal em sua concentração correspondente de exposição. Como pode ser visto na **Figura 2C**

no intervalo entre 0-150 segundos os animais do grupo PTZ 15 mM apresentaram intensidade elevada, enquanto o grupo 10 mM intermediária e os outros dois grupos intensidade baixa. No intervalo de tempo 150-300s, apenas os peixes imersos em 5 mM apresentaram uma intensidade menor do que os animais expostos as outras concentrações. No terceiro e último momento, todas as concentrações induziram intensidade de crise muito similares entre si. Sendo assim, os animais apresentaram um perfil comportamental e uma intensidade de crise diferente em cada concentração testada. Provavelmente esse perfil se deve a chegada de PTZ ao SNC que depende do tempo e da concentração de exposição de pentilenotetrazol (**Figura 4**).

Embora foi observado esse perfil convulsivo diferente em cada grupo, em relação à latência para score 4 não houve diferença estatística entre as concentrações de 7.5, 10 e 15 mM de PTZ (**Figura 2D**). Isto pode ser resultado da variabilidade vista nos peixes imersos na concentração de 7.5 mM, na qual uma fração dos animais chega ao score 4 próximo ao intervalo de tempo dos animais expostos a 10 mM, enquanto outra parte dos animais leva mais tempo para chegar no mesmo score (**Figura 2B**). Por outro lado, os animais expostos as concentração de 10 mM e 15 mM apresentam um perfil comportamental similar (**Figura S2**). Ambos os grupos apresentam pequena diferença nos dois primeiros minutos de convulsão, o que justifica a diferente intensidade vista no primeiro intervalo de tempo de convulsão (0-150 s). Baseando-se nessas observações pode ser sugerido que talvez o parâmetro de latência não seja tão eficaz para medir diferenças convulsivas neste modelo, e que uma abordagem mais completa das manifestações convulsivas seja essencial em triagens de DAEs ao utilizar o *zebrafish* adulto.

Em relação ao período pós-ictal, é importante ressaltar que foi feita somente uma análise comportamental e não eletrofisiológica, estimando o tempo que os animais levariam para retornar a um comportamento basal (score 0) após ser retirado das soluções de PTZ. Não houve diferença entre os grupos 5–10 mM de PTZ (**Figura 3**). Contraditoriamente, ao avaliar a concentração de PTZ no SNC, os animais tanto do grupo 5 mM, quanto do grupo 10 mM, apresentaram concentrações similares as vistas no período convulsivo para cada concentração de exposição respectiva de PTZ (**Figura 4**). Estudos envolvendo o uso de roedores injetados s.c. com PTZ, apresentam um pico de chegada no sistema nervoso central, levando horas para que as concentrações de PTZ não sejam mais detectadas. Uma hora após a injeção as concentrações são similares as vistas no momento da convulsão, mas neste período os animais não apresentam crises motoras (Ramzan and Levy, 1985; Soto-Otero et al., 1987). Isto resulta-se de que um SNC submetido a convulsões apresenta, após as mesmas, mudanças neuroquímicas relevantes (Yonekawa et al., 1980). Tais mudanças levam a depressão do SNC após um período de hiperativação, tornando-o refratário a novas crises mesmo na presença de concentrações convulsivas de PTZ (Goodman et al., 1953; Ramzan and Levy, 1985; Yonekawa et al., 1980). Por este motivo mesmo apresentando níveis de PTZ no SNC suficientes para iniciar as convulsões, o *zebrafish* apresenta um comportamento normal 1 h após ser retirado do PTZ.

Ao avaliar a mortalidade dos animais procurou-se distinguir intensidade e severidade da crise convulsiva. Doravante, definiu-se severidade como um conjunto entre intensidade, latência para o retorno ao comportamento basal e mortalidade da crise induzida por uma determinada concentração de exposição de PTZ. Neste contexto, os animais expostos a concentração de 15 mM de

PTZ apresentaram uma crise de alta severidade (50% de mortalidade). Os animais imersos na concentração de 10 mM de PTZ apresentaram uma crise de severidade intermediária (33% de mortalidade). Já os animais imersos nas concentrações de 7.5 mM e 5 mM de PTZ apresentaram uma crise de baixa severidade (0% de mortalidade). Embora a alta mortalidade em modelos de convulsão seja  um fator limitante para futuros testes neste modelo (Goodman et al., 1953), é importante ressaltar que os 50% de mortalidade vistos nos animais expostos a concentração de 15 mM de PTZ são provavelmente resultantes do tempo de exposição que foi utilizado  nesse estudo (chegada de PTZ ao cérebro é tempo dependente). Em registros eletrofisiológicos realizados por Pineda et. al., (2011), não houve mortalidade nos animais que foram expostos apenas por 3 min a 15 mM de PTZ. Diferente deste estudo mencionado anteriormente, nesse trabalho optou-se por utilizar 20 min porque outros trabalhos utilizaram esse tempo (Lee et al., 2010; Siebel et al., 2011; Stewart et al., 2012; Wong et al., 2010), e o perfil de comportamento visto neste tempo engloba os 3 minutos iniciais, servindo desta forma como base comportamental para ambos os tempos.

Finalmente, como um último ponto deste estudo, testou-se as ferramentas comportamentais propostas frente a utilização de um fármaco anti-convulsivo clássico em uma concentração que induzisse uma mortalidade relevante, mas não uma alta severidade. Neste contexto, os animais foram imersos em diazepam e posteriormente submetidos a concentração de 10 mM de PTZ. Os resultados demonstraram que as ferramentas testadas foram sensíveis a utilização do fármaco anti-convulsivante, mas não houve diferenças no tempo necessário para o animal retornar ao nado normal.

## **CONCLUSÃO**

Os resultados deste estudo permitiram uma caracterização temporal detalhada das manifestações comportamentais do zebrafish adulto ao serem imersos em diferentes concentrações de um agente convulsivante amplamente utilizado para induzir convulsões agudas (PTZ). Neste estudo, descreveu-se curva de perfil comportamental de exposição ao PTZ, curva cumulativa de escores, intensidade das crises convulsivas, latência para o score 4, latência para retorno ao score 0 após a retirada da solução de PTZ, taxa de mortalidade, e compreensão da severidade das crises convulsivas. Além disso, foi determinada pela primeira vez que as concentrações cerebrais do PTZ no *zebrafish* adulto com curvas de tempo e concentração de exposição dependente, indicando um perfil similar ao visto em trabalhos com roedores. Esse trabalho permitirá futuras análises comportamentais em trabalhos que utilizem esse modelo como propósito para a busca de novos fármacos anticonvulsivantes. Além disso, o trabalho otimizou um método de quantificação cerebral de PTZ. Os resultados apresentados nessa dissertação sirvem de base para futuras pesquisas translacionais.

## PERSPECTIVAS

Podemos dividir nossas perspectivas em 3 focos:

O primeiro poderia vir a ser uma abordagem neuroquímica e molecular das modificações que ocorrem no SNC do peixe após ele ser retirado da solução de PTZ. Valendo-se de sistemas de neurotransmissores já caracterizados neste animal, pretendemos mapear mapear temporalmente a convulsão do ponto de vista neuroquímico.

O segundo seria realizar abordagens comportamentais. Tendo em vista que o peixe-zebra já apresenta ferramentas para avaliar alterações a nível de ansiedade, agressividade e memória, seria interessante submeter animais que convulsionaram aos protocolos destes testes. Neste ponto, poderíamos utilizar uma escala de tempo logo após a convulsão, ou ver o impacto no comportamento do animal após um a dois meses da convulsão. Humanos que convulsionam jovens podem vir a apresentar alterações comportamentais na fase adulta, e buscar por fármacos que atenuem as crises convulsivas e essas alterações comportamentais seria de grande valia.

O terceiro tende a ser o ponto mais difícil por ser o mais carente em relação ao peixe-zebra. É de extremo interesse mapear a convulsão do ponto de vista eletrofisiológico. Contudo, as técnicas até o momento só possibilitam avaliar a mesma de forma generalizada e não como ela ocorre em diferentes estruturas e como ela se propaga até tornar-se generalizada.

Partindo dessas premissas, gostaríamos de avaliar o sistema GABAérgico, as alterações comportamentais e se possível a eletrofisiologia durante e após a convulsão induzida por PTZ em peixe-zebra adulto.

**PERSPECTIVAS**

Afrikanova, T., Serruys, A.S., Buenafe, O.E., Clinckers, R., Smolders, I., de Witte, P.A., Crawford, A.D., Esguerra, C.V., 2013. Validation of the Zebrafish Pentylenetetrazol Seizure Model: Locomotor versus Electrographic Responses to Antiepileptic Drugs. PLoS One. 8, e54166.

Alfaro, J.M., Ripoll-Gomez, J., Burgos, J.S., 2011. Kainate administered to adult zebrafish causes seizures similar to those in rodent models. Eur J Neurosci. 33, 1252-5.

Bao, Y.H., Bramlett, H.M., Atkins, C.M., Truettner, J.S., Lotocki, G., Alonso, O.F., Dietrich, W.D., 2011. Post-traumatic seizures exacerbate histopathological damage after fluid-percussion brain injury. J Neurotrauma. 28, 35-42.

Baraban, S.C., Taylor, M.R., Castro, P.A., Baier, H., 2005. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience. 131, 759-68.

Baraban, S.C., 2007. Emerging epilepsy models: insights from mice, flies, worms and fish. Curr Opin Neurol. 20, 164-8.

Baraban, S.C., Dinday, M.T., Castro, P.A., Chege, S., Guyenet, S., Taylor, M.R., 2007. A large-scale mutagenesis screen to identify seizure-resistant zebrafish. Epilepsia. 48, 1151-7.

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, 1351-8.

Bassuk, A.G., Wallace, R.H., Buhr, A., Buller, A.R., Afawi, Z., Shimojo, M., Miyata, S., Chen, S., Gonzalez-Alegre, P., Griesbach, H.L., Wu, S., Nashelsky, M., Vladar, E.K., Antic, D., Ferguson, P.J., Cirak, S., Voit, T., Scott, M.P., Axelrod, J.D., Gurnett, C., Daoud, A.S., Kivity, S., Neufeld, M.Y., Mazarib, A., Straussberg, R., Walid, S., Korczyn, A.D., Slusarski, D.C., Berkovic, S.F., El-Shanti, H.I., 2008. A homozygous mutation in human PRICKLE1 causes an autosomal-recessive progressive myoclonus epilepsy-ataxia syndrome. Am J Hum Genet. 83, 572-81.

Bell, B., Lin, J.J., Seidenberg, M., Hermann, B., 2011. The neurobiology of cognitive disorders in temporal lobe epilepsy. Nat Rev Neurol. 7, 154-64.

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

Blaser, R.E., Rosemberg, D.B., 2012. Measures of anxiety in zebrafish (Danio rerio): dissociation of black/white preference and novel tank test. PLoS One. 7, e36931.

Bloss, E.B., Hunter, R.G., 2010. Hippocampal kainate receptors. Vitam Horm. 82, 167-84.

Boison, D., Chen, J.F., Fredholm, B.B., 2010. Adenosine signaling and function in glial cells. Cell Death Differ. 17, 1071-82.

Bowery, N.G., 1997. Metabotropic GABA(B) receptors cloned at last. Trends Pharmacol Sci. 18, 103.

Brito, V.B., Folmer, V., Puntel, G.O., Fachinetto, R., Soares, J.C., Zeni, G., Nogueira, C.W., Rocha, J.B., 2006. Diphenyl diselenide and 2,3-dimercaptopropanol increase the PTZ-induced chemical seizure and mortality in mice. Brain Res Bull. 68, 414-8.

Buckingham, S.C., Robel, S., 2013. Glutamate and tumor-associated epilepsy: Glial cell dysfunction in the peritumoral environment. Neurochem Int.

Burns, J.D., Green, D.M., Metivier, K., DeFusco, C., 2012. Intensive care management of acute ischemic stroke. Emerg Med Clin North Am. 30, 713-44.

Byler, D.L., Grageda, M.R., Halstead, E.S., Kanekar, S., 2013. Rapid Onset of Hippocampal Atrophy in Febrile-Infection Related Epilepsy Syndrome (FIRES). J Child Neurol.

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

Caplan, R., Siddarth, P., Stahl, L., Lanphier, E., Vona, P., Gurbani, S., Koh, S., Sankar, R., Shields, W.D., 2008. Childhood absence epilepsy: behavioral, cognitive, and linguistic comorbidities. Epilepsia. 49, 1838-46.

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

Coppola, A., Moshe, S.L., 2012. Animal models. Handb Clin Neurol. 107, 63-98.

Daniel Desmond, E.K., Siddharth Gaikwad, Jeremy Green, Russell Riehl, Andrew Roth, Adam Stewart and Allan V. Kalueff., 2012. Assessing epilepsy-related behavioral phenotypes in adult zebrafish., Vol., New York.

DiBella, L.M., Park, A., Sun, Z., 2009. Zebrafish Tsc1 reveals functional interactions between the cilium and the TOR pathway. Hum Mol Genet. 18, 595-606.

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

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

Eliceiri, B.P., Gonzalez, A.M., Baird, A., 2011. Zebrafish model of the blood-brain barrier: morphological and permeability studies. Methods Mol Biol. 686, 371-8.

Ferando, I., Mody, I., 2012. GABAA receptor modulation by neurosteroids in models of temporal lobe epilepsies. Epilepsia. 53 Suppl 9, 89-101.

Fisher, R.S., van Emde Boas, W., Blume, W., Elger, C., Genton, P., Lee, P., Engel, J., Jr., 2005. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia. 46, 470-2.

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

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

Goodman, L.S., Grewal, M.S., Brown, W.C., Swinyard, E.A., 1953. Comparison of maximal seizures evoked by pentylenetetrazol (metrazol) and electroshock in mice, and their modification by anticonvulsants. J Pharmacol Exp Ther. 108, 168-76.

Gowers, W.R., 1881. Epilepsy and other chronic convulsive diseases. J. & A. CHURCHILL, NEW BURLINGTON STREET.

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

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

Hammes, T.O., Pedroso, G.L., Hartmann, C.R., Escobar, T.D., Fracasso, L.B., da Rosa, D.P., Marroni, N.P., Porawski, M., da Silveira, T.R., 2012. The effect of taurine

on hepatic steatosis induced by thioacetamide in zebrafish (Danio rerio). Dig Dis Sci. 57, 675-82.

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

Hortopan, G.A., Dinday, M.T., Baraban, S.C., 2010b. Zebrafish as a model for studying genetic aspects of epilepsy. Dis Model Mech. 3, 144-8.

Jeong, J.Y., Kwon, H.B., Ahn, J.C., Kang, D., Kwon, S.H., Park, J.A., Kim, K.W., 2008. Functional and developmental analysis of the blood-brain barrier in zebrafish. Brain Res Bull. 75, 619-28.

Kavros, P.M., Clarke, T., Strug, L.J., Halperin, J.M., Dorta, N.J., Pal, D.K., 2008. Attention impairment in rolandic epilepsy: systematic review. Epilepsia. 49, 1570-80.

Kwan, P., Arzimanoglou, A., Berg, A.T., Brodie, M.J., Allen Hauser, W., Mathern, G., Moshe, S.L., Perucca, E., Wiebe, S., French, J., 2010. Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. Epilepsia. 51, 1069-77.

Laurie, D.J., Wisden, W., Seeburg, P.H., 1992. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci. 12, 4151-72.

Lee, Y., Kim, D., Kim, Y.H., Lee, H., Lee, C.J., 2010. Improvement of pentylenetetrazol-induced learning deficits by valproic acid in the adult zebrafish. Eur J Pharmacol. 643, 225-31.

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

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

Lin, J.J., Mula, M., Hermann, B.P., 2012. Uncovering the neurobehavioural comorbidities of epilepsy over the lifespan. Lancet. 380, 1180-1192.

Loscher, W., 1984. Genetic animal models of epilepsy as a unique resource for the evaluation of anticonvulsant drugs. A review. Methods Find Exp Clin Pharmacol. 6, 531-47.

Loscher, W., 2011. Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. Seizure. 20, 359-68.

Loscher, W., Schmidt, D., 2011. Modern antiepileptic drug development has failed to deliver: ways out of the current dilemma. Epilepsia. 52, 657-78.

Muller, U.K., van Leeuwen, J.L., 2004. Swimming of larval zebrafish: ontogeny of body waves and implications for locomotory development. J Exp Biol. 207, 853-68.

Newton, C.R., Garcia, H.H., 2012. Epilepsy in poor regions of the world. Lancet. 380, 1193-201.

Oliveira, R.F., Silva, J.F., Simoes, J.M., 2011. Fighting zebrafish: characterization of aggressive behavior and winner-loser effects. Zebrafish. 8, 73-81.

Pandis, D., Scarmeas, N., 2012. Seizures in Alzheimer disease: clinical and epidemiological data. Epilepsy Curr. 12, 184-7.

Perfilova, V.N., Tiurenkov, I.N., 2011. [GABAC receptors: structure and functions]. Eksp Klin Farmakol. 74, 45-9.

Pineda, R., Beattie, C.E., Hall, C.W., 2011. Recording the adult zebrafish cerebral field potential during pentylenetetrazole seizures. J Neurosci Methods. 200, 20-8.

Racine, R.J., 1972. Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol. 32**,** 281-94.

Ramanjaneyulu, R., Ticku, M.K., 1984. Interactions of pentamethylenetetrazole and tetrazole analogues with the picrotoxinin site of the benzodiazepine-GABA receptor-ionophore complex. Eur J Pharmacol. 98**,** 337-45.

Ramzan, I.M., Levy, G., 1985. Kinetics of drug action in disease states. XIV. Effect of infusion rate on pentylenetetrazol concentrations in serum, brain and cerebrospinal fluid of rats at onset of convulsions. J Pharmacol Exp Ther. 234**,** 624-8.

Rauca, C., Wiswedel, I., Zerbe, R., Keilhoff, G., Krug, M., 2004. The role of superoxide dismutase and alpha-tocopherol in the development of seizures and kindling induced by pentylenetetrazol - influence of the radical scavenger alpha-phenyl-N-tert-butyl nitrone. Brain Research. 1009**,** 203-12.

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., 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., Seibt, K.J., Capiotti, K.M., Da Silva, R.S., Bonan, C.D., 2011. Zebrafish neurotransmitter systems as potential pharmacological and toxicological targets. Neurotoxicol Teratol. 33**,** 608-17.

Robbins, C.A., Tempel, B.L., 2012. Kv1.1 and Kv1.2: similar channels, different seizure models. Epilepsia. 53 Suppl 1**,** 134-41.

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

Rosemberg, D.B., Braga, M.M., Rico, E.P., Loss, C.M., Cordova, S.D., Mussulini, B.H., Blaser, R.E., Leite, C.E., Campos, M.M., Dias, R.D., Calcagnotto, M.E., de Oliveira, D.L., Souza, D.O., 2012. Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol. Neuropharmacology. 63**,** 613-23.

Rowley, N.M., Madsen, K.K., Schousboe, A., Steve White, H., 2012. Glutamate and GABA synthesis, release, transport and metabolism as targets for seizure control. Neurochem Int. 61**,** 546-58.

Rubio, C., Rubio-Osornio, M., Retana-Marquez, S., Veronica Custodio, M.L., Paz, C., 2010. In vivo experimental models of epilepsy. Cent Nerv Syst Agents Med Chem. 10**,** 298-309.

Shorvon, S.D., 2009a. Drug treatment of epilepsy in the century of the ILAE: the first 50 years, 1909-1958. Epilepsia. 50 Suppl 3**,** 69-92.

Shorvon, S.D., 2009b. Drug treatment of epilepsy in the century of the ILAE: the second 50 years, 1959-2009. Epilepsia. 50 Suppl 3**,** 93-130.

Siebel, A.M., Piato, A.L., Capiotti, K.M., Seibt, K.J., Bogo, M.R., Bonan, C.D., 2011. PTZ-induced seizures inhibit adenosine deamination in adult zebrafish brain membranes. Brain Res Bull. 86**,** 385-9.

Sieghart, W., Sperk, G., 2002. Subunit composition, distribution and function of GABA(A) receptor subtypes. Curr Top Med Chem. 2**,** 795-816.

Soto-Otero, R., Mendez-Alvarez, E., Sierra-Paredes, G., Galan-Valiente, J., Aguilar-Veiga, E., Sierra-Marcuno, G., 1987. High-performance liquid chromatographic procedure for quantitative determination of pentylenetetrazol in serum and discrete areas of rat brain. Anal Biochem. 165**,** 331-6.

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

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

Stewart, A.M., Desmond, D., Kyzar, E., Gaikwad, S., Roth, A., Riehl, R., Collins, C., Monnig, L., Green, J., Kalueff, A.V., 2012. Perspectives of zebrafish models of epilepsy: what, how and where next? Brain Res Bull. 87**,** 135-43.

Takechi, K., Suemaru, K., Kawasaki, H., Araki, H., 2012. [Impaired memory following repeated pentylenetetrazol treatments in kindled mice]. Yakugaku Zasshi. 132**,** 179-82.

Tang, F.R., Loke, W.K., Ling, E.A., 2011. Comparison of status epilepticus models induced by pilocarpine and nerve agents - a systematic review of the underlying aetiology and adopted therapeutic approaches. Current Medicinal Chemistry. 18**,** 886-99.

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

Teng, Y., Xie, X., Walker, S., Rempala, G., Kozlowski, D.J., Mumm, J.S., Cowell, J.K., 2010. Knockdown of zebrafish Lgi1a results in abnormal development, brain defects and a seizure-like behavioral phenotype. Hum Mol Genet. 19**,** 4409-20.

Teng, Y., Xie, X., Walker, S., Saxena, M., Kozlowski, D.J., Mumm, J.S., Cowell, J.K., 2011. Loss of zebrafish lgi1b leads to hydrocephalus and sensitization to pentylenetetrazol induced seizure-like behavior. PLoS One. 6**,** e24596.

Verrotti, A., Scaparrotta, A., Olivieri, C., Chiarelli, F., 2012. Seizures and type 1 diabetes mellitus: current state of knowledge. Eur J Endocrinol. 167**,** 749-58.

Vogel, G., 2000. Genomics. Sanger will sequence zebrafish genome. Science. 290**,** 1671.

Wong, K., Stewart, A., Gilder, T., Wu, N., Frank, K., Gaikwad, S., Suciu, C., Dileo, J., Utterback, E., Chang, K., Grossman, L., Cachat, J., Kalueff, A.V., 2010. Modeling seizure-related behavioral and endocrine phenotypes in adult zebrafish. Brain Res. 1348**,** 209-15.

Yonekawa, W.D., Kupferberg, H.J., Woodbury, D.M., 1980. Relationship between pentylenetetrazol-induced seizures and brain pentylenetetrazol levels in mice. J Pharmacol Exp Ther. 214**,** 589-93.