UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

CURSO DE GRADUAÇÃO EM BIOMEDICINA

Luana Moro

EFEITOS COMPORTAMENTAIS DA PREGABALINA E GABA-CAPRÓICO EM PEIXE-ZEBRA ADULTO FRENTE AO TESTE DE *NOVEL TANK* E A CRISES EPILÉPTICAS INDUZIDAS POR PENTILENOTETRAZOL

Porto Alegre

2015

Luana Moro

EFEITOS COMPORTAMENTAIS DA PREGABALINA E GABA-CAPRÓICO EM PEIXE-ZEBRA ADULTO FRENTE AO TESTE DE *NOVEL TANK* E A CRISES EPILÉPTICAS INDUZIDAS POR PENTILENOTETRAZOL

Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Bacharela em Biomedicina.

Orientador: Prof. Dr. Diogo Losch de Oliveira

Co-orientador: Me. Ben Hur Marins Mussilini

Porto Alegre

2015

CIP - Catalogação na Publicação

Moro, Luana
Efeitos comportamentais da Pregabalina e GABA-Capróico em peixe-zebra adulto frente ao teste novel tank e a crises epilépticas induzidas por pentilenotetrazol / Luana Moro. -- 2015.
58 f.

Orientador: Diogo Losch de Oliveira.
Coorientador: Ben Hur Marins Mussulini.

Trabalho de conclusão de curso (Graduação) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Curso de Biomedicina, Porto Alegre, BR-RS, 2015.

1. Ansiedade. 2. Epilepsia. 3. Comportamento animal. I. Losch de Oliveira, Diogo, orient. II. Marins Mussulini, Ben Hur, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

EFEITOS COMPORTAMENTAIS DA PREGABALINA E GABA-CAPRÓICO EM PEIXE-ZEBRA ADULTO FRENTE AO TESTE DE *NOVEL TANK* E A CRISES EPILÉPTICAS INDUZIDAS POR PENTILENOTETRAZOL

Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharel(a) em Biomedicina.

Aprovado em: ____ de _____ de _____.

BANCA EXAMINADORA

_____

Nome do professor - instituição

_____

Nome do professor - instituição

_____

Nome do professor - instituição (orientador)

AGRADECIMENTOS

Agradeço, de maneira geral, as pessoas que possibilitaram a realização desse trabalho. Ao Departameto de Bioquímica e ao Departamento de Química Orgânica da Universidade de Federal do Rio Grande do Sul por concretizar a realização desse trabalho.

À minha família, pela confiança que sempre me depositaram ao longo desses anos e pelo apoio incondicional a todas as decisões que tomei durante esse percurso. Sem eles, nada disso seria possível.

Ao meu namorado Paulo, por toda paciência e compreensão durante esse semestre, por sempre me acalmar, me fazer rir e ser meu companheiro em todos os momentos.

Ao meu orientador, Diogo, e ao meu coorientador, Ben hur, pela atenção, ensinamentos e pelo bom exemplo de cientista, não apenas durante esse semestre, mas desde o início da minha iniciação científica, que fizeram meu pensamento e vida acadêmica evoluir de maneira intensa.

Agradeço também meus colegas que constituem ou já passaram pelo laboratório 24, seja pelas discussões científicas, pela amizade e pelas conversas informais na salinha do café: Thainá, Suelen, Emerson, Kami, Mery, Denis, Rico, Gabi, Natã, Diego, Lucimara e Cássio.

Aos meus colegas e bons amigos que encontrei na Biomedicina, Andréia, Giana, Maria Eduarda, Patrícia, Jéssica, Eduardo e Leonardo, por tornar meu dia-a-dia mais divertido e pela ajuda durante esses anos.

Aos meus demais amigos, da vida, do intercâmbio e do meu estágio no HCPA, por tornarem minha vida tão feliz e pela descontração nos momentos de estresse.

Obrigada, vocês são essenciais!

LISTA DE FIGURAS

SUMÁRIO

1. Lista de abreviaturas

AMPA α-amino-3-hidroxil-5-metil-4-isoxazol-propionato

DMSO Dimetilsulfóxido

GABA Ácido gamma-aminobutírico

$GABA_A$ Receptor GABAérgico ionotrópico

KA Cainato

NMDA N-metil D-Aspartato

PTZ Pentilenotetrazol

SNC Sistema nervoso central

SV2A Synaptic vesicle glycoprotein 2A (Proteína da vesícula sináptica 2A)

2. Resumo

A epilepsia é uma desordem neurológica que afeta aproximadamente 50 milhões de pessoas no mundo. Muitos pacientes diagnosticados com epilepsia sofrem de comorbidades psiquiátricas, como por exemplo a ansiedade, e sabe-se que isso pode levar a diminuição na qualidade de vida e na resposta aos fármacos antiepilépticos. Nós modificamos a estrutura química da pregabalina que deu origem a um derivado chamado GABA-capróico. Nosso objetivo foi analisar os efeitos comportamentais causados pelo tratamento de pregabalina e GABA-capróico no modelo de peixe-zebra adulto expostos a um aparato que representa novidade e também contra crises epilépticas induzidas por pentileotetrazol (PTZ). Os animais foram pré-tratados com pregabalina (10, 20, 60 mM) ou GABA-capróio (60 mM) dissolvidos em DMSO 1%) via injeção intraperitoneal. Após 30 min, os animais foram transferidos para o aquário trapezoidal (*novel tank*) a fim de avaliar parâmetros de locomoção e exploração dos animais e em aquários retangulares com solução de pentilenotetrazol na concentração de 10 mM para analisar o comportamento frente a crises epilépticas. A locomoção geral dos animais não apresentou alterações significativas. Os parâmetros exploratórios analisados não mostraram diferença estatística, porém, os animais demonstram uma tendência de aumentar o tempo de permanência no topo do aparato e diminuir o tempo de latência para a subida ao topo quando comparado com grupo controle. Os dados sobre crises epilépticas demonstraram que a pregabalina é capaz de diminuir a severidade das crises na dose de 60 mM. A pregabalina na dose de 20 mM e o GABA-caproico 60 mM tendem a diminuir a intensidade da crise e a latência para a crise epiléptica clônica, mas não apresentam diferença estatística significativa. Concluindo, a pregabalina e seu derivado tendem a apresentem efeitos tipo ansiolíticos no peixe-zebra, não causando efeitos adversos na locomoção. Além disso, a pregabalina é capaz de reduzir a intensidade das crises epilépticas induzidas por PTZ na concentração de 60 mM.

3 Introdução compreensiva

3.1 Epilepsia

A epilepsia é definida como uma desordem cerebral na qual os neurônios encontram-se excessivamente ativos de forma sincronizada, e essa atividade anormal pode gerar alterações neurobiológicas e cognitivas, bem como transtornos psicológicos e problemas sociais (Fisher *et al.*, 2014). Essa desordem está entre as mais comuns, afetando pessoas de todas as idades. Dados epidemiológicos estimam que 50 milhões de indivíduos no mundo tenham epilepsia (Banerjee *et al.*, 2009). Diferentes históricos de pacientes podem levar ao diagnóstico de epilepsia. Segundo definições recentes da Liga Internacional Contra Epilepsia (ILAE), um indivíduo pode ser considerado epiléptico após a ocorrência de uma única crise não provocada, se esse indivíduo apresentar alto risco de ter outra crise similar dentro de 10 anos, como no caso de indivíduos que sofreram alguma injúria cerebral, por exemplo, infecções, traumas ou acidente vascular cerebral (Fisher *et al.*, 2014). Também pode ser caracterizado com essa condição o indivíduo que apresentar crises epilépticas recorrentes (duas ou mais) com 24h de diferença, cuja causa não seja identificada, ou pacientes com diagnóstico de síndrome epiléptica (Fisher *et al.*, 2005; Banerjee *et al.*, 2009).

Na última década, muitos pacientes com epilepsia foram diagnosticados também com variadas comorbidades psiquiátricas. As comorbidades mais reportadas em estudos são desordens de humor, psicoses, neuroses e desordem de personalidade. Dentre essas, as desordens de humor, particularmente a depressão e a ansiedade, são as mais frequentes desordens relatadas (Tellez-Zenteno *et al.*, 2007). A depressão em pacientes com epilepsia vem sendo muito estudada e, segundo a literatura, esses indivíduos apresentam diminuição na qualidade de vida, aumento do risco de suicídio e uma piora na resposta a tratamentos farmacológicos; portanto, esses pacientes devem ser devidamente diagnosticados e tratados (Kanner, 2011). Em contraste, a ansiedade é um tema pouco abordado, mas capaz de causar impactos negativos tanto quanto a depressão na vida desses pacientes.

3.2 Fármacos antiepilépticos

Ao final da década de 80, o tratamento para epilepsia era restrito aos medicamentos fenobarbital, fenitoína, carbamazepina e ácido valpróico, que até hoje estão entre os mais receitados mundialmente (Bialer, 2002). Apartir da década de 90, deu-se início a revolução no tratamento dessa desordem. Levando em consideração o grande número de pacientes epilépticos que não respondem aos tratamentos farmacológicos existentes (aproximadamente 30%) e a lista de efeitos colaterais causados por esses medicamentos medicamentos (**Tabela 1**), notou-se a necessidade de buscar novas terapias com maior eficácia e tolerância (Brodie e Dichter, 1996; Loscher, 2011).

Tabela 1: Efeitos colaterais importantes dos fármacos antiepiléptico (Brodie e Dichter, 1996).

Fármaco	Efeito colateral dose-dependente
Fenobarbital	Fadiga, desatenção, depressão; especifico para crianças: insônia, distração, hipercinesia, irritabilidade
Fenitoína	Nistagmo, ataxia, náusea, vômito, depressão, sonolência, aumento paradoxal na crise epiléptica, anemia megaloblástica
Carbamazepina	Visão dupla, tontura, dor de cabeça, náusea, sonolência, neutropenia, hiponatremia
Ácido valproico	Tremor, ganho de peso, dispepsia, náusea, vômito, alopécia, edema periférico

Atualmente existem diversos grupos de fármacos antiepilépticos, por exemplo: (1) fármacos que modulam predominantemente canais Na^+ (e Ca^{2+}) como a fenitoína, carbamazepina, oxcarbamazepina, lamotrigina e a zonisamida; (2) fármacos que modulam predominantemente canais de Ca^{2+}: etosuximida; (3) fármacos que modulam o sistema GABAérgico: benzodiazepínicos, vigabatrina, tiagabina; (4) ações mistas:

valproato (diminuição da ação de canais de Na^+ dependentes de voltagem e inibição da degradação do GABA aumentando seus níveis na fenda sináptica, entre outras), felbamato (bloqueia canais de Na^+ e o receptor NMDA, modulador do receptor $GABA_A$), topiramato (bloqueia canais de Na^+, Ca^{2+}, potencia a ação do GABA, bloqueia receptores AMPA/KA e aumenta correntes de potássio), fenobarbital (aumento da afinidade do receptor GABAa pelo neurotransmissor GABA, ação sobre o glutamato e bloqueio de canais de Na^+); (5) fármacos recentes com novos alvos, com mecanismos pouco conhecidos: gabapentina (bloqueio de canais Ca^{2+}), pregabalina (canais de Ca^{2+}), levetiracetam (se liga a SV2A que pode estar envolvida na liberação de neurotransmissores) , lacosamida (aumenta a inativação lenta dos canais de Na^+) e retigabina (Czapinski *et al.*, 2005; Bialer e White, 2010; Loscher, 2011) (**Figura 1**).

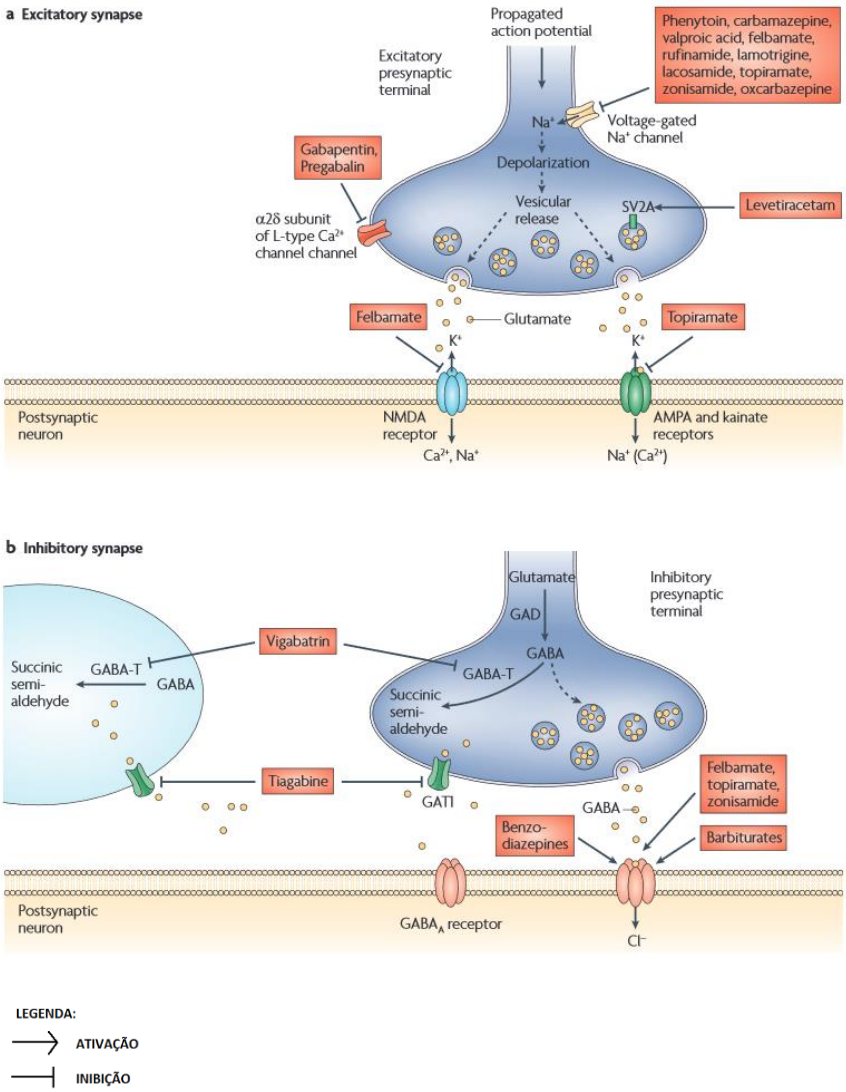

Figura 1. Mecanismo de ação sobre as sinapses excitatórias e inibitórias proposto para os fármacos antiepilépticos atuais (Czapinski *et al.*, 2005). Num balanço global, fármacos antiepilépticos atuam diminuindo o sistema excitatório e aumentando o sistema inibitório dos neurônios.

Na busca por novos fármacos, podemos encontrar compostos que foram descobertos de forma empírica e possuem estrutura química completamente nova, e outros que derivam de fármacos antiepilépticos pré-existentes. O objetivo de desenvolver fármacos com base em algum pré-existente, é, além de melhorar a eficácia, segurança e tolerância do fármaco, aumentar a biodisponibilidade cerebral, eliminar a toxicidade dos metabólitos do composto original e evitar os elementos estruturais que tornam a molécula original possivelmente teratogênica (Bialer e White, 2010). Mesmo com todos os novos fármacos introduzidos no mercado e seus novos alvos, os problemas enfrentados anteriormente não desapareceram por completo; no entanto, pode-se dizer que em termos de segurança, tolerância e farmacocinética melhorias consideráveis foram alcançadas (Perucca *et al.*, 2007).

A escolha do fármaco antiepiléptico deve ser feita com cuidado, após análise dos sintomas do paciente. O diagnóstico correto do tipo de epilepsia é essencial para a escolha da terapia mais eficaz (Brodie e Dichter, 1996). A Liga Internacional Contra Epilepsia propôs um guia para o tratamento de pacientes epilépticos de acordo com o tipo de crise e idade, e quais seriam as melhores opções de substituição de terapia caso o fármaco de primeira escolha não seja eficaz para aquele indivíduo, baseado em estudos da literatura que indicam a capacidade de cada fármaco antiepiléptico como monoterapia (Glauser *et al.*, 2006).

3.2.1 Pregabalina

A pregabalina é um fármaco desenvolvido recentemente e destaca-se na clínica com seu espectro de indicações. Utilizada no tratamento de crises parciais epilépticas, dor neuropática e transtornos de ansiedade generalizada, a pregabalina tornou-se amplamente prescrita (Kustermann *et al.*, 2014). Apesar de ser análoga estrutural do GABA, a pregabalina exerce sua ação ao se ligar à subunidade alpha2-delta de canais de

cálcio dependentes de voltagem na região pré-sináptica dos neurônios (Lotarski *et al.*, 2014). A ligação a esse sítio atenua a despolarização induzida pelo influxo de Ca^{2+}nos terminais nervosos, levando à diminuição na liberação de diversos neurotransmissores, como o glutamato, noradrenalina e substância P (Arroyo *et al.*, 2004). Além disso, apresenta caráter hidrofílico, mas atravessa a barreira hematoencefálica (Dooley *et al.*, 2000).

Tanto em crianças quando adultos, a pregabalina é utilizada em pacientes como tratamento adjunto em casos de crises parciais (com ou sem generalização secundária) refratários ao tratamento clássico, e apresenta perfil de tolerância e segurança aceitáveis (Arroyo *et al.*, 2004; Mann *et al.*, 2014). Os relatos mais comuns de efeitos adversos foram de sonolência e tontura (em adultos e crianças), visão dupla e aumento de peso (em adultos), vômitos, falta de coordenação motora, constipação, irritabilidade, febre (em crianças) (Arroyo *et al.*, 2004; Mann *et al.*, 2014).

Alguns estudos recentes apontam efeitos adversos mais graves relacionados ao uso da pregabalina. Dois casos de depressão acompanhados de ideação e prática suicída já foram relatados na clínica, alertando sobre esse possível efeito adverso, mesmo que raro, causado pelo uso de pregabalina (Kustermann *et al.*, 2014). Além disso, em ratos, já se tem estudos demonstrando um possível efeito teratogênico do medicamento mesmo em doses baixas (Etemad *et al.*, 2013). Portanto, apesar dos estudos clínicos sugerirem a pregabalina como uma das terapias adjuntas mais promissoras devido a sua alta eficiência, segurança e perfil farmacocinético vantajoso, estudos mais aprofundados ainda são necessários (Arroyo *et al.*, 2004).

3.3 Modelo animal peixe-zebra

O peixe-zebra(*Danio rerio)*, chamado também de *zebrafish* ou paulistinha, é um pequeno teleósteo (3-4 cm) da família dos Cyprinidae. No final da década de 60, a biologia do peixe-zebra começou a ser abordada por George Streisinger, que dentre diversas espécies de peixes tropicais estudadas, destacou o peixe-zebra por apresentar vantagens de criação, reprodução e de manipulação gênica (Grunwald e Eisen, 2002). Esse modelo animal tornou-se popular rapidamente, e, como uma espécie de vertebrado, o zebrafish tem uma alta homologia genética e fisiológica quando comparado a

humanos, além de possuir neuroanatomia e neurofisiologia conservada(Panula *et al.*, 2006; Alsop e Vijayan, 2008; Panula *et al.*, 2010).

Desde então, no cenário de pesquisas, o peixe-zebra vem ganhando cada vez mais espaço e aplicações, e dentre essas se destaca a utilização do modelo para varreduras farmacológicas. O peixe-zebra possui a vantagem de ter reprodução que chega a gerar centenas de ovos a cada fecundação e, devido ao seu rápido desenvolvimento embrionário (em 24h apresenta estrutura básica corporal), em pouco tempo já é possível realizar ensaios biológicos em larga escala (Goldsmith, 2004). Além disso, o pequeno espaço requerido para manutenção dos animais e a quantidade reduzida de fármacos gastos são pontos de interesse quando se procura testar diversas moléculas com grande amostra experimental (Lieschke e Currie, 2007). Portanto, ao comparar modelos animais utilizados para reproduzir doenças humanas, percebemos a vantagem no modelo do peixe-zebra que apesar da similaridade anatômica conservada entre vertebrados, as condições experimentais são realizadas na escala de modelos invertebrados (Kalueff *et al.*, 2015).

3.4 Teste de open/novel tank

Semelhante ao teste de *open field* realizado em roedores, o teste *novel tank*, também conhecido como *open tank*, é utilizado para avaliar o comportamento do peixe-zebra. Esse teste fornece uma avaliação espaço-temporal do perfil locomotor e exploratório do animal sujeito a um ambiente que representa novidade (Rosemberg *et al.*, 2011). Através desse aparato experimental trapezoidal e da sua divisão virtual em diferentes zonas (Figura 2), é possível inferir, por exemplo, o perfil geral de locomoção através de parâmetros como distância total percorrida, tempo móvel, tempo imóvel, imobilidade e velocidade média e máxima. O repertório de resultados sobre a locomoção geral dos animais pode ser correlacionado com a presença ou ausência de efeitos colaterais em modelos animais comumente encontrados em diversos fármacos, como letargia, episódios de imobilidade e comportamento de *burst* (Ibrahim *et al.*, 2014).

O perfil exploratório horizontal e vertical pode ser analisado através de diversas medidas como latência para entrada em áreas, tempo em cada área e transições entre as áreas delimitadas virtualmente (Rosemberg *et al.*, 2011). Quando expostos ao aparato

novel tank, o peixe-zebra tende a explorar pouco o aparato, demorar mais para atingir a área do topo, e entrar menos vezes na área do topo (Levin et al., 2007). Esse perfil exploratório que o animal apresenta frente a um ambiente novo é reconhecido como um comportamento do tipo ansiogêncio (Levin et al. 2007). A correlação entre exploração do aparato e o comportamento tipo ansiolítico dos animais foi proposto em roedores, e mais recentemente para o modelo de peixe-zebra, baseando-se no fato de que animais tratados com fármacos ansiolíticos clássicos geram um aumento no comportamento exploratório dos animais pelas áreas delimitadas do aparato, menor latência para entrada na área do topo, tempo de permanência na área do topo aumentada, aumento no número de transições na área do topo (Cryan e Holmes, 2005; Stewart *et al.*, 2012).

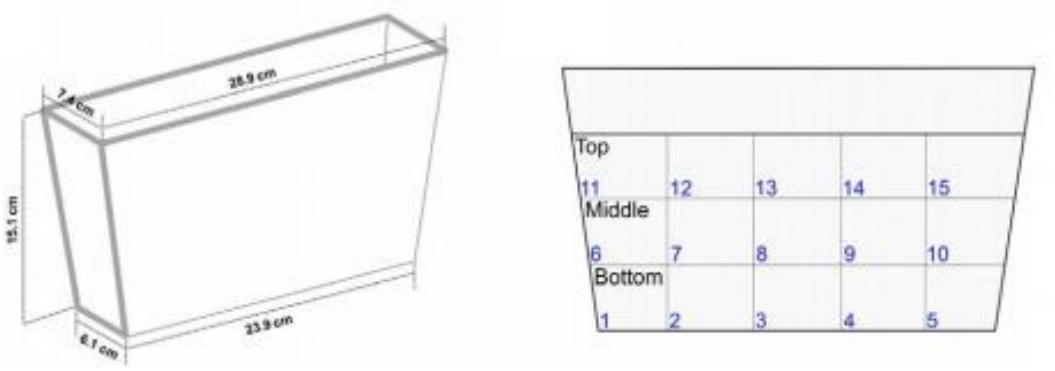

Figura 2. ***Novel tank*.** Tanque trapezoidal com divisões virtuais utilizadas para avaliar a atividade natatória do peixe-zebra, com divisão de três áreas verticais (topo, meio e fundo) e quinze áreas horizontais.

3.5 Modelos de indução de crises epilépticas em peixe-zebra

O peixe-zebra apresenta um grande potencial como modelo animal para estudos sobre a epilepsia, tanto com animais geneticamente modificados para essa patologia ou com modelo de crises epilépticas agudas provocadas (Grone e Baraban, 2015). Para que um modelo animal de uma patologia ou de uma desordem seja considerado válido, ele precisa reproduzir características essenciais das condições humanas (Sarkisian, 2001), no caso das epilepsias, por exemplo, apresentar descargas elétricas cerebrais anormais e comportamentos estereotipados (Baraban *et al.*, 2005).

Para gerar mutações no peixe-zebra, a técnica de injeção de morfolinos é realizada para gerar *gene knockdown*, que são oligonucleotídeos modificados que se ligam e bloqueiam a tradução do mRNA endógeno, diminuindo a expressão do gene alvo relacionado à epilepsia (Teng *et al.*, 2010; Suls *et al.*, 2013). Outro método mais eficaz utilizado é o uso do plasmídeo CRISPR*Cas9 gerando deleções (*knockout*) ou inserções (*knock-in*) em alelos específicos do DNA do peixe-zebra (Grone e Baraban, 2015). Esses animais geneticamente modificados apresentam crises epilépticas espontâneas.

Quanto a modelos de indução de crises aguda, temos o modelo de hipertermia e modelos químicos. No modelo de hipertermia, a crise epiléptica é induzida pelo aumento controlado na temperatura do banho em que se encontram as larvas de peixe-zebra, que produz registros eletrofisiológicos semelhantes aos dos animais com crises epilépticas induzidas pelo agente químico pentilenotetrazol (Hunt *et al.*, 2012). Quanto aos modelos químicos, temos os clássicos agentes pró-convulsivos: pentilenotetrazol (PTZ), antaganonista não-competitivo do receptores $GABA_A$, e ácido caínico (KA), agonista de receptores glutamatérgicos. O modelo químico de PTZ vem sendo amplamente utilizado para descoberta de novos fármacos antiepilépticos (Loscher, 2011). Em um sistema equilibrado, o neurotransmissor GABA ativa o receptor $GABA_A$, levando ao influxo de Cl^- através desse canal iônico e à hiperpolarização neuronal. Na presença do agente pró-convulsivo, ocorre redução no influxo de Cl-, diminuindo a inibição neuronal, gerando um desequilíbrio entre o sistema nervoso excitatório e inibitório, o que desencadeia a crise epiléptica (Hansen *et al.*, 2004). É bem estabelecido que o agente químico PTZ provoca, tanto em peixe-zebra larvas quanto em adultos, um aumento no sistema alterações nos registros eletroencefalográficos e padrões comportamentais estereotipados que já estão bem descritos na literatura (Baraban *et al.*, 2005; Pineda *et al.*, 2011; Mussulini *et al.*, 2013). Em relação ao ácido caínico, as alterações elétricas cerebrais devido a exposição das larvas de peixe-zebra a esse agente químico também estão descritas, bem como o comportamento estereotipado apresentado tanto em larvas quanto em adultos (Kim *et al.*, 2010; Alfaro *et al.*, 2011; Menezes *et al.*, 2014).

4. OBJETIVO GERAL

O presente projeto visa avaliar os possíveis efeitos promovidos pela pregabalina e seu análogo sintético, GABA-capróico, frente ao teste baseado no paradigma de novidade e também frente à indução de crises epilépticas induzidas por pentilenotetrazol no modelo de peixe-zebra adulto.

4.1 Objetivos específicos

4.1.1 Buscar a concentração ideal de aplicação da pregabalina e seus derivado sintético no peixe-zebra a ser utilizada como pré-tratamento em crises epilépticas induzidas pelo PTZ;

4.1.2 Investigar os efeitos comportamentais (atividade locomotora e exploratória) induzidos pela pregabalina e GABA-capróico isoladamente no peixe-zebra durante 6 min após passados 30 minutos da injeção do pré-tratamento;

4.1.3 Investigar os efeitos comportamentais do pré-tratamento com pregabalina e GABA-capróico nas concentrações ideias no comportamento do peixe-zebra frente a crises epilépticas induzidas por pentilenotetrazol durante 20 minutos após 30 min da administração do pré-tratamento;

4.1.4 Avaliar a taxa de mortalidade dos animais pré-tratados com as moléculas durante 5 dias posteriores à indução de crise epiléptica.

5. Trabalho experimental na forma de artigo científico

Este trabalho foi escrito em formato de artigo científico, seguindo as normas para submissão de artigo da revista Epilepsy Research, as quais estão contidas no anexo 1.

Behavioral effects of pregabalin and GABA-caproic on adult zebrafish in the novelty paradigm and pentylenetetrazole induced epileptic seizure.

Luana Moro[1], Caroline Da Ros Montes D'Oca[2], Ben Hur Marins Mussulini[1], Marcelo G. Montes D'Oca[3], Dennis Russowsky[2], Diogo Losch de Oliveira[1].

[1]Programa de pós-graduação em Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil.

[2]Programa de pós-graduação em Química, Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, 91501-970, Porto Alegre, RS, Brazil.

[3]Programa de pós-graduação em Química Tecnológica e Ambiental, Escola de Química e Alimentos, Universidade Federal do Rio Grande, Av. Italia, Km 08, s/ n°, 96203-900, Rio Grande, RS, Brazil.

Corresponding author: Luana Moro Tel.: + 55 51 33085555;

E-mail address:luanamoro222@gmail.com

HIGHLIGHTS

• Pregabalin treatment reduced seizure intensity induced by pentylenetetrazole in adult zebrafish.

• GABA-caproic showed a tendency to decrease seizure intensity induced by pentylenetetrazole.

•Pregabalin and GABA-caproic tend to display anxiolytic profile in the novel tank test and both did not modify locomotor parameters in adult zebrafish.

ABSTRACT

Epilepsy is a common brain disorder affecting approximately 50 million people worldwide. Several studies have assessed psychiatric comorbidities in epilepsy, such as anxiety. Despite AED therapies progression, new compounds have been studied. Pregabalin, a recent drug developed to treat epilepsy, was chemically modified generating a derivative called GABA-caproic. We evaluate the behavioral effect of pregabalin and GABA-caproic in adult zebrafish model in the novelty test paradigm and also against epileptic seizures induced by pentylenetetrazole. Fish were pretreated with an intraperitoneal (i.p) injection of pregabalin (10, 20, 60 mM) or GABA-caproic (60 mM) dissolved in DMSO 1% (*n*= 8 in each group). After 30 min of pretreatment, animals were transferred to the trapezoidal apparatus (novel tank) to assess locomotion and exploratory behavior or to rectangular apparatus filled with pentylenetetrazole 10 mM to analyze seizure behavior. General locomotion did not show modification between pretreated animals and control group. Parameters analyzed for exploratory behavior did not show statistical difference; although, animals exhibit a tendency to spend more time in the top area and decrease latency to the top compared to control. Seizure parameters analyzed demonstrated that only pregabalin at 60 mM concentration is able to reduce seizure intensity. Pregabalin 20 mM and GABA-caproic 60 mM tend to decrease seizure intensity and latency to clonic seizure-like behavior onset in lower levels. In conclusion, pregabalin and GABA-caproic tend to present anxyolitic-like effects in adult zebrafish, but it did not cause locomotor side effects. Pregabalin exhibit anticonvulsant effect at 60 mM in PTZ-induced seizure model in adult zebrafish, while pregabalin 20 mM and GABA-caproic 60 mM were able to prevent epileptic seizure in a certain degree.

Key words: Zebrafish, Pregabalin, Anxiety, Epilepsy, GABA-caproic.

1. Introduction

Epilepsy is characterized as a brain disorder in which neurons exhibit an abnormal excessive or synchronized activity, and this abnormal activity can generate neurobiological and cognitive modifications, as well as psychological and social disorders(Fisher et al., 2005). Epilepsy is the most common disorder, affecting people of all ages, and epidemiologic data estimates that over 50 million people worldwide have a diagnosis of the disorder (Banerjee et al., 2009).Despite therapeutic advances, studies have shown that approximately 30% of patients diagnosed epileptic do not respond to the proposed pharmacological treatments(Brodie and Dichter, 1996). Moreover, in the last decades, many epileptic patients were diagnosed with psychiatric comorbidities (Gaitatzis et al., 2004). Among them, anxiety is one of the most common disorder related (Tellez-Zenteno et al., 2007).

In last decades, new drugs with anticonvulsant activity were successfully developed, and many compounds are resultant of structural modification of a pre-existing molecule. Despite AED therapies progression in the last few decades, new compounds have been studied in order to enhance treatment quality (Loscher, 2011). Recently, a gamma aminobutyric acid (GABA) structural analog, pregabalin, was accepted for refractory epilepsy treatment (Hamandi and Sander, 2006).

Although pregabalin and GABA share structural similarity, pregabalin binds to the auxiliary $\alpha_2\delta$ subunit of voltage-sensitive calcium channels at presynaptic terminals (Dooley et al., 2000). Pregabalin is largely used in cases of partial epileptic seizures and generalized anxiety disorders. It showed to be efficient, well tolerated and with a safety acceptable profile as add-on treatment for partial seizures in adults and children and generalized anxiety disorder in adults (Alvarez et al., 2015; Arroyo et al., 2004; Mann et al., 2014). Although it displays high efficiency, studies suggest that pregabalin should not be prescribed in treatment of idiopathic generalized epilepsy(Hamandi and Sander, 2006). In addition, some recent studies in mice have been shown that pregabalin can be teratogenic even in lower doses (Etemad et al., 2013) and some severe adverse effects have been related, such as depression with suicidal attempt (Kustermann et al., 2014). At this point, it seems that more research about pregabalin effects is needed and

chemical modifications in pregabalin molecule seem to be an interesting goal to develop new compounds.

The development of new AED face a problem to be discovered by the same animal models (Loscher, 2011). It seems to be essential to improve preclinical development of AED and zebrafish emerges as an alternative approach for pharmacological screening in biomedical research. We chose to work with adult model instead of larval model due to its brain complexity, including neurotransmitter systems and blood-brain barrier well developed, which are extremely important in AED research (Ullmann et al., 2010). Furthermore, drugs with low aqueous solubility are difficult to tested in larval zebrafish because of precipitation and lack of body absorption, while in adult zebrafish another administration, such as i.p injection can be performed (Banote et al., 2013). Besides, these animal model fulfill some prerequisites for emergent animal model utilized when testing large number of investigational compounds, like handling and storage convenience and time- and cost-efficient (Loscher, 2011). These characteristics turn the zebrafish model attractive and it could be as an alternative for the homogeneity in AED discovery. Thus, to detect antiepileptic efficacy in drug development, pentylenetetrazole is a chemical model of induce epileptic seizure largely indicated and this model is well described in zebrafish (Baraban et al., 2005; Mussulini et al., 2013).

In accordance to literature, long alkyl chain derivative compounds have shown several biological activities, suggesting the potential application in the construction of new chemical entities or by incorporation in the molecular structure of commercial available drugs (D'Oca Cda et al., 2010; Rodrigues et al., 2013). Thus, pregabalin was chemically modified with an insertion of a fatty acid chain from caproic acid, generating a new derivative that we called GABA-caproic. Therefore, to assess the behavioral effects of GABA-caproic in zebrafish adult model, we tested the locomotor profile and anxiolytic-like behavior in the novel test paradigm and also we tested GABA-caproic as a pharmacological pretreatment against epileptic seizures induced by pentylenetetrazole. To accomplish this goal, we first needed to characterize the effect of pregabalin in zebrafish adult model.

2. Materials and Methods

2.1. Ethics statement

All procedures with animal subjects have been approved by the Ethics Committee for Use of Animals- CEUA from Universidade Federal do Rio Grande do Sul (protocol number 27006).

2.2. Reagents

Ethyl 3-aminobenzoate methanesulfonic acid salt, pentylenetetrazole and dimethyl sufoxide was purchased from Sigma-Aldrich (St. Louis, MO, USA. Pregabalin and GABA-caproic were synthesized by Instituto de Química from Universidade Federal do Rio Grande do Sul, RS, Brazil. Their chemical structures were elucidated and patented (BR102013012934-A2).

2.3. Chemical

The compounds were synthesized in a four step strategy from isovaleral and caproic aldehydes (**Fig. 1**). First, the aldehydes were submitted to Knoevenagel condensation with Meldrum's acid (2) in the presence of piperidinium acetate to afford de alkylidene derivatives (3a-b) (Milite et al., 2011).The intermediates (3a-b) were converted to nitroderivatives (4a-b) by Michael addition of CH_3NO_2, that after opening Meldrum's portion and decarboxylation domino process lead to the nitroacid intermediates (5a-b) (Kimmel et al., 2012). Finally, the reduction of nitro group resulted in the Pregabalin (6) or GABA-caproic derivative (7) in global yields 60-65% respectively. The compounds were characterized by NMR ^{1}H and ^{13}C, IR and elemental analysis.

2.4. Animals

Adult male and female zebrafish (6-8 months-old) of heterogeneous wild-type stock (standard short-fin phenotype) were obtained from a local commercial supplier (Delphis, RS, Brazil). Fish were housed in 40-L aquariums (70-100 fish per aquarium) for at least 2 weeks prior to the experiments in order to acclimate to the animal facility. They were maintained on a constant 14/10-h light/dark cycle (lights on at 8:00 am). Water from all tanks was aerated, maintained with mechanical and chemical filtration at 26±2°C and water pH at 7.0-8.0. Animals were fed twice a day with commercial flake fish food (alcon, BASICH, Alcon®, Brazil) and twice a day with artemia cysts (3h

intervals). 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).

2.5. Treatments with pregabalin and GABA-caproic

Animals were removed from their tanks, and individually transferred to beakers filled with 300 mL of home system water in which they were weighed. After, animals were anesthetized by immersion in tricainesolution160 μg/ml (Alfaro et al., 2011) and in the absence of reflex response to slight mechanical pressure on the tail, fish were pretreated with an intraperitoneal (i.p) injection of pregabalin (10, 20, 60 mM) or GABA-caproic (60 mM) dissolved in DMSO 1%(Siebel et al., 2015).Control group received vehicle DMSO 1%. The volume injected was 10μl/g of body weight. The concentrations of pregabalin tested in zebrafish were based on the dose capable to prevent seizure in 50% of mice (Lotarski et al., 2014).

Fish were subsequently transferred back to their individual beakers where they remained during 30 minutes. Fish were placed into the apparatus to run the experiments.

In order to perform experiments with similar conditions, all tests were conducted during the same period each day (8:30 am to 1:00 p.m.) and the animals were handled carefully to reduce stress and avoid interference in locomotor activity.

2.6. Behavioral analysis

2.6.1. General locomotion and exploratory behavior

After pretreatment with pregabalin (10, 20 and 60 mM) and GABA-caproic (60mM), fish (n=8 in each group) were individually transferred to a trapezoidal tank (23 cm along the bottom x 28.3 cm at the top x 16 cm in height x 7 cm width), dimensions similar to those previously described, filled with 1.5L home system water called novel tank(Rosemberg et al., 2011). A webcam (HD Pro, C920–Logitech) was placed (40 cm) front of the novel tank to monitor locomotor activity during 6 minutes.

In order to ensure a uniform background for the video analysis and also prevent animals' distraction due to environmental factors, yellow sheets of paper were placed

around (10 cm near) novel tank. Furthermore, one 60-W light bulb was placed behind the yellow sheet to enhance contrast between background and fish and improve quality of the animal's tracking. The webcam was connected to a laptop and the video analyses were performed by ANY-maze® software (Stoelting, CO, USA) at the rate of 20 positions/second. General locomotion was analyzed by the total distance travelled and time immobile. Exploratory behavior was analyzed by time spent in the three horizontal areas, entries to the top area, and latency to enter the top area.

2.6.2 Pentylenetetrazole induced seizure

Animals (n=8 in each group) received pregabalin (20 and 60 mM) and GABA-caproic (60 mM) prior exposure to the proconvulsant agent pentylenetetrazole (PTZ).

To assess possible antiepileptic effect of pregabalin and GABA-caproic, fish were individually transferred to a rectangular tank (20 cm long x 13 cm high x 7 cm width) filled with 1L of PTZ at a concentration of 10 mM dissolved in water. Behavioral phenotype of the PTZ seizure model in adult zebrafish was evaluated by scores: (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 tank walls were coated with white cover, in order to avoid the reflex of the animal in the glass walls, and to standardize background for the video recording. A webcam (HD Pro, C920– Logitech) was placed 25 cm in front of the rectangular tank coupled to a laptop in order to record animal behavior during 20 minutes. All behavioral data were evaluated by 1 trained observer according to the behavioral characterization of PTZ induced seizure in adult zebrafish well established in the literature (Mussulini et al., 2013). After exposure to PTZ, mortality was assessed during 5 days.

2.7. Statistics

Locomotion was expressed by mean±S.E.M and analyzed by the one-way ANOVA followed by the Bonferroni's test as post hoc. Entries to the top area were

expressed as median±interquartile range and it was analyzed by Kruskal Wallis test. Non-parametric data of seizure scores were expressed as median±interquartile range. 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) and latency were represented as mean±S.E.M and analyzed by the one-way ANOVA followed by the Bonferroni's test as post hoc. In all analyses, the significance level was taken as $p \leq 0.05$.

3. Results

Animals treated with pregabalin (6, 20 and 60 mM) did not show significant statistical differences compared to control group in general locomotor parameters measured, such as distance travelled (**Fig. 2A**) and time immobile (**Fig. 2B**). However, animals pretreated with pregabalin at concentration 6 mM have a tendency to spend more time immobile. As the highest concentration of pregabalin tested did not alter locomotor parameters, we selected 60 mM to test the same parameters with GABA-caproic (**Fig. 2A, 2B**) and the animals behave similar to control group.

Endpoint analysis used to measure potential anxiolytic-like effects of molecules were analyzed for pregabalin and GABA-caproic treatment. (**Fig. 3, 4)**. Although no statistical differences were founded in time spent in each horizontal area of the apparatus, animals treated with pregabalin in highest concentrations (20 and 60 mM) and GABA-caproic (60 mM) exhibit a tendency to spend more time in the top area instead of bottom area (**Fig. 3 A, C**). We did not find differences statistically significant in the number of entries to the top area, although there is a tendency in animals pretreated with pregabalin 20 mM and GABA-caproic 60 mM to enter more times in the top area of the novel tank (**Fig. 4A**). The latency to enter top area seems to decrease in the groups treated with pregabalin and GABA-caproic at concentration of 60 mM (**Fig. 5A**).

After analyze pretreatments effects separately, we tested the effects of pregabalin (20 and 60 mM) and GABA-caproic (60 mM) as pretreatment in PTZ-induced seizure. All groups exhibited stereotyped movements when exposed to PTZ 10 mM, classified in different scores (**Fig. 6**). Control group (DMSO 1%) shown behavioral profile with

rapid score progression in first 5 min(**Fig. 6A**). Fish which received pregabalin 20 mM and GABA-caproic 60 mM exhibit seizure profile similar to control group, although both showed scores increasing slower during the first 5 min (**Fig. 6B, 6D**). Animals pretreated with pregabalin 60 mM showed remarkable difference during the first 5 min, exhibiting low score progression (**Fig. 6C**). In the last 15 min of PTZ immersion, animal of all groups displayed a stable similar seizure profile, predominantly clonic seizure-like behavior or fall to the bottom of the tank and loss of body posture with tonic seizure-like behavior, the highest scores in the behavioral characterization of PTZ seizures in adult zebrafish (**Fig. 6 A, B, C, D**).

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 group. In the first interval (0-150 s), fish pretreated with pregabalin 60 mM exhibited lower seizure intensity when compared to control (one-way ANOVA, $F_{[3,28]} = 3,26$, $p < 0.05$; Bonferroni test $p < 0.05$ (**Fig. 6E**). The other two groups, pregabalin 20 mM and GABA-caproic 60 mM did not show statistical difference, although both seizure intensity are lower when compared to control (**Fig. 6E**). In the second interval (150 – 300 s), no statistical difference was found, but the pretreatment with the highest concentration of pregabalin and GABA-caproic tend do decrease seizure intensity (**Fig. 6F**).In the last interval (300–1200 s), as the animals showed almost the same behavior in seizure score curve, seizure intensity are very similar and it did not showed significant statistic difference (**Fig. 6G**).

Cumulative scores frequency shows that control group which exhibit the most intense seizure curve and intensity, rapidly change from score 2 to score 4, with just few animals presenting score 3 (25%) (**Fig. 7A**). As the seizure intensity in pretreated animals decreases, more animals reach score 3, pregabalin 20 mM (50%), pregabalin 60 mM (87.5%) and GABA-caproic (62.5%) (**Fig. 7B, C, D**). Also, 100% of the control group and pregabalin 20 mM reached score 4 before 300 s and almost all, 75% and 50%, respectively, reached score 5 (**Fig. 7A,B**). In contrast, this stereotype score is showed by 100% of pregabalin 60 mM and GABA-caproic60 mM after 300 s and just 12.5% and 25% of the animals exhibited score 5 (**Fig. 7C, D**).An end point measure for AEDs, the latency to clonic seizure behavior, was measured for each group pretreated during PTZ exposure. Only pregabalin at the concentration 60 mM showed statistical

difference (one-way ANOVA $F_{[3,28]} = 6.832$, $p < 0.001$; Bonferroni test $p < 0.05$ (**Fig. 8A**).

Mortality was assessed during 5 days after PTZ expousure. Only animals exposed to highest concentration of pregabalin (60 mM/kg) and GABA-caproic (60 mM) died (score 6), 12.5% and 25%, respectively.

4. Discussion

Zebrafish became a very time-efficient model used in biomedical drugs discovery and it is an emerging model in order to understand complex brain disorders(Kalueff et al., 2014).To develop new therapies based on pregabalin structure in the zebrafish model, a description of pregabalin is necessary, and there are no previously evidence of pregabalin effects in adult zebrafish in the literature. In our study, we showed the effects of anticonvulsant pretreatment on chemoconvulsant-induced seizure-like behavior in adult zebrafish an anxiolytic-like effects by behavioral assessment.

Similar to the open field apparatus used for rodents, the open tank for fish is used to assess locomotor and exploratory behavior in zebrafish (Rosemberg et al., 2011).Locomotion endpoint analysis can be used to measure potential toxic effects of the compounds. In this study, we measured the swimming performance of fish by the distance travelled and time immobile. Although no statistical difference was found, animals pretreated with pregabalin (20 and 60 mM) tend to enhance swimming activity and reduce time immobile when compared to control, while animals treated with 60 mM GABA-caproic showed similar behavior profile to control group. Hyperlocomotion has been reported in zebrafish in response to MK-801 treatment, which showed anxiolytic-like effects at the novel tank test (Swain et al. 2004; Stewart et al., 2012).These results show that the pretreatments did not evoke side effects such as lethargy or increase in time immobile(Ibrahim et al., 2014).

Correlation between exploratory behavior with anxiolytic-like behavior was proposed for rodents and zebreafish (Cryan and Holmes, 2005; Stewart et al., 2012).Our results about exploratory profile showed that animals which received pregabalin and GABA-caproic tend to increase exploratory behavior in the open tank, spending more time in the top area, increasing entries to the top area and reducing latency to enter the

top when compared to control when compared to control group. Zebrafish naturally exhibit a reduced exploratory profile and preference to the bottom area in response to exposure to an apparatus which represent novelty and could induce anxiogenic-like behavior, so this results could indicate reduced anxiety levels in the fish as it was previously reported(Levin et al., 2007). We believe that absence of statistical significance is due to small number of animal in each group, and also some animals inside each group exhibit very distinct behavior compared to majority; therefore, it is important to perform an additional open tank experiment increasing number of animals to find true effects of both compounds.

As it would be valuable to develop therapies with both anxiolytic and antiepileptic action, we performed the behavioral sequence score profile of seizure induced by PTZ as described in the literature (Mussulini et al., 2013).The score-system during 20 min analysis showed a control group pattern similar to the one found in literature, with rapid score progression in first 5 min, and animals presenting high seizure scores in the last 15 min, leading to a high intensity seizure profile. Lower seizure intensity found in group pretreated with pregabalin 60 mM in first 150 s is a sign found in fish pretreated by immersion in anticonvulsant solution(Mussulini et al., 2013). Literature data about absence or presence of circular movements (score 3) is controversial, but in our experiments manifestation of low scores, such as score 3, become more prominent in all groups which received pretreatment, and it seems that proportional increase as seizure intensity decrease.(Banote et al., 2013).

Moreover, classical parameter of latency to clonic seizure-like behavior largely used in development of new AED was measured for all pretreatments (Bachiega et al., 2008; Ben-Menachem et al., 2010). Statistical significance was found for group pretreated with pregabalin at concentration of 60 mM, while pregabalin at 20 mM and GABA-caproic 60 mM showed, despite no statistical significance, increase in latency to score 4 onset. Percentage mortality found in groups pretreated with pregabalin and GABA-caproic 60 mM suggest that may be compounds are toxic in a certain level, but more studies about toxic mechanism are needed. In contrast, our control mortality did not follow mortality rate of 33% in control group. With all data together, we can infer that pregabalin reduce seizure severity in a concentration dependent way.

Conclusion

Summarizing, pregabalin and GABA-caproic tend to present anxyolitic-like effects in zebrafish adult, but did not cause locomotor side effects. Pregabalin exhibit anticonvulsant effect at 60 mM in PTZ-induced seizure model in adult zebrafish, while pregabalin 20 mM and GABA-caproic 60 mM were able to prevent epileptic seizure in a certain degree. These findings turn adult zebrafish into an animal model able to be used in AED mechanism comprehension and development using pregabalin as pharmacological base.

5. Conflict of interest

None of the authors have any conflict of interest to disclose.

Fig. 1. Synthesis of tested compounds Pregabalin (6) and GABA-Caproic (7). Aldehydes (1a, 1b)submitted to Knoevenagel condensation with Meldrum's acid (2) in the presence of piperidinium acetate to afford de alkylidene derivatives (3a-b) and then they were converted to nitroderivatives (4a-b) by Michael addition of CH_3NO_2, that after opening Meldrum's portion and decarboxylation domino process lead to the nitroacidintermediates (5a-b). The reduction of nitro group resulted in the pregabalin (6) or GABA-caproic derivative (7).

Fig. 2. Endpoint behaviors used to assess locomotor pattern resultant of different concentrations of pregabalin and GABA-caproic for adult zebrafish. The graphs show the total distance traveled (A) and time immobile (B) during 6 min. Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test, considering $p \leq 0.05$ as significant. No statistical differences were found.

Fig. 3.Endpoint analysis for the assessment of anxiolytic-like behavior. Graphs show time spent at the top (A), middle (B) and bottom (C) areas. Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test, considering $p \leq 0.05$ as significant. No statistical differences were found.

Fig. 4. Entries to the top area. Graph shows the number of entries to the top zone of novel tank (A). Data were analyzed Kruskal Wallis test, considering $p \leq 0.05$ as significant. No statistical differences were found.

Fig. 5. Latency to enter the top area. Graph show time (s) to first entry to the top area (A). Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test, considering $p \leq 0.05$ as significant. No statistical differences were found.

Fig. 6.Behavioral profile of PTZ-induced seizures in adult zebrafish. Seizure scores curve (the highest score reached in each interval) during total 20 min for control (A), pregabalin 20 mM (B), pregabalin 60 mM (C), GABA-caproic 60 mM (D). Seizure intensitywas evaluated by the area under curve observed for animal in each group in three separated moments: 0–150 s (E), 150–300 s (F), and 300–1200 s (G). Seizure scores are represented as median±interquartile range. The area under the curve (AUC) is represented as mean±S.E.M and analyzed by the one-way ANOVA followed by the Bonferroni's test as post hoc. * indicates statistical differences between control group

(black) and the other groups (gray bars).In all analyses, the significance level was taken as $p \leq 0.05$.

Fig. 7. Cumulative score frequency. Cumulative curve of animals (%) which reached scores (0 to 5) during 1200 s observed for each treatment: control (A), pregabalin 20 mM (B), pregabalin 60 mM (C), GABA-caproic 60 mM (D).

Fig. 8. Latency to score 4 onset (A). Latency for each group (control, pregabalin 20, pregabalin 60, GABA-caproic 60 mM).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 between control group (black) and the other groups (gray bars).In all analyses, the significance level was taken as $p \leq 0.05$.

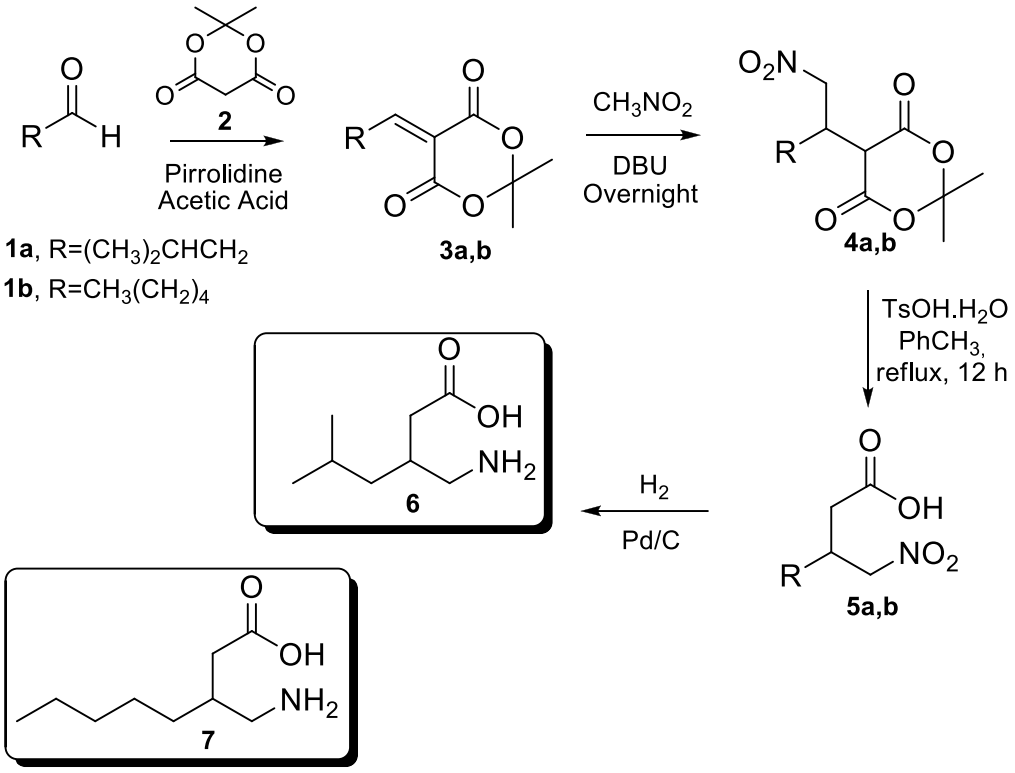

Fig. 1. Synthesis of tested compounds Pregabalin (6) and GABA-Caproic.

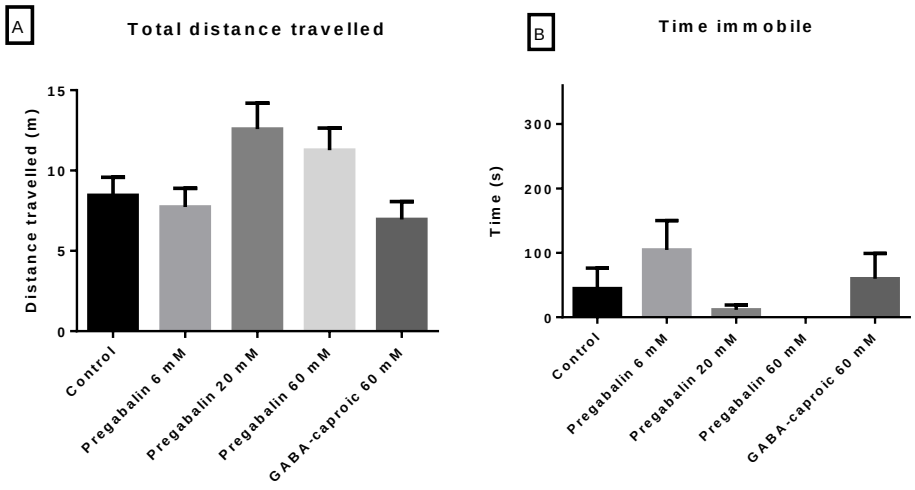

Fig. 2. Endpoint behaviors used to assess locomotor pattern resultant of different concentrations of pregabalin and GABA-caproic for adult zebrafish.

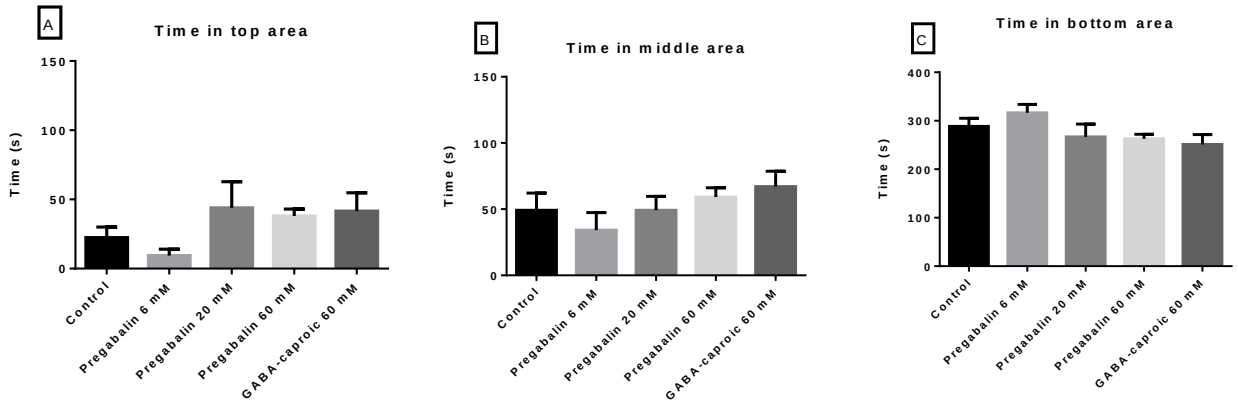

Fig. 3.Endpoint analysis for the assessment of anxiolytic-like behavior.

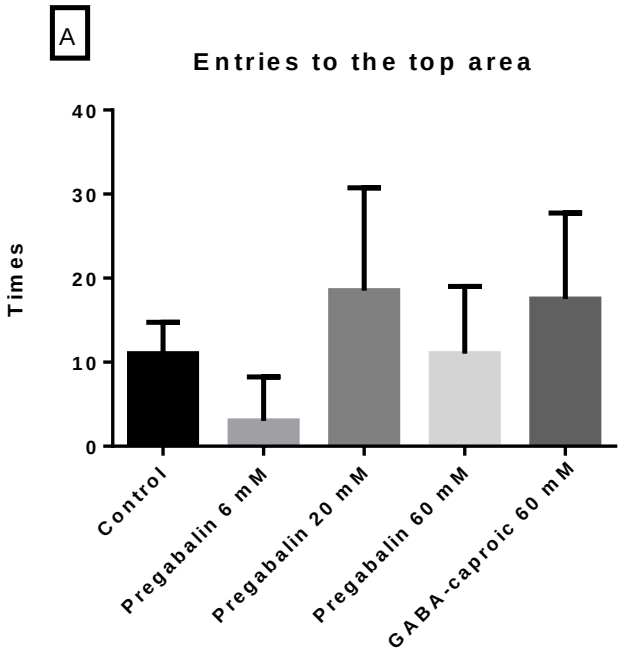

Fig. 4. Entries to the top area.

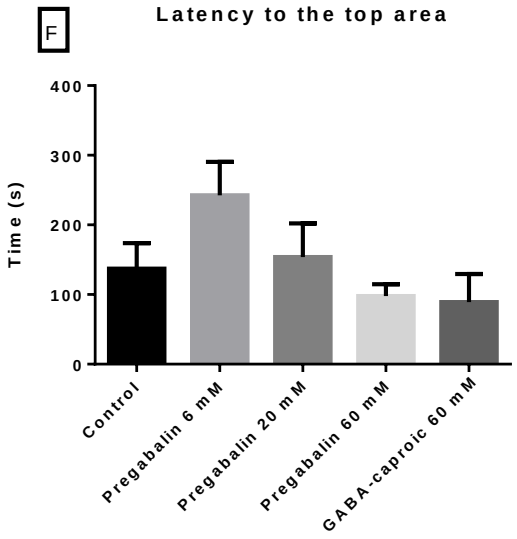

Fig. 5. Latency to enter the top area.

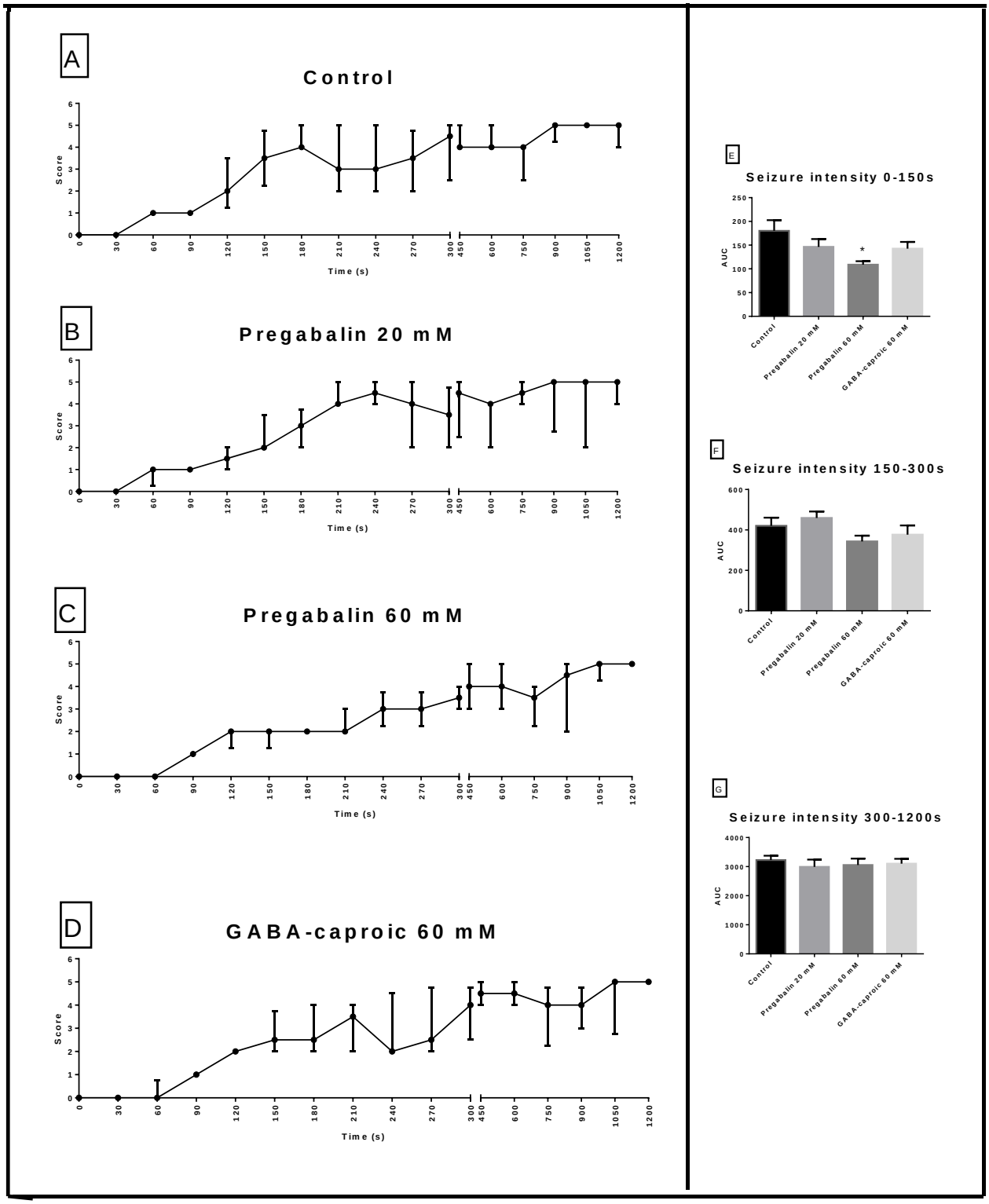

Fig. 6. Behavioral profile of PTZ-induced seizures in adult zebrafish.

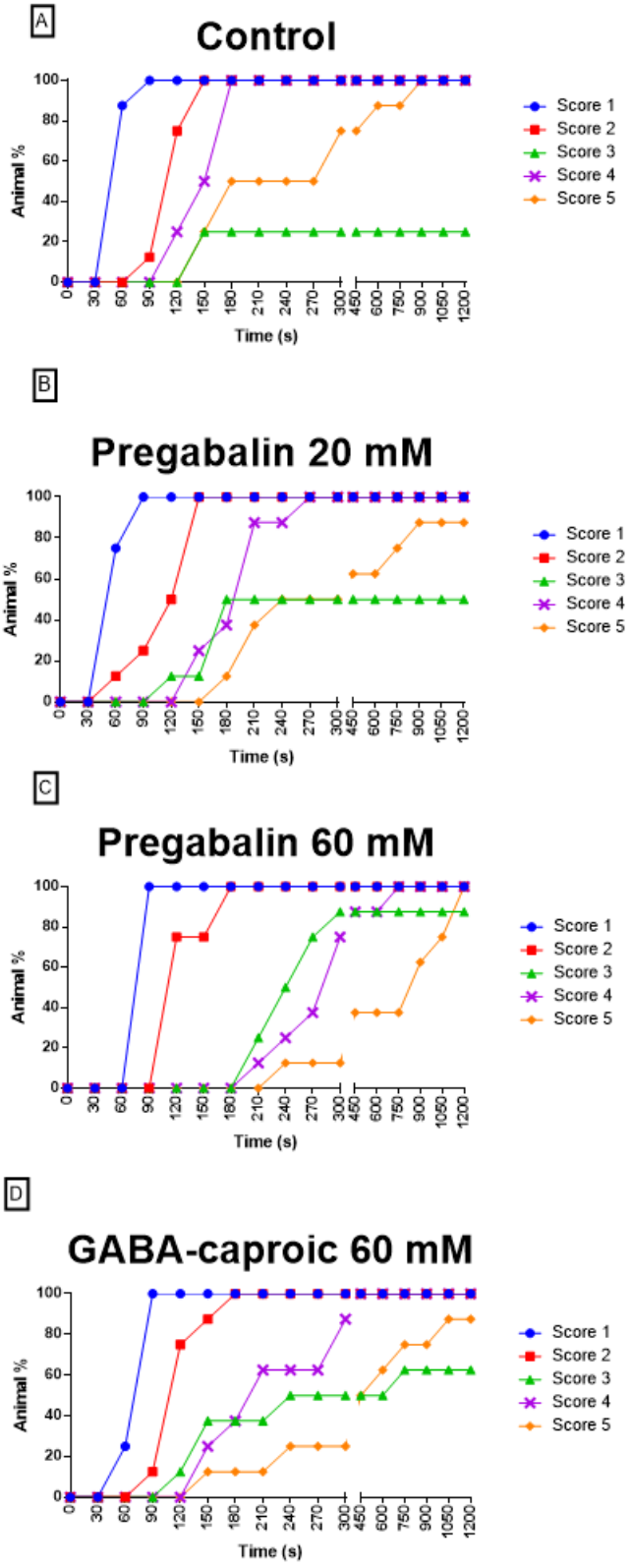

Fig. 7. Cumulative score frequency.

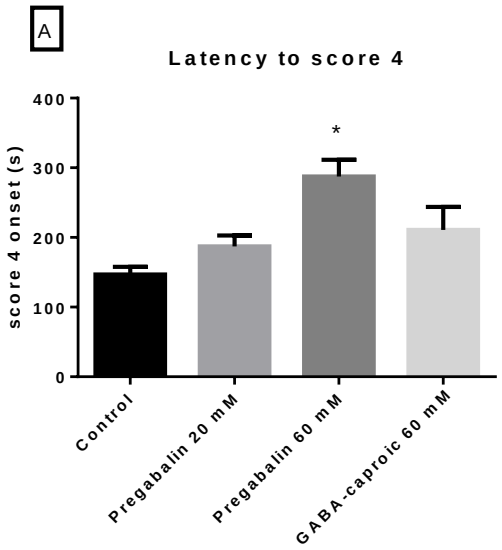

Fig. 8. Latency to score 4 onset (A).

REFERENCES

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, 1252-1255.
Alvarez, E., Olivares, J.M., Carrasco, J.L., Lopez-Gomez, V., Rejas, J., 2015. Clinical and economic outcomes of adjunctive therapy with pregabalin or usual care in generalized anxiety disorder patients with partial response to selective serotonin reuptake inhibitors. Annals of general psychiatry 14, 2.
Arroyo, S., Anhut, H., Kugler, A.R., Lee, C.M., Knapp, L.E., Garofalo, E.A., Messmer, S., 2004. Pregabalin add-on treatment: a randomized, double-blind, placebo-controlled, dose-response study in adults with partial seizures. Epilepsia 45, 20-27.
Bachiega, J.C., Blanco, M.M., Perez-Mendes, P., Cinini, S.M., Covolan, L., Mello, L.E., 2008. Behavioral characterization of pentylenetetrazol-induced seizures in the marmoset. Epilepsy & behavior : E&B 13, 70-76.
Banerjee, P.N., Filippi, D., Allen Hauser, W., 2009. The descriptive epidemiology of epilepsy-a review. Epilepsy Res 85, 31-45.
Banote, R.K., Koutarapu, S., Chennubhotla, K.S., Chatti, K., Kulkarni, P., 2013. Oral gabapentin suppresses pentylenetetrazole-induced seizure-like behavior and cephalic field potential in adult zebrafish. Epilepsy & behavior : E&B 27, 212-219.
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, 759-768.
Ben-Menachem, E., Sander, J.W., Privitera, M., Gilliam, F., 2010. Measuring outcomes of treatment with antiepileptic drugs in clinical trials. Epilepsy & behavior : E&B 18, 24-30.
Brodie, M.J., Dichter, M.A., 1996. Antiepileptic drugs. N Engl J Med 334, 168-175.
Cryan, J.F., Holmes, A., 2005. The ascent of mouse: advances in modelling human depression and anxiety. Nature reviews. Drug discovery 4, 775-790.
D'Oca Cda, R., Coelho, T., Marinho, T.G., Hack, C.R., Duarte Rda, C., da Silva, P.A., D'Oca, M.G., 2010. Synthesis and antituberculosis activity of new fatty acid amides. Bioorganic & medicinal chemistry letters 20, 5255-5257.
Dooley, D.J., Donovan, C.M., Pugsley, T.A., 2000. Stimulus-dependent modulation of [(3)H]norepinephrine release from rat neocortical slices by gabapentin and pregabalin. J Pharmacol Exp Ther 295, 1086-1093.
Etemad, L., Mohammad, A., Mohammadpour, A.H., Vahdati Mashhadi, N., Moallem, S.A., 2013. Teratogenic effects of pregabalin in mice. Iran J Basic Med Sci 16, 1065-1070.
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, 470-472.
Gaitatzis, A., Trimble, M.R., Sander, J.W., 2004. The psychiatric comorbidity of epilepsy. Acta Neurol Scand 110, 207-220.
Hamandi, K., Sander, J.W., 2006. Pregabalin: a new antiepileptic drug for refractory epilepsy. Seizure 15, 73-78.
Ibrahim, M., Mussulini, B.H., Moro, L., de Assis, A.M., Rosemberg, D.B., de Oliveira, D.L., Rocha, J.B., Schwab, R.S., Schneider, P.H., Souza, D.O., Rico, E.P., 2014. Anxiolytic effects of diphenyl diselenide on adult zebrafish in a novelty paradigm. Progress in neuro-psychopharmacology & biological psychiatry 54, 187-194.
Kalueff, A.V., Stewart, A.M., Gerlai, R., 2014. Zebrafish as an emerging model for studying complex brain disorders. Trends in pharmacological sciences 35, 63-75.

Kimmel, K.L., Weaver, J.D., Lee, M., Ellman, J.A., 2012. Catalytic enantioselective protonation of nitronates utilizing an organocatalyst chiral only at sulfur. Journal of the American Chemical Society 134, 9058-9061.
Kustermann, A., Mobius, C., Oberstein, T., Muller, H.H., Kornhuber, J., 2014. Depression and attempted suicide under pregabalin therapy. Annals of general psychiatry 13, 37.
Levin, E.D., Bencan, Z., Cerutti, D.T., 2007. Anxiolytic effects of nicotine in zebrafish. Physiology & behavior 90, 54-58.
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.
Lotarski, S., Hain, H., Peterson, J., Galvin, S., Strenkowski, B., Donevan, S., Offord, J., 2014. Anticonvulsant activity of pregabalin in the maximal electroshock-induced seizure assay in alpha2delta1 (R217A) and alpha2delta2 (R279A) mouse mutants. Epilepsy Res 108, 833-842.
Mann, D., Liu, J., Chew, M.L., Bockbrader, H., Alvey, C.W., Zegarac, E., Pellock, J., Pitman, V.W., 2014. Safety, tolerability, and pharmacokinetics of pregabalin in children with refractory partial seizures: a phase 1, randomized controlled study. Epilepsia 55, 1934-1943.
Milite, C., Castellano, S., Benedetti, R., Tosco, A., Ciliberti, C., Vicidomini, C., Boully, L., Franci, G., Altucci, L., Mai, A., Sbardella, G., 2011. Modulation of the activity of histone acetyltransferases by long chain alkylidenemalonates (LoCAMs). Bioorganic & medicinal chemistry 19, 3690-3701.
Mussulini, B.H., Leite, C.E., Zenki, K.C., Moro, L., Baggio, S., Rico, E.P., Rosemberg, D.B., Dias, R.D., Souza, T.M., Calcagnotto, M.E., Campos, M.M., Battastini, A.M., de Oliveira, D.L., 2013. Seizures induced by pentylenetetrazole in the adult zebrafish: a detailed behavioral characterization. PloS one 8, e54515.
Rodrigues, M.O., Cantos, J.B., D'Oca, C.R., Soares, K.L., Coelho, T.S., Piovesan, L.A., Russowsky, D., da Silva, P.A., D'Oca, M.G., 2013. Synthesis and antimycobacterial activity of isoniazid derivatives from renewable fatty acids. Bioorganic & medicinal chemistry 21, 6910-6914.
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.
Siebel, A.M., Menezes, F.P., da Costa Schaefer, I., Petersen, B.D., Bonan, C.D., 2015. Rapamycin suppresses PTZ-induced seizures at different developmental stages of zebrafish. Pharmacology, biochemistry, and behavior.
Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., Kalueff, A.V., 2012. Modeling anxiety using adult zebrafish: a conceptual review. Neuropharmacology 62, 135-143.
Tellez-Zenteno, J.F., Patten, S.B., Jette, N., Williams, J., Wiebe, S., 2007. Psychiatric comorbidity in epilepsy: a population-based analysis. Epilepsia 48, 2336-2344.
Ullmann, J.F., Cowin, G., Kurniawan, N.D., Collin, S.P., 2010. A three-dimensional digital atlas of the zebrafish brain. NeuroImage 51, 76-82.

6. Conclusões gerais

Concluindo, tanto os animais pré-tratado com pregabalina na concentração de 20 e 60 mM quanto seu derivado sintético GABA-capróico na concentração de 60 mM tendem a aumentar o seu perfil locomotor e exploratório quando submetidos ao teste *novel tank*. Segundo dados da literatura, isso representaria uma tendencia das moléculas apresentarem efeito ansiolítico no modelo animal de peixe-zebra adulto. Efeitos colotarais relacionados à locomoção dos animais não foram encontrados nessas concentrações. A pregabalin demonstrou efeito anticonvulsivo na concentração de 60 mM frente a crises epilépticas induzidas por pentilenotetrazol em modelo de peixe-zebra adulto. Apregabalina 20 mM e o GABA-capróico 60 mM foram capazes de diminuir a intensidade das crises epilépticas em níveis menores que a pregabalina 60 mM, porém, ainda exibindo resultados de intensidade de crises epilépticas menores que o grupo controle.

7. Perspectivas

- Aumentar o número de animais por grupo no experimento de *open tank*, a fim de definir um padrão mais claro sobre a exploração dos animais no aparato comportamental;

- Aumentar o número de animais por grupo no experimento de indução de crises epilépticas;

- Calcular o tempo total de duração de estágios 4 (crises clônicas) e 5 (perda de postura, queda ao fundo do aquário e convulsões predominantemente tônicas) em todos os grupos experimentais;

- Testar outras concentrações de GABA-capróico;

- Realizar um teste de preferência animal pelo claro/escuro. Também seria interessante realizar novos experimentos com uma concentração elevada do composto derivado da pregabalina;

- Realizar testes para solubilizar dos demais análogos sintetizados, tendo em vista que a alta hidrofobicidade das cadeias graxas desses compostos dificultam sua solubilidade, mesmo na concentração máxima utilizada pela literatura de DMSO (1%).

8. Referências gerais

ALFARO, J. M.; RIPOLL-GOMEZ, J.; BURGOS, J. S. Kainate administered to adult zebrafish causes seizures similar to those in rodent models. **Eur J Neurosci,** v. 33, n. 7, p. 1252-5, Apr 2011. ISSN 0953-816x.

ALSOP, D.; VIJAYAN, M. M. Development of the corticosteroid stress axis and receptor expression in zebrafish. **Am J Physiol Regul Integr Comp Physiol,** v. 294, n. 3, p. R711-9, Mar 2008. ISSN 0363-6119 (Print) 0363-6119.

ARROYO, S. et al. Pregabalin add-on treatment: a randomized, double-blind, placebo-controlled, dose-response study in adults with partial seizures. **Epilepsia,** v. 45, n. 1, p. 20-7, Jan 2004. ISSN 0013-9580 (Print) 0013-9580.

BANERJEE, P. N.; FILIPPI, D.; ALLEN HAUSER, W. The descriptive epidemiology of epilepsy-a review. **Epilepsy Res,** v. 85, n. 1, p. 31-45, Jul 2009. ISSN 0920-1211.

BARABAN, S. C. et al. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. **Neuroscience,** v. 131, n. 3, p. 759-68, 2005. ISSN 0306-4522 (Print) 0306-4522.

BIALER, M. New antiepileptic drugs currently in clinical trials: is there a strategy in their development? **Ther Drug Monit,** v. 24, n. 1, p. 85-90, Feb 2002. ISSN 0163-4356 (Print) 0163-4356.

BIALER, M.; WHITE, H. S. Key factors in the discovery and development of new antiepileptic drugs. **Nat Rev Drug Discov,** v. 9, n. 1, p. 68-82, Jan 2010. ISSN 1474-1776.

BRODIE, M. J.; DICHTER, M. A. Antiepileptic drugs. **N Engl J Med,** v. 334, n. 3, p. 168-75, Jan 18 1996. ISSN 0028-4793 (Print) 0028-4793.

CRYAN, J. F.; HOLMES, A. The ascent of mouse: advances in modelling human depression and anxiety. **Nat Rev Drug Discov,** v. 4, n. 9, p. 775-90, Sep 2005. ISSN 1474-1776 (Print)

1474-1776 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16138108 >.

CZAPINSKI, P.; BLASZCZYK, B.; CZUCZWAR, S. J. Mechanisms of action of antiepileptic drugs. **Curr Top Med Chem,** v. 5, n. 1, p. 3-14, 2005. ISSN 1568-0266 (Print) 1568-0266.

DOOLEY, D. J.; DONOVAN, C. M.; PUGSLEY, T. A. Stimulus-dependent modulation of [(3)H]norepinephrine release from rat neocortical slices by gabapentin and pregabalin. **J Pharmacol Exp Ther,** v. 295, n. 3, p. 1086-93, Dec 2000. ISSN 0022-3565 (Print) 0022-3565.

ETEMAD, L. et al. Teratogenic effects of pregabalin in mice. **Iran J Basic Med Sci,** v. 16, n. 10, p. 1065-70, Oct 2013. ISSN 2008-3866 (Print) 2008-3866.

FISHER, R. S. et al. ILAE official report: a practical clinical definition of epilepsy. **Epilepsia,** v. 55, n. 4, p. 475-82, Apr 2014. ISSN 1528-1167 (Electronic)

0013-9580 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24730690 >.

FISHER, R. S. et al. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). **Epilepsia,** v. 46, n. 4, p. 470-2, Apr 2005. ISSN 0013-9580 (Print) 0013-9580.

GAITATZIS, A.; TRIMBLE, M. R.; SANDER, J. W. The psychiatric comorbidity of epilepsy. **Acta Neurol Scand,** v. 110, n. 4, p. 207-20, Oct 2004. ISSN 0001-6314 (Print) 0001-6314.

GLAUSER, T. et al. ILAE treatment guidelines: evidence-based analysis of antiepileptic drug efficacy and effectiveness as initial monotherapy for epileptic seizures and syndromes. **Epilepsia,** v. 47, n. 7, p. 1094-120, Jul 2006. ISSN 0013-9580 (Print) 0013-9580.

GOLDSMITH, P. Zebrafish as a pharmacological tool: the how, why and when. **Curr Opin Pharmacol,** v. 4, n. 5, p. 504-12, Oct 2004. ISSN 1471-4892 (Print) 1471-4892.

GRONE, B. P.; BARABAN, S. C. Animal models in epilepsy research: legacies and new directions. **Nat Neurosci,** v. 18, n. 3, p. 339-43, Mar 2015. ISSN 1097-6256.

GRUNWALD, D. J.; EISEN, J. S. Headwaters of the zebrafish -- emergence of a new model vertebrate. **Nat Rev Genet,** v. 3, n. 9, p. 717-24, Sep 2002. ISSN 1471-0056 (Print) 1471-0056.

HANSEN, S. L.; SPERLING, B. B.; SANCHEZ, C. Anticonvulsant and antiepileptogenic effects of GABAA receptor ligands in pentylenetetrazole-kindled mice. **Prog Neuropsychopharmacol Biol Psychiatry,** v. 28, n. 1, p. 105-13, Jan 2004. ISSN 0278-5846 (Print) 0278-5846.

HUNT, R. F. et al. A novel zebrafish model of hyperthermia-induced seizures reveals a role for TRPV4 channels and NMDA-type glutamate receptors. **Exp Neurol,** v. 237, n. 1, p. 199-206, Sep 2012. ISSN 0014-4886.

IBRAHIM, M. et al. Anxiolytic effects of diphenyl diselenide on adult zebrafish in a novelty paradigm. **Prog Neuropsychopharmacol Biol Psychiatry,** v. 54, p. 187-94, Oct 3 2014. ISSN 0278-5846.

KALUEFF, A. V. et al. Zebrafish neurobehavioral phenomics for aquatic neuropharmacology and toxicology research. **Aquat Toxicol**, Aug 24 2015. ISSN 0166-445x.

KANNER, A. M. Anxiety disorders in epilepsy: the forgotten psychiatric comorbidity. **Epilepsy Curr,** v. 11, n. 3, p. 90-1, May 2011. ISSN 1535-7511.

KIM, Y. H. et al. Reduced neuronal proliferation by proconvulsant drugs in the developing zebrafish brain. **Neurotoxicol Teratol,** v. 32, n. 5, p. 551-7, Sep-Oct 2010. ISSN 0892-0362.

KUSTERMANN, A. et al. Depression and attempted suicide under pregabalin therapy. **Ann Gen Psychiatry,** v. 13, n. 1, p. 37, 2014. ISSN 1744-859x.

LIESCHKE, G. J.; CURRIE, P. D. Animal models of human disease: zebrafish swim into view. **Nat Rev Genet,** v. 8, n. 5, p. 353-67, May 2007. ISSN 1471-0056 (Print) 1471-0056.

LOSCHER, W. Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. **Seizure,** v. 20, n. 5, p. 359-68, Jun 2011. ISSN 1059-1311.

LOTARSKI, S. et al. Anticonvulsant activity of pregabalin in the maximal electroshock-induced seizure assay in alpha2delta1 (R217A) and alpha2delta2 (R279A) mouse mutants. **Epilepsy Res,** v. 108, n. 5, p. 833-42, Jul 2014. ISSN 0920-1211.

MANN, D. et al. Safety, tolerability, and pharmacokinetics of pregabalin in children with refractory partial seizures: a phase 1, randomized controlled study. **Epilepsia,** v. 55, n. 12, p. 1934-43, Dec 2014. ISSN 0013-9580.

MENEZES, F. P.; RICO, E. P.; DA SILVA, R. S. Tolerance to seizure induced by kainic acid is produced in a specific period of zebrafish development. **Prog Neuropsychopharmacol Biol Psychiatry,** v. 55, p. 109-12, Dec 3 2014. ISSN 0278-5846.

MUSSULINI, B. H. et al. Seizures induced by pentylenetetrazole in the adult zebrafish: a detailed behavioral characterization. **PLoS One,** v. 8, n. 1, p. e54515, 2013. ISSN 1932-6203.

PANULA, P. et al. The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. **Neurobiol Dis,** v. 40, n. 1, p. 46-57, Oct 2010. ISSN 0969-9961.

PANULA, P. et al. Modulatory neurotransmitter systems and behavior: towards zebrafish models of neurodegenerative diseases. **Zebrafish,** v. 3, n. 2, p. 235-47, 2006. ISSN 1545-8547.

PERUCCA, E.; FRENCH, J.; BIALER, M. Development of new antiepileptic drugs: challenges, incentives, and recent advances. **Lancet Neurol,** v. 6, n. 9, p. 793-804, Sep 2007. ISSN 1474-4422 (Print) 1474-4422.

PINEDA, R.; BEATTIE, C. E.; HALL, C. W. Recording the adult zebrafish cerebral field potential during pentylenetetrazole seizures. **J Neurosci Methods,** v. 200, n. 1, p. 20-8, Aug 30 2011. ISSN 0165-0270.

ROSEMBERG, D. B. et al. Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments. **PLoS One,** v. 6, n. 5, p. e19397, 2011. ISSN 1932-6203.

SARKISIAN, M. R. Overview of the Current Animal Models for Human Seizure and Epileptic Disorders. **Epilepsy Behav,** v. 2, n. 3, p. 201-216, Jun 2001. ISSN 1525-5050.

STEWART, A. et al. Modeling anxiety using adult zebrafish: a conceptual review. **Neuropharmacology,** v. 62, n. 1, p. 135-43, Jan 2012. ISSN 1873-7064 (Electronic)

0028-3908 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21843537 >.

SULS, A. et al. De novo loss-of-function mutations in CHD2 cause a fever-sensitive myoclonic epileptic encephalopathy sharing features with Dravet syndrome. **Am J Hum Genet,** v. 93, n. 5, p. 967-75, Nov 7 2013. ISSN 0002-9297.

TELLEZ-ZENTENO, J. F. et al. Psychiatric comorbidity in epilepsy: a population-based analysis. **Epilepsia,** v. 48, n. 12, p. 2336-44, Dec 2007. ISSN 0013-9580 (Print) 0013-9580.

TENG, Y. et al. Knockdown of zebrafish Lgi1a results in abnormal development, brain defects and a seizure-like behavioral phenotype. **Hum Mol Genet,** v. 19, n. 22, p. 4409-20, Nov 15 2010. ISSN 0964-6906.

9. Anexo 1- Normas de publicação da revista

Guide for authors

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the

authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.
Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images and in accordance with all technical requirements: Illustration Service.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for

example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Math formulae

Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.

• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions.
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. For further information on the preparation of electronic artwork, please see
http://www.elsevier.com/artworkinstructions.

Figure captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes

below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles (http://citationstyles.org), such as Mendeley (http://www.mendeley.com/features/reference-manager) and Zotero (https://www.zotero.org/), as well as EndNote (http://endnote.com/downloads/styles). Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link:
http://open.mendeley.com/use-citation-style/epilepsy-research
When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Reference style

Text: All citations in the text should refer to:
1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;

2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.
Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.
Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations: http://www.issn.org/services/online-services/access-to-the-ltwa/.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 150 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

AudioSlides

The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at http://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary material

Supplementary material can support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Please note that such items are published online exactly as they are submitted; there is no typesetting involved (supplementary data supplied as an Excel file or as a PowerPoint slide will appear as such online). Please submit the material together with the article and supply a concise and descriptive caption for each file. If you wish to make any changes to supplementary data during any stage of the process, then please make sure to provide an updated file, and do not annotate any corrections on a previous version. Please also make sure to switch off the 'Track Changes' option in any Microsoft Office files as these will appear in the published supplementary file(s). For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

3D neuroimaging

You can enrich your online articles by providing 3D neuroimaging data in NIfTI format. This will be visualized for readers using the interactive viewer embedded within your article, and will enable them to: browse through available neuroimaging datasets; zoom, rotate and pan the 3D brain reconstruction; cut through the volume; change opacity and color mapping; switch between 3D and 2D projected views; and download the data. The viewer supports both single (.nii) and dual (.hdr and .img) NIfTI file formats. Recommended size of a single uncompressed dataset is maximum 150 MB. Multiple datasets can be submitted. Each dataset will have to be zipped and uploaded to the online submission system via the '3D neuroimaging data' submission category. Please provide a short informative description for each dataset by filling in the 'Description' field when uploading a dataset. Note: all datasets will be available for downloading from the online article on ScienceDirect. If you have concerns about your data being downloadable, please provide a video instead. For more information see: http://www.elsevier.com/3DNeuroimaging.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address

All necessary files have been uploaded, and contain:
• Keywords
• All figure captions
• All tables (including title, description, footnotes)
Further considerations
• Manuscript has been 'spell-checked' and 'grammar-checked'
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Internet)
Printed version of figures (if applicable) in color or black-and-white
• Indicate clearly whether or not color or black-and-white in print is required.
For any further information please visit our customer support site at
http://support.elsevier.com