



Pontifícia Universidade Católica do Rio Grande do Sul
Faculdade de Biociências
Programa de Pós-Graduação em Biologia Celular e Molecular

**Efeito de Crises Convulsivas e Fármacos Antiepilépticos em Parâmetros
Neuroquímicos e Moleculares em Peixe Zebra (*Danio rerio*)**

Autor
Anna Maria Siebel

Orientador
Profª Drª Carla Denise Bonan

Porto Alegre
2011



Pontifícia Universidade Católica do Rio Grande do Sul
Faculdade de Biociências
Programa de Pós-Graduação em Biologia Celular e Molecular

Efeito de crises convulsivas e fármacos antiepilépticos em parâmetros neuroquímicos e moleculares em peixe zebra (*Danio rerio*)

Dissertação apresentada como requisito para obtenção do grau de Mestre pelo Programa de Pós-graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

Autor
Anna Maria Siebel

Orientador
Profª Drª Carla Denise Bonan

Porto Alegre
2011

AGRADECIMENTOS

Aos meus pais e aos meus irmãos pelo indispensável carinho, apoio e incentivo na busca da minha realização pessoal e profissional.

À minha orientadora Prof^a. Dra. Carla D. Bonan pela orientação científica e não-científica, amizade e grande exemplo profissional. Muito obrigada pela disponibilidade, paciência (imensa), ensinamentos e muitos conselhos essenciais.

Aos professores do Laboratório de Neuroquímica e Psicofarmacologia, Laboratório de Biologia e Desenvolvimento do Sistema Nervoso e Laboratório de Biologia Genômica e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul pelo auxílio e cooperação.

A todos os colegas de laboratório pela colaboração, ensinamentos, conselhos e amizade.

Ao meu querido amigo Ângelo L. Piato, um agradecimento especial pelo apoio, incentivo e grande amizade. Muito obrigada pelas muitas críticas e alguns (poucos) elogios, sempre buscando me ajudar. Muito obrigada pelos vários ensinamentos (acredito ter aprendido alguns) e dedicação durante este trabalho.

Ao meu querido amigo Denis B. Rosemberg, um agradecimento especial pelos ensinamentos bioquímicos, seus gráficos sempre esclarecedores, disponibilidade em revisar artigos alterados e reescritos diversas vezes e indispensável descontração em nosso dia-a-dia.

Às minhas amigas da Pós-Graduação pela grande amizade e por nossas alegres e inspiradoras jantas, essenciais neste período.

Aos meus amigos e minhas amigas de Novo Hamburgo, que sempre me apoiaram e compreenderam minha ausência, agradeço pelos bons (e maus) exemplos, que têm contribuído muito para minha formação pessoal.

Aos professores do Programa de Pós-Graduação em Biologia Celular e Molecular da PUCRS pela contribuição na minha formação.

Ao CNPq pela bolsa de estudo e aos demais órgãos que têm apoiado e possibilitado a pesquisa científica no Brasil.

A todos que de alguma forma contribuíram para a realização desse trabalho.

RESUMO

A epilepsia é uma desordem neuronal caracterizada pela ocorrência de convulsões espontâneas e recorrentes. Essa patologia e seu tratamento interferem em diversos mecanismos neurológicos. O sistema purinérgico é uma importante rota de sinalização celular que emprega nucleotídeos e nucleosídeos extracelulares como moléculas sinalizadoras. O neurotransmissor ATP atua através de receptores do tipo P2Y, acoplados à proteína G e receptores P2X, que são ionotrópicos. A degradação do ATP extracelular e a conseqüente produção de adenosina é realizada por uma família de enzimas de superfície celular conhecidas como ectonucleotidases, que inclui as NTPDases (nucleosídeo trifosfato difosfohidrolases) e a ecto-5'-nucleotidase. A adenosina é um neuromodulador que atua através da ativação de receptores metabotrópicos do tipo P1 (A_1 , A_{2A} , A_{2B} , A_3). Esse nucleosídeo pode agir como um anticonvulsivante endógeno, principalmente via receptores A_1 . As NTPDases hidrolisam nucleotídeos tri- e difosfatados originando a adenosina, que é hidrolisada pela adenosina deaminase (ADA). Assim, as NTPDases, ecto-5'-nucleotidase e ADA controlam os níveis de nucleotídeos e nucleosídeos, modulando o sistema purinérgico. No sistema colinérgico, a acetilcolina (ACh) atua através de receptores muscarínicos (metabotrópicos) e nicotínicos (ionotrópicos). Sua ação é encerrada através de sua hidrólise catalisada pela acetilcolinesterase (AChE). O peixe zebra é um pequeno teleosteo de água doce que vem sendo amplamente utilizado como modelo experimental em pesquisa. Estudos mostram que o peixe zebra pode ser uma ferramenta importante para o entendimento da epilepsia, bem como para o *screening* de fármacos antiepilépticos. Considerando que as sinalizações purinérgica e colinérgica têm importante participação no sistema nervoso e que essas vias de neurotransmissão estão identificadas e caracterizadas em peixe zebra, o objetivo desse estudo foi avaliar nesse teleosteo o efeito de convulsões induzidas por pentilenotetrazol (PTZ), bem como de fármacos antiepilépticos na atividade das ectonucleotidases, ADA e AChE, enzimas essenciais na modulação destas vias de sinalização. Foram avaliados os efeitos *in vitro* da carbamazepina, fenitoína e gabapentina na atividade das ectonucleotidases e AChE. A carbamazepina diminuiu a hidrólise de ATP e também de ACh. A fenitoína aumentou a hidrólise de AMP e a gabapentina não provocou alterações enzimáticas. Foi analisado também o efeito de crises convulsivas induzidas por PTZ na atividade das ectonucleotidases e da ADA. Os resultados não mostraram alterações nas ectonucleotidases e ADA nas frações extracelular e intracelular, respectivamente. No entanto, a atividade extracelular da ADA foi inibida em animais expostos ao PTZ. As análises mostraram que os fármacos antiepilépticos podem influenciar a atividade das enzimas envolvidas na degradação extracelular de nucleotídeos, bem como na hidrólise de ACh. Além disso, a diminuição na degradação de adenosina observada em nosso modelo de estudo pode sugerir a participação da ADA na modulação nos níveis de adenosina durante as crises convulsivas em peixe zebra.

Palavras-chave: epilepsia, fármacos antiepilépticos, adenosina deaminase, ectonucleotidases, acetilcolinesterase, PTZ, peixe zebra.

ABSTRACT

Epilepsy is a neurological disorder characterized by the occurrence of spontaneous and recurrent seizures. This disease and its treatment influence diverse neurological pathways. The purinergic system is an essential route for cell-to-cell communication and employs extracellular nucleotides and nucleosides as signaling molecules. The neurotransmitter ATP mediates synaptic currents through activation of G-protein-coupled P2Y receptors and P2X ionotropic receptors. The ATP degradation and consequent production of adenosine is promoted by a family of enzymes named ectonucleotidases, which includes NTPDases (nucleoside triphosphate diphosphohydrolases) and ecto-5'-nucleotidase. Adenosine is a neuromodulator that acts through the activation of P1 metabotropic receptors (A₁, A_{2A}, A_{2B}, and A₃). Adenosine can act as an endogenous anticonvulsant, mainly by A₁ receptors. The NTPDases hydrolyze both tri- and diphosphonucleosides whereas ecto-5'-nucleotidase hydrolyzes nucleoside monophosphates producing the nucleoside adenosine, that is hydrolyzed by adenosine deaminase (ADA). Therefore, NTPDases, ecto-5'-nucleotidase, and ADA control nucleotide and nucleoside levels, modulating the purinergic signaling. In the cholinergic system, acetylcholine (ACh) acts through muscarinic (metabotropic) and nicotinic (ionotropic) receptors. ACh is inactivated by its hydrolysis, that is catalyzed by acetylcholinesterase (AChE). Zebrafish is a small freshwater teleost which is becoming widely used as an experimental organism. Studies have demonstrated that zebrafish could be an important model to study seizures and to screen antiepileptic drugs. Considering that the purinergic and cholinergic systems exert an important role in the nervous system and that these neurotransmitter pathways have been already described in zebrafish, the aim of this study was to evaluate in this teleost the effect of pentylenetetrazole (PTZ)-induced seizures and antiepileptic drugs on the ectonucleotidases, ADA, and AChE activities. We have tested the *in vitro* effects of carbamazepine, phenytoin, and gabapentine at different concentrations on ectonucleotidase and AChE activities. Our results have shown that carbamazepine reduced ATP and ACh hydrolysis whereas phenytoin enhanced AMP hydrolysis. Gabapentin did not promote any changes on enzymatic activities. In addition, we tested the effect of PTZ-induced seizures on ectonucleotidases and ADA activities in adult zebrafish brain. Our results have demonstrated that PTZ treatments did not alter ectonucleotidase and ADA activities in membranes and soluble fraction, respectively. However, ecto-ADA activity was significantly decreased in brain membranes of animals exposed to PTZ treatments. These results have shown that antiepileptic drugs can influence the enzyme activities involved in the degradation of ACh and extracellular nucleotides. Furthermore, the decreased adenosine deamination observed in our study suggests the ADA participation on the modulation of extracellular adenosine levels during PTZ-induced seizures in zebrafish.

Keywords: epilepsy, antiepileptic drugs, adenosine deaminase, ectonucleotidase, acetylcholinesterase, PTZ, zebrafish.

LISTA DE ABREVIATURAS

Acetil-CoA: acetil-coenzima A
ACh: acetilcolina
AChE: acetilcolinesterase
ADA: adenosina deaminase
ADP: adenosina 5´ - difosfato
AK: adenosina quinase
AMP: adenosina 5´ - monofosfato
AMPC: adenosina 5´ - monofosfato cíclico
ATP: adenosina 5´ - trifosfato
BuChE: butirilcolinesterase
Ca²⁺: cálcio
ChAT: colina acetiltransferase
Cl⁻: cloro
E-NPP: ectonucleotídeo pirofosfatase/fosfodiesterase
GABA: ácido gama-aminobutírico
GPI: glicosilfosfatidilinositol
K⁺: potássio
Na⁺: sódio
NTPDase: nucleosídeo trifosfato difosfohidrolase
PTZ: pentilenotetrazol
SNC: sistema nervoso central
SNP: sistema nervoso periférico

LISTA DE FIGURAS

Figura 1: Peixe zebra (<i>Danio rerio</i>).....	10
Figura 2: Mecanismos de ação de fármacos antiepilépticos em sinapses excitatórias e inibitórias.....	16
Figura 3: Representação esquemática da sinalização purinérgica no sistema nervoso central.....	19
Figura 4: Representação esquemática da degradação extracelular de purinas.....	22
Figura 5: Representação esquemática da sinalização colinérgica.....	25
Figura 6: Estruturas moleculares da AChE.....	26

SUMÁRIO

RESUMO	4
ABSTRACT	5
LISTA DE ABREVIATURAS	6
LISTA DE FIGURAS	7
CAPÍTULO 1: INTRODUÇÃO E OBJETIVOS	9
1. INTRODUÇÃO	10
1.1 Peixe zebra.....	10
1.2. Epilepsia.....	13
1.3 Fármacos antiepilépticos.....	14
1.4 Sistema purinérgico.....	16
1.4.1 ATP.....	16
1.4.2 Adenosina.....	17
1.4.3 Ectonucleotidasas e ADA.....	19
1.4.4 Sistema purinérgico e epilepsia.....	22
1.5 Sistema colinérgico.....	23
1.5.1 Acetilcolinesterase.....	25
1.5.2 Sistema colinérgico e epilepsia.....	27
2. OBJETIVOS	28
2.1 Objetivo geral.....	28
2.2 Objetivos específicos.....	28
CAPÍTULO 2: ARTIGO CIENTÍFICO	29
CAPÍTULO 3: ARTIGO CIENTÍFICO	36
CAPÍTULO 4: CONSIDERAÇÕES FINAIS	63
REFERÊNCIAS	69
ANEXO : Comprovante de aprovação do protocolo pelo Comitê de Ética da PUCRS.....	80

CAPÍTULO 1

INTRODUÇÃO E OBJETIVOS

1. INTRODUÇÃO

1.1 Peixe zebra

O peixe zebra, *Danio rerio* (Figura 1), é um pequeno teleósteo de água doce pertencente à família Cyprinidae. Seu habitat natural é o sul e sudeste da Ásia, principalmente Índia, Bangladesh e Nepal. Esse peixe é caracterizado por seu tamanho pequeno (3-5 cm) e um padrão de coloração distinto, baseado em listras horizontais, claras e escuras e alternadas (Spence et al., 2008).



Figura 1: peixe zebra (*Danio rerio*)

Aspectos como seu baixo custo e pouco espaço necessário para manutenção, bem como o crescimento do número de metodologias e ferramentas disponíveis para seu estudo e sua melhor caracterização, tornaram o peixe zebra amplamente utilizado como modelo experimental em diversas linhas de pesquisa (Brittijn et al., 2009; Tsang, 2010). Isso pode ser confirmado pelo crescimento exponencial do número de publicações a partir de estudos com esta espécie e o grande número de laboratórios que têm utilizado este teleósteo como modelo experimental (Bradford et al., 2010). Atualmente, o peixe zebra vem sendo utilizado em estudos de bioquímica, farmacologia, toxicologia, neurobiologia, fisiologia, entre outros (Goldsmith, 2004; Levin & Chen, 2004; Zon & Peterson, 2005; Egan et al., 2009; Grossman et al., 2010; Lee et al., 2010). Além disso, modelos de diversas doenças humanas como, por exemplo, neurológicas, cardíacas, e câncer, já foram desenvolvidos em peixe zebra (Shepard et al., 2005; Lieschke & Currie, 2007; Dahme et al., 2009; Hortopan et al., 2010).

Inicialmente, o peixe zebra foi empregado no estudo da biologia do desenvolvimento e genética molecular, devido a vantagens apresentadas quando comparado a outros organismos (Feitsma & Cuppen, 2008). Seus embriões são

suficientemente resistentes para manipulações experimentais como microinjeção e transplante celular (Gilmour et al., 2002). Além disso, a transparência desses embriões permite a visualização dos processos do desenvolvimento nos seus diferentes estágios e também o *screening* por fenótipo (Lieschke & Currie, 2007). O ciclo de desenvolvimento do peixe zebra é bastante rápido, sendo que a embriogênese possui uma duração aproximada de 24 horas e a organogênese leva em torno de cinco dias (Dahm & Geisler, 2006). Outro atrativo para o uso do peixe zebra, é sua capacidade de gerar um grande número de descendentes. Quando mantida em condições ideais, uma fêmea pode gerar de 100 a 400 ovos por semana (Dahm & Geisler, 2006).

Suas características embriológicas e de desenvolvimento conferem ao peixe zebra grande vantagem em relação a outros modelos de vertebrados, nos quais aspectos patológicos e da organogênese não podem ser estudados sem intervenções cirúrgicas ou análises *post-mortem* (Lieschke & Currie, 2007). Os avanços nos estudos com peixe zebra permitiram *screening* genéticos em larga-escala que tornaram possível entender processos específicos de vertebrados. Anteriormente, esse tipo de estudo era restrito a moscas, vermes e fungos (Zon & Peterson, 2005). Além disso, o rápido desenvolvimento desse teleósteo permite o estudo de aspectos do desenvolvimento em apenas algumas horas ou dias (Dahm & Geisler, 2006).

Em estudos farmacológicos, sua capacidade de absorver compostos adicionados à água dispensa a necessidade de tratamentos através de protocolos invasivos. Uma vez que as larvas de peixe zebra podem viver em meios com volume em torno de 50 µl, pouca quantidade de droga é necessária para a realização de testes. Essas características permitem realizar estudos *in vivo* em larga escala e também a reprodução do tratamento em um grande número de animais, bem como em diferentes linhagens (Goldsmith, 2004; Berghmans et al., 2007).

O estudo do genoma do peixe zebra teve considerável progresso nos últimos anos, principalmente devido ao seu seqüenciamento iniciado em 2001 pelo Instituto Sanger (Spence et al., 2008). O genoma da espécie foi seqüenciado e estão sendo feitas revisões e atualizações em sua anotação (sanger.ac.uk/Projects/D_zebra). Muitos genes associados a doenças humanas, bem como seu padrão de expressão, já são conhecidos ou estão sendo estudados nesse teleósteo. Ortólogos envolvidos em patologias neurológicas, como Parkinson, Alzheimer e Huntington são alguns

exemplos de genes já identificados (Sager et al., 2010). Além disso, já foram desenvolvidos modelos transgênicos expressando genes humanos mutados e modelos *knockout* que reproduzem patologias humanas, bem como técnicas para controle de expressão gênica (Bandmann & Burton, 2010).

Recentemente, o peixe zebra emergiu também como um importante modelo para estudos neurológicos e comportamentais (Egan et al., 2009; Piatto et al., 2010; Stewart et al., 2010). O sistema nervoso central (SNC) do peixe zebra apresenta organização geral, células neuronais especializadas, células gliais, mielina e circuitos neuronais semelhantes ao que é observado em mamíferos (Sager et al., 2010). A ocorrência dos principais neurotransmissores encontrados em mamíferos, incluindo aminoácidos (glutamato, GABA, glicina), monoaminas (histamina, dopamina, norepinefrina, epinefrina, serotonina, melatonina) e acetilcolina (ACh), entre outros, bem como seus mecanismos de ação já foram descritos (Rinkwitz et al., 2010). Esse teleósteo apresenta os sistemas motor, sensitivo e endócrino bem desenvolvidos, alta sensibilidade a alterações ambientais e manipulações farmacológicas e um amplo espectro de fenótipos comportamentais conhecidos (Egan et al., 2009; Cachat et al., 2010; Sager et al., 2010; Burne et al., 2011). Agentes farmacológicos que interferem na transmissão sináptica e na estabilidade da membrana neuronal mostraram, em peixe zebra, efeitos semelhantes aos verificados em humanos, sugerindo a existência de mecanismos de controle neurológico semelhantes (Lieschke & Currie, 2007).

O peixe zebra tem se mostrado um eficiente modelo experimental para epilepsia, permitindo estudos bioquímicos relacionados a essa patologia e seu tratamento (Berghmans et al., 2007). Larvas expostas ao pentilenotetrazol (PTZ) apresentaram alterações comportamentais, eletroencefalográficas e moleculares semelhantes às alterações verificadas em modelos de convulsão em roedores (Baraban et al., 2005). Além disso, a exposição de peixe zebra adultos a cafeína, picrotoxina e PTZ provocou respostas comportamentais características de crise convulsiva (Wong et al., 2010). Finalmente, a sua capacidade de absorver compostos adicionados à água fez com que o esse teleósteo se tornasse uma boa ferramenta para o estudo em grande escala de fármacos antiepiléticos (Berghmans et al., 2007).

1.2 Epilepsia

A epilepsia, caracterizada pela ocorrência espontânea e recorrente de convulsões, é uma das principais desordens crônicas do SNC, afetando aproximadamente 1% da população mundial (Fisher et al., 2005; Elger & Schmidt, 2008). Essas convulsões têm origem a partir de descargas neuronais excessivas e sincrônicas e são evidenciadas pela manifestação transitória de sintomas como alterações sensitivas ou sensoriais, perda de consciência e manifestações motoras (Fisher et al., 2005). Crises convulsivas prolongadas ou repetidas em intervalos curtos de tempo que resultam em um estado epiléptico contínuo e duradouro caracterizam o *status epilepticus* (Banerjee et al., 2009).

De acordo com a *International League Against Epilepsy* (ILAE), as crises epiléticas podem ser classificadas como parciais ou generalizadas. Nas crises denominadas parciais, a descarga neuronal é restrita a um hemisfério do cérebro e tem seu início em regiões focais. Nesse tipo de convulsão o indivíduo pode ou não apresentar alteração de consciência. As crises generalizadas acometem a área cerebral como um todo e incluem crises clônicas, tônicas, tônico-clônicas, mioclônicas e de ausência. Em alguns casos, ocorrem variados tipos de crises, não sendo possível sua classificação (Berg et al., 2010).

A epilepsia pode ser desenvolvida a partir de insultos cerebrais como tumores, traumatismos, infecções, derrames e convulsões febris. A partir desses insultos ocorre uma série de alterações morfológicas e funcionais na área cerebral afetada que pode levar ao desenvolvimento da epilepsia. Esse processo, conhecido como epileptogênese, é longo, contínuo e progressivo, podendo levar de meses a anos até que se inicie a manifestação de crises convulsivas decorrentes do dano cerebral inicial (Pitkanen, 2004; Acharya et al., 2008). A epileptogênese envolve perda neuronal, plasticidade axonal e dendrítica e reorganização das membranas celulares e da matriz extracelular (Jutila et al., 2002; Pitkanen, 2002). Pode ocorrer a formação de novos circuitos neuronais excitatórios e a perda seletiva de neurônios gabaérgicos (Dudek & Sutula, 2007; Dudek & Staley, 2010).

A epilepsia também pode ser originada por alterações genéticas que causam, principalmente, disfunções nos canais de Na^+ , Ca^{2+} e K^+ . Muitas crises convulsivas são causadas por uma combinação de fatores genéticos e ambientais. Além disso, muitos pacientes que desenvolvem convulsões recorrentes não possuem um motivo

óbvio ou uma anormalidade neurológica aparente (Badawi et al., 2009; Bialer & White, 2010).

Diversos estudos têm mostrado significativa influência dessa patologia e seu tratamento nas propriedades dinâmicas e funcionais da atividade cerebral. A atividade neuronal excessiva característica das crises convulsivas pode causar alterações na plasticidade sináptica que levam ao agravamento da doença a longo prazo (Rakhade & Jensen, 2009). Essa desordem afeta o encéfalo como um todo e seus pacientes podem ter problemas ou diminuição em sua atenção, memória e aprendizado (Hamed et al., 2009).

1.3 Fármacos antiepilépticos

O tratamento da epilepsia é sintomático, ou seja, visa o controle das crises convulsivas e não a progressão da doença (Acharya et al., 2008). Esses tratamentos garantem a satisfatória supressão de convulsões em cerca de 60-70% dos pacientes. Em situações nas quais não é possível evitar completamente, busca-se a diminuição na frequência e também da severidade das crises (Stefan & Hopfengärtner, 2009). Recentes descobertas revelando as alterações neurobiológicas que levam a essa patologia levantaram a questão de como esse processo pode ser prevenido ou então modificado de maneira que a epilepsia seja mais branda, mais fácil de tratar, não progressiva e sem prejuízos cognitivos ou ocorrência de resistência a medicamentos (Löscher & Schmidt, 2004). Nas últimas décadas, houve uma considerável evolução nos tratamentos farmacológicos para epilepsia, devido à melhora e à inserção de novos medicamentos anticonvulsivantes (Blanco et al., 2009).

Os fármacos antiepilépticos têm como principais mecanismos de ação (Figura 2) a diminuição dos disparos neuronais repetitivos, através do bloqueio de canais iônicos dependentes de voltagem, a potencialização da neurotransmissão gabaérgica (inibitória) e a diminuição da neurotransmissão excitatória glutamatérgica (Sitges et al., 2007). Entre os principais fármacos antiepilépticos utilizados atualmente, encontram-se a carbamazepina, a fenitoína e a gabapentina.

A carbamazepina é utilizada no tratamento de pacientes que apresentam crises parciais e generalizadas. Esse fármaco também tem sido utilizado nos tratamentos de transtorno bipolar, miotonia, distonia e de diversos tipos de dor

neuropática, como neuralgia do trigêmeo (Rogawski & Löscher, 2004a,b). A carbamazepina atua através da modulação de canais de Na^+ dependentes de voltagem. Esse fármaco reconhece e bloqueia canais de Na^+ abertos, impedindo assim, as descargas neuronais excessivas (Rogawski & Löscher 2004a). Além disso, a carbamazepina pode atuar modulando canais de Ca^{+2} e inibindo a liberação de glutamato (Ambrósio et al., 1999; Sitges et al., 2007).

A fenitoína é um fármaco amplamente utilizado no tratamento da epilepsia, em casos de ocorrência de crises parciais e também generalizadas. Esse fármaco é utilizado também em casos de neuralgia do trigêmeo, miotonia, distonia e mioquimia (Rogawski & Löscher 2004a,b). A fenitoína tem como principal mecanismo de ação a modulação dos canais de Na^+ voltagem-dependentes e, assim como a carbamazepina, reconhece canais de Na^+ abertos e os bloqueia impedindo a propagação de descargas neuronais excessivas (Rogawski & Löscher 2004a,b). Também pode agir através da ativação da liberação de ácido gama-aminobutírico (GABA) e diminuição da liberação de glutamato (Cunningham et al., 2000; Yang et al., 2007).

A gabapentina apresenta propriedades antiepiléptica, analgésica e ansiolítica, sendo empregada em diversos tipos de tratamentos (Dooley et al., 2007). Na epilepsia, é utilizada no controle de crises parciais e generalizadas (Rogawski & Löscher 2004a,b). Apesar de sua eficiência clínica ter sido comprovada, o mecanismo de ação da gabapentina não está elucidado. De um modo geral, esse fármaco atua aumentando a sinalização gabaérgica e modulando canais de Ca^{+2} . Estudos mostram que a gabapentina poderia ligar-se a receptores GABA_B e controlar a síntese, liberação, degradação e recaptação do neurotransmissor GABA (Petroff et al., 2000; Bertrand et al., 2001; Errante et al., 2002; Parker et al., 2004). No entanto, outros estudos mostram que esse fármaco não se liga a receptores GABA e questionam sua interação com outros componentes do sistema gabaérgico (Lanneau et al., 2001; Jensen et al., 2002; Sills, 2006). A atuação da gabapentina através da modulação de canais de Ca^{+2} é melhor esclarecida. Sabe-se a gabapentina liga-se à subunidade $\alpha_2\delta$ de canais de Ca^{2+} pré-sinápticos, inibindo o influxo de Ca^{+2} e restaurando a liberação de neurotransmissores à níveis fisiológicos, através da diminuição de sua liberação excessiva, característica em crises convulsivas (Bayer et al., 2004; Dooley et al., 2007; Rogawski & Bazil, 2008).

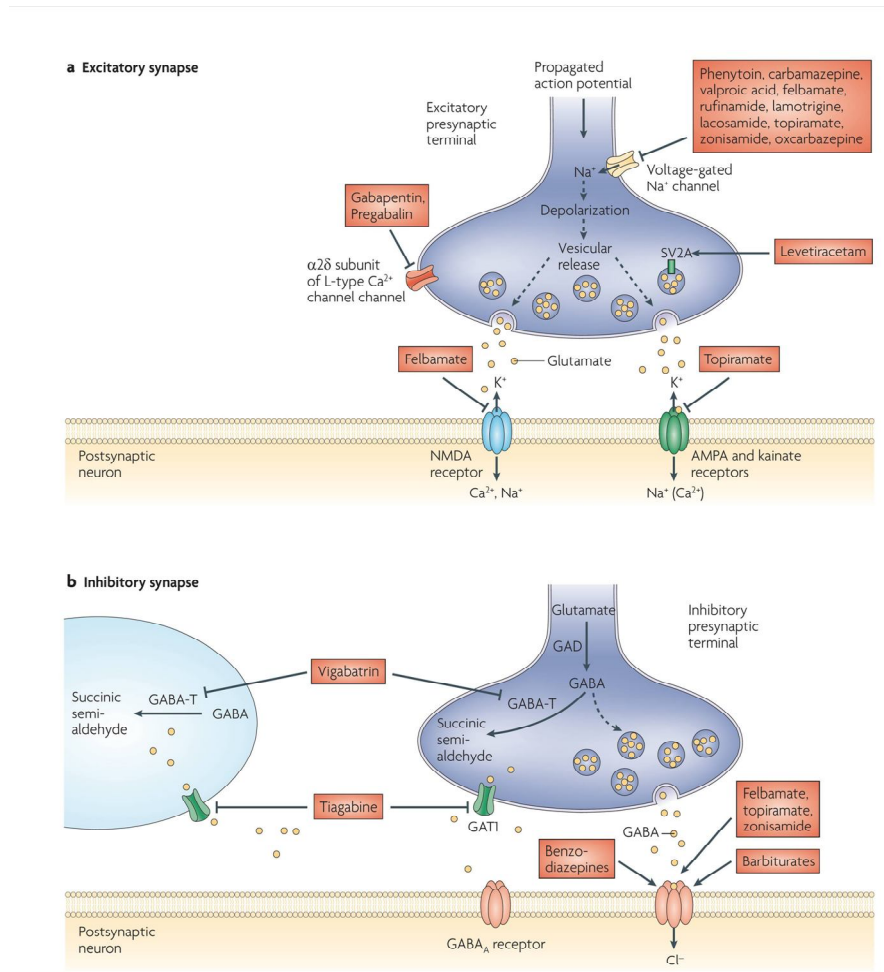


Figura 2: Mecanismo de ação de fármacos antiepilépticos em sinapses excitatórias e inibitórias. Figura obtida de Bialer & White, 2010.

1.4 Sistema purinérgico

1.4.1 ATP

A existência da neurotransmissão purinérgica foi proposta pela primeira vez em 1972 por Geoffrey Burnstock. No sistema purinérgico (Figura 3), os nucleotídeos de adenina são empregados como neurotransmissores, sendo o ATP (adenosina 5'-trifosfato) e a adenosina as principais moléculas sinalizadoras (Burnstock, 1972). Receptores de membrana para adenosina e ATP foram identificados em 1978 e denominados P1 e P2, respectivamente (Burnstock, 1978). O sistema purinérgico constitui uma rota comum de comunicação célula-célula, estando envolvido em

muitos mecanismos, neuronais e não neuronais, como respostas imunes, inflamação, dor, proliferação e morte celular (Abbracchio et al., 2009; Burnstock & Knight, 2004). Os receptores purinérgicos encontram-se amplamente distribuídos por todo o SNC e sistema nervoso periférico (SNP) (Ralevic & Burnstock, 2008).

O ATP é sintetizado e armazenado em terminais pré-sinápticos e liberado na fenda sináptica sob estímulos nervosos, podendo ser co-liberado juntamente com outros neurotransmissores, como ACh, glutamato, noradrenalina e serotonina (Burnstock, 2004; North & Verkhratsky, 2006). Uma vez liberado no espaço extracelular, o ATP pode modular a sinalização purinérgica através da ativação de receptores do tipo P2 (Zimmermann, 2001; Robson et al., 2006).

Os receptores do tipo P2 são divididos em dois grupos, P2X e P2Y, tendo como base seu mecanismo de ação, farmacologia e clonagem molecular (Abbracchio et al., 2009). O grupo P2X é composto por sete (P2X₁₋₇) subtipos de receptores ionotrópicos com permeabilidade rápida e seletiva para cátions (Na⁺, K⁺ e Ca²⁺). Esses receptores encontram-se amplamente distribuídos em neurônios, células gliais e músculo liso e são ativados por ATP (Burnstock, 2004). O grupo P2Y consiste em oito subtipos de receptores metabotrópicos (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ e P2Y₁₄) que podem ser ativados por diversos nucleotídeos: ATP, ADP, UTP e UDP (Abbracchio et al., 2006). Os receptores P2Y estão presentes em vários tecidos e sistemas, como epitelial, muscular, cardíaco, nervoso e imunológico (Burnstock, 2006, 2007). No sistema nervoso, controlam canais de Ca²⁺ e K⁺ e a liberação de outros neurotransmissores (Hussl & Boehm, 2006). Além de atuar em receptores do tipo P2, o ATP liberado em resposta a uma atividade cerebral pode ser convertido em adenosina, através de sua hidrólise pelas ectonucleotidasas (Zimmermann, 2001; Robson et al., 2006).

1.4.2 Adenosina

A adenosina é um neuromodulador endógeno que está envolvido no controle de vários mecanismos do SNC (Boison, 2007). Essa purina não é armazenada em vesículas e não é liberada por exocitose como outros neurotransmissores (Fredholm, 2003). A adenosina pode ser produzida no meio intracelular e transportada para o meio extracelular através de transportadores específicos bidirecionais (que mantém os níveis intracelulares e extracelulares de adenosina em

equilíbrio), pode ser formada a partir da hidrólise do ATP pela cascata enzimática das ectonucleotidases ou ainda a partir da degradação do AMP cíclico (AMPC) (Latini & Pedata, 2001).

A função neuromoduladora da adenosina é exercida através dos receptores purinérgicos do tipo P1, subdivididos em A_1 , A_{2A} , A_{2B} e A_3 . Esses receptores encontram-se acoplados à proteína G e têm sete domínios transmembrana formados por aminoácidos hidrofóbicos (Fredholm et al., 2001).

Os receptores A_1 , que apresentam alta afinidade pela adenosina, são amplamente distribuídos no encéfalo, principalmente no córtex, hipocampo, cerebelo e medula espinhal (Ribeiro et al. 2003). A ativação de receptores A_1 pré-sinápticos diminui o funcionamento das sinapses excitatórias através da inibição da liberação de glutamato. Sua ativação pós-sináptica leva à estabilização do potencial de membrana através do controle do fluxo de Ca^{2+} e K^+ (Boison, 2005; Fredholm et al., 2005).

Os receptores A_2 são subdivididos em A_{2A} e A_{2B} , sendo que os receptores A_{2A} apresentam alta afinidade pela adenosina. A ativação dos purinoreceptores A_2 pode estimular a adenilato ciclase, podendo levar ao aumento da produção de AMPC, aumento no influxo de Ca^{2+} e inibição dos canais de Na^+ (Ribeiro et al., 2003). Os receptores A_{2A} estão localizados em altos níveis no bulbo olfatório e em neurônios gabaérgicos estriato-palidais e em menor nível em outras regiões cerebrais (Ribeiro et al., 2003). Embora em pequena densidade, os receptores A_{2B} são encontrados na maioria dos neurônios e células gliais (Etherington & Frenguelli, 2004; Fredholm et al., 2005).

Os receptores do tipo A_3 , de baixa afinidade pela adenosina, são expressos em neurônios e células gliais e distribuídos por todo o encéfalo, com menor densidade em relação aos outros receptores P1 (Fredholm et al., 2001; Hammarberg et al., 2003; Wittendorp et al., 2004). Estão claramente envolvidos em processos de injúria cerebral, como hipóxia e inflamação (Chen et al., 2006; Lee et al., 2006; Pugliese et al., 2007).

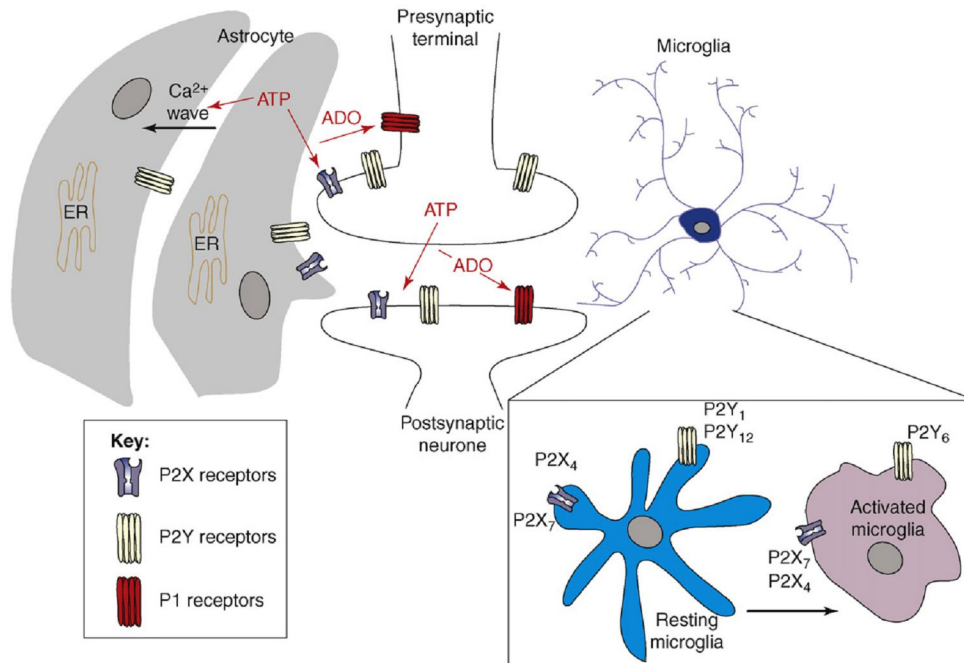


Figura 3: Representação esquemática da sinalização purinérgica no sistema nervoso central. O ATP liberado liga-se a receptores P2 localizados em membranas pós-sinápticas ou pré-sinápticas e astrócitos. A degradação do ATP origina a adenosina, que atua em receptores P1. O ATP também pode ser liberado a partir de astrócitos, onde inicia e propaga o fluxo de Ca^{+2} na sinalização glia-neurônio, que também pode ser controlada pela adenosina. Figura obtida de Abbracchio et al., 2009.

1.4.3 Ectonucleotidases e ADA

A hidrólise dos nucleotídeos extracelulares ocorre por uma cascata de reações que resulta na formação do respectivo nucleosídeo e fosfato livre (Figura 4). As enzimas que catalisam essa reação são as ectonucleotidases, dentre as quais destacam-se as NTPDases (nucleosídeo trifosfato difosfohidrolases) e a 5'-nucleotidase. Esse grupo de enzimas inclui também a família das E-NPP (ectonucleotídeo pirofosfatase/fosfodiesterase) e as fosfatases alcalinas (Zimmermann, 2006). As ectonucleotidases desempenham uma função essencial na neurotransmissão purinérgica, controlando a disponibilidade e os níveis de nucleotídeos e nucleosídeos extracelulares e, conseqüentemente a ativação dos purinoreceptores P2 e P1. A via das ectonucleotidases catalisa a hidrólise de ATP em ADP, AMP e adenosina, que é então hidrolisada pela adenosina deaminase (ADA) (Zimmermann, 2001; Robson et al., 2006).

As NTPDases realizam a hidrólise de nucleotídeos tri e difosfatados (Zimmermann, 2001). Essa família de enzimas é composta por oito membros (NTPDases 1-8) sendo que quatro das NTPDases (1, 2, 3, 8) estão localizadas na superfície das células, com um sítio catalítico extracelular, as NTPDases 5 e 6 apresentam localização intracelular e as NTPDases 4 e 7 estão localizadas no meio intracelular com seus sítios ativos direcionados para o lúmen das organelas citoplasmáticas. As NTPDases compartilham 5 domínios altamente conservados, denominados regiões conservadas de apirase (Zimmermann, 2001; Robson et al., 2006). Em termos de hidrólise de nucleotídeos, a NTPDase 1 hidrolisa ATP e ADP igualmente (Heine et al., 1999). A enzima NTPDase 2 hidrolisa 30 vezes mais ATP do que o ADP. A NTPDase 3 e a NTPDase 8 preferem o ATP em relação ao ADP numa razão de hidrólise de aproximadamente 3:1 e 2:1, respectivamente (Chadwick et al., 1998; Bigonnesse et al., 2004). A NTPDase 4 α tem uma alta preferência por UTP e TTP, enquanto que a NTPDase 4 β apresenta alta preferência por CTP e UDP. A função dessas NTPDases ainda não é clara (Zimmermann, 2001). A NTPDase 5 tem uma preferência na hidrólise de nucleotídeos na seguinte ordem: UDP > GDP = IDP >> ADP = CDP, enquanto que a NTPDase 6 tem a seguinte preferência: GDP > IDP >> UDP = CDP >> ADP. Acredita-se que a NTPDase 5 e a NTPDase 6 participam das reações de glicosilação envolvidas nos processos de dobramento de glicoproteínas (Zimmermann, 2001). A NTPDase 7 prefere nucleosídeos trifosfatados como substratos (Zimmermann, 2001). As NTPDases hidrolisam tanto ATP como ADP, formando AMP na presença de íons Ca⁺² e Mg⁺² (Robson et al., 2006).

A 5'-nucleotidase encontra-se presente na maioria dos tecidos e hidrolisa os nucleotídeos monofosfatados extracelulares AMP, GMP ou UMP, a seus respectivos nucleosídeos. Essa enzima é ancorada à membrana plasmática por um glicosilfosfatidilinositol (GPI) e possui a forma estrutural de dímero com pontes dissulfeto entre as cadeias. O ancoramento por GPI pode ser clivado pela ação de uma fosfolipase C específica, dando origem a formas solúveis desta enzima (Zimmermann, 2000; Hunsucker et al., 2005). A 5'-nucleotidase realiza a hidrólise de AMP em adenosina, tendo como principais funções a formação desta molécula e a regulação dos níveis extracelulares de nucleotídeos e nucleosídeos, permitindo o controle da função dos purinoreceptores (Zimmermann, 2001).

A adenosina extracelular pode ser liberada pelas células ou ser proveniente da hidrólise do ATP. A sua concentração pode ser controlada via transportadores

celulares, sua fosforilação à AMP pela adenosina quinase (AK) ou através de sua desaminação à inosina pela ADA (Figura 4) (Latini & Pedata, 2001). A ADA catalisa a desaminação hidrolítica da adenosina e também da 2'-deoxiadenosina em inosina e 2'-deoxinosina, respectivamente, em nível citosólico e também na superfície celular (Franco et al., 1997).

Existem dois subtipos identificados e bem caracterizados de ADA, denominados ADA1 e ADA2, além de um grupo similar de proteína, denominado ADAL (adenosina deaminase "like"). A ADA1 é uma enzima monomérica encontrada em forma solúvel ou associada a uma proteína (CD26). O complexo ADA1/CD26 constitui uma ecto-ADA, que se encontra ancorada à membrana celular e é responsável pela hidrólise da adenosina extracelular. Além de sua função enzimática, a ADA1 pode interagir com receptores de adenosina do tipo A₁, aumentando sua afinidade de ligação a adenosina e facilitando assim a transdução de sinal através desses receptores (Ciruela et al., 1996). A ADA2 é encontrada principalmente no plasma sanguíneo. Outro tipo de ADA, a ADAL também participa da desaminação da adenosina, porém não está bem caracterizada (Maier et al., 2005).

Estudos do nosso laboratório identificaram a presença e caracterizaram a atividade das enzimas NTPDase, 5'-nucleotidase e ADA em peixe-zebra. As enzimas NTPDase e 5'-nucleotidase foram caracterizadas como cátion-dependentes, apresentando atividade máxima à temperatura de 37 °C, pH ótimo entre 7,2 e 8,0, K_M na faixa do micromolar e uma ampla especificidade por outros nucleotídeos (Rico et al., 2003; Senger et al., 2004). Rosemberg et al. (2010) verificou a presença de diferentes membros da família das NTPDases em peixe zebra. Foi evidenciada a expressão destas enzimas (NTPDases 1, 2, 3, 4, 5, 6, 8) em amostras de cérebro, coração e fígado. As NTPDase1 e NTPDase2 também foram encontradas em fotorreceptores, células horizontais e células ganglionares em retina de peixe zebra (Ricatti et al., 2009). Estudos do nosso laboratório evidenciaram a presença de atividade de desaminação de adenosina em níveis citosólicos e de membrana em diversos tecidos (cérebro, brânquias, coração, fígado, esqueleto, músculo e rim) de peixe zebra. Além disso, foi verificada a expressão de distintos genes relacionados à ADA (ADA1, ADAL e dois ortólogos da ADA2) em tecidos de peixe zebra (Rosemberg et al., 2007,2008).

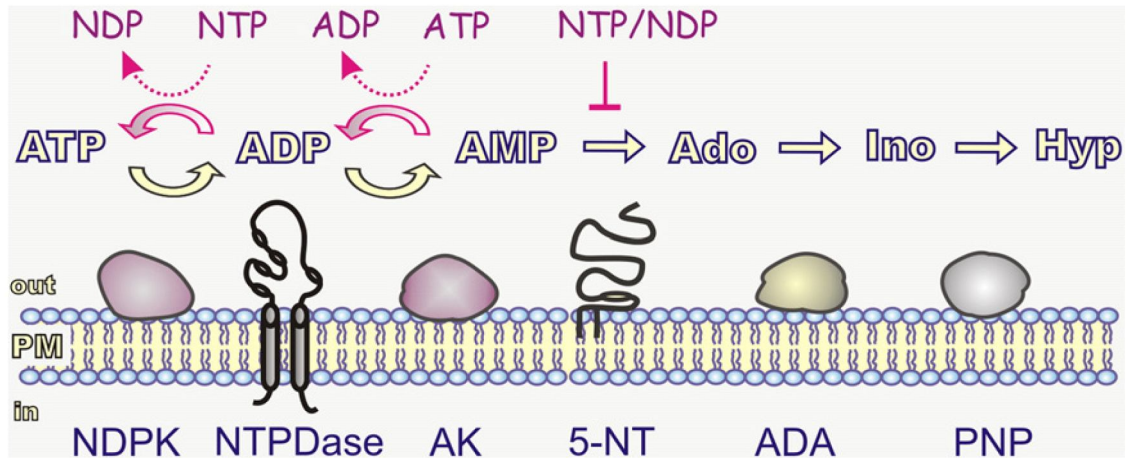


Figura 4: Representação esquemática da degradação extracelular de purinas. As enzimas que atuam na conversão de ATP à adenosina incluem as NTPDases, 5' nucleotidase e ADA. A PNP (purina nucleosídeo fosforilase) hidrolisa a adenosina, produzindo hipoxantina. A AK fosforila a adenosina em AMP. Figura obtida de Yegutkin, 2008.

1.4.4 Sistema purinérgico e epilepsia

Diversos estudos têm demonstrado que a adenosina tem forte ação depressora sobre a transmissão sináptica excitatória, podendo atuar como um importante anticonvulsivante endógeno, principalmente via receptores A_1 (Boison, 2005; Cunha, 2005). A adenosina em níveis aumentados pode contribuir para impedir a propagação da descarga neuronal focal e para o encerramento da crise convulsiva (Berman et al., 2000). Em indivíduos com epilepsia, testes demonstraram concentrações aumentadas de adenosina extracelular durante e após crises convulsivas (During & Spencer, 1992). Em ratos, foram verificados níveis aumentados de adenosina no hipocampo após convulsões induzidas por bicuculina, ácido caínico e PTZ (Berman et al., 2000). Agonistas seletivos de receptores A_1 já foram usados para inibir convulsões em modelos de epilepsia e a inibição da AK também se mostrou uma ferramenta efetiva no controle de convulsões (Huber et al., 2002; Gouder et al., 2004). A inibição da desaminação de adenosina se mostrou uma estratégia de ação anticonvulsivante eficaz em diversos modelos de epilepsia (Dupere et al, 1999; Southam et al, 2002). Porém, a aplicação sistêmica de agonistas A_1 ou drogas que induzem o aumento dos níveis de adenosina podem

implicar em efeitos adversos, como sedação e diminuição na atividade cardíaca (Gouder et al., 2004).

Estudos têm relatado que as crises convulsivas podem alterar a via das ectonucleotidases e ADA. Alterações tardias e prolongadas na hidrólise dos nucleotídeos foram relatadas após a indução de *status epilepticus* por pilocarpina ou cainato e em modelo de *kindling* induzido por PTZ em ratos (Bonan et al., 2000a,b). O tratamento de ratos com pilocarpina induziu o aumento na hidrólise de ATP, ADP e AMP em hipocampo e córtex cerebral de ratos, efeito que foi inibido por tratamentos prévios com fenitoína e carbamazepina (Cognato et al., 2007).

1.5 Sistema Colinérgico

A neurotransmissão colinérgica (Figura 5) está relacionada a diversos mecanismos celulares, como mitose, diferenciação, agregação, migração e secreção (Zimmermann, 2008). Além disso, a neurotransmissão mediada pela ACh é essencial para o correto funcionamento do SNC e SNP, sendo que o comprometimento deste sistema está associado a prejuízos na cognição, aprendizado, memória e funções musculares (Pirondi et al., 2007; Tsai et al., 2007). O sistema colinérgico é verificado também em células não neuronais, como epiteliais, endoteliais e linfócitos (Fujii & Kawashima, 2001; Kirkpatrick et al., 2001).

A ACh é sintetizada a partir dos substratos acetil-coenzima A (acetil-CoA) e colina através de uma reação catalisada pela enzima colina acetiltransferase (ChAT). A colina, importante produto do metabolismo de lipídeos, é captada da corrente sanguínea, e o acetil-CoA é produzido a partir do piruvato, durante o metabolismo mitocondrial. Após sua síntese, que ocorre no citoplasma, a ACh é transportada para o interior de vesículas sinápticas (Zimmermann, 2008). Esse neurotransmissor é liberado em vesículas na fenda sináptica a partir da alteração no potencial elétrico da membrana celular em um processo que depende da concentração de Ca^{2+} intracelular. Em células não neuronais, a ACh também pode ser liberada através de transportadores ativos (Kirkpatrick et al., 2001; Racke et al., 2006; Zimmermann, 2008). Ao ser liberada na fenda sináptica, a ACh pode ligar-se a receptores muscarínicos ou nicotínicos.

Os receptores muscarínicos são metabotrópicos e podem ser ativados pela ACh e muscarina (toxina presente no cogumelo *Amanita muscaria*). Esses

receptores medeiam a ação da ACh no SNC, SNP e em órgãos autonômicos. São encontrados em neurônios, coração, músculo liso e glândulas exócrinas (Sarter & Parikh, 2005). Existem cinco subtipos de receptores muscarínicos (M1-M5) identificados e classificados conforme a proteína G a qual estão acoplados. Os receptores M1, M3 e M5 estão acoplados a uma proteína G_q e alteram a atividade celular pela estimulação da fosfolipase C, e pela geração do segundo mensageiro inositol 1,4,5-trifosfato (IP3), o qual induz a liberação de Ca^{2+} intracelular e diacilglicerol (DAG). Estão localizados principalmente no córtex, hipocampo, estriado e substância negra. Os receptores M2 e M4 estão acoplados a uma proteína G_i que induz sua resposta via inibição da adenilato ciclase. Além do SNC, são encontrados em outros tecidos, onde participam no controle, por exemplo, do tônus das células musculares do coração, pulmões, intestino e bexiga (Nathanson, 2008).

Os receptores nicotínicos são ionotrópicos e sensíveis à ACh e a ligantes exógenos como a nicotina. Tais receptores se localizam no SNC, medula adrenal, nos gânglios autonômicos e na junção neuromuscular (Sarter & Parikh, 2005). Os receptores nicotínicos de células musculares são altamente permeáveis ao Na^+ e K^+ . Os neuronais quando ativados, abrem canais de Ca^{2+} , alterando a polaridade da membrana e influenciando a liberação de neurotransmissores e cascatas de mensageiros intracelulares controlados por Ca^{2+} (Dasgupta & Chellappan, 2006; Gahring & Rogers, 2006).

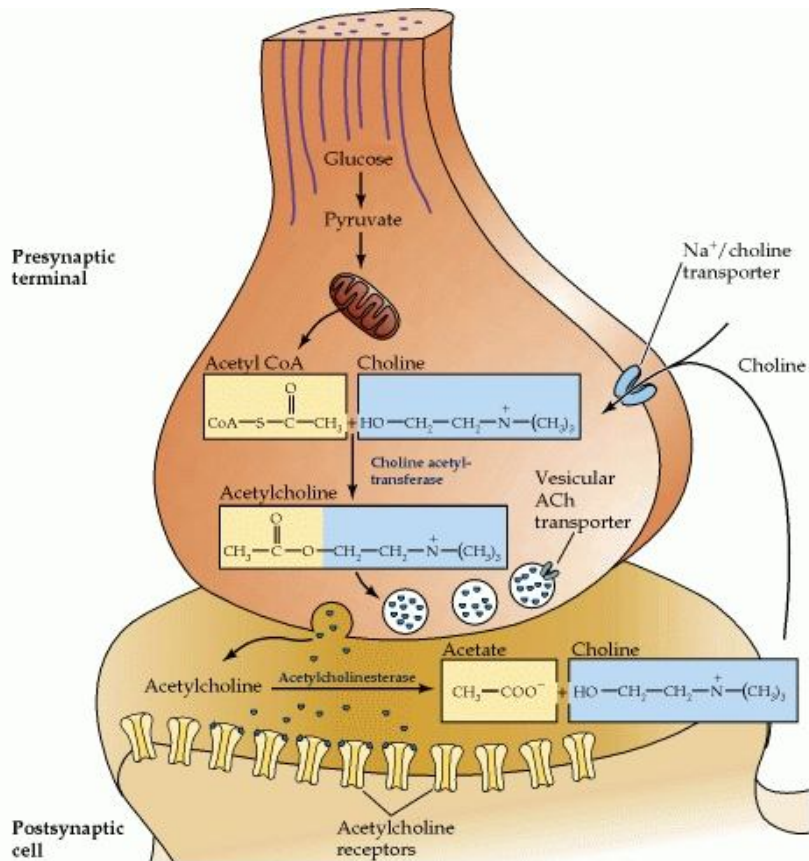


Figura 5: Representação esquemática da sinalização colinérgica. A ACh é sintetizada, pela ChAT, a partir dos substratos acetil-CoA e colina. O acetil-CoA provém do piruvato produzido no metabolismo mitocondrial e a colina é transportada para o interior celular através de transportadores dependentes de Na⁺. Após sua liberação, a ACh é hidrolisada em acetato e colina, sendo a colina recaptada pelo terminal pré-sináptico. Figura obtida de Neuroscience, 2nd edition, 2001.

1.5.1 Acetilcolinesterase

A ação sináptica da ACh é encerrada fundamentalmente por sua hidrólise e as colinesterases garantem a rápida regulação dos níveis desse neurotransmissor na fenda sináptica. Após sua liberação dos receptores, a ACh é degradada pelas colinesterases em acetato e colina. Transportadores recaptam para o terminal pré-sináptico a colina resultante da hidrólise (Soreq & Seidman, 2001).

As colinesterases são encontradas em tecidos neuronais e não neuronais. Diferenciadas por suas propriedades catalíticas e especificidade de inibidores, essas enzimas são divididas em dois tipos: acetilcolinesterase (E.C.3.1.1.7, AChE) e butirilcolinesterase (E.C. 3.1.1.8, BuChE). A AChE hidrolisa preferencialmente

ésteres como grupamento acetil e é encontrada no sistema nervoso e em junções neuromusculares. A BuChe é menos específica e hidrolisa outros tipos de ésteres, como a butirilcolina (Zimmermann, 2008). As colinesterases são serina hidrolases e seus sítios ativos são compostos por uma tríade catalítica que contém resíduos de aminoácidos de serina, histidina e de um grupo ácido (glutamato). O mecanismo de hidrólise da AChE envolve o ataque nucleofílico da serina ao carbono carbonílico da ACh, gerando um intermediário tetraédrico estabilizado por ligações de hidrogênio, o qual produz colina livre e serina acetilada. Ao final, a hidrólise do grupo acetil da serina recupera o sítio catalítico da enzima (Soreq & Seidman, 2001).

A AChE pode ter forma estrutural globular ou assimétrica (Figura 6). A conformação globular pode ser encontrada solúvel na célula e é composta por monômeros (G1), dímeros (G2) e tetrâmeros (G4) da subunidade catalítica. A unidade G1 está ligada ao citosol, enquanto que a G4 à membrana da célula. As formas heteroméricas da AChE estão associadas à lâmina basal externa por proteínas que determinam sua localização sináptica. Os tipos assimétricos consistem de um (A4), dois (A8) e três (A12) tetrâmeros catalíticos ligados ao colágeno Q (ColQ) que é predominante nas junções musculares. O tetrâmero ligado à âncora de membrana rica em prolina (PRIMA) é abundante nas sinapses cerebrais (Figura 6) (Zimmerman & Soreq, 2006).

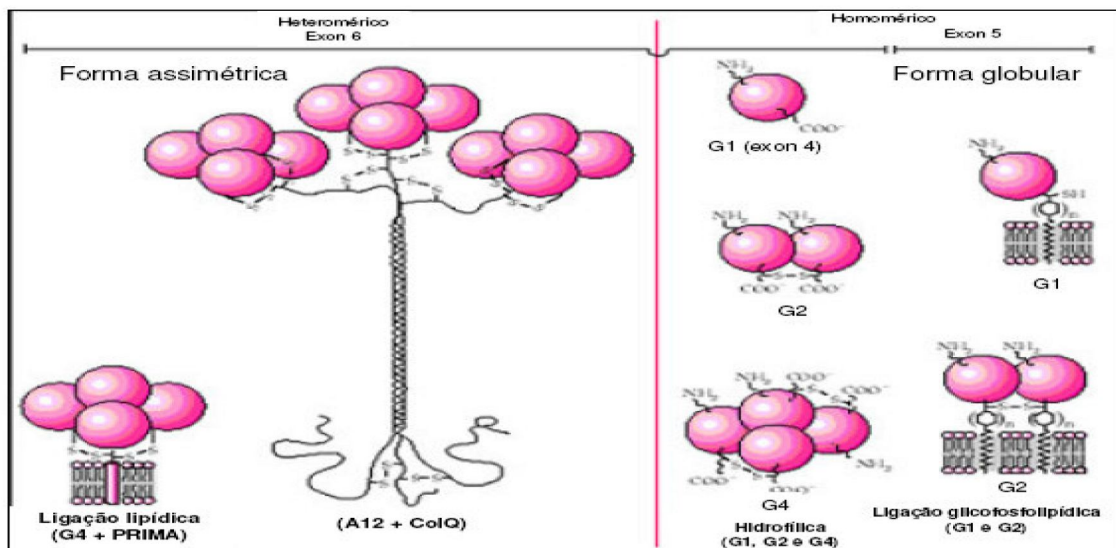


Figura 6: Estruturas moleculares da AChE. Estruturas globulares e assimétricas. Figura adaptada de Feldman & Quenzer, 1984.

1.5.2 Sistema colinérgico e epilepsia

O aumento na neurotransmissão colinérgica, principalmente via receptores muscarínicos está fortemente relacionado à ocorrência de crises convulsivas e epileptogênese (Hamilton et al., 1997; Friedman et al., 2007). Estudos mostram que a liberação de ACh e a ativação de receptores muscarínicos potencializa a neurotransmissão excitatória glutamatérgica, um possível mecanismo envolvido na indução de crises convulsivas através do sistema colinérgico (Marino et al., 1998; Kozhemyakin et al., 2010). Aumentos focais na neurotransmissão colinérgica foram suficientes para provocar epilepsia em camundongos (Pernot et al., 2009). A ativação de receptores muscarínicos, a partir da inibição da AChE e conseqüente elevação nos níveis de ACh, levou ao aumento da transmissão glutamatérgica em células granulares hipocâmpais (Kozhemyakin et al., 2010). A estimulação de receptores nicotínicos também induziu o aumento da liberação de glutamato em células hipocâmpais (Alkondon & Albuquerque 2004). Potier & Psarropoulou (2001) demonstraram que níveis aumentados de ACh podem agravar o processo de epileptogênese em neocórtex de ratos imaturos. A estimulação de receptores muscarínicos é uma metodologia frequentemente aplicada na indução de crises convulsivas em modelos animais. Tratamentos com pilocarpina e outros agonistas muscarínicos ou inibidores da AChE podem induzir convulsões, *status epilepticus* e epileptogênese, que leva à ocorrência de crises espontâneas após um período de latência (Friedman et al., 2007).

2 OBJETIVOS

2.1 Objetivo geral

Considerando que: (1) os sistemas purinérgico e colinérgico têm importante participação na sinalização celular no SNC, (2) essas vias de sinalização podem estar envolvidas no mecanismo da epilepsia e (3) os sistemas purinérgico e colinérgico estão identificados e caracterizados em peixe zebra, o objetivo geral deste estudo foi avaliar o efeito de crises convulsivas e fármacos antiepilépticos sobre as ectonucleotidasas, ADA e AChE, enzimas que têm fundamental participação na modulação dos sistemas purinérgico e colinérgico.

2.2 Objetivos específicos

2.2.1 Verificar o efeito *in vitro* de fármacos antiepilépticos, tais como fenitoína, carbamazepina e gabapentina, sobre a atividade enzimática das ectonucleotidasas e da AChE em SNC do peixe zebra;

2.2.2 Avaliar o efeito *in vitro* da exposição ao PTZ sobre a atividade enzimática das ectonucleotidasas e da ADA em SNC do peixe zebra;

2.2.3 Investigar possíveis alterações sobre a expressão e atividade das ectonucleotidasas e da ADA em membranas cerebrais de peixe zebra submetidos ao modelo de crise convulsiva induzido por PTZ.

CAPÍTULO 2

ARTIGO CIENTÍFICO

In vitro effects of antiepileptic drugs on acetylcholinesterase and
ectonucleotidase activities in zebrafish (*Danio rerio*) brain

Anna M. Siebel, Eduardo P. Rico, Katiucia M. Capiotti, Ângelo L. Piato, Catiane T.
Cusinato, Tathyana A. M. Franco, Maurício R. Bogo, Carla D. Bonan

Artigo publicado no periódico Toxicology in Vitro (FI 2.06)
(doi:10.1016/j.tiv.2010.03.018)



Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

In vitro effects of antiepileptic drugs on acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain

A.M. Siebel^{a,b}, E.P. Rico^c, K.M. Capiotti^{a,b}, A.L. Piato^{a,b}, C.T. Cusinato^a, T.M.A. Franco^a, M.R. Bogo^{b,d}, C.D. Bonan^{a,b,*}

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

^bInstituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-IM), 90035-003 Porto Alegre, RS, Brazil

^cDepartamento de Bioquímica, Programa de Pós-Graduação em 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

^dLaboratório de Biologia Celular e Molecular, Departamento de Biologia Celular e Molecular, Programa de Pós-Graduação em Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:

Received 18 January 2010

Accepted 28 March 2010

Available online 31 March 2010

Keywords:

Acetylcholinesterase
Antiepileptic drugs
Nucleoside triphosphate
diphosphohydrolase
Ecto-5'-nucleotidase
Zebrafish

ABSTRACT

Carbamazepine (CBZ), phenytoin (PHT), and gabapentine (GBP) are classical antiepileptic drugs (AEDs) that act through a variety of mechanisms. We have tested the *in vitro* effects of CBZ, PHT, and GBP at different concentrations on ectonucleotidase and acetylcholinesterase activities in zebrafish brain. CBZ inhibited ATP hydrolysis at 1000 μ M (32%) whereas acetylcholine hydrolysis decreased at 500 μ M (25.2%) and 1000 μ M (38.7%). PHT increased AMP hydrolysis both at 500 μ M (65%) and 1000 μ M (64.8%). GBP did not promote any significant changes on ectonucleotidase and acetylcholinesterase activities. These results have shown that CBZ can reduce NTPDase (nucleoside triphosphate diphosphohydrolase) and PHT enhance ecto 5'-nucleotidase activities. Therefore, it is possible to suggest that the AEDs induced-effects on ectonucleotidases are related to enzyme anchorage form. Our findings have also shown that high CBZ concentrations inhibit acetylcholinesterase activity, which can induce an increase of acetylcholine levels. Taken together, these results showed a complex interaction among AEDs, purinergic, and cholinergic systems, providing a better understanding of the AEDs pharmacodynamics.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Epilepsy, a neurological disorder characterized by the occurrence of spontaneous recurrent seizures, is one of the most common pathologies of the central nervous system (CNS), affecting individuals of all ages (Badawy et al., 2009; Banerjee et al., 2009). Epilepsy damages the brain as a whole showing significant influence on dynamic and functional properties, mental development, cognition, and behavior (Hamed, 2009). Classical AEDs such as GBP, PHT, and CBZ work through a variety of mechanisms, often

acting to suppress ion channels, promote gabaergic neurotransmission, and/or decrease glutamatergic neurotransmission. AEDs may be used for the treatment of several types of epilepsy and GBP, PHT and CBZ are treatments for partial and generalized tonic-clonic seizures (Rogawski and Löscher, 2004). Studies have suggested that AEDs can interfere in the purinergic (Borowicz et al., 1997, 2002) and cholinergic systems in CNS (Boccia et al., 2001; D'Antuono et al., 2007).

The purinergic system employs extracellular nucleotides as signaling molecules. ATP is a neurotransmitter co-released with other signaling molecules, such as glutamate, GABA, and acetylcholine in different subpopulations of neurons in CNS. ATP acts through activation of G-protein-coupled P2Y receptors and P2X ionotropic receptors, linked to Ca²⁺ channels (Burnstock, 2004, 2009; Pankratov et al., 2009). Extracellular nucleotides can be hydrolyzed by a variety of soluble or cell-surface-located enzymes named ectonucleotidases (Zimmermann et al., 1998). Nucleoside 5'-tri- and diphosphates may be hydrolyzed by members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase), ectonucleoside pyrophosphatase/phosphodiesterase (E-NPP) and alkaline

Abbreviations: AChE, acetylcholinesterase; AEDs, antiepileptic drugs; BuChE, butyrylcholinesterase; CBZ, carbamazepine; CNS, central nervous system; GBP, gabapentine; NTPDase, nucleoside triphosphate diphosphohydrolase; PHT, phenytoin.

* Corresponding author at: Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Programa de Pós-Graduação em Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil. Tel.: +55 51 3353 4158; fax: +55 51 3320 3568.

E-mail address: cbonan@puccs.br (C.D. Bonan).

phosphatase protein families. AMP may be hydrolyzed by the ecto-5'-nucleotidase family and alkaline phosphatases to produce the nucleoside adenosine (for review see Abbracchio et al. (2009)). In this way, ectonucleotidases control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors and, consequently, the extent and duration of receptor activation (Chen and Guidotti, 2001). Therefore, this is an enzymatic pathway with double function of removing one signaling molecule, ATP, and generating a second one, adenosine, a well-known neuromodulator (Zimmermann, 2006; Abbracchio et al., 2009; Burnstock and Verkhratsky, 2009). Adenosine acts through four known subtypes of P1 metabotropic receptors (A_1 , A_{2A} , A_{2B} and A_3) and reduces excessive neuronal activity through inhibitory A_1 receptors, acting as an endogenous anticonvulsant (Fredholm et al., 2001; Sebastião and Ribeiro, 2009). Adenosine modulates the acetylcholine release through A_1 receptor-mediated inhibition or by A_{2A} receptor-mediated facilitation of release (Cunha, 2001).

In cholinergic neurotransmission, acetylcholine is synthesized by cholineacetyltransferase in the presynaptic neuron, then released into the synaptic cleft and bound to muscarinic (metabotropic) and nicotinic (ionotropic) acetylcholine receptors. After its release, acetylcholine is degraded by the cholinesterases that cleave acetylcholine into choline and acetate. Two different types of cholinesterases are able to hydrolyze acetylcholine: acetylcholinesterase (AChE) (E.C.3.1.1.7) and butyrylcholinesterase (BuChE) (E.C.3.1.1.8) (Soreq and Seidman, 2001). Cholinergic mechanisms, in particular those related to the activation of muscarinic receptors, regulate brain excitability and can promote and maintain synchronous epileptiform discharges (Liu et al., 1994; Nagao et al., 1996; Dickson and Alonso, 1997). Moreover, cholinergic agents, such as pilocarpine, produce limbic seizures that are followed by histopathological changes resembling that encountered in temporal lobe epileptic patients (Turski et al., 1989; Mello et al., 1993).

PHT and CBZ blocked the pilocarpine-induced increase on ATP, ADP, and AMP hydrolysis in synaptosomes from hippocampus and cerebral cortex in rats (Cognato et al., 2007). Moreover, CBZ inhibited *in vitro* ATP hydrolysis from synaptosomal plasma membranes (Horvat et al., 2006). GBP and other AEDs, when administered together with adenosine receptor antagonists, showed decreased effect in mice models of seizure (Zuchora et al., 2004). In rats, the supraeffective dose of CBZ (100 mg/kg) inhibited the activities of acetylcholinesterase (9.5%) and butyrylcholinesterase (24.7%) whereas the effective dose of CBZ (25 mg/kg) did not alter acetylcholine degradation (Mizuno et al., 2000). Moreover, Sudha et al. (1995) showed that PHT decreased acetylcholinesterase activity in the hippocampus (50 and 75 mg/kg) and in the striatum (75 mg/kg).

Zebrafish is a small freshwater teleost fish that has been used to study learning and memory process, development, pharmacology, toxicology, behavior, and teratology (Kosmehl et al., 2008; Bencan et al., 2009; Gerlai et al., 2009; Ingham, 2009; Yang et al., 2009). This specie has been used as a tool for the study of seizure and to screen potential novel AEDs (Baraban et al., 2005; Baraban, 2007; Berghmans et al., 2007). NTPDase and ecto-5'-nucleotidase activities have been characterized in zebrafish in our laboratory (Rico et al., 2003; Senger et al., 2004). Acetylcholinesterase is encoded by a single gene and butyrylcholinesterase was not found in zebrafish genome (Clemente et al., 2004). Other drugs used for neurological disorder treatments were tested *in vitro* (haloperidol, olanzapine, and sulpiride) and inhibited NTPDase and acetylcholinesterase activities whereas did not change ecto-5'-nucleotidase activity in zebrafish brain (Seibt et al., 2009a,b). However, haloperidol significantly increased the acetylcholinesterase activity after *in vivo* treatments (Seibt et al., 2009b).

Considering that zebrafish may be a model organism to study human diseases and drug mechanisms, and purinergic and cholin-

ergic systems have been described in this specie, the aim of this study was to evaluate the *in vitro* effects of different concentrations of the antiepileptic drugs GBP, PHT, and CBZ on ectonucleotidase and acetylcholinesterase activities of zebrafish brain.

2. Materials and methods

2.1. Animals

Adult wild-type zebrafish (*Danio rerio*) of both sexes were obtained from a commercial supplier (Red Fish, RS, Brazil) and acclimated for 2 weeks before the experiments in a 50 l thermostated aquarium filled with continuously aerated and unchlorinated water. The fish were conditioned at $26 \pm 2^\circ\text{C}$ under a 14–10 h light/dark cycle photoperiod. The animals were maintained healthy and free of any signs of disease and fed twice a day with commercial food for fish. The use and maintenance of zebrafish were according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. The protocol was approved by the Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCKS) under the number 085/06-CEP.

2.2. Chemicals

PHT, GBP, CBZ, acetylthiocholine, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Trizma Base, EDTA, EGTA, sodium citrate, Coomassie blue, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, calcium, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were from analytical grade.

2.3. *In vitro* treatments

PHT, GBP, and CBZ were tested at 10, 50, 100, 500, and 1000 μM . PHT and GBP were diluted in deionized water. CBZ was diluted in 2% ethanol. Control treatments with equal volume of vehicle were performed to exclude the ethanol effect on the enzyme activities. Antiepileptic drugs were added to the reaction medium before the preincubation with the enzyme and were maintained during the enzyme assays.

2.4. Determination of ectonucleotidase activities

The brain membranes for the ectonucleotidase assays were prepared as described previously by Barnes et al. (1993). For each membrane preparation, a pool of five whole brains was obtained and briefly homogenized in 60 vol. (v/w) of chilled Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4) in a glass-Teflon homogenizer. This homogenate was centrifuged at 1000g for 10 min and the pellet was discarded in order to remove cell debris. The supernatant was centrifuged for 25 min at 40,000g. The resultant pellet was frozen in liquid nitrogen and thawed in order to ensure the lysis of the brain membranes. The pellet was resuspended in Tris–citrate buffer and centrifuged for 20 min at 40,000g. The final pellet was resuspended and used in the enzyme assays. The samples were maintained at $2-4^\circ\text{C}$ throughout preparation.

NTPDase and 5'-nucleotidase assays were performed as described previously (Rico et al., 2003; Senger et al., 2004). Brain membranes of zebrafish (3 μg protein for NTPDase and 5 μg protein for 5'-nucleotidase) were added to the reaction medium containing 50 mM Tris–HCl (pH 8.0) and 5 mM CaCl_2 (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM MgCl_2 (for the 5'-nucleotidase activity) at a total volume of 200 μl . The samples were preincubated for 10 min at 37°C and the reaction

was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. After 30 min the reaction was stopped by the addition of 200 μ l 10% trichloroacetic acid and the samples were kept on ice during 10 min. In order to determine the inorganic phosphate released (Pi) 1 ml of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added to the samples for 20 min (Chan et al., 1986). The quantification of inorganic phosphate (Pi) released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nanomole of Pi released per minute per milligram of protein. In order to correct non-enzymatic hydrolysis of the substrates we used controls with the addition of the enzyme preparation after the addition of trichloroacetic acid. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. All enzyme assays were performed in at least four different experiments, each one performed in triplicate.

2.5. Determination of acetylcholinesterase activity

Zebrafish were euthanized and their whole brains were removed by dissection. The brains (five whole brains for each sample) were homogenized on ice in 60 vol. (v/w) of 50 mM Tris-HCl, pH 8.0, in a glass-Teflon homogenizer. Acetylcholinesterase activity was measured as the method described previously (Ellman et al., 1961) determining the rate of hydrolysis of acetylthiocholine (ACSch, 0.8 mM) in 2 ml assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB. Samples containing protein (10 μ g) and the reaction medium described above were preincubated during 10 min at 25 °C followed by starting of reaction with addition of substrate. The hydrolysis of substrate was monitored by the formation of thiolate dianion of DTNB at 412 nm every 30 s for 2–3 min. The linearity of absorbance towards time and protein concentration was previously determined. Acetylcholinesterase activity was expressed as micromole of thiocholine (Sch) released per hour per milligram of protein. All enzyme assays were performed in at least four different experiments, each one performed in triplicate.

2.6. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976) and bovine serum albumin was used as standard.

2.7. Statistical analysis

Results are expressed as means \pm S.D. Data were analyzed by one-way ANOVA followed by Duncan post-hoc test, considering $P < 0.05$ as significant. SPSS 16.0 was used for statistical analysis.

3. Results

The *in vitro* effect of GBP, PHT, and CBZ (at concentrations ranging from 10 to 1000 μ M) was tested on acetylcholinesterase, NTP-Dase, and ecto-5'-nucleotidase activities in zebrafish brain. CBZ significantly decreased (32%; $P < 0.05$) ATP hydrolysis at 1000 μ M (285 ± 63.06 nmol Pi min⁻¹ mg⁻¹ of protein) when compared to the ethanol group (419.54 ± 111.17 nmol Pi min⁻¹ mg⁻¹ of protein) (Fig. 1) whereas this drug did not alter both ADP and AMP hydrolysis (data not shown). The results demonstrated that CBZ inhibited (25.2% and 38.7%, respectively; $P < 0.05$) acetylcholinesterase activity at 500 μ M (24.13 ± 5.35 μ mol Sch h⁻¹ mg⁻¹ of protein) and at 1000 μ M (19.75 ± 3.76 μ mol Sch h⁻¹ mg⁻¹ of protein) when compared to the ethanol group (32.25 ± 6.49 μ mol Sch h⁻¹ mg⁻¹ of protein; Fig. 2).

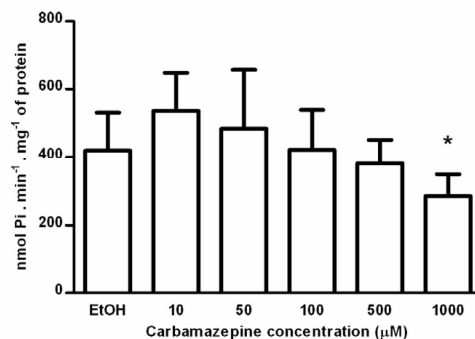


Fig. 1. *In vitro* effect of carbamazepine on ATP hydrolysis evaluated in different concentrations (10–1000 μ M). Bars represent the mean \pm S.D. The symbol (*) indicates significantly difference when compared to the ethanol group. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein.

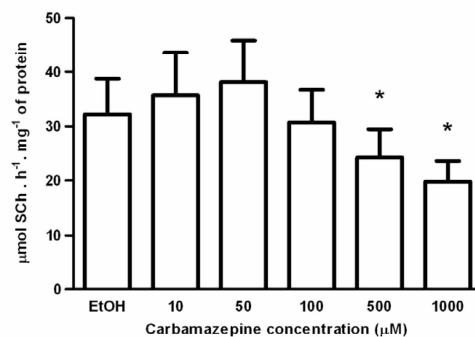


Fig. 2. *In vitro* effect of carbamazepine on acetylcholine hydrolysis evaluated in different concentrations (10–1000 μ M). Bars represent the mean \pm S.D. The symbol (*) indicates significantly difference when compared to the ethanol group. The specific enzyme activity is reported as micromole of thiocholine released per hour per milligram of protein.

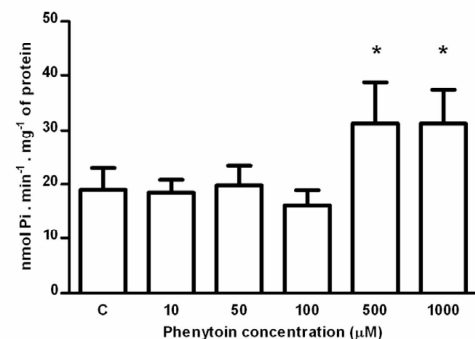


Fig. 3. *In vitro* effect of phenytoin on AMP hydrolysis evaluated in different concentrations (10–1000 μ M). Bars represent the mean \pm S.D. The symbol (*) indicates significantly difference when compared to the control group. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein.

PHT significantly increased (65%; $P < 0.05$ and 64.8%; $P < 0.05$) AMP hydrolysis at 500 μM ($31.31 \pm 7.42 \text{ nmol Pi min}^{-1} \text{ mg}^{-1}$ of protein) and 1000 μM ($31.28 \pm 6.14 \text{ nmol Pi min}^{-1} \text{ mg}^{-1}$ of protein) when compared to the control group ($18.98 \pm 3.98 \text{ nmol Pi min}^{-1} \text{ mg}^{-1}$ of protein; Fig. 3). However, PHT did not change ATP, ADP, and acetylcholine hydrolysis (data not shown). Similarly, GBP did not alter ATP, ADP, AMP, and acetylcholine hydrolysis (data not shown).

4. Discussion

The interactions of AEDs and ectonucleotidases have been investigated and these drugs have demonstrated different effects in several studies. Cognato et al. (2007) observed the *in vitro* and *in vivo* effects of PHT, valproate, and CBZ on ectonucleotidase activities in synaptosomes from hippocampus and cerebral cortex of rats. These results have shown that these drugs did not alter the ectonucleotidase activities both *in vitro* and *in vivo* experiments in hippocampus and cerebral cortex. However, the authors observed that previous treatment with CBZ and PHT can modulate plastic events associated to the nucleotidase activities preventing the pilocarpine-induced increase on ATP, ADP, and AMP hydrolysis (Cognato et al., 2007). Horvat et al. (2006) observed ecto-ATPase inhibition in rat brain synaptosomal plasma membranes treated with CBZ. This effect may represent a potential neuromodulatory action of this drug given that by inhibiting ecto-ATPase activities, CBZ may increase the extracellular ATP content and decrease the production of adenosine.

Ectonucleotidases modulate activation of P2 or P1 receptors by controlling extracellular concentrations of ATP and adenosine. NTPDase family, which hydrolyzes both nucleoside triphosphates and diphosphates, comprises cell-surface-enzymes with an extracellular active site, anchored to the membrane via two transmembrane domains that are essential to catalytic activity and substrate specificity (Grinthal and Guidotti, 2006; Robson et al., 2006). The ectonucleotidase chain is also constituted by ecto-5'-nucleotidase, which is able to promote the hydrolysis of nucleoside monophosphates to adenosine. Unlike NTPDases, ecto-5'-nucleotidase is attached to the extracellular membrane by a glycosyl phosphatidylinositol (GPI) anchor (Sträter, 2006).

Changes in membrane constituents can affect membrane-bound enzymes activity. Cholesterol depletion from membranes of NTPDase1-expressing cells reduces ATPase activity to the same extent as solubilization does (Papanikolaou et al., 2005). Intra and intermolecular oxidative cross-linking decreases ATPase activity (Chiang and Knowles, 2008). These results showed that changes at the balance between stability and mobility of the transmembrane domains can alter NTPDase activity (Grinthal and Guidotti, 2006).

Our findings demonstrated that CBZ decreased NTPDase whereas did not change ecto-5'-nucleotidase activities. A study with human erythrocyte have shown that CBZ perturbed outer moiety lipids inducing a disordering effect on the polar head groups and acyl chains of the membrane lipid showing that CBZ can lead interactions in membrane bilayer (Suwalsky et al., 2006). Therefore, it is possible to suggest that the effects on NTPDase activities induced by CBZ may be due to changes promoted in the bilayer membrane. Previous studies from our laboratory have shown that other drug classes, such as antidepressant (Pedrazza et al., 2007) and antipsychotic drugs (Seibt et al., 2009a) also promoted different effects on NTPDase when compared with ecto-5'-nucleotidase activities, probably by modifying plasma membranes.

PHT did not change NTPDase while significant increase of ecto-5'-nucleotidase activity was observed. Ecto-5'-nucleotidase has

several functions, including generation of adenosine leading to the activation of adenosine receptors (Hunsucker et al., 2005). This enzymatic effect may represent further ways by PHT modulate the neuronal activity. Adenosine is known to be very effective in the suppression of seizures. Binding of adenosine to the high affinity A_1 receptor reduces excitability of the cells by modulation of Ca^{2+} and K^+ fluxes and inhibits the release of various neurotransmitters, such as glutamate, dopamine, serotonin, and acetylcholine (Boison, 2005, 2008).

Our results showed that CBZ and PHT can increase ATP and adenosine levels, respectively. It is possible to suggest that these effects are involved, at least partially, in the antiepileptic mechanisms of these AEDs. Although it has been shown that adenosine inhibits neurotransmission (Dunwiddie and Masino, 2001; Fredholm et al., 2005; Boison, 2008), some studies indicate that ATP can also inhibit synaptic transmission (Yoshioka and Nakata, 2004; Nakata et al., 2005). The colocalization of A_1 and $P2Y_1$ receptors in several regions in the brain suggests a potential heterodimerization and functional interaction mechanism between these receptors. $A_1/P2Y_1$ heterodimerization forms an ATP-sensitive adenosine receptor and ATP can work as an A_1 agonist to inhibit neurotransmission (Yoshioka and Nakata, 2004; Nakata et al., 2005). Furthermore, a cross-talk of A_1 - $P2Y_1$ receptors might be a mechanism by which low and high concentrations of adenosine or purines could regulate glutamate release (Tonazzini et al., 2007).

Acetylcholine is a neurotransmitter involved in essential brain functions, including memory and learning (Shaked et al., 2008). Acetylcholinesterase, the key enzyme that hydrolyzes and inactivates acetylcholine, modulates also non-cholinergic functions, such as glutamatergic and dopaminergic systems (Soreq and Seidman, 2001; Zimmermann and Soreq, 2006; Shaked et al., 2008). Several studies have shown the cholinergic system is also involved in the mechanisms related to epilepsy and acetylcholinesterase inhibitors lead to an increase in acetylcholine levels inducing seizures (Friedman et al., 2007; Pernot et al., 2009). Different studies have demonstrated the effect of AEDs on acetylcholinesterase activity and acetylcholine levels. Previous studies showed that cortical injury and enhanced neural excitability are associated with modifications in the isoform of acetylcholinesterase. The variation in distribution patterns suggested a possible isoform shift of acetylcholinesterase enzyme in epileptic brains from membrane adhered AChE-S protein, located in cellular layers, to the soluble AChE-R monomers, which may diffuse to extrasynaptic regions. The results suggested an increased production of AChE-R monomers, which may access the synaptic microenvironment and potentially protect the epileptic tissue from cholinergic hyperexcitation (Zimmermann et al., 2008). In another study with rats, chronic treatment with PHT (ranging from 5 to 75 mg/kg) did not modify significantly the acetylcholinesterase activity in the motor cortex, pyriform cortex, and olfactory bulb when compared to control group. On the other hand, PHT decreased acetylcholinesterase activity in the hippocampus (50 and 75 mg/kg) and also in the striatum (75 mg/kg) (Sudha et al., 1995). Likewise, in rats, acute treatment with effective dose of CBZ (25 mg/kg) enhances acetylcholine synthesis without affecting the degradation, increasing acetylcholine levels. The supraeffective tested dose of CBZ (100 mg/kg) decreased the activity of acetylcholinesterase and butyrylcholinesterase and also inhibited acetylcholine synthesis, reducing the levels of this neurotransmitter. Therefore, effective dose of CBZ increased acetylcholine levels, which were reduced by increasing of CBZ dose (Mizuno et al., 2000). It also has been reported that CBZ had biphasic effects on acetylcholine release and synthesis (Zhu et al., 2002).

Previous studies have shown the IC_{50} of CBZ for acetylcholinesterase and butyrylcholinesterase activities were more than 300 μM (Mizuno et al., 2000). The authors suggest that the inhibitory ef-

ffects of supraeffective CBZ concentrations on cholinesterase activities may not play a role in acetylcholine transmission (Mizuno et al., 2000). In contrast, both toxic concentration of acetylcholine receptor agonists, antagonists and cholinesterase inhibitor have been shown to have proconvulsant activities, whereas low doses of cholinesterase inhibitor reduced seizure activities (Bhattacharya et al., 1991; Cruickshank et al., 1994). This evidence supports the hypothesis that drastic elevation of cholinergic function induces seizure activity. The therapeutic ranges of serum concentration of CBZ are from 15 to 45 μM (Loiseau and Duché, 1995). Our results showed an inhibitory effect on acetylcholinesterase only at high CBZ concentrations (500 and 1000 μM). Therefore, the inhibitory effect on acetylcholinesterase at these high doses of CBZ could promote an increase of acetylcholine levels, which could induce a seizure activity or a neurotoxic effect promoted by CBZ high concentrations. Here, the CBZ biphasic profile also was observed in purinergic system. Our results demonstrated a trend to increase ATP hydrolysis in the presence of low doses of CBZ (10 and 50 μM) and a decreased hydrolysis of this neurotransmitter in high CBZ dose (1000 μM). Such results demonstrate that CBZ induces a complex set of effects characterized by a biphasic profile. Additional studies are required to verify the *in vivo* effect of the high CBZ doses in the seizure activity and purinergic and cholinergic transmission.

These findings indicate that AEDs could show different pharmacokinetic profile depending on relevant end points, such as route of administration, dose of the drug tested and animal model used in the study. However, there are some limitations about the side effects and resistance developed of AEDs. The discovery and screening of new molecular targets and AEDs may increase the treatment spectrum, which will be reached with pharmacology studies focusing to this issue (Stefan and Feuerstein, 2007; Bialer and White, 2010; Luna-Tortós et al., 2010). In summary, this study highlight that enzymes related to purinergic and cholinergic systems can be modulated by antiepileptic drugs in zebrafish. Furthermore, these findings can contribute to a better understanding about the pharmacology of classical AEDs and their interaction with purinergic and cholinergic neurotransmission.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and by the FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" #01.06.0842-00. A.M.S., A.L.P., and E.P.R. were recipient of fellowship from CNPq.

References

- Abbraccio, M.P., Burnstock, G., Verkhatsky, A., Zimmermann, H., 2009. Purinergic signalling in the nervous system: an overview. *Trends in Neurosciences* 32 (1), 19–29.
- Badawy, R.A.B., Harvey, A.S., Macdonell, R.A.L., 2009. Cortical hyperexcitability and epileptogenesis: understanding the mechanisms of epilepsy – part 1. *Journal of Clinical Neuroscience* 16, 355–365.
- Banerjee, P.N., Filippi, D., Hauser, W.A., 2009. The descriptive epidemiology of epilepsy: a review. *Epilepsy Research* 85 (1), 31–45.
- Baraban, S.C., 2007. Emerging epilepsy models: insights from mice, flies, worms and fish. *Current Opinion in Neurology* 20 (2), 164–168.
- Baraban, S.C., Taylor, M.R., Castro, P.A., Bialer, H., 2005. Pentylentetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. *Neuroscience* 131 (3), 759–768.
- Barnes, J.M., Murphy, P.A., Kirkham, D., Henley, J., 1993. Interaction of guanine nucleotides with [3H] kainate and 6-[3H]cyano-7-nitroquinoxaline-2,3-dione binding in goldfish brain. *Journal of Neurochemistry* 61 (5), 1685–1691.
- Bencan, Z., Sledge, D., Levin, E.D., 2009. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacology, Biochemistry and Behavior* 94 (1), 75–80.
- 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 Research* 75, 18–28.
- Bhattacharya, S.K., Sen, A.P., Mitra, S.K., 1991. Effects of muscarinic receptor agonists and antagonists on response of non-extensor rats to maximal electroshock. *Indian Journal of Experimental Biology* 29 (3), 237–240.
- Bialer, M., White, H.S., 2010. Key factors in the discovery and development of new antiepileptic drugs. *Nature Reviews. Drug Discovery* 9 (1), 68–82.
- Boccia, M.M., Acosta, G.B., Baratti, C.M., 2001. Memory improving actions of gabapentine in mice; possible involvement of central muscarinic cholinergic mechanism. *Neuroscience Letters* 311, 153–156.
- Boison, D., 2005. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. *The Neuroscientist* 11 (1), 25–36.
- Boison, D., 2008. Adenosine as a neuromodulator in neurological diseases. *Current Opinion in Pharmacology* 8 (1), 2–7.
- Borowicz, K.K., Kleinrok, K., Czuczwar, S.J., 1997. N⁶-2-4-aminophenyl/ethyladenosine enhances the anticonvulsive activity of antiepileptic drugs. *European Journal of Pharmacology* 372 (2–3), 125–133.
- Borowicz, K.K., Luszczyk, J., Czuczwar, S.J., 2002. 2-Chloroadenosine, a preferential agonist of adenosine A₁ receptors enhances the anticonvulsant activity of carbamazepine and clonazepam in mice. *European Neuropsychopharmacology* 12, 173–179.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* 72, 248–254.
- Burnstock, G., 2004. Cotransmission. *Current Opinion on Pharmacology* 4, 47–52.
- Burnstock, G., 2009. Purinergic signalling: past, present and future. *Brazilian Journal of Medical and Biological Research* 42, 3–8.
- Burnstock, G., Verkhatsky, A., 2009. Evolutionary origins of the purinergic signalling system. *Acta Physiologica* 195, 415–447.
- Chan, K.M., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca²⁺-stimulated ATPase activity. *Analytical Biochemistry* 157, 375–380.
- Chen, W., Guidotti, G., 2001. Soluble apyrases release ADP during ATP hydrolysis. *Biochemical and Biophysical Research Communications* 282, 90–95.
- Chiang, W.C., Knowles, A.F., 2008. Inhibition of human NTPDase₂ by modification of an intramembrane cysteine by p-chloromercuriphenylsulfonate and oxidative cross-linking of the transmembrane domains. *Biochemistry* 47 (33), 8775–8785.
- Clemente, D., Porteros, A., Weruaga, E., Alonso, J.R., Arenzana, F.J., Aijon, J., Arevalo, R., 2004. Cholinergic elements in the zebrafish central nervous system: histochemical and immunohistochemical analysis. *Journal of Comparative Neurology* 474, 75–107.
- Cognato, G.P., Bruno, A.N., da Silva, R.S., Bog, M.R., Sarkis, J.J.F., Bonan, C.D., 2007. Antiepileptic drugs prevent changes induced by pilocarpine model of epilepsy in brain ectonucleotidases. *Neurochemical Research* 32, 1046–1055.
- Cruickshank, J.W., Brudzynski, S.M., McLachlan, R.S., 1994. Involvement of M1 muscarinic receptors in the initiation of cholinergically induced epileptic seizures in the rat brain. *Brain Research* 643, 125–129.
- Cunha, R.A., 2001. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochemistry International* 38, 107–125.
- D'Antuono, M., Kawasaki, H., Palmieri, C., Cúria, G., Biagini, G., Avoli, M., 2007. Antiepileptic drugs and muscarinic receptor-dependent excitation in the rat subiculum. *Neuropharmacology* 52, 1291–1302.
- Dickson, C.T., Alonso, A., 1997. Muscarinic induction of synchronous population activity in the entorhinal cortex. *The Journal of Neuroscience* 17, 6729–6744.
- Dunwiddie, T.V., Masino, S.A., 2001. The role and regulation of adenosine in the central nervous system. *Annual Review of Neuroscience* 24, 31–55.
- Ellman, G.L., Courtney, K.D., Andrés, J.V., Fearherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* 7, 88–95.
- Fredholm, B.B., Ijzerman, A.P., Jacobson, J.K., Klotz, K., Linden, J., 2001. International union of pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacological Reviews* 53 (4), 527–552.
- Fredholm, B.B., Chen, J.F., Cunha, R.A., Svenningsson, P., Vaugeois, J.M., 2005. Adenosine and brain function. *International Review of Neurobiology* 63, 191–270.
- Friedman, A., Behrens, C.J., Heinemann, U., 2007. Cholinergic dysfunction in temporal lobe epilepsy. *Epilepsia* 48 (5), 126–130.
- Gerlai, R., Fernandes, Y., Pereira, T., 2009. Zebrafish (*Danio rerio*) responds to the animated image of a predator: towards the development of an automated aversive task. *Behavioural Brain Research* 201, 318–324.
- Grinthal, A., Guidotti, G., 2006. CD39, NTPDase₁, is attached to the plasma membrane by two transmembrane domains. Why? *Purinergic Signalling* 2, 391–398.
- Hamed, S.A., 2009. The aspects and mechanisms of cognitive alterations in epilepsy: the role of antiepileptic medication. *CNS Neuroscience and Therapeutics* 15, 134–156.
- Horvat, A., Orlic, T., Banjac, A., Momic, T., Petrovic, S., Demajo, M., 2006. Inhibition of rat brain ecto-atpase activity by various drugs. *General Physiology and Biophysics* 25, 91–105.

- Hunsucker, S.A., Mitchell, B.S., Spychala, J., 2005. The 5'-nucleotidases as regulators of nucleotide and drug metabolism. *Pharmacology and Therapeutics* 107, 1–30.
- Ingham, P.W., 2009. The power of the zebrafish for disease analysis. *Human Molecular Genetics* 18, 107–112.
- Kosmehl, T., Hallare, A.V., Braunbeck, T., Hollert, H., 2008. DNA damage induced by genotoxicants in zebrafish (*Danio rerio*) embryos after contact exposure to freeze-dried sediment and sediment extracts from Laguna Lake (The Philippines) as measured by the comet assay. *Mutation Research* 650, 1–14.
- Liu, Z., Nagao, T., Desjardins, C.G., Gloor, P., Avoli, M., 1994. Quantitative evaluation of neuronal loss in the dorsal hippocampus in rats with longterm pilocarpine seizures. *Epilepsy Research* 17, 237–247.
- Loiseau, P., Duché, B., 1995. Carbamazepine clinical use. In: Levy, R.H., Mattson, R.H., Meldrum, B.S. (Eds.), *Antiepileptic Drugs*, fourth ed. Raven Press, New York, pp. 555–566.
- Luna-Tortós, C., Fedrowitz, M., Löscher, W., 2010. Evaluation of transport of common antiepileptic drugs by human multidrug resistance-associated proteins (MRP1, 2 and 5) that are overexpressed in pharmacoresistant epilepsy. *Neuropharmacology* 18, 1–14.
- Mello, L.E., Cavalheiro, E.A., Tan, A.M., Kupfer, W.R., Pretorius, J.K., Babb, T.L., Finch, D.M., 1993. Circuit mechanisms of seizures in the pilocarpine model of chronic epilepsy: cell loss and mossy fiber sprouting. *Epilepsia* 34, 985–995.
- Mizuno, K., Okada, M., Murakami, T., Kamata, G.Z., Kawata, Y., Wada, K., Kaneko, S., 2000. Effects of carbamazepine on acetylcholine release and metabolism. *Epilepsy Research* 40, 187–195.
- Nagao, T., Alonso, A., Avoli, M., 1996. Epileptiform activity induced by pilocarpine in the rat hippocampal-entorhinal slice preparation. *Neuroscience* 72, 399–408.
- Nakata, H., Yoshioka, K., Kamiya, T., Tsuga, H., Oyanagi, K., 2005. Functions of heteromeric association between adenosine and P2Y receptors. *Journal of Molecular Neuroscience* 26 (2–3), 233–238.
- Pankratov, Y., Lalo, U., Krishtal, O.A., Verkhratsky, A., 2009. P2X receptors and synaptic plasticity. *Neuroscience* 158, 137–148.
- Papanikolaou, A., Papafotika, A., Murphy, C., Papamarcaki, T., Tsolas, O., Drab, M., Kurzchalia, T.V., Kasper, M., Christoforidis, S., 2005. Cholesterol-dependent lipid assemblies regulate the activity of the ectonucleotidase CD39. *Journal of Biological Chemistry* 15, 26406–26414.
- Pedraza, E.L., Senger, M.R., Pedraza, L., Zimmermann, F.F., Sarkis, J.J.F., Bonan, C.D., 2007. Sertraline and clomipramine inhibit nucleotide catabolism in rat brain synaptosomes. *Toxicology In Vitro* 21, 671–676.
- Pernot, F., Carpentier, P., Baille, V., Testylier, G.A., Beaup, C., Foquin, A., Filliat, P., Liscia, P., Coutan, M., Piérard, C., Béracochea, D., Dorandeu, F., 2009. Intrahippocampal cholinesterase inhibition induces epileptogenesis in mice without evidence of neurodegenerative events. *Neuroscience* 162 (4), 351–365.
- Rico, E.P., Senger, M.R., Fauth, M.G., Dias, R.D., Bogo, M.R., Bonan, C.D., 2003. ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*). *Life Science* 73, 2071–2082.
- Robson, S.C., Sévigny, J., Zimmermann, H., 2006. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. *Purinergic Signalling* 2, 409–430.
- Rogawski, M.A., Löscher, W., 2004. The neurobiology of antiepileptic drugs. *Nature Reviews Neuroscience* 5 (7), 553–564.
- Sebastião, A.M., Ribeiro, J.A., 2009. Adenosine receptors and the central nervous system. *Handbook of Experimental Pharmacology* 193, 471–534.
- Seibt, K.J., Oliveira, R.L., Rico, E.P., Dias, R.D., Bogo, M.R., Bonan, C.D., 2009a. Antipsychotic drugs inhibit nucleotide hydrolysis in zebrafish (*Danio rerio*) brain membranes. *Toxicology In Vitro* 23, 78–82.
- Seibt, K.J., Oliveira, R.L., Rico, E.P., Dias, R.D., Bogo, M.R., Bonan, C.D., 2009b. Typical and atypical antipsychotics alter acetylcholinesterase activity and AChE expression in zebrafish (*Danio rerio*) brain. *Comparative Biochemistry and Physiology, Toxicology and Pharmacology* 150 (1), 10–15.
- Senger, M.R., Rico, E.P., Dias, R.D., Bogo, M.R., Bonan, C.D., 2004. Ecto-5'-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*). *Comparative Biochemistry and Physiology* 139 (2), 203–207.
- Shaked, I., Zimmermann, G., Soreq, H., 2008. Stress-induced alternative splicing modulations in brain and periphery. *Annals of the New York Academy of Science* 1148, 269–281.
- Soreq, H., Seidman, S., 2001. Acetylcholinesterase: news roles for old actor. *Nature Reviews Neuroscience* 2 (4), 294–302.
- Stefan, H., Feuerstein, T.J., 2007. Novel anticonvulsant drugs. *Pharmacology and Therapeutics* 113, 165–183.
- Sträter, N., 2006. Ecto-5'-nucleotidase: structure function relationships. *Purinergic Signalling* 2, 343–350.
- Sudha, S., Lakshmanan, M.K., Pradhan, N., 1995. Chronic phenytoin induced impairment of learning and memory with associated changes in brain acetylcholine esterase activity and monoamine levels. *Pharmacology Biochemistry and Behavior* 52 (1), 119–124.
- Suwalsky, M., Mennicken, S., Norris, B., Villena, F., Sotomayor, C.P., 2006. Effects of the antiepileptic drug carbamazepine on human erythrocytes. *Toxicology In Vitro* 20, 1363–1369.
- Tonazzini, I., Trincavelli, M.L., Storm-Mathisen, J., Martini, C., Bergersen, L.H., 2007. Co-localization and functional cross-talk between A1 and P2Y1 purine receptors in rat hippocampus. *European Journal of Neuroscience* 26, 890–902.
- Turski, L., Ikonomidou, C., Turski, W.A., Bortolotto, Z.A., Cavalheiro, E.A., 1989. Review: cholinergic mechanisms and epileptogenesis. The seizures induced by pilocarpine: a novel experimental model of intractable epilepsy. *Synapse* 3, 154–171.
- Yang, L., Ho, N.Y., Alshut, R., Legradi, J., Weiss, C., Reischl, M., Mikut, R., Liebel, U., Müller, F., Strähle, U., 2009. Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. *Reproductive Toxicology* 28 (2), 245–253.
- Yoshioka, K., Nakata, H., 2004. ATP- and adenosine-mediated signaling in the central nervous system purinergic receptor complex: generating adenosine nucleotide-sensitive adenosine receptors. *Journal of Pharmacological Sciences* 94, 88–94.
- Zhu, G., Okada, M., Murakami, T., Kawata, Y., Kamata, A., Kaneko, S., 2002. Interaction between carbamazepine, zonisamide and voltage-sensitive Ca²⁺ channel on acetylcholine release in rat frontal cortex. *Epilepsy Research* 49 (1), 49–60.
- Zimmermann, H., 2006. Nucleotide signaling in nervous system development. *European Journal of Physiology* 452, 573–588.
- Zimmermann, G., Soreq, H., 2006. Termination and beyond: acetylcholinesterase as a modulator of synaptic transmission. *Cell and Tissue Research* 326, 655–669.
- Zimmermann, H., Braun, N., Kegel, B., Heine, P., 1998. New insights into molecular structure and function of ecto-nucleotidases in the nervous system. *Neurochemistry International* 32, 421–425.
- Zimmermann, G., Njunting, M., Ivens, S., Tolner, E., Behrens, C.J., Gross, M., Soreq, M., Heinemann, U., Friedman, A., 2008. Acetylcholine-induced seizure-like activity and modified cholinergic gene expression in chronically epileptic rats. *European Journal of Neuroscience* 27, 965–975.
- Zuchora, B., Wielosz, M., Urbanska, E.M., 2004. Adenosine A1 receptors and the anticonvulsant potential of drugs effective in the model of 3-nitropropionic acid-induced seizures in mice. *European Neuropsychopharmacology* 15, 85–93.

CAPÍTULO 3

ARTIGO CIENTÍFICO

PTZ-induced seizures inhibit adenosine deamination in
adult zebrafish brain membranes

Anna M. Siebel, Ângelo L. Piato, Katiucia M. Capiotti, Kelly J. Seibt,
Maurício R. Bogo, Carla D. Bonan

Artigo sendo escrito para ser submetido ao periódico Brain Research (FI 2.46)

PTZ-induced seizures inhibit adenosine deamination in adult zebrafish brain membranes

Anna Maria Siebel^{a,b}, Ângelo Luis Piato^{a,b,c}, Katiucia Marques Capiotti^{a,b}, Kelly Juliana Seibt^{a,b}, Maurício Reis Bogo^{b,d}, Carla Denise Bonan^{a,b,*}

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

^bInstituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), 90035-003 Porto Alegre, RS, Brazil

^cPrograma de Pós-Graduação em Medicina e Ciências da Saúde, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6690, 90610-000 Porto Alegre, RS, Brazil

^dLaboratório de Biologia Celular e Molecular, Departamento de Biologia Celular e Molecular, Programa de Pós-Graduação em Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil

*Corresponding author. Tel.: +55 51 3353 4158; Fax: +55 51 3320 3568.

E-mail address: cbonan@pucrs.br (C.D. Bonan).

ABSTRACT

Adenosine exerts neuromodulatory functions with mostly inhibitory effects, being considered an endogenous anticonvulsant. The hydrolysis of ATP by ectonucleotidases is an important source of adenosine and adenosine deaminase (ADA) regulates the concentration of this nucleoside through its deamination. In this study, we tested the effect of pentylenetetrazole (PTZ)-induced seizures on ectonucleotidases and ADA activities in adult zebrafish brain. Our results have demonstrated that PTZ treatments did not alter ectonucleotidase and ADA activities in membranes and soluble fraction, respectively. However, ecto-ADA activity was significantly decreased in brain membranes of animals exposed to 5 mM and 15 mM PTZ treatments when compared to the control group. Semiquantitative RT-PCR analysis did not show significant changes after the PTZ exposure on ADA gene expression. The decreased adenosine deamination observed in this study suggests a modulation of extracellular adenosine levels during PTZ-induced seizures. Our data support the use of zebrafish as model for studying purinergic signaling system, which can be an interesting approach for the identification of pharmacological targets that may increase the treatment spectrum to epilepsy.

Keywords: Adenosine; Adenosine deaminase; ectonucleotidases; seizures; pentylenetetrazole; zebrafish.

Abbreviations: adenosine deaminase (ADA); nucleoside triphosphate diphosphohydrolase (NTPDase); pentylenetetrazole (PTZ).

1. Introduction

Spontaneous and recurrent seizures characterize epilepsy (Fisher et al., 2005), which is one of the most common neurological diseases worldwide (Elger and Schmidt, 2008). These epileptic seizures occur due to an imbalance between synaptic excitation and inhibition that results in hypersynchronous electrical activity of neuronal networks (Boison and Stewart, 2009). The zebrafish is a small freshwater teleost which is becoming widely used in genetic, toxicological, behavioral, and neurochemical studies (Lieschke and Currie, 2007; Gerlai et al., 2009; Grossman et al., 2010; Siebel et al., 2010). Also, the genome of this fish is well-characterized and shares many similarities with the human genome (Barbazuk et al., 2000). Studies have demonstrated that zebrafish larvae and adults could be potential models to study seizures (Baraban et al., 2005; Berghmans et al., 2007; Wong et al., 2010). Zebrafish larvae exposed to PTZ, a convulsant agent, presented stereotyped behavioral, electrographic, and molecular changes, similar to the alterations reported in rodent seizure models (Baraban et al., 2005). Furthermore, exposure to PTZ, caffeine, and picrotoxin in adult zebrafish also leads to seizure-like behavioral responses (Wong et al., 2010). As reported in rodent models, chemically-induced seizures in zebrafish can be suppressed by classical antiepileptic drugs, such as valproate, diazepam, and carbamazepine (Baraban et al., 2005; Berghmans et al., 2007).

Adenosine, which is a modulator of neuronal activity, is effective in seizure suppression and neuroprotection (Cunha, 2005; Lado and Moshé, 2008). This nucleoside regulates many physiological and pathophysiological processes through activation of G protein-coupled receptors named A_1 , A_{2A} , A_{2B} , and A_3 , (Fredholm et

al., 2001; Ribeiro et al., 2003). Anticonvulsant properties of adenosine are mediated mainly by activation of A₁ receptors, which reduces the presynaptic neurotransmitter release and stabilizes the postsynaptic membrane potential (Dunwiddie and Masino, 2001; Fredholm et al., 2001). Several studies have shown the anticonvulsant action of adenosine and its analogues in different seizure models (Malhorta and Gupta, 1997, 1999; Zuchora et al., 2001; Borowicz et al., 2002). Extracellular adenosine can be provided by its cellular release or as a result of ATP hydrolysis by ectonucleotidases, such as NTPDases and ecto-5'-nucleotidase (Zimmermann, 2001). ATP is a signalling molecule that acts as a neurotransmitter and/or neuromodulator through ionotropic P2X and metabotropic P2Y receptors (Ralevic and Burnstock, 1998; Cunha and Ribeiro, 2000). The NTPDases family members are cell surface-located enzymes that hydrolyze both tri- and diphosphonucleosides whereas ecto-5'-nucleotidase hydrolyzes nucleoside monophosphates producing the nucleoside adenosine (Zimmermann, 1996, 2001). Therefore, ectonucleotidases control ATP, ADP, AMP and adenosine levels, modulating nucleotide and nucleoside-mediated signaling (Abbracchio et al., 2009). Extracellular adenosine concentrations can be also regulated via cell transporters, phosphorylation to AMP by adenosine kinase or deamination to inosine by ADA (Latini and Pedata, 2001). ADA catalyzes the adenosine deamination both in the cytosol and at the cell surface (Franco et al., 1997). Beyond its enzymatic function, ADA interacts with adenosine A₁ receptors, allowing the high-affinity binding of adenosine, facilitating the signal transduction through these receptors (Ciruela et al., 1996). Biochemical and molecular studies have already characterized NTPDase, ecto-5'-nucleotidase, and ADA in zebrafish brain (Rico et al., 2003; Senger et al., 2004; Rosemberg et al., 2007, 2008).

Several studies have reported that seizure episodes can alter ectonucleotidase pathway. Late and prolonged changes in nucleotide hydrolysis were reported after status epilepticus induction by pilocarpine and kainate or PTZ-kindling model (Bonan et al., 2000a, b). After induction of pilocarpine model, it has been observed an increase in ectonucleotidase activities, which was suppressed by previous treatment with antiepileptic drugs (Cognato et al., 2007). In addition, an enhancement in ADA activity was observed in PTZ-kindled mice (Ilhan et al., 2005, 2006). Despite the important role of the ectonucleotidases and ADA activities on the regulation of adenosine levels, little is known about the behavior of these enzymes during acute epileptic seizures. Considering that zebrafish may be a suitable model organism to evaluate seizure-induced changes in neurochemical parameters and that adenosine is an important endogenous anticonvulsant, the aim of this study was to evaluate the effect of PTZ-induced seizure episodes on ectonucleotidase and ADA activities in zebrafish brain, which are enzymes involved in the adenosine production and degradation.

2. Materials and methods

2.1 Animals

Adult wild type zebrafish (*Danio rerio*) of both sexes were obtained from a commercial supplier (Red Fish, RS, Brazil) and acclimated for 2 weeks before the experiments in a 50 L thermostated aquarium filled with unchlorinated water constantly aerated. Fish were kept at 26 ± 2 °C under a 14 -10 h light/dark cycle photoperiod and fed three times daily with commercial fish flake. The use and

maintenance of zebrafish were according to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health. The protocol was approved by the Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the number 09/00117-CEUA.

2.2 Materials

Trizma base, ammonium molybdate, polyvinyl alcohol, malachite green, nucleotides, adenosine, EDTA, EGTA, sodium citrate, Coomassie blue G, bovine serum albumin, calcium chloride, and PTZ were purchased from Sigma (St. Louis, MO, USA). Magnesium chloride, phenol, and sodium nitroprusside were purchased from Merck (Darmstadt, Germany). TRIzol, SuperScript™ III First-Strand Synthesis SuperMix, Taq Platinum, GelRed and Low DNA Mass Ladder were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents used were from analytical grade.

2.3 *In vitro* assays

Different concentrations of PTZ (2.5, 5, and 15 mM) were tested on ectonucleotidase and ADA activities. PTZ was diluted in deionized water and added to the reaction medium before the preincubation with the enzyme and was maintained through the enzyme assays.

2.4 PTZ model

To induce seizures, zebrafish were individually exposed to 2.5, 5, and 15 mM PTZ in a 250 ml beaker. PTZ treatments were chosen based on previous study with zebrafish (Baraban et al., 2005). The animals were submitted to the treatment for 20 minutes and the behavior was classified according each stage: stage I – increased swimming activity (2.5 mM), stage II – circling swimming behavior (5 mM) and stage III – clonus-like seizures followed by loss of posture (fish falls to one side and remains immobile for 1–3 s) (15 mM) as previously reported for zebrafish larvae (Baraban et al., 2005; Berghmans et al., 2007) and adults (Wong et al., 2010). Control group animals were maintained in a 250 ml beaker with tank water for the same period and conditions as the PTZ -treated groups.

2.5 Preparation of soluble and membrane fractions

Brain samples were obtained as described previously (Rico et al., 2003; Senger et al., 2004; Rosemberg et al., 2008). First, zebrafish were cryoanaesthetized, euthanized, and brains were removed by dissection (Wilson et al., 2009). Samples were then further homogenized in a glass-Teflon homogenizer according to the protocol for each enzyme assay. For NTPDase and ecto-5'-nucleotidase assays zebrafish brains were homogenized in 60 vol. (v/w) of chilled Tris–citrate buffer (50mM Tris–citrate, 2mM EDTA, 2mM EGTA, pH 7.4). For ADA experiments, brains were homogenized in 20 vol. (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4. The brain membranes were prepared as described previously by Barnes et al. (1993). In brief, the homogenates

were centrifuged at $800 \times g$ for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at $40\,000 \times g$. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. For soluble ADA activity experiments, the supernatant was collected and kept on ice for enzyme assays. The pellets of membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers and centrifuged for 20 min at $40\,000 \times g$. This freeze–thaw–wash procedure was used to ensure the lysis of the brain vesicles membranes. The final pellets were resuspended and used for enzyme assays. All samples were maintained at $2\text{--}4^{\circ}\text{C}$ throughout preparation.

2.6 Ectonucleotidase assays

NTPDase and 5'-nucleotidase assays were performed as described previously (Rico et al., 2003; Senger et al., 2004). Zebrafish brain membranes ($3 \mu\text{g}$ protein for NTPDase and $5 \mu\text{g}$ protein for 5'-nucleotidase) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM CaCl_2 (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM MgCl_2 (for the 5'-nucleotidase activity) at a total volume of 200 μL . The samples were preincubated for 10 min at 37°C before starting the reaction by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was terminated after 30 min with 200 μL trichloroacetic acid at a final concentration of 5%. The samples were chilled on ice for 10 min and 1 ml of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added in order to determine the inorganic phosphate released (Pi) (Chan et al., 1986). The quantification of Pi released was determined spectrophotometrically at 630 nm and the specific activity

was expressed as nmol of Pi min⁻¹ mg⁻¹ of protein. In order to correct non-enzymatic hydrolysis of the substrates, controls with the addition of the enzyme preparation after the addition of trichloroacetic acid were used. All enzyme assays were performed in at least five different experiments, each one performed in triplicate.

2.7 Adenosine deaminase assays

Ecto- and cytosolic-ADA activities were determined as described previously (Rosemberg et al., 2008). The brain fractions (5–10 µg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for soluble and membrane fractions, respectively, in a final volume of 200 µL. The samples were preincubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate (adenosine) to a final concentration of 1.5 mM. The reaction was stopped after 75 min (soluble fraction) and 120 min (membrane fraction) by the addition of 500 µl phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml). ADA activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported (Weisman et al., 1988). In order to correct non-enzymatic hydrolysis of the substrates controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used. The reaction mixtures were immediately mixed to 500 µl of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine in 0.6M NaOH) and vortexed. Samples were incubated at 37°C for 15 min and the colorimetric assay was carried out at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. The ADA activity was expressed as nmol of NH₃

min⁻¹ mg⁻¹ of protein. All enzyme assays were performed in at least five different experiments, each one performed in triplicate.

2.8 Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard.

2.9 Reverse transcription-polymerase chain reaction (RT-PCR)

The expression of ADA-related genes *ada1*, *ada2.1*, *ada2.2*, and *adal* was analyzed by a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) assay. TRIzol[®] reagent (Invitrogen) was employed to isolate total zebrafish brain RNA in accordance with manufacturer instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values at 260 and 280 nm. Afterwards, all samples were adjusted to 160 ng/μL and cDNA species were synthesized with SuperScript[™] First-Strand Synthesis SuperMix Kit (Invitrogen, USA), following the supplier's instructions. The *β-actin* primers were designed as described previously (Chen et al., 2004). Primer sequences of ADA-related genes were designed and PCR reactions were performed according to Rosemberg et al. (2007). PCR products were separated on a 1.0% agarose gel with GelRed 10x and visualized with ultraviolet light. The fragment lengths expected for the PCR reactions were confirmed using Low DNA Mass Ladder and *β-actin* was determined as an internal standard. Band intensities were analyzed by optical

densitometry using the software ImageJ 1.37 for Windows after running all PCR products in a single gel.

2.10 Statistical analysis

Results were expressed as means \pm S.E.M. Data were analyzed by one-way ANOVA followed by Duncan post-hoc test. P values ≤ 0.05 were considered as significant.

3. Results

The *in vivo* effect of PTZ (2.5, 5, and 15 mM) was tested on NTPDase, ecto-5'-nucleotidase, and ADA activities in adult zebrafish brain. The animals remained exposed to PTZ treatments for 20 minutes, enough time for all animals reach stage I – increased swimming activity (2.5 mM), stage II – circling swimming behavior (5 mM) and stage III – clonus-like seizures followed by loss of posture (15 mM) (data not shown). Our results have demonstrated that PTZ treatment in all doses tested did not alter ATP, ADP, and AMP hydrolysis in zebrafish brain membranes (Table 1). In contrast, PTZ inhibited ecto-ADA activity (22.38% and 29.55%, respectively; $P < 0.05$) at 5 mM (10.61 ± 2.07 nmol of $\text{NH}_3 \text{ min}^{-1} \text{ mg}^{-1}$ of protein) and at 15 mM (9.63 ± 0.77 nmol of $\text{NH}_3 \text{ min}^{-1} \text{ mg}^{-1}$ of protein) when compared to the control group (13.67 ± 1.81 nmol of $\text{NH}_3 \text{ min}^{-1} \text{ mg}^{-1}$ of protein) (Fig. 1A). However, the soluble ADA activity was not altered by PTZ exposure (Fig. 1B).

In order to elucidate whether PTZ has any direct effect on these enzymes that could influence the *in vivo* experiments, we also tested the *in vitro* effect of PTZ on

NTPDase, ecto-5'-nucleotidase and ADA activities. No changes were observed in the enzyme activities in the presence of this drug (data not shown). Semiquantitative RT-PCR experiments were performed to verify whether the PTZ treatments have altered the expression of ADA-related genes. The results did not show significant effects of the PTZ treatments on ADA gene expression in zebrafish brain (Fig. 2).

4. Discussion

The findings of the present study demonstrated that ecto-ADA was altered in zebrafish brain during PTZ exposure. Soluble ADA, NTPDase, and 5'-nucleotidase activities did not show significant changes. The decreased ecto-ADA activity suggests the existence of a modulation of extracellular adenosine levels during PTZ-induced seizures.

Besides the adenosine release, the hydrolysis of ATP by ectonucleotidases is another important source of extracellular adenosine. This enzymatic pathway includes the NTPDase family, which promotes ATP and ADP hydrolysis, and 5'-nucleotidase, which cleaves AMP to adenosine (Zimmermann, 2001). Therefore, this enzyme cascade has a dual function, because it removes the excitatory signaling molecule ATP and concomitantly generates the nucleoside adenosine, which has important inhibitory effects (Abbracchio et al., 2009). Our study showed that NTPDase and 5'-nucleotidase activities were not altered during acute seizures induced by PTZ. Previous studies reported changes in ectonucleotidase activities at different periods after chemically-induced seizures. In brain samples of adult rats, the adenine nucleotide hydrolysis was increased in different times (45 min, 5h, 12h) after quinolinic acid-induced seizures (Nicolaidis et al., 2005) and at 7 days after

pilocarpine-induced epilepsy (Cognato et al., 2007). After a single convulsive PTZ injection in rats, no differences in ATP, ADP and AMP hydrolysis were observed on ectonucleotidase activities from synaptosomal-enriched fractions. However, after PTZ-kindling, an increased ATP hydrolysis in the rats that were more resistant to seizure development was reported (Bonan et al., 2000b). The authors suggest that recurrent seizures could lead to late and prolonged changes in ectonucleotidase activity, which is not observed after a single seizure episode (Bonan et al., 2000b). Therefore, our findings are in agreement with this hypothesis since a single PTZ exposure did not show significant changes in ATP, ADP, and AMP hydrolysis in zebrafish. In addition, our results demonstrated that ADA activity is altered during a single PTZ exposure, suggesting a modulation of adenosine levels, which may represent a compensatory response in order to promote a neuroprotective and anticonvulsant effect of adenosine.

Studies report that the extracellular adenosine levels rises markedly during epileptic seizures. In rats, hippocampal adenosine levels increased following seizures produced by bicuculline, kainic acid, and PTZ (Berman et al., 2000). In humans with intractable complex partial epilepsy, microdialysis measurements demonstrated that seizures increase extracellular adenosine range and also that the adenosine levels remain elevated above basal values after the seizure episodes (During and Spencer, 1992). These findings support the hypothesis that adenosine has been implicated in the arrest of seizures and postictal refractoriness (During and Spencer 1992; Boison, 2005). As previously reported, besides the cell uptake, and phosphorylation to AMP, extracellular adenosine concentrations can be regulated through its deamination (Latini and Pedata, 2001). Studies suggest an important role of this mechanism during pathophysiological conditions (Lloyd and Fredholm, 1995), since the inhibition

of adenosine deamination has been shown an effective anticonvulsant strategy in diverse epilepsy models (Dupere et al., 1999; Southam et al., 2002).

In this study, we showed that PTZ treatments significantly decreased ecto-ADA activity, whereas did not change adenosine deamination in soluble fraction. Previous studies reported that the regulation of adenosine levels in intracellular and extracellular fractions in the zebrafish brain might be promoted by distinct ADA members (ADA1, ADA2, and ADAL), which have diverse gene expression pattern and activity properties (Rosemberg et al., 2007, 2008) and might contribute in different manners for the regulation of adenosine levels. Adenosine deamination has been attributed mainly to ADA1 activity, a typical cytosolic enzyme that also acts as an ecto-ADA, cleaving extracellular adenosine (Franco et al., 1997; Zavialov and Engström, 2005). Together with ADA1, the ADA2 enzyme also contributes to adenosine deamination. However, it seems to act specifically in the extracellular fraction (Zavialov and Engström, 2005; Zavialov et al., 2010). Beyond its enzymatic function, there is evidence that ecto-ADA is bound with adenosine receptors, modulating their affinity (Ciruela et al., 1996; Herrera et al., 2001). Although the ADAL functionality still remains unclear, the presence of ADAL mRNA transcripts in zebrafish brain (Rosemberg et al., 2007) might indicate a possible role for this ADA member in this species. Considering that the inhibition of adenosine deamination caused by PTZ exposure was observed exclusively for the ecto-ADA activity, our results suggest that ADA-related members are differentially modulated by PTZ treatments.

Previous study from our group has already reported the expression profile of ADA-related genes (*ada1*, *ada2.1*, *ada2.2*, and *adal*) in zebrafish brain (Rosemberg et al., 2007). Our RT-PCR results showed that PTZ-induced seizures did not interfere

in the expression of these ADA-related genes. Therefore, the decreased adenosine deamination observed cannot be associated with alterations in transcriptional pattern of ADA family enzymes. Since no direct effect of PTZ on the enzyme activities was shown by *in vitro* assays, as well as any alterations in transcriptional pattern of ADA-related genes were observed, we suggest that the modulation of ADA activities might involve posttranslational events. Rosemberg et al. (2007) showed that the ADA-related enzymes have putative regulatory sites for posttranslational mechanisms, such as phosphorylation/desphosphorylation. Therefore, further studies are required to evaluate the role of these mechanisms in the modulation of ADA activity of zebrafish.

Wong et al. (2010) showed that caffeine, a nonselective antagonist of adenosine receptors, was able to induce robust seizure-like behavior in adult zebrafish. This result is in agreement with our suggested hypothesis that adenosine signaling is related with epileptic seizures in zebrafish. Despite the extensive use of rodents, zebrafish has been increasingly recognized as an important model organism to study the neurobiology of epilepsy (Baraban, 2007; Hortopan et al., 2010). Recent studies have shown the antiepileptic effects of valproic acid in the adult zebrafish, which coincides with reduced heat shock protein 70 (HSP70) mRNA expression, preventing learning impairment promoted by PTZ treatment (Lee et al., 2010). In addition, PTZ-induced seizures result in a massive reduction in cell proliferation in wide-ranging areas of the zebrafish developing brain (Kim et al., 2010). Several behavioral, electrographic, molecular, and endocrine responses to induced-seizures have been characterized in this species (Baraban et al., 2005; Berghmans et al., 2007; Wong et al., 2010) and these tools can also be a tempting strategy for further studies related to epileptic seizures in zebrafish.

In summary, our findings showed that adenosine deaminase activity is altered during PTZ-induced seizures whereas ectonucleotidase activities are not affected by this condition. Such effects have showed that the catabolism of adenosine may be modulated by seizure development in zebrafish. These results clearly contribute to the demonstration that the enzyme pathway involved in the control of adenosine levels may participate of the mechanism of seizure induction and development. Furthermore, our data suggest that the use of zebrafish as model of the adenosine signaling system can be an interesting approach for the identification of pharmacological targets that may increase the treatment spectrum to epilepsy.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by DECIT/SCTIE-MS through Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (Proc. 10/0036-5 – PRONEX) and by the FINEP research grant “Rede Instituto Brasileiro de Neurociência (IBN-Net)” # 01.06.0842-00. A.M.S, A.L.P, and K.M.C were recipient of fellowship from CNPq. K.J.S. was recipient of fellowship from Programa PROBOLSAS/PUCRS.

References

- Abbracchio, M. P., Burnstock, G., Verkhratsky, A., Zimmermann, H., 2009. Purinergic signalling in the nervous system: an overview. *Trends Neurosci.* 32 (1), 19–29.
- Baraban, S.C., Taylor, M. R., Castro, P.A., Baier, H., 2005. Pentylentetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. *Neuroscience* 131 (3), 759–768.
- Baraban, S.C., 2007. Emerging epilepsy models: insights from mice, flies, worms and fish. *Curr. Opin. Neurol.* 20 (2), 164–168.
- Barbazuk, W.B., Korf, I., Kadavi, C., Heyen, J., Tate, S., Wun, E., Bedell, J.A., McPherson, J.D., Johnson, S.L., 2000. The syntenic relationship of the zebrafish and human genomes. *Genome Res.* 10 (9), 1351-1358.
- Barnes, J.M., Murphy, P.A., Kirkham, D., Henley, J., 1993. Interaction of guanine nucleotides with [3H] kainate and 6-[3H] cyano-7-nitroquinoxaline -2, 3 -dione binding in goldfish brain. *J. Neurochem.* 61 (5), 1685–1691.
- 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 (1), 18–28.
- Berman, R.F., Fredholm, B.B., Aden, A., O'Connor, W.T., 2000. Evidence for increased dorsal hippocampal adenosine release and metabolism during pharmacologically induced seizures in rats. *Brain Res.* 872 (1-2), 44–53.
- Boison, D., 2005. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. *Neuroscientist* 11 (1), 25–36.
- Boison, D., Stewart, K., 2009. Therapeutic epilepsy research: from pharmacological rationale to focal adenosine augmentation. *Biochem. Pharmacol.* 78 (12), 1428–1437.

- Bonan, C.D., Walz, R., Pereira, G.S., Worm, P.V., Battastini, A.M.O., Cavalheiro, E.A., Izquierdo, I., Sarkis, J.J.F., 2000a. Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy. *Epilepsy Res.* 39 (3), 229-238.
- Bonan, C.D., Amaral, O.B., Rockenbach, I.C., Walz, R., Battastini, A.M.O., Izquierdo, I., Sarkis, J.J.F., 2000b. Altered ATP hydrolysis induced by pentylentetrazol kindling in rat brain synaptosomes. *Neurochem. Res.* 25 (6), 775-779.
- Borowicz, K.K., Luszczki, J., Czuczwar, S.J., 2002. 2-Chloroadenosine, a preferential agonist of adenosine A₁ receptors enhances the anticonvulsant activity of carbamazepine and clonazepam in mice. *Eur. Neuropsychopharmacol.* 12 (2), 173–179.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Analy. Biochem.* 72, 248–254.
- Chan, K.M., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca²⁺ - stimulated ATPase activity. *Anal. Biochem.* 157 (2), 375–380.
- Chen, W.Y., John, J.A.C., Lin, C.H., Lin, H.F., Wu, S.C., 2004. Expression of metallothionein gene during embryonic and early larval development in zebrafish. *Aquat. Toxicol.* 69 (3), 215–227.
- Ciruela, F., Saura, C., Canela, E.I., Mallol, J., Lluís, C., Franco, R., 1996. Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. *FEBS Lett.* 380 (3), 219-223.
- Cognato, G.P., Bruno, A.N., da Silva, R.S., Bogo, M.R., Sarkis, J.J.F., Bonan, C.D., 2007. Antiepileptic drugs prevent changes induced by pilocarpine model of epilepsy in brain ecto-nucleotidases. *Neurochem. Res.* 32 (6), 1046–1055.

- Cunha, R.A., Ribeiro, J.A., 2000. Purinergic modulation of [3H] GABA release from rat hippocampal nerve terminals. *Neuropharmacology* 39 (7), 1156–1167.
- Cunha, R.A., 2005. Neuroprotection by adenosine in the brain: from A₁ receptor activation to A_{2A} receptor blockade. *Purinergic Signal.* 1 (2), 111–134.
- Dunwiddie, T.V., Masino, S.A., 2001. The role and regulation of adenosine in the central nervous system. *Annu. Rev. of Neurosci.* 24, 31–55.
- Dupere, J.R., Dale, T.J., Starkey, S.J., Xie, X., 1999. The anticonvulsant BW534U87 depresses epileptiform activity in rat hippocampal slices by an adenosine-dependent mechanism and through inhibition of voltage-gated Na⁺ channels. *Br. J. Pharmacol.* 128 (5), 1011-1020.
- During, M.J., Spencer, D.D., 1992. Adenosine: a potential mediator of seizure arrest and postictal refractoriness. *Ann. Neurol.* 32 (5), 618–624.
- Elger, C.E., Schmidt, D., 2008. Modern management of epilepsy: a practical approach. *Epilepsy & Behav.* 12 (4), 501-539.
- Feldman RS, Quenzer LF. *Fundamentals of neuropsychopharmacology*. Sunderland: Sinauer Associates, 1984. 528p.
- 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 (4), 470–472.
- Franco, R., Casado, V., Ciruela, F., Saura, C., Mallol, J., Canela, E.I., Lluís, C., 1997. Cell surface adenosine deaminase: much more than an ectoenzyme. *Prog. Neurobiol.* 52 (4), 283-294.

- Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Klotz, K.N., Linden, J., 2001. International Union of Pharmacology. XXV. Nomenclature and Classification of Adenosine Receptors. *Pharmacol. Rev.* 53 (4), 527-552.
- Gerlai, R., Fernandes, Y., Pereira, T., 2009. Zebrafish (*Danio rerio*) responds to the animated image of a predator: towards the development of an automated aversive task. *Behav. Brain Res.* 201 (2), 318–324.
- Grossman, L., Utterback, E., Stewart, A., Gaikwad, S., Chung, K.M., Suci, C., Wong, K., Elegante, M., Elkhayat, S., Tan, J., Gilder, T., Wu, N., DiLeo, J., Cachat, J., Kalueff, A.V., 2010. Characterization of behavioral and endocrine effects of LSD on zebrafish. *Behav. Brain Res.* 214 (2), 277-284.
- Herrera, C., Casadó, V., Ciruela, F., Schofield, P., Mallol, J., Lluís, C., Franco, R., 2001. Adenosine A_{2B} receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. *Mol. Pharmacol.* 59 (1), 127-34.
- Hortopan, G.A., Dinday, M.T., Baraban, S.C., 2010. Zebrafish as a model for studying genetic aspects of Epilepsy. *Dis. Model. Mech.* 3 (3-4), 144-148.
- Ilhan, A., Gurel, A., Armutcu, F., Kamisli, S., Iraz, M., 2005. Antiepileptogenic and antioxidant effects of *Nigella sativa* oil against pentylenetetrazol-induced kindling in mice. *Neuropharmacology* 49 (4), 456-464.
- Ilhan, A., Iraz, M., Kamisli, S., Yigitoglu, R., 2006. Pentylenetetrazol-induced kindling seizure attenuated by *Ginkgo biloba* extract (EGb 761) in mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 30 (8), 1504-1510.
- Kim, Y.H., Lee, Y., Lee, K., Lee, T., Kim, Y.J., Lee, C.J., 2010. Reduced neuronal proliferation by proconvulsant drugs in the developing zebrafish brain. *Neurotoxicol Teratol.* 32(5):551-557.

- 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(2-3):225-231.
- Lado, F.A., Moshé, S.L., 2008. How do seizures stop. *Epilepsia* 49 (10), 1651-1664.
- Latini, S., Pedata, F., 2001. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J. Neurochem.* 79 (3), 463-484.
- Lieschke, G.J., Currie, P.D., 2007. Animal models of human disease: zebrafish swim into view. *Nat. Rev., Genet.* 8 (5), 353–367.
- Löscher W, Schmidt D. New horizons in the development of antiepileptic drugs: the search for new targets. *Epilepsy Res.* 2004; 60(2-3):77-159.
- Lloyd, H.G.E., Fredholm, B.B., 1995. Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem. Int.* 26 (4), 387-395.
- Malhorta, J., Gupta, Y.K., 1997. Effect of adenosine receptor modulation on pentylenetetrazol-induced seizures in rats. *Br. J. Pharmacol.* 120 (2), 282–288.
- Malhorta, J., Gupta, Y.K., 1999. Effect of adenosinergic modulation on the anticonvulsant effect of phenobarbital and carbamazepine. *Methods Find. Exp. Clin. Pharmacol.* 21 (2), 79-83.
- Nicolaidis, R., Bruno, A.N., Sarkis, J.J.F, Souza, D.O., 2005. Increase of adenine nucleotide hydrolysis in rat hippocampal slices after seizures induced by quinolinic acid. *Neurochem. Res.* 30 (3), 385–390.
- Pagonopoulou, O., Efthimiadou, A., Asimakopoulos, B., Nikolettos, N.N., 2006. Modulatory role of adenosine and its receptors in epilepsy: possible therapeutic approaches. *Neurosci. Res.* 56 (1), 14–20.

- Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. *Pharmacol. Rev.* 50 (3), 413–492.
- Ribeiro, J. A., Sebastião, A. M., Mendonça, A., 2003. Adenosine receptors in the nervous system: pathophysiological implications. *Prog. Neurobiol.* 68 (6), 377–392.
- Rico, E.P., Senger, M.R., Fauth, M.G., Dias, R.D., Bogo, M.R., Bonan, C.D., 2003. ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*). *Life Sci.* 73 (16), 2071–2082.
- Rosemberg, D.B., Rico, E.P., Guidoti, M.R., Dias, R.D., Souza, D.O., Bonan, C.D., Bogo, M.R., 2007. Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (*Danio rerio*). *Life Sci.* 81 (21–22), 1526–1534.
- 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* 151 (1), 96–101.
- Senger, M.R., Rico, E.P., Dias, R.D., Bogo, M.R., Bonan, C.D., 2004. Ecto-5'-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*). *Comp. Biochem. Physiol., B* 139 (2), 203–207.
- Siebel, A.M., Rico, E.P., Capiotti, K.M., Piato, A.L., Cusinato, C.T., Franco, T.M.A., Bogo, M.R., Bonan, C.D., 2010. *In vitro* effects of antiepileptic drugs on acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain. *Toxicol. in Vitro* 24 (4), 1279-1284.
- Southam, E., Stratton, S.C., Sargent, R.S., Brackenborough, K.T., Duffy, C., Hagan, R.M., Pratt, G.D., Jones, S.A., Morgan, P.F., 2002. Broad spectrum

- anticonvulsant activity of BW534U87: possible role of an adenosine-dependent mechanism. *Pharmacol. Biochem. Behav.* 74 (2), 111–118.
- Weisman, M.I., Caiolfa, V.R., Parola, A.H., 1988. Adenosine deaminase-complexing protein from bovine kidney. Isolation of two distinct subunits. *J. Biol. Chem.* 263 (11), 5266–5270.
- Wilson, J.M., Bunte, R.M., Carty, A.J., 2009. Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). *J. Am. Assoc. Lab. Anim. Sci.* 48 (6), 785-789.
- Wong, K., Stewart, A., Gilder, T., Wu, N., Frank, K., 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, 209-215.
- Zavialov, A.V., Engström, A., 2005. Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity. *Biochem. J.* 391, 51–57.
- Zavialov, A.V., Yu, X., Spillmann, D., Lauvau, G., Zavialov, A.V., 2010. Structural basis for the growth factor activity of human adenosine deaminase ADA2. *J. Biol. Chem.* 285 (16), 12367–12377.
- Zimmermann, H., 1996. Biochemistry, localization and functional roles of ectonucleotidases in the nervous system. *Prog. Neurobiol.* 49 (6), 589–618.
- Zimmermann, H., 2001. Ectonucleotidases: some recent developments and note on nomenclature. *Drug Dev. Res.* 52 (1-2), 44-56.
- Zuchora, B., Turski, W.A., Wielosz, M., Urbńska, E.M., 2001. Protective effect of adenosine receptor agonists in a new model of epilepsy: seizures evoked by mitochondrial toxin, 3-nitropropionic acid, in mice. *Neurosci. Lett.* 305 (2), 91-94.

Figure Legends

Figure 1: Effect of PTZ treatments (2.5, 5, and 15 mM) on membrane-bound (A) and soluble (B) ADA activity from zebrafish brain. Bars represent the mean \pm S.E.M. The symbol (*) represents a significant difference from control group (one-way ANOVA, followed by Duncan test as post hoc, $P \leq 0.05$). The specific enzyme activity is reported as nmol of $\text{NH}_3 \text{ min}^{-1} \text{ mg}^{-1}$ of protein.

Figure 2: Effect of PTZ treatments (2.5, 5, and 15 mM) on ADA gene expression pattern. The figure shows *β -actin*, *ada1*, *adal*, *ada2.1*, and *ada2.2* expression in adult zebrafish brain (A) and the enzyme/ β -actin mRNA ratios obtained by optical densitometry analysis (B). The results were expressed as optical densitometry of the ADA-related genes versus *β -actin* expression (mean \pm S.E.M) of four independent replicate RT-PCR experiments.

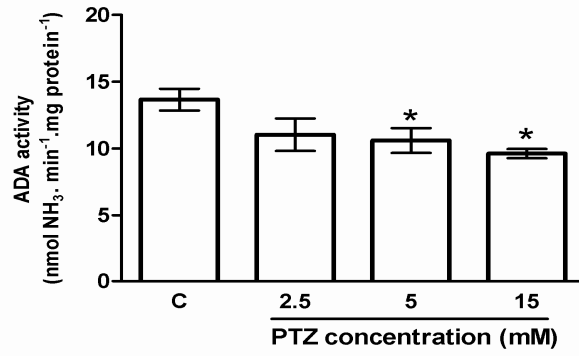
Table 1: Effect of PTZ treatments on ectonucleotidase activities in adult zebrafish brain membranes

Group	<i>n</i>	ATP	ADP	AMP
Control	5	475.9 \pm 101.6	68.6 \pm 13.4	12.83 \pm 2.3
2.5 mM	5	421.3 \pm 92.1	64 \pm 8.5	11.14 \pm 1.7
5 mM	5	419.2 \pm 87.7	72.2 \pm 14	12.20 \pm 1.6
15 mM	5	488.5 \pm 104.5	68.2 \pm 11.7	13.07 \pm 1.3

The specific activity was expressed as nmol Pi. $\text{min}^{-1} \cdot \text{mg}^{-1}$ protein.
Data are expressed as mean \pm S.E.M.

Figure 1

A



B

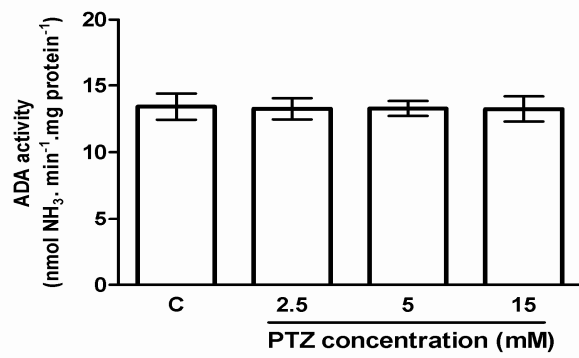
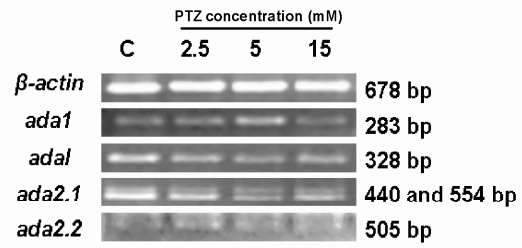
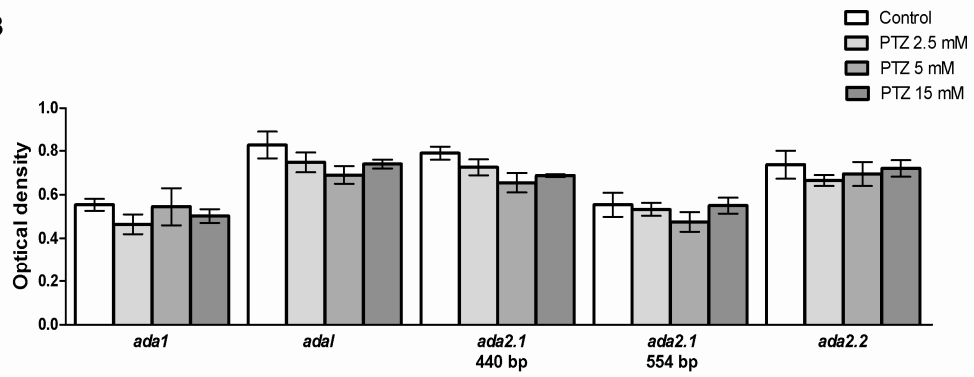


Figure 2

A



B



CAPÍTULO 4

CONSIDERAÇÕES FINAIS

A epilepsia encontra-se hoje entre as principais doenças neuronais crônicas. Conforme a Organização Mundial de Saúde existem em torno de 50 milhões de pacientes epiléticos no mundo, sendo que de 0,4 a 1% da população mundial tem o que se considera epilepsia ativa, ou seja, apresenta convulsões recorrentes ou necessita de tratamento crônico (Pitkanen, 2010). Os fármacos antiepiléticos constituem a principal opção no tratamento da epilepsia. Alternativas não-farmacológicas (cirurgias, dieta cetogênica, entre outras) são viáveis em poucos casos e aplicadas somente quando o uso de fármacos não é efetivo (Duncan et al., 2006).

Indivíduos com epilepsia apresentam predisposição crônica à ocorrência de crises convulsivas, causadas por descargas súbitas, excessivas e sincrônicas em um grupo neuronal (Fisher et al., 2005; Elger & Schmidt, 2008). Os mecanismos que envolvem a ocorrência dessas descargas neuronais não estão completamente esclarecidos. Sabe-se que ocorre o aumento súbito de sinapse excitatória e falhas nos mecanismos inibitórios, que podem envolver alterações em propriedades neuronais intrínsecas, como instabilidade da membrana, disfunções nos canais iônicos, e capacidade neuronal de manter e propagar o estímulo (Elger & Schmidt, 2008). Os fármacos antiepiléticos atuam impedindo essas descargas neuronais excessivas. Agem principalmente reduzindo a excitabilidade elétrica das membranas celulares, controlando canais de Na^+ e através da potencialização da neurotransmissão inibitória gabaérgica (Rogawski & Löscher 2004a,b). Assim, os medicamentos antiepiléticos são paliativos, atuam prevenindo convulsões, mas não impedem o agravamento da doença e não tem efeitos na epileptogênese, ou seja, não impedem a epilepsia decorrente de insultos como traumatismos e hipóxia (Bialer & White, 2010). Mesmo com o desenvolvimento de novos fármacos nos últimos anos, ainda não se tem um tratamento totalmente eficaz que controle a epileptogênese e não cause efeitos adversos. Além disso, em torno de 30% dos pacientes epiléticos não respondem aos tratamentos farmacológicos disponíveis (Stefan & Hopfengärtner, 2009).

Para que se possa obter o efetivo controle da epilepsia é necessário o melhor entendimento dos mecanismos envolvidos na predisposição à ocorrência de descargas neuronais descontroladas e no mecanismo de ação dos fármacos antiepiléticos. É necessária a melhor caracterização do envolvimento de diferentes vias de sinalização nos processos que envolvem a epilepsia. Identificando os

mecanismos envolvidos, pode-se, por exemplo, buscar novos alvos de ação para fármacos antiepilépticos. Os sistemas purinérgico e colinérgico são importantes vias de sinalização no SNC, estando amplamente distribuídos e envolvidos em diversos mecanismos de controle neuronal (Zimmermann, 2008). Disfunções no sistema colinérgico já estão evidentemente relacionadas a diferentes patologias (Alzheimer, Parkinson, déficit de atenção e distúrbios de ansiedade, entre outros), e mais recentemente vem se estabelecendo a ligação do sistema purinérgico com algumas doenças humanas, como neurodegenerativas e inflamação (Zimmermann, 2008; Khakh & Burnstock, 2009).

A sinalização através da ACh pode promover a excitabilidade neuronal e também descargas neuronais excessivas, características de crises convulsivas. Esse efeito ocorre principalmente através da ativação de receptores muscarínicos (D'Antuono, 2007). A ativação do sistema colinérgico pode levar à liberação excessiva de glutamato, causando convulsões (Friedman et al., 2007). A AChE é fundamental no controle da sinalização colinérgica, uma vez que a inativação da ACh ocorre fundamentalmente através de sua hidrólise (Zimmermann & Soreq, 2006). A injeção, em hipocampo de camundongos, de inibidor da AChE, induziu crises convulsivas crônicas e mostrou que a ativação focal do sistema colinérgico pode causar epilepsia (Pernot et al., 2009). Em ratos, tratamentos com inibidor da AChE causaram *status epilepticus* e posterior ocorrência crônica de convulsões (Furtado et al., 2011).

Em nosso estudo, testamos o efeito *in vitro* de diversas concentrações (10-1000 μM) de carbamazepina, fenitoína e gabapentina na atividade da AChE, em membranas cerebrais de peixe zebra. A fenitoína e a gabapentina não causaram efeito na atividade enzimática. A carbamazepina, nas concentrações 500 μM (25,2%) e 1000 μM (38,7%), diminuiu a hidrólise de ACh. A faixa terapêutica de concentração sérica de carbamazepina está entre 15 a 45 μM (Loiseau & Duche, 1995). Nossos resultados mostraram efeitos inibitórios da carbamazepina na atividade da AChE apenas em altas concentrações (500 e 1000 μM). Esses dados indicam que altas concentrações de carbamazepina podem levar ao aumento dos níveis de ACh, uma vez que inibem sua degradação. Essa elevação nos níveis de ACh pode ter efeitos neurotóxicos, além de causar crises convulsivas (Friedman et al., 2007).

No sistema purinérgico, o ATP atua como um neurotransmissor excitatório (principalmente ativando receptores P2X) e também tem envolvimento na proliferação, crescimento e diferenciação celular (atuando em receptores P2Y) (Fields & Burnstock, 2006; Burnstock, 2008). Imediatamente após algum insulto, como traumatismo ou hipóxia, ocorre a elevada liberação de ATP, que atua como um sinalizador, ativando mecanismos de defesa (Abbracchio et al., 2009). O ATP liberado em resposta a uma atividade neuronal pode ser rapidamente degradado pelas ectonucleotidases, gerando o neuromodulador inibitório adenosina (Burnstock, 2008). Assim, o ATP tem propriedades excitatórias, mas é também substrato para a produção de um modulador com efeitos inibitórios. As ectonucleotidases, família de enzimas que inclui as NTPDases e a 5'nucleotidase, são responsáveis pela regulação dos níveis de ATP e adenosina, controlando a sua ação e modulando o sistema purinérgico. Estão localizadas na superfície celular e atuam especificamente na hidrólise de nucleotídeos (Yegutkin, 2008).

Considerando que as ectonucleotidases controlam os níveis do neurotransmissor excitatório ATP e do neuromodulador inibitório adenosina, essas enzimas tornam-se importantes na epilepsia. Diversos estudos verificaram alterações na atividade destas enzimas em modelos animais de crises convulsivas e epilepsia. A indução de epilepsia através de tratamentos com pilocarpina e ácido caínico causou alterações tardias e prolongadas na atividade das ectonucleotidases (Bonan et al., 2000a). Em ratos submetidos ao modelo *kindling* com PTZ, não houve alterações enzimáticas após uma única convulsão, porém após a ocorrência de diversas crises, foi verificado o aumento da hidrólise de ATP (Bonan et al., 2000b). Crises convulsivas sucessivas provocaram o aumento na atividade das ectonucleotidases por até 48 horas em soro de ratos tratados com PTZ. Após uma única crise convulsiva, foi verificado o aumento da atividade dessas enzimas por até 24 horas (Bruno et al., 2002, 2003).

Estudos mostram que fármacos antiepilépticos podem alterar a atividade enzimática das ectonucleotidases. Em nossas análises, a carbamazepina (1000 μ M) inibiu a hidrólise de ATP e a fenitoína (500 e 1000 μ M) aumentou a hidrólise de AMP. A alteração provocada pela carbamazepina coincide com os efeitos verificados por Horvat et al. (2006), que evidenciou a inibição induzida por carbamazepina na atividade *in vitro* das Ecto-ATPases em sinaptossomas cerebrais de ratos. Em modelos *in vivo*, a carbamazepina e a fenitoína preveniram o aumento induzido por

pilocarpina na hidrólise de ATP, ADP e AMP em sinaptossomas de hipocampo e córtex cerebral de ratos. O ácido valpróico impediu o aumento da hidrólise de ATP e ADP em sinaptossomas de hipocampo (Cognato et al., 2007). O aumento na hidrólise de AMP induzida pela fenitoína pode indicar um potencial mecanismo de ação desse fármaco antiepilético. A 5'-nucleotidase tem papel importante na produção de adenosina, e o aumento na sua atividade pode levar à maior produção desse nucleosídeo neuroprotetor (Robson, 2006).

A ação neuroprotetora da adenosina foi verificada há mais de 20 anos, quando estudos mostraram que seus níveis aumentavam rapidamente durante crises convulsivas e contribuíam para o término da convulsão (Dragunow et al., 1985; Dragunow, 1991; During & Spencer, 1992). A adenosina apresenta ação anticonvulsivante principalmente através da ativação de receptores A₁. A ativação desses receptores leva à inibição de canais de Ca²⁺ e à diminuição da liberação de diversos neurotransmissores, principalmente glutamato, dopamina, serotonina e acetilcolina (Fredholm et al., 2005; Boison, 2007).

Devido às promissoras propriedades anticonvulsivantes da adenosina, assim como a importância das ectonucleotidases e da ADA no controle dos níveis desse modulador, consideramos importante o estudo da atividade dessas enzimas em crises convulsivas induzidas por PTZ em peixe zebra. Nossos resultados mostraram que não houve alteração enzimática nas ectonucleotidases e na ADA intracelular. Porém, houve diminuição significativa na hidrólise de adenosina extracelular. Os testes *in vitro* não mostraram alterações de atividade em nenhuma das enzimas. A significativa diminuição na degradação de adenosina verificada em nossos experimentos pode sugerir a ocorrência de modulação extracelular dos níveis de adenosina durante crises convulsivas em peixe-zebra. Além disso, esse resultado está de acordo com estudos que verificaram níveis aumentados de adenosina durante crises convulsivas (During & Spencer, 1992).

Durante a realização desse estudo verificamos que fármacos antiepiléticos podem alterar a atividade *in vitro* das ectonucleotidases e da AChE, demonstrando sua influência nos sistemas purinérgico e colinérgico. Esses resultados sugerem a importância da realização de estudos *in vivo* para o melhor entendimento da ação dos fármacos antiepiléticos nestes importantes sistemas de neurotransmissão.

A ausência de alterações nas atividades enzimáticas das ectonucleotidases após crise convulsiva induzida por PTZ está de acordo com estudos prévios que

observaram aumento na hidrólise de nucleotídeos após crises convulsivas recorrentes, sem verificar alterações após um único episódio. Esses resultados sugerem a ocorrência de adaptações duradouras nas ectonucleotidases, que levam ao aumento da hidrólise de ATP e conseqüente produção de adenosina, mostrando que essas enzimas podem ter um importante papel neuromodulador na epileptogênese (Bonan et al., 2000a,b; Bruno et al., 2002,2003). A inibição da hidrólise extracelular de adenosina verificada em nosso modelo de crise convulsiva enfatiza a ocorrência da modulação dos níveis desse nucleosídeo, levando ao aumento de sua ação inibitória, que impede a propagação das descargas neuronais excessivas, contribui para o término da crise convulsiva e previne a epileptogênese (Fedele et al., 2008; Li et al., 2008).

REFERÊNCIAS

- Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev.* 2006; 58(3):281-341.
- Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signalling in the nervous system: an overview. *Trends Neurosci.* 2009; 32(1):19–29.
- Acharya MM, Hattiangady B, Shetty AK. Progress in neuroprotective strategies for preventing epilepsy. *Prog Neurobiol.* 2008; 84(4): 363–404.
- Alkondon M, Albuquerque EX. The nicotinic acetylcholine receptor subtypes and their function in the hippocampus and cerebral cortex. *Prog Brain Res.* 2004; 145:109-20.
- Ambrósio AF, Silva AP, Malva JO, Soares-da-Silva P, Carvalho AP, Carvalho CM. Carbamazepine inhibits L-type Ca²⁺ channels in cultured rat hippocampal neurons stimulated with glutamate receptor agonists. *Neuropharmacology* 1999; 38(9):1349-59.
- Badawy, RAB, Harvey AS, Macdonell RAL. Cortical hyperexcitability and epileptogenesis: understanding the mechanisms of epilepsy – Part 1. *J Clin Neurosci* 2009; 16: 355-65.
- Bandmann O, Burton EA. Genetic zebrafish models of neurodegenerative. *Neurobiol Dis.* 2010; 40:58-65.
- Banerjee PN, Filippi D, Hauser WA. The descriptive epidemiology of epilepsy. *Epilepsy Res.* 2009; 85(1):31-45.
- Baraban SC, Taylor MR, Castro PA, Baier H. Pentylentetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. *Neuroscience* 2005; 131(3): 759–68.
- Bayer K, Ahmadi S, Zeilhofer HU. Gabapentin may inhibit synaptic transmission in the mouse spinal cord dorsal horn through a preferential block of P/Q-type Ca²⁺ channels. *Neuropharmacology* 2004; 46(5): 743-9.
- Berg AT, Berkovic SF, Brodie MJ, Buchhalter J, Cross JH, van Emde Boas W, Engel J, French J, Glauser TA, Mathern GW, Moshé SL, Nordli D, Plouin P, Scheffer IE. Revised terminology and concepts for organization of seizures and epilepsies: Report of the ILAE Commission on Classification and Terminology, 2005–2009. *Epilepsia* 2010; 51 (4):676–85.
- Berghmans S, Hunt J, Roach A, Goldsmith P. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. *Epilepsy Res.* 2007; 75 (1): 18–28.

Berman RF, Fredholm BB, Aden A, O'Connor WT. Evidence for increased dorsal hippocampal adenosine release and metabolism during pharmacologically induced seizures in rats. *Brain Res.* 2000; 872 (1-2): 44–53.

Bertrand S, Ng GY, Purisai MG, Wolfe SE, Severidt MW, Nouel D, Robitaille R, Low MJ, O'Neill GP, Metters K, Lacaille JC, Chronwall BM, Morris SJ. The anticonvulsant, antihyperalgesic agent gabapentin is an agonist at brain gamma-aminobutyric acid type B receptors negatively coupled to voltage-dependent calcium channels. *J Pharmacol Exp Ther.* 2001; 298(1):15-24.

Bialer M, White HS. Key factors in the discovery and development of new antiepileptic drugs. *Nat Rev Drug Discov.* 2010; 9 (1): 68–82.

Bigonnesse F, Lévesque SA, Kukulski F, Lecka J, Robson SC, Fernandes MJ, Sévigny J. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-8. *Biochemistry* 2004; 43(18):5511-19.

Blanco MM, dos Santos JG Jr, Perez-Mendes P, Kohek SR, Cavarsan CF, Hummel M, Albuquerque C, Mello LE. Assessment of seizure susceptibility in pilocarpine epileptic and nonepileptic Wistar rats and of seizure reinduction with pentylentetrazole and electroshock models. *Epilepsia* 2009; 50(4):824-31.

Boison D, 2005. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. *Neuroscientist* 11 (1): 25–36.

Boison D. Adenosine as a modulator of brain activity. *Drug News Perspect.* 2007; 20(10):607-11.

Bonan CD, Walz R, Pereira GS, Worm PV, Battastini AMO, Cavalheiro EA, Izquierdo I, Sarkis JJF. Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy. *Epilepsy Res.* 2000a; 39 (3): 229-38.

Bonan CD, Amaral OB, Rockenbach IC, Walz R, Battastini AMO, Izquierdo I, Sarkis JJF. Altered ATP hydrolysis induced by pentylentetrazol kindling in rat brain synaptosomes. *Neurochem Res.* 2000b; 25 (6): 775-79.

Bradford Y, Conlin T, Dunn N, Fashena D, Frazer K, Howe DG, Knight J, Mani P, Martin R, Moxon SA, Paddock H, Pich C, Ramachandran S, Ruef BJ, Ruzicka L, Bauer Schaper H, Schaper K, Shao X, Singer A, Sprague J, Sprunger B, Van Slyke C, Westerfield M. ZFIN: enhancements and updates to the zebrafish model organism database. *Nucleic Acids Res.* 2010; 39: 283-89.

Brittijn SA, Duivesteyn SJ, Belmamoune M, Bertens LF, Bitter W, de Bruijn JD, Champagne DL, Cuppen E, Flik G, Vandenbroucke-Grauls CM, Janssen RA, de Jong IM, de Kloet ER, Kros A, Meijer AH, Metz JR, van der Sar AM, Schaaf MJ, Schulte-Merker S, Spaink HP, Tak PP, Verbeek FJ, Vervoordeldonk MJ, Vonk FJ, Witte F, Yuan H, Richardson MK. Zebrafish development and regeneration: new tools for biomedical research. *Int J Dev Biol.* 2009; 53: 835-50.

Bruno AN, Osés JP, Bonan CD, Walz R, Battastini AM, Sarkis JJ. Increase of nucleotidase activities in rat blood serum after a single convulsive injection of pentylentetrazol. *Neurosci Res.* 2002; 43(3): 283-8.

Bruno AN, Osés JP, Amaral O, *et al.* Changes in nucleotide hydrolysis in rat blood serum induced by pentylentetrazol-kindling. *Brain Res Mol Brain Res.* 2003; 114(2): 140-5.

Burne T, Scott A, van Swinderen B, Hilliard M, Reinhard J, Claudianos C, Eyles D, McGrath J. Big ideas for small brains: what can psychiatry learn from worms, flies, bees and fish? *Mol Psychiatry* 2011; 16:7–16.

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

Burnstock G. A basis for distinguishing two types of purinergic receptor. In: Straub RW, Bolis L (Editors), *Cell membrane receptors for drugs and hormones: A multidisciplinary approach.* New York: Raven Press 1978; 107-18.

Burnstock G. Cotransmission. *Curr Opin Pharmacol.* 2004; 4:47-52.

Burnstock G, Knight GE. Cellular distribution and functions of P2 receptor subtypes in different systems. *Int Rev Cytol.* 2004; 240:231–304.

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

Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev.* 2007; 87(2): 659-797.

Burnstock G. Purinergic signalling and disorders of the central nervous system. *Nat Rev Drug Discov.* 2008; 7(7):575-90.

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

Chadwick BP, Frischauf AM. The CD39-like gene family: identification of three new human members (CD39L2, CD39L3, and CD39L4), their murine homologues, and a member of the gene family from *Drosophila melanogaster*. *Genomics* 1998; 50(3):357-67.

Chen GJ, Harvey BK, Shen H, Chou J, Victor A, Wang Y. Activation of adenosine A3 receptors reduces ischemic brain injury in rodents. *J Neurosci Res.* 2006; 84(8):1848-55.

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

- Cognato GP, Bruno AN, da Silva RS, Bogo MR, Sarkis JJF, Bonan CD. Antiepileptic drugs prevent changes induced by pilocarpine model of epilepsy in brain ectonucleotidases. *Neurochem Res.* 2007; 32 (6): 1046–55.
- Cunha RA. Neuroprotection by adenosine in the brain: from A₁ receptor activation to A_{2A} receptor blockade. *Purinergic Signal* 2005; 1 (2): 111–34.
- Cunningham MO, Dhillon A, Wood SJ, Jones RS. Reciprocal modulation of glutamate and GABA release may underlie the anticonvulsant effect of phenytoin. *Neuroscience* 2000; 95(2):343-51.
- D'Antuono M, Kawasaki H, Palmieri C, Cúria G, Biagini G, Avoli M. Antiepileptic drugs and muscarinic receptor-dependent excitation in the rat subiculum. *Neuropharmacology* 2007; 52:1291–1302.
- Dahm R, Geisler R. Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. *Mar Biotechnol (NY)* 2006; 8(4):329-45.
- Dahme T, Katus HA, Rottbauer W. Fishing for the genetic basis of cardiovascular disease. *Dis Model Mech.* 2009; 2(1-2):18-22.
- Dasgupta P, Chellappan SP. Nicotine-mediated cell proliferation and angiogenesis: new twists to an old story. *Cell Cycle* 2006; 5(20):2324-8.
- Dooley DJ, Taylor CP, Donevan S, Feltner D. Ca²⁺ channel $\alpha_2\delta$ ligands: novel modulators of neurotransmission. *Trends Pharmacol Sci.* 2007; 28(2):75-82.
- Dragunow M, Goddard GV, Lavery R. Is adenosine an endogenous anticonvulsant? *Epilepsia* 1985; 26(5):480-7.
- Dragunow M. Adenosine and seizure termination. *Ann Neurol.* 1991; 29(5):575.
- Dudek FE, Staley KJ. How does the balance of excitation and inhibition shift during epileptogenesis? *Epilepsy Curr.* 2007; 7(3):86-8.
- Dudek FE, Sutula TP. Epileptogenesis in the dentate gyrus: a critical perspective. *Prog Brain Res.* 2007; 163:755-73.
- Duncan JS, Sander JW, Sisodiya SM, Walker MC. Adult epilepsy. *Lancet* 2006; 367(9516):1087-100.
- Dupere JR, Dale TJ, Starkey SJ, Xie X. The anticonvulsant BW534U87 depresses epileptiform activity in rat hippocampal slices by an adenosine-dependent mechanism and through inhibition of voltage-gated Na⁺ channels. *Br J Pharmacol.* 1999; 128 (5): 1011-20.
- During MJ, Spencer DD. Adenosine: a potential mediator of seizure arrest and postictal refractoriness. *Ann Neurol.* 1992; 32 (5): 618–24.

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav Brain Res.* 2009; 205(1):38-44.

Elger CE, Schmidt D. Modern management of epilepsy: a practical approach. *Epilepsy Behav.* 2008; 12(4):501-39. Erratum in: *Epilepsy Behav.* 2008; 13(3):575.

Errante LD, Williamson A, Spencer DD, Petroff OA. Gabapentin and vigabatrin increase GABA in the human neocortical slice. *Epilepsy Res.* 2002; 49 (3):203-10.

Etherington LA, Frenguelli BG. Endogenous adenosine modulates epileptiform activity in rat hippocampus in a receptor subtype-dependent manner. *Eur J Neurosci.* 2004; 19(9):2539-50.

Fedele DE, Li T, Lan JQ, Fredholm BB, Boison D. Adenosine A₁ receptors are crucial in keeping an epileptic focus localized. *Exp Neurol.* 2006; 200(1):184-90.

Feitsma H, Cuppen E. Zebrafish as a cancer model. *Mol Cancer Res.* 2008; 6(5):685-94.

Feldman RS, Quenzer LF. *Fundamentals of neuropsychopharmacology.* Sunderland: Sinauer Associates, 1984. 528p.

Fields RD, Burnstock G. Purinergic signalling in neuron-glia interactions. *Nat Rev Neurosci* 2006; 7(6): 423-36.

Fisher RS, van Emde Boas W, Blume W, Elger C, Genton P, Lee P, Engel J. Jr. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia* 2005; 46 (4): 470–2.

Franco R, Casado V, Ciruela F, Saura C, Mallol J, Canela EI, Lluís C. Cell surface adenosine deaminase: much more than an ectoenzyme. *Prog Neurobiol.* 1997; 52 (4): 283-94.

Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and Classification of Adenosine Receptors. *Pharmacol.* 2001; 53 (4): 527-52.

Fredholm BB. Adenosine receptors as targets for drug development. *Drug News Perspect.* 2003; 16(5):283-9.

Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM. Adenosine and brain function. *Int Rev Neurobiol* 2005; 63: 191–270.

Friedman A, Behrens CJ, Heinemann U. Cholinergic dysfunction in temporal lobe epilepsy. *Epilepsia* 2007; 48 (5): 126–30.

- Fujii T, Kawashima K. An independent non-neuronal cholinergic system in lymphocytes. *Jpn J Pharmacol.* 2001; 85(1):11-15.
- Furtado MA, Castro OW, Del Vecchio F, de Oliveira JA, Garcia-Cairasco N. Study of spontaneous recurrent seizures and morphological alterations after status epilepticus induced by intrahippocampal injection of pilocarpine. *Epilepsy Behav.* 2011. [Epub ahead of print]
- Gahring LC, Rogers SW. Neuronal nicotinic acetylcholine receptor expression and function on nonneuronal cells. *AAPS J.* 2006; 7(4):885-94.
- Gilmour DT, Maischein HM, Nüsslein-Volhard C. Migration and function of a glial subtype in the vertebrate peripheral nervous system. *Neuron.* 2002; 34(4):577-88.
- Gouder N, Scheurer L, Fritschy JM, Boison D. Overexpression of adenosine kinase in epileptic hippocampus contributes to epileptogenesis. *J Neurosci.* 2004; 24(3):692-701.
- Goldsmith P. Zebrafish as a pharmacological tool: the how, why and when. *Curr Opin Pharmacol.* 2004; 4: 504–12.
- Grossman L, Utterback E, Stewart A, Gaikwad S, Chung KM, Suciu C, Wong K, Elegante M, Elkhayat S, Tan J, Gilder T, Wu N, DiLeo J, Cachat J, Kalueff AV. Characterization of behavioral and endocrine effects of LSD on zebrafish. *Behav Brain Res.* 2010; 214 (2): 277-84.
- Hamed SA. The aspects and mechanisms of cognitive alterations in epilepsy: the role of antiepileptic medication. *CNS Neurosci Ther.* 2009; 15:134–56.
- Hamilton SE, Loose MD, Qi M, Levey AI, Hille B, McKnight GS, Idzerda RL, Nathanson NM. Disruption of the M1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. *Proc Natl Acad Sci USA* 1997; 94(24):13311-6.
- Hammarberg C, Schulte G, Fredholm BB. Evidence for functional adenosine A₃ receptors in microglia cells. *J Neurochem.* 2003; 86:1051–54.
- Heine P, Braun N, Heilbronn A, Zimmermann H. Functional characterization of rat ecto-ATPase and ecto-ATP diphosphohydrolase after heterologous expression in CHO cells. *Eur J Biochem.* 1999; 262(1):102-7.
- Hortopan GA, Dinday MT, Baraban SC. Zebrafish as a model for studying genetic aspects of Epilepsy. *Dis Model Mech.* 2010; 3 (3-4): 144-8.
- Horvat A, Orlic T, Banjac A, Momic T, Petrovic S, Demajo M. Inhibition of rat brain ecto-ATPase activity by various drugs. *Gene Physiol Biophys.* 2006; 25, 91–105.
- Huber A, Güttinger M, Möhler H, Boison D. Seizure suppression by adenosine A(2A) receptor activation in a rat model of audiogenic brainstem epilepsy. *Neurosci Lett.* 2002; 329(3):289-92.

Hunsucker SA, Mitchell BS, Spychala J. The 5'-nucleotidases as regulators of nucleotide and drug metabolism. *Pharmacol Ther.* 2005; 107(1):1-30.

Hussl S, Boehm S. Functions of neuronal P2Y receptors. *Pflugers Arch.* 2006; 452(5):538-51.

Jensen AA, Mosbacher J, Elg S, Lingenhoehl K, Lohmann T, Johansen TN, Abrahamsen B, Mattsson JP, Lehmann A, Bettler B, Bräuner-Osborne H. The anticonvulsant gabapentin (neurontin) does not act through gamma-aminobutyric acid-B receptors. *Mol Pharmacol.* 2002; 61(6):1377-84.

Jutila L, Immonen A, Partanen K, Partanen J, Mervaala E, Ylinen A, Alafuzoff I, Paljärvi L, Karkola K, Vapalahti M, Pitkänen A. Neurobiology of epileptogenesis in the temporal lobe. *Adv Tech Stand Neurosurg.* 2002; 27:5-22.

Khakh BS, Burnstock G. The double life of ATP. *Sci Am.* 2009; 301(6):84-90, 92.

Kirkpatrick CJ, Bittinger F, Unger RE, Kriegsmann J, Kilbinger H, Wessler I. The non-neuronal cholinergic system in the endothelium: evidence and possible pathobiological significance. *Jpn J Pharmacol.* 2001; 85(1):24-8.

Kozhemyakin M, Rajasekaran K, Kapur J. Central cholinesterase inhibition enhances glutamatergic synaptic transmission. *J Neurophysiol.* 2010; 103(4):1748-57.

Lanneau C, Green A, Hirst WD, Wise A, Brown JT, Donnier E, Charles KJ, Wood M, Davies CH, Pangalos MN. Gabapentin is not a GABA_B receptor agonist. *Neuropharmacol.* 2001; 41(8):965-75.

Latini, S., Pedata, F. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem.* 2001; 79 (3): 463-84.

Lee HT, Kim M, Joo JD, Gallos G, Chen JF, Emala CW. A₃ adenosine receptor activation decreases mortality and renal and hepatic injury in murine septic peritonitis. *Am J Physiol Regul Integr Comp Physiol.* 2006; 291(4):959-69.

Lee Y, Kim D, Kim YH, Lee H, Lee CJ. Improvement of pentylentetrazol-induced learning deficits by valproic acid in the adult zebrafish. *Eur J Pharmacol.* 2010; 643(2-3): 225-31.

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

Li T, Ren G, Lusardi T, Wilz A, Lan JQ, Iwasato T, et al. Adenosine kinase is a target for the prediction and prevention of epileptogenesis in mice. *J Clin Inv* 2008; 118:571–82.

Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet.* 2007; 8 (5): 353–67.

- Löscher W, Schmidt D. New horizons in the development of antiepileptic drugs: the search for new targets. *Epilepsy Res.* 2004; 60(2-3):77-159.
- Maier SA, Galellis JR, McDermid HE. Phylogenetic analysis reveals a novel protein family closely related to adenosine deaminase. *J Mol Evol.* 2005; 61(6):776-94.
- Marino MJ, Rouse ST, Levey AI, Potter LT, Conn PJ. Activation of the genetically defined M1 muscarinic receptor potentiates N-methyl-D-aspartate (NMDA) receptor currents in hippocampal pyramidal cells. *Proc Natl Acad Sci USA.* 1998; 95(19):11465-70.
- Nathanson NM. Synthesis, trafficking, and localization of muscarinic acetylcholine receptors. *Pharmacol Ther.* 2008; 119(1):33-43.
- North RA, Verkhratsky A. Purinergic transmission in the central nervous system. *Pflugers Arch.* 2006; 452(5):479-85.
- Parker DA, Ong J, Marino V, Kerr DI. Gabapentin activates presynaptic GABA_B heteroreceptors in rat cortical slices. *Eur J Pharmacol.* 2004; 495(2-3):137-43.
- Pernot F, Carpentier P, Baille V, Testylier GA, Beaup C, Foqui A, Filliat P, Liscia P, Coutan M, Piérard C, Béracocha D, Dorandeu F. Intrahippocampal cholinesterase inhibition induces epileptogenesis in mice without evidence of neurodegenerative events. *Neurosci.* 2009; 162 (4), 351–65.
- Petroff OA, Hyder F, Rothman DL, Mattson RH. Effects of gabapentin on brain GABA, homocarnosine, and pyrrolidinone in epilepsy patients. *Epilepsia* 2000; 41(6):675-80.
- Piato AL, Capiotti KM, Tamborski AR, Oses JP, Barcellos LJ, Bogo MR, Lara DR, Vianna MR, Bonan CD. Unpredictable chronic stress model in Zebrafish (*Danio rerio*): Behavioral and physiological responses. *Prog Neuropsychopharmacol Biol Psychiatry* 2010. [Epub ahead of print]
- Pirondi S, D'Intino G, Gusciglio M, Massella A, Giardino L, Kuteeva E, Ogren SO, Hökfelt T, Calzà L. Changes in brain cholinergic markers and spatial learning in old galanin-overexpressing mice. *Brain Res.* 2007; 1138:10-20.
- Pitkanen A. Efficacy of current antiepileptics to prevent neurodegeneration in epilepsy models. *Epilepsy Res* 2002; 50:141–60.
- Pitkanen A, Kubova H. Antiepileptic drugs in neuroprotection. *Expert Opin Pharmacother.* 2004; 5:777–98.
- Pitkanen A. Therapeutic approaches to epileptogenesis-Hope on the horizon. *Epilepsia* 2010; 51: 2–17.
- Potier S, Psarropoulou C. Endogenous acetylcholine facilitates epileptogenesis in immature rat neocortex. *Neurosci Lett.* 2001; 302(1):25-8.

- Pugliese AM, Coppi E, Volpini R, Cristalli G, Corradetti R, Jeong LS, Jacobson KA, Pedata F. Role of adenosine A₃ receptors on CA1 hippocampal neurotransmission during oxygen-glucose deprivation episodes of different duration. *Biochem Pharmacol.* 2007; 74(5):768-79.
- Racké K, Juergens UR, Matthiesen S. Control by cholinergic mechanisms. *Eur J Pharmacol.* 2006; 533(1-3):57-68.
- Rakhade SN, Jensen FE. Epileptogenesis in the immature brain: emerging mechanisms. *Nat Rev Neurol.* 2009; 5(7):380-91.
- Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev.* 1998; 50 (3), 413–92.
- Ribeiro JA, Sebastião AM, Mendonça A. Adenosine receptors in the nervous system: pathophysiological implications. *Prog Neurobiol.* 2003; 68 (6): 377–92.
- Ricatti MJ, Alfie LD, Lavoie EG, Sévigny J, Schwarzbaum PJ, Faillace MP. Immunocytochemical localization of NTPDases1 and 2 in the neural retina of mouse and zebrafish. *Synapse* 2009; 63(4):291-307.
- Rico EP, Senger MR, Fauth MG, Dias RD, Bogo MR, Bonan, CD. ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*). *Life Sci.* 2003; 73 (16), 2071–82.
- Rinkwitz S, Mourrain P, Becker TS. Zebrafish: An integrative system for neurogenomics and neurosciences. *Prog Neurobiol.* 2010. [Epub ahead of print]
- Robson SC, Sévigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. *Purinergic Sig* 2006; 2: 409–30.
- Rogawski MA, Löscher W. The neurobiology of antiepileptic drugs. *Nat Rev Neurosci.* 2004a; 5 (7): 553–64.
- Rogawski MA, Löscher W. The neurobiology of antiepileptic drugs for the treatment of nonepileptic conditions. *Nat Med* 2004b; 10(7):685-92.
- Rogawski MA, Bazil CW. New molecular targets for antiepileptic drugs: alpha(2)delta, SV2A, and K(v)7/KCNQ/M potassium channels. *Curr Neurol Neurosci Rep.* 2008; 8(4):345-52.
- Rosemberg DB, Rico EP, Guidoti MR, Dias RD, Souza DO, Bonan CD, Bogo MR. Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (*Danio rerio*). *Life Sci.* 2007; 81 (21–22): 1526–34.
- Rosemberg DB, Rico EP, Senger MR, Dias RD, Bogo MR, Bonan CD, Souza DO. Kinetic characterization of adenosine deaminase activity in zebrafish (*Danio rerio*) brain. *Comp Biochem Physiol B* 2008; 151 (1): 96–101.

Rosemberg DB, Rico EP, Langoni AS, Spinelli JT, Pereira TC, Dias RD, Souza DO, Bonan CD, Bogo MR. NTPDase family in zebrafish: Nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart. *Comp Biochem Physiol B Biochem Mol Biol*. 2010; 155(3):230-40.

Sager JJ, Bai O, Burton EA. Transgenic zebrafish models of neurodegenerative diseases. *Brain Struct Funct*. 2010; 214: 285–302.

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

Senger MR, Rico EP, Dias RD, Bogo MR, Bonan CD. Ecto-5'-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*). *Comp Biochem Physiol B* 2004; 139 (2): 203–7.

Shepard JL, Amatruda JF, Stern HM, Subramanian A, Finkelstein D, Ziai J, Finley KR, Pfaff KL, Hersey C, Zhou Y, Barut B, Freedman M, Lee C, Spitsbergen J, Neuberg D, Weber G, Golub TR, Glickman JN, Kutok JL, Aster JC, Zon LI. A zebrafish *bmyb* mutation causes genome instability and increased cancer susceptibility. *Proc Natl Acad Sci USA* 2005; 102(37):13194-9.

Sills GJ. The mechanisms of action of gabapentin and pregabalin. *Curr Opin Pharmacol*. 2006; 6(1):108-13.

Sitges M, Guarneros A, Nekrassov V. Effects of carbamazepine, phenytoin, valproic acid, oxcarbazepine, lamotrigine, topiramate and vinpocetine on the presynaptic Ca^{2+} channel-mediated release of [3H]glutamate: Comparison with the Na^+ channel-mediated release. *Neuropharmacol*. 2007; 53:854-62.

Soreq H, Seidman S. Acetylcholinesterase: news roles for old actor. *Nat Rev Neurosci*. 2001; (4): 294–302.

Southam E, Stratton SC, Sargent RS, Brackenborough KT, Duffy C, Hagan RM, Pratt GD, Jones SA, Morgan PF. Broad spectrum anticonvulsant activity of BW534U87: possible role of an adenosine-dependent mechanism. *Pharmacol Biochem Behav*. 2002; 74(1):111-8.

Spence R, Gerlach G, Lawrence C, Smith C. The behaviour and ecology of the zebrafish, *Danio rerio*. *Biol Rev*. 2008; 83: 13–34.

Stefan H, Hopfengärtner R. Epilepsy monitoring for therapy: Challenges and perspectives. *Clin Neurophysiol*. 2009; 120: 653–58.

Stewart A, Wu N, Cachat J, Hart P, Gaikwad S, Wong K, Utterback E, Gilder T, Kyzar E, Newman A, Carlos D, Chang K, Hook M, Rhymes C, Caffery M, Greenberg M, Zadina J, Kalueff AV. Pharmacological modulation of anxiety-like phenotypes in adult zebrafish behavioral models. *Prog Neuropsychopharmacol Biol Psychiatry* 2010 [Epub ahead of print]

Tsang M. Zebrafish: A Tool for Chemical Screens. *Birth Defects Research (Part C)* 2010; 90: 185–92.

Wittendorp MC, von Frijtag Drabbe Künzel J, Ijzerman AP, Boddeke HW, Biber K. The mouse brain adenosine A1 receptor: functional expression and pharmacology. *Eur J Pharmacol.* 2004; 487(1-3):73-9.

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

Yang J, Wetterstrand C, Jones RS. Felbamate but not phenytoin or gabapentin reduces glutamate release by blocking presynaptic NMDA receptors in the entorhinal cortex. *Epilepsy Res.* 2007; 77(2-3):157-64.

Yegutkin GG. Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. *Biochim Biophys Acta* 2008; 1783(5): 673-94.

Zimmermann H. Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol.* 2000; 362:299–309.

Zimmermann H. Ectonucleotidases: some recent developments and note on nomenclature. *Drug Dev.* 2001; 52 (1-2): 44-56.

Zimmermann H. Nucleotide signaling in nervous system development. *Eur J Physiol.* 2006; 452:573–88.

Zimmermann G, Soreq H. Termination and beyond: acetylcholinesterase as a modulator of synaptic transmission. *Cell Tissue Res.* 2006; 326: 655–69.

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

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

ANEXO

Comprovante de aprovação do protocolo pelo Comitê de Ética para o Uso de Animais (CEUA) da PUCRS



Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 007/10 – CEUA

Porto Alegre, 07 de janeiro de 2010.

Senhora Pesquisadora:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 09/00117, intitulado: **"Efeito de crises convulsivas e fármacos antiepilépticos em parâmetros neuroquímicos e moleculares em peixe zebra (*Danio rerio*)"**.

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Prof. Dr. Paulo Márcio C. Pitrez
Coordenador-adjunto do CEUA – PUCRS

Ilma. Sra.
Profa. Dra. Carla Denise Bonan
N/Universidade

PUCRS

Campus Central

Av. Ipiranga, 6690 – Prédio 60, sala 314

CEP: 90610-000

Fone/Fax: (51) 3320-3345

E-mail: ceua@pucrs.br