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INTERFERÊNCIAS NA SINALIZAÇÃO ADENOSINÉRGICA DURANTE A EMBRIOGÊNESE ACARRETAM EM ALTERAÇÕES DURADOURAS NA MORFOLOGIA E NA SENSIBILIDADE A PRÓ-CONVULSIVANTES EM PEIXE-ZEBRA (D*anio rerio*)

Porto Alegre
2018

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Interferências na sinalização adenosinérgica durante a embriogênese acarretam em alterações duradouras na morfologia e na sensibilidade a pró-convulsivantes em peixe-zebra (*Danio rerio*)

Orientadora: Profª Drª ROSANE SOUZA DA SILVA

Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Escola de Ciências da Pontifícia Universidade Católica do Rio Grande do Sul.

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Porto Alegre
2018

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“A educação é a arma mais poderosa que você pode usar para mudar o mundo”

Nelson Mandela

Resumo

A epilepsia é a condição neurológica grave de maior incidência no mundo. É caracterizada por crises convulsivas recorrentes, provenientes de descargas neuronais sincrônicas. Distúrbios na sinalização neuronal na fase inicial do desenvolvimento podem acarretar em aumento na suscetibilidade a crises convulsivas na fase adulta, assim como crises convulsivas na fase inicial do desenvolvimento podem acarretar em alterações nos sistemas de neurotransmissão. A sinalização adenosinérgica reconhecidamente é capaz de agir como um anticonvulsivante endógeno, através de sua função neuromoduladora. Perturbações na sinalização adenosinérgica em fases inicias do desenvolvimento acarretam em alterações na suscetibilidade a crises convulsivas de forma condicional ao estágio de desenvolvimento em que a perturbação ocorre e tempo de exposição ao agente perturbador.

Nos quatro capítulos integrantes dessa tese foram abordados, sob diferentes aspectos, fatores que influenciam a susceptibilidade a crise convulsiva provocada pela exposição ao pentilenotetrazol (PTZ) utilizando peixe-zebra. No primeiro capítulo, foi analisada a influência da temperatura na sensibilidade do peixe-zebra ao PTZ, bem como a capacidade do antagonista MK-801 de reverter os efeitos provocados pela hipertermia na suscetibilidade a crises convulsivas induzidas por PTZ. Além de serem verificadas as possíveis diferenças na suscetibilidade a crises convulsivas em função do gênero ou peso.

No segundo capítulo, foi descrito o uso do bloqueio molecular transitório através da técnica de morfolinos para bloquear a tradução dos transcritos correspondentes aos receptores adenosinérgicos A_1 e A_{2A} no inicio da embriogênese. Os animais que sofreram o bloqueio transitório foram avaliados quanto a taxa de sobrevivência e morfologia até os 7 dias pós-fertilização (dpf) e atividade locomotora e suscetibilidade a crises convulsivas provocadas por PTZ aos 7 dpf e na fase adulta.

No terceiro capítulo, foi descrito o uso da técnica de morfolinos para bloquear a tradução dos transcritos correspondentes a enzima ecto-5'-nucleotidase (e5'nt) e transportadores concentrativos de nucleosídeo tipo 2 (CNT2) no inicio da embriogênese. Os animais que sofreram o bloqueio transitório foram avaliados quanto a taxa de sobrevivência e morfologia aos 7 dpf e atividade locomotora e suscetibilidade a crises convulsivas provocadas por PTZ aos 7dpf e na fase adulta.

No quarto capítulo, foi abordado o efeito da microinjeção de 8-Ciclopentil-1,3-dipropilxantina (DPCPX), antagonista do receptor A_1; ZM241385 antagonista do receptor A_{2A}; cafeína, antagonista não-seletivo dos receptores de adenosina; dipiridamol, bloqueador do transportador equilibrativo de nucleosídeo (ENT) e Adenosina 5′-(α,β-metileno)difosfato (AMPCP), inibidor da enzima ecto-5'-nucleotidase, nos ovos do peixe-zebra (1 hora pós-fertilização). Os animais expostos a estes fármacos foram avaliados quanto a taxa de

sobrevivência, morfologia, atividade locomotora aos 7 dpf e suscetibilidade a crises convulsivas provocadas por PTZ aos 7dpf e na fase adulta.

Nossos resultados apontam que a hipertermia aumenta a suscetibilidade do peixe-zebra a crises convulsivas provocadas por PTZ e que esse efeito é prevenido pela administração de MK-801. Além disso, não houve diferença na suscetibilidade do PTZ dependente de gênero ou massa corporal.

Nossos resultados indicam que perturbações na sinalização adenosinérgica através de bloqueio via morfolinos ou nas doses mais altas dos fármacos acima citados, provocaram diminuição na taxa de sobrevivência e altas taxas de alterações morfológicas. Nenhuma das abordagens provocou alterações na atividade locomotora na fase inicial do desenvolvimento, enquanto que na fase adulta foram verificadas alterações pontuais. Aos 7dpf nenhum dos alvos bloqueados por morfolinos provocou alteração na suscetibilidade a crises convulsivas provocadas por PTZ, enquanto que entre os alvos bloqueados por fármacos houve alteração principalmente em animais microinjetados com DPCPX, Cafeína e Dipiridamol. Já na fase adulta todos os alvos bloqueados por morfolinos desencadearam em maior suscetibilidade a crises convulsivas enquanto os bloqueados por fármacos exibiram alterações em doses e estágio de convulsão específicos.

Esses resultados corroboram com uma série de estudos que reportam a importância da sinalização adenosinérgica na fase inicial do desenvolvimento, bem como os efeitos deletérios provenientes de perturbações tanto exógenas quanto endógenas nessa via de sinalização.

Palavras – Chave: Adenosina, Crise convulsiva, Desenvolvimento e Hipertermia

Abstract

Epilepsy is the most serious neurological condition in the world. It is characterized by recurrent seizures from synchronous neuronal discharges. Disturbances in neuronal signaling in the early stages of development may lead to increased susceptibility to seizures in adulthood, as well as seizures in the early stages of development may lead to alterations in neurotransmission systems.

Adenosinergic signaling is known to act as an endogenous anticonvulsant through its neuromodulatory function. Disturbances in adenosinergic signaling in early stages of development lead to changes in the susceptibility to seizures conditionally at the stage of development in which the disturbance occurs, and time of exposure to the disturbing agent.

In the four chapters of this thesis, it was discussed about factors that influence the susceptibility to pentylenetetrazole (PTZ)-induced seizure under different aspects using zebrafish. In the first chapter, it was analyzed the influence of temperature on zebrafish sensitivity to PTZ as well as the ability of the MK-801 antagonist to reverse the effects of hyperthermia on susceptibility to PTZ-induced seizures. In addition, it was verifyed possible differences in the susceptibility to seizures according to gender or weight.

In the second chapter, it was used transient molecular blockade through the morpholine technique to block the translation of the transcripts corresponding to the adenosinergic A_1 and A_{2A} receptors at the beginning of embryogenesis. The animals that underwent transient blockade were evaluated for survival rate and morphology, at 7 days post-fertilization (dpf) and locomotor activity and susceptibility to seizures caused by PTZ at 7 dpf and in adulthood.

In the third chapter, it was used the morpholine technique to block the translation of the transcripts corresponding to the enzyme ecto-5'-nucleotidase and concentrative nucleoside transporters type 2 (CNT2) at the beginning of embryogenesis. The animals that underwent transient blockade were evaluated for survival rate and morphology at 7 days post-fertilization (dpf) and locomotor activity and susceptibility to seizures caused by PTZ at 7 dpf and in adulthood.

In the fourth chapter, it was performed microinjection of the 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), antagonist of A_1 receptor; ZM241385, A_{2A} antagonist; caffeine, non-selective adenosine receptor antagonist; dipyridamole, equilibrative nucleoside transporter blocker (ENT) and Adenosine 5 '- (α, β-methylene) diphosphate (AMPCP), ecto-5'-nucleotidase enzyme inhibitor, in zebrafish eggs (1 hour post -fertilization). The animals exposed to these drugs were evaluated for survival rate, morphology and locomotor activity at 7 dpf and susceptibility to seizures caused by PTZ at 7 dpf and in adulthood.

These results indicated that hyperthermia increases the susceptibility of zebrafish to PTZ-induced seizures and that this effect is prevented by the

administration of MK-801. In addition, there was no difference in susceptibility to PTZ dependent on gender or body mass.

These results indicated that disturbances in adenosinergic signaling through blockade via morpholine or in the higher doses of the drugs mentioned above, caused a decrease in the survival rate and high rates of morphological changes. None of the approaches caused alterations in the locomotor activity in the initial phase of development, whereas in the adult phase, there were occasional changes. At 7dpf, none of the targets blocked by morpholine caused alterations in the susceptibility to seizures caused by PTZ, whereas among the targets blocked by drugs there was alteration mainly in animals microinjected with DPCPX, Caffeine and Dipyridamole. However, in the adult phase all the targets blocked by morpholine triggered in greater susceptibility to seizures, while those blocked by drugs showed changes in specific doses and seizure stage.

These results corroborate a series of studies that report the importance of adenosinergic signaling in the early stages of development as well as the deleterious effects of both exogenous and endogenous perturbations in this signaling pathway.

Key-words: Adenosine; Development; Hyperthermia; Seizure.

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ADA- Adenosina deaminase

ADK – Adenosina quinase

AMP - Adenosina 5´-monofosfato

AMPc - Adenosina 5´-monofosfato cíclico

ATP - Adenosina 5´-trifosfato

CPCA- 5'-(N-ciclopropil)-carboxamido-adenosina

CNT2- Transportador concentrativo de nucleosídeos

DPF- dias-pós-fertilização

Ecto-ADA- Ecto-adenosina deaminase

ENT- Transportador equilibrativo de nucleosídeos

E-NPP - Ectonucleotídeo Pirofosfatase/Fosfodiesterase

E-NTPDases - Ecto-nucleosídeo trifosfato difosfohidrolases

e5’nt - ecto-5’-nucleotidase

GABA – Ácido gama-aminobutírico

HIV- Vírus da imunodeficiência humana

HPF- horas-pós-fertilização

PTZ- Pentilenotetrazol

Sumário

1 Introdução

1.1 Crise Convulsiva

Crises convulsivas são manifestações anormais transitórias da sinalização neurológica causada por despolarização sincrônica, rítmica ou excessiva da rede neuronal (BROMFIELD; CAVAZOS; SIRVEN, 2006; FISHER et al., 2014).

De forma geral, as crises convulsivas podem ser classificadas de acordo com sua abrangência no sistema nervoso central: 1) Focal (parcial) com prevalência em uma área de ativação delimitada a uma pequena porção, geralmente localizada no córtex de um dos hemisférios cerebrais, o que implica em sintomas relacionados a processos efetuados por essa região; 2) Generalizada atinge simultaneamente várias regiões do cérebro nos dois hemisférios cerebrais, caracterizando-se por uma hiperexcitabilidade que abrange todo o cérebro; 3) Não-classificada ou desconhecida, destina-se aos casos em que as outras manifestações são conhecidas, porém o momento inicial da crise é desconhecido (FALCO-WALTER; SCHEFFER; FISHER, 2017; SHORVON, 2011). (Figura 1).

Durante a comunicação sináptica, o balanço entre os sistemas inibitórios e excitatórios garantem ao sistema uma resposta apropriada aos estímulos, sem que haja uma exacerbação na sinalização. Desequilíbrios nas atividades dos sistemas de neurotransmissão são observados durante as crises convulsivas, levando a alterações no fluxo dos íons de sódio, potássio, cloro e cálcio (SOMJEN, 2002). Os neurotransmissores glutamato e ácido gama aminobutírico (GABA) são respectivamente os neurotransmissores excitatórios e inibitórios de maior

abundância no sistema nervoso, sendo assim os maiores responsáveis na manutenção do balanço entre excitação e inibição através do influxo e efluxo de íons (Na^+, K^+, Ca^{2+} ou Cl^-) através de seus receptores ionotrópicos (RAIMONDO et al., 2015). A magnitude dos efeitos de crises convulsivas está diretamente relacionada à plasticidade do sistema nervoso central e a capacidade dos sistemas de neuromodulação em controlar a sinalização exacerbada (BATEUP et al., 2013; ILIE; RAIMONDO; AKERMAN, 2012a; TCHEKALAROVA; KUBOVÁ; MARES, 2007, 2010).

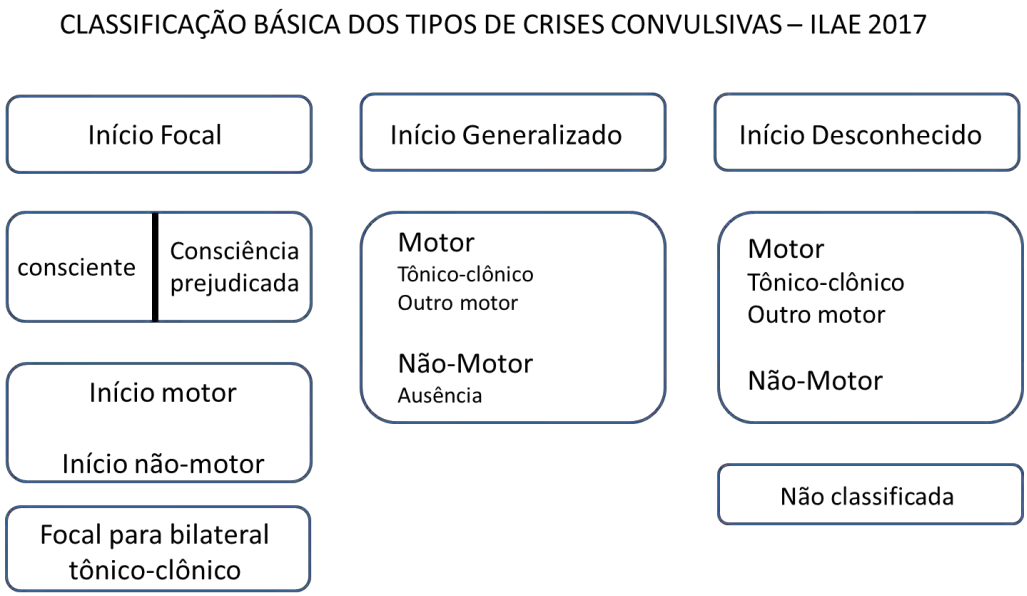

FIGURA 1 - CLASSIFICAÇÃO DOS TIPOS DE CRISES CONVULSIVAS. MODIFICADO DE FALCO-WALTER ET AL. (FALCO-WALTER; SCHEFFER; FISHER, 2017). ILAE-INTERNATIONAL LEAGUE AGAINST EPILEPSY

1.1.1 Epilepsia

Epilepsia é o nome dado para uma doença neural caracterizada predominantemente por crises convulsivas recorrentes e imprevisíveis, capazes de interromper o funcionamento normal do cérebro (FISHER et al., 2005, 2014).

Segundo Falco-Walter e colaboradores, a epilepsia existe quando há uma crise epilética e o cérebro demonstra uma patológica e duradora tendência a ter crises convulsivas recorrente (FALCO-WALTER; SCHEFFER; FISHER, 2017).

A epilepsia está entre as condições neurológicas grave mais comum no mundo, e uma importante causa de mortalidade e incapacidade nos países em desenvolvimento (BURNEO; TELLEZ-ZENTENO; WIEBE, 2005). A epilepsia afeta cerca de 50 milhões de pessoas em todo o mundo, sendo que 80% desses vivem em países em desenvolvimento. Embora em alguns casos o uso de fármacos antiepiléticos não se mostre efetivo contra as crises epiléticas, cerca de 70% dos pacientes que recebem tratamento, apresentam remissão em longo prazo ou ausência de convulsões dentro do prazo de 5 anos após o diagnóstico (MEYER et al., 2010). Apesar do tratamento para epilepsia não apresentar um alto custo e seu diagnóstico não demandar tecnologias avançadas, a maioria dos indivíduos com epilepsia, nos países pobres, não recebe o tratamento adequado (BURNEO; TELLEZ-ZENTENO; WIEBE, 2005; CHISHOLM, 2005; MEYER et al., 2010).

Assim como as crises convulsivas, as epilepsias podem ser classificadas como: 1) parcial (focal); 2) generalizada; 3) uma combinação de parcial e generalizada; e 4) desconhecida. As epilepsias ainda podem ser classificadas de acordo com sua etiologia: 1) Estrutural, causada por uma anormalidade estrutural do cérebro decorrentes de cistos, tumores, entre outros (ETTINGER, 1994); 2) Genética, variações geneticas ou um conjunto de polimorfismos geralmente relacionados a genes codificantes de proteínas fundamentais no processo de neurogênese e formação de rede neural (SÁNCHEZ-CARPINTERO ABAD; SANMARTÍ VILAPLANA; SERRATOSA FERNÁNDEZ, 2007); 3) Metabólica, causada por doenças metabólicas (O'BRIEN, 1998); 4) Imune, mediada por ação de anticorpos

(DEVINSKY; SCHEIN; NAJJAR, 2013); 5) Infecciosa, causada por doenças infecciosas crônicas como HIV e toxoplasmose (VEZZANI et al., 2016); e 6) Não-conhecida, pacientes com etiologia não muito clara (FALCO-WALTER; SCHEFFER; FISHER, 2017).

1.1.2 Crises convulsivas no desenvolvimento do Sistema Nervoso Central

Durante a fase inicial de desenvolvimento do cérebro pode-se destacar processos como proliferação, migração, organização morfofuncional e sinaptogênese (URBÁN; GUILLEMOT, 2014). O glutamato, assim como o GABA, desempenha um papel fundamental, agindo como um fator parácrino, sendo liberado no meio extracelular de forma não convencional, ou seja, não envolvendo uma comunicação sináptica, e se difundindo de forma a atuar na maturação e migração neuronal (MANENT et al., 2005). O equilíbrio entre esses sistemas de neurotransmissão é fundamental nesses processos, e pode sofrer grande influência das atividades moduladoras exercidas por receptores adenosinérgicos (ZIMMERMANN, 2006). Durante a fase inicial de desenvolvimento, o sistema nervoso está passando por um período crítico de maturação e organização da rede neural que, ao ser perturbado, pode acarretar em danos que podem ser permanentes, tais como, atrofia hipocampal retardada e edema hipocampal, além da alteração na motilidade dos dendritos (SHI et al., 2007; VANLANDINGHAM et al., 1998).

Distúrbios na sinalização neuronal em cérebros imaturos, ocasionados por episódios de convulsão, também podem acarretar em uma maior tendência para

desenvolvimento de um quadro epiléptico na fase adulta (DULAC et al., 2007; KOH et al., 1999). Além da suscetibilidade a agentes convulsivos, modelos que sofreram crises convulsivas na fase inicial do desenvolvimento, apresentam algumas disfunções comportamentais, como prejuízo na cognição, memória e um grau maior de ansiedade (HOLMES, 2009; HOLMES et al., 1998; HUANG et al., 2002; SAYIN; SUTULA; STAFSTROM, 2004; SHI et al., 2007).

Existe uma série de diferenças na ocorrência de episódios epilépticos convulsivos em cérebros imaturos e cérebros adultos, como a etiologia, a suscetibilidade e as consequências. Estudos anteriores reportam a existência de uma idade-dependência quanto à ocorrência das crises convulsivas, onde nos primeiros estágios do desenvolvimento há uma maior suscetibilidade que diminui ao longo do desenvolvimento (DULAC et al., 2007). Tanto as causas das convulsões quanto as regiões cerebrais atingidas também diferem entre cérebros imaturos e adultos, podendo ser resultado das mudanças na plasticidade cerebral e ontogenia dos sistemas de neurotransmissão (WEISSINGER et al., 2005). Em modelos animais que mimetizam essa patologia foi observado que diferente do padrão apresentado em animais adultos, animais mais jovens não apresentam redução na proliferação neural, dando indicativos que animais com a rede neural ainda imatura apresentam maior resistência aos danos provocados pela crise convulsiva (HAAS et al., 2001; NITECKA et al., 1984; SADGROVE; CHAD; GRAY, 2005; TANDON et al., 1999).

1.2 Adenosina e crises convulsivas

1.2.1 Adenosina

A adenosina é um nucleosídeo endógeno do grupo das purinas, é constituída de uma adenina e uma ribose (SMOLENSKI et al., 1998). Presente tanto intra quanto extracelularmente em uma variedade de tipos celulares, a adenosina pode desempenhar importante papel como sinalizador metabólico e modulador neuroquímico (CUNHA, 2001; FENSTER et al., 1998).

A sinalização realizada pela adenosina dá-se através de sua ligação aos receptores purinérgicos do tipo P1 (A_1, A_{2A}, A_{2B} e $A_{3)}$ (RIBEIRO; SEBASTIÃO, 2010). Os receptores de adenosina são proteínas transmembrana ligadas a proteínas Gi (inibitórias) ou proteínas Gs (facilitatórias) (CUNHA, 2001; FREDHOLM et al., 2005).

Dentre os quatro subtipos de receptores purinérgicos P1, os receptores A_1 e A_3 são de caráter inibidor e os receptores A_{2A} e A_{2B} facilitador (FREDHOLM et al., 2005). Os receptores A_1 e A_{2A}, além de apresentarem maior distribuição no corpo, apresentam alta afinidade pelo ligante, enquanto os receptores A_{2B} e A_3 são de menor afinidade (SEBASTIÃO; RIBEIRO, 2000). A determinação de prevalência de ação inibitória ou facilitatória da adenosina é dependente de sua concentração no meio extracelular. Receptores A_1 são predominantemente ativados em concentrações basais de adenosina enquanto que a facilitação exercida pelos receptores A_{2A} prevalece em concentrações mais elevadas de adenosina, uma consequência dos valores distantes de afinidade pela adenosina para cada receptor, 70 nM e 150 nM, respectivamente (CORREIA-DE-SÁ; TIMÓTEO;

RIBEIRO, 1996; DUNWIDDIE; MASINO, 2001). A disponibilidade de adenosina extracelular pode ser mediada pela cascata da hidrólise do ATP, ADP e AMP até adenosina realizada por ação das enzimas da família das ecto-nucleosídeo trifosfato difosfohidrolases (E-NTPDases 1, 2, 3 e 8) que se encontram ancoradas na membrana celular, possuindo seu sítio ativo voltado para o meio extracelular, ectonucleotídeo pirofosfatase/fosfodiesterase (E-NPP), fosfatase alcalina, e como último passo a Ecto-5'-nucleotidase responsável pela desfosforilação do nucleotídeo monofosfatado, AMP, gerando fosfato livre e adenosina (ROBSON; SÉVIGNY; ZIMMERMANN, 2006; ZIMMERMANN; ZEBISCH; STRÄTER, 2012). Os transportadores de nucleosídeos equilibrativos (ENT) transportam a adenosina de forma bidirecional e independente de concentração, podendo tanto liberar quanto recaptar a adenosina (DUNWIDDIE; DIAO, 2000; PINTO-DUARTE et al., 2005; YOUNG et al., 2008). Além de transportadores concentrativos (CNT2) responsáveis pela recaptação de adenosina para o meio intracelular (GRAY; OWEN; GIACOMINI, 2004). A recaptação da adenosina para o meio interno pode ser influenciada por ação da adenosina quinase (ADK) que fosforila a adenosina a AMP (ETHERINGTON et al., 2009; PARK; GUPTA, 2008) ou por ação da adenosina deaminase (ADA) convertendo adenosina em inosina, diminuindo assim a concentração intracelular de adenosina, o que provoca a recaptação para o restabelecimento do equilíbrio das concentrações intra e extracelulares (ANTONIOLI et al., 2012). Além da recaptação da adenosina, esta pode ser hidrolisada por ação de uma ecto-adenosina deaminase (Ecto-ADA), a qual converte extracelularmente a adenosina à inosina (GRACIA et al., 2011). (Figura 2).

Alguns estudos reportam ainda que o receptor a ser ativado pela adenosina pode sofrer influência do mecanismo provedor de adenosina no meio extracelular (MELANI et al., 2012). Sendo que, sob condições basais, inibidores da enzima ecto-5'-nucleotidase acarretam em aumento dos níveis de ATP, sem diminuir a concentração de adenosina no meio extracelular, a qual é mantida via transportadores bidirecionais ENTs, além de ativar preponderantemente receptores A_1 (MELANI et al., 2012; WALL; DALE, 2008). Sob condições de hipóxia ou de isquemia as concentrações extracelulares de adenosina exibem um aumento significativo, tendo como sua principal fonte a cascata de hidrolise de ATP, realizada pelas enzimas E-NTPDases, das quais a enzima ecto-5'-nucleotidase é a considerada a enzima marca-passo da formação de adenosina (HART et al., 2008; PEDATA et al., 2001; ZIMMERMANN, 2001, 2006). Altas concentrações de adenosina no meio extracelular geram significativo aumento da ativação dos receptores A_{2A} e que em alguns tecidos como estriado e válvulas aórticas esta maior ativação está vinculada com a relação de proximidade entre a ecto-5'-nucleotidase e o receptor A_{2A} (AUGUSTO et al., 2013; MAHMUT et al., 2015; PEDATA et al., 2001).

Uma das principais funções celulares desempenhadas pela adenosina é a de mensageiro intracelular de desbalanço energético, tendo assim um importante papel homeostático (CUNHA, 2001; YAO et al., 2010). A adenosina tem sua formação determinada pelo balanço entre as reações que formam e utilizam ATP, principal moeda energética do metabolismo, sendo assim, a concentração intracelular de adenosina é consequência do estado metabólico (LOMAX; HENDERSON, 1973).

No sistema nervoso, a adenosina atua como um neuromodulador capaz de influenciar diretamente na sinalização exercida por alguns neurotransmissores como dopamina, serotonina, acetilcolina e glutamato, e na diminuição da atividade neural via hiperpolarização pós-sináptica (DUNWIDDIE; MASINO, 2001). A inibição da excitabilidade neuronal e transmissão sináptica, exercida via receptores A_1, pode ser realizada na região pré-sináptica, inibindo a entrada de cálcio, evitando assim a liberação de neurotransmissores para a fenda (SEBASTIÃO; RIBEIRO, 2000). Outra forma de inibição da excitabilidade é via terminais pós-sinápticos, abrindo os canais de potássio, levando assim a uma hiperpolarização (SEBASTIÃO; RIBEIRO, 2009a, 2009b). Por estarem presentes em uma maior quantidade de estruturas neurais, as ações inibitórias da ativação de receptores A_1 parecem ser preponderantes (DIXON et al., 1996). No entanto, a ativação de receptores A_{2A} exerce grande influência na neuromodulação, através da ativação da Adenilato ciclase, aumento do influxo de cálcio e ativação paralela da Proteína quinase C (DIXON et al., 1996). De fato, na região cerebral estriatopalidal, a abundância dos receptores A_{2A}, em neurônios gabaérgicos, promove preponderância da ação da adenosina neste subtipo de receptor (SEBASTIÃO; RIBEIRO, 1996). Quando ambos receptores estão presentes na mesma terminação sináptica pode ocorrer formação de heterômeros, em tal situação o receptor A_{2A} pode agir como um modulador dos receptores A_1, e assim, diminuir a afinidade do receptor A_1 pelo ligante (CIRUELA et al., 2006; FERRE et al., 2008). Tais interações estruturais para o controle da neurotransmissão não estão restritas aos receptores de mesmo ligante. A formação de heterômeros envolvendo receptores P1 inclui os heterômeros A_{2A}/D_2 e $A_{2A}/mGlu_5$, os quais tem a afinidade

por seus ligantes modificada devido a ativação dos receptores A_{2A} (FERRÉ et al., 2007a, 2007b).

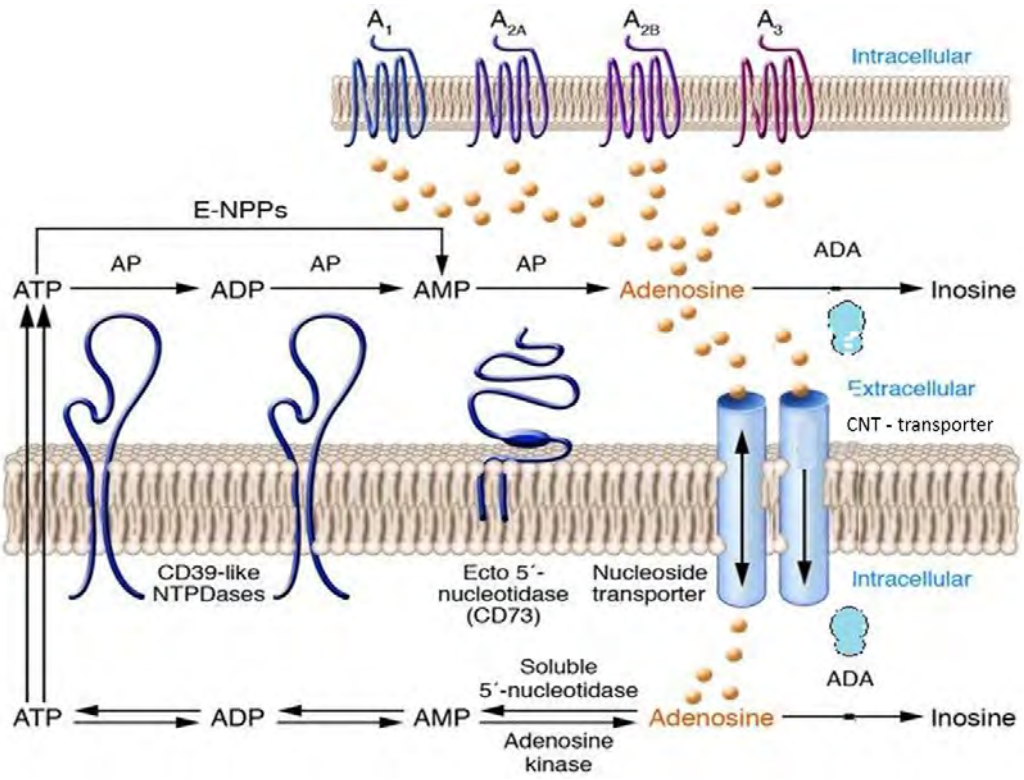

FIGURA 2 - MECANISMOS ENVOLVIDOS NA CONCENTRAÇÃO EXTRACELULAR DE ADENOSINA. MODIFICADO DE TILLEY E BOUCHER (TILLEY; BOUCHER, 2005)**.**

1.2.2 Adenosina e sua influência no desenvolvimento

A fase inicial do desenvolvimento embrionário é uma delicada e suscetível janela temporal a qual intervenções causadas por agentes exógenos ou endógenos podem acarretar em alterações que podem ser de natureza morfológica, cognitiva ou fisiológica (LANE; GARDNER, 2005; SHIOTA, 2009).

Estas alterações, em sua maioria de caráter considerado negativo, podem ser de pequena escala, sendo quase que indetectáveis ou de grande escala, podendo perdurar até a fase adulta ou acarretar em interrupção do desenvolvimento do embrião (GLOVER; AHMED-SALIM; CAPRON, 2016; PERERA; HERBSTMAN, 2011). Eventos celulares que são altamente relacionadas com o desenvolvimento normal do embrião, tais como síntese proteica, diferenciação celular, metabolismo e dinâmica do citoesqueleto, sofrem grande influência da regulação homeostática, regulação essa em grande parte controlada pela ação da adenosina (LANE; GARDNER, 2000, 2005; RIVKEES; WENDLER, 2017; RIVKEES et al., 2001). As perturbações capazes de alterar a sinalização adenosinérgica durante a fase embrionária podem ser tanto de natureza endógena quanto exógena, tais como estresse, consumo de álcool ou altas doses de cafeína (LUTTE et al., 2015; RIVKEES et al., 2001; SILVA et al., 2013; ZHAO; RIVKEES, 2001)

1.2.3 Sinalização adenosinérgica na crise convulsiva

Os efeitos anticonvulsivantes mediados por receptores adenosinérgicos foram observados quando o uso de agonistas ou antagonistas adenosinérgicos diminuíram ou retardaram os efeitos produzidos por fármacos pró-convulsivantes (GEORGIEV; JOHANSSON; FREDHOLM, 1993; GUILLET; DUNHAM, 1995). Além disto, foi visto que a administração de adenosina exógena diminuiu a extensão das descargas neuronais pós-crises, por atenuar a despolarização de membrana provocada por receptores $GABA_A$ (ILIE; RAIMONDO; AKERMAN, 2012b). A ativação dos receptores A_{2A}, pelo agonista 5'-(N-ciclopropil)-carboxamido-adenosina (CPCA), suprime os efeitos convulsivos em ratos propensos a crises

epiléticas por modificação genética (HUBER et al., 2002). A exposição a antagonistas purinérgicos, como a cafeína, durante a fase inicial de desenvolvimento, parece ter uma variada gama de fatores que podem influenciar em seus efeitos tardios, além de provocar diferentes graus de sensibilidade de acordo com o pró-convulsivante utilizado na fase adulta (GUILLET; DUNHAM, 1995). Outros fatores como a fase de desenvolvimento e o tempo de exposição a antagonistas adenosinérgicos podem determinar o tipo de resposta obtida na fase adulta, podendo aumentar a sensibilidade a convulsivantes ou aumentar o limiar de tolerância a estes (GEORGIEV; JOHANSSON; FREDHOLM, 1993; GUILLET, 1995; SZOT; SANDERS; MURRAY, 1987)

A redução na concentração de adenosina na fenda sináptica através do uso de um inibidor da enzima ecto-5'-nucleotidase promove um efeito pró-convulsivante (ZHANG; FRANKLIN; MURRAY, 1993). Ainda assim, após a indução de crises convulsivas por terapia eletroconvulsiva ou utilizando pentilenotetrazol (PTZ), não foi observada nenhuma mudança na atividade de hidrólise de AMP (SIEBEL et al., 2011; ZHANG; FRANKLIN; MURRAY, 1993). No entanto, em avaliações de sinaptossomas de hipocampo e córtex de ratos que sofreram indução de crises convulsivas por exposição à pilocarpina ou ácido caínico foi observado um aumento na atividade da enzima ecto-5'-nucleotidase (BONAN et al., 2000; DE PAULA COGNATO et al., 2005)

Os níveis de adenosina na fenda sináptica podem variar conforme a região do cérebro e o estado metabólico (AUGUSTO et al., 2013). Sob condições fisiológicas, a adenosina disponível na fenda pode ter origem via transportadores equilibrativos, através da cascata de hidrólise de ATP e mais recentemente tem

sido sugerido que a adenosina pode ser liberada de forma vesicular (CORTI et al., 2013; MELANI et al., 2012), enquanto que, durante situações de estresse fisiológico, como isquemia e crises convulsivas epiléticas, a adenosina liberada na fenda é preponderantemente fruto da hidrólise do ATP por ectoenzimas (AUGUSTO et al., 2013; MELANI et al., 2012).

Em crises convulsivas provocadas por hipertermia foi observada uma variação na quantidade de receptores de adenosina (LEÓN-NAVARRO; ALBASANZ; MARTÍN, 2015). Entretanto, em modelos de crise convulsivas utilizando ácido caínico foi observada uma diminuição dos receptores A_1 na região CA1 e CA3 do hipocampo de ratos e nenhuma diferença significativa em outras áreas (EKONOMOU et al., 2000). Já em modelos de crises convulsivas, utilizando pentilenotetrazol, a densidade de receptores A_1 teve um aumento na região hipocampal e uma diminuição na região do estriado (ANGELATOU; PAGONOPOULOU; KOSTOPOULOS, 1991). Além disto, a bicuculina, um antagonista dos receptores $GABA_A$, foi capaz de diminuir densidade dos receptores de adenosina A_{2A} em ratos (DORIAT et al., 1999).

1.3 Modelos de Crise convulsiva em peixe-zebra

A magnitude das crises convulsivas em animais modelos geralmente são medidas pela perda neuronal, eletroencefalogramas ou por escores comportamentais, que se sucedem progressivamente conforme a intensidade da crise (imobilidade, rigidez, movimentos circulares, clônus dos membros anteriores, quedas e Tônico-clônico) (MCKHANN et al., 2003). Por um longo tempo, roedores vem sendo usados para modelar a patologia da epilepsia, no entanto, em estudos

recentes, o peixe-zebra, tanto na fase larval quanto na adulta, tem se mostrado efetivo em virtude dos fenótipos comparáveis a mamíferos (Tabela 1) (ALFARO; RIPOLL-GÓMEZ; BURGOS, 2011; STEWART et al., 2012). O uso do peixe-zebra como animal modelo de crise epilética convulsiva tem se mostrado uma promissora ferramenta para o entendimento dos mecanismos bioquímicos, celulares e moleculares envolvidos na gênese dessa patologia.

TABELA 1 - EXEMPLOS DE FENÓTIPOS REGISTRADOS EM MODELOS DE ROEDORES E PEIXE-ZEBRA RELACIONADOS À EPILEPSIA EM HUMANOS. MODIFICADO DE STEWART ET AL. (STEWART ET AL., 2012)

Quadro Clínico	Modelos em roedores	Modelos em peixe-zebra
Sintomas Neurofisiológicos - Hiperatividade cerebral	Resposta neurofisiológica aumentada em ratos e camundongos Aumento da expressão de *c-fos* em cérebro de ratos e camundongos	Resposta neurofisiológica aumentada em larvas e adultos Aumento da expressão de *c-fos* em encéfalo de larvas e adultos
Sintomas Comportamentais - Convulsões/Crises	Crise convulsiva em ratos e camundongos	Hiperatividade/comportamento convulsivo em larvas e adultos
Prejuízos Comportamentais	Perda da postura em camundongos e ratos Epilepsia não-motora e crise de ausência em ratos e camundongos	Imobilidade com perda de postura corporal e insensibilidade ao toque

O peixe-zebra exibe um repertório de respostas a indutores de convulsão que podem facilmente ser comparados com as respostas observadas em roedores. O antagonista GABAérgico, pentilenotetrazol, é o fármaco mais utilizado para indução de crises convulsivas em peixe-zebra, tendo sido caracterizado em uma série de

trabalhos, onde foi possível determinar diferentes graus de severidade das crises ao longo do tempo de exposição (BARABAN et al., 2005; DA SILVA; BONAN; VIANNA, 2016; KUNDAP et al., 2017; MUSSULINI et al., 2013). Outros pró-convulsivantes classicamente utilizados em mamíferos também se mostraram eficientes na indução de crises convulsivas em peixe-zebra. Animais expostos ao ácido caínico, via injeção intraperitoneal, exibiram um repertório comportamental bem semelhante aos observados com indução via PTZ, com o grau de severidade e o tempo para alcançar cada estágio convulsivo seguindo uma escala progressiva de acordo com a dose aplicada (ALFARO; RIPOLL-GÓMEZ; BURGOS, 2011; MENEZES; RICO; DA SILVA, 2014).

Larvas expostas ao ácido domóico, um agonista do receptor caínico, durante a primeira hora pós-fertilização, exibiram maior suscetibilidade a crises convulsivas provocadas por exposição ao PTZ aos 7 dias-pós-fertilização (dpf) (TIEDEKEN; RAMSDELL, 2007). Têm sido evidenciado a existência de um estágio específico no desenvolvimento em que larvas pré-expostas a doses sub-convulsivantes de ácido caínico exibiram maior resistência a doses convulsivantes de ácido caínico na fase adulta (MENEZES; RICO; DA SILVA, 2014).

A exposição do peixe-zebra a estímulos convulsivantes durante a fase inicial de desenvolvimento tem exibido uma variada gama de respostas, que podem diferir em virtude da fase do desenvolvimento escolhida, tamanho da janela de exposição, agente convulsivante, entre outros fatores (BARABAN et al., 2005; HUNT et al., 2012b; MENEZES; RICO; DA SILVA, 2014). Modelos de indução à convulsão por hipertermia em larvas de peixe-zebra mimetizam o mais comum tipo de crise convulsiva ocorrente em crianças e demonstram através de registros eletroencefalográficos uma resposta idade-dependente (HUNT et al., 2012b).

2 Objetivo

2.1 Objetivo Geral

Avaliar os efeitos do bloqueio da sinalização adenosinérgica na fase inicial de desenvolvimento do peixe-zebra sobre o estágio convulsivo na fase larval e adulta e avaliar se tais intervenções alteram parâmetros morfológicos e locomotores.

2.1.1 Objetivos Específicos

Avaliar a influência do gênero e da massa corporal na suscetibilidade do peixe-zebra a crises convulsivas mediada por pentilenotetrazol.

Avaliar a influência da temperatura na suscetibilidade do peixe-zebra a crises convulsivas mediada por pentilenotetrazol.

Avaliar o efeito do pré-tratamento com antagonistas glutamatérgicos sobre a suscetibilidade do peixe-zebra a crises convulsivas mediada por pentilenotetrazol em temperatura elevada.

Avaliar os efeitos do bloqueio da tradução de receptores adenosinérgicos A_1 e $A_{2A,}$ da ecto-5'-nucleotidase e do transportador concentrativo de nucleosídeo em embriões de peixe-zebra sobre a taxa de sobrevivência, morfologia geral e locomoção na fase larval e adulta.

Avaliar os efeitos do bloqueio da tradução de receptores adenosinérgicos A_1 e $A_{2A,}$ da ecto-5'-nucleotidase e do transportador concentrativo de nucleosídeo em embriões de peixe-zebra sobre a convulsão mediada por pentilenotetrazol na fase larval e adulta.

Avaliar os efeitos do antagonismo de receptores adenosinérgicos A_1 e $A_{2A,}$ da inibição da ecto-5'-nucleotidase e da inibição do transportador equilibrativo de nucleosídeo em embriões de peixe-zebra sobre a convulsão mediada por pentilenotetrazol na fase larval e adulta.

3 Resultados

3.1 Capítulo I

Artigo Científico Original

The influence of temperature on adult zebrafish sensitivity to pentylenetetrazole.

Autores: Fabiano Peres Menezes, Rosane Souza Da Silva

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The influence of temperature on adult zebrafish sensitivity to pentylenetetrazole

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ABSTRACT

Pentylenetetrazole (PTZ) is one of the most valuable drugs used to induce seizure-like state in zebrafish especially considering the pharmacological screening for anticonvulsants and the study of basic mechanisms of epilepsy. Here, the effect of gender, weight and changes in temperature on latency to adult zebrafish reach classical seizure states induced by PTZ (10 mM) was evaluated. Gender and weight (200–250 mg *versus* 400–500 mg) did not affect the profile of response to PTZ. When water temperature was changed from 22 to 30 °C the lower temperature increased the latency time to reach seizure states and the higher temperature significantly decreased it, in comparison to the control group maintained at 26 °C. The blockage of kainate receptors by DNQX (10 μM) were unable to prevent the increased susceptibility of adult zebrafish exposed to hyperthermia and PTZ-induced seizures. The NMDA block by MK-801 (2.5 μM) prevented the additive effect of hyperthermia on PTZ effects in adult zebrafish. This report emphasize that PTZ model in adult zebrafish exhibits no confounder factors from gender and weight, but water temperature is able to directly affect the response to PTZ, especially through a mechanism related to NMDA receptors.

1. Introduction

The search for models that mimic diseases and/or manifestation of symptoms observed in specific diseases has been the subject of many studies, since as more accurate the model becomes more effective is that tool to the search for treatments. In diseases such as epilepsy, which affects approximately 50 million people in worldwide (Prilipko et al., 2005; World Health Organization, 2016), there is a number of models that seek to emulate in a reliable way the characteristics found in this disease (Albala et al., 1984; Bonan et al., 2000; Haas et al., 2001; Porter et al., 2006).

The use of zebrafish to model seizure has proved to be very efficient as regards the understanding of the various mechanisms involved in the etiology of this pathology, by the possibility of use in large-scale of the genetic and pharmacologic screening (Cunliffe, 2016; Hortopan et al., 2010). Pentylenetetrazole (PTZ) is the widest validated pharmacological proconvulsant used in zebrafish to perform anticovulsivant screenings and elucidate the pathogenetic mechanism of epilepsy (Baraban et al., 2005; Baxendale et al., 2012; Cunliffe, 2016; Da Silva et al., 2016) PTZ-based models offer behavioral changes similar to clonic-like convulsion (Mussulini et al., 2013; Orellana-paucar et al., 2013), epileptiform discharges (Baraban et al., 2005), reduced neurogenesis (Kim et al., 2010) and c-*fos* expression (Baraban et al., 2005; Baxendale et al., 2012). The basis of PTZ mechanism is the block of GABAA receptors, which is soon expressed in zebrafish embryos promoting responsiveness to PTZ as early as 50 hours post-fertilization (Baxendale et al., 2012).

To promote accuracy in the translation of research findings using zebrafish, several particularities of the zebrafish biology, and also must be considered. Here, we evaluated the effect of gender, weight and changes in water temperature on PTZ-sensitivity of adult zebrafish. Additionally, we evaluated the influence of antagonists of glutamatergic receptors on the PTZ effects under hyperthermia conditions.

2. Methods

2.1. Animals

All animals were from the local breeding of Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. They were kept on a shelf of aquariums with circulating water system and automated controllers of water quality ZEBTEC (Tecniplast Group Buguggiate (VA), Italy). The light/dark cycle was 14/10 h, the water temperature was 27 °C ± 1 and a maximum density of 5 animals/L was maintained. A total of 169 animals were used in the range of 5-7 months post-fertilization. No deaths were registered during the experi-

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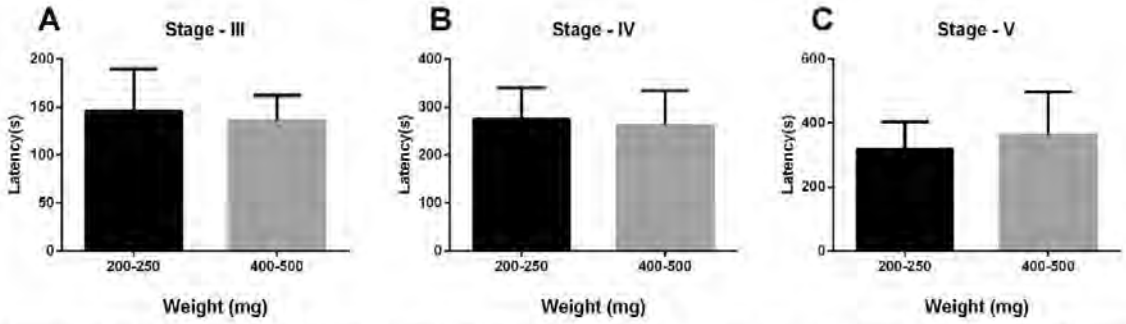

Fig. 1. Latency time of male zebrafish weighted 200–250 mg (N = 13) and 400–500 mg (N = 10) to reach the seizure stages caused by exposure to PTZ (10 mM) at 26 °C. Latency times to reach the stage III (A), IV (B) and V (C) of seizure are expressed as mean ± C.I. N represents the number of animals per group.

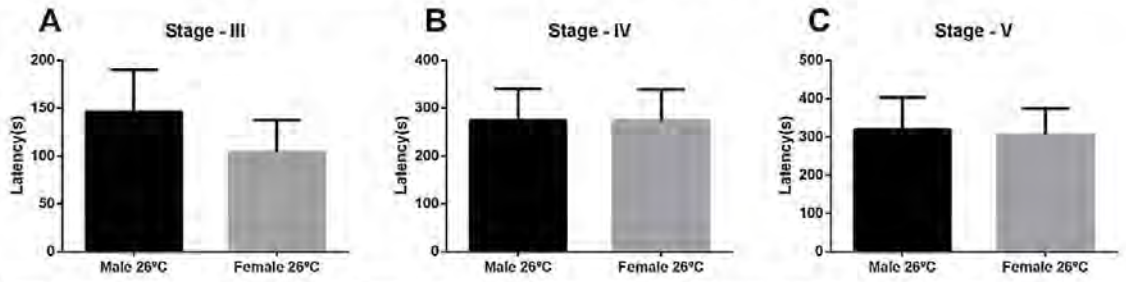

Fig. 2. Latency time of male (N = 13) and female (N = 10) zebrafish to reach the seizure stages caused by exposure to PTZ (10 mM) at 26 °C. Latency times of animals to reach the stage III (A), IV (B) and V (C) of seizure are expressed as mean ± C.I. N represents the number of animals per group.

ments. All animal experiments were conform with the ARRIVE guidelines and carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the Brazilian legislation. The experimental protocols were approved by the Institutional Animal Care Committee of PUCRS registered under the protocol number 14/00416 – CEUA/PUCRS.

2.2. PTZ exposure

The exposure to PTZ (10 mM) (Sigma-St. Louis, Missouri, EUA) was performed by 10 minutes in an aquarium in dimensions 13 × 11.5 × 8 cm (length × height × width) and volume of 500 mL. Control animals were kept in the water system free of drugs. PTZ exposure was performed between females *versus* males with similar weight (200–250 mg), males weighted 200–250 mg *versus* males weighted 400–500 mg and males (200–250 mg) in three water temperatures (22, 26 and 30 °C). The temperature was monitored with a digital thermometer and the solution was changed after each test. To check if the highest temperature was able to promote convulsive behavior *per se*, an additional control group was kept at 30 °C without the presence of PTZ.

2.3. Seizure scores

The differences in susceptibility to PTZ seizure were measured by the latency (seconds) for the animals reach the three most obvious seizure stages observed in zebrafish, standardized by Mussulini et al. (2013) as stage III: a circular motion; stage IV: behavior convulsive clonic type; and stage V: fall to the bottom of the aquarium and convulsive behavior of tonic type. The animals were exposed to PTZ until to reach the score V, or up to 10 minutes of exposure.

2.4. Pretreatment with antagonist of glutamatergic receptors

To assess the role of NMDA and Kainic receptors in PTZ-induced seizure in combination with hyperthermia, two groups were considered, the animals pretreated with NMDA receptor antagonist, MK-801 (2.5 μM), and animals pretreated with Kainic receptor antagonist, DNQX (10 μM) (Sigma-St. Louis, Missouri, EUA). The exposure was carried out for 10 minutes in an aquarium in the dimensions of 13 × 11.5 × 8 cm containing 400 ml of solution of MK-801, DNQX or water-free drug at 26 °C. Locomotor activity during the pretreatment period was recorded by Logitech cam (Romanel-sur-Morges, Switzerland), located frontally to the apparatus. The analysis of locomotor activity was performed considering the digitally division of aquarium into upper and lower part, using any-maze software. The analyzed period was 5 minutes between the third and the eighth minute of exposure. The parameters measured were; Average speed (m/s), distance traveled (m) and time at the bottom (s) of the aquarium.

After 10 minutes of pretreatment, the animals were transferred immediately to the aquarium containing the PTZ solution (10 mM) at temperatures of 26 °C or 30C. The analysis of seizure score was performed as described above. For these tests only males between 200–250 mg were used.

2.5. Statistical analysis

Statistical tests were performed using Graphpad-Prism software version 6.0 (La Jolla, CA 92037 USA). Normality test was performed in all groups through the test D'Agostino & Pearson normality test. To check for significant differences between groups One-way-ANOVA was used followed by Dunnett's test post-hoc for multiple comparisons. To test difference among the genders or weights unpaired t-test was used with Welch's correction. Statistical significant levels considered $p < 0.05$. Values were expressed as means with confidence intervals.

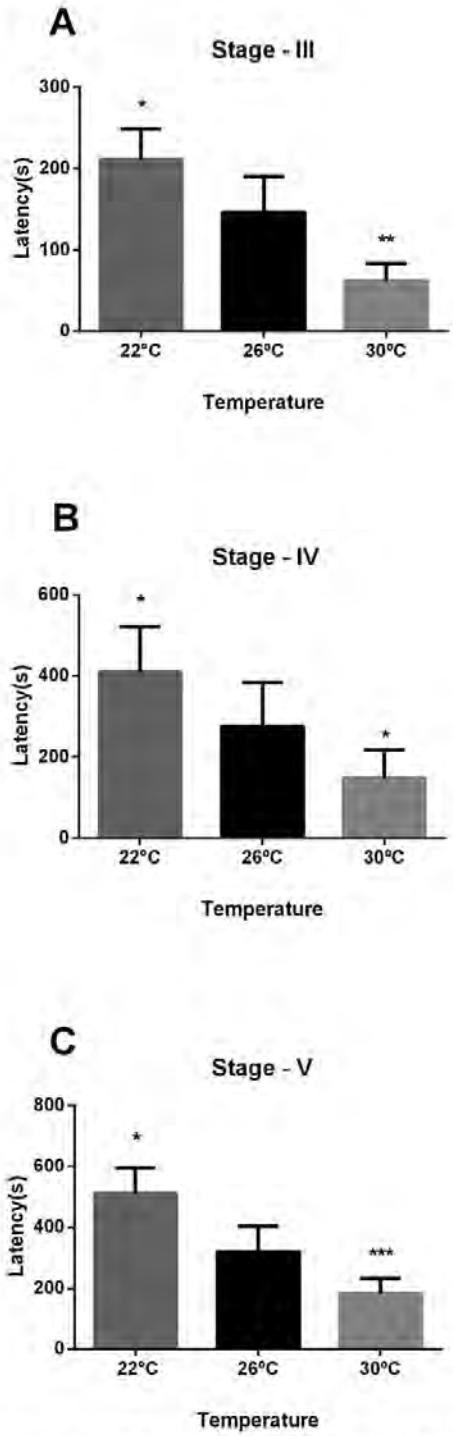

Fig. 3. Latency time to reach the seizure stages caused by exposure to PTZ (10 mM) at different temperatures. (A) Latency time of animals to reach the stage III of seizure. Temperatures 22 °C (N = 10; p = 0.011) and 30 °C (N = 10; p = 0.003) were compared to the standard temperature (26 °C, N = 13); (B) Latency time for stage IV at 22 °C (p = 0.018) and 30 °C (p = 0.018) compared to 26 °C; (C) Latency time for the stage V at 22 °C (p = 0.015) and 30 °C (p = 0.0009) compared to 26 °C. The bars represent the mean ± C.I. N represents the number of animals per group. * means statistical significance at $p < 0.05$, ** means statistical significance at $p < 0.01$, *** means statistical significance at $p < 0.001$.

3. Results

In order to check if animal weight or gender affects the sensitivity to PTZ, males weighted 200–250 mg and 400–500 mg were compared, and males compared to females with the same weight (200–250 mg). No significant difference in the time required to reach the stages of seizure was observed between animals from different gender or weight (Figs. 1 and 2).

The time latency for zebrafish achieve seizure stages caused by exposure to PTZ was directly influenced by the decrease and the increase in water temperature. Animals that were exposed to PTZ at 22 °C showed greater resistance, having tolerated longer without showing seizure stages. The mean latency times to reach the stage III, IV and V of animals submitted to 22 °C were 226 ± 38.3, 409.8 ± 111.2 and 510.1 ± 83.8 seconds (s), respectively in comparison to 26 °C (Fig. 3). During the tests, 50% of animals exposed to PTZ at 22 °C reached the set time limit (600 s) without displaying the stage V. At 30 °C, the average times to reach the stages III, IV and V were 61.7 ± 21.19, 149.2 ± 48.8 and 182.3 ± 50.9 s, respectively, for both temperature in stage III [$F_{(2,30)} = 16,99$], stage IV [$F_{(2,30)} = 12,75$] and stage V [$F_{(2,30)} = 20,42$], ($p < 0,001$) significantly less when compared to the group at 26 °C (Fig. 3). None of the tested animals exposed to water free of drugs at 30 °C showed any standardized convulsion stages (Data not shown).

In the test with MK-801 and DNQX gave to animals previously to the exposure to PTZ at 26 °C was observed that DNQX did not present any significant effect on locomotor activity (distance traveled and average speed) and anxiety parameter (time at the bottom of aquarium). MK-801 caused significant changes in locomotor and behavioral parameters. The animals exposed to MK-801 increased significantly the distance travelled (11.57 ± 1.9 m) in comparison to animals exposed to drug-free water (8.54 ± 0.9 m) [$p < 0.05$; $F_{(2,28)} = 4.723$] (Fig. 4A). In addition, they also had a significant increase in mean velocity (0.039 ± 0.006 m/s) in relation to control animals (0.028 ± 0.003 m/s) [$p < 0.05$; $F_{(2,28)} = 5.363$] (Fig. 4B). Animals treated with MK-801 remained in the bottom of the aquarium longer (224.3 ± 32.5 s) than the control animals (111.3 ± 41.64 s), [$p < 0.01$; $F_{(2,28)} = 8.648$] (Fig. 4C).

DNQX gave to animals previously to the exposure to PTZ at 26 and 30 °C did not cause any significant influence on the time of manifestation of any of the seizure stages evaluated (Figs. 5 and 6).

MK-801 gave to animals previously to the exposure to PTZ at 26 °C did not cause any effect on the response to PTZ (Fig. 5), while at 30 °C was able to increase the time latency to reach seizure stages when compared to drug-free animals exposed to PTZ at 30 °C (Fig. 6). Animals that were previously exposed to MK-801 for 10 minutes had a longer latency time to reach stage III (168.3 ± 30.01s) when compared to the drug-free group, both exposed to PTZ at 30 °C, [$p < 0.01$; $F_{(2,25)} = 29,18$] (Fig. 6A). In stage IV, animals previously treated with MK-801 showed longer latency to reach this stage (339.7 ± 116.1s) when compared to animals without previous exposure to drugs, and also exposed to PTZ at 30° C [$p < 0.01$ $F_{(2,25)} = 11,93$](Fig. 6B). The latency time to reach stage V was increased in animals previously treated with MK-801 and exposed to PTZ at 30 °C (370.9 ± 113.5 s) when compared to control animals exposed to PTZ at 30° C (182.3 ± 71.1 s) [$p < 0.01$; $F_{(2,25)} = 11,69$] (Fig. 6C).

4. Discussion

PTZ, as one of the main pharmacological proconvulsant used in animal models, has its pharmacologic mechanisms and behavioral effects well characterized. In zebrafish, the exposure to PTZ directly through the water is one of the hallmarks of the successes in the pharmacological screening to antiepileptic drugs and search for basic biological mechanisms of epilepsy. Here, we verified that adult zebra-

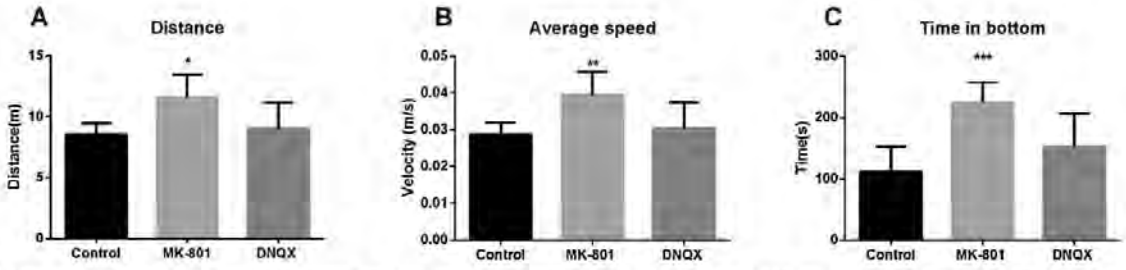

Fig. 4. Locomotor activity between 3–8 minutes of exposure to glutamatergic antagonists. (A) Distance traveled (m) for 5 minutes of animal treated with 2.5 μM MK-801 (N = 10, p = 0.014), 10 μM DNQX (N = 10, p = 0.87) or control (drug-free; N = 11). (B) Mean velocity of animals treated with 2.5 μM MK-801 (p = 0.009) or 10 μM DNQX (p = 0.86) in comparison to control animals (drug-free). (C) Time on the bottom of aquarium of animals treated with MK-801 2.5 μM (p = 0.0006) and DNQX 10 μM (p = 0.25) in comparison to control group (drug-free). The bars represent the mean ± C.I. N represents the number of animals per group. * means statistical significance at $p < 0.05$; *** means statistical significance at $p < 0.001$.

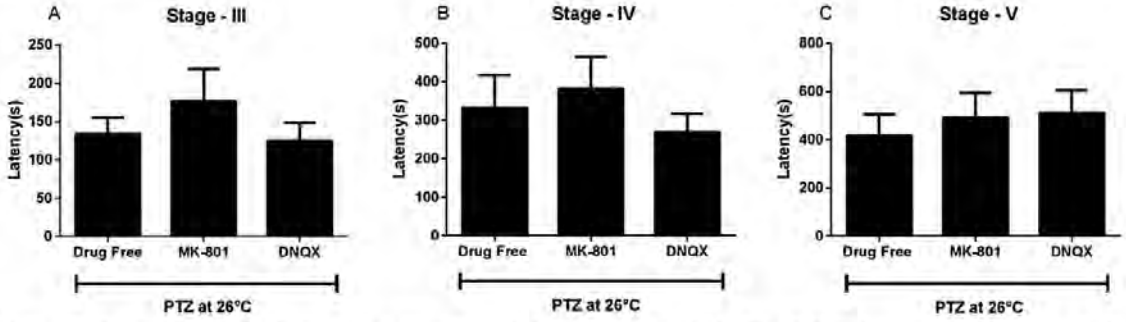

Fig. 5. Latency time for animals pretreated with glutamatergic antagonists to reach the seizure stages provoked by PTZ at 26 °C. (A) Latency time to reach the stage III of animals pretreated with 2.5 μM MK-801 (N = 9, p = 0.053), 10 μM DNQX (N = 12, p = 0.79) or control animals (drug-free, N = 12). (B) Latency time for stage IV of animals pretreated with 2.5 μM MK-801 (N = 9, p = 0.46), 10 μM DNQX (N = 12, p = 0.29) or control animals (drug-free, N = 12). (C) Latency time for stage V of animals pretreated with 2.5 μM MK-801 (N = 9, p = 0.41), 10 μM DNQX (N = 12, p = 0.21) or control animals (drug-free, N = 12).

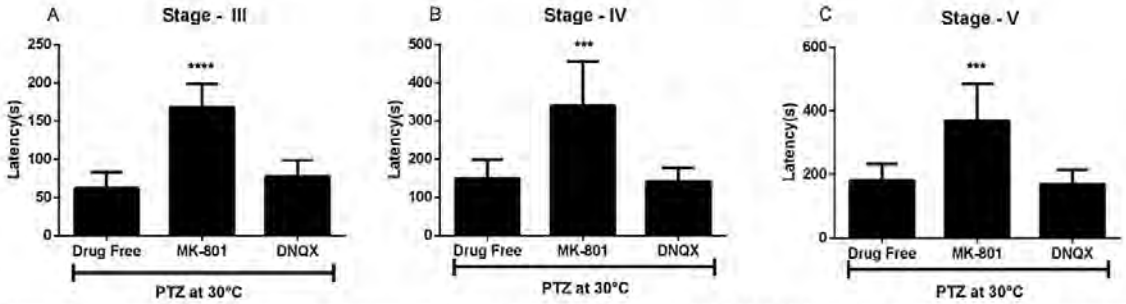

Fig. 6. Latency time for animals pretreated with glutamatergic antagonists to reach the seizure stages caused by PTZ at 30 °C. (A) Latency time to reach the stage III of animals pretreated with 2.5 μM MK-801 (N = 9, p = 0.0001), 10 μM DNQX (N = 9, p = 0.465) or control animals (drug-free, N = 10). (B) Latency time for stage IV of animals pretreated with 2.5 μM MK-801 (N = 9, p = 0.0006), 10 μM DNQX (N = 9; = 0.9760) or control animals (drug-free, N = 10). (C) Latency time for stage V of animals pretreated with 2.5 μM MK-801 (N = 9, p = 0.0007), 10 μM DNQX (N = 9; = 0.946) or control animals (drug-free, N = 10). *** means statistical significance at $p < 0.001$, **** means statistical significance at $p < 0.0001$.

fish offers few confounder factors to the evaluation of PTZ effects, since gender and weight did not affect the response to this proconvulsant. Although the influence of the temperature about the spread dynamic and pharmacokinetics of PTZ in zebrafish has no report, the results found here corroborate to the findings in rats, which showed no differences in absorption, distribution and sensitivity to PTZ between genders (Haberer and Pollack, 1991; McLean et al., 2004). However, in the mentioned study, a state of hyperthermia combined with exposure to PTZ increased the susceptibility to seizures of female in relation to male rats (Dai et al., 2014).

The 26 °C is considered the standard temperature to keep zebrafish colonies from laboratories around the world (Westerfield, 2007; Wilson, 2012) and the maintenance of this parameter through the zebrafish manipulation is a mandatory fact. Studies demonstrated that increasing temperature is enough to obtain electroencephalographic recordings in zebrafish larvae related to those observed in epilepsy crisis, while in terms of behavior and locomotor activity no changes are registered using only the temperature as an inductor of seizure (Baraban et al., 2005; Hunt et al., 2012). Here, our results show that also adult zebrafish did not show locomotor activity resembling seizures stages when submitted to the temperature of 30 °C. However, when testing different temperatures concomitantly to PTZ, we observed a decrease of latency of animals to reach the seizure stages at the highest temperature and higher resistance to the effects of PTZ at the lowest temperature.

Previous works reported that the temperature exerts no influence on the distribution and absorption of PTZ in the nervous system (MacKintosh et al., 1984; Turker et al., 2011; Walker and Levy,

1991). However, the increasing temperature causes a number of other neurophysiological responses that can influence the standard response to PTZ (Gonzalez-Ramirez et al., 2009; Hunt et al., 2012; León-Navarro et al., 2015). It has been observed that rat pups afflicted with febrile seizures have lower levels of GABA in the cerebrospinal fluid, due to a decrease in glutamate decarboxylase activity induced by hyperthermia (Arias et al., 1992). In rats previously exposed to GABA antagonists or hyperthermia, the hyperthermia increased the susceptible to seizures induced by PTZ, thus evidencing a relationship between the actions of both factors (Fukuda et al., 1997; Gonzalez-Ramirez et al., 2009).

Another factor that may be linked to increased susceptibility to seizures induced by concomitantly PTZ and hyperthermia exposure is the effect that increased temperature produces in Ca^{2+} channels. It is possible to reduce seizures caused by hyperthermia using antagonists for NMDA receptors, as well as, antagonists of the thermosensitive cationic channels, the transient receptor potential vanilloid (TRPV4) (Hunt et al., 2012; Laorden et al., 1990). Previous work has shown that increasing the temperature of the environment can lead to an increase in the internal temperature of the brain, which leads to an increase in extracellular glutamate, thus decreasing the threshold for induction of seizures (Kiyatkin, 2014, 2007; Morimoto et al., 1993). Other studies evidence the participation of the NMDA receptor in episodes of seizures, in which they show that the administration of MK-801 suppressed or increased the threshold to hyperthermia-induced seizure kindling (Morimoto et al., 1995).

Here, NMDA and kainate receptors were evaluated through pharmacological approach to be participants of the incremented effect of hyperthermia on latency to reach seizure states induced by GABA receptor inhibition. Kainate inhibition did not prevent hyperthermia effects or promoted locomotor or behavioral effects on the dose tested. Although the MK-801 was not able to inhibit the effects of PTZ at the temperature of 26 °C, considered the ideal temperature of zebrafish (Westerfield, 2007), the MK-801 was able to attenuate the potentiation of these effects caused by a combination of PTZ and hyperthermia (30 °C).

Through our experiments we have observed that, like other convulsive crisis models using PTZ, the convulsive effects of hyperthermia in zebrafish may be strongly linked to the actions performed by neurotransmission mechanisms that control the influx of calcium in the cell, such as NMDA receptors. Also, the induction of seizure by PTZ exhibit few confounder factors since gender and weight did not alter the response profile to this proconvulsant.

Disclosure Statement

No competing financial interests.

Acknowledgment

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3.2 Capítulo II

Artigo Científico Original

Transient Disruption of Adenosine Signaling During Embryogenesis Triggers a Pro-epileptic Phenotype in Adult Zebrafish

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Transient Disruption of Adenosine Signaling During Embryogenesis Triggers a Pro-epileptic Phenotype in Adult Zebrafish

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Abstract
Adenosinergic signaling has important effects on brain function, anatomy, and physiology in both late and early stages of development. Exposure to caffeine, a non-specific blocker of adenosine receptor, has been indicated as a developmental risk factor. Disruption of adenosinergic signaling during early stages of development can change the normal neural network formation and possibly lead to an increase in susceptibility to seizures. In this work, morpholinos (MO) temporarily blocked the translation of adenosine receptor transcripts, *adora1*, *adora2aa*, and *adora2ab*, during the embryonic phase of zebrafish. It was observed that the block of *adora2aa* and *adora2aa* + *adora2ab* transcripts increased the mortality rate and caused high rate of malformations. To test the susceptibility of MO *adora1*, MO *adora2aa*, MO *adora2ab*, and MO *adora2aa* + *adora2ab* animals to seizure, pentylenetetrazole (10 mM) was used as a convulsant agent in larval and adult stages of zebrafish development. Although no MO promoted significant differences in latency time to reach the seizures stages in 7-day-old larvae, during the adult stage, all MO animals showed a decrease in the latency time to reach stages III, IV, and V of seizure. These results indicated that transient interventions in the adenosinergic signaling through high affinity adenosine receptors during embryonic development promote strong outcomes on survival and morphology. Additionally, long-term effects on neural development can lead to permanent impairment on neural signaling resulting in increased susceptibility to seizure.

Keywords Adenosine · Development · Morphology · Seizure · Zebrafish

Introduction

The role of adenosine during the initial phase of development has been the subject of a series of studies that seek to clarify the possible consequences of disturbances of this important signaling pathway mainly in the embryonic phase [1–4]. Caffeine, one of the most common psychoactive drugs consumed word widely, at typical concentration ranging of human consumption, acts as a non-specific blocker of the adenosine receptors [5]. Caffeine freely crosses biological barriers, being able to reach fetus during the entire gestation and through breast milk in breastfeeding neonates [5]. High caffeine intake (> 300 mg/day) during pregnancy is associated with a significant increase in the risk of low birth weight, while spontaneous abortion and preterm delivery are also major concerns [6]. From studies using rat dams, the exposure of fetus to high doses of caffeine promoted teratogenesis, mainly related to cardiac function [7, 8]. These evidences indicate that adenosine signaling plays an important role in early morphological development [9]. Considering the neural impact of early caffeine exposure, the disturbances in adenosinergic signaling during the development may lead to severe consequences that could be transient or permanent, such as seizure susceptibility [10, 11]. Some studies attribute the morphological and neural damages caused by high doses of caffeine to the decreased protective effect exerted by the A_1 adenosine receptor [2, 12].

In rodents, early exposure to caffeine promotes latent effects to several proconvulsants. These latent effects appeared only in adulthood, probably as a result of alterations in the ontogeny of brain excitability [10, 13]. While still inconclusive, the effects of adenosine receptors blockage during early development over brain excitability and behavior have been extensively investigated and a long-lasting imbalance of

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excitatory and inhibitory transmission appears to be related to delayed GABAergic neurons migration [14].

Adenosine is tightly implicated in the modulation of stimulatory and inhibitory neurotransmission, angiogenesis, and acts as a trophic factor [15]. The control of adenosine availability and action can suffer intervention in many steps, such as enzymatic production and degradation [16], active release [17], transport through membrane [18], receptor activation [19], and internal cascade of signaling [20]. Here, we investigated the developmental effects of transiently disruption of adenosine receptors signaling using zebrafish embryos.

Materials and Methods

Animals

The animals used in the experiments came from the breeding wild-type zebrafish colony held at the ZEBLAB laboratories of the Pontifical Catholic University of Rio Grande do Sul, Brazil. All animals were maintained on a light cycle of 14 h light/10 h dark and temperature of water at 27 ± 1 °C. Before the day of mate, the female and male zebrafishes were kept on an automated shelf of aquariums, with system water quality controllers (ZEBTEC, Tecniplast, Buguggiate, VA, Italy) in a ratio of two male/one female, at a density of 2 animals/L, separated by a barrier. The barriers were removed in the first hour of the light cycle, and the eggs were collected 10 min after the beginning of the reproductive ritual and forwarded to microinjection. After microinjection, eggs were kept in plates in a B.O.D. incubator (bio-oxygen demand) until reached 7 days post-fertilization (dpf), when they were relocated to the aquarium system. After morphological outcomes and rate survival determination, 7-day-old larvae or 4–7-month-old adult animals were submitted to locomotor evaluation and seizure induction. After the tests, animals were euthanized by tricaine (MS-222, MERK, Darmstadt, Germany) overdose (500 mg/L) and discarded in biological residues container. All protocols followed National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the Brazilian legislation and were approved by the Institutional Animal Care Committee of PUCRS registered under the protocol number 14/00416—CEUA/PUCRS.

Morpholino Targets and Injection

The targets chosen for temporary knockdown by morpholinos technique were transcripts of adenosinergic receptors, *adora1*, *adora2aa*, and *adora2ab* (Table 1), all obtained from Gene Tools LLC (Oregon, USA). Control animals were non-injected embryos and scrambled oligonucleotide-injected embryos at the same stage of development. Lyophilized morpholinos were resuspended in 300 μL Milli-Q water RNAase free at a temperature of 65 °C for further storage in a freezer at − 20 °C at 1 mM concentration. For application in embryos, small aliquots of stock solutions were diluted in solution (e.g., solution Danieau [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)$, 25 mM HEPES, pH 7.6]), as described in The Zebrafish Book [21], and stained with 0.5% phenol red in DPBS (Sigma-Aldrich, cat. no. P-0290). The final dose of morpholino injected was 3 ng/embryo; animals were co-injected with *adora2aa* and *adora2abMO*, receiving 3 ng/target totaling 6 ng/embryo [22].

For microinjection, Narishige micromanipulation system coupled to a stereomicroscope SMZ1500 (NIKON, Melville, USA) was used. The microcapillary needles were previously prepared, containing 1.5 μL of the agent to be microinjected. The calibration of injection was performed by immersion in oil drop and calculation of the released volume $\left(V = \frac{4}{3}\pi r^3\right)$ to reach a suitable pressure (≈ 3–6 psi) to deliver a total volume of 6 nL/pulse. The eggs were lined up on a microscope slide adjusted to a Petri dish to perform the microinjection series. All eggs were injected into the yolk sac region of the embryo, with maximum development stage of four cells.

Survival Rate and Malformations

The analysis of the survival rate was performed between 0 and 7 days post-fertilization (dpf) by daily conference and removal of dead animals. At least four sets of animals per group were analyzed to confirm survival rate. Unfertilized or damaged eggs were discarded few hours after the microinjection and not accounted.

For teratogenic evaluation, 5 dpf animals were used. The animals were placed on the lid of a 96-well plate, which were individualized by the grooves of each well containing approximately 40 μL of system water. The morphological parameters analyzed were based on the most affected structures: eyes,

Table 1 Sequence of morpholino antisense

Reference number	Target	Sequence
–	Scramble	5′-CCTCCTACCTCAGTTACAATTTATA-3′
ENSDART00000112926	*adora1*	5′-GAGAGATCCTCGGGCATTCTTGCAC-3′
ENSDART00000043902	*adora2aa*	5′-AAACATTGTTCAGCATGGTGAGGTC - 3′
ENSDART00000081368	*adora2ab*	5′-GAACAATGTAGACGAGAGAAGACAT-3′

bladder swim, pericardium, and tail. For all the changes found in each one of these structures scores were assigned: 0 for absence of malformation, 1 for presence of any malformation, and 2 for the accumulation of more than one malformation in the same structure. Each animal had its score calculated based on the sum of malformations observed in each of the structures analyzed, revealing a degree of teratogenic severity for each target (Fig. 1). A rate of malformations was performed by the sum of the absolute number of individuals that shown malformations in each group.

Locomotor Activity

For animals 7 dpf, the test apparatus (open field) for locomotor activity assessment consisted of a 24-well culture plate, containing the water system (drug-free), with a water column of 1.5 cm, where the animals were placed individually and the locomotor activity recorded by a digital camera (Logitech™, Romanel-sur-Morges, Switzerland) located at the top of the apparatus. After 30 s of acclimation, animals were observed for 5 min, where the mobile time (s) and the average speed during the moving time (s) were registered.

Animals aged 4–7 months post-fertilization (mpf) were allocated for habituation in the behavioral testing room during 24 h before testing. Locomotor activity tests in adult animals were performed in an aquarium with internal dimensions of 13 cm × 11.5 cm × 8 cm (height × length × width) using system water (drug-free) in a 5.5 cm water column (500 mL). The animals were placed individually in the test tank, where they had their performance recorded by a digital front camera (Logitech™, Romanel-sur-Morges, Switzerland). The parameters measured were as follows: average speed (m/s), mobile time(s), and time at the bottom of the aquarium (s), used as anxiety parameter in zebrafish [23, 24]. All tests were performed in the early afternoon, between 1:00 p.m.–4:30 p.m., when there is less variability in behavior over time [25]. Only animals with no malformation were used to assess the locomotor activity.

Sensitivity to Pentylenetetrazole

Animals microinjected with morpholinos and controls were kept under normal handling until reaching 7 dpf or 4–7 mpf when they were exposed to pentylenetetrazole (PTZ) by immersion. The exposure of larvae and adults to 10 mM PTZ diluted in the water of system was performed for 10 min [26–28]. This PTZ dose is able to provoke convulsive stages in a distinguishable way over time [26–28]. Only animals with no malformation were used to assess the sensibility to PTZ.

Zebrafish larvae exposed to PTZ in a 24-well culture plate had their seizure stages measured over 10 min of exposure. The seizure stages were adapted from previous works of Baraban et al. (2005) considering stage II: fast circular swimming behavior and stage III: series of short clonic convulsions, leading to loss of posture, and fall to one side remained still for 1–3 s.

For adult zebrafish, seizure stages were measured over the 10 min of exposure in a test tank with internal dimensions of 13 cm × 11.5 cm × 8 cm (length × height × wide). For the purpose of this study, the seizure stages used were adapted

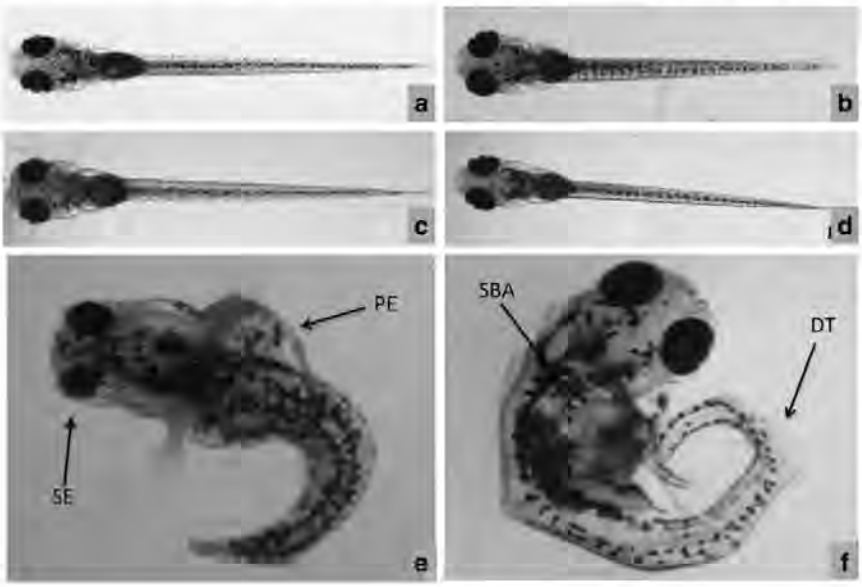

Fig. 1 Illustration of malformations observed in larval zebrafish at 5 days post-fertilization. PE pericardial edema, SBA swimming bladder absent, SE small eyes, DT deformed Tail. **a** Control. **b** Scramble. **c** MO *adora1*. **d** MO *adora2aa*. **e** MO *adora2ab*. **f** Both MO *adora2aa* + MO *adora2ab*

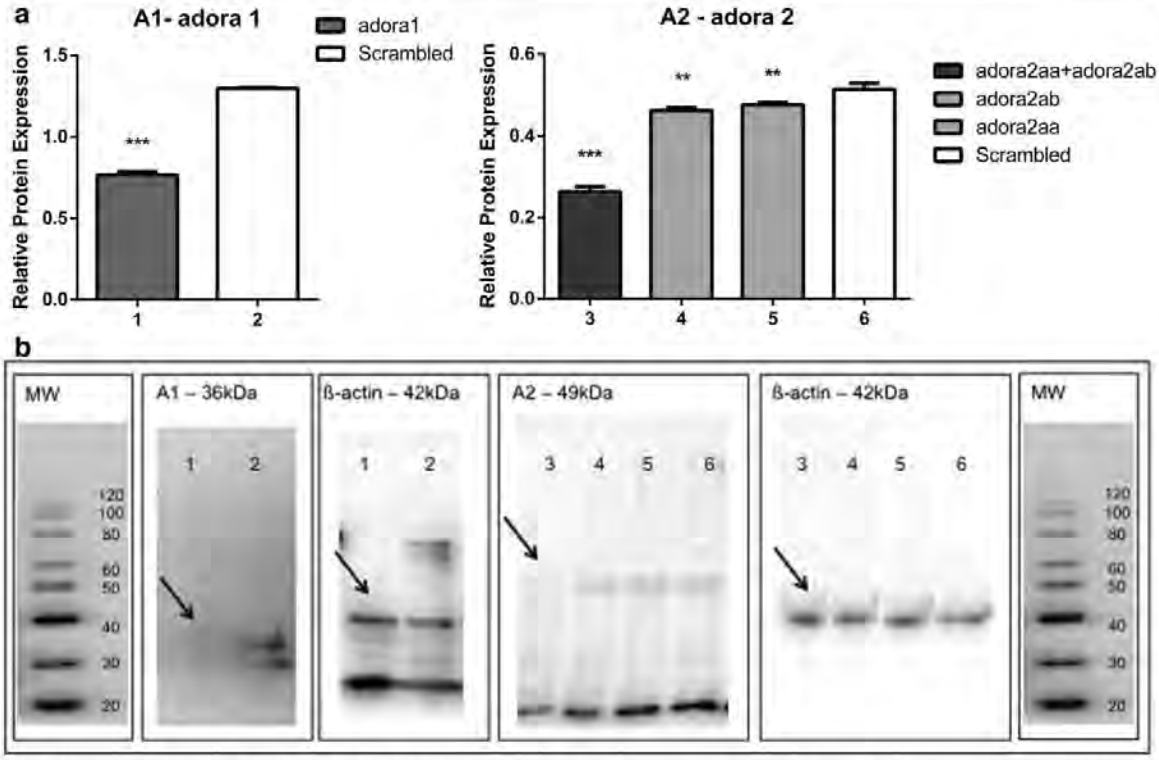

Fig. 2 Morpholino injection efficacy of 48 hpf zebrafish embryos. **a** Representative western blot membranes, where numbers 1 to 6 indicate samples submitted to MO scrambled (2 and 6), morpholino targeted adenosine receptors adora1 (1), both adora2aa + adora2ab (3), adora2ab (4), and adora2aa (5). **b** Protein expression of A1 adenosine receptor and A2 adenosine receptors relative to ß-actin protein expression. Student's *t* test for A1 adenosine receptor or One-way ANOVA for A2 receptor ($F(3,8)=328.5$) followed by Tukey's post-hoc test; Double and triple asterisks represent $p < 0.01$ and $p < 0.001$, respectively, different from MO scrambled (6)

from previous works as stage III: a circular motion, stage IV: convulsive behavior clonic type, and stage V: fall to the bottom of the aquarium and convulsive behavior of tonic type [27].

Western Blot

To check the impairment on the protein synthesis by morpholino injection, we performed immunodetection using antibodies against to adenosine receptors. A set of 20 embryos of 48 hpf per group in triplicated was euthanized, and whole embryos were placed in a cooled protease inhibitor solution (Complete Mini; Roche Applied Science, Indianapolis, USA) and stored at − 80 °C for subsequent analysis as previously described with few modifications. The protein extract was prepared in RIPA buffer (Sigma-Aldrich, USA). Total protein (25 mg) was separated on a 12% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane.

Next, the membrane was blocked with 5% albumin (Sigma-Aldrich, USA) in TBS containing 0.05% Tween-

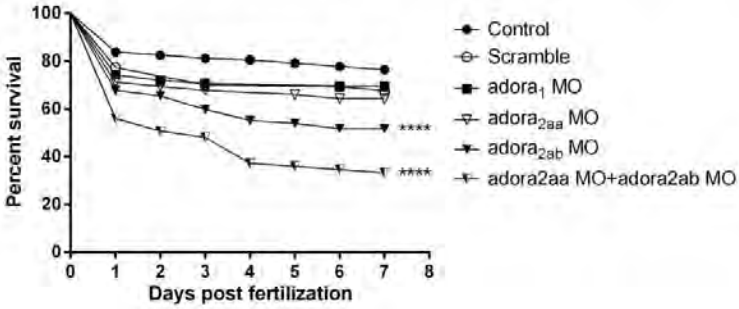

Fig. 3 Survival rate through 7 days post-fertilization of zebrafish larvae submitted to yolk injection (< 1 h post-fertilization) of morpholino targeted to adenosine receptors *adora1*, *adora2aa*, *adora2ab*, and both *adora2aa* + *adora2ab*. Each group was compared to control group using log-rank test; four asterisks represent a significant level $p < 0.0001$

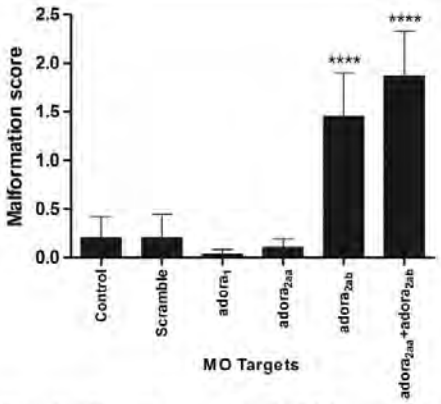

Fig. 4 General malformations score for zebrafish larvae of 5-days post-fertilization submitted to morpholino targeted to adenosine receptors *adora1*, *adora2aa*, *adora2ab* and both *adora2aa* + *adora2ab*. The score was established considering 0 for absence of malformation; 1 for presence of any malformation; and 2 for the accumulation of more than one malformation in the same structure. Each group consisted of 60 animals. Quadruple asterisks represent a significant level at $p < 0.0001$

20 and incubated overnight with rabbit polyclonal antibody against Adora1 (ab82477, 1:500; Abcam) and mouse polyclonal Adora2a (ab79714, 1:500; Abcam) receptors or mouse monoclonal anti-ß-actin antibody (ab8226, 1:1000; Abcam) that served as primary antibodies. Anti-mouse (G-21040, 1:2000; Molecular Probes) and anti-rabbit (ab97069, 1:2000; Abcam) horseradish peroxidase-conjugated secondary antibodies were used to detect the primary antibodies, and the resulting signal was measured with the Western Lighting-Enhanced Chemiluminescence Detection Kit (NEL 104001EA; PerkinElmer). Prestained molecular weight protein markers (Magicmarker; Invitrogen) were used to determine the molecular weight of each detected band and to confirm antibody target specificity. Densitometry quantification of each replicated gel was performed using Carestream software (Carestream Health, Rochester, USA). Total protein levels were normalized for each sample relative to ß-actin protein levels.

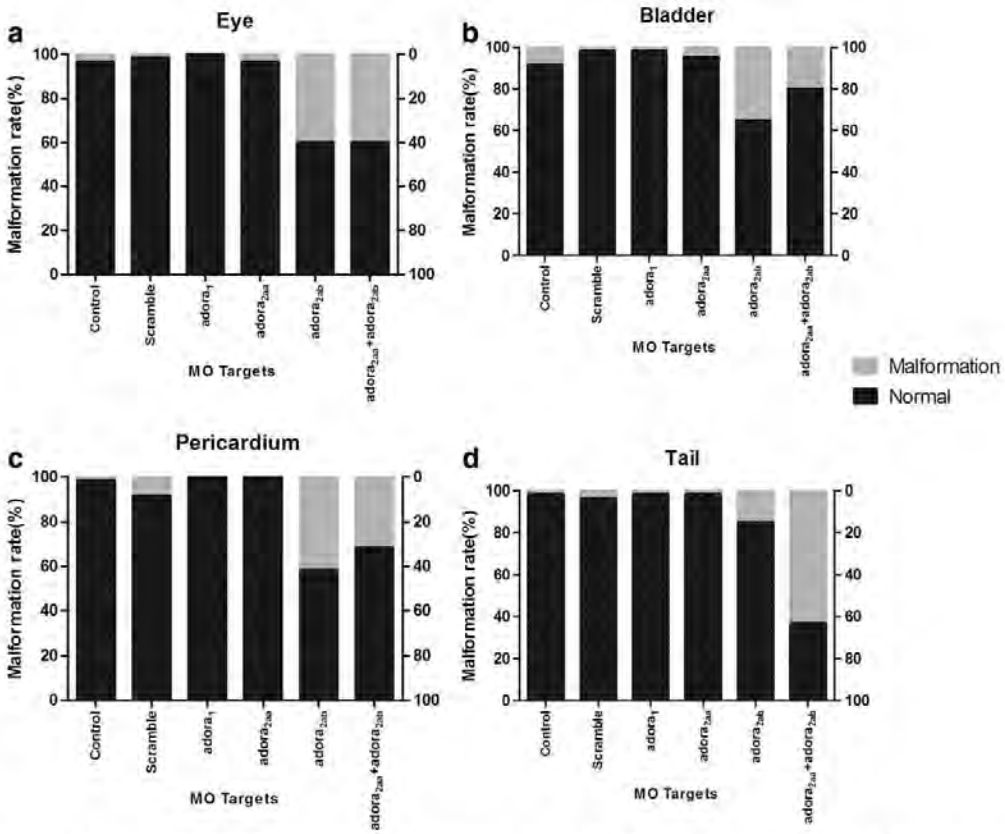

Fig. 5 Percentage of animals showing malformation in **a** eye, **b** bladder, **c** pericardium, and **d** tail for zebrafish larvae of 5 days post-fertilization submitted to morpholino targeted to adenosine receptors *adora1*, *adora2aa*, *adora2ab*, and both *adora2aa* + *adora2ab*. Each group consisted of 60 animals

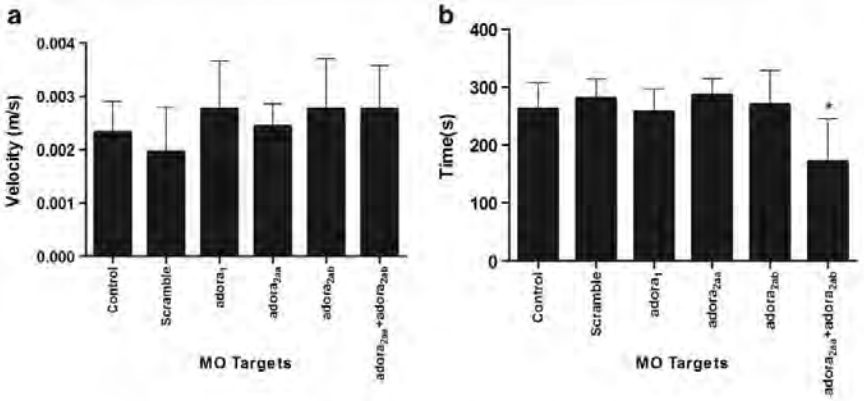

Fig. 6 Locomotor activity of 7-day-old zebrafish larvae after embryonic exposure to morpholino targeted to adenosine receptors *adora1*, *adora2aa*, *adora2ab*, and both *adora2aa* + *adora2ab*. **a** Average speed (m/s). **b** Mobile time (s). Single asterisk represents $p < 0.05$

Statistical Analysis

The survival rate was estimated according to the log-rank (Mantel-Cox) test. Morphological aspects, locomotor activity, and seizures latency evaluation were first analyzed by normality through D'Agostino and Pearson omnibus normality test. For those data where the variance had no normal distribution the analysis was performed by Kruskal-Wallis test followed by Dunn's test, while data with normal distribution of variance followed one-way ANOVA, when appropriated, followed by Dunnet's test as post hoc analysis. All the analyses were performed using GraphPad prism 6.0. The significance level was established at $p < 0.05$. The results were expressed as means ± 95% CI, except for survival determination and malformations where the data were expressed as percentages.

Results

Morpholino Injection Efficacy

Morpholino protein impairment was observed through immunodetection. MO *adora1* injection significantly blocked A_1 adenosine receptor expression at 48 hpf ($n = 3$; $p < 0.001$). Embryos injected with MO *adora2aa* ($n = 3$; $p < 0.01$), MO *adora2ab* ($n = 3$; $p < 0.01$), and MO *adora2aa* + *adora2ab* ($n = 3$; $p < 0.001$) showed significant decrease in A_2 adenosine receptor expression when compared to MO Scrambled (Fig. 2).

Survival Rate

Survival rate was compared for each MO target to the control group (survival rate of 76.5%). Embryos injected with scrambled oligonucleotide ($n = 241$; 67.75%; $p = 0.1062$), MO *adora1* ($n = 492$; 69.5%; $p = 0.226$) and MO *adora2aa* ($n = 405$; 64.4%; $p = 0.067$) showed no significant difference in survival rate in relation to the control group. Embryos injected with MO *adora2ab* (51.7%; $n = 348$; $p < 0.0001$) showed significant decrease in survival rate compared to control group. The co-injection of MO *adora2aa* + MO *adora2ab* (33%; $n = 589$; $p < 0.0001$) showed the most significant reduction on survival rate in relation to control ($n = 406$) (Fig. 3).

Malformations

The mean values of the number of animals that showed any malformation and the mean of accumulated malformations *per* larvae, based on severity scores for each anomaly on morphology, showed no differences between scramble ($n = 60$), MO *adora1* ($n = 60$), MO *adora2aa* ($n = 60$) groups, and the control group. However, high scores of malformations were registered from embryos injected with MO *adora2ab* ($n = 60$; $p < 0.0001$) and *adora2aa* + *adora2ab* ($n = 60$; $p < 0.0001$) (Fig. 4). The animals that showed malformation was high to MO *adora2ab*, mainly associated to eyes (40%), bladder (35%), pericardium (41%), and tail (15%) (Fig. 5). Co-injected animals with MO *adora2a* + MO *adora2ab* also displayed high scores of malformations ($n = 60$; $p < 0.0001$) (Fig. 4) that are also associated to eyes (40% of animals), bladder (20%), pericardium (31%), and tail (63%) malformation (Fig. 5).

Locomotor Activity

In the parameter average speed of 7-day-old larvae, no group showed a significant difference from the control group ($n = 8$) (Fig. 6a). Considering the parameter mobile time, the animals injected with MO *adora2aa* + MO *adora2ab* had a decrease in relation to the control group ($n = 8$; $p < 0.05$; $F_{(5, 41)} = 3.15$) (Fig. 6b).

Adult animals injected with MO *adora2aa* ($n = 12$) had their average speed augmented in relation to the control group ($n = 12$; $p < 0.01$; $F_{(5, 64)} = 7.325$), while animals injected with MO *adora1* ($n = 15$), *adora2ab* MO ($n = 11$), and MO *adora2aa* + MO *adora2ab* ($n =$

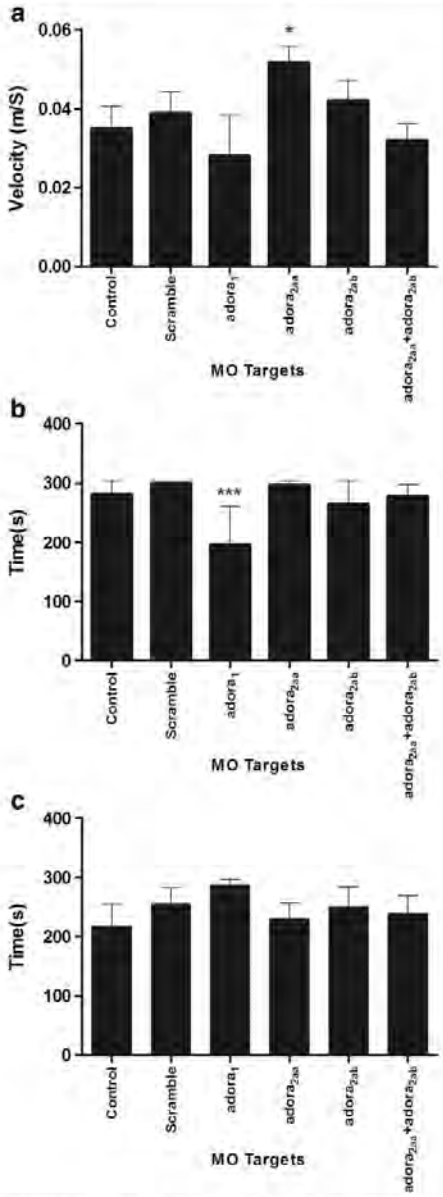

Fig. 7 Locomotor activity of adult zebrafish after embryonic exposure to morpholino targeted to adenosine receptors *adora1*, *adora2aa*, *adora2ab*, and both *adora2aa* + *adora2ab*. **a** Average speed (m/s). **b** Mobile time (s). **c** Time at the bottom of the aquarium (s). Single, double, and triple asterisks represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

9) had no difference in the average speed when compared to control group (Fig. 7a). The MO *adora1* animals were the only group that presented significant difference in time at the bottom of the aquarium ($p < 0.001$; $F_{(5,\ 64)} = 4.415$) (Fig. 7b). For mobile time parameter, MO *adora1* group ($n = 15$) had a decreased mobile time in relation to control group ($p < 0.05$; $F_{(5,\ 64)} = 4.715$) (Fig. 7c).

Susceptibility to Seizures

During testing of susceptibility to seizures by exposure to PTZ (10 mM), zebrafish larvae from scramble ($n = 8$), MO *adora1* ($n = 12$), MO *adora2aa* ($n = 11$), MO *adora2ab* ($n = 8$), and MO *adora2aa* + MO *adora2ab* ($n = 8$) groups did not differ their latency to reach seizure scores when compared to control group ($n = 8$) (Fig. 8a, b).

Considering the susceptibility of adult animals to PTZ, the latency time for the animals to reach the stages III, IV, and V was registered. Control ($n = 10$) and scramble ($n = 10$) groups had no difference to reach each stages of the seizure scale (Fig. 9). The time to reach the stage III was significantly lower in adult animals from MO *adora1* ($n = 10$; $p < 0.01$), MO *adora2aa* ($n = 10$; $p < 0.0001$), and MO *adora2ab* ($n = 10$; $p = 0.0001$) and MO *adora*2aa + MO *adora*2ab ($n = 9$; $p < 0.01$) groups when compared to the control group ($n = 10$; $F_{(5,\ 53)} = 12.74$) (Fig. 9a).

The results observed in the latency time to reach stage IV of seizures showed a marked decrease of the groups that suffered temporary blocking of adenosine receptor genes in relation to the control group (Fig. 9b). With the exception of the scramble group ($n = 10$; 305.3 s; CI ± 75.7), all MO *adora* groups took an average of less than 200 s to reach the stage IV of seizure, while the control group took an average of 327.9 s (CI ± 84.8) (MO *adora1*: $n = 10$, $p < 0.01$; MO *adora2aa*: $n = 10$, $p < 0.0001$; MO *adora2ab*: $n = 9$, $p < 0.0001$; and MO *adora2aa* + MO $adora_{2ab}$: $n = 9$, $p < 0.001$) ($F_{(5,\ 53)} = 14.59$) (Fig. 9b).

The latency time to reach stage V of seizure was considerably lower comparing all groups tested to the control group. The average latency times showed by the groups which suffered specific temporary adenosine receptor genes blockage did not exceed 230 s, while the averages of the scramble group and the control group were 367.9 s (CI ± 101.1) and 421.8 s (CI ± 116.2), respectively, thereby achieving a significant difference (MO *adora1*: $n = 10$, $p < 0.01$; MO *adora2aa*: $n = 10$, $p < 0.0001$; MO *adora2ab*: $n = 9$, $p < 0.0001$; and MO *adora2aa* + MO *adora2ab*: $n = 9$, $p = 0.001$) ($F_{(5,\ 53)} = 13.05$) (Fig. 8c).

Discussion

The results presented in this work were caused by the temporary blocking of transcripts related to adenosinergic function. This disruption of the key role performed by adenosine in early development influenced neurochemical aspects, general morphology, and survival. The transient influence on adenosine receptor function during the period of formation and

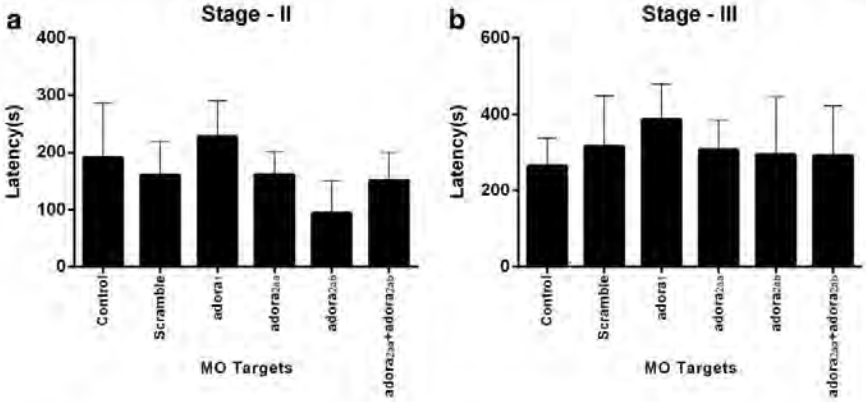

Fig. 8 Latency to reach seizure stages in 7-day-old zebrafish larvae after embryonic exposure to morpholino targeted to adenosine receptors *adora1*, *adora2aa*, *adora2ab*, and both *adora2aa* + *adora2ab*. **a** Latency (s) to reach seizure stage II established as fast circular swimming behavior. **b** Latency (s) to reach seizure stage III established as a series of short clonic convulsions, leading to loss of posture, and fall to one side remained still for 1–3 s

maturation of neural network promoted later consequences on the susceptibility of adult zebrafish to the proconvulsant pentylenetetrazole.

In rodents, global A_1 and A_{2a} adenosine receptor knockout revealed no significant impact on viability and morphology [29–31]. Here, we showed that zebrafish exhibits low survival rate observed in animals injected with MO to the transcripts of receptor *adora2ab* and co-injected *adora2aa* plus *adora2ab*, suggesting that adenosine plays a key role in early stages of zebrafish development. The increased mortality, caused by the blockade of these targets in the embryonic stage, may be related to changes in the development of the cardiac region and vasculature [2, 32, 33], since Adora2aa are expressed in the blood and vasculature and Adora2ab are also detectable in vasculature in early stages of development [34]. All animals that have been identified with malformation of pericardium during our observations died before reaching 10 dpf (data not shown).

Because zebrafish presents paralogous genes for the Adora2a receptor, identified as *adora2aa* and *adora2ab* (formerly as *adora2a*.1 and *adora2a*.2) by Boehmler et al. (2009), we decided to conduct the independent and simultaneous genetic block of these genes. In terms of effectiveness of knockdown, the double-targeted MO appeared more efficient, since the detected protein amount was strongly reduced when compared to the single-targeted MO. While few is known about the specific function of each A2a clones in zebrafish, possible a compensatory protein expression could occur when one target was blocked alone or a superposition of targets could occur in the protein detection method. Animals receiving MO *adora2aa* + *adora2b* presented a higher mortality associated to several morphological alterations mainly related to pericardium, eye, and tail. This pattern of malformations was strongly related to MO *adora2ab* group, since MO *adora2aa* animals did not present malformations. These differences may be a response to the distribution of transcripts for these receptors in the initial phase of development. The distribution of *adora2ab* gene transcripts shows a higher

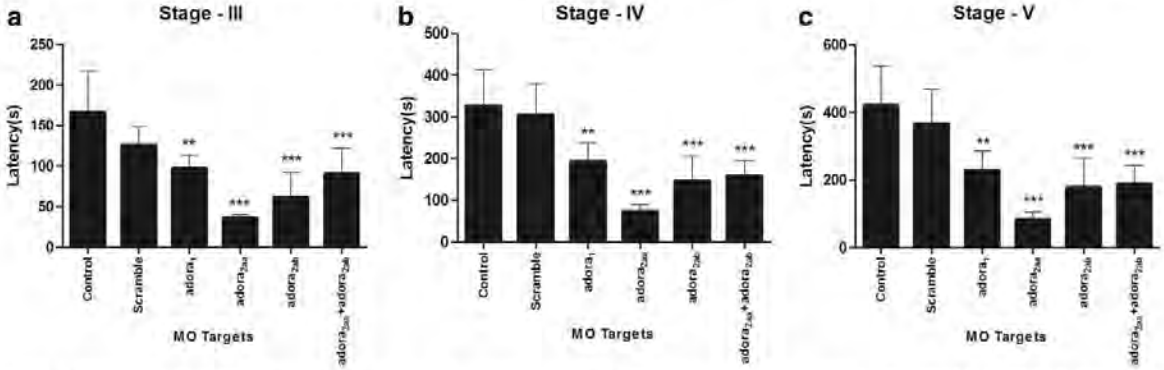

Fig. 9 Latency to reach seizure stages in adult zebrafish after embryonic exposure to morpholino targeted to adenosine receptors *adora1*, *adora2aa*, *adora2ab*, and both *adora2aa* + *adora2ab*. **a** Latency (s) to reach seizure stage III established as a circular motion. **b** Latency (s) to reach seizure stage IV established as convulsive behavior clonic type. **c** Latency (s) to reach seizure stage V established as fall to the bottom of the aquarium and convulsive behavior of tonic type. Double, triple, and quadruple asterisks represent $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively

concentration in areas relating to the central nervous system, vasculature, and interrenal tissue, this later suggesting a role of protection against renal injury in zebrafish [34].

Locomotor activity of 7dpf zebrafish larvae was not affected by transitory disruption of adenosine receptor genes transcription, except for the double disruption of *adora2aa* + *adora2b*, which had a reduced mobile time. The double-gene disrupted animal group, as already mentioned, had the most severe morphological outcomes. Even though animals with malformation were not considered for locomotion and PTZ susceptibility assessment, subtle effects on locomotor machinery could not be discarded. In fact, reports using pharmacological blockage of adenosine receptors of zebrafish larvae by caffeine showed no evident morphological outcomes but muscle fibers misalignment [35].

The analysis of seizures susceptibility in zebrafish larvae did not show significant differences between the treated groups compared to the control group. On the other hand, at adulthood, the animals that suffered disruption of *adora1* gene transcription had reduced mobile time associated to a higher susceptibility to reach seizure stages. Also, animals that suffered disruption of *adora2aa* gene transcription had reduced average speed associated to a higher susceptibility to reach seizure stages. Curiously, MO *adora2ab* and MO *adora2aa* + MO *adora2b* groups, which had no effects on locomotor activity, also reduced their latencies to reach seizure stages. Previous studies suggested that changes in adenosinergic signaling in some phases of development may interfere with maturation of several neurotransmitter systems [36–38]. During this critical period, the control of glutamate and GABA concentrations plays a key role in the formation of the neural network [39, 40]. The signaling exerted by both glutamate and GABA participates in processes such as migration and neuronal maturation and the activity of these neurotransmitters varies throughout development, which could promote differential effects according to the stage of development affected [39, 41–44]. This could explain the controversial results about the effect of caffeine exposition in early stages of development in the susceptibility to seizures in adulthood [45–47]. Neuromodulation exerted by adenosine on glutamate and GABA is dependent upon a delicate balance and control of its own concentration in the extracellular medium [16, 48]. At least in rodents, both A_1 and A_{2a} adenosine receptors are widely distributed in the nervous system and can be found together in the form of heteromers [13, 49]. This uneven distribution can be one of the factors contributing to the observed responses. These changes induced in the initial phase of development may be involved in the increased susceptibility of zebrafish to PTZ in adulthood, since they can result in modification on gene expression of several components of neurotransmitter systems, such as glutamate receptor subunits that are closely related to seizures [50–52]. Additionally, adjustments on transporters and enzymes related to the control of adenosine levels, as well as other neurotransmitters involved in locomotor control, could also be indirect targets. In fact, early exposure to caffeine during gestational period appears to affect nucleotide and acetylcholine metabolizing enzymes [53, 54].

In summary, the transitory intervention in adenosine receptors promotes a widely effect on morphology, locomotion, and susceptibility to proconvulsants in a long-lasting manner. The strong impact of this transitory intervention on adenosine signaling reinforces the aspects of awareness about caffeine exposure during embryonic development.

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Compliance with Ethical Standards All protocols followed National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the Brazilian legislation and were approved by the Institutional Animal Care Committee of PUCRS registered under the protocol number 14/00416—CEUA/PUCRS.

Conflict of Interest The authors declare that they have no conflict of interest.

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3.3 Capítulo III

Artigo Científico Original

Early and transitory block of concentrative nucleoside transporter type 2 and ecto-5'-nucleotidase increases the susceptibility of adult zebrafish to seizure.

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Title Page

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Early and transitory gene blockade of concentrative nucleoside transporter type 2 and ecto-5'-nucleotidase increases the susceptibility of adult zebrafish to seizure

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Abstract

To evaluate the impacts of adenosine signaling impairment during early development over adult susceptibility to seizure we performed knockdown by the morpholino technique of the transcripts for concentrative nucleoside transporter type 2 and ecto-5'-nucleotidase of zebrafish embryos. One hour-old zebrafish embryos received morfolino oligonucleotides against concentrative nucleoside transporter type 2 (CNT2) and ecto-5'-nucleotidase (5'nt). The survival rate, morphology and sensitivity to pentylenetetrazole were assessed at larval and adult period. CNT2 and 5'nt block decreased to 43% and 38% the survival rate, respectively. The transitory block developed high number of malformations, 82% for CNT2 and 58% for 5'nt. At larval stage, the sensitivity to pentylenetetrazole (10 mM), measured as the latency time to reach the seizure stages, was not affected, while adult animals presented higher sensitivity to pentylenetetrazole, compared to control group mainly to reach the stage V at seizure scale (*cnt2 MO*: $p<0.001$ and *5'nt MO*: $p<0.01$). Our results corroborate previous studies that demonstrate the relevance of adenosinergic signaling in the early stages of development, since interventions mechanisms of control of adenosine concentration trigger severe morphological outcomes and proepiletic profile in adult life.

Key words: adenosine; concentrative nucleoside transporter; development; ecto-5'-nucleotidase; seizure.

Introduction

Adenosine is an important intracellular signal of energetic imbalance, thus having a key role in homeostatic control, which is closely related to fundamental processes to the embryonic development, such as cell differentiation, protein synthesis, cytoskeletal dynamics and others(Lomax and Henderson, 1973; Lane and Gardner, 2000, 2005; Rivkees et al., 2001; Rivkees and Wendler, 2017).

The adenosinergic signaling plays an important morphological role, especially in the initial phase of development (Massé and Dale, 2012). Rats exposed during embryonic stage to high doses of caffeine, an unspecific adenosine receptor antagonist, exhibited morphological changes in the cardiac region (Christian and Brent, 2001; Souza et al., 2016). Early and temporary blockade of adenosinergic receptors from zebrafish using morpholinos (MO), especially A_{2A} adenosine receptor, caused a series of malformations in structures such as heart, bladder and tail (Menezes et al., 2018). Adenosine is widely spread throughout the body and is an important marker of energetic demands and cellular damage caused by hypoxia or ischemia (Hart et al., 2008; Boehmler et al., 2009). Additionally, adenosine also plays an important role as a neuromodulator in the nervous system (Cunha, 2001; Boehmler et al., 2009)

The adenosine is a modulator capable of interfering with neuronal excitability and is an important endogenous anticonvulsant (Siebel et al., 2015). The neuromodulation exerted by adenosine occurs by the activation of P1 receptors (A_1, A_{2A}, A_{2B} and A_3), which demonstrate differentiated affinity for adenosine and tissue expression (Sebastião and Ribeiro, 2009). The enzyme ecto-5'-nucleotidase, a major source of adenosine through AMP hydrolysis, plays a key role in the availability of adenosine to inhibit epileptic seizure initiation, spread and duration, through the A_{2A} adenosine receptor (Barros-Barbosa et al., 2016). It was observed that 48 h after an induction of *status epilepticus* in rats, there was a significant increase in the enzymatic activity of the ecto-5'-nucleotidase (Bonan et al., 2000). Additionally, the ecto-5'-nucleotidase expression is increased in patients with mesial temporal lobe epilepsy (Barros-Barbosa et al., 2016). The concentrative nucleoside transporter-2 (CNT2), as well as the ecto-5'-nucleotidase, plays a significant role in the control of extracellular adenosine concentration by reuptake it into the cell (Rose and Coe, 2008). Previous works have shown the attenuating effect of inhibitors of adenosine reuptake via CNT2 against proconvulsant agents in animal models (George and Kulkarni, 1997; Akula et al., 2008). Some studies have shown that disturbances in adenosinergic signaling during

the early stage of development may lead to transient or permanent severe consequences, such as seizure susceptibility (Guillet and Dunham, 1995; Silva et al., 2013; Menezes et al., 2018).

Considering the early period of brain development as a susceptible period and the anticonvulsants properties of adenosine, in the current study, we aimed to evaluate the effects of early and transitory block of CNT2 and ecto-5'- nucleotidase translation in the zebrafish embryos. This approach allows verify the long-term effects of a disrupted adenosine signaling, emphasizing the influence on morphological outcomes and proconvulsant sensitivity, contributing to the study of adenosine signaling in the context of seizure investigation.

Experimental procedures

Animals

The animals used in the experiments came from the wild type zebrafish breeding colony held at the ZEBLAB laboratories of the Pontifical Catholic University of Rio Grande do Sul, Brazil. All animals were maintained on a light cycle of 14h Light / 10h Dark, and temperature of water at 27±1°C. Before the day of mate, the female and male zebrafish were kept on an automated shelf of aquariums, with system water quality controllers (ZEBTEC, Tecniplast, Buguggiate (VA) Italy) in a ratio of 2 male/1 female, at a density of 2 animals/L, separated by a barrier. The barrier was removed in the first hour of the light cycle and the eggs were collected 10 minutes after the beginning of the reproductive ritual, and forwarded to microinjection. After microinjection, eggs were kept in plates in a B.O.D. (Bio-Oxygen Demand) incubator until reach 7 days post-fertilization (dpf), when were relocated to the aquarium system. After morphological outcomes and rate survival determination, 7-day old larvae or 4-7-month old adult animals were submitted to locomotor evaluation and seizure induction. After experiments, animals were euthanized by tricaine (MS-222, MERK, Darmstadt, German) overdose (500 mg/L). The planning of the experiments followed the principles outlined in the ARRIVE guidelines and the Basel declaration (http://www. basel.declaration.org) including the 3R concept. All protocols followed National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 80-23, revised 1996) and the Brazilian legislation and were approved by the

Institutional Animal Care Committee of PUCRS registered under the protocol number 14/00416 - CEUA/PUCRS.

Morpholino targets and injection

The morpholinos targets were chosen for transcript sequences of the nucleoside transporter *cnt2* and the ecto-5'-nucleotidase *5'nt*, extracted from the database *Ensembl*. All morfolinos sequences were design and purchased from Gene Tools LLC (Oregon, USA) (Table 1). Non-injected embryos (control) and scrambled oligonucleotide-injected embryos were used at the same stage of development. Lyophilized morpholinos were resuspended in 300 µL Milli-Q water RNAase free at a temperature of 65 °C for further storage in a freezer at -20 °C at 1 mM concentration. For application in embryos, small aliquots of stock solutions were diluted in Danieau's solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)$, 25 mM HEPES, pH 7.6) as described in The Zebrafish Book (Westerfield, 2007), and stained with 0.5% phenol red in DPBS (Sigma-Aldrich, cat. no. P-0290). The final dose of morpholino injected was 3 ng/embryo (Bill et al., 2009).

For microinjection was used Narishige micromanipulation system coupled to a stereomicroscope SMZ1500 (NIKON, Melville, USA). The microcapillary needles were previously prepared, containing 1.5 µL of the solution to be microinjected and calibrated by injection in immersion oil. The released volume was calculated ($V = \frac{4}{3}\pi r^3$) to reach a suitable pressure (≈3- 6 PSI) for delivering a total volume of 6 nL/pulse. The eggs were lined up on a microscope slide adjusted to a Petri dish, to perform the microinjection series. All eggs, with maximum development stage of 4 cells, received injection into the yolk sac region of the embryo.

Survival rate and malformations

The survival rate was measured between 0 and 7 days post-fertilization (dpf) by daily conference and removal of dead animals. Unfertilized or damaged eggs were discarded few hours after the microinjection and not accounted. At least four sets of animals per group were analyzed to confirm survival rate.

The teratogenicity evaluation was performed at 5 dpf following the protocol of Menezes et al. (Menezes et al., 2018). Briefly, the animals were placed on the lid of a 96-well plate, which were individualized by the grooves of each well containing approximately 40 µL of system water. The morphological parameters analyzed were

based on the most affected structures: eyes, bladder swim, pericardium and tail. Scores were assigned for each malformation found in each single structure studied: 0 for absence of malformation; 1 for presence of any malformation and 2 for the accumulation of more than one malformation in the same structure. Each animal had its score calculated based on the sum of malformations observed in each of the structures analyzed, revealing a degree of teratogenicity severity for each target (Menezes et al., 2018) (Figure 1). A rate of malformations was performed by the sum of the absolute number of individuals that shown malformations in each group.

Locomotor activity

To measure the locomotor activity, larvae 7dpf were tested at 24-well culture plate apparatus (open field) containing water system (drug-free), with a water column of 1.5 cm (2.5 ml), where the animals were placed individually and the locomotor activity recorded by a digital camera (Logitech™, Romanel-sur-Morges, Switzerland) located at the top of the apparatus. After acclimation time (30 seconds), animals were recorded during 5 minutes, the mobile time (s) and the average speed(m/s) were analyzed over moving periods(Menezes et al., 2015).

Animals aged 4-7 months post-fertilization (mpf) were placed for habituation in the behavioral testing room 24h before testing. Locomotor activity tests in adult animals were performed over 330 seconds, of which the first 30 seconds was for acclimatization, in an aquarium with dimensions of 13 cm x 11.5 cm x 8 cm (height x length x width) using system water (drug-free) in a 5.5 cm water column (500 mL). The animals were placed individually in the test tank, where their performance was recorded by a digital front camera (Logitech™, Romanel-sur-Morges, Switzerland). The parameters measured were: Average speed (m/s), mobile time(s), and time at the inferior zone of the aquarium (s), used as anxiety parameter in zebrafish (Riehl et al., 2011; Kalueff et al., 2013). All tests were performed in the early afternoon, between 1:00PM-16:30PM, when there is less variability behavior over the time (MacPhail et al., 2009). Animals with any malformation were not used to assess the locomotor activity.

Sensitivity to Pentylenetetrazole

Microinjected animals and controls were kept under normal handling until reaching 7 dpf or 4-7 mpf, when they were exposed to Pentylenetetrazole (PTZ) by immersion. The exposure of larvae and adults to 10 mM PTZ diluted in the water of system was performed up to 10 minutes (Baraban et al., 2005; Mussulini et al., 2013;

Menezes and Da Silva, 2017). Animals with any malformation were not used to assess the sensibility to PTZ.

Zebrafish larvae exposed to PTZ in a 24-well culture plate had their seizure stages measured over 10 minutes of exposure. The seizure stages were adapted from Baraban et al. (2005)(Baraban et al., 2005), considering: stage II= fast circular swimming behavior; and stage III= series of short clonic convulsions, leading to loss of posture, and fall to one side remained still for 1-3 seconds.

For adult zebrafish, seizure stages were analyzed over the 10 minutes of exposure in a test tank with dimensions of 13 cm x 11.5 cm x 8 cm (length x height x wide). For the purpose of this study the seizure stages used were adapted from Mussulini et al. (2013)(Mussulini et al., 2013) as: stage III= a circular motion; stage IV= convulsive behavior clonic type; and stage V= fall to the bottom of the aquarium and convulsive behavior of tonic type (Mussulini et al., 2013).

Statistical analysis

The survival rate was estimated according to the Log-rank (Mantel-Cox) Test. Morphological aspects, locomotor activity and seizures latency evaluation were first analyzed by normality through D'Agostino & Pearson omnibus normality test. For those data where the variance had no normal distribution the analysis was performed by Kruskal-Wallis test followed by Dunn's test, while data with normal distribution of variance followed One-way ANOVA, when appropriated, followed by Dunnet's test as post hoc analysis. All the analysis was performed using Graphpad prism 6.0. The significance level was established at $p<0.05$. The results were expressed as means ± 95% CI, except for survival determination and malformations where the data were expressed as percentages.

Results

Survival

Our results from survival demonstrated significant difference between control and scrambled *MO* larvae, with survival rate of 89% and 72%, respectively ($p<0.05$), which could be in response to a possible physical insult promoted by the needle's perforation. The animals injected with morpholinos for both *cnt2* and *5'nt* showed a survival rate lowest than observed in no-injected control and scramble, 43% and 38%, respectively, both with significant differences ($p<0.001$) (Figure 2).

Morphology

Control and scrambled *MO* embryos showed similar low incidence of malformations. In the other hand, embryos injected with morpholino against *cnt2* and *5'nt* transcripts exhibited great numbers of larvae with high scores of the malformations at 5dpf. The *cnt2 MO* promoted malformation in 82% of animals, and the *5'nt MO* affected 58% of animals, while control and scrambled *MO* showed 10% and 8%, respectively (Figure 3 insert). Animals injected with *cnt2 MO* showed a mean score of the malformations significantly elevated compared to control and scramble groups ($p<0.001$) for both (Figure 3). Animals injected with *5'nt MO* showed mean score above the level 2 from the assumed malformation scale, showing an elevated malformations level when compared with scrambled *MO* and control groups ($p<0.001$ for both) (Figure 3). The malformations observed in *cnt2* MO and *5'nt MO* groups occurred in all structures analyzed. For eyes malformations, while the control and the scrambled *MO* groups had occurrence in 3.33% and 1.66% of animals, respectively, the *cnt2 MO* and *5'nt MO* had 50% and 33.3%, respectively (Figure 4.A). The rate of malformation for swimming bladder was 8.33% in control, 1.66% in scrambled *MO*, 40% in *cnt2 MO* and 40% in *5'nt MO* (Figure 4.B). The rate of malformations for pericardium was 1.66% in control, 8.33% in scrambled *MO*, 50% in *cnt2 MO* and 53.3% in *5'nt MO* (Figure 4.C). The tail malformations rate was 1.66% in control, 3.33% in scrambled *MO* 71.6% in *cnt2 MO* and 46% in *5'nt MO* (Figure 4.D).

Locomotor Activity

For the parameters average speed and mobile time from 7-day old larvae, no group showed a significant difference from the control group or when compared with scrambled *MO* group (Figure 5 A and B).

Adult animals injected with *cnt2 MO* and scrambled *MO* showed no significant difference in locomotor parameters in relation to control group, while *5'nt MO* animals ($n=10$) had their average speed decreased in relation to the control group ($n=13$) [$p<0.05$; $F(3; 39) = 5.580$] and scramble group [$p<0.01$; $F(3; 39) = 5.580$] (Figure 6A). For time in inferior zone parameter, only the *5'nt MO* group showed significant difference from control group, increasing the total time in this zone [$p< 0.01$], but not in relation to scrambled *MO* group (Figure 6B). The *5'nt MO* group showed significant difference for mobile time in relation to scrambled *MO* group [$p<0.05$; $F(3; 39) = 5.580$], but no significant difference in relation to control group (Figure 6C).

Susceptibility to seizures

During testing of susceptibility to seizures by exposure to PTZ (10 mM) in zebrafish larvae 7dpf old, no significant alteration was observed for latency time to reach the stages II and III of seizure behavior, compared to scramble and control groups (Figure 7A and 7B).

Considering the susceptibility of adult animal to PTZ, was evaluated latency time to animals to reach the stages III, IV and V from the scale standardized by Mussulini et al., (2013). For latency time to reach stage III, scramble group showed no significant difference from control group. The time to reach the stage III was significantly lower in adult animals that received *cnt2 MO* (n=10; $p<0.01$) or *5'nt MO* (n=10; $p<0.01$) at the embryonic stage, when compared to the control group [n=10; F (3; 36) = 6.713] but not different from scrambled *MO* group (Figure 8A).

To reach the stage IV, the control group latency time was 327.9s (±84.8 CI) and the scrambled *MO* group had 305.3 seconds (±75.7 CI), showing no significant difference between them. Groups, *cnt2* MO and *5'nt* MO, showed decreased time to reach the stage IV from seizure behavioral scale. For *cnt2* MO group the latency time was 178.3 seconds (±27.4 CI) and *5'nt* MO group the time was 195.9 seconds (± 43.8 CI), both significantly different from scramble and control groups [$p< 0.01$; F (3; 36) = 7.522] (Figure 8B).

The average latency times presented by the groups who suffered specific temporary blockage for transcripts of the adenosinergic system were considerably lower comparing to the scrambled *MO* and control groups to reach the stage V of seizure. While the average of the control group was 421.8 seconds (CI ±116.2), and scrambled *MO* group was 367.9 seconds (±101.1 CI), the latency time for *cnt2 MO* group was 207.7 seconds (±54.7 CI) and *5'nt MO* group was 215.3 seconds (± 40.4 CI) thereby achieving a significant difference (*cnt2 MO*: $p<0.001$ and *5'nt MO*: $p<0.01$) [F (3; 36) = 8.450] (Figure 8C).

Discussion

The results found in this work corroborate previous studies that report the important role of adenosine in embryonic development (Massé and Dale, 2012; Rivkees and Wendler, 2017). Disturbances in adenosine signaling at early stage of development can lead to a series of morphological or neurological damages that can last until

adulthood (Wendler et al., 2009; Massé and Dale, 2012; Souza et al., 2016; Rivkees and Wendler, 2017; Menezes et al., 2018).

The survival rate of the larvae that underwent temporary gene blockade for both the CNT2 and the ecto-5-nucleotidase enzyme were considerably lower than the scrambled *MO* and control groups. This low survival may be linked to the important role of adenosine in development during the embryonic phase, being related to the formation of cardiac structures, angiogenesis and control of blood flow (Adair et al., 1989; Montesinos et al., 2004; Thompson et al., 2004; Robin et al., 2013).

The transient changes in the availability of key controllers of adenosine concentration in the extracellular medium were able to provoke a high teratogenicity. Both targets induced malformation rates distributed in a very similar way between the analyzed structures, making evident the participation of adenosine in the formation of these structures. These results corroborate with a series of previous studies showing that intervention in adenosinergic signaling during the embryonic stage leads to severe morphological damage in rodents and zebrafish (Clark et al., 1987; Rana et al., 2010; Souza et al., 2016; Menezes et al., 2018). The mechanism by which adenosine influenced all these malformations is still not entirely clear, although some studies attribute to the protective effect exerted by the A_1 receptor, against stressor agents, as hypoxia (Buscariollo et al., 2011; Souza et al., 2016). However, in studies with zebrafish in which the A_1 receptor was temporarily blocked, during the embryonic stage, no evident teratogenic effect was observed (Menezes et al., 2018).

Open field tests were performed using only animals that did not have any of the malformations analyzed previously. 7dpf larvae did not have any significant difference in locomotor activity in relation to the control and the scrambled *MO* groups. In tests of locomotor activity during the adult phase it was possible to observe that the animals that underwent temporary blockade of the enzyme ecto-5'-nucleotidase showed a significant increase both in the speed and in the time spent in the inferior zone of the aquarium. In zebrafish, these parameters are associated to anxiety, and are affected by exposure to adenosinergic antagonists (Maximino et al., 2011; Kalueff et al., 2013). These results may be associated with the control exerted by the ecto-5'-nucleotidase over adenosine levels, which is assumed to be the main source of adenosine to A_{2A} receptors (Augusto et al., 2013). In fact, A_{2A} receptors knockout animals has been shown display motor disturbance and cognitive alterations (Moscoso-Castro et al., 2017).

Among the animals tested at 7dpf, none showed a significant difference in their means regarding the latency time to reach the seizure stages analyzed. The decrease in the latency time to reach the stages of seizure in adults shows that, although during a short term, the intervention in the adenosinergic system had evident long-term consequences on PTZ sensitivity. The interventions seemed here occurs immediately before neurulation, when the neural plate is converted, in fish, in a solid structure named neural keel, which occurs precisely during gastrulation (Blader and Strähle, 2000). Tchekalarova et al 2010 and 2013 reported that the effects of interventions in the adenosinergic system are extremely sensitive to period in which the exposure is performed (Tchekalarova et al., 2010, 2013). In fact, rodents exposed to caffeine in the neonatal period had increased latency time for PTZ-induced convulsive episodes in adulthood (Guillet and Dunham, 1995). A myriad of factors involved in a complex cellular integration could be influenced by adenosine disturbance. As well as revised elsewhere, extrinsic signaling factors, such as bone morphogenetic protein, wingless-integrated and fibroblast growth factor families and the intrinsic transcription factor program, such as SRY-box containing genes B1 family are important factor during this early phase of development (Schmidt et al., 2013). Additionally, neurotransmitters and neuromodulators, including adenosine, have been proposed act as important factors in the neurogenesis (Zimmermann, 2011). The long-term response to the adenosinergic system disturbance could be related to the influence of adenosinergic signaling on, for example, glutamatergic and gabaergic neurotransmission throughout development (Safiulina et al., 2005). The signaling exerted by both glutamate and GABA is extremely important for formation of the neural network, since they participate in processes such as migration and neuronal maturation (Manent et al., 2005; Manent and Represa, 2007). However the activity of these neurotransmitters varies throughout development, thus impacting the responses exerted by these neurotransmitters according to the stage of development (Sanchez and Jensen, 2001; Menezes et al., 2015). There are studies that hypothesize other pathways by which adenosinergic signaling may cause disturbances in normal cellular development, such as transcription factors, or acting as a morphogen (Massé and Dale, 2012; Rivkees and Wendler, 2017).

Our results reaffirm that the intervention in the mechanisms of control of adenosine concentration has prominent and long-term effects in morphological aspects and neural response to proconvulsant. However, the mechanisms by which interventions on the main contributors of adenosine tonus has all these long-lasting effects are not

fully understood. Further studies involving disruption of adenosine signaling during early development stage and its relationships with other cellular components and signaling systems need to be performed.

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Figures

Figure 1

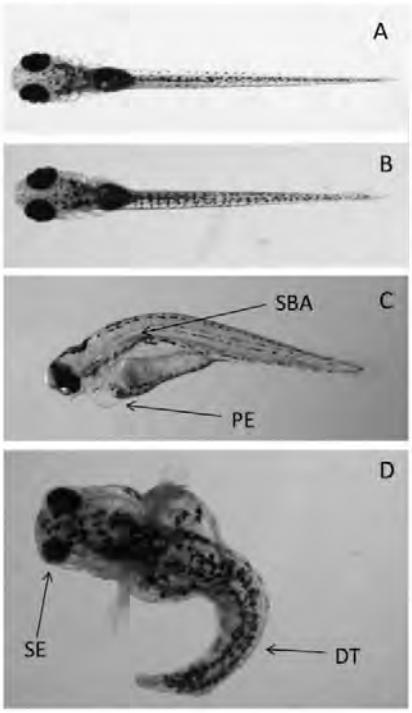


Figure 2

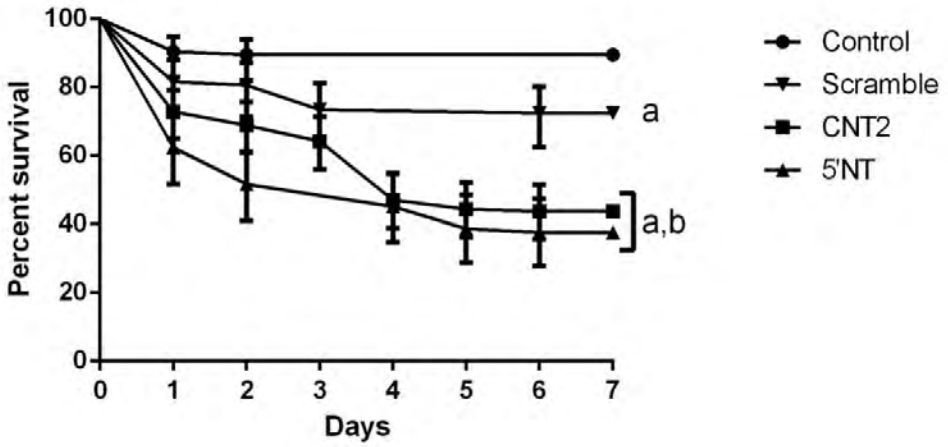


Figure 3

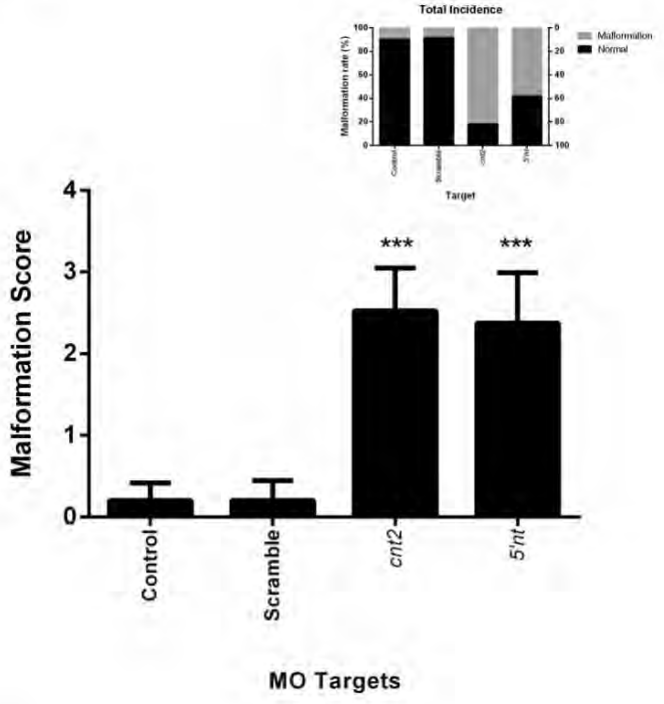


Figure 4

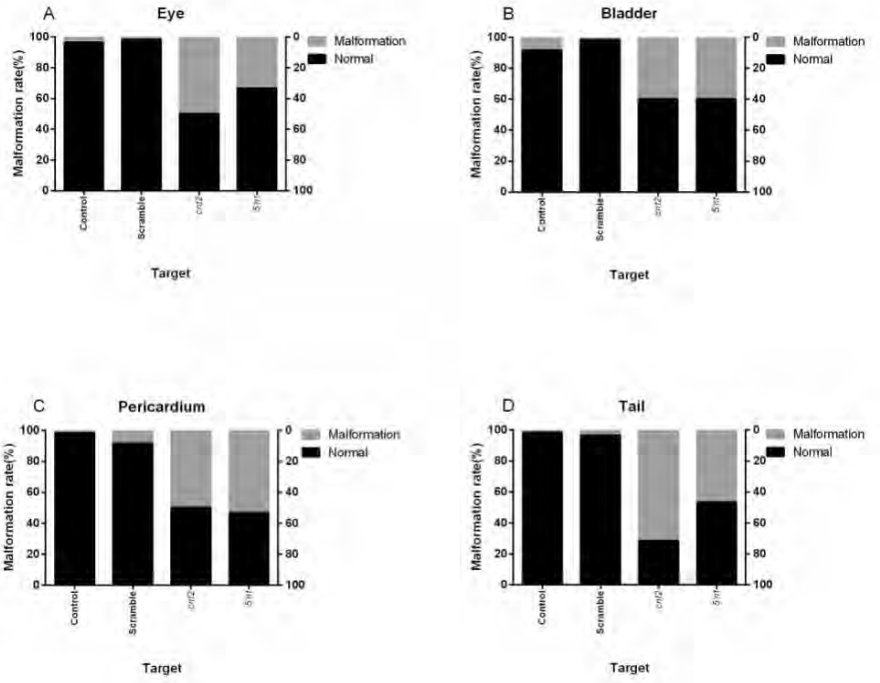
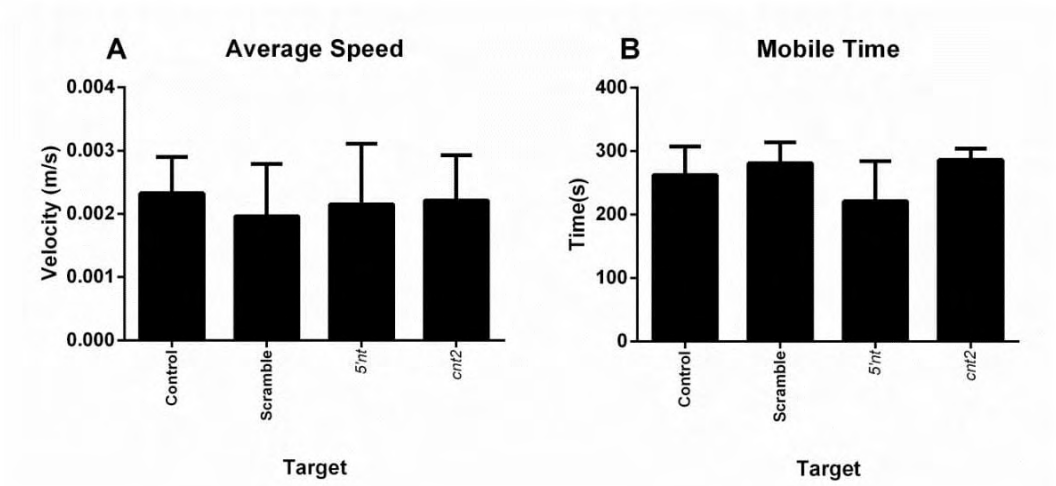


Figure 5



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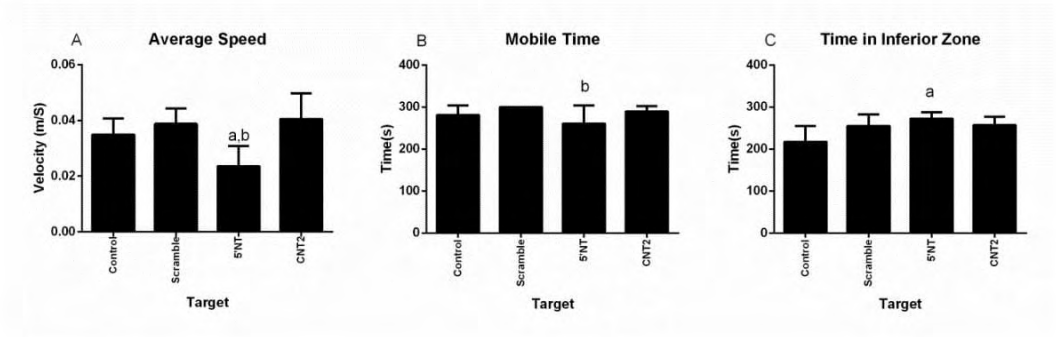

Figure 7

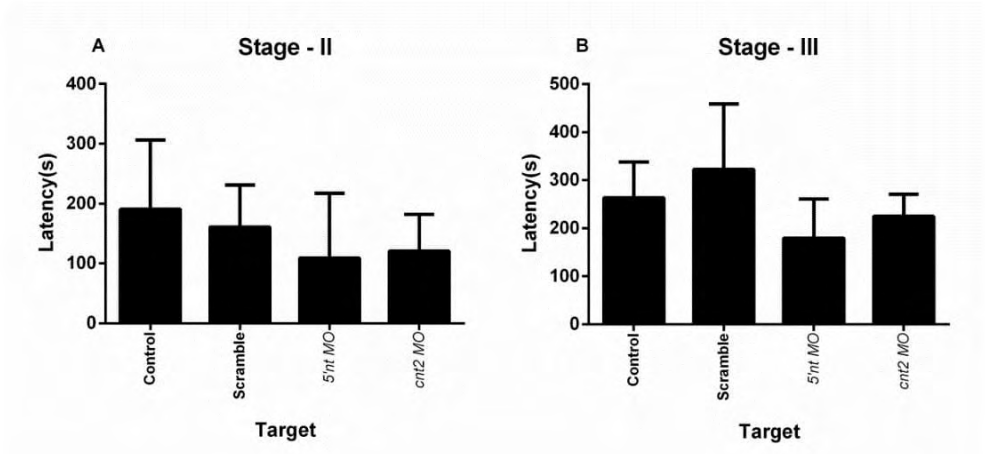

Figure 8

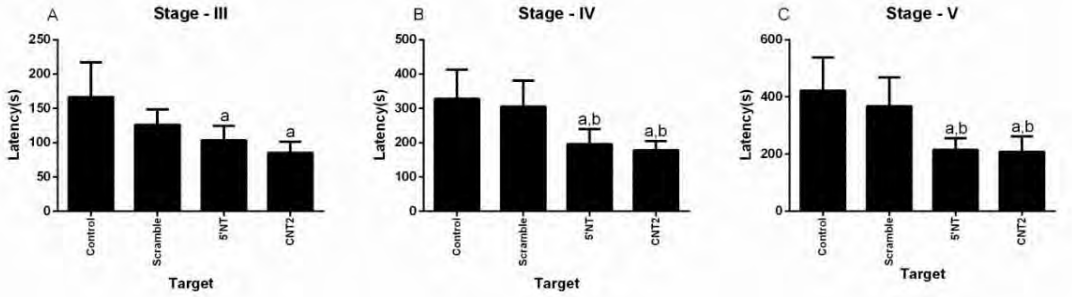

Table 1: Sequence of morpholino antisense

-	scrambled	5'-CCTCCTACCTCAGTTACAATTTATA-3'
ENSDART00000098356	CNT2	5' -CTTCTTTGTCCTTCATTCTGCGTTC - 3'
ENSDART00000104740	5'NT	5'-CATCATCATCATCCTCCTACTGTAA- 3'

Legend to Figures:

Figure 1: Illustration of malformations observed in larval zebrafish at 5 days post-fertilization. PE: Pericardial edema, SBA: Swimming bladder absent, SE: Small eyes, DT: Deformed Tail. (A) Control, (B) Scrambled *MO*, (C) *cnt2 MO* and (D)*5'nt MO.*

Figure 2: Survival rate through 7 days post-fertilization of zebrafish larvae submitted to intra egg injection (~1hour post-fertilization) of morpholino (MO) targeted to transcripts of concentrative nucleoside transporter (*cnt2*) and enzyme ecto-5'-nucleotidase (*5'nt*) . Each group was compared to control group or scrambled *MO* group using Log-rank test. "a" and "b" represent a significant level at $p<0.05$, in relation to control or scrambled *MO* group, respectively.

Figure 3: General malformations score for zebrafish larvae at 5-days post-fertilization submitted to morpholino targeted to transcripts of concentrative nucleoside transporter (*cnt2*) and enzyme ecto-5'-nucleotidase (*5'nt*). The score was established considering 0 for absence of malformation; 1 for presence of any malformation; and 2 for the accumulation of more than one malformation in the same structure. *** represents a significant level at $p<0.001$. (Insert) Total of the malformed animals in each group in percentage (n=60).

Figure 4: Percentage of animals showing malformation in (A) Eye; (B) Bladder; (C) Pericardium; and (D) Tail for zebrafish larvae at 5-days post-fertilization submitted to morpholino targeted to transcripts of concentrative nucleoside transporter (*cnt2*) and enzyme ecto-5'-nucleotidase (*5'nt*). Each group consisted of 60 animals.

Figure 5: Locomotor activity of 7-day old zebrafish larvae after embryonic exposure to morpholino targeted to concentrative nucleoside transporter (*cnt2*) and enzyme ecto-5'-nucleotidase (*5'nt*) (A) Average speed (meter/seconds) and (B) Mobile time (seconds).

Figure 6: Locomotor activity of adult zebrafish after embryonic exposure to morpholino targeted concentrative nucleoside transporter (*cnt2*) and enzyme ecto-5'-nucleotidase (*5'nt*) (A) Average speed (meters/seconds), (B) Mobile time (seconds), and (C) time at the inferior zone of the aquarium (seconds). "a" and "b" represent a significant level at $p<0.05$, in relation to control or scrambled *MO* group, respectively.

Figure 7: Latency to reach seizure stages in 7-day old zebrafish larvae after embryonic exposure to morpholino targeted to transcripts of concentrative nucleoside

transporter (*cnt2*) (n:10) and enzyme ecto-5'-nucleotidase (*5'nt*) (n:10). (A) Latency (seconds) to reach seizure stage II established as fast circular swimming behavior and (B) Latency (seconds) to reach seizure stage III established as a series of short clonic convulsions, leading to loss of posture, and fall to one side remained still for 1-3 seconds.

Figure 8: Latency time to reach seizure stages in adult zebrafish after embryonic exposure to morpholino targeted to to transcripts of concentrative nucleotide transporter (*cnt2*) (n:10) and enzyme ecto-5'-nucleotidase (*5'nt*) (n:10). (A) Latency (seconds) to reach seizure stage III established as a circular motion, (B) Latency (seconds) to reach seizure stage IV established as convulsive behavior clonic type, and (C) Latency (seconds) to reach seizure stage V established as fall to the bottom of the aquarium and convulsive behavior of tonic type. "a" and "b" represent a significant level at $p<0.005$, in relation to control or scrambled *MO* group, respectively.

3.4 Capítulo IV

Artigo Científico Original

Pharmacological manipulation of extracellular adenosine metabolism during early development affects seizure susceptibility in adult zebrafish

Autores: Fabiano Peres Menezes, Felipe Machado Torresini e Rosane Souza da Silva

A ser submetido ao periódico: Neurochemistry International

Qualis CAPES: B1

Title Page

Title: Pharmacological manipulation of extracellular adenosine metabolism during early development affects seizure susceptibility in adult zebrafish

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Key words: adenosine receptors; nucleoside transporters; development; ecto-5'-nucleotidase; seizure.

Abstract

Endogenous adenosine has anticonvulsive properties based on its ability to modulate neural excitability. Perturbations in adenosinergic signaling in the early stage of development may lead to permanent changes in susceptibility to seizures induced by pentylenetetrazole (PTZ). We performed microinjections of A_1 adenosine receptor antagonist (DPCPX; 0.01, 0.02, 0.5 mg/kg), A_{2A} adenosine receptor antagonist (ZM241385; 0.02, 0.05, 1 mg/kg), caffeine (0.1, 0.5, 1 mg/kg), nucleoside transporter inhibitor (Dipyridamole; 0.05, 0.1, 2.5 mg/kg) and ecto-5'-nucleotidase inhibitor (AMPCP; 1.5, 2, 10 mg/kg) during the embryogenesis of zebrafish. Later effects were searched through analysis of survival rate, malformation, locomotion and sensitivity to PTZ in the larval and adult phase. With the exception of DPCPX, all treatments resulted in decreased survival rate and malformation rate above 20% at the highest doses. Locomotor activity was not affected by any of the treatments. Embryos microinjected with DPCPX (0.02 and 0.5 mg/kg), caffeine (0.1, 0.5, 1 mg/kg), Dipyridamole (0.05, and 2.5 mg/kg) or AMPCP (1.5 and 2 mg/kg) showed decreased latency time to reach seizure scores compared to the control group at larval stage ($p<0.05$). In the adult stage, embryos microinjected with DPCPX (0.5 mg/kg), ZM241385 (0.05 and 1 mg/kg), caffeine (1 mg/kg), Dipyridamole (0.05 and 0.1 mg/kg) and AMPCP (1.5 mg/kg) showed lower latency time to reach seizure scores compared to the control groups ($p<0.05$). In addition to causing deleterious effects to embryonic development, early adenosinergic signaling disorders cause changes in PTZ sensitivity in the short and long term.

1. Introduction

Adenosine plays an important role as an endogenous anticonvulsant (Boison, 2013; Dunwiddie, 1999). In studies using microdialysis or HPLC, an increase in extracellular adenosine levels was observed in individuals following spontaneous seizure disorder (Doná et al., 2016; During and Spencer, 1992). By increasing its extracellular levels, adenosine is able to perform both pre-synaptic and post-synaptic signals that contribute to decrease the frequency and duration of seizures (Ilie et al., 2012; Sebastião and Ribeiro, 2009). The relationship between adenosine and epilepsy may not be related only to neuromodulation during seizure episodes, but also its signaling during embryogenesis (Doriat et al., 1999). The adenosinergic system is able to interfere in the signaling of other neurotransmitters, or to interact with transcription factors, acting as a key element during the embryonic phase, which can significantly affect morphological and neurological development with long or short-term consequences (Ciruela et al., 2001; Rivkees and Wendler, 2017; Rivkees et al., 2001).

Disturbance of adenosine signaling during early development can be commonly associated with risk of low birth weight (Chen et al., 2014). In rodents, pro and anticonvulsant effects of early caffeine exposure have been demonstrated to be age, model and dose-dependent (Tchekalarova et al., 2010, 2007). The mechanism of caffeine action appears to affect both inhibitory and excitatory neurotransmitter systems (Tchekalarova et al., 2010, 2007). During brain development, a hypoxic event also disturbs adenosine metabolism, since adenosine levels increase drastically as a way to decrease brain metabolism (Pearce, 2006). The hypoxic event itself can promote seizure, which in neonates can promote preconditioning in brain involving purinergic system (Zgodziński et al., n.d.).

The impacts in adenosine availability and action can also receive contribution from a myriad of cellular components involved in the control of this nucleoside, such as the enzymes involved in the production and degradation of adenosine and the nucleoside transporters. Accumulation of adenosine in the extracellular medium by adenosine kinase inhibition and nucleoside transport blockade seems to contribute to seizure suppression, while inhibition of adenosine production by ecto-5'-nucleotidase inhibition produces generalized seizures (Zhang et al., 1993).

There measureless influences that an immature brain can be exposed and possible promote impacts with long-term consequences as a response to maladaptations. In this sense, we surrounded the extracellular adenosine metabolism through the use of specific and unspecific antagonists of adenosine receptors, an inhibitor of ecto-5'nucleotidase and an inhibitor of nucleoside transporter to evaluate how the early exposure to these agents affects the morphology and the susceptibility to seizures induced by pentylenotetrazole in the larval and adult phase.

2. Methods

2.1 Animals

For these experiments, animals came from the breeding colony of wild type zebrafish held at the ZEBLAB laboratories of the Pontifical Catholic University of Rio Grande do Sul, Brazil. Breeding matrices were kept on a shelf of automated aquariums, with system water quality controllers (ZEBTEC, Tecniplast, Buguggiate (VA) Italy) in a ratio of 2 male / 1 female, at a density of 2 / L. The day before the reproduction, the matrices were re-allocated and separated by a barrier. The barriers were removed in the first hour of the light cycle and the eggs were collected 10 minutes after the beginning of the reproductive ritual, and forwarded to microinjection. After microinjection,

animals followed standardized protocol for growth, until the day of the experiments. All animals were maintained on a light cycle of 14h Light / Dark 10h, and temperature of water at 27± 1 ° C. After the behavioral testing, the animals were euthanized with (MS-222) tricaine overdose (500 mg/L) according to Brazilian Legislation. All protocols followed Brazilian legislation and were approved by the Institutional Animal Care Committee of PUCRS registered under the protocol number 14/00416 - CEUA/PUCRS.

2.2 Drugs and microinjection

To evaluate the influence from interventions on adenosinergic signaling during the early development was used antagonists and blockers, as follow: 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; A_1 adenosine receptor antagonist; Merck KGaA, Darmstadt, Germany)(Anna Maria Siebel et al., 2015), ZM-241385 (A_{2A}/A_{2B} receptor antagonist; Tocris Bioscience, Bristol, United Kingdom) (Lasley et al., 2007; Anna Maria Siebel et al., 2015), Caffeine (unspecific adenosine receptor blocker; Merck KGaA, Darmstadt, Germany), (Fredholm et al., 1999), Dipyridamole (Equilibrative Nucleoside Transporters (ENT) inhibitor; Merck KGaA, Darmstadt, Germany)(A.M. Siebel et al., 2015), and Adenosine 5′-(α,β-methylene)diphosphate (AMP-CP) (CD73/ecto-5′-nucleotidase enzyme inhibitor; Merck KGaA, Darmstadt, Germany) (Lutte et al., 2015; Anna Maria Siebel et al., 2015). A curve of dose was performed for each drug (Table 1). The microinjection was chosen to guarantee the concentration of each drug. Injected control were animals injected with vehicle (saline or DMSO 2%) and non-injected controls were naïve embryos at the same stage of development. For microinjection in embryos, small aliquots of stock solutions were diluted in Danieau's solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)$, 25 mM HEPES, pH 7.6] as described in The Zebrafish Book (Westerfield, 2007), and stained

with 0.5% phenol red in Dulbecco's Phosphate-Buffered Saline (DPBS;Sigma, cat. no. P-0290).

For microinjection was used Narishige micromanipulation system coupled to a stereomicroscope NIKON SMZ1500™. The microcapillary needles were previously prepared, containing 2μL of the agent to be microinjected and calibrated by injection in immersion oil and calculation of the released volume ($V = 4/3\ \pi r^3$) reaching a suitable pressure (≈3- 6 PSI) for delivering a total volume of 10 nL/pulse. Eggs at 1 hour post-fertilization were lined up on a microscope slide adjusted to a petri dish, to perform the microinjection series. All eggs were injected into the yolk sac region of the embryo, with maximum development stage of 4 cells.

2.3 **Survival rate and malformations**

The survival analysis rate was performed between 0 and 7 days post-fertilization (dpf) by daily conference and removal of dead animals. Unfertilized or damaged eggs were discarded few hours after the microinjection and not accounted.

For teratogenic evaluation 5 dpf animals were used. The animals were placed on the lid of a 96-well plate, which were individualized by the grooves of each well containing approximately 40 μL of fish-system water (reverse osmosis water reconditioned with instant ocean salt, pH 7.5 and 500μS-micro siemens). The morphological parameters analyzed were based on the most affected structures: eyes, bladder swim, pericardium and tail. For all the changes found in each one of these structures scores were assigned: 0 for absence of malformation; 1 for presence of any malformation and 2 for the accumulation of more than one malformation in the same structure. Each animal had its score calculated based on the sum of malformations observed in each of the structures analyzed, revealing a degree of teratogenic severity

for each target. A rate of malformations was performed by the sum of the absolute number of individuals that shown malformations in each group.

2.4 Locomotor activity

For 7dpf animals, the test apparatus for locomotor activity analysis (open field) consisted of a 24-well culture plate, containing the fish-system water (drug-free), with a column of 1.5 cm, where the animals were placed individually and the performance recorded by a digital camera (Logitech ©) located under o apparatus, at 20 cm distance.

The activity evaluation was performed during 5 minutes for assessment of mobile time (s) and the average speed during the moving time (s). These parameters were registered after 30 seconds of acclimation.

2.5 Sensitivity to Pentylenetetrazole

Animals microinjected with drugs and vehicle were created under normal handling until reaching 7 dpf or 4-7 months post-fertilization (mpf) when those that did not present malformation were exposed to Pentylenetetrazole (PTZ) by immersion.

The exposure of larvae and adults to 10 mM PTZ diluted in the fish-system water was performed up to 10 minutes (Baraban et al., 2005; Mussulini et al., 2013). The larvae undergoing the PTZ exposure had their stages measured over 10 minutes of exposure in a 24-well culture plate. The seizure stages were adapted from previous works of the (Baraban et al., 2005) considering stage II: fast circular swimming behavior; and stage III: series of short clonic convulsions, leading to loss of posture, and fall to one side remained still for 1-3 seconds (Baraban et al., 2005). The seizure stages of adult animals were measured over the 10 minutes of exposure in a test tank with internal dimensions of 13 cm x 11.5 cm x 8 cm (length x height x wide). For the purpose of this study the seizure stages used for adult animals were adapted from previous works as stage III: a circular motion; stage IV: convulsive behavior clonic

type; and stage V: fall to the bottom of the aquarium and convulsive behavior of tonic type (Mussulini et al., 2013).

2.6 Statistical analysis

The survival rate was estimated according to the Log-rank (Mantel-Cox) Test. Morphological aspects, locomotor activity and seizures latency evaluation were first analyzed by normality through D'Agostino & Pearson omnibus normality test. Kruskal-Wallis test followed by Dunn's test was used for data with no normal distribution, while data with normal distribution of variance followed One-way ANOVA followed by Dunnet's test as post hoc analysis. All the analysis was performed using Graphpad prism 6.0. The significance level was established at $p<0.05$. The results were expressed as means ± 95% CI, except for survival determination and malformations where the data were expressed as percentages.

3. Results

3.1 Survival rate

The survival rate was not significantly different between non-injected control and injected control (Saline and 2% DMSO) (Figure 1). Animals microinjected with DPCPX did not present a significant difference in the survival rate on the seventh day when compared to the control group (Figure 1A). Among the microinjected embryos with ZM241385, only the dose of 0.05 mg/kg showed a decrease in the survival rate at the end of the seventh day after fertilization ($p<0.05$) (Figure 1B).

Embryos microinjected with doses 0.1, 0.5 and 1 mg/kg of caffeine showed lower survival rate than the non-injected control group at the end of 7dpf ($p<0.05$, $p<0.0001$ and $p<0.0001$, respectively) (Figure 1C).

Microinjected larvae with dipyridamole presented a significant difference from non-injected control in survival rate only at the dose of 2.5 mg / kg ($p<0.0001$) (Figure 1D). The survival rate of the microinjected larvae with AMPCP presented a difference from non-injected control only at the 2 mg/kg dose ($p<0.001$) (Figure 1E).

3.2 Malformation

To reveal the severity of malformation we used a score of severity, indicating that animals presented more than one anomalous organ morphology presented here as a mean for each dose of each treatment (Figure 2). Non-injected control and injected control (Saline and 2% DMSO) had no differences on malformation rate (Figure 2). Larvae injected with ZM241385 at the doses of 0.05 and 1 mg/kg showed high scores significantly different from the non-injected control group ($p<0.05$) (Figure 2B). The microinjected larvae with caffeine 0.5 mg/kg had a high malformation score compared to the non-injected control group ($p<0.01$) (Figure 2C). Dipyridamole and AMPCP had no significantly malformation score (Figures 2D and 2E).

In the observations of the percentage of animals that showed some malformation in each treatment, we could see that the non-injected control and injected control with saline and 2% DMSO presented rates of 8%, 7% and 15%, respectively. The embryos microinjected with DPCPX at doses 0.01, 0.02 and 0.5 mg/kg presented malformation rates of 8%, 15% and 7%, respectively (Figure 3A). The embryos microinjected with ZM241385 at doses 0.02, 0.05 and 1 mg/kg presented 27%, 30% and 28% of malformation rate, respectively (Figure 3B). Caffeine caused 33%, 50% and 20% of malformation rate at the doses 0.01, 0.5 and 1 mg/kg, respectively (Figure 3C). The embryos microinjected with dipyridamole at doses 0.05, 0.2 and 2.5 mg/Kg, showed total malformation rates of 0%, 17% and 27%, respectively (Figure 3D). The

malformation rates of larvae microinjected with AMPCP at doses 1.5, 2 and 10 mg/kg were 0%, 0% and 22%, respectively (Figure 3E).

3.3 Locomotor Activity

Locomotor activity of 7 dpf larva was not affected by any of the drugs microinjected during embryonic phase, including the vehicles, when compared to non-injected control group (Supplementary fig. 1).

3.4 Sensitivity to seizure induced by PTZ

Microinjected embryos with different doses of adenosine receptor antagonists or inhibitors of extracellular adenosine concentration controllers tested at 7dpf exhibited a unpredictable degree of sensitivity to pentylenetetrazole, measured on the basis of latency time to reach seizure stages.

Embryos microinjected with A_1 adenosine receptor antagonist (DPCPX) at the dose 0.01 mg/kg, as well as the injected control, did not present a significant difference of latency of larvae to reach seizure stages in relation to the non-injected control (Figure 4A). The microinjected larvae at the 0.02 mg/ kg dose showed a decrease in the latency time to reach stages II and III in relation to the non-injected control group ($p<0.05$ and $p<0.01$, respectively) (Figure 4 A and B). Also, microinjected larvae at the dose 0.5 mg/kg showed a decrease in latency time to reach stages II and III in relation to the non-injected control group ($p<0.05$ and $p<0.001$, respectively) (Figure 5 A and B).

Embryos microinjected with A_{2A} adenosine receptor antagonist (ZM241385) showed no significant difference in latency time of larvae to reach seizure scores in relation to non-injected control group (Figure 4 C and D).

Caffeine microinjection, a non-selective antagonist for adenosine receptors, at the dose of 0.1 mg/kg exhibited an increase in latency time to reach stage II ($p<0.05$),

but not to stage III (Figure 4 E and F). At the doses 0.5 and 1 mg/kg, caffeine microinjection did not show significant difference from non-injected control group in the latency to reach stage II (Figure 4 E), but increased the latency time to reach stage III at the dose 0.5 mg/kg ($p<0.001$) and decreased the latency time to reach stage III at the dose 1 mg/kg ($p<0.001$) (Figure 4 F).

Embryos microinjected with dipyridamole did not show a significant difference in latency to reach seizure stage II in relation to the non-injected control group for any of the doses tested (Figure 4 G). However, microinjected embryos with 0.05 mg/kg of dipyridamole showed an increase in latency time of larvae to reach stage III ($p<0.01$), while at the dose 2.5 mg/kg a decrease in latency time occurred ($p<0.05$) (Figure 4 H).

The microinjection of AMPCP, an ecto-5'-nucleotidase inhibitor, decreased the latency of larvae to reach stages II and III at the doses 1.5 and 2 mg/Kg, respectively, in relation to non-injected ($p <0.05$ and $p<0.01$, respectively) (Figure 4 I and J).

The results observed in adult animals for latency time to reach stages III, IV and V show that some drugs cause long-lasting effects. However, embryos microinjected with vehicles and DPCPX at the doses 0.01 and 0.02 mg/kg did not present significant differences in relation to the non-injected control group to reach seizure stages. In the latency time to achieve stage V, only the dose 0.5 mg/kg of DPCPX showed a significant difference, decreasing the time to reach this stage ($p<0.05$) (Figure 5 A,B and C).

Microinjection of ZM241385 at the dose 1 mg/kg presented a decrease of the latency time of adult animals to reach the stage III in relation to non-injected control group ($p<0.05$). The animals injected at doses 0.02 and 1 mg/kg presented a decrease in time to reach stages IV ($p<0.05$ and $p<0.01$, respectively) and V ($p<0.05$ and $p<0.01$, respectively) in relation to non-injected control (Figure 6 A,B and C).

Among the animals microinjected with caffeine, those animals exposed to the dose 1 mg/kg showed a significant decrease of latency time of adults to reach stage IV ($p<0.05$) in relation to non-injected control group (Figure 7 A, B and C).

The results of the latency time of adults animals injected with dipyridamole during embryonic phase showed that the dose of 0.05 mg/kg decreased the time to reach stage III ($p<0.01$), IV ($p<0.01$) and V ($p<0.001$). The dose 0.1 mg/kg of dipyridamole decreased the latency time of adult animals to reach stages IV ($p<0.05$) and V ($p<0.01$), while the dose 2.5 mg/kg had no effect in latency time in relation to non-injected control group (Figure 8 A, B and C).

In the tests performed with animals microinjected with AMPCP a significant decrease in the latency time of adult animals to reach stages IV and V was promoted by the dose 1.5 mg/kg ($p<0.05$) (Figure 9 A, B and C).

4. Discussion

Here we found that selective pharmacological intervention on adenosine metabolism during early life has different short- and long-term effects depends on the target of adenosine metabolism. The survival rates decreased and the morphology was affected mainly in embryos submitted to the unspecific block of adenosine receptors or specific antagonism of A_{2A} adenosine receptor at higher doses. Additionally, the decrease of adenosine production by inhibition of ecto-5'-nucleotidase at intermediate doses of AMPCP was also detrimental to survival rate. In the other hand, the possible increase of adenosine levels promoted by blockade of nucleoside reuptake also impacted survival and morphology at higher doses, while the blockage of A_1 adenosine receptor did not affect survival and morphology.

Considering the particularities of zebrafish to shed light upon the results, it has been shown that zebrafish exhibits paralogs for the A_{2A} adenosine receptor, termed $Adora_{2AA}$ and $Adora_{2AB}$, and that these different genes are expressed in different regions from the gastrulation phase and following during the larval phase (Boehmler et al., 2009). In our previous work we have observed that the specific blockade of $A2_{AA}$ adenosine receptors did not cause significant morphological changes, whereas $A2_{AB}$ receptor blockade caused severe malformations in many larvae, and the joint blockade of $A2_{AA}$ and $A2_{AB}$, caused slightly more prominent morphological changes than those observed in animals that only blocked $A2_{AB}$ transcripts (Menezes et al., 2018). The blockage performed on both receptors A_2 and A_1 adenosine receptors, using caffeine in the embryonic stage of rats, provokes a high index of malformations (Souza et al., 2016). The low survival rate and the high morphological outcomes at the higher dose of A_{2A} adenosine receptor antagonists, considering specific and unspecific drugs, indicate an important participation of A_{2A} receptors in embryonic development, which could be related for example, to the role of this receptor in the hemodynamics. In fact, it was shown that caffeine exposure during early development of mice affects embryonic arterial blood flow and induced intrauterine growth retardation, which was reproduced by a specific block of A_{2A} adenosine receptor blockade (Momoi et al., 2008). The possible reduction of adenosine availability by inhibition of ecto-5'-nucleotidase promoted effects on survival only at the intermediate concentrations of AMPCP. While no significant morphological differences were observed, some studies report that interventions in the mechanisms responsible for the control of the extracellular concentration of adenosine can also trigger in physiological and neuronal changes in the initial phase of development (Klyuch et al., 2012; Koszalka et al., 2004; Zernecke et al., 2006). Previous studies show that disturbances in the signaling exerted by adenosine,

even when punctual, can entail severe damages to the embryonic development becoming determinants for the total interruption of development (Menezes et al., 2018; Souza et al., 2016).

However, it was observed that A_1 receptor blockade did not significantly alter the survival rate and morphology, which is in agreement with our previous work in that the blocking of transcripts to the A_1 adenosine receptor, through the morpholinos technique, did not cause alteration in the survival rate of zebrafish larvae (Menezes et al., 2018). Some studies highlight that knockout animals for the A_1 adenosine receptor showed no difference in the heart rate in normal situations, but in a state of hypoxia or stress during embryogenesis, significant changes were observed in cardiac function, suggesting a protective role of this receptor (Buscariollo et al., 2011; Rivkees and Wendler, 2017; Wendler et al., 2010, 2007). However, other studies report that the activation of A_1 receptors in the initial phase of development causes severe damage to the formation of the cardiac structure (Zhao and Rivkees, 2001).

Although locomotion tests performed on 7dpf larvae did not show any significant difference in relation to the control group, other possible neurological consequences provoked by the intervention in the adenosinergic signaling in the embryonic phase cannot be ruled out, since previous works report changes in the normal development of the brain, caused by adenosinergic antagonists (Silva et al., 2013), which could underline the responses to PTZ. Previous studies showed disturbances caused on the adenosinergic signaling during the early stages of development are related to the susceptibility to seizures, but a series of factors such as (i) the stage of development in which exposure is performed, (ii)) time of exposure, (iii) model of induction of seizures, can lead to different levels of susceptibility(Georgiev et al., 1993;

Guillet, 1995; Guillet and Dunham, 1995; Menezes et al., 2018; Tchekalarova et al., 2010).

The action of adenosine as an endogenous tonic for seizures has been widely recorded in a series of studies (Boison, 2013; León-Navarro et al., 2015; Murray et al., 1985; Anna Maria Siebel et al., 2015). In the results observed in our study, the seizure crisis were obtained long after the exposure, it was evidenced that impairments in the adenosinergic signaling during the development of the neural network may entail modifications in the nervous system. At 7dpf, microinjected during embryonic phase with the highest doses of the A_1 receptor antagonist (DPCPX) demonstrated greater sensitivity to pentylenetetrazole, while the A_2 adenosine receptor antagonism showed no significant difference. However, the A_2 adenosine receptor antagonism during embryonic phase promoted great sensitivity to PTZ in the adult phase. In our previous work, the temporary knockdown of A_1 and A_2 adenosine receptor in the early stage of development promoted prominent PTZ sensitivity at adulthood, but no significant effect when tested at 7dpf (Menezes et al., 2018). These differences may be related to the dynamics of action of each of the approaches, such as half-life, affinity and compensatory effect. The role of A_1 and A_{2A} adenosine receptors in neurogenesis demonstrates adenosine actively participating in processes such as cell differentiation, migration and proliferation (Canals et al., 2005; Silva et al., 2013; Weaver, 1996), or still interfering in the signaling of neurotransmitters like GABA and glutamate that are directly involved in the formation of neural network (Manent and Represa, 2007; Obrietan et al., 1995; Silva et al., 2013). Like the previous antagonists, the microinjected animals with caffeine exhibited divergent results between the periods tested. In the larval phase lower dose decreased susceptibility to PTZ, while the higher dose increased susceptibility to PTZ, while in adult phase, only one dose showed

difference in stage II seizure. The administration of caffeine in the early stages of development has a number of effects that may vary due to small differences such as dose or stage of development in which the exposure was performed(Hughes and Beveridge, 1990; Poole et al., 2016; Tchekalarova et al., 2010).

Animals exposed to inhibitors of mechanisms regulating the concentrations of extracellular adenosine, dipyridamole and AMPCP, exhibited slightly similar results. The interventions via these mechanisms caused alterations in the susceptibility provoked by PTZ in the two developmental phases tested, although this was not observed as a dose-dependent effect. An increase in the extracellular concentrations of adenosine caused by the administration of dipyridamole has been reported in adult animals as anticonvulsant (Akula et al., 2008; Park and Gidday, 1990; Anna Maria Siebel et al., 2015; Xu et al., 2015). However, in the early stages of development the exacerbated activation of A_1 receptors by increase of extracellular adenosine may contribute to permanent changes in the nervous system (Turner et al., 2002). On the other hand, inhibition of the action of the enzyme ecto-5'-nucleotidase under basal conditions does not have a great influence on the extracellular concentrations of adenosine, however in some regions like striatum is the main source of adenosine responsible for the activation of $A2_A$ receptors (Augusto et al., 2013). The relationship between adenosine from ecto-5'-nucleotidase and $A2_A$ was also observed in in vitro experiments using mouse mesenchymal cells, in which the absence of ecto-5'-nucleotidase directly reflected in the expression of $A2_A$ receptor and its actions in processes differentiation and proliferation (Katebi et al., 2009). In our results it was possible to observe that the blockade of the ecto-5'-nucleotidase using AMPCP was able to promote disorders in the adenosinergic signaling during the embryogenesis able to influence the susceptibility of the zebrafish to the PTZ.

While we had several information on long-term effects of adenosine metabolism disturbance on early development, there are some limitations to be overcome, such as the availability of information on drug specificity to the cellular targets in zebrafish. In summary, it is still not entirely clear the mechanisms by which interventions in adenosinergic signaling is able to interfere in proconvulsive susceptibility in adulthood. It has been observed that adenosine receptor antagonists impair the migration of gabaergic neurons in the hippocampus region (Silva et al., 2013), which could be strong related to our findings. The study of the development of other signaling pathways that are fundamental in the formation of the neural network, such as the glutamatergic, in animals that underwent interventions in adenosinergic signaling during the embryonic phase should be an imminent future investigation of this phenomenon.

Figures:

Figure 1

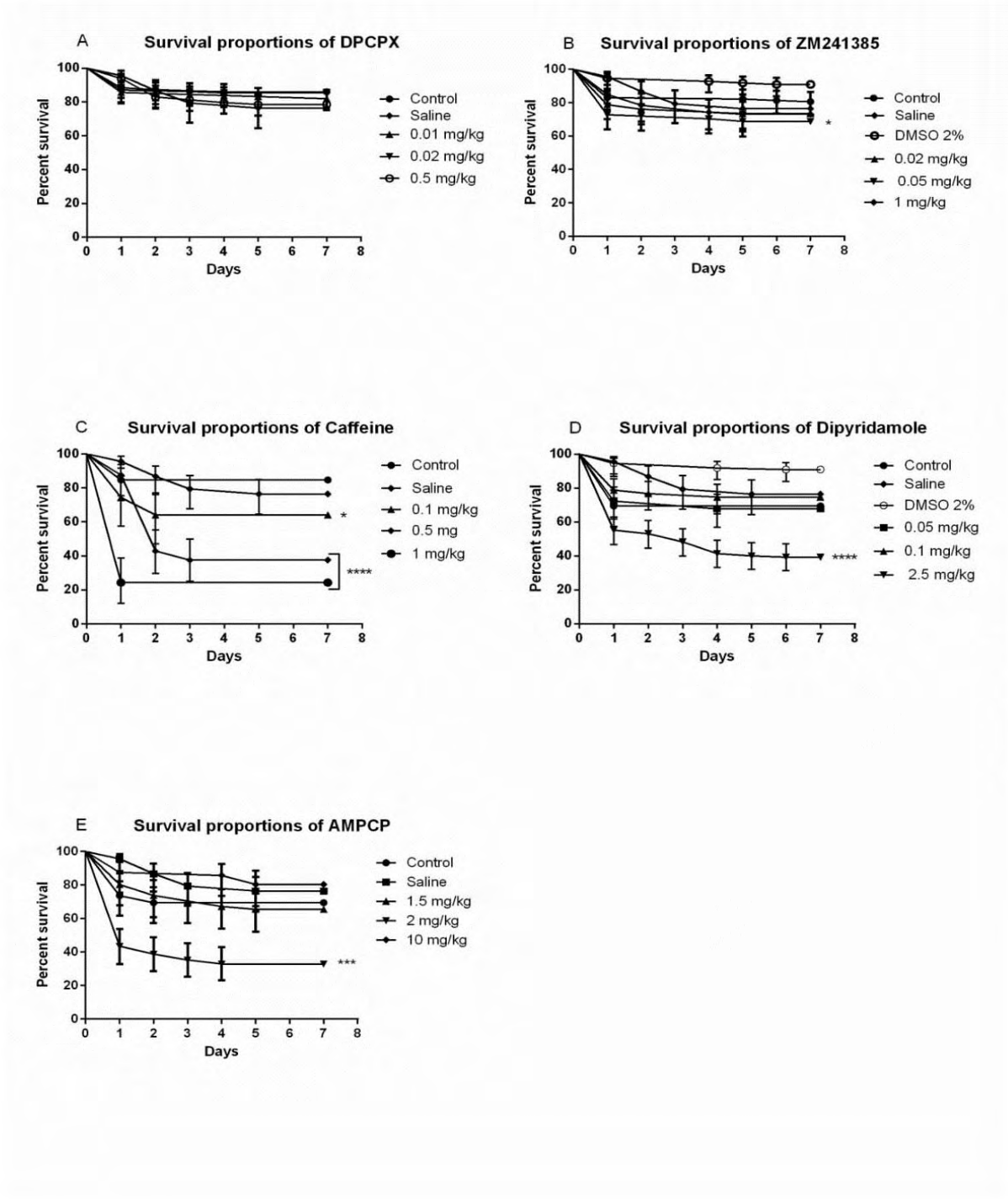


Figure 2

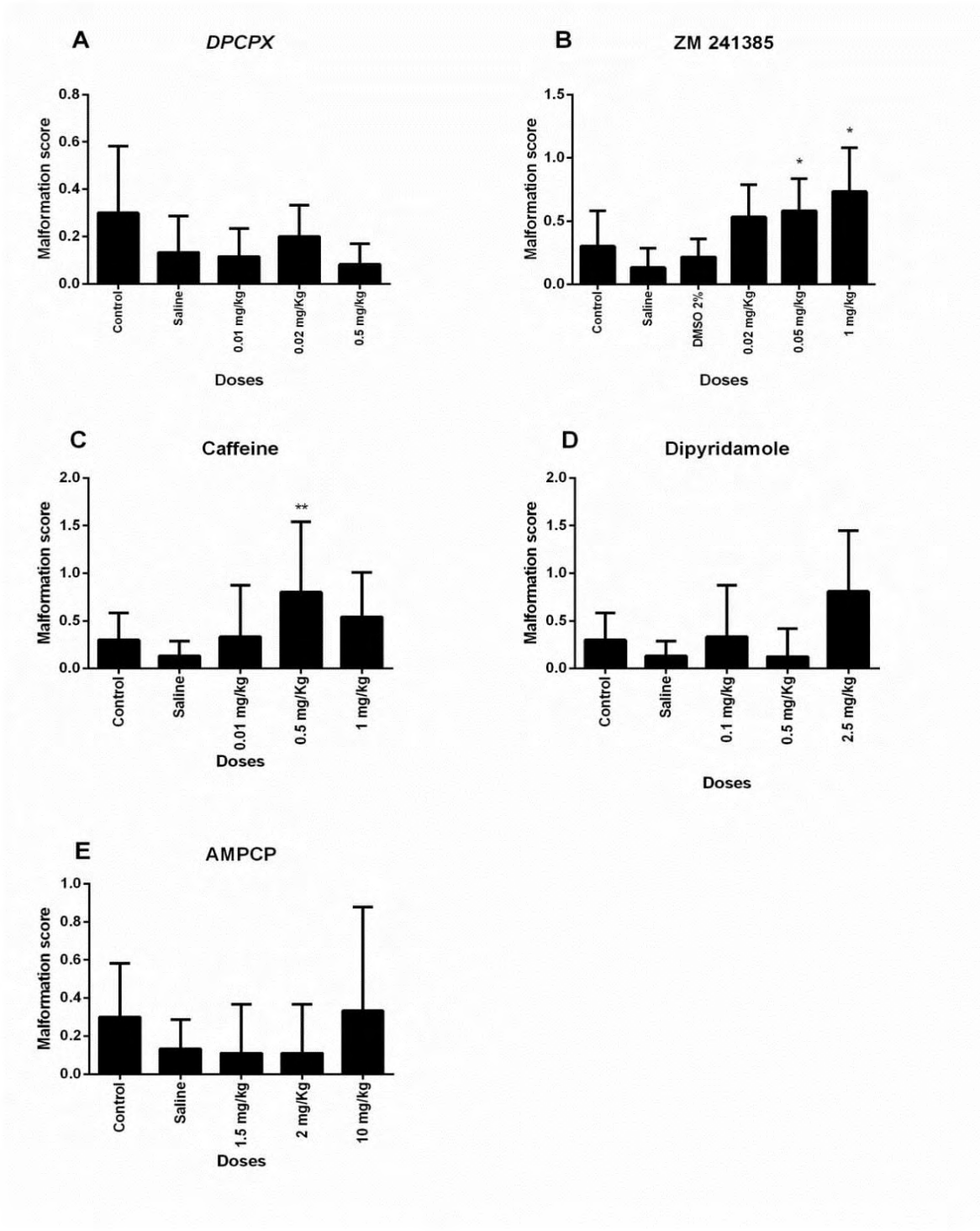

A
DPCPX
Malformation score
0.0
0.2
0.4
0.6
0.8
Control
Saline
0.01 mg/kg
0.02 mg/Kg
0.5 mg/kg
Doses
B
ZM 241385
Malformation score
0.0
0.5
1.0
1.5
Control
Saline
DMSO 2%
0.02 mg/Kg
0.05 mg/kg
1 mg/kg
*
*
Doses
C
Caffeine
Malformation score
0.0
0.5
1.0
1.5
2.0
Control
Saline
0.01 mg/kg
0.5 mg/Kg
1 mg/kg
**
Doses
D
Dipyridamole
Malformation score
0.0
0.5
1.0
1.5
2.0
Control
Saline
0.1 mg/kg
0.5 mg/Kg
2.5 mg/kg
Doses
E
AMPCP
Malformation score
0.0
0.2
0.4
0.6
0.8
1.0
Control
Saline
1.5 mg/kg
2 mg/Kg
10 mg/kg
Doses

Figure 3

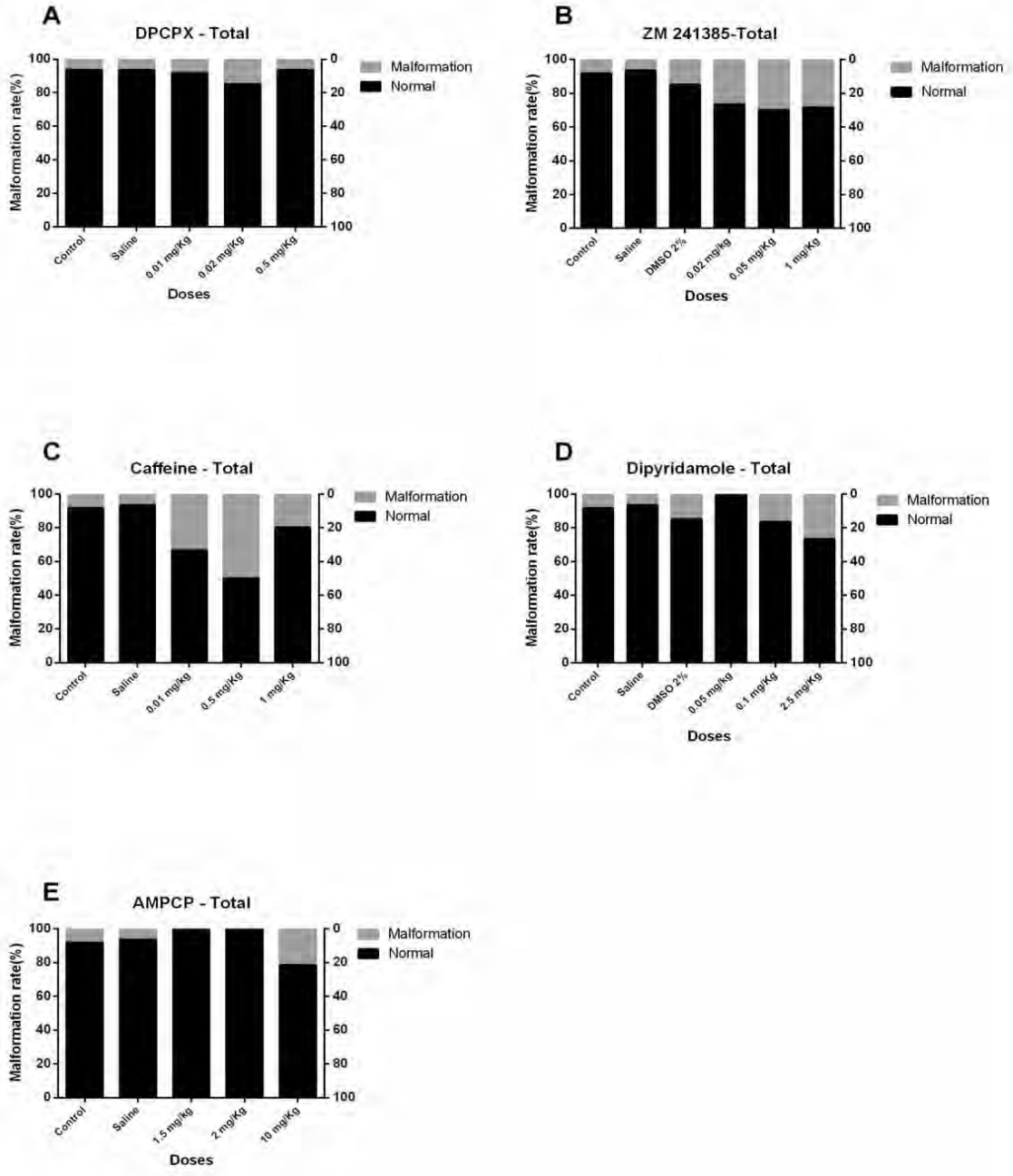

A
DPCPX - Total
Malformation rate(%)
Malformation
Normal
Control
Saline
0.01 mg/Kg
0.02 mg/Kg
0.5 mg/Kg
Doses
B
ZM 241385-Total
Malformation rate(%)
Malformation
Normal
Control
Saline
DMSO 2%
0.02 mg/Kg
0.05 mg/Kg
1 mg/Kg
Doses
C
Caffeine - Total
Malformation rate(%)
Malformation
Normal
Control
Saline
0.01 mg/Kg
0.5 mg/Kg
1 mg/Kg
D
Dipyridamole - Total
Malformation rate(%)
Malformation
Normal
Control
Saline
DMSO 2%
0.05 mg/Kg
0.1 mg/Kg
2.5 mg/Kg
Doses
E
AMPCP - Total
Malformation rate(%)
Malformation
Normal
Control
Saline
1.5 mg/Kg
2 mg/Kg
10 mg/Kg
Doses

Figure 4

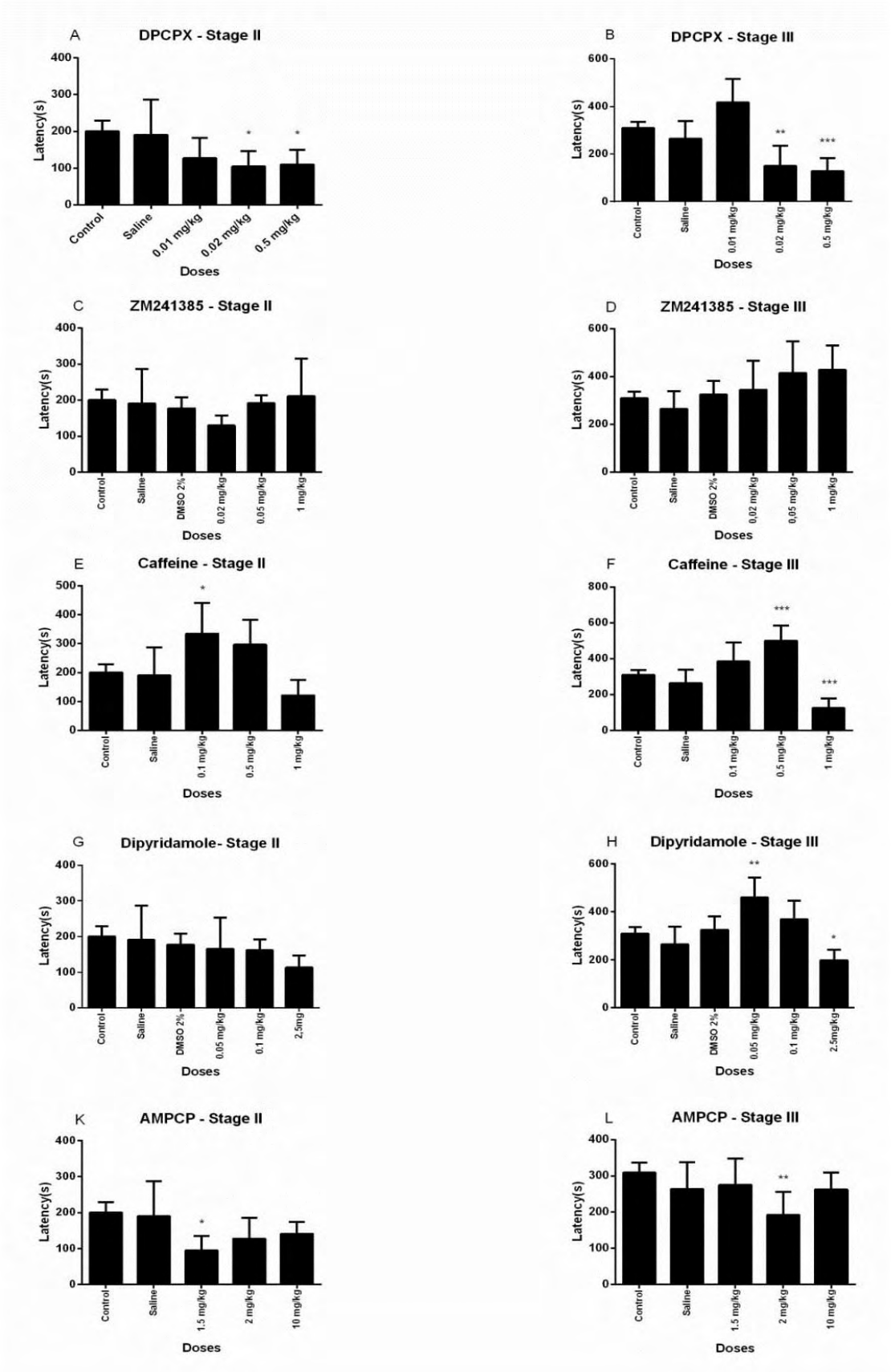

A
DPCPX - Stage II
Latency(s)
Control
Saline
0.01 mg/kg
0.02 mg/kg
0.5 mg/kg
Doses
B
DPCPX - Stage III
Latency(s)
Control
Saline
0.01 mg/kg
0.02 mg/kg
0.5 mg/kg
Doses
C
ZM241385 - Stage II
Latency(s)
Control
Saline
DMSO 2%
0.02 mg/kg
0.05 mg/kg
1 mg/kg
Doses
D
ZM241385 - Stage III
Latency(s)
Control
Saline
DMSO 2%
0,02 mg/kg
0,05 mg/kg
1 mg/kg
Doses
E
Caffeine - Stage II
Latency(s)
Control
Saline
0.1 mg/kg
0.5 mg/kg
1 mg/kg
Doses
F
Caffeine - Stage III
Latency(s)
Control
Saline
0.1 mg/kg
0.5 mg/kg
1 mg/kg
Doses
G
Dipyridamole- Stage II
Latency(s)
Control
Saline
DMSO 2%
0.05 mg/kg
0.1 mg/kg
2.5mg
Doses
H
Dipyridamole - Stage III
Latency(s)
Control
Saline
DMSO 2%
0.05 mg/kg
0.1 mg/kg
2.5mg/kg
Doses
K
AMPCP - Stage II
Latency(s)
Control
Saline
1.5 mg/kg
2 mg/kg
10 mg/kg
Doses
L
AMPCP - Stage III
Latency(s)
Control
Saline
1.5 mg/kg
2 mg/kg
10 mg/kg
Doses

Figure 5

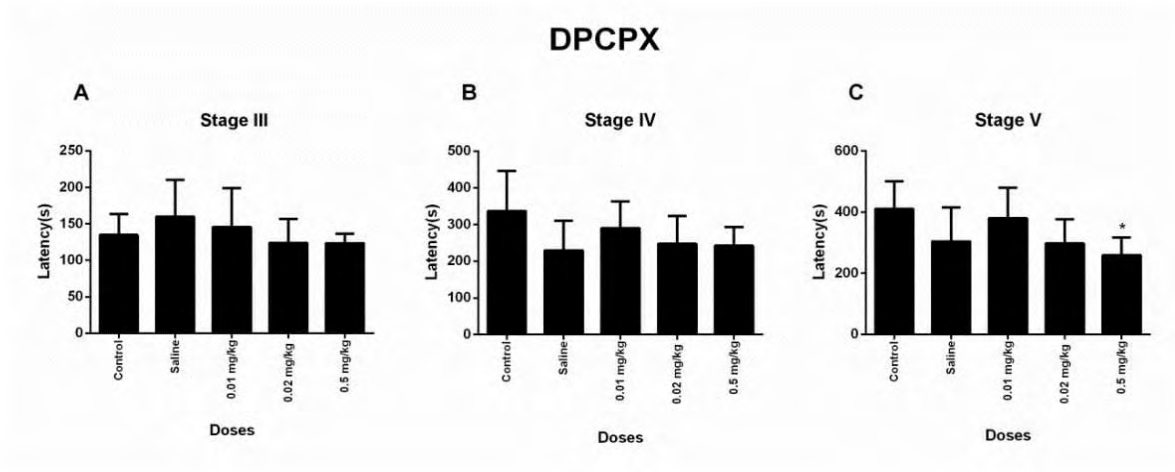


Figure 6

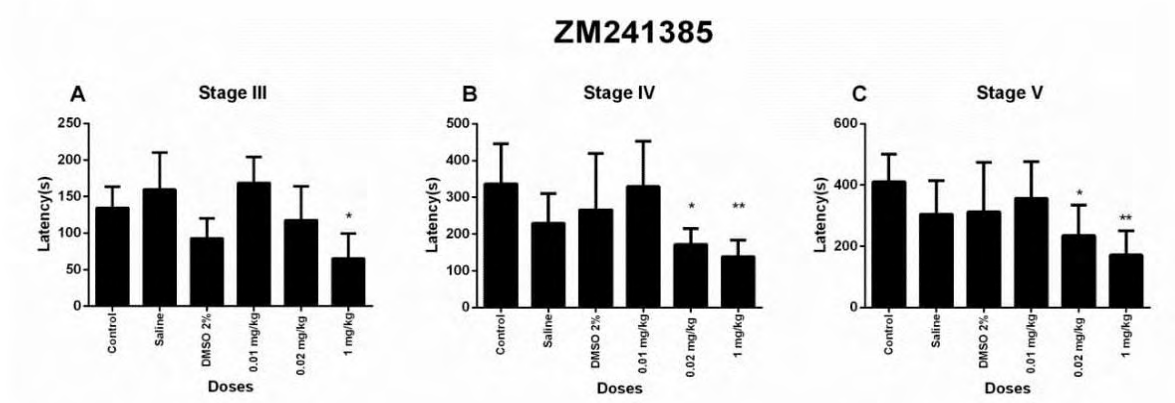


Figure 7

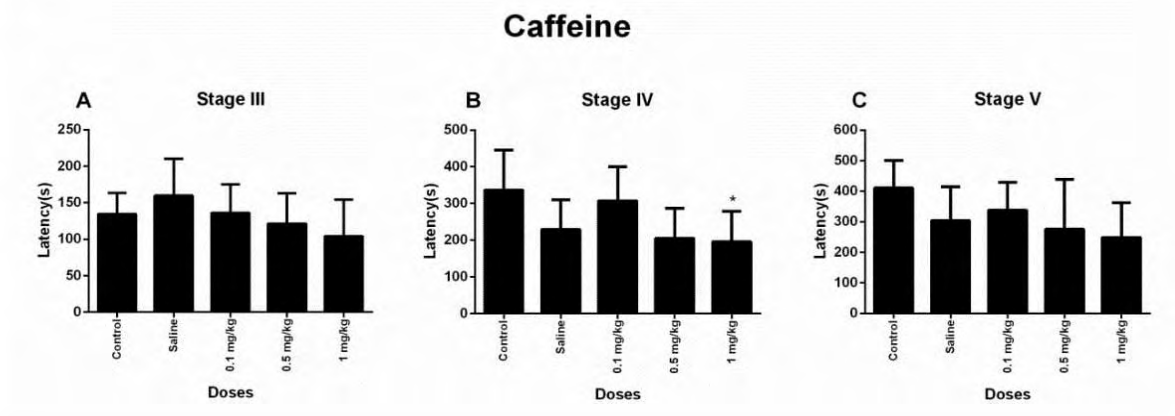


Figure 8

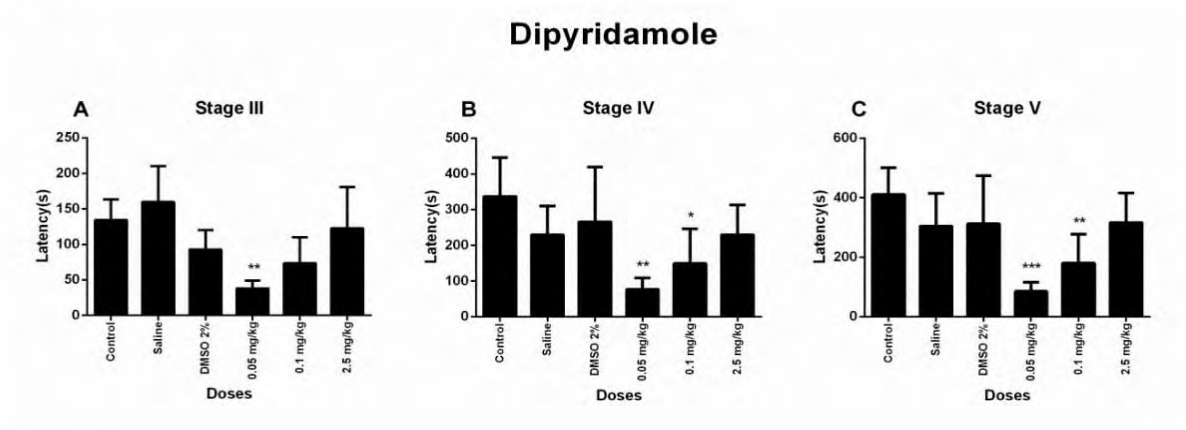


Figure 9

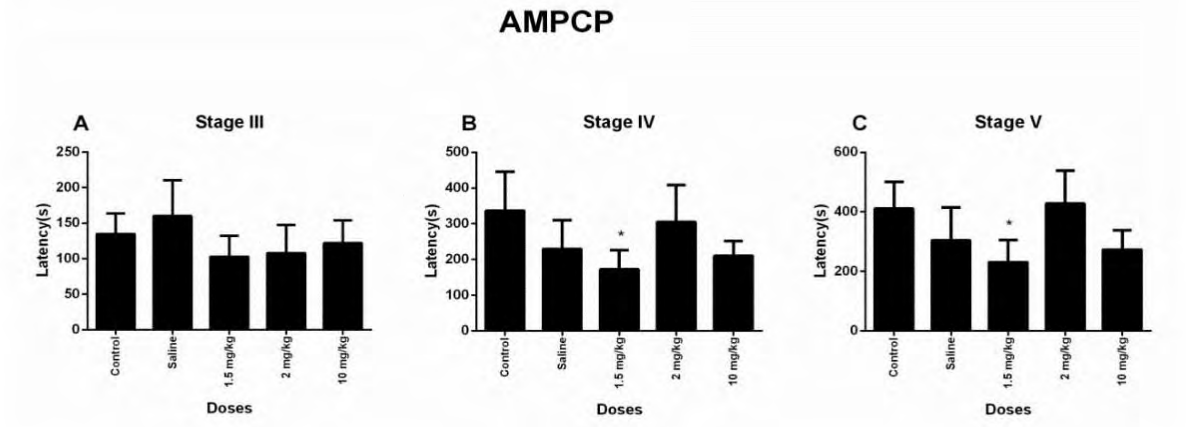
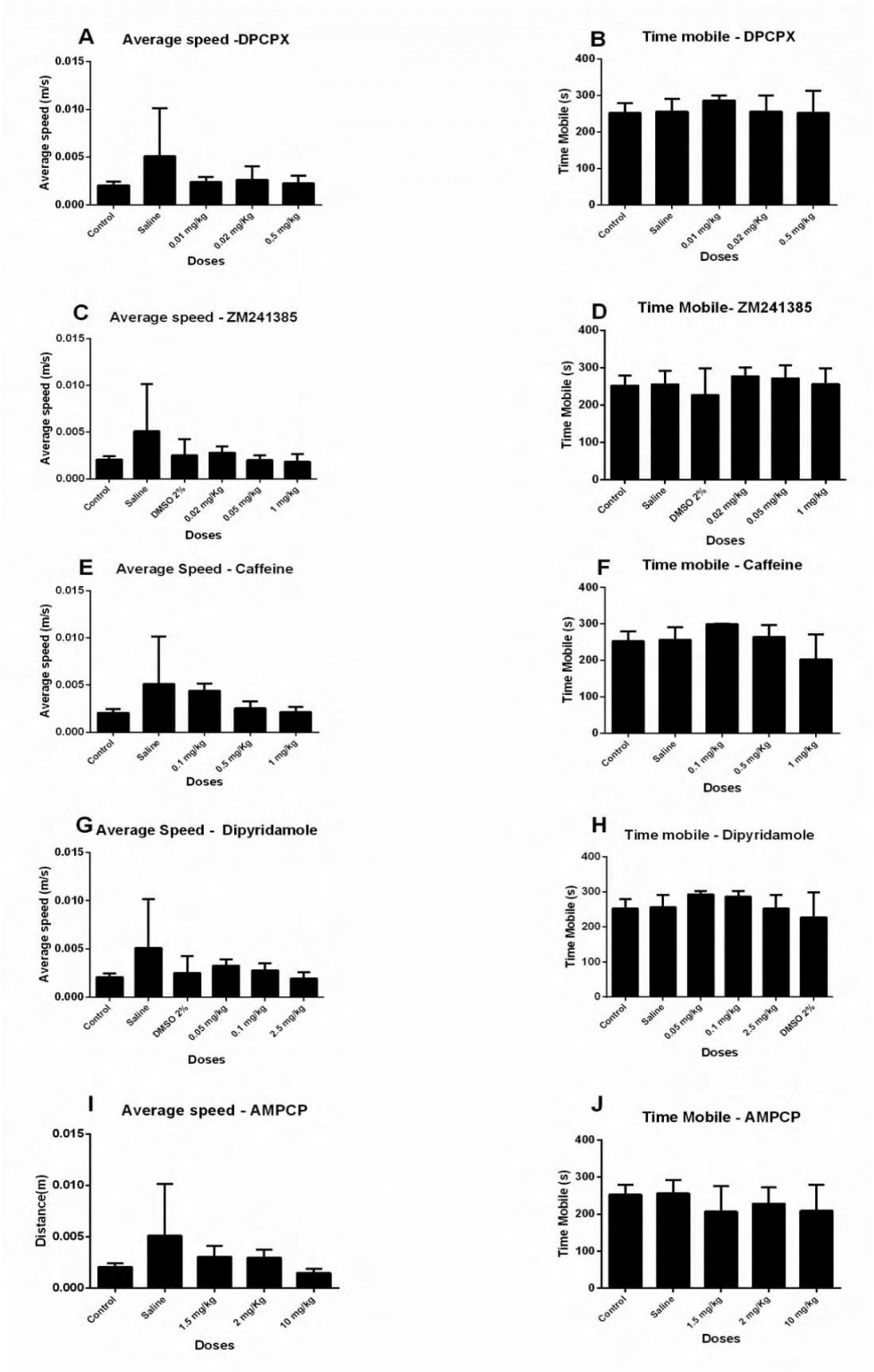


Table 1- Doses Performed

Drugs	Dose 1	Dose 2	Dose 3
DPCPX	0.01 mg/kg	0.02 mg/kg	0.5 mg/kg
ZM-241385	0.02 mg/kg	0.05 mg/kg	1 mg/kg
Caffeine	0.1 mg/kg	0.5 mg/kg	1 mg/kg
Dipyridamole	0.05 mg/kg	0.1 mg/kg	2.5 mg/kg
AMPCP	1.5 mg/kg	2 mg/kg	10 mg/kg

Supplementary Fig. 1



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Legends

Figure 1: Survival rate through 7 days post-fertilization of zebrafish larvae submitted to yolk microinjection (~1hour post-fertilization) of (a) DPCPX (0.01, 0.02, 0.5 mg/kg), (b) ZM241385 (0.02, 0.05, 1 mg/kg) (c) caffeine (0.1, 0.5, 1 mg/kg), (d) Dipyridamole (0.05, 0.1, 2.5 mg/kg and (e) AMPCP (1.5, 2, 10 mg/kg). 2% DMSO was used as a vehicle for ZM241385 and Dipyridamole. Each group was compared to control group (saline) using Log-rank test. *; *** and **** represent a significant level at $p<0.05$, $p< 0.001$ and $p<0.0001$ in relation to control group, respectively.

Figure 2: Mean of general malformations score found in each zebrafish larvae at 5 days post-fertilization submitted to (a) DPCPX (0.01, 0.02, 0.5 mg/kg), (b) ZM241385 (0.02, 0.05, 1 mg/kg) (c) caffeine (0.1, 0.5, 1 mg/kg), (d) Dipyridamole (0.05, 0.1, 2.5 mg/kg) and (e) AMPCP (1.5, 2, 10 mg/kg) yolk microinjection at ~1hour post-fertilization. 2% DMSO was used as a vehicle for ZM241385 and Dipyridamole. The score was established considering 0 for absence of malformation; 1 for presence of any malformation; and 2 for the accumulation of more than one malformation in the same structure. * represents a significant level at $p<0.05$.

Figure 3: Total percentage of animals showing some malformation in response to (a) DPCPX (0.01, 0.02, 0.5 mg/kg), (b) ZM241385 (0.02, 0.05, 1 mg/kg) (c) caffeine (0.1, 0.5, 1 mg/kg), (d) Dipyridamole (0.05, 0.1, 2.5 mg/kg) and (e) AMPCP (1.5, 2, 10 mg/kg) yolk microinjection at ~1hour post-fertilization. 2% DMSO was

used as a vehicle for ZM241385 and Dipyridamole. Each group consisted of 8 animals.

Figure 4: Latency time to zebrafish embryo microinjected with (A and B) DPCPX (0.01, 0.02, 0.5 mg/kg), (C and D) ZM241385 (0.02, 0.05, 1 mg/kg and DMSO 2%) (E and F) Caffeine (0.1, 0.5, 1 mg/kg), (G and H) Dipyridamole (0.05, 0.1, 2.5 mg/kg and DMSO 2%) and (I and J) AMPCP (1.5, 2, 10 mg/kg) reach seizure stages in 7-day old zebrafish larvae after to PTZ exposure. (A, C, E, G and I). (A, C, E, G and I) Latency (seconds) to reach seizure stage II established as fast circular swimming behavior and (B, D, F, H and J) Latency (seconds) to reach seizure stage III established as a series of short clonic convulsions, leading to loss of posture, and fall to one side remained still for 1-3 seconds. Each group was compared to control group using one-way-ANOVA followed by Dunnett's post hoc test. *;**; and *** represent a significant level at $p<0.05$, $p< 0.01$ and $p<0.001$ in relation to control group, respectively.

Figure 5: Latency time to zebrafish embryo microinjected with DPCPX (0.01, 0.02, 0.5 mg/kg) reach seizure stages in adult after to PTZ exposure (n: 8;10;10 respctively), (A) Latency (seconds) to reach seizure stage III established as a circular motion, (B) Latency (seconds) to reach seizure stage IV established as convulsive behavior clonic type, and (C) Latency (seconds) to reach seizure stage V established as fall to the bottom of the aquarium and convulsive behavior of tonic type. Each group was compared to control group using One-way-ANOVA followed by Dunnett's post hoc test. * represent a significant level at $p<0.05$ in relation to control group (n:10).

Figure 6: Latency time to zebrafish embryo microinjected with ZM241385 (0.02, 0.05, 1 mg/kg) reach seizure stages in adult after to PTZ exposure (n:9;10;8 respectively) (A) Latency (seconds) to reach seizure stage III established as a circular motion, (B) Latency (seconds) to reach seizure stage IV established as convulsive behavior clonic type, and (C) Latency (seconds) to reach seizure stage V established as fall to the bottom of the aquarium and convulsive behavior of tonic type. Each group was compared to control group using One-way-ANOVA followed by Dunnett's post hoc test. *; and ** represent a significant level at $p<0.05$ and $p< 0.01$ in relation to control group (n:10), respectively.

Figure 7: Latency time to zebrafish embryo microinjected with Caffeine (0.1, 0.5, 1 mg/kg) reach seizure stages in adult after to PTZ exposure (n:8;8;9 respectively) (A) Latency (seconds) to reach seizure stage III established as a circular motion, (B) Latency (seconds) to reach seizure stage IV established as convulsive behavior clonic type, and (C) Latency (seconds) to reach seizure stage V established as fall to the bottom of the aquarium and convulsive behavior of tonic type. Each group was compared to control group using One-way-ANOVA followed by Dunnett's post hoc test. * represent a significant level at $p<0.05$ in relation to control group (n:10).

Figure 8: Latency time to zebrafish embryo microinjected with Dipyridamole (0.05, 0.1, 2.5 mg/kg) reach seizure stages in adult after to PTZ exposure (n:6; 8; 8 respectively) (A) Latency (seconds) to reach seizure stage III established as a

circular motion, (B) Latency (seconds) to reach seizure stage IV established as convulsive behavior clonic type, and (C) Latency (seconds) to reach seizure stage V established as fall to the bottom of the aquarium and convulsive behavior of tonic type. Each group was compared to control group using One-way-ANOVA followed by Dunnett's post hoc test. *; **; and *** represent a significant level at $p<0.05$, $p< 0.01$ and $p<0.001$ in relation to control group (n:10), respectively.

Figure 9: Latency time to zebrafish embryo microinjected with AMPCP (1.5, 2, 10 mg/kg) reach seizure stages in adult after to PTZ exposure (n: 8; 8; 8 respectively) (A) Latency (seconds) to reach seizure stage III established as a circular motion, (B) Latency (seconds) to reach seizure stage IV established as convulsive behavior clonic type, and (C) Latency (seconds) to reach seizure stage V established as fall to the bottom of the aquarium and convulsive behavior of tonic type. Each group was compared to control group using One-way-ANOVA followed by Dunnett's post hoc test. * represent a significant level at $p<0.05$ in relation to control group (n:10).

Supplementary Fig. 1 : Locomotor activity of 7-day old zebrafish larvae after embryonic microinjection of (A and B) DPCPX (0.01, 0.02, 0.5 mg/kg), (C and D) ZM241385 (0.02, 0.05, 1 mg/kg) (E and F) Caffeine (0.1, 0.5, 1 mg/kg), (G and H) Dipyridamole (0.05, 0.1, 2.5 mg/kg) and (I and J) AMPCP (1.5, 2, 10 mg/kg). 2% DMSO was used as a vehicle for ZM241385 and Dipyridamole. Average speed (meter/seconds) (A, C, E, G and I). and Mobile time (seconds) (B, D, F, H and J).

4 Discussão e Conclusão

As crises convulsivas são a manifestação resultante de uma exacerbação na sinalização neuronal, podendo ser resultado de um trauma, uma patologia, uma intoxicação ou uma pré-disposição genética (FISHER et al., 2005). Durante a fase infantil, a convulsão febril é o distúrbio convulsivo mais comum em todo o mundo (WHO, 2017). Embora não sejam totalmente claro quais são os mecanismos pelos quais a hipertermia é capaz de desencadear crises convulsivas, alguns trabalhos reportam que a inibição de canais de cálcio TRPV4 foi capaz de atenuar as crises convulsivas provocadas por hipertermia, devido à inibição do influxo do cálcio via TRPV4 e pela influência que esse canal exerce sobre os receptores NMDA (HUNT et al., 2012a; SHIBASAKI et al., 2007). Nos resultados exibidos no primeiro capítulo desse documento foi possível verificar que o gênero e uma pequena variação na massa corporal não foram capazes de mudar comportamentalmente a sensibilidade do peixe-zebra ao PTZ. Enquanto os testes para verificar a influência da temperatura na resposta do peixe-zebra a exposição ao PTZ demonstram que, em uma faixa de 22°C até 30°C, houve um aumento crescente na sensibilidade do peixe-zebra ao PTZ, mensurada a partir do tempo necessário para alcançar os escores pré-estabelecidos (MUSSULINI et al., 2013). Considerando que a temperatura ideal do ambiente de criação do peixe-zebra fica em torno de 26-27°C, pode-se dizer que a hipertermia causou um efeito pró-convulsivo (BARAM; GERTH; SCHULTZ, 1997; WESTERFIELD, 2007). Estudos prévios em ratos e peixe-zebra demonstram que canais de cálcio participam ativamente nos processos desencadeadores de crises

convulsivas provocadas por hipertermia (GHADIMKHANI et al., 2016; HUNT et al., 2012a; SHIBASAKI et al., 2007)

O tratamento prévio com antagonistas glutamatérgicos corrobora com a ideia de que canais de cálcio tem grande influência nas crises convulsivas provocadas por hipertermia, visto que enquanto o antagonista caínico (DNQX) não causou efeito na maior sensibilidade ao PTZ provocada pelo aumento de temperatura, o antagonista NMDA (MK-801) foi capaz de abolir totalmente esse efeito (HUNT et al., 2012a; MORIMOTO et al., 1995).

Esse trabalho além de apresentar resultados contundentes quanto à participação dos receptores NMDA no efeito potencializador da hipertermia sobre crises convulsivas provocados via exposição ao PTZ serviu de suporte para que fosse possível realizar os testes de sensibilidade ao PTZ nos trabalhos seguintes sem que fosse necessária a separação dos animais por gênero. Uma vez que os resultados apontaram para inexistência de diferença entre os gêneros e massa corporal para suscebilidade a crises convulsivas provocadas pelo PTZ.

Nos três últimos capítulos apresentados aqui, nos quais foram mensuradas a letalidade e a teratogenicidade da intervenção na sinalização adenosinérgica por diferentes métodos, foi possível evidenciar a participação dos receptores A_1 e A_{2A}, além da enzima ecto-5'-nucleotidase e dos transportadores ENT e CNT2 referente a esses parâmetros.

O bloqueio dos receptores A_1 em ambas as abordagens, molecular e farmacológica, não causou diminuição na taxa de sobrevivência das larvas, tão pouco causou prejuízos morfológicos evidentes. Esses achados corroboram com trabalhos anteriores que demonstram que sob condições normais, a ausência do receptor A_1 durante o desenvolvimento embrionário não causa alterações deletérias evidentes

(BUSCARIOLLO et al., 2011; WENDLER et al., 2007). No entanto, em testes usando agonistas do receptor A_1 em ratos, foram observados efeitos deletérios ao embrião, principalmente na formação da estrutura cardíaca (RITCHIE et al., 2016; ZHAO; RIVKEES, 2001).

O bloqueio dos receptores $A_{2A,}$ tanto via fármacos quanto molecular, através da técnica de morfolino, exibiu resultados altamente prejudiciais ao desenvolvimento embrionário. Embora uma série de estudos aponte a participação dos receptores A_{2A} em processos fundamentais para o desenvolvimento embrionário como proliferação e diferenciação, até o momento em nossas pesquisas foram encontrados poucos relatos científicos que suportem que a inibição ou bloqueio gênico especifico do receptor A_{2A} tenha causado elevada taxa de mortalidade ou má-formação (ESCUDERO et al., 2013; KAEBISCH et al., 2015; MOMOI et al., 2008). Trabalhos que reportam inibição do receptor A_{2A} na fase embrionária indicam efeitos prejudiciais na função e formação cardiovascular (MOMOI et al., 2008). Entretanto, BOEHMLER et al (2009) demonstrou que no peixe-zebra o receptor A_{2A} possui genes parálogos, o que poderia ser um fator determinante para os resultados encontrados. Nos testes utilizando morfolinos, em que foi possível bloquear individualmente e de forma específica cada um dos transcritos para o receptor A_{2A}, os resultados apontam para papeis diferentes entre os receptores A_{2AA} e A_{2AB}, uma vez que o bloqueio dos transcritos do gene *Adora2aa* não exibiu prejuízos ao desenvolvimento das larvas enquanto o bloqueio dos transcritos do gene *Adora$_2$ab* foi extremamente danoso. O bloqueio de ambos A_{2AA} e A_{2AB} por co-injeção, resultou em efeitos semelhantes aos observados em A_{2AB} individualmente. Estes achados acerca dos efeitos dos receptores A_1 e A_{2A} na embriogênese se

inter-relacionam quando observamos os efeitos provocados pela exposição de embriões a cafeína, um antagonista não seletivo para os receptores de adenosina.

Nos resultados em embriões microinjetados com cafeína, antagonista não seletivo dos receptores de adenosina, exibiram uma taxa de sobrevivência dose dependente, além de significativa alteração morfológica nas larvas. O caráter teratogênico exibido pela cafeína tem sido encontrado em outros trabalhos usando diferentes modelos animais, incluindo o peixe-zebra (LI et al., 2012; MA et al., 2014; SOUZA et al., 2016). De forma linear, o conjunto de dados encontrados até aqui, além de relatos de trabalhos anteriores, poderia levar a hipótese de que os efeitos teratogênicos causados pela cafeína na fase embrionária são causados via inibição dos receptores A_{2A}. Contudo não podemos descartar uma série de fatores como compensação por *upregulation* (regulação ascendente) desses receptores (HUANG et al., 2005; LOPES; CUNHA; RIBEIRO, 1999).

A ecto-5'-nucleotidase e transportadores concentrativos de nucleosideo tipo 2, proteínas envolvidos no controle extracelular da adenosina, avaliados quanto a influência no desenvolvimento embrionário, apresentaram um alto nível de nocividade em larvas que sofreram o bloqueio via morfolinos. No entanto esses efeitos não foram tão acentuados, quando da utilização de fármacos inibidores da ecto-5'-nucleotidase, e bloqueadores do transportador equilibrativo de nucleosídeo. A enzima ecto-5'-nucleotidase é relatada como sendo a principal fonte de adenosina para ativação dos receptores A_{2A} (AUGUSTO et al., 2013). Os resultados encontrados com a inibição da enzima ecto-5'-nucleotidase demonstram que a e5'nt foi capaz de causar efeitos próximos aos observados em larvas que sofreram bloqueio dos receptores A_{2A}.

A inibição específica do transportador CNT2, o qual nesse trabalho foi realizada apenas através do bloqueio via morfolinos provocou baixas taxas de sobrevivência e

altos índice de má-formação. O bloqueio do CNT2 impede a recaptação da adenosina para o meio intracelular, aumentando assim as concentrações de adenosina no meio extracelular. No entanto, alguns eventos não podem ser descartados na explicação para os resultados encontrados, como por exemplo, uma compensação no transporte via outros transportadores, CNT3 e ENTs, uma maior atividade da enzima ecto-adenosine deaminase, ou ainda um aumento na ativação de receptores A_1 devido ao elevado nível de adenosina extracelular (RITCHIE et al., 2016). Efeitos semelhantes foram vistos em embriões microinjetados com a dose mais elevada de dipiridamol. O bloqueio dos transportadores ENTs utilizando dipiridamol é capaz de aumentar significativamente a concentração de adenosina extracelular (PARK; GIDDAY, 1990). Reforçando a ideia de que um aumento exacerbado da adenosina extracelular na fase embrionária pode acarretar em efeito danoso para o desenvolvimento do embrião (RIVKEES; WENDLER, 2011; TURNER et al., 2002).

As consequências para o sistema nervoso, a longo e curto prazo, provenientes de perturbações na sinalização adenosinergica durante as fases inicias do desenvolvimento tem sido alvo de muitos estudos (RIVKEES; WENDLER, 2017; TCHEKALAROVA; KUBOVÁ; MAREŠ, 2014). Os testes de atividade locomotora realizados em peixe-zebra têm como uma das finalidades avaliar um possível estado de ansiedade, levando em conta os resultados obtidos de parâmetros como, velocidade de nado, tempo no fundo do aquário e mobilidade, que em conjunto podem atestar o estado de ansiedade. Além disto, estes dados atestam a não manifestação de qualquer prejuízo na locomoção capaz de interferir na avaliação dos estágios de crise convulsiva (BLASER; ROSEMBERG, 2012; KALUEFF et al., 2013). Nos testes de atividade locomotora realizados em larvas, apenas nos animais em que

foi efetuado o bloqueio por meio de co-injeção de morfolinos para os transcritos dos receptores A_{2AA} e $A_{2AB,}$ obteve-se uma mobilidade reduzida em relação aos animais utilizados como controle. Embora, os animais que apresentaram qualquer tipo visível de limitação física tenham sido descartados para os testes motores, não pode ser descartado algum prejuízo muscular sutil como explicação para esse resultado. Entre os animais adultos, foram realizados testes locomotores apenas em animais com bloqueio via morfolinos, dentre os quais os A_1MO apresentaram mobilidade reduzida e maior tempo no fundo do aquário, e os A_{2AA}MO exibiram velocidade média elevada. Em animais com bloqueio gênico direcionado à ecto-5'-nucleotidase, a velocidade média e a mobilidade foram reduzidas e o tempo no fundo do aquário foi maior em comparação aos animais controle. Embora estes resultados reafirmem os efeitos em longo prazo provocados pelas alterações na sinalização adenosinérgica, não é possível atribuir a esses resultados um comportamento de ansiedade.

O papel da adenosina como um anticonvulsivante endógeno tem sido amplamente estudado nos últimos anos devido a sua capacidade de interferir na liberação e atividade pós-sináptica de outros neurotransmissores (DUNWIDDIE; MASINO, 2001; GOUDER; FRITSCHY; BOISON, 2003; ILIE; RAIMONDO; AKERMAN, 2012b; SIEBEL et al., 2015). Os efeitos tardios de intervenções da sinalização adenosinérgica durante a fase inicial de desenvolvimento também têm sido alvo de muitos estudos (GEORGIEV; JOHANSSON; FREDHOLM, 1993; GUILLET, 1995; TCHEKALAROVA; KUBOVÁ; MARES, 2007).

No presente estudo foi possível observar que as intervenções na sinalização adenosinérgica na fase embrionária resultaram em alterações no sistema nervoso capazes de causar, no peixe-zebra, maior suscetibilidade a crises convulsivas induzidas pela exposição ao PTZ, mesmo em um longo período após a interrupção da

sinalização. Nos testes de suscetibilidade ao PTZ aos 7dpf os animais microinjetados com morfolinos não se mostraram mais sensíveis ao PTZ, enquanto que as larvas expostas a fármacos apresentaram alterações na suscetibilidade a crises convulsivas, alcançando os estágios de convulsão em menos tempo naqueles animais tratados com antagonista A_1 (DPCPX) e inibidor da ecto-5'-nucleotidase. Além disso, larvas tratadas com cafeína (0.5 mg/kg) e dipiridamol (0.05 mg/kg) apresentaram aumento no tempo de latência para crise convulsiva e larvas tratadas com cafeína (1 mg/kg) e dipiridamol (2.5 mg/kg), apresentaram maior sensibilidade, observada pela diminuição do tempo de latência para alcançar os estágios de convulsão.

Em animais adultos expostos ao PTZ foi possível observar que o bloqueio dos transcritos dos receptores A_1 e A_{2A}, e da e5'nt e CNT2 provocou um contundente aumento na suscetibilidade a crises convulsivas. Em animais adultos microinjetados na fase embrionária com fármacos de ação nos receptores A_1 e A_{2A}, na enzima e5'nt e do transportador ENT1, os efeitos não foram tão proeminentes. As alterações mais contundentes foram observadas principalmente em animais expostos ao ZM241385 e ao dipiridamol, onde houve claramente uma diminuição no tempo de latência para alcançar os estágios de convulsão.

A capacidade da adenosina de interferir na liberação e sinalização de outros sistemas de neurotransmissão como gabaérgico, glutamatérgico e dopaminérgico faz com que perturbações em suas concentrações possam causar alterações significativas na sinalização exercida por esses neurotransmissores, principalmente na fase inicial do desenvolvimento (ACQUAS; TANDA; DI CHIARA, 2002; DELIC; ZIMMERMANN, 2010; FERREIRA et al., 2014; FREDHOLM; SVENNINGSSON, 2003; POPOLI et al., 2003). A sinalização exercida pelo GABA e glutamato durante a fase inicial de desenvolvimento está diretamente relacionada a processos de extrema

importância para formação da rede neural, tais como migração e proliferação (MANENT et al., 2005; MANENT; REPRESA, 2007; SILVA et al., 2013).

Alguns estudos relacionam crises convulsivas na fase inicial do desenvolvimento e suscetibilidade a crises convulsivas a alterações nas sinalizações glutamatergica, adenosinérgica e gabaérgicas (HORTOPAN; DINDAY; BARABAN, 2010; LEÓN-NAVARRO; ALBASANZ; MARTÍN, 2015; ZHANG et al., 2004).

O conjunto de dados apresentados ao longo desse documento demostram que houve algumas discrepâncias nos efeitos entre as duas abordagens escolhidas para o bloqueio dos mecanismos envolvidos na sinalização adenosinérgica. A diferença encontrada entre as duas abordagens pode estar relacionada à diferença de especificidade e afinidade aos alvos selecionados em peixe-zebra e ao tempo que cada tratamento permanece ativo no embrião. Alguns estudos demonstram que a ação de morfolinos no bloqueio da tradução de proteínas já pode ser observada no período entre 24-48h após a aplicação, atingindo seu ápice no terceiro dia. A partir do dia 5, embora o bloqueio ainda ocorra, a eficácia do morfolino começa a diminuir, com a normalização ocorrendo cerca de 6 dias após a aplicação (BILL et al., 2009; NASEVICIUS; EKKER, 2000). Enquanto os fármacos possuem meia-vida de horas, que pode variar em função do metabolismo (Tabela 2). Essa diferença entre as duas abordagens é extremamente significativa, em virtude da velocidade que o peixe-zebra se desenvolve durante a embriogênese, alcançando o estágio de segmentação entre 10 e 24 horas após a fecundação (KIMMEL et al., 1995; WESTERFIELD, 2007).

TABELA 2 - MEIA-VIDA DOS FARMACOS MICROINJETADOS

Fármaco	Meia-vida	Referência
Dipiridamol	12h (humanos)	(MAHONY; COX; BJORNSSON, 1983)
AMPCP (AOPCP)	5h (Ratos)	(BHATTARAI et al., 2015)
DPCPX	2.36h (Humanos)	(LU et al., 2014)
Cafeína	5.7h (Humanos)	(STATLAND; DEMAS, 1980)
Zm241385	4h (Cachorro e Gato)	(POUCHER et al., 1996)

Mesmo apresentando algumas diferenças, há concordância entre os resultados encontrados em ambas as abordagens, ou seja, distúrbios na sinalização adenosinérgica durante a fase inicial do desenvolvimento resultam em consequências danosas à longo prazo. As alterações provocadas pela alteração na sinalização normal exercida pela adenosina durante essa fase, tem efeitos duradouros suficiente para de influenciar no comportamento de animais adultos, além de modificar a sensibilidade ao pentilenotetrazol, fármaco utilizado para indução de crises convulsivas.

Tais dados poderão contribuir para o embasamento a cerca da suscetibilidade do cérebro imaturo a intervenções no tônus adenosinérgico, as quais podem ocorrer em

situações corriqueiras, tais como a exposição à cafeína ou situações emergenciais, tais como uma hipóxia neonatal.

Uma série de estudos tem demonstrado uma crescente conscientização de que a cafeína pode ser um fator de risco para o desenvolvimento anormal do cérebro, em grande parte por sua ação nos receptores adenosinérgicos (DOYLE et al., 2010; SILVA et al., 2013). O aumento da suscetibilidade a crises convulsivas em peixe-zebra adultos, em virtude da perturbação na via adenosinérgica, suporta diretamente a importância de não interferir na sinalização adenosinérgica durante desenvolvimento cerebral, mesmo que não seja observada nenhuma anormalidade fenotípica evidente. Este trabalho traz à tona a importância de uma reavaliação das consequências, do consumo de produtos, por gestantes, como a cafeína, bem como exacerbado estresse fisiológico, que podem interferir na sinalização adenosinérgica durante a gestação. A avaliação de possíveis danos à capacidade cognitiva ao longo do desenvolvimento em indivíduos que foram expostos de alguma forma a agentes exógenos, de ação adenosinergica, durante a fase embrionária podem ajudar a elucidar de forma mais criteriosa a real extensão dos efeitos dessa abordagem. Por todo conteúdo discutido nesse trabalho, fica evidente que a adenosina tem um importante papel na formação da rede neural e que perturbações na sua sinalização durante esse processo é altamente danoso e permanente.

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Anexo A - Aprovação do Comitê de Ética

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 67/2015 - CEUA

Porto Alegre, 24 de setembro de 2015.

Senhor (a) Pesquisador (a),

Informamos que a Comissão de Ética no uso de Animais apreciou e **aprovou** sua solicitação datada de 17 de setembro do corrente ano, para acréscimo de 48 animais adultos e 195 larvas de peixe-zebra no projeto 14/00416, intitulado: "**Efeito de intervenções na sinalização adenosinérgica e caínica na fase inicial do desenvolvimento sobre a crise convulsiva e parâmetros comportamentais em peixe-zebra (Danio rerio)**".

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilma. Sra.
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Anexo B – Trabalhos realizados durante o doutorado, que não compõem a tese.

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Research report

Hyperglycemia induces memory impairment linked to increased acetylcholinesterase activity in zebrafish (*Danio rerio*)

Katiucia Marques Capiotti[a], Daiani Almeida De Moraes[a], Fabiano Peres Menezes[a], Luiza Wilges Kist[b,c], Maurício Reis Bogo[b,c], Rosane Souza Da Silva[a,c,*]

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HIGHLIGHTS

- The hyperglycemia promoted memory deficit in adult zebrafish.
- Memory deficit is linked to increased acetylcholinesterase activity.
- Galantamine reverses memory deficit caused by hyperglycemia.

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ABSTRACT

Diabetes mellitus, which causes hyperglycemia, affects the central nervous system and can impairs cognitive functions, such as memory. The aim of this study was to investigate the effects of hyperglycemia on memory as well as on the activity of acethylcholinesterase. Hyperglycemia was induced in adult zebrafish by immersion in glucose 111 mM by 14 days. The animals were divided in 4 groups: control, glucose-treated, glucose-washout 7-days and glucose-washout 14-days. We evaluated the performance in inhibitory avoidance task and locomotor activity. We also determined acethylcholinesterase activity and gene expression from whole brain. In order to counteract the effect of hyperglycemia underlined by effects on acethylcholinesterase activity, we treated the animals with galantamine (0.05 ng/g), an inhibitor of this enzyme. Also we evaluated the gene expression of insulin receptor and glucose transporter from zebrafish brain. The hyperglycemia promoted memory deficit in adult zebrafish, which can be explained by increased AChE activity. The *ache* mRNA levels from zebrafish brain were decrease in 111 mM glucose group and returned to normal levels after 7 days of glucose withdrawal. Insulin receptors (*insra-1*, *insra-2*, *insrb*-1 and *insrb*-2) and *glut-3* mRNA levels were not significantly changed. Our results also demonstrated that galantamine was able to reverse the memory deficit caused by hyperglycemia, demonstrating that these effects involve modulation of AChE activity. These data suggest that the memory impairment induced by hyperglycemia is underlined by the cholinergic dysfunction caused by the mechanisms involving the control of acetylcholinesterase function and gene expression.

1. Introduction

Diabetes mellitus (DM) is a complex disorder that can cause damage to multiple organs in the body, due to changes caused by dysfunctional glucose metabolism, as hyperglycemia and hypoglycemia [1]. The DM itself has been recognized as an independent risk factor for development of cognitive impairment [2,3], and has been suggested by several authors that this diabetes-related cognitive dysfunction is largely a consequence of changes within the central nervous system (CNS) that are secondary to chronic hyperglycemia [4,5]. Kodl and Seaquist [1] also addressed the causes

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Original Article

Role of Adenosine Signaling on Pentylenetetrazole-Induced Seizures in Zebrafish

Anna Maria Siebel,[1] Fabiano Peres Menezes,[1] Katiucia Marques Capiotti,[1] Luiza Wilges Kist,[2] Isabel da Costa Schaefer,[1] Juliana Zanetti Frantz,[1] Maurício Reis Bogo,[2] Rosane Souza Da Silva,[1] and Carla Denise Bonan[1]

Abstract

Adenosine is a well-known endogenous modulator of neuronal excitability with anticonvulsant properties. Thus, the modulation exerted by adenosine might be an effective tool to control seizures. In this study, we investigated the effects of drugs that are able to modulate adenosinergic signaling on pentylenetetrazole (PTZ)-induced seizures in adult zebrafish. The adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) decreased the latency to the onset of the tonic-clonic seizure stage. The adenosine A_1 receptor agonist cyclopentyladenosine (CPA) increased the latency to reach the tonic-clonic seizure stage. Both the adenosine A_{2A} receptor agonist and antagonist, CGS 21680 and ZM 241385, respectively, did not promote changes in seizure parameters. Pretreatment with the ecto-5′nucleotidase inhibitor adenosine 5′-(α,β-methylene) diphosphate (AMPCP) decreased the latency to the onset of the tonic-clonic seizure stage. However, when pretreated with the adenosine deaminase (ADA) inhibitor, erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA), or with the nucleoside transporter (NT) inhibitors, dipyridamole and *S*-(4-Nitrobenzyl)-6-thioinosine (NBTI), animals showed longer latency to reach the tonic-clonic seizure status. Finally, our molecular analysis of the *c-fos* gene expression corroborates these behavioral results. Our findings indicate that the activation of adenosine A_1 receptors is an important mechanism to control the development of seizures in zebrafish. Furthermore, the actions of ecto-5′-nucleotidase, ADA, and NTs are directly involved in the control of extracellular adenosine levels and have an important role in the development of seizure episodes in zebrafish.

Introduction

EPILEPSY IS A COMMON neurological disorder characterized by the occurrence of recurrent and unpredictable seizures.[1] In many cases, conventional antiepileptic drugs (AEDs) are not able to provide satisfying control of seizures.[2] Adenosine is a well-known endogenous modulator of neuronal excitability and provides anticonvulsant effects.[3] Therefore, adenosine modulation might be an effective tool to control epileptic seizures in patients resistant to conventional AEDs.[4–6]

Adenosine is a purine nucleoside that can be produced by extracellular nucleotide hydrolysis or be cell released through NTs.[3] Once released in the extracellular space, ATP is rapidly dephosphorylated into adenosine by ectonucleotidases, an enzyme cascade system that catalyzes the successive hydrolysis of purine and pyrimidine nucleoside tri-, di-, and monophosphates to their respective nucleosides. Nucleoside triphosphates and diphosphates may be hydrolyzed by the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family members, whereas nucleoside monophosphates are hydrolyzed by ecto-5′-nucleotidase, generating adenosine.[7] Adenosine is metabolized by two possible pathways: deamination to inosine through adenosine deaminase (ADA) and phosphorylation to AMP through adenosine kinase.[8] Finally, adenosine can be cell released through nucleoside transporters (NTs) in bidirectional equilibrative processes driven by chemical gradients and unidirectional concentrative processes driven by sodium electrochemical gradients.[9,10]

Adenosine exerts its effects through the activation of the specific G-protein-coupled receptors: A_1, A_{2A}, A_{2B}, and A_3 receptors. The A_1 and A_{2A} receptors are the most sensitive to adenosine and the most abundant in the central nervous system.[11] During a seizure, extracellular adenosine levels rapidly rise and are believed to play an important role in the arrest and

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Rapamycin suppresses PTZ-induced seizures at different developmental stages of zebrafish

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Zebrafish

ABSTRACT

The mTORC1 complex integrates different inputs from intracellular and extracellular signals to control various cellular processes. Therefore, any disruption in the mTORC1 pathway could promote different neurological disorders. mTORC1 overactivation has been verified in different genetic and acquired epilepsy animal models. Therefore, inhibitors of this complex could have both antiepileptogenic and antiseizure effects. In our study, we investigated the effects of rapamycin pretreatment on pentylenetetrazole (PTZ)-induced seizures in zebrafish. Our results have shown that the latency to reach the tonic–clonic stage (stage III) of progressive behavioral alterations shown during PTZ-induced seizures was prolonged in larval (7 days post fertilization, 7 dpf), juvenile (45 days post fertilization, 45 dpf) and adult (6–8 months) zebrafish after pretreatment with rapamycin. Furthermore, rapamycin pretreatment did not alter the locomotor activity in zebrafish. Therefore, the results obtained in our study indicate that rapamycin pretreatment is an important mechanism to control the progress of seizures in zebrafish throughout different developmental stages (larval, juvenile, and adult). Taken as a whole, our data support that rapamycin has immediate antiseizure effects and could be a potential alternative therapy for seizure control in epilepsy.

1. Introduction

Epilepsy is a common neurological disorder, affecting around 65 million people worldwide. This disorder is characterized by the occurrence of recurrent and unpredictable seizures, which occur due to abnormal activity of neuronal cells (Moshé et al., 2015; Lin and Baines, 2015). The mechanist target of rapamycin (mTOR) protein is a 289-kDa serine-threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and forms two distinct multi-protein complexes: mTORC1 and mTORC2. mTOR complexes integrate intracellular and extracellular signals and act as central regulators of cell activity, metabolism, growth, proliferation, and survival (Laplante and Sabatini, 2012, 2013). Rapamycin is an anti-fungal macrolide compound that acts by inhibiting mTOR signaling and is produced by the soil bacterium *Streptomyces hygroscopicus*, which was isolated from a soil sample obtained in Rapa Nui (Vézina et al., 1975).

mTORC1 is the best characterized of the two mTOR complexes and integrates different inputs from intracellular and extracellular signals: growth factors, stress, energy status, hormones, and amino acids in the control of various cellular processes, including protein and lipid synthesis, differentiation, and autophagy (Laplante and Sabatini, 2013; Maiese et al., 2013). Regarding the central nervous system (CNS), the mTORC1 complex regulates a variety of neuronal functions: cell proliferation, survival, growth, and plasticity. Disruption of the mTORC1 pathway has been implicated in different neurological disorders, and previous studies have shown that the mTORC1 signaling overactivation is related to epilepsy occurrence (Wong, 2010). The mTORC1 overactivation has been verified in genetic and acquired epilepsies: tuberous sclerosis complex, focal cortical dysplasias, and animal models of epilepsy acquired after *status epilepticus* or trauma (Wong, 2010). Therefore, as a mTORC1 inhibitor, rapamycin could be an important alternative in epilepsy treatment (Ryther and Wong, 2012).

Several studies have shown that rapamycin has seizure suppressive and antiepileptogenic effects in animal models of epilepsy. Rapamycin treatment attenuated the development of posttraumatic epilepsy in a mouse model of traumatic brain injury, decreasing the frequency of seizure occurrence (Guo et al., 2013). Furthermore, rapamycin treatment showed protective effect following *status epilepticus* since rats treated with this compound presented less seizure frequency when compared

Abbreviations: AED, antiepileptic drug; CNS, central nervous system; mTOR, mechanist target of rapamycin; PTZ, pentylenetetrazole; VPA, valproate.

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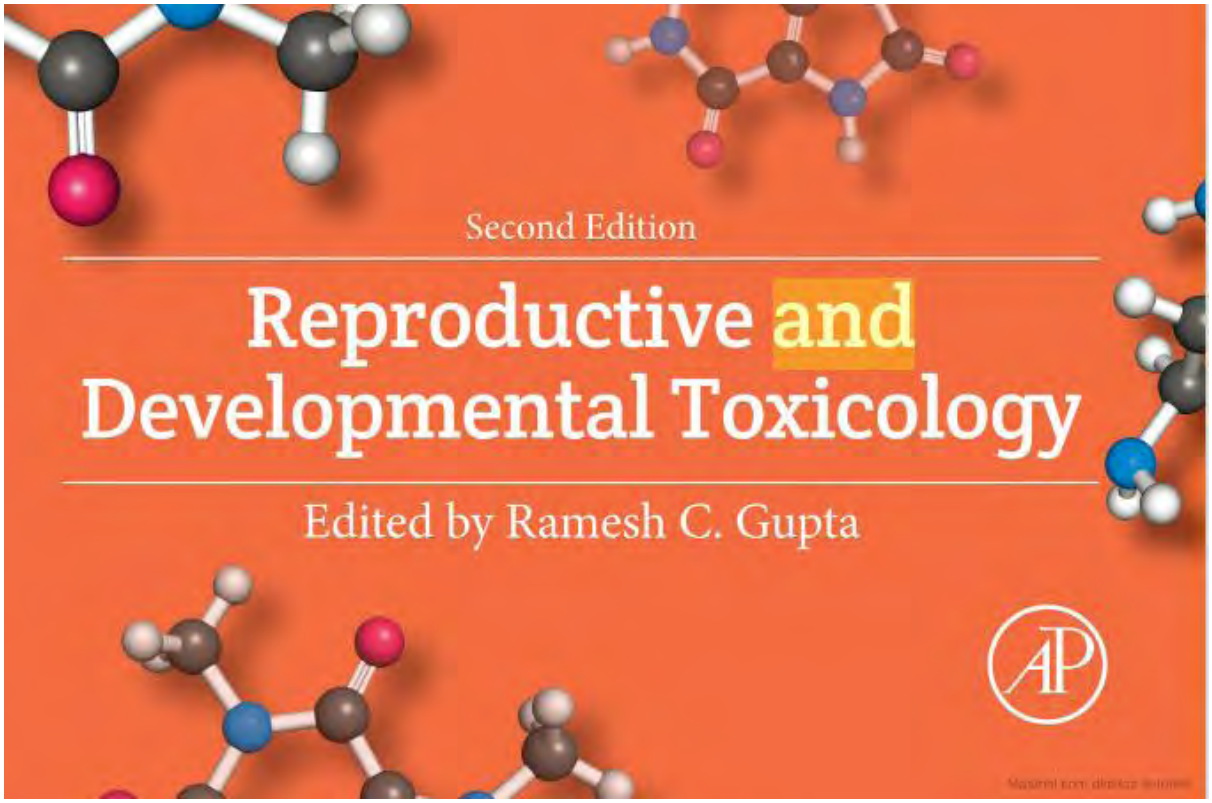

CHAPTER

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Caffeine

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