PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

STEFANI ALTENHOFEN

AVALIAÇÃO DOS EFEITOS DE FÁRMACOS BENZODIAZEPÍNICOS SOBRE O CATABOLISMO DE NUCLEOTÍDEOS, NUCLEOSÍDEOS E ACETILCOLINA EM ENCÉFALO DE ZEBRAFISH ADULTO (*Danio rerio*)

Orientadora: Prof. Dra. Carla Denise Bonan

PORTO ALEGRE – RS

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

PORTO ALEGRE – RS

Janeiro, 2013

Aos meus pais e eternos incentivadores, Denise e Ademir.

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Ao meu melhor amigo e namorado, Felipe.

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.”

Arthur Schopenhauer

RESUMO

Fármacos benzodiazepínicos, como diazepam e midazolam, são muito usados na prática clínica para o tratamento da ansiedade, possuindo propriedades ansiolíticas, hipnóticas e anticonvulsivantes. O uso do *zebrafish* (*Danio rerio*) como modelo para avaliar mecanismos farmacológicos tem ganhado grande importância devido ao rápido desenvolvimento e alta sensibilidade a drogas que essa espécie possui. Estudos têm demonstrado que parâmetros comportamentais mostraram-se alterados em *zebrafish* após tratamento com benzodiazepínicos. Muitos sistemas de neurotransmissão foram identificados nessa espécie, incluindo os sistemas purinérgico e colinérgico. O sistema purinérgico é caracterizado pela ação do ATP e adenosina (ADO) nos purinoreceptores P2 e P1, respectivamente. Os níveis dessas moléculas são regulados pela ação das ectonucleotidases, especialmente as nucleosídeo trifosfato difosfoidrolases (NTPDases) e a ecto-5'-nucleotidase, que catalisam a hidrólise do ATP a adenosina. A adenosina pode ser desaminada a inosina pela ação da adenosina desaminase (ADA). O ATP é coliberado com outros neurotransmissores, entre eles a acetilcolina, e tem sido demonstrado que a adenosina pode controlar a liberação de acetilcolina. O sistema colinérgico é caracterizado pela ação da acetilcolina (ACh) nos receptores muscarínicos e nicotínicos. O nível dessa molécula é regulado pela acetilcolinesterase (AChE), que catalisa a degradação da ACh em colina e acetato. Uma vez que existem poucos relatos relacionando esses sistemas enzimáticos e a ação de fármacos benzodiazepínicos, o objetivo deste estudo foi avaliar o efeito *in vitro* e *ex vivo* do tratamento com fármacos benzodiazepínicos, tais como diazepam e midazolam, sobre a atividade das NTPDases, ecto-5'-nucleotidase, ADA and AChE no encéfalo de *zebrafish* e o padrão de expressão gênica nos tratamentos que induziram alterações na atividade enzimática nos experimentos *ex vivo*. A fim de elucidar se o diazepam e o midazolam têm efeitos diretos nessas enzimas, experimentos *in vitro* foram realizados. Na concentração de 500 µM, o diazepam diminuiu a hidrólise de ATP (66%) e, nas concentrações de 10-500 µM, este fármaco reduziu a hidrólise de ADP (40-54%, respectivamente). O midazolam também diminuiu a hidrólise do ATP (16-71% para 10-500 µM, respectivamente), ADP (48-73% para 250-500 µM, respectivamente) e a atividade da ecto-ADA (26-27,5% para 10-500 µM, respectivamente). Diazepam e midazolam não induziram alterações significativas sobre a atividade da ecto-5´-nucleotidase nas concentrações testadas. Com relação à atividade da AChE, o diazepam, 500 µM, promoveu uma diminuição na hidrólise de ACh (19%) e o midazolam, nas concentrações de 50-500 µM, reduziu a atividade da AChE (18-79%, respectivamente). Nos experimentos *ex vivo*, as exposições ao diazepam e midazolam não alteraram a atividade enzimática das NTPDases em membranas cerebrais de *zebrafish*. A hidrólise do AMP diminuiu em animais tratados com 0.5 mg/L e 1 mg/L de midazolam (31.5% e 36.1%, respectivamente) quando comparados com o grupo controle. Entretanto, o diazepam foi incapaz de alterar a atividade da ecto-5'-nucleotidase. Ambos os fármacos diminuíram significativamente a atividade da ecto-ADA, sendo que o diazepam e o midazolam reduziram a hidrólise da adenosina na concentração de 1.25 mg/L (30.85%) e 1 mg/L (32.8%), respectivamente. O diazepam não alterou a atividade da ADA citosólica, no entanto a exposição a 0.1 mg/L de midazolam induziu um significativo aumento na atividade dessa enzima (39.9%) quando comparado ao grupo controle. O padrão de expressão gênica demonstrou que os níveis

de transcritos do *CD73* apresentaram-se reduzidos (41,7%) após o tratamento com 0.5 mg/L de midazolam. Com relação a sinalização colinérgica, diazepam diminuiu a hidrólise da ACh na concentração de 1.25 mg/L (30.7%) quando comparado ao grupo controle. Similarmente, a exposição à concentração de 0.5 mg/L de midazolam também alterou a atividade enzimática da AChE, promovendo um aumento na hidrólise da ACh (36.7%). É possível sugerir que essas drogas podem induzir um efeito direto na atividade enzimática, uma vez que foi observada uma diminuição na hidrólise de nucleotídeos e nucleosídeos após a exposição *in vitro*. Além disso, as alterações na hidrólise do AMP e atividade da ADA e da AChE sugerem uma modulação dos níveis extracelulares de adenosina e acetilcolina induzidos pela exposição aos fármacos benzodiazepínicos.

Palavras chaves: Benzodiazepínicos, ansiedade, ectonucleotidases, acetilcolinesterase, adenosina desaminase, *zebrafish*.

ABSTRACT

Benzodiazepines, such as diazepam and midazolam, are a widely used class of drugs for anxiety treatment, with anxiolytic, hypnotic, and anticonvulsant properties. The use of zebrafish (*Danio rerio*) as a model for evaluating pharmacological mechanisms has gained importance due to their rapid development and high sensitivity to drugs. Studies have shown that behavioral parameters were altered in zebrafish after benzodiazepine treatment. Many neurotransmitter systems have been identified in this species, including purinergic and cholinergic system. Purinergic system is characterized by the action of ATP and adenosine on purinoreceptor P2 and P1, respectively. The levels of these molecules are regulated by ectonucleotidases, especially nucleoside triphosphate diphosphohydrolase (NTPDases) and ecto-5'-nucleotidase, which constitute the extracellular cascade for ATP hydrolysis to adenosine. Adenosine can be subsequently deaminated to inosine by action of adenosine deaminase (ADA). ATP is coreleased with other neurotransmitters, including acetylcholine, and has been demonstrated that adenosine can control the release of acetylcholine. Cholinergic system is characterized by the action of acetylcholine (ACh) on muscarinic and nicotinic receptors. The level of this molecule is regulated by acetylcholinesterase (AChE), which catalyzes degradation of ACh into choline and acetate. Since there are few reports relating these enzyme activities and the action mechanism of benzodiazepines, the aim of this study was evaluated the *in vitro* and *ex vivo* effects of classical benzodiazepines, such as diazepam and midazolam, on NTPDase, ecto-5'nucleotidase, ADA, and AChE activities in zebrafish brain and gene expression pattern in treatments that induced changes in enzyme activity in the *ex vivo* experiments. In order to elucidate whether diazepam or midazolam has direct effects on these enzymes, we performed *in vitro* experiments. Diazepam, at 500 µM, promoted a decrease on ATP hydrolysis (66%), whereas this drug, at 10-500 µM, reduced ADP hydrolysis (40-54%, respectively). Midazolam also decreased ATP (16-71% for 10-500 µM, respectively) and ADP hydrolysis (48-73% for 250-500 µM, respectively), and ecto-ADA activity (26-27.5% for 10-500 µM, respectively). Diazepam and midazolam did not induce significant changes on ecto-5´-nucleotidase activity at the concentrations tested. Concerning to AChE activity, 500 µM diazepam promoted a decrease on ACh hydrolysis (19%), whereas midazolam, at 50-500 µM, reduced AChE activity (18-79%, respectively). For *ex vivo* experiments, diazepam or midazolam exposures did not alter NTPDase activities in zebrafish brain membranes. AMP hydrolysis was decreased in animals treated with of 0.5 and 1mg/L midazolam (31.5% and 36.1%, respectively) when compared to the control group. However, diazepam was unable to alter ecto-5'-nucleotidase. Both drugs significantly decreased the ecto-ADA activity, whereas diazepam and midazolam reduced the adenosine hydrolysis at a concentration of 1.25 mg/L (30.85%) and 1 mg/L (32.8%), respectively. Diazepam did not alter cytosolic-ADA activity; however, the exposure to 0.1 mg/L midazolam induced a significant increase in cytosolic-ADA (39.9%) when compared with the control group. The gene expression pattern demonstrated that the CD73 transcript levels were increased (41.7%) after treatment with 0.5 mg/L midazolam. Moreover, the changes caused by diazepam and midazolam in the ADA activity are not related to the transcriptional control. Concerning the cholinerg signaling, diazepam decreased ACh hydrolysis at 1.25 mg/L (30.7%) when compared to the control group. Similarly, the exposure to 0.5 mg/L midazolam also changed the enzymatic activity of

AChE promoting an increase in the ACh hydrolysis (36.7%). It is possible to suggest that these drugs can induce a direct effect on the enzyme activities, since we observed a decreased on nucleotide and nucleoside hydrolysis after *in vitro* exposure. In addition, the alteration on AMP hydrolysis, ADA and AChE activities suggest a modulation of extracellular adenosine and ACh levels induced by benzodiazepine exposure.

Keywords: Benzodiazepine, anxiety, ectonucleotidases, acetylcholinesterase, adenosine deaminase, zebrafish.

LISTA DE ABREVIATURAS

Acetil-CoA: acetilcoenzima A
ACh: acetilcolina
AChE: acetilcolinesterase
ADA: adenosina desaminase
ADO: adenosina
ADP: adenosina 5’-difosfato
AMP: adenosina 5’-monofosfato
AMPc: adenosina 5´- monofosfato cíclico
ATP: adenosina 5’-trifosfato
BuChE: butirilcolinesterase
ChAT: colina acetiltransferase
Ecto-5´-NT: ecto-5´-nucleotidase
E-NPP: ectonucleotídeo pirofosfatase/fosfodiesterase
E-NTPDase: ectonucleosídeo trifosfato difosfoidrolase
GABA: ácido gama-aminobutírico
mAChRs: receptores muscarínicos
nAChRs: receptores nicotínicos
NTPDase: nucleosídeo trifosfato difosfoidrolase
SNC: sistema nervoso central

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INTRODUÇÃO E OBJETIVOS

1. INTRODUÇÃO

1.1. *ZEBRAFISH*

O *zebrafish*, *Danio rerio* (Figura 1), é um pequeno teleósteo (3-4 cm) de água doce que vem sendo considerado um modelo ideal para estudos sobre o desenvolvimento de vertebrados (Bai e Burton, 2011; Málaga-Trillo et al., 2011), de numerosas doenças humanas (Ackermann e Paw, 2003; Best e Alderton, 2008; Sloman et al., 2003), e ainda para a triagem e descoberta de novos fármacos (Chakraborty et al., 2009; Málaga-Trillo et al., 2011; Takaki et al., 2012; Yu et al., 2012). Essa espécie tem sido utilizada como uma importante ferramenta para a realização de estudos nas áreas de bioquímica (Seibt et al., 2009; Siebel et al., 2011; Taylor et al., 2004), comportamento (Buske e Gerlai, 2010; Cognato et al., 2012; Gerlai et al., 2009), toxicologia (Hill et al., 2005; Pereira et al., 2012; Senger et al., 2006), pesquisa transgênica, teratologia e neurociências (Cachat et al. 2010; Edwards e Michel, 2002; Ivetac et al., 2000). O interesse pela espécie pode ser observado pelo número crescente de grupos de pesquisa que têm utilizado este teleósteo como um modelo experimental (Heur et al., 2012; Schärer et al., 2012; Sprague et al., 2003; Tessadori et al., 2012).

Figura 1: *Zebrafish*. Disponível em *www.zfin.org*.

Por ser pequeno e de fácil manipulação, o zebrafish tornou-se atrativo para o desenvolvimento de pesquisas, uma vez que pode ser armazenado em grande quantidade em um espaço pequeno e com baixos custos de manutenção laboratorial (Málaga-Trillo et al., 2011; Shin e Fishman, 2002). Esta espécie é bastante utilizada para estudos de biologia do desenvolvimento por apresentar fecundação e reprodução externas, possuindo um ciclo biológico de desenvolvimento rápido e ao longo de todo o ano. Seus ovos são relativamente grandes e transparentes, podendo observar-se em tempo real a divisão celular e a formação de um novo organismo (Bai e Burton, 2011; Langheirich, 2003; Shin e Fishman, 2002). Além disso, estudos têm demonstrado que o genoma do zebrafish é muito similar ao genoma de mamíferos (70–80%), incluindo a espécie humana, apresentando marcos neuroanatômicos e sistemas de neurotransmissão muito similares (Barbazuk et al., 2000; Maximino et al., 2011a).

A utilização do zebrafish como modelo para o estudo de mecanismos farmacológicos e toxicológicos vem ganhando importância significativa (Froehlicher et al., 2009; Yang et al., 2009). Isso se deve ao fato dessa espécie possuir um rápido metabolismo e uma grande sensibilidade a fármacos (Karlovich et al., 1998; Goldsmith, 2004), absorvendo os componentes diretamente da água pelas suas brânquias (Grosell

e Wood, 2002), mostrando assim ser um modelo útil para pesquisas em neurociência comportamental (Bencan et al., 2009; Gerlai et al., 2009; Ng et al. 2012).

Atualmente, muitos estudos são realizados nesta espécie para avaliar as bases moleculares da neurobiologia, identificando genes envolvidos na formação de circuitos neuronais, no comportamento e nos mecanismos envolvidos na neuropatogênese (Guo, 2004).

1.2. SISTEMA PURINÉRGICO

A sinalização purinérgica é uma rota comum de comunicação célula-célula envolvida em muitos mecanismos neuronais e não neuronais e em eventos de curta e longa duração, incluindo respostas imunes, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (Agteresch et al., 1999; Burnstock e Knight, 2004; Hoebertz et al., 2003).

O ATP, molécula sinalizadora do sistema purinérgico, é um nucleotídeo trifosfatado existente em todas as células e está envolvido na regulação de vários processos fisiopatológicos no meio extracelular. Este nucleotídeo é armazenado em vesículas nas terminações sinápticas e, após despolarização neuronal, é liberado atuando em receptores específicos na membrana pós-sináptica, denominados purinoreceptores (Burnstock, 1972; 1976; Ralevic e Burnstock, 1998). O ATP pode ser coliberado juntamente com vários neurotransmissores, tais como acetilcolina, glutamato, noradrenalina, serotonina e ácido γ-amino butírico (GABA) (Burnstock, 2004; 2009; Holton, 1959; Nakanishi e Takeda, 1973; Pankratov et al., 2009; Zimmermann,

2008). A liberação de ATP nos terminais pré e pós-sinápticos pode ocorrer como um mecanismo fisiológico ou em resposta a danos celulares, como hipóxia e injúrias (Burnstock, 2008).

O ATP pode atuar tanto como transmissor quanto como co-transmissor, agindo através de purinoreceptores do tipo P2, divididos em duas famílias distintas de acordo com a base do mecanismo de ação, farmacologia e clonagem molecular, sendo eles P2X e P2Y (Burnstock e Kennedy, 1985; Burnstock, 2012).

A família P2X consiste de receptores ionotrópicos ligados a canais iônicos que quando ativados resultam na abertura de um poro na membrana celular que permite a passagem de cátions Na^{+}, K^{+} e Ca^{+2}. Ela está dividida em sete membros (P2X1-7), os quais estão distribuídos em neurônios, células gliais e no músculo liso (Fields e Burnstock, 2006; Kirischuk et al., 1995a,b; Moller et al., 2000; North, 2002; North and Verkhratsky, 2006). A família P2Y consiste em receptores metabotrópicos acoplados a uma proteína G e foram funcionalmente descritos oito membros (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 e P2Y14), que apresentam uma ampla distribuição nos tecidos e sistemas, tais como vascular, nervoso e cardíaco (Burnstock, 2007; Díaz-Hernandez et al., 2002; Erb et al., 2006; Zimmermann, 2011).

Em situações patofisiológicas, a liberação de ATP e a expressão de receptores purinérgicos pelas células são consideravelmente aumentadas (Guido et al., 2008). Como este nucleotídeo não é capaz de atravessar as membranas biológicas por difusão ou transporte ativo, o controle de sua concentração extracelular é realizado pela ação das ectonucleotidases que catalisam sua conversão até ADO (Bonan et al., 2000; Goding e Howard, 1998; Robson et al., 2006).

A ADO está envolvida na síntese de ácidos nucléicos, metabolismo de aminoácidos, modulação do estado metabólico da célula e, diferente do ATP, não é considerada um neurotransmissor clássico, uma vez que não é armazenada em vesículas ou liberada por exocitose, sendo então classificada como neuromodulador (Fredholm e Dunwiddie, 1988; Shen e Chen, 2009; von Lubitz, 1999). Devido a esse papel de neuromodulação, ela está envolvida na regulação de importantes mecanismos no SNC, como estados de ansiedade (El Yacoubi et al., 2000; Maximino et al., 2011b), sono (Carús-Cadavieco e de Andrés, 2012; Porkka-Heiskanen, 1999), cognição e memória (Ribeiro et al., 2003; Shen et al., 2012; Wei et al., 2011).

A concentração extracelular de ADO é um fator determinante dos efeitos neuromoduladores desta molécula. Ela exerce seus efeitos através da ativação de receptores purinérgicos de membrana específicos do tipo P1. Estes receptores são divididos em quatro subtipos de acordo com suas características, como estrutura molecular, distribuição tecidual e afinidade pelo seu ligante. São eles: os receptores A_1, A_{2A}, A_{2B} e A_3, sendo todos acoplados a proteína G e exibindo sete domínios transmembrana formados por aminoácidos hidrofóbicos (Fredholm et al., 2000; Libert et al., 1989; Maenhaut et al. 1990; Stehle et al. 1992). Os receptores A_1 e A_3 se ligam à família das proteínas Gi/o, responsáveis pela inibição da produção do segundo mensageiro AMPc. Os receptores A_{2A} e A_{2B} estimulam a produção de AMPc via ativação de proteínas Gs (Ralevic e Burnstock, 1998).

A clonagem e caracterização molecular dos receptores P2X em zebrafish já foram realizadas (Díaz-Hernandez et al., 2002; Norton et al., 2000). A subunidade P2X possui nove membros, sendo destes seis ortólogos aos genes dos receptores P2X de mamíferos (zfP2X$_1$, zfP2X$_2$, zfP2X$_3$, zfP2X$_4$, zfP2X$_5$ and zfP2X$_7$), dois parálogos (P2X$_{3.2}$

and $P2X_{4.2}$) e um gene ainda precisa ser devidamente classificado (514) (Kucenas et al., 2003). Até o momento, foram identificados apenas receptores P2Y1 em trombócitos de zebrafish (Gregory e Jagadeeswaran, 2002). Estudos também identificaram os receptores de ADO A_1, A_{2A}, e A_{2B} neste teleósteo (Boehmler et al., 2009; Capiotti et al., 2011).

1.2.1 Ectonucleotidases

Os nucleotídeos e nucleosídeos extracelulares atuam como moléculas sinalizadoras envolvidas em uma ampla gama de efeitos biológicos. Estes nucleotídeos extracelulares são degradados por uma cascata de hidrólise constituída por uma variedade de enzimas que estão localizadas na superfície celular, chamadas de ectonucleotidases. As ectonucleotidases estão ancoradas na membrana celular, possuindo seu sítio ativo voltado para o meio extracelular, ou estão presentes na forma solúvel no meio intersticial (Zimmermann, 2011). Este grupo de enzimas é constituído pelas famílias das ectonucleotídeo pirofosfatase/fosfodiesterase (NPP), ectonucleosideo trifosfato difosfoidrolases (NTPDases), ecto-5'-nucleotidase e fosfatases alcalinas. Estas enzimas são capazes de controlar a disponibilidade de ligantes como ATP e ADO aos seus receptores específicos (Zimmermann, 1992; 1996a; 1996b; 2011). Neste estudo, daremos maior ênfase a família das NTPDases e ecto-5'-nucleotidase.

As NTPDases estão presentes em vertebrados, invertebrados, plantas, leveduras e protozoários (Handa e Guidotti, 1996; Vasconcelos et al., 1996; Rosemberg et al., 2010; Smith et al., 1997; Zimmermann, 1999; Zimmermann e Braun, 1999). Os membros da família das NTPDases são codificados por oito genes diferentes. Quatro

membros desta família de enzimas estão localizados na superfície das células, com um sítio catalítico extracelular, sendo eles NTPDases 1, 2, 3 e 8. A NTPDase1 hidrolisa ATP e ADP igualmente bem, enquanto a NTPDase3 e a NTPDase8 apresentam preferência por ATP em relação ao ADP como substrato. A NTPDase2 se caracteriza por possuir uma alta preferência por nucleosídeos trifosfatados e foi previamente classificada como uma ecto-ATPase (Chadwick e Frischauf, 1997; Kaczmarek et al., 1996; Robson et al., 2006; Sévigny et al., 2000; Smith e Kirley, 1998). Outros dois membros conhecidos como NTPDases 5 e 6 apresentam localização intracelular, porém, são secretadas após expressão heteróloga (Braun et al., 2000; Mulero et al., 1999; Trombetta e Helenius, 1999). As NTPDases 4 e 7 apresentam localização intracelular com o sítio ativo voltado para o lúmen de organelas citoplasmáticas (Biederbick et al., 2000; Shi et al., 2001; Wang e Guidotti, 1998). Estas enzimas hidrolisam tanto ATP como ADP, formando AMP na presença de íons Ca^{2+} e Mg^{2+} (Bigonnesse et al. 2004; Robson et al., 2006; Rosemberg et al., 2010).

A ecto-5´-nucleotidase (Ecto-5´-NT) desfosforila nucleosídeos monofosfatados não cíclicos, através da hidrólise da ligação fosfodiéster de 5´-ribonucleotídeos, levando à formação do correspondente ribonucleosídeo e fosfato. A principal função em animais é a hidrólise de AMP até ADO. As ecto-5´-nucleotidases apresentam uma ampla distribuição tecidual e fazem parte da cascata enzimática para finalizar a ação de nucleotídeos que agem em receptores P2X e P2Y, sendo a principal enzima responsável pela produção de ADO extracelular (Cunha, 2001; Kluge et al., 1972; Robson et al., 2006; Zimmermann, 1992; 1996a; 2011).

Desta forma, as ectonucleotidases controlam a disponibilidade de ligantes (ATP, ADP, AMP e ADO) para ambos os receptores de nucleotídeos e nucleosídeos e,

consequentemente, a extensão e a duração da ativação do receptor (Chen e Guidotti, 2001). Portanto, essa é uma via enzimática com função dupla de remoção de uma molécula sinalizadora, ATP, e geração de uma segunda molécula, a ADO (Abbracchio et al.,2009; Burnstock e Verkhratsky, 2009; Zimmermann, 1996a; 1996b).

Em zebrafish, estudos demonstraram a presença de uma NTPDase e uma ecto-5'-nucleotidase em membranas cerebrais, sendo estas caracterizadas como cátion-dependentes (Rico et al., 2003; Rosemberg et al., 2010; Senger et al., 2004). Recentemente, estudos clonaram e caracterizaram o padrão de expressão de dez ortólogos de NTPDases, sendo elas: NTPDase1 (Rosemberg et al., 2010), três isoformas da NTPDase2, nomeadas como NTPDase2_mv, NTPDase2_mq e NTPDase2_mg (Rico et al., 2006; Rosemberg et al., 2010), NTPDase 3 (Appelbaum et al., 2007; Rosemberg et al., 2010), NTPDase4, duas isoformas da NTPDase5, sendo NTPDase5_ms e NTPDase5_me, NTPDase6 e NTPDase8 nesta espécie (Rosemberg et al., 2010). Estudos realizados na retina deste animal demonstraram a presença de isoformas das NTPDase1 e 3, bem como do receptor P2Y1. Também foram encontradas três isoformas da NTPDase2, sendo elas também classificadas como NTPDase2_mv, NTPDase2_mq e NTPDase2_mg (Ricatti et al. 2011). As NTPDase1 e 2 parecem ser expressas na margem germinal da retina do zebrafish, onde contém células em proliferação e diferenciação (Ricatti et al., 2009).

1.2.2 Adenosina desaminase

A adenosina desaminase (ADA) (EC 3.5.4.4) é uma enzima envolvida no metabolismo das purinas, promovendo a desaminação hidrolítica da ADO e da deoxiadenosina até inosina e deoxiinosina, respectivamente. Ela é encontrada como

uma enzima citosólica, pode ser expressa na superfície celular como uma ectoenzima e possui uma função importante no sistema imune em processos inflamatórios, controlando os níveis de ADO (Franco et al., 1997; Haskó et al., 2000; Hirschhorn e Ratech, 1980; Iwaki-Egawa et al., 2004; Ratech et al., 1981; Zavialov e Engström, 2005).

Diferentes membros da ADA, apresentando características cinéticas distintas, já foram descritos nas células animais. Essas diferenças cinéticas sugerem uma função diferenciada de cada membro no organismo (Iwaki-Egawa et al., 2004; Maier et al., 2005; Ratech et al., 1981; Schrader et al., 1979; Zavialov Engström, 2005). Dois subtipos identificados já estão bem caracterizados, denominados ADA1 e ADA2, e existe ainda um grupo similar de proteína, denominado ADAL.

A ADA1 é uma enzima monomérica importante na resposta imune mediada por linfócitos T, cuja massa molecular é de aproximadamente 3-40 kDa. Sua deficiência pode levar a imunodeficiência combinada grave em crianças (Daddona e Kelley, 1977; Pacheco et al, 2005;. Ozdemir, 2006). Tecidos como fígado e rins apresentam tanto a ADA1 solúvel quanto a forma associada a uma proteína de ligação. O complexo ADA-proteína de ligação constitui uma ecto-ADA, a qual é responsável pelo controle dos níveis de ADO extracelular (Iwaki-Egawa et al., 2004; Torvinen et al., 2002). Além de sua função enzimática, a ADA1 pode facilitar a transdução de sinal através do receptor de adenosina do tipo A1 através da sua interação com esse receptor (Ciruela et al., 1996).

A ADA2 possui massa molecular de aproximadamente 100 kDa e representa uma menor parte da atividade de desaminação da adenosina em tecidos, sendo abundante no plasma e o seu aumento está associado com casos de doenças

hepáticas (Iwaki-Egawa et al., 2006; Kobayashi et al., 1993). Sabe-se que a ADAL também participa da desaminação da adenosina, porém ela ainda não está bem caracterizada (Maier et al., 2005).

A existência de diferentes genes relacionados à ADA, com um padrão de expressão ubíquo em zebrafish já foi caracterizada (Rosemberg et al. 2007). Além disso, a cinética enzimática e propriedades da desaminação de ADO a partir do cérebro de zebrafish também já foram descritas (Rosemberg et al., 2008). A desaminação da ADO no SNC de zebrafish promovida por diferentes membros da família da ADA pode ser um elemento-chave para o controle da ADO/inosina no meio intracelular e extracelular (Rosemberg et al., 2008).

1.3. SISTEMA COLINÉRGICO

O sistema colinérgico tem um papel fundamental em várias funções vitais (Mesulam et al., 2002), sendo a acetilcolina (ACh) o neurotransmissor mais importante desse sistema (Descarries et al., 1997; Geffard et al., 1985). A ACh desempenha um papel fundamental no SNC e está relacionada à modulação da resposta neuronal por estímulos sensoriais (Murphy e Sillito, 1991), ao comportamento, à participação em circuitos neurais do controle do sono, ao aprendizado e memória (Shaked et al., 2008).

Este sistema pode modular funções cognitivas de maneira eficiente no cérebro, agindo em receptores metabotrópicos e ionotrópicos (Edwards et al., 2007; Schröder et al., 1989; van der Zee et al., 1989). A ACh é sintetizada nos neurônios pré-sinápticos, a partir da acetilcoenzima A (acetil-CoA), e da colina, pela colina acetiltransferase (ChAT), enzima responsável por transferir um grupamento acetil da acetil-CoA para a colina

(Crawford et al., 1982; Eckenstein e Thoenen, 1982; Levey et al., 1983). Após a síntese, a ACh é transportada dentro de vesículas para os terminais dos axônios colinérgicos, onde é armazenada. Outra importante fonte de acetilcolina é a quebra de fosfatidilcolina (Picciotto et al., 1998).

A acetil-CoA usada na síntese de ACh é formada na membrana interna da mitocôndria após o metabolismo de transformação da glicose em piruvato. A colina provém diretamente da reciclagem da ACh, que é hidrolisada pela acetilcolinesterase (AChE) na fenda sináptica, ou a partir da fosfatidilcolina. Essas duas fontes de colina são particularmente importantes para o SNC, pois a colina presente no plasma não ultrapassa a barreira hematoencefálica. A liberação de ACh depende das variações no potencial elétrico das membranas dos terminais nervosos e este processo é dependente da concentração de cálcio intracelular (Oda, 1999; Picciotto et al., 1998).

Ao ser liberada na fenda sináptica, a ACh interage com receptores específicos causando despolarização e propagação do potencial de ação na célula pós-sináptica. Seus efeitos são mediados pela ativação de receptores nicotínicos e muscarínicos (Edwards et al., 2007; Park et al., 2008; Schröder et al., 1989; Soreq e Seidman, 2001; van der Zee et al., 1989). Os receptores nicotínicos (nAChRs) consistem de cinco subunidades designadas α, β, γ e δ, sendo que a subunidade α é expressa em duas formas. A ACh se liga normalmente a subunidade α, produzindo mudanças conformacionais que permitem a passagem principalmente de cátions, sendo responsáveis pelo aumento do influxo de íons como Na^{+}, K^{+} e Ca^{+2}. A dessensibilização do receptor aumenta quando o mesmo é fosforilado por proteína quinase dependente de AMPc ou tirosina quinase (Castro e Albuquerque, 1995; Díaz-Hernandez et al., 2002; MacDermott et al., 1999; Rogers e Dani, 1995; Sargent, 1993; Wonnacott, 1997).

Os nAChRs estão envolvidos em mecanismos de recompensa no SNC, o que explica em grande parte o mecanismo do uso de tabaco e nicotina (Picciotto et al., 1998). Os receptores muscarínicos (mAChRs) se associam à proteínas G e consistem em cinco tipos diferentes de receptores (M1-M5). Assim como os nAChRs, um único neurônio colinérgico pode expressar mais de um tipo de subtipo de mAChR (Anagnostaras et al., 2003; Fischer at el., 1998). Os mAChRs estão envolvidos na neurotransmissão e neuromodulação (Castillo et al., 1999; Ghatpande et al., 2006), memória olfatória (Ravel et al., 1994), aquisição de tarefas de discriminação de odores (De Rosa e Hasselmo, 2000), e discriminação de odores similares (Edwards et al., 2007; Fletcher e Wilson, 2002; Linster et al., 2001; Prediger et al., 2006). Muitas evidências também os relacionam a processos de aprendizado e memória, entre elas a observação de déficits cognitivos em ratos *knockout* para o gene do receptor M1 (Anagnostaras et al., 2003).

A ACh que permanece na fenda sináptica é degradada pelas colinesterases que a clivam em colina e acetato, eliminando os efeitos desencadeados por esta molécula. Grande parte da colina resultante é captada pelo terminal do axônio colinérgico por um transportador de colina e reutilizada na síntese de nova ACh (Mesulam et al., 2002; Soreq e Seidman, 2001).

1.3.1 Acetilcolinesterase

As colinesterases hidrolisam a ACh na fenda sináptica. Existem dois diferentes tipos, que são classificados de acordo com suas propriedades catalíticas, especificidade de inibidores e distribuição nos tecidos: a acetilcolinesterase (AChE) (E.C.3.1.1.7) e a butirilcolinesterase (BuChE) (E.C.3.1.1.8). A AChE hidrolisa preferencialmente ésteres com grupamento acetil, presente principalmente nas

sinapses dos sistemas nervoso central e periférico parassimpático e ainda junção neuromuscular; e a BuChE hidrolisa outros tipos de ésteres como a butirilcolina (Alles e Hawes, 1940; Augustinsson e Nachmansohn, 1949; Massoulié et al., 2008; Mendel et al., 1943; Soreq e Seidman, 2001). Ambas as colinesterases são amplamente distribuídas no organismo.

A AChE é uma serina hidrolase sintetizada no retículo endoplasmático, processada e transportada para o meio extracelular pela presença de um peptídeo sinal na região N-terminal. Ela desempenha um papel essencial no mecanismo colinérgico, catalisando a hidrólise natural do substrato acetilcolina em acetato e colina (Massoulié et al., 2008). Esta enzima também pode modular funções não colinérgicas, tais como glutamatérgicas e dopaminérgicas (Shaked et al., 2008; Soreq e Seidman, 2001; Zimmermann e Soreq, 2006). Os níveis de AChE parecem ser controlados pela interação da ACh com seus receptores, sendo que quando a interação é acentuada, aumentam os níveis de AChE. No entanto, a AChE pode ser usada como um marcador da função colinérgica, e mudanças na atividade da enzima podem indicar alterações na disponibilidade de ACh e do nível de seus receptores (Fernandez e Hodges-Savola, 1992).

Tem sido demonstrado que BuChE não está presente no genoma de zebrafish. No entanto, o gene da AChE já foi clonado e sequenciado e sua atividade enzimática já foi detectada no cérebro deste teleósteo (Bertrand et al., 2001; Rico et al., 2007). O zebrafish apresenta AChE codificada por um único gene, porém várias formas moleculares são observadas (monômeros, dímeros, trímeros e tetrâmeros) como resultado da ocorrência de splicing alternativo nos éxons da região C-terminal

(Massoulié et al., 2008). Além disso, subunidades de nAChRs e mAChR também são expressos nesta espécie (Zirger et al., 2003).

1.4. BENZODIAZEPÍNICOS

Os fármacos hipnóticos e ansiolíticos são bastante utilizados na prática clínica, sendo superados apenas em prescrições médicas para medicamentos utilizados em doenças cardiovasculares. Os fármacos ansiolíticos diminuem a ansiedade, moderam a excitação e acalmam o paciente. Os hipnóticos induzem e mantêm o sono (Ashton, 1994; Woods e Winger, 1992). Estudos realizados por Ashton (1994) e Woods e Winger (1992), demonstram que cerca de 10% a 20% da população faz uso de hipnóticos ou ansiolíticos em algum momento da vida e estima-se que o consumo desses fármacos dobra a cada cinco anos (Auchewski et al., 2004).

Em 1957, iniciou-se a era dos benzodiazepínicos, uma família de fármacos psicoativos com estrutura básica formada a partir da fusão de um anel de benzina com um anel de diazepina, como mostra a Figura 2 (Anderson, 2010; Ashton, 1994; Woods e Winger, 1992). Esta classe de fármacos possui variação ansiolítica, hipnótica e anticonvulsivante, podendo provocar amnésia anterógrada e relaxamento muscular (Anderson, 2010; Fahey et al., 2006; Listos et al., 2005; Mandrioli et al., 2010).

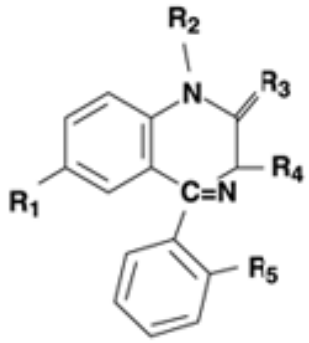

Figura 2: Estrutura geral dos benzodiazepínicos

Além da sua ação no SNC, os benzodiazepínicos também possuem efeito depressor dose-dependente, causando uma modesta redução na pressão sanguínea arterial e um aumento na frequência cardíaca (Colussi et al., 2011; Olkkola e Ahonen, 2008).

Apesar das similaridades neurofarmacológicas, existem diferenças entre as classes de benzodiazepínicos. As diferentes estruturais apresentadas pelo diazepam e midazolam (Figura 3) e as diferenças na sua afinidade para subtipos de receptores, em combinação com a ampla variedade de perfis farmacocinéticos, são responsáveis por diversos efeitos farmacológicos (Anderson, 2010; Nelson e Chouinard, 1999). Cada membro da família de benzodiazepínicos tem diferentes propriedades, sendo que um exemplo é a solubilidade lipídica onde cada componente possuirá diferente impacto na sua absorção, distribuição nos compartimentos teciduais, metabolismo e excreção. Esse perfil farmacocinético único tem um maior impacto na escolha de um benzodiazepínico específico para uma condição particular, principalmente em relação à rota de administração, taxa e amplitude de absorção. Essas diferenças farmacocinéticas muitas vezes estabelecem formulações específicas para membros individuais da família dos benzodiazepínicos (Anderson, 2010).

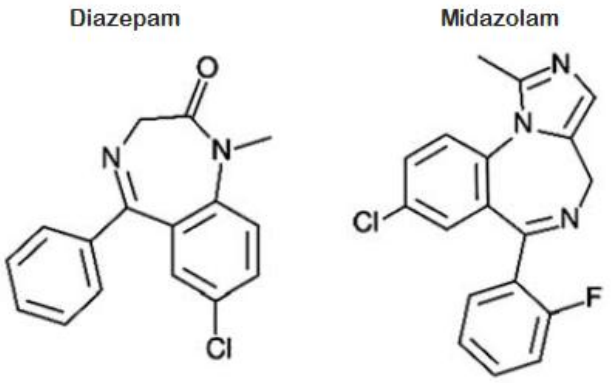

Figura 3: Estruturas do diazepam e midazolam

Essa família de fármacos pode atravessar a barreira hematoencefálica e a duração de sua ação está fortemente associada com a duração da sua administração. A taxa de declínio da concentração dessa classe de fármacos no plasma pode ser um importante fator na determinação do número de doses necessárias para manter os efeitos ótimos no tratamento das desordens do pânico, além de mínimo efeito rebote de ansiedade e de abstinência (Anderson, 2010; Olkkola e Ahonen, 2008).

Embora sejam fármacos relativamente seguros, restrições à sua utilização têm sido cada vez maiores, devido à incidência dos efeitos adversos, relacionados à depressão do SNC. Dentre eles, os principais são a dano psicomotor e cognitivo, tolerância, dependência e potencialização do efeito depressor pela interação com outras drogas depressoras, principalmente o álcool (Anderson, 2010; Barbui et al., 2011; Longo e Johnson, 2000). Compostos de meia-vida média ou curta carregam um maior risco de dependência e de reações de efeito rebote e abstinência do que os agentes de longa ação (Nelson e Chouinard, 1999). Outra característica relevante deste tipo de medicamento é o aparecimento da tolerância e dependência (Barbui et al., 2011). Um estudo realizado por Fahey e colaboradores (2001) analisou os aspectos farmacodinâmicos e neuroquímicos da tolerância aos benzodiazepínicos em

camundongos, utilizando o fármaco lorazepam. Seus resultados mostraram que uma diminuição na regulação dos receptores de benzodiazepínicos está associada com a tolerância comportamental a esses fármacos, uma vez que os camundongos mostraram baixa atividade locomotora em teste de campo aberto no primeiro dia de avaliação, enquanto esse perfil não se manifestou no 14º dia de avaliação (Fahey et al., 2001).

A ação dos benzodiazepínicos se dá devido aos efeitos mediados pelo ácido gama-aminobutírico (GABA), sendo o sistema GABAérgico o principal sistema de neurotransmissão inibitória do SNC. Os agonistas de GABA, como os benzodiazepínicos, agem em uma estrutura transmembrana no receptor do GABA denominado complexo $GABA_A$ (Campo-Soria et al., 2006; Nelson and Chouinard, 1999; Olkkola e Ahonen, 2008; Rifkin, 1990). Até o momento, dezenove diferentes subtipos do receptor $GABA_A$ foram identificados (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3). A maioria dos subtipos de receptores $GABA_A$ expressos no encéfalo de ratos são α1β2γ2, α3β3γ2, e α2β3γ2 (Whiting, 2003). Os sítios de ligação dos benzodiazepínicos clássicos são encontrados comumente em receptores $GABA_A$ compostos de cinco subunidades, duas α, duas β e uma γ2 (Figura 4). O ponto de ligação dos benzodiazepínicos está situado na interface das subunidades γ2 e α (α1, α2, α3, α5) (Harrison, 2007; Rudolph et al., 2001; Sigel e Buhr, 1997; Smith e Olsen, 1995). Essas subunidades α possuem um resíduo de histidina no domínio de ligação da droga, resultando em uma alta afinidade para os benzodiazepínicos. Ao contrário, as subunidades α4 e α6 contêm um resíduo de arginina, não demonstrando afinidade para benzodiazepínicos (Fritschy e Mohler , 1995; Harrison, 2007; Rudolph et al., 2001; Whiting, 2003).

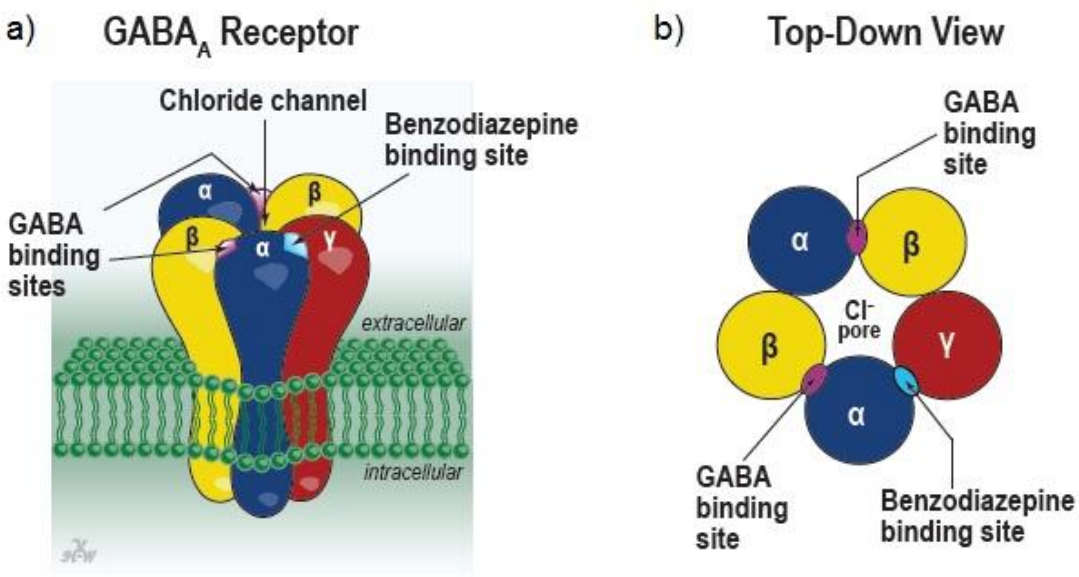

Figura 4: Representação esquemática resumida do receptor $GABA_A$. a) Complexo pentamérico do receptor $GABA_A$ abrangendo a bicamada lipídica. b) Vista de cima do receptor $GABA_A$ mostrando o canal de íons Cl^- circundado pelas subunidades α, β, e γ. Figura obtida de Hambrecht-Wiedbusch et al., 2010.

A atuação dos benzodiazepínicos no receptor $GABA_A$ se dá devido a um aumento na frequência da abertura dos canais de cloreto (Cl^-), resultando em um influxo desse íon para dentro do neurônio com consequente hiperpolarização da célula, causando uma corrente inibitória aumentada e potenciais inibitórios pós-sinápticos mais fortes, expressando o seu efeito de neurotransmissor inibitório (Anderson, 2010; DeMicco et al., 2010; Olkkola e Ahonen, 2008; Rifkin, 1990).

Hawkins e colaboradores (1988) realizaram um estudo avaliando a função dos receptores de ADO em específicas estruturas cerebrais de ratos tratados durante sete dias com diazepam, um fármaco da família dos benzodiazepínicos. Como resultado, o tratamento com esta droga não alterou a ligação do receptor A_1 de ADO nas áreas estudadas do cérebro. No entanto, a ligação dos receptores A_2 e estímulos mediados

por tal receptor, foram significantemente atenuados, indicando que este tipo de receptor apresenta-se dessensibilizado após tratamento prolongado com diazepam (Hawkins et al., 1988). Foi também demonstrado que o sistema adenosinérgico atenua os sinais de abstinência ao diazepam em camundongos, demonstrando que o receptor A_{2A} exerce um importante papel neste processo (Listos et al., 2008).

Outras linhas de evidência demonstraram que benzodiazepínicos administrados *in vitro* inibem a captação de ADO pelos sinaptossomas corticais cerebrais de ratos (Phillis, 1981). Experimentos ex *vivo* também mostraram que os receptores de ADO são capazes de antagonizar as ações centrais dos benzodiazepínicos em ratos (Phillis et al., 1980) e reverter o quadro de sedação em humanos (Arvidsson et al., 1982).

Um estudo realizado por Barcellos e colaboradores (1998) avaliou o efeito *in vitro* de psicofármacos na atividade de ATPase-ADPase e acetilcolinesterase no SNC de ratos adultos. O diazepam mostrou ser capaz de inibir a atividade dessas enzimas (Barcellos et al., 1998). Além disso, o diazepam inibiu a atividade ecto-ATPásica em concentrações de 0,06-1,5 mM em membrana plasmática sináptica (Horvat et al., 2006). Evidências destacam que os benzodiazepínicos alteram a atividade da acetilcolinesterase, inibindo o seu efeito no córtex cerebral de ratos adultos (Schetinger et al., 2000). Outro estudo relata ainda que existe uma leve diminuição na neurotransmissão colinérgica, também nessa espécie, quando em tratamento crônico com drogas psicotrópicas, incluindo o diazepam (Bekpinar et al., 1994). Foi também relatada a efetividade dos benzodiazepínicos no tratamento de intoxicação com pesticidas organofosforados (Gilat et al., 2003; Tuovinen, 2004), que são tóxicos ambientais conhecidos por inibir a atividade catalítica da acetilcolinesterase, resultando em sintomas de toxicidade hipercolinérgica (Fukuto, 1990). Os efeitos dos

organofosforados se apresentam como quadros de crises convulsivas e *status epilepticus*, que levam a danos cerebrais. Os fármacos benzodiazepínicos agem diminuindo essas consequências, prevenindo assim danos causados por esse tipo de agente tóxico (Gilat et al., 2003; Tuovinen, 2004).

O zebrafish tem se tornado útil como modelo animal para estudos das bases moleculares da neurobiologia com aplicações na neurofarmacologia e neurotoxicologia. Receptores benzodiazepínicos têm sido encontrados em uma variedade de espécies. Vários estudos têm identificado receptores de benzodiazepínicos em peixes com características de ligação similares a roedores e humanos (Anzelius et al., 1995; Carr et al., 1999; Friedl et al., 1988; Wilkinson et al., 1983). Análises do comportamento do zebrafish na presença de fármacos ansiolíticos já estão bem documentadas (Bencan et al., 2009; Cachat et al., 2010; Gebauer et al., 2011; Mathur e Guo, 2010). No entanto, pouco se sabe com relação ao mecanismo de ação dessas drogas nas enzimas que compõem os sistemas purinérgico e colinérgico. Portanto, torna-se necessário um estudo mais aprofundado, avaliando a interação entre os benzodiazepínicos e esses sistemas de neurotransmissão no encéfalo de zebrafish.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar o efeito *in vitro* e *ex vivo* da administração de benzodiazepínicos sobre as enzimas envolvidas no controle da sinalização purinérgica e colinérgica em cérebro de zebrafish.

2.2. OBJETIVOS ESPECÍFICOS

- Verificar o efeito *in vitro* dos fármacos benzodiazepínicos diazepam e midazolam sobre a atividade enzimática das NTPDases, ecto-5'-nucleotidase, adenosina desaminase em membranas cerebrais de zebrafish, e da acetilcolinesterase no homogenato encefálico de zebrafish.

- Verificar o efeito *ex vivo* dos fármacos benzodiazepínicos diazepam e midazolam sobre a atividade enzimática das NTPDases, ecto-5'-nucleotidase e adenosina desaminase em membranas cerebrais, e da acetilcolinesterase no homogenato encefálico de zebrafish.

- Verificar o efeito *ex vivo* do diazepam e midazolam sobre o padrão de expressão gênica das NTPDases, ecto-5'-nucleotidase, adenosina desaminase e acetilcolinesterase em homogenato encefálico de zebrafish.

CAPITULO 2

ARTIGO CIENTÍFICO

ALTENHOFEN S, ZIMMERMANN FF, BONAN CD. Benzodiazepines alter acetylcholine, nucleotide, and nucleoside hydrolysis in zebrafish (*Danio rerio*) brain.

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Benzodiazepines alter acetylcholine, nucleotide, and nucleoside hydrolysis in zebrafish (*Danio rerio*) brain

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Abstract

Diazepam and midazolam are benzodiazepines with anxiolytic and hypnotic effects, respectively. Their actions are due to the potentiation of the neural inhibition that is mediated by gamma-aminobutyric acid (GABA). ATP is co-released with several neurotransmitters, such as GABA and acetylcholine, and its metabolite adenosine is a neuromodulator playing a role in the benzodiazepine effects. We have tested the *in vitro* benzodiazepine effects on extracellular nucleotide, nucleoside, and acetylcholine hydrolysis promoted by nucleoside triphosphate diphosphohydrolases (NTPDases), ecto-5'-nucleotidase, adenosine deaminase (ADA), and acetylcholinesterase (AChE) in zebrafish brain. Diazepam, at 500 µM, decreased ATP hydrolysis (66%), whereas, at10-500 µM, it reduced ADP hydrolysis (40-54%, respectively). Midazolam also decreased ATP (16-71% for 10-500 µM, respectively) and ADP (48-73.5% for 250-500 µM, respectively) hydrolysis and ecto-ADA activity (26-27.5% for 10-500 µM, respectively). Diazepam and midazolam did not alter ecto-5´-nucleotidase activities at all concentrations tested. Concerning to AChE activity, 500 µM diazepam, promoted a decrease on acetylcholine (ACh) hydrolysis (19%), whereas midazolam, at 50-500 µM, reduced ACh hydrolysis (18-79%, respectively). It is possible to suggest that benzodiazepines induce a direct effect on these enzyme activities, which shows a complex interaction among benzodiazepines, purinergic, and cholinergic systems, providing a better understanding of the benzodiazepine pharmacodynamics.

Keywords: Benzodiazepine, anxiety, ectonucleotidases, acetylcholinesterase, adenosine deaminase, zebrafish.

1. Introduction

The purinergic signaling has important role in the central nervous system (CNS) in both physiological and pathological conditions. ATP is considered as a neurotransmitter in the CNS and performs its functions when it is released into the synaptic cleft in a calcium-dependent manner (Burnstock, 1972; Cunha and Ribeiro, 2000). This signaling molecule is stored in presynaptic vesicles and is released after depolarization acting through activation of G-protein-coupled P2Y receptors and P2X ionotropic receptors (Burnstock and Kennedy, 1985). After its release to synaptic cleft, ATP is hydrolyzed by the cell-surface-located enzymes named ectonucleotidases (Zimmermann, 2001). The hydrolysis of ATP to AMP is catalyzed mainly by nucleoside triphosphate diphosphohydrolases (NTPDases) whereas the nucleotide AMP is hydrolyzed to adenosine by the action of an ecto-5´-nucleotidase (CD73, EC 3.1.3.5) (Robson et al., 2006; Bonan, 2012). Adenosine is an important signaling molecule, acting as a neuromodulator in the CNS through four subtypes of P1 metabotropic receptors (A_1, A_{2A}, A_{2B} and A_3) (Fredholm et al., 1994; Sebastião and Ribeiro, 2009). Extracellular adenosine concentrations can be also regulated by neural cell uptake through bi-directional nucleoside transporters followed by phosphorylation to AMP by adenosine kinase, or deamination to inosine by adenosine deaminase (ADA) at the intracellular medium (Fredholm et al., 2005; Rosemberg et al., 2007). ADA (E.C.3.5.4.4) is an enzyme which catalyzes the hydrolytic deamination of adenosine to inosine both in the cytosol and at the cell membrane (Franco et al., 1997; Rosemberg et al., 2008). Furthermore, studies have shown that extracellular concentrations of adenosine may also be regulated by ecto-ADA activity (Franco et al., 1998; Romanowska et al., 2007).

ATP can be coreleased with various neurotransmitters such as acetylcholine (ACh), glutamate, noradrenaline, serotonin and gamma-aminobutyric acid (GABA) (Burnstock, 2007). ACh and ATP act both as fast neurotransmitters and neuromodulators and a modulatory function prevails in the brain (Zimmermann, 2008). Adenosine A_1 receptors are known to mediate the actions of adenosine on the release of many neurotransmitters in the CNS, including ACh, noradrenaline, and dopamine (Fredholm and Dunwiddie, 1988). There is evidence demonstrating that adenosine A_1 and A_{2A} receptors modulate Ach release at cerebral cortex and prefrontal (Broad and Fredholm, 1996; van Dort et al., 2009). In contrast, ACh release was shown to be enhanced by adenosine A_{2A} agonists in striatal synaptosomes (Kirkpatrick and Richardson, 1993).

Studies have demonstrated the participation of cholinergic signaling in depression, sleep and wakefulness, anxiety, and stress (Chen et al., 2011; Hambrecht-Wiedbusch et al., 2010; Martinowich et al., 2012; Zarrindast et al., 2011). Likewise, decreased brain levels of ACh are associated with deficits in cognitive performances, as learning, behavior, and memory processes (Hasselmo, 2006; Schliebs and Arendt, 2011). ACh is widely distributed in the nervous system and provokes its effects via muscarinic (metabotropic) and nicotinic (ionotropic) ACh receptors. The control of extracellular ACh levels is catalyzed by acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) through the degradation of ACh into choline and acetate, allowing its reuptake through the choline transporter (Soreq and Seidman, 2001).

Benzodiazepines, such as diazepam and midazolam, are widely used in clinical practice to treat anxiety and panic disorders. Anxiolytic drugs reduce anxiety, temper the excitement and calm the patient whereas hypnotics induce and maintain sleep (Ashton, 1994; Woods and Winger, 1992). Benzodiazepines are GABA agonists, acting in specific transmembrane receptor called $GABA_A$ (Campo-Soria et al., 2006; Nelson and Chouinard, 1999; Olkkola e Ahonen,

2008; Rifkin, 1990). The $GABA_A$ receptor has a pentameric structure composed of different types of subunits: α, β, γ, δ, ε, θ, π, ρ (Allison and Pratt, 2003; Wafford, 2005). The combination of different subunits determines the pharmacological characteristics of each individual receptor (Anderson, 2010). The ligand site of benzodiazepines is located at the interface of α and γ subunits of the $GABA_A$ receptor (Buhr and Sigel, 1997). Previous studies have already reported adenosinergic system may play an important role in mechanisms underlying development of benzodiazepine tolerance and physical dependence (Listos et al., 2010). In addition, diazepam is able to inhibit the NTPDases and AChE activities in the CNS of adult rats (Barcellos et al., 1998).

The use of zebrafish as a model for studying pharmacological mechanisms is gaining significant importance (Froehlicher et al., 2009; Yang et al., 2009). This is due to this species has a fast metabolism and a high sensitivity to drugs (Goldsmith, 2004; Rihel and Schier, 2012), thus proving to be a useful model for biochemical (Rico et al., 2011; Seibt et al., 2009a) and behavioral studies (Bencan et al., 2009; Brennan, 2011). The presence of NTPDases, ecto-5'-nucleotidase, and ADA activities has been characterized in zebrafish brain (Rico et al., 2003; Rosemberg et al., 2008; Senger et al., 2004). It has been demonstrated that BuChE is not encoded in the zebrafish genome, but AChE is encoded by a single gene, although several molecular forms were observed as a result of alternative splicing of exons in the C-terminal region (Bertrand et al., 2001; Massoulié et al., 2008). Analysis of the behavior of zebrafish in the presence of benzodiazepines and anxiolytic drugs has been well documented, with similar effects than observed in rodents and humans (Bencan et al., 2009; Cachat et al., 2010; Gebauer et al., 2011; Mathur and Guo, 2010).

Considering purinergic and colinergic system have been described in zebrafish and this species may be a model organism to study human diseases and drug mechanisms, the aim of this

study was to evaluate the *in vitro* effects of different concentrations of diazepam and midazolam on ectonucleotidases, ADA and AChE activities in zebrafish brain.

2. Materials and methods

2.1 Animals

Adult wild-type zebrafish (*Danio rerio*) of both sexes were obtained from a commercial supplier (Red Fish, RS, Brazil) and acclimated for 2 weeks before the experiments in a 50 L-thermostated aquarium filled with continuously aerated and unchlorinated water. The fish were conditioned at 26 ± 2° C under a 14-10 h light/dark cycle photoperiod. The animals were maintained healthy and free of any signs of disease and fed twice a day with commercial food for fish. The use and maintenance of zebrafish were according to the ‘‘Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health. The protocol was approved by the Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the number 11/00256.

2.2 Chemicals

Midazolam (Roche, São Paulo, Brazil) and Diazepam (União Química, Embu-Guaçu, Brazil) were purchased from common commercial suppliers. Acetylthiocholine, 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB), adenosine, Trizma Base, EDTA, EGTA, sodium citrate, Coomassie blue, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, calcium, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were from analytical grade.

2.3 In vitro treatments

Diazepam and midazolam were diluted in water and tested at 10, 50, 100, 250, and 500 µM. Controls without the drug were performed under the same experimental conditions. Diazepam and midazolam were added to the reaction medium before the preincubation with the enzyme and were maintained during the enzyme assays.

2.4 Preparation of soluble and membrane fractions

Brain samples were obtained as described previously (Rico et al., 2003; Senger et al., 2004; Rosemberg et al., 2008). Each independent experiment was performed using biological preparations consisted of a set of ten brains. First, zebrafish were cryoanaesthetized, euthanized, and brains were removed by dissection (Wilson et al., 2009). Samples were then further homogenized in a glass-Teflon homogenizer according to the protocol for each enzyme assay. For NTPDases and ecto-5'-nucleotidase assays, zebrafish brains were homogenized in 60 vol. (v/w) of chilled Tris-citrate buffer (50 mM Tris-citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4). For ADA experiments, brains were homogenized in 20 vol (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4. The brain membranes were prepared as described previously (Barnes et al., 1993). In brief, the homogenates were centrifuged at 800 *g* for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at 40000 *g*. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. For soluble ADA experiments, the supernatant was collected and kept on ice for enzyme assays. The pellets of membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers and centrifuged for 20 min at 40000 *g*. This freeze-thaw-wash procedure was used to ensure the lysis of the brain vesicles membranes. The final pellets

were resuspended and used for enzyme assays. All samples were maintained at 2-4° C throughout preparation.

2.5 Ectonucleotidase assays

NTPDases and ecto-5'-nucleotidase assays were performed as described previously (Rico et al., 2003; Senger et al., 2004). Brain membranes of zebrafish (3 µg protein for NTPDase and 5 µg protein for ecto-5'-nucleotidase) were added to the reaction medium containing 50 mM Tris-HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris-HCl (pH 7.2) and 5 mM $MgCl_2$ (for the ecto-5'-nucleotidase activity) at a total volume of 200 µl. The samples were preincubated for 10 min at 37° C and the reaction was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. After 30 min the reaction was stopped by the addition of 200 µl 10% trichloroacetic acid and the samples were kept on ice during 10 min. In order to determine the inorganic phosphate released (Pi) 1 ml of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added to the samples for 20 min (Chan et al., 1986). The quantification of inorganic phosphate (Pi) released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nmol of Pi min^{-1} mg^{-1} of protein. In order to correct non-enzymatic hydrolysis of the substrates we used controls with the addition of the enzyme preparation after the addition of trichloroacetic acid. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. All enzyme assays were performed in at least four different experiments, each one performed in triplicate.

2.6 Adenosine deaminase assays

Ecto- and cytosolic-ADA activities were determined as described previously (Rosemberg et al., 2008). The brain fractions (5-10 µg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for soluble and membrane fractions, respectively, in a final volume of 200 µL. The samples were preincubated for 10 min at 37° C and the reaction was initiated by the addition of substrate (adenosine) to a final concentration of 1.5 mM. The reaction was stopped after 75 min (soluble fraction) and 120 min (membrane fraction) by the addition of 500 µL phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL). ADA activities were determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported (Weisman et al., 1988). In order to correct non-enzymatic hydrolysis of the substrates controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used. The reaction mixtures were immediately mixed to 500 µL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed. Samples were incubated at 37° C for 15 min and the colorimetric assay was carried out at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. The ADA activities were expressed as nmol of NH_3 min^{-1} mg^{-1} of protein. All enzyme assays were performed in five independent experiments carried out in triplicate.

2.7 Acetylcholinesterase assay

Zebrafish were euthanized and their whole brains were removed by dissection. The brains (a set of three whole brains for each sample) were homogenized on ice in 60 vol. (v/w) of 50 mM Tris-HCl, pH 8.0, in a glass-Teflon homogenizer. AChE activity was determined according to the

method of Ellman et al. (1961) with minor modifications. Briefly, the activity in the homogenate was measured by determining the rate of hydrolysis of acetylthiocholine iodide (ACSCh, 0.88 mM) in a final volume of 300 µL, with 33 µL of 100 mM phosphate buffer, pH 7.5 mixed to 33 µL of 2.0 mM 5,50-dithionitrobis2-nitrobenzoic acid (DTNB). In this solution, 5 µg of protein of each sample were added and preincubated at 25° C for 10 min. The reaction was started with the addition of the substrate acetylthiocholine, and as soon as the substrate was added the hydrolysis and the formation of the dianion of DTNB were analyzed in 412 nm for 3 min (in intervals of 30 s) using a microplate reader. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. All enzyme assays were performed in at least four different experiments, each one performed in triplicate.

2.8 Protein determination

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

2.9 Statistical analysis

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

3. Results

The *in vitro* effect of diazepam and midazolam (at concentrations of 10, 50, 100, 250 and 500 µM) was tested on NTPDases, ecto-5'nucleotidase, ADA, and ACh activities in zebrafish brain.

Diazepam and midazolam were able to modulate the NTPDase activity. Diazepam significantly decreased ATP hydrolysis at 500 µM (66%; $P<0.05$) and ADP hydrolysis at all concentrations (40-54% for 10-500 µM, respectively; $P<0.05$) when compared to the control group (Fig. 1). Similarly, midazolam significantly decreased ATP hydrolysis at all concentration (16-71% for 10-500 µM, respectively; $P<0.05$) and ADP hydrolysis at 250 (48%; $P<0.05$) and 500 µM (73.5%; $P<0.05$) when compared to the control group (Fig. 2). However, both midazolam and diazepam did not alter the ecto-5'-nucleotidase activity (data not show).

In relation to ADA activity, midazolam significantly decreased the ecto-ADA activity (26-27.5% for 10-500 µM, respectively; $P<0.05$) at all concentrations when compared to the control group (Fig. 3). However, midazolam did not alter cytosolic ADA activities whereas diazepam was not able to change both ecto- and cytosolic-ADA activities in zebrafish brain (data not show).

The results shown in Fig. 4 demonstrated that diazepam inhibited AChE activity at 500 µM (19%) when compared to the control group. Similarly, midazolan inhibited this enzyme activity, except at a concentration of 10 µM (18.1-78.8% for 50-500 µM, respectively; $P<0.05$).

4. Discussion

This study demonstrated that the benzodiazepines diazepam and midazolan are able to alter ATP, ADP, and ACh hydrolysis in zebrafish brain membranes. However, only midazolan decreased the ecto-ADA activity. Such findings indicate that these drugs promote a modulatory effect on purinergic and cholinergic signaling.

Hypnotic and anxiolytic drugs of the benzodiazepines family are widely used in clinical practice. These drugs are prescribed as sedatives, anticonvulsants, and muscle relaxants, and

share in common an ability to interact with the $GABA_A$ receptor (Bateson, 2004; Saunders and Ho, 1990). The effects of these drugs have been widely discussed in several behavioral studies, including with the use of zebrafish as a model. Bencan et al. (2009) evaluate the effects of diazepam at a dose of 1.25 mg/L on zebrafish behavior and observed a decrease in time spent on the bottom zone of the tank. These data suggest a decrease in anxiety, since the animal increased the exploration on the novel tank. However, Gebauer et al. (2011) showed that diazepam significantly reduced shoal cohesion with no changes on locomotion, probably due to the lower dose tested (0.16 mg/L) (Gebauer et al., 2011).

Considering the involvement of ATP and adenosine in anxiety, stress, learning, and sleep, the modulation of nucleotide and nucleoside levels can represent an important mechanism related to benzodiazepine effects. ATP signaling is inactivated by an enzyme cascade, which consists of cell surface-located enzymes named ectonucleotidases, having its active site facing the extracellular medium or present in soluble form in the cytosol. Among these enzymes, there are the NTPDase family and ecto-5'-nucleotidase, which are able to control the availability of ATP and adenosine ligands to their specific receptors (Bonan, 2012; Schetinger et al., 2007). Studies have demonstrated the ability of diazepam for inhibiting NTPDase activity in rat brain, directly affecting ATP and ADP hydrolysis (Barcellos et al., 1998, Horvat et al., 2006). These data are in agreement with the findings observed in our study, since midazolam and diazepam inhibited NTPDase activities in zebrafish brain membranes. Midazolam affected ATP hydrolysis at all concentrations tested whereas diazepam has changed only at the highest concentration (500 μM). Concerning to the ADP hydrolysis, midazolam showed significant inhibition only at the highest doses, while diazepam was able to inhibit it at all concentrations tested. Studies from our laboratory showed the action of antipsychotic drugs, such as olanzapine and sulpiride, are able to inhibit the ATP and ADP hydrolysis, respectively, and haloperidol was capable of decreasing the

NTPDase activities in the zebrafish brain membranes (Seibt et al., 2009a, 2009b). These findings reinforce the hypothesis that several families of drugs, including benzodiazepines, have the ability to modulate purinergic neurotransmission, affecting the enzymes responsible for the nucleotide hydrolysis. Benzodiazepines are reported to potentiate the depressant actions of AMP and adenosine on cerebral cortical neurons (Phillis, 1979). Korotkina et al. (1985) observed that phenazepam, diazepam, and midazolam inhibited ecto-5'-nucleotidase in brain homogenates of male albino rats, modulating extracellular AMP catabolism. However, we did not observe similar effects since both diazepam and midazolam were not able to alter the ecto-5'-nucleotidase activity in zebrafish brain membranes.

Some drugs can alter the structure of lipid membranes. Drug interaction with the biomembrane influences the lipid bilayer, consequently modulating membrane-bound enzyme activities, receptor binding to membrane, permeability, and transport (Carfagna and Muhoberac, 1993). The interaction of benzodiazepine drugs with the lipid bilayer could alter membrane fluidity, promoting changes in the function of the membrane proteins. The inhibitory effect produced by diazepam and midazolam on NTPDase activity could be related with these modifications at lipid membrane, since these enzymes are firmly anchored to the membrane by two transmembrane domains (Grinthal and Guidotti, 2006;). This effect could also explain the fact that diazepam and midazolam did not alter ecto-5'-nucleotidase activity, which is attached via a glycosylphosphatidylinositol anchor at the extracellular membrane (Sträter, 2006). Thus, the different effects induced by benzodiazepines on ectonucleotidase activities maybe related to the different forms of anchorage of these enzymes.

Adenosine, a product of ATP catabolism, exerts its effects through activation of P1 purinoceptors that are divided according to their characteristics: adenosine A_1 and A_3 receptors induce inhibitory actions while A_{2A} and A_{2B} receptors promote stimulatory effects (Ralevic and

Burnstock, 1998). Hawkins et al. (1989) developed a study evaluating the role of adenosine receptors in brain structures of rats treated for 10 and 20 days with triazolam (0.5, 1, 2 mg/day). As a result, the treatment with this drug did not alter the binding of adenosine in hippocampus and cortex cerebral. However, the binding of A_2 receptors, and their stimuli mediated by this receptor were significantly reduced and increased in the striatum of rats treated for 10 days at a concentration of 2 mg/day and 0.5 mg/day, respectively, indicating that this type of receptor is desensitized after prolonged treatment with diazepam. The control of the adenosinergic signaling can be exerted by adenosine uptake via bi-directional transporters, followed by intracellular phosphorylation to AMP by adenosine kinase or deamination to ADA (Fredholm et al., 2005). ADA is an enzyme which catalyzes the hydrolytic deamination of adenosine to inosine. It is found as a cytosolic enzyme and can also be expressed on the cell surface as an ecto-enzyme (Franco et al., 1997; Rosemberg et al., 2008). Benzodiazepines drugs, such as phenazepam and diazepam, administered by intraperitoneal injection were able to increase the ADA activity in rats (Korotkina et al., 1986). These data differ from those found in our study. Our findings demonstrated that diazepam was not able to change the ADA, whereas midazolam at all concentrations tested was capable of inhibiting the enzymatic activity of ecto-ADA in zebrafish brain membranes. Despite the neuropharmacological similarities, the different influence of diazepam and midazolam for the maintenance of adenosine levels in zebrafish brain can be explained by differences in their affinities to receptor subtypes, in combination with a wide variety of pharmacokinetic profiles (Anderson, 2010; Nelson and Chouinard, 1999). Each member of benzodiazepine family has different properties, where each component possesses different lipid solubility, with impacts on absorption, distribution in tissue compartments, metabolism, and excretion (Anderson, 2010). These different properties can be responsible for the effect caused by midazolam, but not by diazepam, on adenosine deaminase activity.

ACh is a neuromotransmitter capable of being released along with ATP in the synaptic cleft and it is involved in essential brain functions, including memory and learning (Burnstock and Verkhratsky, 2009; Pankratov et al., 2009; Shaked et al., 2008). AChE, the key enzyme that hydrolyzes and inactivates ACh, modulates also non-cholinergic functions, such as glutamatergic and dopaminergic systems (Shaked et al., 2008; Soreq and Seidman, 2001; Zimmermann and Soreq, 2006). Studies have shown that some antidepressant and antipsychotic drugs induce changes on the AChE activity. An analysis in rat cerebral cortex has shown that long-term administration of both amitriptyline and chlorpromazine increased the ACh hydrolysis (Bekpinar et al., 1994). Seibt et al. (2009b) also found an increased brain AChE activity in zebrafish exposed in vivo to haloperidol. However, in this same study, both haloperidol, olanzapine, and sulpiride, when administered *in vitro*, showed an opposite effect, inhibiting ACh hydrolysis (Seibt et al., 2009b). Our data showed that AChE activity is differentially altered by the action of both diazepam and midazolam in zebrafish brain. While diazepam was able to inhibit AChE only the highest dose, midazolam at 50-500μM significantly reduced AChE in a dose-dependent manner. Other studies have also demonstrated that diazepam is an inhibitor of AChE activity and ACh synthesis in mouse brain (Appleyard et al.,1990; Barcellos et al., 1998;Nordgren et al.,1992). These data suggest that the benzodiazepine drugs are able to control ACh levels and, consequently, might modulate the cholinergic neurotransmission in zebrafish brain.

Thus, it is possible to suggest that benzodiazepine drugs can induce a direct effect on the NTPDases, ecto-ADA, and AChE activities, since we observed a decreased on nucleotide, nucleoside and ACh hydrolysis after *in vitro* exposure. Therefore, the enzymes related to the control of purinergic and cholinergic signaling can be modulated by anxiolytic and hypnotic drugs in zebrafish. Moreover, our findings may contribute to a better understanding about the action of benzodiazepines on cholinergic and purinergic neurotransmission.

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Figure legends

Fig. 1. *In vitro* effect of diazepam on ATP and ADP hydrolysis at different concentrations (10-500µM) in zebrafish brain membranes. Bars represent the mean ± S.D. The symbol (∗) indicates significantly difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$).

Fig. 2. *In vitro* effect of midazolam on ATP and ADP hydrolysis at different concentrations (10-500µM) in zebrafish brain membranes.Bars represent the mean ± S.D. The symbol (∗) indicates significantly difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0,05$).

Fig. 3. *In vitro* effect of midazolam on ecto-ADA activity at different concentrations (10-500µM) in zebrafish brain membranes. Bars represent the mean ± S.D. The symbol (∗) indicates significantly difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0,05$).

Fig.4. *In vitro* effect of diazepam and midazolam on acetylthiocholine hydrolysis evaluated at different concentrations (10-500µM) in zebrafish brain homogenates. Bars represent the mean ± S.D. The symbol (∗) indicates significantly difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0,05$).

Figure 1

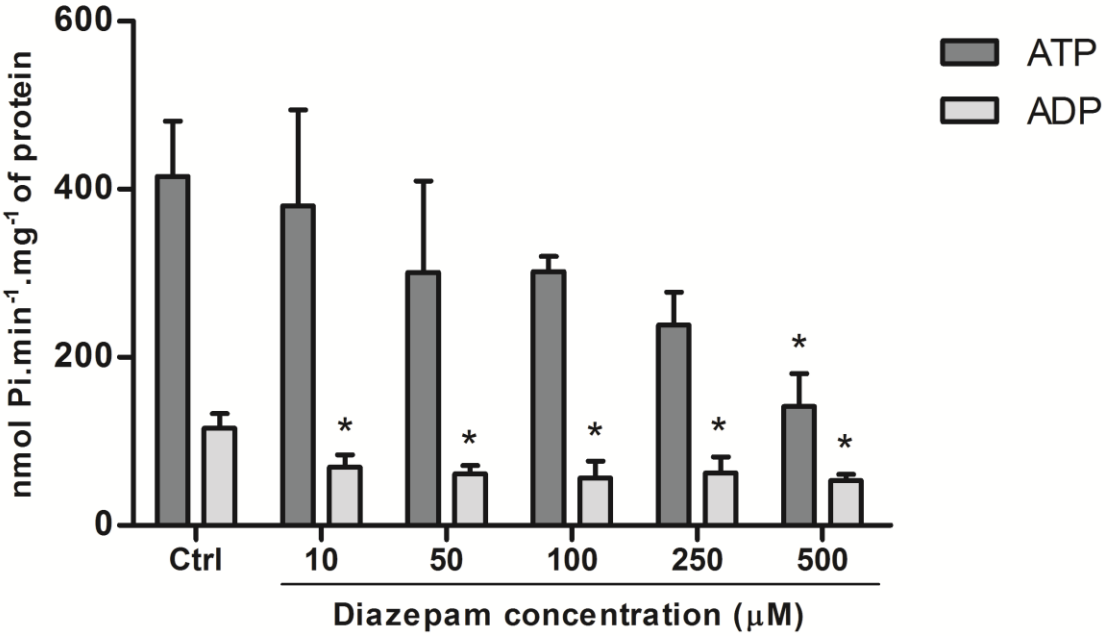


Figure 2

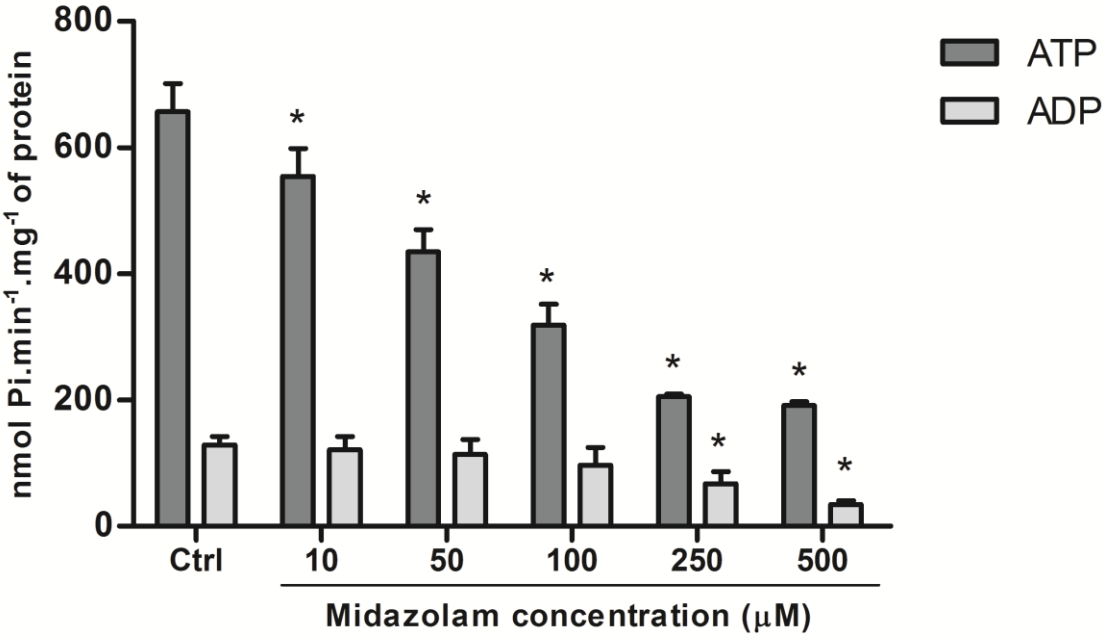


Figure 3

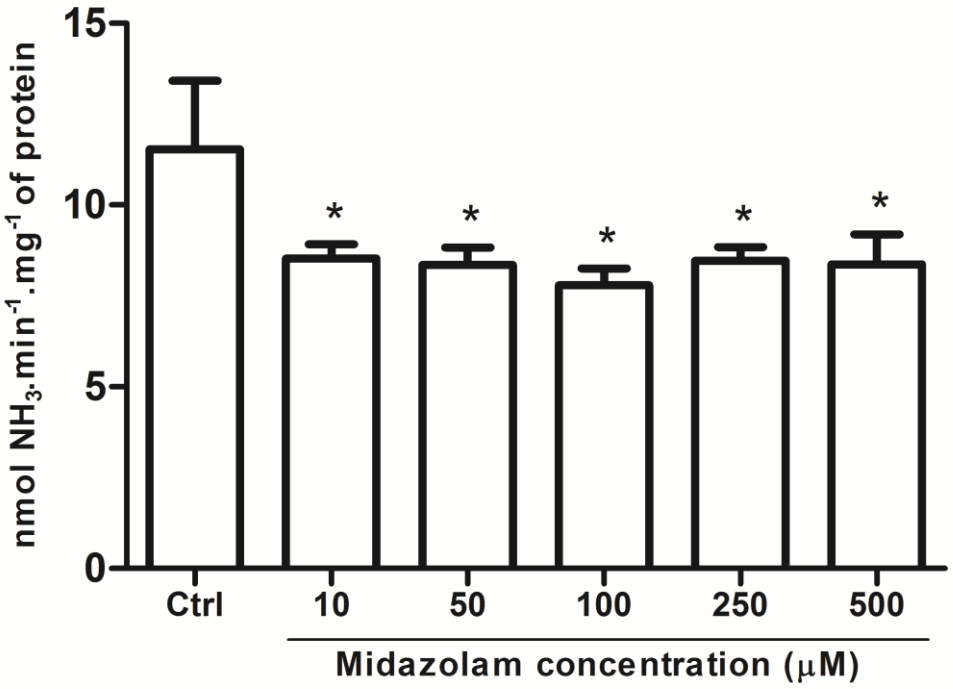
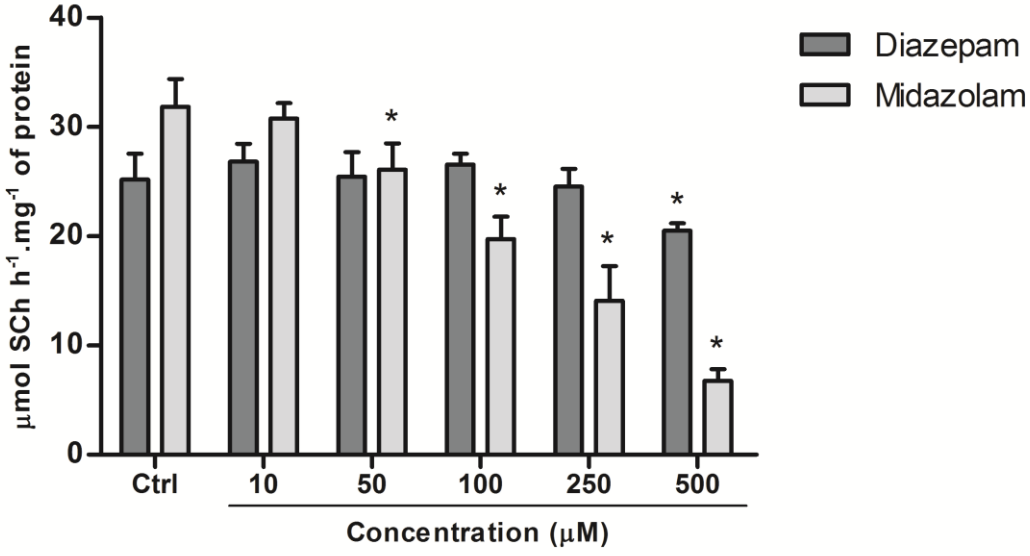


Figure 4



CAPITULO 3

ARTIGO CIENTÍFICO

ALTENHOFEN S, ZIMMERMANN FF, BARRETO LS, BORTOLOTTO JW, KIST LW, BOGO MR, BONAN CD. Benzodiazepines alter adenosine metabolism on zebrafish (*Danio rerio*) brain

Artigo em preparação que será submetido ao periódico

Comparative Biochemistry and Physiology - Part C Toxicology & Pharmacology

Benzodiazepines alter the adenosine metabolism on zebrafish (*Danio rerio*) brain

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Abstract

Anxiety is a physiological and psychological state which is characterized by emotional, cognitive and behavioral components. The classical therapy for anxiety involves the administration of benzodiazepine drugs. Studies have shown that behavioral parameters were altered in zebrafish after benzodiazepine treatments. Purinergic signaling participates in the induction of several behavioral patterns and their actions are inactivated by ectonucleotidases and adenosine deaminase (ADA). Since there is evidence about the involvement of purinergic system in the actions mediated by benzodiazepines, we evaluated the effects of the acute exposure to diazepam or midazolam on nucleoside triphosphate diphosphohydrolases (NTPDases), ecto-5'-nucleotidase, and ADA activities in zebrafish brain, followed by the analysis of gene expression pattern. There were no changes on NTPDase activities after exposure to diazepam and midazolam in zebrafish brain membranes. AMP hydrolysis was decreased in animals treated with of 0.5 and 1 mg/L midazolam (32% and 36%, respectively). Diazepam and midazolam decreased the ecto-ADA activity at 1.25 mg/L and 1 mg/L (31% and 33%, respectively), but only 0.1 mg/L midazolam induced an increase (40%) in cytosolic ADA. The gene expression analysis demonstrated an increase in ecto-5’-nucleotidase mRNA transcript levels after treatment with 0.5 mg/L midazolam. These findings demonstrated a modulation of extracellular adenosine metabolism induced by benzodiazepine exposure.

Keywords: Benzodiazepines, anxiety, ectonucleotidases, adenosine deaminase, zebrafish.

1. Introduction

Anxiety is a biological characteristic which antecedes moments of real or imagined danger, characterized by unpleasant bodily sensations, such as pounding heart, sweating and intense fear. Benzodiazepines, such as diazepam and midazolam, are a widely used class of drugs for anxiety and panic disorders treatment, with anxiolytic, hypnotic, and anticonvulsant properties (Ashton, 1994; Woods and Winger, 1992). This family of psychoactive drugs is formed from the fusion of a benzene ring with a diazepine ring (Anderson, 2010). Despite neuropharmacological similarities, there are differences between benzodiazepines classes. The differences in their affinity to receptor subtypes, in combination with a variety of pharmacokinetic profiles, are responsible for various pharmacological effects, such as sedation, hypnosis, decreased anxiety, anterograde amnesia, muscle relaxation and anticonvulsive activity. Apart from its action on the central nervous system (CNS), they also possess depressant dose-dependent effect, causing a modest reduction in arterial blood pressure and increased heart rate (Anderson, 2010; Colussi et al., 2011; Nelson and Chouinard, 1999; Olkkola and Ahonen, 2008).

The action of benzodiazepines is due to the potentiation of the neural inhibition that is mediated by gamma-aminobutyric acid (GABA). These classical drugs act on a specific transmembrane receptor called $GABA_A$ consisting of nineteen different types of subunits: α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3 (Allison and Pratt, 2003; Wafford, 2005). The binding sites of classical benzodiazepines are commonly found in $GABA_A$ receptors composed of five subunits, two α, β and γ2 two. The binding site of benzodiazepines is located at the interface of α and γ2 subunits (α1, α2, α3, α5) (Harrison, 2007; Rudolph et al. 2001; Sigel and Buhr, 1997; Smith and Olsen, 1995). It is known that the

neurotransmitter GABA can be coreleased with others neurotransmitters, such as ATP, glutamate, noradrenaline, and serotonin (Burnstock, 2004; Holton, 1959; Nakanishi and Takeda, 1973; Zimmermann, 2008). ATP is stored in presynaptic vesicles and is released into the synaptic cleft after depolarization in a calcium-dependent manner acting through activation of G-protein-coupled P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) and ionotropic P2X (P2X1-7) receptors (Burnstock, 1972, 1976, 2012; Burnstock and Kennedy, 1985). As this nucleotide is not able to cross biological membranes by diffusion or active transport, control of its extracellular concentration is accomplished by the action of ectonucleotidases that catalyze its conversion to adenosine (Bonan et al., 2000; Goding and Howard, 1998; Robson et al., 2006). The hydrolysis of ATP to AMP is catalyzed mainly by a family of ectonucleotidases, named nucleoside triphosphate diphosphohydrolases (NTPDases) and the nucleotide AMP is hydrolyzed to adenosine by the action of an ecto-5´-nucleotidase (CD73, EC 3.1.3.5) (Schetinger et al., 2007; Zimmermann,1992, 1996, 2011).

Adenosine is involved in nucleic acid synthesis, amino acid metabolism, and modulation of metabolic state of the cell and is classified as a neuromodulator, since it is not stored in vesicles or released by exocytosis as a classical neurotransmitter. It exerts its effects through the activation of specific G-protein-coupled P1 purinoceptors (A_1, A_{2A}, A_{2B} and A_3) (Fredholm and Dunwiddie, 1988; Libert et al., 1989; Shen and Chen, 2009; von Lubitz, 1999). The adenosine that remains in the synaptic cleft can be reuptake through bi-directional transporters or be deaminated by the action of adenosine deaminase. Adenosine deaminase (ADA) (EC 3.5.4.4) promotes the hydrolytic deamination of ADO and deoxyadenosine to inosine and deoxyinosine, respectively. It is

found as a cytosolic enzyme and can also be expressed on the cell surface as an ectoenzyme, It has an important role in the immune system in inflammatory processes, controlling the adenosine levels (Franco et al., 1997; Hirschhorn and Ratech, 1980; Ratech et al., 1981; Zavialov and Engström, 2005).

The zebrafish is a small teleost (3-4 cm) of fresh water that has been considered an ideal model for studies of vertebrate development (Bai and Burton, 2011; Málaga-Trillo et al., 2011), human diseases (Ackermann and Paw, 2003; Best and Alderton, 2008; Sloman et al., 2003), and for the screening and drug discovery (Chakraborty et al., 2009; Goldsmith, 2004; Kari et al., 2007; Malaga-Trillo et al., 2011; Rubinstein, 2006). This species have a fast metabolism and a high sensitivity to drugs (Karlovich et al., 1998; Goldsmith, 2004). Zebrafish absorbs components directly from the water through their gills and accumulates them in different tissues, especially in central nervous system (CNS) (Grosell and Wood, 2002), showing thus be a useful model for research in behavioral neuroscience (Bencan et al., 2009; Gerlai et al., 2009; Ng et al. 2012). Studies demonstrated the presence of NTPDases, ecto-5'-nucleotidase, and ADA activities in zebrafish brain (Rico et al., 2003; Rosemberg et al., 2008; Senger et al., 2004). Several studies have identified benzodiazepine receptors in fish with binding characteristics similar to rodents and humans (Anzelius et al., 1995; Carr et al., 1999; Friedl et al., 1988; Wilkinson et al., 1983).

The behavior of zebrafish in the presence of different anxiolytic drugs belonging to the benzodiazepine family is already well documented, including the role of these drugs in addiction and withdrawal (Bencan et al., 2009; Cachat et al., 2010; Gebauer et al., 2011; Mathur and Guo, 2010). Therefore, it becomes relevant to investigate the effects on anxiolytic drugs on neurotransmitter systems in this species, such as the

purinergic system, since adenosine has an important role as neuromodulator. Thus, the aim of this study was to evaluate the ex vivo effects of different concentrations of diazepam and midazolam on NTPDases, ecto-5'-nucleotidase and ADA activities in zebrafish brain followed by a gene expression pattern analysis.

2. Materials and methods

2.1 Animals

Adult wild-type zebrafish (*Danio rerio*) of both sexes were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for 2 weeks before the experiments in a 50 L thermostated aquarium filled with continuously aerated and unchlorinated water. The fish were conditioned at 26 ± 2° C under a 14-10 h light/dark cycle photoperiod. The animals were maintained healthy and free of any signs of disease and fed twice a day with commercial food for fish. The use and maintenance of zebrafish were according to the ‘‘Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. The protocol was approved by the Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the number 11/00256.

2.2 Chemicals

Midazolam (Dormonid®) and diazepam (União Química, Brazil) were purchased from common commercial suppliers. ATP, ADP AMP, adenosine, Trizma Base, EDTA, EGTA, sodium citrate, Coomassie blue, bovine serum albumin, malachite green,

ammonium molybdate, polyvinyl alcohol, calcium, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were from analytical grade.

2.3 Treatments

For treatment, three fish were kept in a 600 mL beaker and exposed to water with diazepam (0.2, 1.25, 5 mg/L) or midazolam (0.1, 0.5, 1 mg/L) during 10 minutes and, immediately after the exposure, the fish were euthanized. The drug solutions were changed for each experiment. For the control group, the animals were exposed only to water in a 600mL beaker during 10 minutes and, after this time period, the fish were euthanized. The diazepam dose and time of *ex vivo* treatment were chosen based on previous studies with zebrafish (Bencan et al., 2009). The concentrations of midazolam used in this study were chosen based on drug potencies observed in rat study (Koch et al., 2008).

2.4 Preparation of soluble and membrane fractions

Brain samples were obtained as described previously (Rico et al., 2003; Senger et al., 2004; Rosemberg et al., 2008). Each independent experiment was performed using biological preparations consisted of a “pool” of four and six brains for ectonucleotidases and adenosine deaminase, respectively. First, zebrafish were cryoanaesthetized, euthanized, and brains were removed by dissection (Wilson et al., 2009). Samples were then further homogenized in a glass-Teflon homogenizer according to the protocol for each enzyme assay. For NTPDase and ecto-5’-nucleotidase assays zebrafish brains were homogenized in 60 vol. (v/w) of chilled Tris-

citrate buffer (50 mM Tris-citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4). For ADA experiments, brains were homogenized in 20 vol (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4. The brain membranes were prepared as described previously (Barnes et al., 1993). In brief, the homogenates were centrifuged at 800 g for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at 40000 g. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. For soluble ADA activity experiments, the supernatant was collected and kept on ice for enzyme assays. The pellets of membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers and centrifuged for 20 min at 40000 g. This freeze-thaw-wash procedure was used to ensure the lysis of the brain vesicles membranes. The final pellets were resuspended and used for enzyme assays. All samples were maintained at 2-4° C throughout preparation.

2.5 Ectonucleotidase assays

NTPDase and ecto-5'-nucleotidase assays were performed as described previously (Rico et al., 2003; Senger et al., 2004). Brain membranes of zebrafish (3 µg protein for NTPDase and 5 µg protein for ecto-5'-nucleotidase) were added to the reaction medium containing 50 mM Tris-HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris-HCl (pH 7.2) and 5 mM $MgCl_2$ (for the 5'-nucleotidase activity) at a total volume of 200 µl. The samples were preincubated for 10 min at 37° C and the reaction was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. After 30 minutes the reaction was stopped by the addition of 200 µl 10% trichloroacetic acid and the samples were kept on ice during 10 min. In order to

determine the inorganic phosphate released (Pi) 1 ml of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added to the samples for 20 min (Chan et al., 1986). The quantification of inorganic phosphate (Pi) released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nanomole of inorganic phosphate released per minute per milligram of protein. In order to correct non-enzymatic hydrolysis of the substrates we used controls with the addition of the enzyme preparation after the addition of trichloroacetic acid. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. All enzyme assays were performed in at least six different experiments, each one performed in triplicate.

2.6 Adenosine deaminase assays

Ecto- and cytosolic-ADA activities were determined as described previously (Rosemberg et al., 2008). The brain fractions (5-10 µg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for soluble and membrane fractions, respectively, in a final volume of 200 µL. The samples were preincubated for 10 min at 37° C and the reaction was initiated by the addition of substrate (adenosine) to a final concentration of 1.5 mM. The reaction was stopped after 75 min (soluble fraction) and 120 min (membrane fraction) by the addition of 500 µL phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL). ADA activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported (Weisman et al., 1988). In order to correct non-enzymatic hydrolysis of the substrates controls with the addition of the enzyme

preparation after mixing with phenol-nitroprusside reagent were used. The reaction mixtures were immediately mixed to 500 µL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed. Samples were incubated at 37° C for 15 min and the colorimetric assay was carried out at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. The ADA activity was expressed as nanomole of ammonia released per minute per milligram of protein. All enzyme assays were performed in five independent experiments carried out in triplicate.

2.7 Protein determination

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

2.8 Gene expression analysis by quantitative real time RT-PCR (RTqPCR)

Gene expression analysis was carried out only when kinetic alteration occurred. Total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, California, USA) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 µg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 µL using 12.5 µL of diluted cDNA (1:50 for *EF1α*, *Rlp13α*, *ada1*, *ada2.1*, *adaL*, *ADAasi*, *CD73* and 1:20 *ada2.2*), containing a final concentration of 0.2 x SYBR® Green I (Invitrogen), 100 µM dNTP, 1 x PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase

(Invitrogen) and 200 nM of each reverse and forward primers (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 minutes at 95 °C, 40 cycles of 15 seconds at 95 °C for denaturation, 35 seconds at 60 °C for annealing and 15 seconds at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl) and the stability of the references genes, *EF1α* and *Rlp13α* (*M-value*) and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

2.9 Statistical analysis

Results from enzyme assays are expressed as means ± S.D and the data were analyzed by one-way ANOVA followed by Tukey's post hoc test, considering $P < 0.05$ as significant. Molecular data were expressed as means ± S.E.M. and analyzed by Student's t-test for unpaired samples or by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test considering $P < 0.05$ as statistical significance.

3. Results

The effect of acute benzodiazepine treatment was tested on NTPDases, ecto-5'-nucleotidase, and ADA activities in zebrafish brain. The experiments were performed

after a 10 min-exposure to different concentrations of diazepam (0.2, 1.25, 5 mg/L) or midazolam (0.1, 0.5, 1 mg/L).

Diazepam and midazolam did not promote any significant difference on NTPDase activities (Table 2). In the same way, diazepam also did not alter the ecto-5'-nucleotidase activity, but midazolam was able to modulate the ecto-5'-nucleotidase activity, promoting a reduction in AMP hydrolysis in animals treated with 0.5 and 1mg/L (32% and 36%, respectively) when compared to the control group (Fig. 1).

In relation to ADA activity, both diazepam and midazolam significantly decreased the ecto-ADA activity. Diazepam reduced adenosine deamination at 1.25 mg/L (31%; Fig. 2a) whereas midazolam induced such effect at 1 mg/L (33%; Fig. 3a). Diazepam did not alter cytosolic-ADA activity (Fig. 2b). However, the exposure to 0.1 mg/L midazolam induced a significant increase in cytosolic ADA (40%; Fig. 3b) when compared with the control group.

The changes in enzyme activity promoted by diazepam or midazolam exposure could be a consequence of transcriptional control. In order to determine if transcriptional regulation has occurred, a RT-qPCR analysis was carried out for the treatments that induced changes in enzyme assay. The results have demonstrated that the relative amount of ecto-5'-nucleotidase (*CD73*) mRNA transcripts significantly decreased (41.7%) after treatment with 0.5 mg/L midazolam (Fig. 4). Moreover, the results did not show significant effects of the diazepam treatments at 1.25 mg/L on *ada1* ($p = 0.353$), *ada2.1* ($p = 0.584$), *ada2.2* ($p = 0.400$), *adaL* ($p = 0.238$) and *ADAasi* ($p = 0.285$). Similarly, there were no significant effects induced by 0.1 and 1 mg/L midazolam treatments on *ada1* ($p = 0.282$ and $p = 0.195$, respectively), *ada2.1* ($p = 0.931$ and $p = 0.930$, respectively), *ada2.2* ($p = 0.852$ and $p = 0.328$, respectively), *adaL* ($p = 0.916$

and p = 0.879, respectively) and *ADAasi* (p = 0.721 and p = 0.574, respectively) gene expression in zebrafish brain (data not shown).

4. Discussion

Our results demonstrated that the acute treatment with benzodiazepine drugs alters the enzyme activities involved in the control of adenosine levels. Both diazepam and midazolam were unable to alter NTPDase activity. Likewise, diazepam did not influence the AMP hydrolysis. However, midazolam promoted a reduction in ecto-5'-nucleotidase activity. Midazolam was able to increase the cytosolic ADA activity, since diazepam showed no effect on this specific enzyme. Both diazepam and midazolam changed the adenosine hydrolysis, showing that both drugs can modulate the effects of ecto-ADA. The results reinforce the idea that benzodiazepines are able to modulate the purinergic signaling.

The effects of benzodiazepine drugs, such as sedation, anxiety reduction, muscle relaxation, and anticonvulsive activity are due to the binding on $GABA_A$ receptor (Anderson, 2010; Bateson, 2004; Saunders and Ho, 1990). The actions of benzodiazepines at $GABA_A$ receptor induce an increase in the frequency of channel opening of chloride (Cl^-), promoting an influx of ions into the neuron with consequent cell hyperpolarization. Such effects cause an increased inhibitory current and strong inhibitory-postsynaptic potential, expressing the effect of this inhibitory neurotransmitter (Anderson, 2010; DeMicco et al., 2010; Olkkola and Ahonen, 2008; Rifkin, 1990). Considering the effect of benzodiazepines on GABAergic system and the possible involvement of other neurotransmitters and neuromodulators coreleased with GABA, such as ATP and adenosine, on the anxiety, fear, and stress, the control of purine

messengers may represent an important mechanism related to the benzodiazepine effects (El Yacoubi et al., 2000; Carús-Cadavieco e de Andrés, 2012; Porkka-Heiskanen, 1999; Ribeiro et al., 2003).

ATP is the signaling molecule of purinergic system, which is stored in vesicles and released in the synaptic cleft after neuronal depolarization, acting on P2X and P2Y specific receptors (Burnstock, 1972, 1976; Burnstock and Kennedy, 1985). It is inactivated by enzymes localized on the cell surface and named ectonucleotidases. Ectonucleotidases, such as NTPDase and ecto-5'-nucleotidase, are anchored in the cell membrane and hydrolyses ATP to adenosine (Goding e Howard, 1998; Schetinger et al., 2007; Zimmermann, 2008, 2011). The NTPDases hydrolyse ATP and ADP, to produce AMP in the presence of Ca^{2+} and Mg^{2+} whereas ecto-5'-nucleotidase hydrolyses AMP to adenosine (Robson et al., 2006; Schetinger et al., 2007; Zimmermann, 1992, 1996, 2011). Studies have shown that diazepam is capable to inhibit the NTPDases activity in rat brain, modulating the purinergic neurotransmission (Barcellos et al., 1998; Horvat et al., 2006). However, our findings showed that both diazepam and midazolam are not able to change the NTPDase activity. Likewise, diazepam was not able to alter the ecto-5'-nucleotidase activity. However, midazolam inhibited the enzyme activity at concentrations of 0.5 and 1 mg/L. Korotkina et al. (1985) evaluated the effect of phenazepam (2.5, 3.75, 5 mg/200 g body weight) diazepam (2, 3, 4 mg/200 g body weight) and midazolam (2, 3, 4 mg/200 g body weight) in brain homogenates from male albino rats. The results showed that lower doses of phenazepam and diazepam, i.e. 2.5 and 2 mg/200 g body weight, respectively, caused no significant changes in enzyme activity. However, lower doses of midazolam (2 mg/200 g body weight) have been able to significantly decrease the ecto-5'-nucleotidase

activity. The subsequent treatment with higher doses of the same drugs showed that all benzodiazepines caused a decrease in the levels of AMP hydrolysis. Our results reinforce the idea that benzodiazepine drugs modulate extracellular catabolism of AMP and the production of adenosine due to inhibition of ecto-5´-nucleotidase induced by midazolam. Such effects could induce a decrease in adenosine levels, which could be related to the neuromodulatory effect induced by this nucleoside.

Adenosine exerts its effects through the activation of P1 purinoreceptores (Fredholm and Dunwiddie, 1988; Libert et al., 1989; Shen and Chen, 2009; von Lubitz, 1999). Hawkins et al. (1988) evaluated, by radioligand, the effects of subcutaneous administration of chronic diazepam (5 mg/kg/day) for 10 and 20 days on adenosine receptors in different brain areas. The results showed that the treatment performed for 10 days were able to reduce binding of adenosine A_2 and A_1 receptors in the striatum and hippocampus, respectively, whereas the 20 day-treatment had no effect. These results showed that adenosine receptors play an important role in support the processes related to benzodiazepines. Adenosine, which remains in the synaptic cleft, can be recaptured by bidirectional transporter or deaminated to inosine by the action of ADA (Franco et al., 1997; Fredholm et al., 2005; Hirschhorn and Ratech, 1980). This enzyme can be found in cytosol, or expressed on the cell surface as an ectoenzyme (Franco et al., 1997; Rosemberg et al., 2008). Korotkina et al. (1986) showed that intraperitoneal administration of benzodiazepine drugs, such as diazepam and phenazepam, was able to increase the ADA activity in rats. These data are consistent with our study where animals treated with 0.1 mg/L of midazolam showed an increase in cytosolic ADA activity. In contrast, our study has shown that both diazepam (1.25 mg/L) and midazolam (1 mg/L) were able to alter differentially the ecto-ADA activity, decreasing the

adenosine hydrolysis after acute treatment. Considering that midazolam inhibit ecto-5´-nucleotidase, it is possible to suggest that a decrease in the ecto-ADA could represent a compensatory mechanism in order to maintain the basal adenosine levels.

Despite the neuropharmacological similarities, the differential effects induced by diazepam and midazolam on the maintenance the adenosine levels can be explained by differences between the drugs in the benzodiazepine family. The differences in their affinity to receptor subtypes, in combination with a wide variety of pharmacokinetic profiles, are responsible for various pharmacological effects (Anderson, 2010; Nelson and Chouinard, 1999). This unique pharmacokinetic profile has a major impact on the choice of a specific benzodiazepine for particular condition, especially in relation to the administration route, rate and absorption. These pharmacokinetic differences often establish specific formulations for individual family members of benzodiazepines (Anderson, 2010).

The changes promoted by midazolam in the ecto-5'-nucleotiadase activity and both diazepam and midazolam on the ADA activity could be a consequence of transcriptional control. To verify that the genes of these enzymes could be modulated after exposure to diazepam and midazolam, we performed RT-qPCR experiments for the treatments that induced changes in enzyme activity. The results showed that changes in ADA mRNA levels were not directly related with the action of benzodiazepine drugs, since there was no change at the transcriptional level. Therefore these findings suggest an involvement of post-transcriptional or post-translational mechanisms for the modulation of these enzyme activities. However, the results showed that the ecto-5'-nucleotidase mRNA levels were significantly decreased after treatment with midazolam

(0.5 mg/L), suggesting that the reduction in ecto-5'-nucleotidase activity observed in this treatment can be directly related to the low *CD73* expression.

In summary, the findings of this study indicated that midazolam treatment leads to changes in ecto-5'-nucleotidase activity and *CD73* expression. Likewise, both diazepam and midazolam can induce a direct effect on the ADA activities, but did not alter the *ada1*, *ada2.1*, *ada2.2, adaL* and *ADAasi* gene expression Thus, our findings may contribute to a better understanding about the role of purinergic signaling on the actions induced by acute treatment with benzodiazepines.

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Figure legends

Fig. 1. Effect of midazolam on AMP hydrolysis in zebrafish brain membranes. Bars represent the mean ± S.D. The symbol (*) indicates significantly difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$).

Fig. 2. Effect of diazepam on ecto-ADA (a) and cytosolic-ADA (b) activities in zebrafish brain membranes. Bars represent the mean ± S.D. The symbol (*) indicates significantly difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0,05$).

Fig. 3. Effect of midazolam on ecto-ADA (a) and cytosolic-ADA (b) activities in zebrafish brain membranes. Bars represent the mean ± S.D. The symbol (*) indicates significantly difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0,05$).

Fig. 4. Effect of Midazolan on CD73 relative gene expression in zebrafish brain. Data represent mean ± SEM of four independent experiments performed in quadruplicate. The symbol (*) indicates difference when compared to the control group. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc, considering a $p \leq 0.05$ significant.

Table 1
PCR primers design

Enzymes	Primer sequences (5'-3')	GenBank accession number (mRNA)
*EF1α**	F – CTGGAGGCCAGCTCAAACAT R – ATCAAGAAGAGTAGTACCGCTAGCATTAC	NSDART00000023156
*Rpl13α**	F – TCTGGAGGACTGTAAGAGGTATGC R – AGACGCACAATCTTGAGAGCAG	NM_212784
*CD73***	F-TGGACGGAGGAGACGGATTCACC R-GGAGCTGCTGAACTGGAAGCGTC	BC055243.1
*ADA1***	F-GCACAGTGAATGAGCCGGCCAC R-AATGAGGACTGTATCTGGCTTCAACG	BC076532.1
*ADA2.1***	F-TTCAACACCACACGTATCGGGCAC R-ATCAGCACTGCAGCCGGATGATC	AF384217.1
*ADA2.2***	F-TTGCAATTGTTCATCATCCCGTAGC R-TCCCGAATAAACTGGGATCATCG	XM_682627.1
*ADAasi***	F-CTTTGTGGTACTTCAAGGACGCTTTG R-TTGTAGCAGATAAAAGAAGCGAGACG	AF384217.1
*ADAL***	F-CTCTAATGTGAAAGGTCAAACCGTGC R-AAGACGCCCTTATCATCCGTGC	NM_001033744.1

* According to Tang et al. (2007).
** Designed by authors.

Table 2
Effects of diazepam and midazolam treatments on NTPDases activities from adult zebrafish brain membranes.

Group	*n*	ATP hydrolysis (nmol Pi. $min^{-1}.mg^{-1}$ protein)	ADP hydrolysis (nmol Pi. $min^{-1}.mg^{-1}$ protein)
Diazepam			
Control	6	325.2 ± 32.2	83.4 ± 10.9
0.2 mg/L	6	316.7 ± 24.5	79.6 ± 4.2
1.25 mg/L	6	312.6 ± 37.3	77.3 ± 8.7
5 mg/L	6	296.5 ± 43.3	76.5 ± 7.1
Midazolam			
Control	6	419.3 ± 52.6	89.2 ± 15.3
0.1 mg/L	6	386.8 ± 22.5	80.7 ± 18.3
0.5 mg/L	6	370 ± 31.1	73.7 ± 16.4
1 mg/L	6	367 ± 16.2	73.8 ± 9

Data are expressed as means ± S.D.

Figure 1

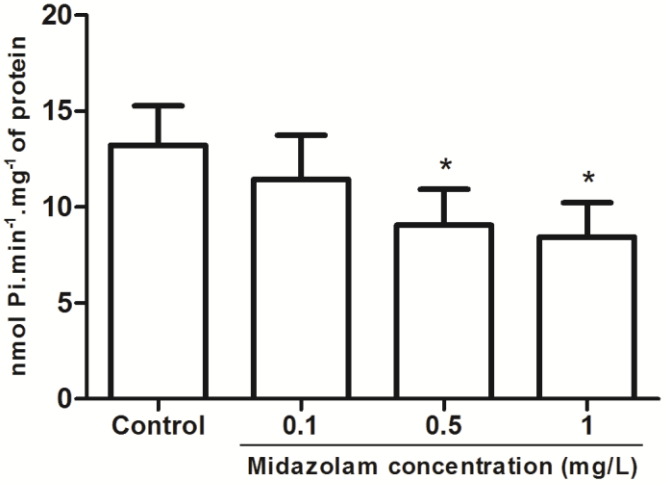


Figure 2

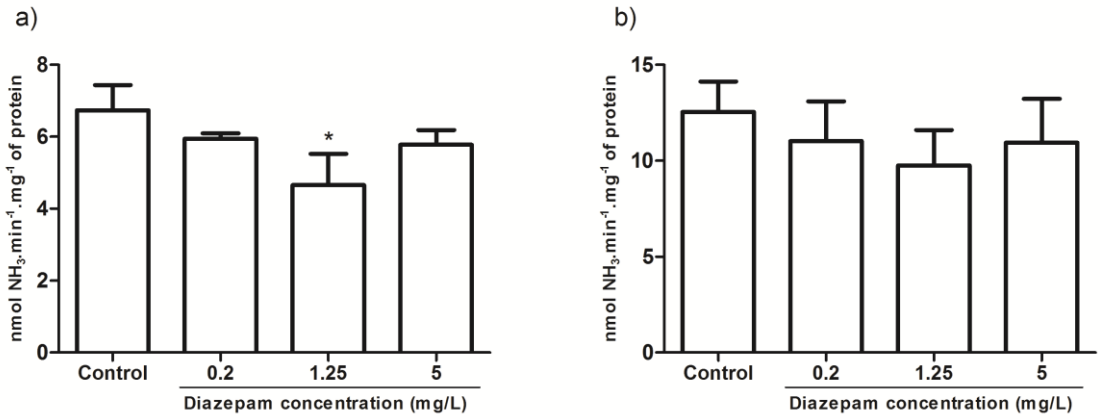


Figure 3

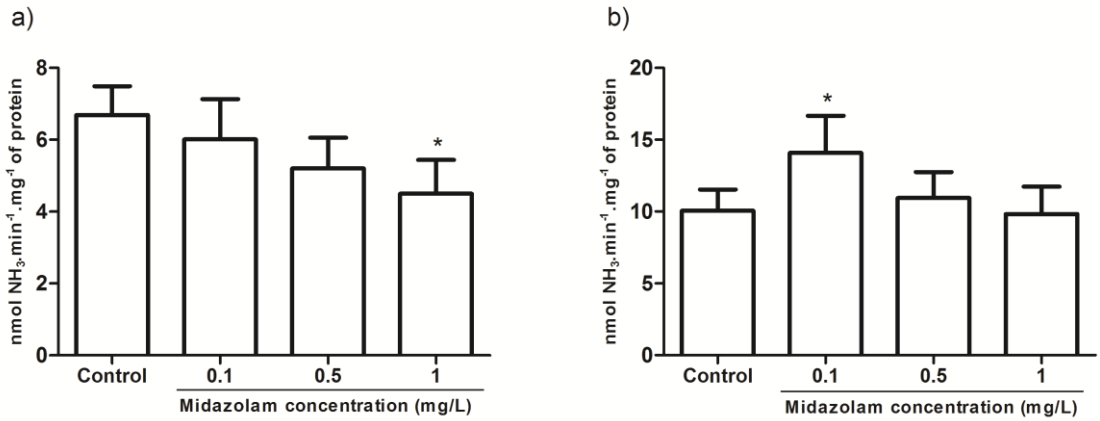
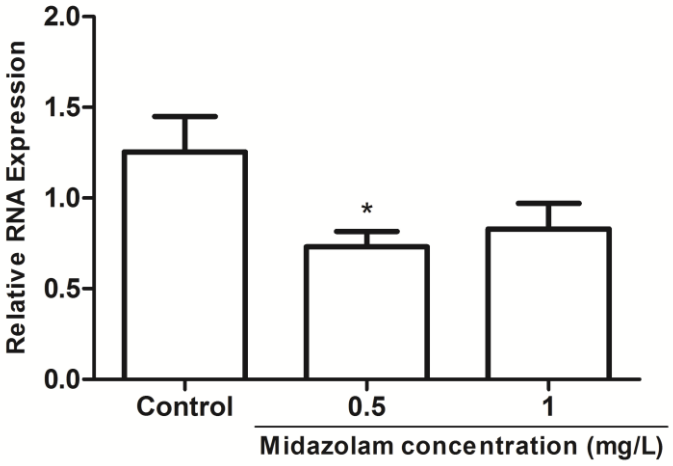


Figure 4



CAPÍTULO 4

RESULTADOS PRELIMINARES E PERSPECTIVAS

Além dos estudos realizados nos capítulos 2 e 3, a análise da atividade da acetilcolinesterase na presença de fármacos benzodiazepínicos também foi realizada. Uma vez que o estudo *in vitro* foi realizado (capítulo 2), a análise *ex vivo* tornou-se importante para um maior entendimento da ação de fármacos benzodiazepínicos, tais como diazepam e midazolam, na hidrólise da acetilcolina.

Portanto, para este conjunto de experimentos, foi utilizado o mesmo tratamento descrito no capítulo 3. Neste tratamento, três zebrafish adultos foram colocados em béquers de 600 mL e expostos ao diazepam (0.2, 1.25, 5 mg/L) ou midazolam (0.1, 0.5, 1 mg/L) durante 10 minutos. Imediatamente após a exposição, os animais foram eutanasiados e seus encéfalos foram dissecados para posterior determinação da atividade da acetilcolinesterase. Os grupos controles foram realizados nas mesmas condições de tratamento, porém na ausência de fármacos. Foram realizados 6 diferentes experimentos (n=6), sendo cada n composto por um pool de 2 encéfalos. Todos os experimentos foram realizados em triplicata.

Os encéfalos obtidos por dissecação foram homogeneizados e a determinação da proteína foi realizada pelo método do Coomassie blue (Bradford, 1976), onde albumina de soro bovino foi utilizada como padrão. A análise da atividade da acetilcolinesterase foi realizada de acordo com o método de Ellman et al. (1961) com algumas modificações. A atividade no homogenato, utilizando uma concentração final de proteína de 5 µg, foi medida por determinação da taxa de hidrólise de iodeto de acetiltiocolina (0,88 mM) onde foi observada a formação do diânion do DTNB em 412 nm durante 3 min (em intervalos de 30 s) utilizando um leitor de microplacas. A atividade da AChE foi expressa como micromole de Tiocolina (SCH) liberado por hora por miligrama de proteína.

Os resultados encontrados mostraram que o diazepam diminuiu significativamente a hidrólise de acetilcolina na concentração de 1,25 mg/L (30,7%) quando comparado ao grupo controle. Similarmente, a exposição à concentração de 0.5 mg/L de midazolam também alterou a atividade da acetilcolinesterase, promovendo um aumento na hidrólise da acetilcolina (36,7%). Esses dados são demonstrados na Figura 1.

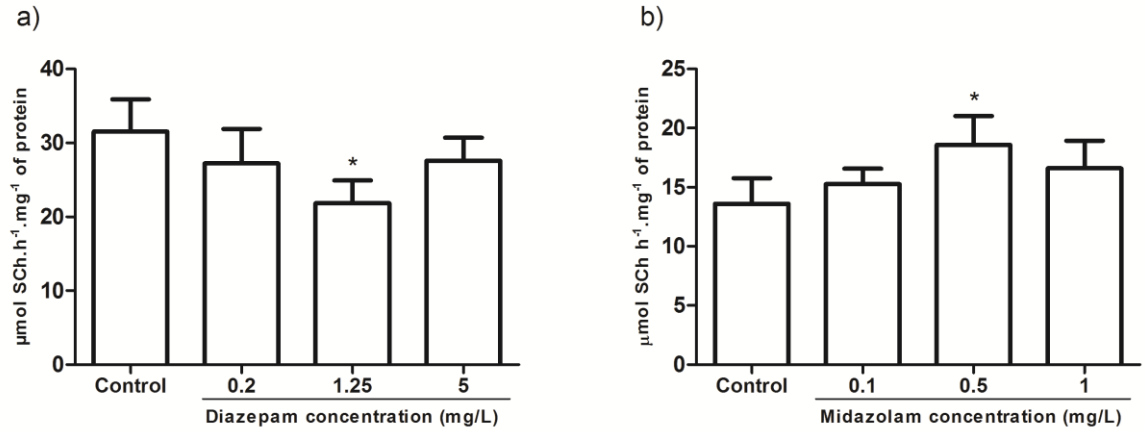


Figura 1: Efeito do diazepam (a) e midazolam (b) na hidrólise da acetilcolinesterase de homogenatos encefálicos de zebrafish. As barras expressam a média ± desvio padrão. Os símbolos (*) indicam diferença significativa em comparação ao grupo controle (one-way ANOVA, seguido pelo teste de Tukey, $P \leq 0,05$). n = 6

A fim de elucidar se as modificações na atividade enzimática da AChE promovidas pela exposição ao diazepam e midazolam poderiam ser consequência do controle transcricional e/ou regulação pós-traducional, foi realizada análise de expressão gênica pelo método quantitativo de real time RT-PCR. As análises foram realizadas para os tratamentos capazes de alterar a atividade desta enzima, utilizando primers específicos para o gene *ache* e constitutivos EF1α e Rpl13α para validar a reação, conforme demonstrado na Tabela 1.

Tabela 1: Primers

Genes	Sequencia dos Primers (5'-3')	Número de acesso GenBank (RNAm)
*EF1α**	F – CTGGAGGCCAGCTCAAACAT R – ATCAAGAAGAGTAGTACCGCTAGCATTAC	NSDART00000023156
*Rpl13α**	F – TCTGGAGGACTGTAAGAGGTATGC R – AGACGCACAATCTTGAGAGCAG	NM_212784
*ache***	F – GCTAATGAGCAAAAGCATGTGGGCTTG R - TATCTGTGATGTTAAGCAGACGAGGCAGG	NP_571921

* De acordo com Tang et al. (2007).
** Desenhados pelos autores.

Os resultados demonstraram que os níveis de transcrição do gene *ache* nas concentrações de 1,25 mg de diazepam/L e 0,5 mg de midazolam/L não mostraram-se alterados quando comparados ao grupo controle (Figura 2), sugerindo que a regulação da acetilcolinesterase pelo diazepam e midazolam no encéfalo de zebrafish não está diretamente relacionada com o controle transcricional.

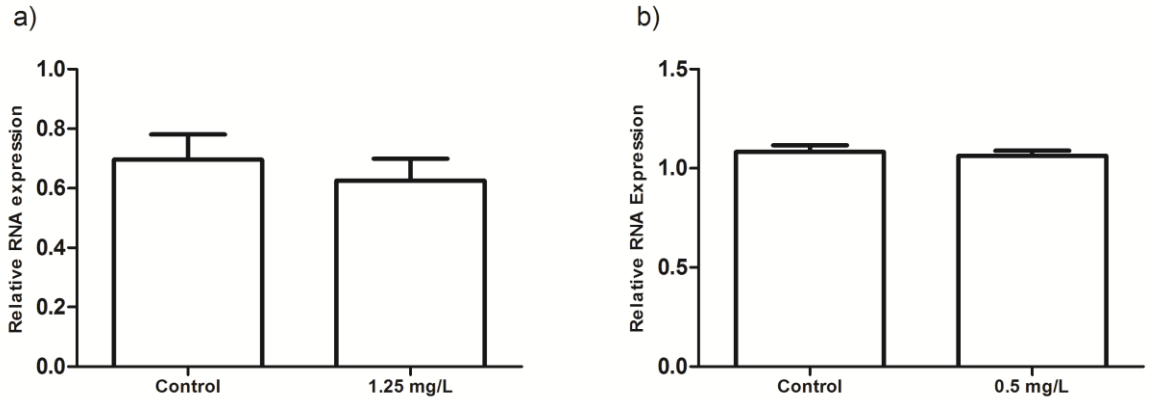


Figura 2: Efeito do diazepam (a) e midazolam (b) na expressão gênica relativa da *ache* de encéfalo de zebrafish. Os dados são expressos como média ± erro padrão e analisados estatisticamente por test-t de amostras não pareadas (P = 0.543 e P = 0.653). n = 4

A principal função da acetilcolinesterase é a hidrólise e subsequente inativação da acetilcolina no sistema nervoso central e periférico (Bajgar, 2010; Layer et al., 1988).

O acúmulo de acetilcolina nas junções sinápticas dessensibiliza e hiperestimula os receptores colinérgicos causando uma crise colinérgica aguda, caracterizada por convulsões, alteração na ventilação e desbalance metabólico (Bajgar, 2004; Barthold e Schier 2005; Jokanovic, 2009).

Compostos químicos, entre eles os organofosforados, são responsáveis por desencadear esse efeito inibitório sobre a acetilcolinesterase, agindo através da fosforilação do grupo hidroxila da serina localizado no sítio ativo dessa enzima (Jokanovic, 2009). Os organofosforados têm sido utilizados desde 1940 para auxiliar no controle de insetos e pragas de lavoura. Eles também são empregados na indústria, medicina humana ou veterinária, além de serem usados como agentes químicos para fins militares, representando uma ameaça persistente para a população em geral como consequência de conflitos armados e ataques terroristas (Bajgar, 2004; Bajgar et al., 2007; Casida e Durkin, 2012; Casida e Quistad, 1998; Eyer, 2003; Mercey et al., 2012). Segundo a Organização Mundial de Saúde, mais de um milhão de acidentes graves e dois milhões de intoxicações suicidas por organofosforados ocorrem no mundo a cada ano e, destes, cerca de 200 mil vem a óbito, com maior ocorrência nos países em desenvolvimento. O envenenamento não intencional possui consequências bem menos graves, mas ainda está relacionado a um grande problema em locais onde esses agentes químicos estão disponíveis (Bajgar, 2004; Bajgar et al., 2007; Eddleston et al., 2008; Eyer, 2003).

Estudos que envolvem o mecanismo de ação, profilaxia e tratamento de intoxicações por organofosforados vêm ganhando muito espaço na comunidade científica (Bajgar, 2010). Sabe-se que o tratamento mais comum para o envenenamento por esses agentes químicos inclui a atropina, um antagonista

colinérgico muscarínico, e as oximas, reativadores da AChE. Atropina bloqueia os sintomas da exposição e as oximas reativam a AChE inibida, restaurando a função normal da enzima (Bajgar, 2004; Eddleston et al., 2005; Tuovinen, 2004). Além disso, diazepam tem sido utilizado como método para proteger contra o envenenamento por organofosforados (Tuovinen, 2004). No sistema nervoso, os benzodiazepínicos parecem ser capazes de diminuir a liberação de ACh sináptica. A principal consequência da ação desses fármacos no SNC é hiperpolarização de neurônios, o que os torna menos suscetíveis à despolarização colinérgica, resultando na cessação da propagação de convulsões (Antonijevic e Stojiljkovic, 2007; Jokanovic, 2009). Em pacientes envenenados com organofosforados, os benzodiazepínicos têm um efeito benéfico na redução da ansiedade e agitação. Quando em uso conjunto com a atropina e uma oxima, eles têm papel significativo na redução da morbidade e mortalidade. O diazepam deve ser dado a pacientes intoxicados com organofosforados sempre que apresentarem convulsões ou contração muscular acentuada (Antonijevic e Stojiljkovic, 2007; Jokanovic, 2009).

Uma vez que os fármacos benzodiazepínicos testados neste estudo alteram a atividade da acetilcoliesterase no encéfalo de zebrafish, este estudo tem como perspectiva investigar o papel do diazepam e midazolam em um quadro de intoxicação por organofosforados, e se estes fármacos benzodiazepínicos seriam capazes de reverter os sintomas causados por esses agentes tóxicos.

CAPÍTULO 5

CONSIDERAÇÕES FINAIS

A ansiedade é um estado psicológico e fisiológico que se caracteriza pela soma de componentes emocionais, cognitivos e comportamentais. Ela é considerada uma reação normal ao estresse, que pode ajudar a superar uma situação difícil na vida. Tanto na presença como em ausência de estresse psicológico, a ansiedade pode criar sentimentos de medo, preocupação, entre outros. Quando a ansiedade se torna excessiva, pode ser classificada como transtorno de ansiedade (Kessler et al., 2005).

Os transtornos de ansiedade afetam cerca de 40 milhões de americanos adultos com idade superior a 18 anos. Ao contrário da ansiedade suave, causada por um leve evento estressante (como falar em público), transtornos de ansiedade duram pelo menos seis meses e podem piorar se não forem tratados. Eles ocorrem geralmente em conjunto com outras doenças, como o abuso do álcool e drogas, podendo esses mascarar os sintomas de ansiedade ou torná-los mais graves (Kessler et al., 2005; Kushner et al., 1990; Regier et al., 1998).

O tratamento para transtornos de ansiedade normalmente é realizado através de medicamentos, podendo variar dependendo do indivíduo e do grau do transtorno apresentado. Caso a desordem de ansiedade seja diagnosticada em combinação com outra doença como depressão, abuso de drogas ou alcoolismo, o tratamento deve ser bem cuidadoso, pois algumas vezes as condições coexistentes possuem um efeito tão forte sobre o indivíduo que o tratamento do transtorno de ansiedade deve esperar até que as condições coexistentes estejam sobre controle (Boyd et al., 1990; Kendler et al., 1995; Yehuda, 1999).

Os principais medicamentos utilizados para tratar transtornos de ansiedade são os fármacos antidepressivos, ansiolíticos e betabloqueadores que possuem a capacidade de controlar alguns dos sintomas físicos causados por essa doença. Os

fármacos ansiolíticos atuam diminuindo a ansiedade, moderando a excitação e, por consequência, acalmando o paciente. Além de suas propriedades ansiolíticas, eles também apresentam características hipnóticas, atuando na indução e manutenção do sono (Anderson, 2010; Ashton, 1994; Fahey et al., 2006; Listos et al., 2005; Mandrioli et al., 2010).

Os fármacos mais comumente utilizados para tratar desordens de ansiedade são os benzodiazepínicos, dentre eles destacam-se o diazepam e o midazolam, sendo que o primeiro possui características ansiolíticas e o segundo apresenta efeitos hipnóticos (Carlo et al., 1989; Olkkola e Ahonen, 2008). Embora sejam fármacos relativamente seguros, com uma alta taxa de eficiência, restrições ao seu uso têm sido cada vez maiores devido à incidência de tolerância e dependência. Uma vez tolerante, o indivíduo necessita de doses cada vez maiores para que os sintomas da ansiedade sejam controlados. Esse aumento contínuo na concentração ingerida pelo paciente pode levar a dependência. Para evitar essas dificuldades de tratamento, os fármacos benzodiazepínicos são geralmente prescritos por curtos períodos de tempo (Ashton, 2005; Martin et al., 2007).

A ação dos fármacos benzodiazepínicos se dá através da sua ligação na interface das subunidades α e γ do receptor $GABA_A$, específico para a ação inibitória do neurotransmissor ácido gama-aminobutírico (GABA) (Anderson, 2010; Campo-Soria et al., 2006; Nelson e Chouinard, 1999; Olkkola e Ahonen, 2008; Rifkin, 1990). Uma vez que essa ligação esteja estabelecida, ocorrerá uma hiperpolarização da célula através do aumento do influxo de íons cloreto (Cl^-) para dentro da célula, causando aumento na corrente inibitória, fazendo com que o receptor expresse seu efeito na

neurotransmissão inibitória (Anderson, 2010; DeMicco et al., 2010; Olkkola e Ahonen, 2008; Rifkin, 1990).

Sabe-se que o neurotransmissor GABA pode ser coliberado com outros neurotransmissores, tais como ATP e acetilcolina (ACh), podendo ambos modular a neurotransmissão sináptica (Burnstock, 2004; Holton, 1959; Nakanishi e Takeda, 1973; Zimmermann, 2008). Na fenda sináptica, o ATP é hidrolisado à adenosina (ADO), e esta subsequentemente à inosina, através da ação de enzimas chamadas ectonucleotidases e adenosina desaminase (ADA), respectivamente (Haskó et al., 2000; Hirschhorn e Ratech, 1980; Zimmermann,1992; 1996a; 1996b; 2011). Já a ACh sofre degradação a colina e acetato pela acetilcolinesterase (AChE) (Massoulié et al., 2008). Os receptores A_1 de ADO são conhecidos por modular a ação da ADO na liberação de muitos neurotransmissores no SNC, incluindo ACh, noradrenalina e dopamina (Fredholm e Dunwiddie, 1988). A existência de receptores A_1 de ADO foi demonstrada em neurônios colinérgicos do córtex de ratos, sendo esses funcionalmente ligados a inibição da liberação de ACh (Broad e Fredholm, 1996). Além disso, foi relatado que a liberação de ACh apresenta-se aumentada na ação de agonistas do receptor A_{2A} de ADO em sinaptossomas do estriado de ratos (Kirkpatrick e Richardson, 1993).

Considerando o envolvimento do ATP e ADO na ansiedade, estresse e sono, a co-liberação existente entre ATP e ACh e a ação neuromoduladora da ADO, a avaliação da atividade das enzimas envolvidas no controle dos níveis desses neurotransmissores e neuromoduladores pode representar um mecanismo importante relacionado aos efeitos dos benzodiazepínicos. Portanto, o objetivo deste estudo foi avaliar o efeito da administração de diazepam e midazolam sobre a atividade

enzimática das NTPDases, ecto-5’-nucleotidase, ADA e AChE em encéfalo de zebrafish. A escolha por estes fármacos benzodiazepínicos se deu pelo fato de ambos possuírem um alto índice de uso, além de serem diferentes em suas características de tratamento, sendo o diazepam um fármaco ansiolítico e o midazolam apresentando características sedativo-hipnóticas (Carlo et al., 1989; Hollister e Csernansky, 1990).

No capítulo 2, foi avaliadoo efeito i*n vitro* dos fármacos benzodiazepínicos diazepam e midazolam sobre a atividade enzimática das NTPDases, ecto-5’-nucleotidase, ADA e AChE em membranas e homogenatos cerebrais de zebrafish. Os resultados demonstraram que o diazepam e o midazolam foram capazes de alterar a atividade das NTPDases, inibindo a hidrólise tanto de ATP como de ADP, mas não modificaram a atividade da ecto-5’nucleotidase. Ambos os fármacos também alteraram a atividade enzimática da AChE, promovendo uma diminuição na hidrólise de ACh. O diazepam não foi capaz de alterar a atividade da ecto-ADA ou ADA solúvel, mas o midazolam causou uma diminuição na atividade da ecto-ADA. As NTPDases, a AChE e a ecto-ADA são enzimas encontradas ancoradas na superfície da membrana plasmática (Grinthal e Guidotti, 2006; Massoulié et al., 2008). Portanto, é possível sugerir que mudanças na bicamada lipídica promovida pela interação com os fármacos benzodiazepínicos poderia explicar, pelo menos em parte, o efeito inibitório observado na atividade dessas enzimas. Sabe-se que algumas famílias de fármacos são capazes de interagir com as membranas lipídicas, influenciando sua estrutura e, consequentemente, modulando processos como a ligação ao receptor que altera a permeabilidade de íons e o transporte através da membrana, bem como a atividade de enzimas que estão ligadas à membrana (Carfagna e Muhoberac, 1993). As diferenças encontradas nos efeitos promovidos pelo diazepam e midazolam sobre a atividade da

ecto-ADA podem ser explicadas pelo fato desses fármacos apresentarem perfis farmacinéticos e estruturas químicas diferentes, acarretando em impactos múltiplos na absorção, distribuição, metabolismo e excreção dessas drogas (Anderson, 2010; Nelson e Chouinard, 1999).

No capítulo 3, foi demonstrada, a influência do tratamento com diazepam e midazolam na atividade das ectonucleotidases e ADA em zebrafish e uma possível alteração a nível transcricional provocada por esses fármacos. Nossos resultados demonstraram que o diazepam e o midazolam não foram capazes de promover alterações significativas na atividade das NTPDases. Com relação à atividade da ecto-5'-nucleotidase, o diazepam não foi capaz de alterar a hidrólise do AMP. No entanto, o midazolam inibiu a atividade dessa enzima em duas concentrações testadas (0.5 e 1 mg/L). A alteração na expressão gênica foi observada no tratamento com 0.5 mg/L de midazolam, indicando uma possível modulação a nível de transcrição induzida por esse fármaco. A concentração de 1 mg/L de midazolam, apesar de modificar a hidrólise do AMP, parece não estar relacionada com possíveis alterações transcricionais. Estudos demonstraram que o fenazepam, diazepam e midazolam são capazes de alterar significativamente a atividade da ecto-5'-nucleotisase em homogenatos cerebrais de ratos adultos (Korotkina et al., 1985), mostrando que o diazepam e o midazolam são capazes de modular o catabolismo extracelular do AMP. Estudos já demonstraram que a administração intraperitoneal de diazepam e fenazepam é capaz de alterar a atividade da ADA em ratos (Korotkina et al., 1986). No nosso estudo, a ecto-ADA mostrou-se alterada em membranas cerebrais de zebrafish tratados tanto por 1,25 mg de diazepam/L quanto por 1 mg de midazolam/L, diminuindo a hidrólise de ADO na fenda sináptica. No caso da atividade da ADA presente no citosol, o diazepam não foi capaz

de modificar a hidrólise de ADO, já o tratamento com 0,1 mg de midazolam/L diminuiu os níveis da ADO no citosol, uma vez que provocou um aumento na atividade da ADA citosólica. Esse perfil bioquímico de alteração na atividade da ADA não foi observado em nível de expressão gênica, sugerindo que a regulação da ADA pelos fármacos benzodiazepínicos não está diretamente relacionada com o controle transcricional.

No capítulo 4, foi abordado o efeito ex vivo dos fármacos benzodiazepínicos sobre a atividade e expressão da AChE. Sabe-se que o acúmulo de ACh na fenda sináptica, devido a exposição a organofosforados, leva a um possível efeito tóxico, causando convulsões, alteração na ventilação e desbalanço metabólico (Bajgar, 2004; Barthold e Schier 2005; Jokanovic, 2009). Nossos resultados demonstraram que o tratamento com 1,25 mg/L de diazepam e 0,5 mg/L de midazolam alterou a atividade enzimática da AChE, promovendo uma diminuição e um aumento na hidrólise de ACh, respectivamente. O diazepam é um dos fármacos utilizados no tratamento da intoxicação por organofosforados (Antonijevic e Stojiljkovic, 2007; Jokanovic, 2009; Tuovinen, 2004). No entanto, em nosso estudo, esse fármaco induziu a um aumento da concentração de ACh na fenda sináptica, o que levaria aos mesmos sintomas encontrados neste tipo de intoxicação. Já o midazolam causou um importante aumento na hidrólise da ACh, sendo esse mecanismo responsável pela reversão dos sintomas da intoxicação por organofosforados, como já foi citado (Antonijevic e Stojiljkovic, 2007; Jokanovic, 2009; Tuovinen, 2004). Portanto, estudos posteriores serão realizados para avaliar um possível quadro de reversão da intoxicação por organofosforados através da administração de midazolam, uma vez que esse fármaco ainda não é utilizado para o tratamento de intoxicações provocadas por estes compostos.

A partir dos resultados obtidos, foi possível concluir que as ectonucleotidases, a ADA e a AChE são enzimas sensíveis a intervenções farmacológicas promovidas por fármacos benzodiazepínicos, apresentando efeitos diferenciados após exposição *in vitro* e *ex vivo* em zebrafish. As alterações demonstradas reforçam a ideia de que, além de agirem através do sistema GABAérgico, os fármacos da família dos benzodiazepínicos afetam também os sistemas de neurotransmissão purinérgico e colinérgico. As alterações observadas contribuíram para um melhor esclarecimento sobre os efeitos neuroquímicos desses fármacos e sugerem que esses sistemas são interessantes alvos para estudos farmacológicos relacionados à ansiedade.

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