Pontifícia Universidade Católica do Rio Grande do Sul

Faculdade de Biociências

Programa de Pós-Graduação em Biologia Celular e Molecular

Efeito de antidepressivos sobre as enzimas envolvidas no controle da sinalização purinérgica e colinérgica em cérebro de peixe-zebra (*Danio rerio*)

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

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Aos meus pais e minha irmã pelo

amor, carinho e incentivo constante durante

*meus estudos, eu dedico este trabalho**!***

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RESUMO

O tratamento clínico da depressão enfrenta sérios obstáculos já que o mecanismo da doença não é totalmente elucidado. Além disso, não existem meios eficazes para prever e prevenir a depressão bem como nenhum método biológico de diagnóstico. O uso de fármacos antidepressivos ainda é a base dos tratamentos para depressão. O lítio tem sido usado clinicamente como fármaco eficaz para tratar todas as fases do transtorno bipolar, incluindo depressão aguda. Os inibidores seletivos da recaptação de serotonina (ISRSs), como fluoxetina e citalopram, e os antidepressivos tricíclicos (TCA), como a clomipramina, são fármacos constantemente utilizados para o tratamento da depressão. Evidências recentes mostram o envolvimento da adenosina e seus receptores na patofisiologia da depressão. O ATP pode ser armazenado e co-liberado, juntamente com outros neurotransmissores, como serotonina e noradrenalina, e pode ser hidrolisado até adenosina por uma família de enzimas de superfície celular, conhecidas como ectonucleotidases. Dentre elas, destacam-se as Nucleosídeo Trifosfato Difosfoidrolases (NTPDases) e a ecto-5´-nucleotidase, capazes de controlar a disponibilidade de ligantes como ATP e adenosina aos seus receptores específicos. A Adenosina desaminase (ADA) pode promover a desaminação hidrolítica da adenosina em inosina, modulando os níveis extracelulares deste neuromodulador. Na sinalização colinérgica, a acetilcolina (ACh), após liberada, promove a ativação de receptores muscarínicos ou nicotínicos, e desta maneira a ACh promove diversas respostas celulares. A ACh que permanece na fenda sináptica é hidrolisada pela acetilcolinesterase (AChE) em acetato e colina. O peixe-zebra tem sido utilizado na pesquisa da neurociência comportamental, sendo também um modelo de escolha para elucidar o desenvolvimento e a função do circuito neuronal. Considerando que as sinalizações purinérgica e colinérgica têm importante participação no sistema nervoso central e que essas vias de neurotransmissão já estão caracterizadas em peixe-zebra, o objetivo desse estudo foi avaliar o efeito de fármacos antidepressivos na atividade das ectonucleotidases, ADA e AChE, enzimas essenciais na modulação destas vias de sinalização em cérebro de peixe-zebra. Foram avaliados os efeitos *ex vivo* da fluoxetina (1-10 µM), clomipramina (1-10 µM) e citalopram (70-300 µM) na atividade das ectonucleotidases e ADA. Foi analisado também o efeito *in vitro* (1 a 1000 µM) e *ex vivo* (1-10mg/L) do lítio sobre a atividade e expressão gênica das ectonucleotidases e AChE.A exposição ao lítio inibiu a hidrólise de ADP nas concentrações de 5 e 10mg/L e inibiu a hidrólise de AMP na concentração de 10mg/L quando comparado ao grupo controle. Este mesmo tratamento diminuiu a atividade da AChE na concentração de 10mg/L.O lítio não induziu alterações significativas na análise do padrão de expressão gênica. No tratamento *in vitro*, não foram observadas alterações na atividade das ectonucleotidases e AChE. O tratamento com a clomipramina mostrou uma inibição na atividade da ecto-5´-nucleotidase na concentração de 5 µM quando comparado ao grupo controle. Na atividade da ADA também observamos uma inibição significativa no tratamento com a clomipramina nas concentrações de 5 e 10 µM em frações de membrana de cérebro de peixe-zebra. Entretanto, o tratamento com fluoxetina e citalopram não alterou a atividade das ectonucleotidases e ADA no cérebro do peixe-zebra. Nossos resultados podem contribuir para uma melhor compreensão da farmacologia dos fármacos antidepressivos e a sua interação com a neurotransmissão colinérgica e purinérgica.

Palavras-chave: Peixe-zebra, fármacos antidepressivos, adenosina desaminase, ectonucleotidases, acetilcolinesterase.

ABSTRACT

The clinical depression treatment faces serious obstacles as the disease mechanism is not fully elucidated. In addition, there are no effective means to predict and prevent depression as well as any biological method of diagnosis. The use of antidepressants is still the basis of the treatments for depression. Lithium has been used clinically as effective drug to treat all phases of bipolar disorder, including major depression. The Selective serotonin re-uptake inhibitors (SSRIs), such as fluoxetine and citalopram, and Tricyclic antidepressant (TCA) as clomipramine are drugs constantly used for depression treatment. Recent evidence has shown an involvement of adenosine and its receptors in the pathophysiology of depression. ATP can be stored and co-released with other neurotransmitters like serotonin and can be hydrolyzed by a cell-surface enzyme family known as ectonucleotidases. Among these members, we highlight the nucleoside triphosphate diphosphohtdrolases (NTPDases) and ecto-5'-nucleotidase. They are able to control the availability of ligands such as ATP and adenosine to its specific receptors. Adenosine deaminase (ADA) can promote the hydrolytic deamination of adenosine to inosine, modulating the extracellulr levels of this neuromodulator. In cholinergic signaling, after its release, acetylcholine (ACh) promotes the activation of specific muscarinic or nicotinic receptors and thus, it promotes diverse cellular responses. ACh is hydrolyzed by acetylcholinesterase (AChE) in acetate and choline in synaptic cleft. The zebrafish has been used in research behavioral neuroscience and is also a choice model for elucidating the development and function of neuronal circuitry. Considering the cholinergic and purinergic signaling are important participation in the CNS and these neurotransmitter pathways have been identified and characterized in zebrafish, the objective of this study was to evaluate the effect of antidepressants on ectonucleotidases, ADA and ACh activities, which are essential enzymes in the modulation of these signaling pathways in the zebrafish brain. We evaluated the *ex vivo* effects of fluoxetine (1-10 µM), clomipramine (1-10 µM), citalopram (70-300 µM) on ectonucleotidases and ADA activities. It has been also analyzed the *in vitro* (1 to 1000 µM) and *ex vivo* (1 to 10mg/L) effect of lithium on ectonucleotidases and AChE activities and gene expression. There was a significant inhibition of ADP hydrolysis after *ex vivo* exposure to lithium at 5 and 10 mg/L, whereas an inhibitory effect was observed for AMP hydrolysis only at 10 mg/L. The same treatment decreased the AChE activity in a concentration of 10mg/L. Lithium did not induce significant changes in the analysis of gene expression patterns in the concentrations tested. In vivo treatment, there were no significant changes inectonucleotidases and AChE activities. Treatment with clomipramine promotes an inhibition ecto-5'-nucleotidase activities at the concentration of 5µM when compared to the control group. For ADA activity, we also observed a significant inhibition in the treatment with clomipramine at concentrations of 5 and 10 µM in membrane fractions of zebrafish brain. However, treatment with fluoxetine and citalopram did not alter ectonucleotidases and ADA activities in the zebrafish brain. Our findings may contribute to a better understanding of pharmacology of antidepressants and their interaction with the cholinergic and purinergic neurotransmission.

Keywords: zebrafish, antidepressants, adenosine deaminase, ectonucleotidases, acetylcholinesterase.

LISTA DE ABREVIATURAS

Acetil CoA: acetil coenzima A

ACh: acetilcolina

AChE: acetilcolinesterase

ADA: adenosina deaminase

ADP: adenosina 5´- difosfato

AMP: adenosina 5´- monofosfato

AP: fosfatases alcalinas

ATP: adenosina 5´- trifosfato

BDNF: fator neurotrófico derivado do cérebro

BuChE: butirilcolinesterase

Ca^{+2}: cálcio

E-NPP: ectonucleotídeo pirofosfatase/fosfodiesterase

E-5´-NT: ecto-5´-nucleotidase

GABA: ácido gama-aminobutírico

GMP- guanosina 5'-monofosfato

GPI: glicosilfosfatidilinositol

IMAO: inibidores da monoaminoxidase

IP3: inositol 1,4,5-trifosfato

ISRNs: inibidores seletivos da recaptação de noradrenalina

ISRSs: inibidores seletivos da recaptação de serotonina

K^{+}: potássio

Mg^{+2}: magnésio

NA: noradrenalina

Na^{+}: sódio

NTPDase: nucleosídeo trifosfato difosfoidrolase

OMS: Organização Mundial da Saúde

SNC: sistema nervoso central

TCA: antidepressivos tricíclicos

UTP – uridina 5´-trifofosfato

UDP – uridina 5’- difosfato

UMP – uridina 5’-monofosfato

5-HT – 5-hidroxitriptamina (serotonina)

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1. INTRODUÇÃO

1.1 Depressão, transtorno bipolar e terapia farmacológica

O transtorno depressivo maior está entre os transtornos psiquiátricos mais comuns (Vanderhasselt et al., 2012), sendo uma preocupação de saúde pública, onde existe a necessidade constante de tratamentos eficazes e aceitáveis pelos pacientes e com menos efeitos colaterais (Connolly & Thase., 2012). Trata-se de uma doença generalizada e de grande impacto socioeconômico, e de acordo com a OMS, será a segunda principal causa de deficiência em termos de carga de morbidade no futuro (Antonioli et al 2012; El-Alfy et al., 2012; Wang et al., 2012).

A depressão é um distúrbio psiquiátrico comum, caracterizado por uma série de sinais e sintomas que podem incluir: humor deprimido, anedonia, insônia, anorexia, dificuldade de concentração e pensamento suicida (Cavanagh et al., 2011; Hashemi et al., 2012). Embora os antidepressivos disponíveis tenham demonstrado sua eficácia e têm melhorado bastante o prognóstico da doença, as atuais abordagens para gerenciamento da depressão ainda não são satisfatórias (Catena-Dell'osso et al., 2012).

O tratamento clínico da depressão enfrenta sérios obstáculos já que o mecanismo da doença não é totalmente compreendido (Haenisch & Bonisch, 2011) e, portanto, não existem meios eficazes para prever e prevenir a depressão bem como nenhum método biológico de diagnóstico (Wang et al., 2012). No entanto, antidepressivos inibidores seletivos da recaptação de serotonina (ISRSs) têm superado os antidepressivos tricíclicos (TCAs) como os medicamentos mais utilizados para a depressão (Mukai & Tampi, 2009). Os medicamentos atuais para tratar os sintomas da depressão apresentam algumas características não favoráveis, como por exemplo, um tempo de ação tardio,

baixa eficácia terapêutica e efeitos colaterais indesejados (El-Alfy et al., 2012). A busca de antidepressivos novos é cada vez maior, bem como se faz necessária uma compreensão da etiologia da depressão. A hipótese monoaminérgica postula que a depressão está relacionada com a deficiência de neurotransmissores em sinapses monoaminérgicas. Atualmente, o desenvolvimento de antidepressivos tem sido principalmente baseado no aumento da neurotransmissão monoaminérgica, por inibição da recaptação neuronal ou da degradação de neurotransmissores, apresentando uma melhora na eficácia terapêutica e diminuição dos efeitos adversos (Connolly & Thase, 2012).

Entretanto, a causa da depressão está longe de ser uma simples deficiência de monoaminas centrais (Krishnan & Nestler, 2008). Além disso, o aumento agudo na quantidade de monoaminas sinápticas induzido por drogas antidepressivas produz alterações neuroplásticas secundárias que envolvem alterações de transcrição e tradução que medeiam a plasticidade celular e molecular (Krishnan & Nestler, 2008; Pittenger et al., 2008).

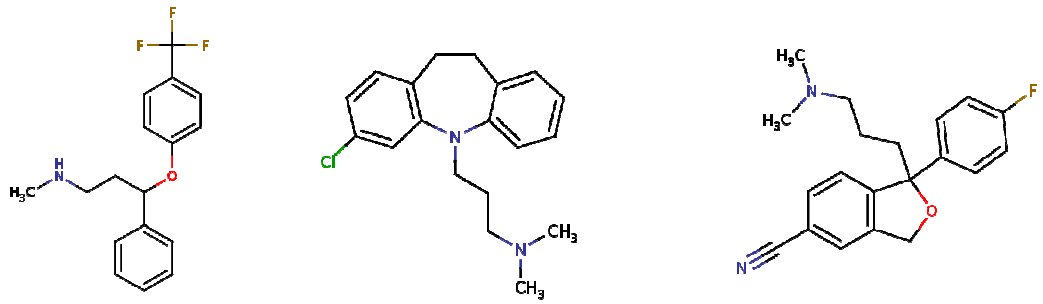
Pesquisas em animais e humanos têm identificado uma série de anormalidades que compõem um modelo psicobiológico da fisiopatologia da depressão. Os principais achados estão relacionados com a diminuição da neurotransmissão monoaminérgica (serotonina e noradrenalina) (Krishnan & Nestler, 2008), baixas concentrações de fator neurotrófico derivado do cérebro (BDNF) (Piccinni et al., 2009; Domschke et al., 2010), citocinas elevadas, desregulação no eixo hipotálamo-pituitária-adrenal e susceptibilidade genética (Ruhe et al., 2007; Frodl et al., 2008; Palazidou et al., 2012). Além disso, a neurogênese adulta é importante para o aprendizado e memória, bem como para os estados de ansiedade e depressão. Estudos mostram que drogas

antidepressivas, antipsicóticas e agonistas de receptores de serotonina aumentam a neurogênese, enquanto que a depressão, esquizofrenia e doenças neurodegenerativas reduzem a neurogênese (Cho & Kim, 2010; Kubesova et al., 2012).

Os primeiros tratamentos para depressão basearam-se na ação de fármacos inibidores da monoaminoxidase (IMAO) e TCA (Dardennes et al., 1999). A descoberta dos ISRSs e dos inibidores seletivos da recaptação de noradrenalina (ISRNs) tem mudado aspectos importantes no tratamento clínico (Rosenzweig-Lipson et al., 2007; Gartlehner et al., 2011). A fluoxetina e o citalopram são fármacos inibidores seletivos da recaptação de serotonina (5-HT), que possuem poucos efeitos sobre outros neurotransmissores (Chen et al., 2007; Gartlehner et al., 2011). A Clomipramina pertence à classe dos TCA que atua com duplo papel, inibindo a recaptação de noradrenalina (NA) e/ou 5-HT (Stoll et al., 2007; Balk et al., 2011) (Figura 1). A principal diferença entre estas duas classes de fármacos é que os ISRSs não possuem efeitos adversos sobre o sistema cardiovascular, o que ocorre com o uso dos TCA (Pacher & Kecskemeti, 2004; Hendron et al., 2011). Além disso, a primeira geração de fármacos pode gerar efeitos secundários múltiplos, sendo considerados intoleráveis pelos pacientes. Por exemplo, TCAs tendem a causar efeitos anticolinérgicos, incluindo boca e olhos secos, hesitação urinária e, às vezes, retenção e constipação (Gartlehner et al., 2011).

O transtorno bipolar é outra desordem psiquiátrica que provoca uma variação de humor, afeta drasticamente a qualidade de vida e aumenta signitivamente a possibilidade de suicídio em pacientes (Altamura et al., 2011; Ludtmann et al., 2011). Esta doença é definida por episódios de mania e hipomania, e sua ocorrência mundial estimada é de aproximadamente 4% (Calabrese et al., 2003; Ketter, 2010).

O lítio é um estabilizador de humor que tem sido usado clinicamente como fármaco eficaz para tratar todas as fases do transtorno bipolar, incluindo depressão aguda, e é bem conhecido por seus efeitos em doenças neuropsiquiátricas e comunicação neuronal (Tkatcheva et al, 2007; Chang & Ha, 2011). Sabe-se que o lítio induz vários efeitos bioquímicos relacionados à captação e liberação de neurotransmissores, envolvendo mecanismos sinápticos e intracelulares (Shaldubina et al., 2001). Evidências recentes também demonstraram que o lítio pode agir através de vários sistemas de sinalização, apresentando efeitos neuroprotetores em uma variedade de insultos em cultura neuronais a partir de modelos animais de doenças neurodegenerativas e em estudos com humanos (Chakraborty et al., 2008; Yucel et al., 2008). Além da hipótese monoaminégica que tem predominado, outros sistemas estão relacionados com os mecanismos neuroquímicos da depressão e com o efeito de fármacos antidepressivos, tais como sistema purinérgico e glutamatérgico (Pedrazza et al., 2008; Pittenger et al., 2008). Além disso, estudos mostram que antidepressivos, como a clomipramina diminuem a atividade da Na^+/K^+-ATPase em ratos, o que poderia estar relacionado com o aumento do déficit cognitivo (Balk et al., 2011).



(A) (B) (C)

Figura 1: Estrutura química dos fármacos antidepressivos. (A) Fluoxetina; (B) Clomipramina; (C) Citalopram (http://www.drugbank.ca/).

1.2 Sistema Purinérgico

1.2.1. Receptores purinérgicos

Os nucleosídeos e nucleotídeos derivados de purinas atuam como moléculas sinalizadoras extracelulares em vários tecidos, por meio da ativação dos receptores purinérgicos (Burnstock & Knight, 2004; Burnstock, 2008).

O ATP está envolvido na regulação de diversos processos fisiológicos e patológicos no meio extracelular (Bours et al., 2006), sendo reconhecido como um neurotransmissor, pois é sintetizado e armazenado em terminais sinápticos e liberado após estímulo destes terminais (Burnstock, 1972; Burnstock, 2007). Além disso, pode ser co-liberado juntamente com vários neurotransmissores, como a acetilcolina, glutamato, noradrenalina, serotonina e GABA (North & Verkhratsky, 2006).

A sinalização extracelular de ATP ocorre via receptores purinérgicos do tipo P2, sendo este grupo subdividido em duas famílias distintas: P2X e P2Y (Ravelic & Burnstock, 1998; Abbracchio et al., 2009). A família P2X consiste de receptores ionotrópicos que apresentam permeabilidade rápida e seletiva para cátions (Na^{+}, K^{+} e Ca^{+2}) e está dividida em sete membros (P2X1-7), os quais estão distribuídos em neurônios, células gliais e no músculo liso (North, 2002; Burnstock, 2004). A ativação de receptores P2X pode resultar na despolarização das células e, para alguns membros da família de genes, diretamente causar aumentos nas concentrações de cálcio intracelular devido à sua permeabilidade relativamente alta para este íon (Egan & Khakh, 2004). Os receptores P2Y, metabotrópicos acoplados a uma proteína G, foram identificados funcionalmente em oito membros ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$) que podem ser ativados por diversos nucleotídeos: ATP, ADP,

UTP e UDP (Abbracchio et al., 2006). Além disso, esses receptores apresentam uma ampla distribuição nos tecidos e sistemas, tais como vascular, nervoso e cardíaco (Burnstock, 2007; Erb et al., 2006). Em situações patofisiológicas, a liberação de ATP e a expressão de receptores purinérgicos pelas células estão consideravelmente aumentadas (Guido et al., 2008). Uma vez liberado no espaço extracelular, o ATP pode ser metabolizado pela ação das ectonucleotidases, que fazem a conversão deste nucleotídeo até adenosina (Zimmermann, 2006; Schetinger et al., 2007).

A adenosina é um metabólito constituinte de todas as células (Cunha, 2001) que desenvolve diversos papéis chave no organismo, bem como o controle de vários mecanismos do SNC (Boison et al., 2010). A adenosina não é considerada um neurotransmissor clássico, como o ATP, pelo fato de não ser armazenada em vesículas, não ser liberada por exocitose e não atuar predominantemente em sinapses (Fredholm, 2003). A adenosina pode ser sintetizada tanto no meio intracelular quanto no espaço extracelular. A síntese intracelular acontece, principalmente, pela degradação do nucleotídeo monofosfatado AMP pela enzima 5´-nucleotidase e pela clivagem da S-adenosil-homocisteína pela enzima S-adenosil-homocisteína hidrolase. A adenosina produzida no meio intracelular pode ser transportada para o meio extracelular através de transportadores específicos bidirecionais, por um mecanismo de difusão facilitada que mantêm os níveis intracelulares e extracelulares de adenosina em equilíbrio (Franco et al., 1997; Latini & Pedata, 2001).

A ação da adenosina se dá através da ativação de receptores purinérgicos, do tipo P1, acoplados a proteínas G (Zimmermann, 2012). Estes receptores são divididos em 4 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 (Burnstock et al., 2011).

A adenosina pode atuar como uma molécula que mantém a homeostase do meio intracelular e como um neuromodulador do sistema nervoso (Snyder et al., 1985; Burnstock et al., 2011). Devido a este papel neuromodulador, a adenosina está envolvida na regulação de importantes mecanismos no SNC (Cunha et al., 2008; Boison, 2007), como estados de ansiedade e desordens psiquiátricas (Ruby et al., 2010; Asatryan et al., 2011), sono (Porkka-Heiskanen, 1999), cognição e memória (Ribeiro et al., 2003). A adenosina é reconhecida como um importante modulador da neurotransmissão excitatória e agente neuroprotetor em diferentes patologias relacionadas ao SNC, tais como na isquemia, hipóxia (Fredholm, 1997; Ribeiro et al., 2003), epilepsia (Boison, 2005; Vianna et al., 2005), doença de Parkinson (Fredduzzi et al., 2002) e na esquizofrenia (Lara et al., 2001; Gomes et al., 2011). Além disso, o receptor de adenosina A_{2A} está no centro de uma rede de neuromoduladores, afetando uma ampla gama de funções neuropsiquiátricas através da interação e integração com vários sistemas de neurotransmissores, especialmente sistema dopaminérgico e glutamatérgico (Shen & Chen, 2009).

A clonagem e caracterização molecular dos receptores P2X do peixe-zebra já foram realizadas (Norton et al., 2000; Diaz-Hernandez et al., 2002). Kucenas e colaboradores (2003) mostraram que a subunidade P2X possui nove membros, sendo destes seis ortólogos aos genes dos receptores P2X de mamíferos, dois parálogos e um gene ainda precisa ser devidamente classificado (Kucenas et al., 2003). Os subtipos dos receptores P2X do peixe-zebra contêm resíduos altamente conservados, os quais são encontrados nas subunidades de mamíferos. Até o momento, na família de receptores

P2Y, foram identificadas oito proteínas funcionais, (Illes & Ribeiro, 2004; Ralevic & Bursntock, 1998) e apenas foram identificados receptores P2Y1 em trombócitos de peixe-zebra (Gregory & Jagadeeswaran, 2002). Um estudo recente identificou os receptores de adenosina A_{2A} em peixe-zebra, que possuem alta similaridade com os receptores adenosinérgicos de humanos (Boehmler et al., 2009). Evidências mostram que a exposição precoce à cafeína, um antagonista não seletivo dos receptores de adenosina A_1 e A_{2A}, foi capaz de alterar a expressão gênica dos receptores de adenosina sem afetar a morfologia do desenvolvimento de embriões de peixe-zebra (Capiotti et al., 2011). Além disso, a cafeína tem apresentado efeitos neuroprotetores em injúrias cerebrais em embriões de peixe-zebra (Boehmler et al., 2009. Outro estudo tem mostrado que a cafeína produz um efeito ansiogênico, provavelment via receptores A_1, e promove um aumento da atividade locomotora mediado via receptores A_2 (Maximino et al., 2011).

1.2.2 Ectonucleotidases

Os nucleotídeos extracelulares são degradados por uma cascata de hidrólise constituída por uma variedade de enzimas que estão localizadas na superfície celular, chamadas de ectonucleotidases, sendo estas ancoradas na membrana celular, possuindo seu sítio ativo voltado para o meio extracelular, ou estão presentes na forma solúvel no meio intersticial. Dentre elas, destacam-se a família das E-NPP, NTPDases, as fosfatases alcalinas (CD73, E.C.3.1.3.5) e a ecto-5'-nucleotidase, capazes de controlar a disponibilidade de ligantes como ATP e adenosina aos seus receptores específicos (Robson et al., 2006; Zimmermann, 2008) (Figura 2).

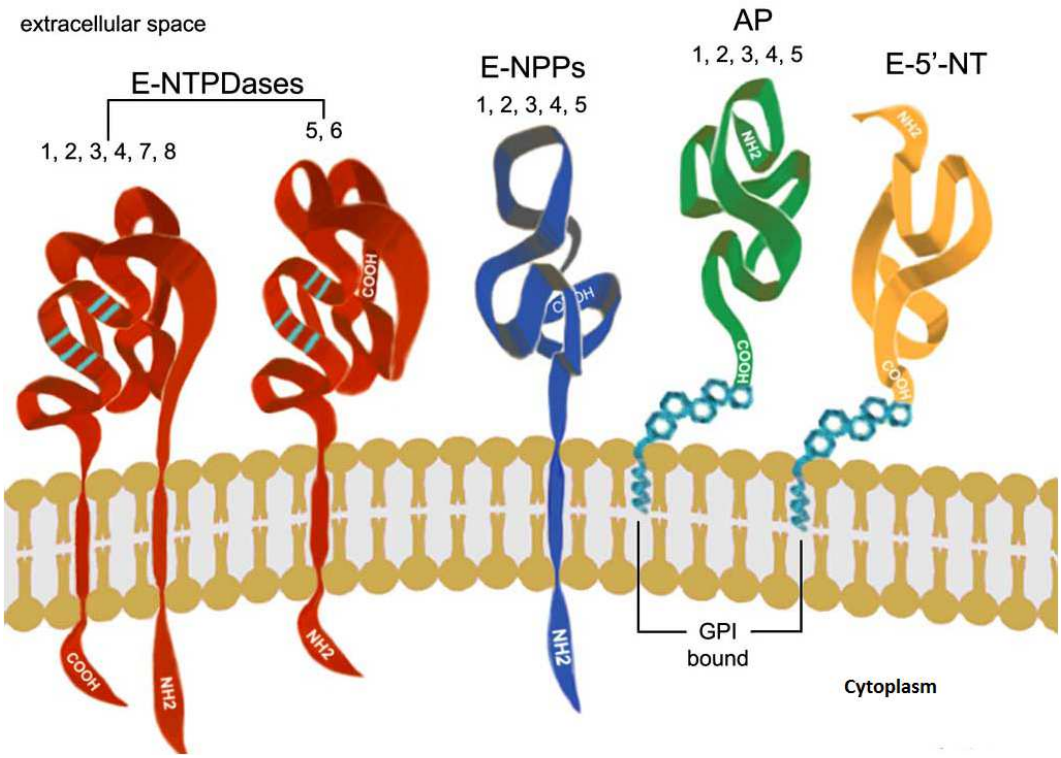


Figura 2: Ectonucleotidases e suas principais características. NTPDases e NPPs são enzimas integrais de membrana, enquanto que as fosfatases alcalinas (AP) e a ecto-5´-nucleotidase (E-5´-NT) estão ancoradas à membrana plasmática por um resíduo de glicosilfosfatidilinositol (GPI). Figura adaptada de Cognato & Bonan, 2010.

Os membros da família das NTPDases são codificados por oito genes diferentes, chamados de genes *Entpd*. Dos oito membros descritos até o momento, quatro estão localizados na membrana celular com o sitio ativo voltado para o meio extracelular (NTPDases 1,2,3 e 8). As NTPDases 5 e 6 se localizam intracelularmente, porém são secretadas após expressão heteróloga. As NTPDases 4 e 7 apresentam localização intracelular com o sítio ativo voltado para o lúmen de organelas citoplasmáticas. As NTPDases compartilham 5 domínios altamente conservados denominados regiões conservadas de apirase (Robson et al., 2006; Zimmermann, 2011). Estas enzimas

hidrolisam tanto ATP como ADP, formando AMP na presença de íons Ca^{+2} e Mg^{+2} (Chan et al., 1986; Zimmermann, 2011). O AMP formado é então convertido a adenosina pela ecto-5'-nucleotidase (Robson et al., 2006).

A ecto-5´-nucleotidase desfosforila nucleotídeos monofosfatados não cíclicos (AMP, GMP ou UMP) (Bianchi e Spychala, 2003), através da hidrólise da ligação fosfodiester de 5´-ribonucleotídeos, levando à formação do correspondente ribonucleosídeo e fosfato. Essa enzima é ancorada à membrana plasmática por um GPI e possui a forma estrutural de dímero com pontes dissulfeto entre as cadeias (Zimmermann, 2000; Hunsucker et al., 2005).

No peixe-zebra, estudos demonstraram a presença de uma NTPDase e uma ecto-5'-nucleotidase em membranas cerebrais. Estas duas enzimas foram caracterizadas como dependentes de cátion divalentes, apresentando atividade máxima à temperatura de 37ºC, pH ótimo entre 7,2 e 8,0, Kм na faixa do micromolar e uma ampla especificidade por outros nucleotídeos (Rico et al., 2003; Senger et al., 2004). A expressão de um gene *Entpd1*, três genes *Entpd2* (isoformas nomeadas como NTPDase2mv, NTPDase2mq e NTPDase2mg) e um gene *Entpd3* têm sido caracterizada em peixe-zebra (Rico et al., 2006) e a localização imunocitoquímica da NTPDase1 e NTPDase2 na retina neural do peixe-zebra sugerem a existência da regulação compartimentalizada da concentração de nucleotídeos/nucleosídeos neste teleósteo (Ricatti et al., 2009). Além disso, Rosemberg e colaboradores (2010) verificaram a presença de diferentes membros da família das NTPDases (NTPDases 1, 2, 3, 4, 5, 6, 8) em cérebro, coração e fígado de peixe-zebra.

As ectonucleotidases desempenham uma função essencial na neurotransmissão purinérgica, controlando a disponibilidade e os níveis de nucleotídeos e nucleosídeos

extracelulares e, conseqüentemente a ativação dos purinoreceptores P2 e P1 (Zimmermann, 2012).

1.2.3 Adenosina Desaminase

A adenosina pode ser substrato de uma enzima denominada adenosina desaminase (ADA, EC 3.5.4.4). Esta enzima promove a desaminação hidrolítica da adenosina em inosina. Além deste nucleosídeo, outro substrato que é capaz de ser clivado pela ADA é a 2'-desoxiadenosina, a qual é convertida a 2'-desoxinosina (Latini & Pedata, 2001; Iwaki-Egawa et al., 2004). Esta enzima possui uma função importante no controle dos níveis de adenosina no sistema imune, mediando processos inflamatórios (Antonioli et al., 2007).

A ADA é encontrada como uma enzima citosólica e também pode ser expressa na superfície celular como uma ecto-enzima. Dois membros clássicos da ADA foram descritos, sendo eles primeiramente denominados como ADA1 e ADA2 (Hirschhorn et al., 1980). Estudos têm demonstrado que ambos os membros apresentam características cinéticas distintas, o que faz com que ambos possam desempenhar uma função diferenciada nos organismos (Zavialov & Engström, 2005).

A ADA1 é uma enzima monomérica, cuja massa molecular é de aproximadamente 3-40 kDa (Daddona & Kelley, 1977). Tecidos como fígado e rins apresentam tanto a ADA1 solúvel quanto a forma associada a uma proteína de ligação (CD26), sendo que esta é constituída por duas moléculas de ADA1 e uma proteína ligante (Iwaki-Egawa et al., 2004). O complexo ADA-proteína de ligação constitui uma

ecto-ADA, que se encontra ancorada à membrana celular, e se torna responsável pelo controle dos níveis de adenosina extracelular (Torvinen et al., 2002).

Estudos envolvendo a sinalização mediada pela adenosina no SNC demonstram que além da interação com CD26, a ADA1 pode funcionar como uma ectoenzima ancorada aos receptores de adenosina A_1 e A_{2B}, mediando os processos de sinalização deste neuromodulador (Romanowska et al., 2007).

Diferentemente da ADA1, a ADA2 apresenta diferenças, tanto estruturais quanto cinéticas. Sua massa molecular é de aproximadamente 100 kDa e representa uma menor parte da atividade da ADA em tecidos, sendo abundante em plasma. A atividade desta enzima encontra-se elevada em casos de doenças hepáticas, mas a fonte celular e a função da ADA2 plasmática ainda não estão completamente esclarecidas (Kobayashi et al., 1993). Embora a existência de dois membros da ADA fosse previamente consolidada, uma análise filogenética das seqüências de diferentes organismos revelou uma nova família de proteína relacionada com a ADA1 e ADA2, a qual foi denominada ADAL (adenosina desaminase “like”) (Rosemberg et al., 2007). Por apresentar sítios de aminoácidos importantes relacionados à desaminação de adenosina e motivos conservados entre as subfamílias da ADA, é sugerido que esta enzima possa participar da clivagem de adenosina à inosina (Maier et al., 2005).

Um estudo desenvolvido por Rosemberg e colaboradores (2007) demonstrou a existência de diferentes genes relacionados à adenosina desaminase (ADA1, ADAL e dois ortólogos da ADA2), com um padrão de expressão ubíquo em peixe-zebra. Outro estudo realizou o mapeamento do padrão de expressão de genes relacionados a adenosina desaminase em diversos tecidos (cérebro, brânquias, coração, fígado, esqueleto, músculo e rim) de peixe-zebra (Rosemberg et al., 2007). Além disso, a

presença de atividade da ADA nas membranas cerebrais do peixe-zebra sugere a existência de uma ecto-ADA neste modelo animal (Rosemberg et al., 2008). Este estudo sugeriu que a desaminação da adenosina no SNC de peixe-zebra promovida por diferentes membros da família da ADA pode ser um elemento-chave para o controle da adenosina/inosina no meio intracelular e extracelular (Rosemberg et al., 2008).

Um estudo recente tem demonstrado a diminuição da atividade da ecto-ADA durante convulsões induzidas por pentilenotetrazol em peixe-zebra, sugerindo a existência de uma modulação dos níveis de adenosina extracelular (Siebel et al., 2011). Piato e colaboradores (2011) mostraram que o estresse por contenção provocou uma diminuição da atividade da adenosina desaminase citosólica em peixe-zebra.

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). A ACh está envolvida com o controle psicomotor, agindo também como importante modulador das funções cognitivas, tais como aprendizagem e memória (Hasselmo, 2006; Tsai et al., 2007).

A síntese da ACh ocorre a partir de Acetil CoA, formada durante o metabolismo celular mitocondrial, e da colina, um importante produto do metabolismo dos lipídios. A etapa final da síntese da ACh ocorre no citoplasma, sendo o neurotransmissor transportado para o interior de vesículas sinápticas. A colina usada na síntese de ACh pode vir diretamente da reciclagem da ACh, que é hidrolisada pela AChE na fenda sináptica ou a partir da fosfatidilcolina (Zimmermann, 2008).

Na neurotransmissão colinérgica, a ACh após liberada promove a ativação de receptores específicos, podendo ser receptores muscarínicos ou nicotínicos, promovendo diversas respostas celulares (Burgen, 1995; Soreq & Seidman, 2001). Os receptores muscarínicos são metabotrópicos e podem ser ativados pela ACh e muscarina. Além disso, a estimulação dos receptores muscarínicos conduzirá à despolarização ou hiperpolarização da membrana e também é capaz de inibir a enzima adenilato ciclase e ativar a enzima fosfolipase C (Cooper et al., 1991). Os receptores nicotínicos são ionotrópicos e sensíveis à ACh e a ligantes exógenos como a nicotina.

A ACh que permanece na fenda sináptica é hidrolisada por uma colinesterase especifica em ácido acético e colina. 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).

As colinesterases são responsáveis pela manutenção dos níveis de ACh no espaço extracelular e são classificadas de acordo com suas propriedades catalíticas, especificidade de inibidores e distribuição nos tecidos. As colinesterases são divididas em pelo menos duas famílias principais: acetilcolinesterases (EC 3.1.1.7, AChE) e butirilcolinesterases (EC 3.1.1.8, BuChE) (Soreq and Seidman, 2001). Ambas as colinesterases são amplamente distribuídas no organismo (Zimmermann, 2008).

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 (Fernandes & Hodges-Savola, 1992).

A sinalização colinérgica já tem sido caracterizada em cérebro de peixe-zebra (Clemente et al., 2004; Rico et al., 2006). Além disso, tem sido demonstrado que a BuChE não é codificada no genoma do peixe-zebra. No entanto, a AChE tem sido detectada funcionalmente no cérebro deste teleósteo, o que mostra que a hidrólise de ACh é realizada preferencialmente pela AChE em peixe-zebra (Bertrand et al., 2001).

1.4 Fármacos antidepressivos e o seu envolvimento com os sistemas de neurotransmissão colinérgico e purinérgico.

Evidências indicam que as pessoas que sofrem de depressão apresentam comprometimento na atenção, memória de trabalho, função executiva, incluindo déficit cognitivo e problemas no planejamento de tarefas (Gohier et al., 2009; Hindmarch & Hashimoto, 2010). Além disso, essas funções são influenciadas pelo sistema de neurotransmissão serotoninérgico que pode ser regulado pela adenosina através de seus receptores A_1 e A_{2A} (Okada et al., 1997; Mossner et al., 2000). Muitos estudos mostram o envolvimento da adenosina e seus receptores na patofisiologia da depressão (Lobato et al., 2008). Okada e colaboradores (1999) demonstraram que a neurotransmissão serotonérgica é modulada pelos subtipos de receptores de adenosina no hipocampo, onde a estimulação dos receptores A_1 levaria a uma diminuição na liberação de serotonina, já a estimulação dos receptores A_{2A}, associada a um bloqueio nos receptores A_1, levaria a um aumento na liberação de serotonina no hipocampo. Outros estudos têm demonstrado que a administração de adenosina produz um efeito antidepressivo em camundongos submetidos ao teste do nado forçado e ao teste de suspensão pela cauda, efeito mediado aparentemente via receptores de adenosina A_1 e A_{2A} (Kaster et al., 2004). Por outro lado, estudos mostram que a adenosina e seus análogos podem induzir

um efeito depressivo e que os antagonistas de receptores A_{2A} de adenosina podem induzir um efeito antidepressivo em animais submetidos a modelos de depressão (El Yacoubi et al., 2000; El Yacoubi et al., 2003). Esta diferença provavelmente depende da dose aplicada e as diferenças no modelo animal e / ou procedimentos utilizados (Kaster et al., 2004).

Estudos também têm mostrado o que fármacos antidepressivos podem modular a degradação de nucleotídeos e, consequentemente, a sinalização purinérgica. Pedrazza e colaboradores (2008) demonstrou que o tratamento agudo com nortriptilina inibiu a hidrólise de ATP e ADP no hipocampo e promoveu a ativação da hidrólise de ADP no córtex de ratos. Além disso, o tratamento crônico com nortriptilina e fluoxetina promoveu uma diminuição da hidrólise de ATP em hipocampo, enquanto que no córtex estas drogas promoveram diferentes efeitos sobre a hidrólise de nucleotídeos (Pedrazza et al., 2008).

Estudos recentes relatam o envolvimento de antidepressivos na via anti-colinérgica, anti-histamínica e alfa-adrenérgica (Hindmarch & Hashimoto, 2010), uma vez que estes estão relacionados com funções cognitivas. Além disso, Bhagya e colaboradores (2011) mostraram que a depressão promoveu a diminuição da atividade da AChE em córtex frontal e hipocampo de ratos e que o tratamento crônico com escitalopram promoveu a restauração da atividade da AChE (Bhagya et al., 2011).

Há evidências que fármacos antidepressivos modulam a sinalização celular e mobilizam segundos mensageiros, como cálcio intracelular e IP3 (Sanganahalli et al., 2000). Yildiz e colaboradores (2005) demonstraram que alterações induzidas por lítio nos níveis de nucleosídeos trifosfatados no cérebro humano causaram uma redução de 25% nos níveis de fosfato inorgânico (Pi). Evidências também demonstraram que o lítio

pode agir através de vários sistemas de sinalização, apresentando efeitos neuroprotetores em uma variedade de insultos em neurônios em cultura a partir de modelos animais de doenças neurodegenerativas e em estudos com humanos (Chakraborty et al, 2008; Yucel et al, 2008). Um aumento da hidrólise de ATP e AMP foi demonstrado no hipocampo de ratos tratados cronicamente com lítio (Wilot et al., 2004). Em contraste, o lítio não afetou a atividade destas enzimas em estudos *in vitro* (Barcellos et al., 1998).

Desta maneira, o estudo da interação entre o sistema purinérgico, colinérgico e fármacos antidepressivos permitirá uma maior compreensão sobre os efeitos neuroquímicos e farmacológicos destes compostos em outros sistemas de neurotransmissão.

1.5 Peixe-zebra (*Danio rerio*) como modelo de estudos comportamentais e neuroquímicos.

O peixe-zebra, também conhecido como paulistinha ou zebrafish é um pequeno teleósteo (3-4 cm) de água doce da família *Cyprinidae* caracterizado por um padrão de coloração distinto, baseado em listras horizontais, claras e escuras e alternadas (Spence et al., 2008).

O número de estudos envolvendo este modelo animal tem aumentado consideravelmente a cada ano, pois o peixe-zebra tem sido utilizado como uma importante ferramenta para a realização de pesquisas nas áreas de bioquímica (Seibt et al., 2012), biologia do comportamento (Mathur & Guo, 2010; Sison & Gerlai, 2010), toxicologia, pesquisa transgênica, evolução do genoma vertebrado, teratologia (Edwards

& Michel, 2002; Yang et al., 2009), mutagênese e neurociências (Becker & Becker, 2008), além de contribuir com estudos farmacológicos (Bencan et al., 2009; Egan et al., 2009) e comportamentais (Mathur & Guo, 2010; Sison & Gerlai, 2011). O uso do peixe-zebra como um modelo para pesquisa possui diversas características favoráveis. Aspectos como seu baixo custo e pouco espaço para manutenção, bem como o seu rápido desenvolvimento e ciclo biológico o tornam um modelo de fácil manipulação (Spence et al., 2008). Outra vantagem do peixe-zebra é a sua alta capacidade de absorver compostos adicionados à água, pois possui sensibilidade para drogas e rápido metabolismo, dispensando assim a necessidade de tratamentos por meio de protocolos invasivos (Karlovich et al., 1998; Berghmans et al., 2007).

Muitos genes associados a doenças humanas, assim como seu padrão de expressão, já são conhecidos ou estão sendo estudados nesse modelo animal. Este teleósteo tem sido alvo para o desenvolvimento de modelos transgênicos que reproduzem patologias humanas, bem como desordens neurológicas (Bandmann & Burton, 2010; Sager et al., 2010). Além disso, o peixe-zebra possui alta homologia genética (70-80%) com roedores e humanos (Barbazuk et al.,2000; Miklósi & Andrew, 2006).

Os modelos animais podem facilitar a compreensão de mecanismos biológicos do comportamento humano, podendo ajudar no desenvolvimento de estratégias de tratamento para o comportamento humano anormal social, um sintoma principal em inúmeras condições clínicas. Estudos mostram que o peixe-zebra pode ser um bom modelo animal para o estudo destas patologias, pois pode ser utilizado para a análise de comportamentos sociais, além das suas características práticas e das inúmeras ferramentas genéticas desenvolvidas envolvendo esta espécie (Blaser & Peñalosa, 2011;

Padilla et al., 2011). Recentemente, o peixe-zebra emergiu como sendo um modelo complementar bastante útil para o estudo das funções neurocomportamentais, incluindo recompensa e funções cognitivas (Piato et al., 2011; Stewart et al., 2011).

Os principais neurotransmissores encontrados em mamíferos, incluindo as monoaminas (histamina, dopamina, norepinefrina, epinefrina, serotonina, melatonina) e ACh, bem como seus mecanismos de ação já foram descritos no peixe-zebra (Rinkwitz et al., 2011). Além disso, estudos demonstraram desordens no movimento e alterações neuroquímicas em peixe-zebra após exposição à fluoxetina (Airhart et al., 2007; Maximino et al., 2011). A análise da atividade locomotora, agressividade, interação social e aprendizado são paradigmas comportamentais que já foram descritos para esta espécie (Egan et al., 2009; Kurta & Palestis, 2010).

Alguns estudos sugerem que o peixe-zebra é uma ferramenta útil para análise das bases genéticas do comportamento e dos mecanismos biológicos induzidos por drogas que podem gerar alterações no cérebro adulto, mostrando que o peixe-zebra é um novo modelo a ser utilizado na pesquisa da neurociência comportamental (Gerlai et al., 2009), sendo também, um modelo de escolha para elucidar o desenvolvimento e a função do circuito neuronal (Rinkwitz et al., 2011).

2. OBJETIVOS

2.1 Objetivo geral

Considerando que (i) a adenosina é capaz de modular a liberação de serotonina, dopamina e noradrenalina, (ii) as ectonucleotidases e a adenosina desaminase representam a rota mais importante de formação e degradação de adenosina extracelular e (iii) o peixe-zebra é um modelo relevante para o estudo de diversas doenças humanas e para a triagem de novos fármacos, o presente trabalho teve como objetivo geral avaliar o efeito de fármacos antidepressivos sobre hidrólise de nucleotídeos e nucleosídeos extracelulares em cérebro de peixe-zebra.

2.2 Objetivos específicos

- Avaliar o efeito *in vitro* do lítio sobre a atividade e expressão gênica das NTPDases, ecto-5`-nucleotidases e acetilcolinesterase em membranas cerebrais de peixe-zebra;

- Avaliar o efeito *ex vivo* (tratamento subcrônico de 7 dias) do lítio sobre a atividade e expressão gênica das NTPDases, ecto-5`-nucleotidases e acetilcolinesterase em membranas cerebrais de peixe-zebra;

- Verificar o efeito do tratamento *ex vivo* agudo (1 hora) com fármacos antidepressivos, como fluoxetina, clomipramina, lítio e citalopram sobre a atividade das NTPDases e ecto-5'-nucleotidase em membranas cerebrais de peixe-zebra;

- Avaliar o efeito do tratamento *ex vivo* agudo (1 hora) com fármacos antidepressivos, como fluoxetina, clomipramina, lítio e citalopram sobre a atividade e expressão gênica da adenosina desaminase em membranas cerebrais e fração solúvel de peixe-zebra.

CAPÍTULO II

ARTIGO CIENTÍFICO

Inhibitory effect of lithium on nucleotide hydrolysis and acetylcholinesterase activity in zebrafish (*Danio rerio*) brain.

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Inhibitory effect of lithium on nucleotide hydrolysis and acetylcholinesterase activity in zebrafish (*Danio rerio*) brain

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ABSTRACT

Lithium has been used as an effective antimanic drug in humans and it is well known for its effects on neuropsychiatric disorders and neuronal communication. ATP and adenosine are important signaling molecules, and most nerves release ATP as a fast co-transmitter together with classical neurotransmitters such as acetylcholine. In this study, we evaluated the *in vitro* and *in vivo* effects of lithium on acetylcholinesterase and ectonucleotidase activities in zebrafish brain. There was a significant inhibition of ADP hydrolysis after *in vivo* exposure to lithium at 5 and 10 mg/l (27.6% and 29% inhibition, respectively), whereas an inhibitory effect was observed for AMP hydrolysis only at 10 mg/l (30%). Lithium treatment *in vivo* also significantly decreased the acetylcholinesterase activity at 10 mg/l (21.9%). The mRNA transcript levels of the genes encoding for these enzymes were unchanged after exposure to 5 and 10 mg/l lithium chloride. In order to directly evaluate the action of lithium on enzyme activities, we tested the *in vitro* effect of lithium at concentrations ranging from 1 to 1000 μM. There were no significant changes in zebrafish brain ectonucleotidase and acetylcholinesterase activities at all concentrations tested *in vitro*. Our findings show that lithium treatment can alter ectonucleotidase and acetylcholinesterase activities, which may regulate extracellular nucleotide, nucleoside, and acetylcholine levels. These data suggest that cholinergic and purinergic signaling may be targets of the pharmacological effects induced by this compound.

1. Introduction

Lithium is widely used in the treatment of bipolar disorder. Recent evidence has demonstrated that lithium can act through several signaling pathways and presents neuroprotective effects against a variety of insults in cultured neurons, in animal models of neurodegenerative diseases, and in human studies (Chakraborty et al., 2008; Yucel et al., 2008). One of the most extensively studied signaling pathways associated with the neuroprotective action of lithium is the inactivation of glycogen synthase kinase-3b (GSK3b), a proapoptotic enzyme responsible for hyperphosphorylation in Alzheimer's disease (Chakraborty et al., 2008).

Bipolar disorder, a neurological condition that causes cyclic variation in mood, drastically affects quality of life and significantly increases the chance of suicide in patients (Altamura et al., 2011; Ludtmann et al., 2011). This disease is defined by episodes of mania and hypomania, and its estimated worldwide occurrence is approximately 4% (Calabrese et al., 2003; Ketter, 2010).

ATP and adenosine are important signaling molecules in the central nervous system (Ralevic and Burnstock, 1998). The adenine nucleotide ATP is released at the synaptic cleft after nerve terminal depolarization, acting as a neurotransmitter or as a co-transmitter (Burnstock, 2009). ATP signaling is mediated by the cell-surface P2 receptors P2X and P2Y, which are a ligand-gated ion channel and metabotropic G protein-coupled receptor, respectively (reviewed in Burnstock, 2006). Extracellular ATP signaling is inactivated by the degradation of this nucleotide to adenosine by the action of ectonucleotidases. This group of enzymes includes the nucleoside triphosphate diphosphohydrolase (NTPDase) family that hydrolyzes both tri- and di-phosphonucleosides, and an ecto-5′-nucleotidase,

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which cleaves monophosphonucleosides to the respective adenosine nucleoside and controls purinergic neurotransmission (Robson et al., 2006; Schetinger et al., 2007). Adenosine may exert its action via the P1 receptors A_1, A_{2A}, A_{2B}, and A_3, which can inhibit (A_1 and A_3) or facilitate (A_{2A} and A_{2B}) neuronal communication (Fredholm et al., 2001;Fredholm, 2010). *In vivo* studies have shown that lithium alters ectonucleotidase activity in hippocampal synaptosomes (Wilot et al., 2004). However, no changes in these enzymes activities were observed in *in vitro* studies (Barcellos et al., 1998).

Most nerves release ATP as a fast co-transmitter together with classical fast transmitters such as acetylcholine, noradrenaline, and glutamate (Burnstock, 2004). Acetylcholine is a neurotransmitter secreted from the presynaptic nerve terminal and binds to acetylcholine receptors, which are clustered in the postsynaptic membrane. After being released, acetylcholine is cleaved into choline and acetate by acetylcholinesterase (AChE, EC 3.1.1.7), a fast serine hydrolase enzyme that regulates the concentration of the transmitter at the synapse (Soreq and Seidman, 2001). Studies have shown that adenosine is able to modulate acetylcholine release through inhibitory A_1 or facilitatory A_{2A} receptors (Rebola et al., 2002). Lithium is known to synergize the action of cholinomimetics in the central nervous system (Chaudhary and Gupta, 2001) and previous studies have shown that lithium treatment may alter the concentration of acetylcholine in the rat brain (Ronai and Vizi, 1975).

There has been growing interest in the development of novel animal models that could mimic human disease features and uncover cellular mechanisms involved in these pathologies (Rubinstein, 2003; Best and Alderton, 2008). The zebrafish, together with forward genetics and pharmacological interventions, has become a promising model to study many human diseases. In addition, drug mechanisms and several neurotransmitter systems, such as the purinergic and cholinergic systems, have been identified in zebrafish (Bertrand et al., 2001; Kucenas et al., 2006; Yi et al., 2006).

Due to the use of lithium for the treatment of mood disorders and the involvement of cholinergic and purinergic systems in several neuropsychiatric diseases, such as depression (Furey and Drevets, 2006; Burnstock, 2008), it is important to investigate whether these neurotransmitter systems may be involved in the therapeutic actions promoted by lithium. Therefore, the aim of this study was to test the *in vivo* and *in vitro* effects of lithium chloride on ectonucleotidase and acetylcholinesterase activities in zebrafish brain followed by a gene expression pattern analysis.

2. Methods

2.1. Animals

Adult (5–7 month-old), outbred, wildtype short-fin zebrafish of both sexes were obtained from a specialized commercial supplier (Redfish Agroloja Ltda., RS, Brazil) from a genetically heterogeneous (randomly bred) stock. The fish were acclimated to the laboratory environment for at least 14 days and housed in a 50-l tank with controlled water quality at 28 ± 2 °C and a density of up to five animals per liter. Animals were kept at a day/night cycle of 14:10 h and fed three times a day with commercial flakes. Fish were manipulated when healthy and free of any signs of disease, according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). The Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) approved the protocol under the number CEP 07/03854.

2.2. Chemicals

Lithium chloride (CAS No. 7447-41-8), Trizma Base, ethylenedioxy-diethylene-dinitrilo-tetraacetic acid (EDTA), ethylene glycol bis (beta-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), sodium citrate, Coomassie blue G, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, calcium, magnesium chloride, acetylthiocholine, and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.3. In vitro and in vivo treatments

For the *in vitro* assays, lithium chloride was added to reaction medium before the preincubation with the enzyme and was maintained throughout the enzyme assays. Lithium chloride was tested at final concentrations of 1, 10, 25, 50, 100 and 1000 μM. The range of doses tested was chosen according to previous studies performed in Wistar rats (Barcellos et al., 1998). For the control group, the enzyme assay was performed in the absence of lithium chloride (no drug added in the reaction medium).

For the *in vivo* assays, fish were kept in 10-l aquariums and exposed to water with 1, 5 and 10 mg/l lithium chloride (corresponding to 23, 118 and 236 μM, respectively) because lithium chloride is highly soluble in water (water solubility: 83.5 g/100 ml at 20 °C). The lithium chloride doses were chosen based on those used in previous studies with aquatic organisms (Kszos et al., 2003). For the control group, animals were exposed only to water. The lithium solution was replaced on the third treatment day, and the animals were maintained in the test aquarium for 7 days. After lithium exposure, the fish were euthanized and the brains were dissected.

2.4. Determination of ectonucleotidase activities

Preparation of brain membranes was performed as described previously by Barnes et al. (1993). For each membrane preparation, a pool of five whole zebrafish brains was used, which were homogenized briefly in 60 volumes (v/w) of chilled Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor-driven Teflon-glass homogenizer. The samples were centrifuged at $1000 \times g$ for 10 min and the pellet was discarded. The supernatant was centrifuged for 25 min at $40{,}000 \times g$. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris-citrate buffer, and centrifuged for 20 min at $40{,}000 \times g$. This freeze–thaw–wash procedure was used to ensure the lysis of the brain membranes. The final pellet, containing a mixture of intra- and extracellular brain membranes, was resuspended and used in the enzyme assays. All samples were maintained at 2–4 °C throughout preparation.

NTPDase and 5′-nucleotidase assays were performed as described previously (Rico et al., 2003; Senger et al., 2004). Zebrafish brain membranes (3 μg protein for NTPDase and 5 μg protein for 5′-nucleotidase) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the 5′-nucleotidase activity) 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 (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min by the addition of 200 μl trichloroacetic acid at a final concentration of 5%. The samples were chilled on ice for 10 min and 1 ml of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added in order to determine the amount of inorganic phosphate released (Pi) (Chan et al., 1986). After 20 min, quantification of Pi released was done spectrophotometrically at 630 nm. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation inactivated with trichloroacetic acid were used to correct for any non-enzymatic hydrolysis of substrates. Specific activity was expressed as nanomoles of Pi released per minute per

milligram of protein. Four different experiments were performed and the assays were run in triplicate.

2.5. Determination of acetylcholinesterase activity

Three whole zebrafish brains were pooled and homogenized on ice in 60 volumes (v/w) of Tris-citrate buffer in a motor-driven Teflon-glass homogenizer. The rate of hydrolysis of 0.8 mM acetylthiocholine was determined in a final volume of 2 ml with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB using a previously described method (Ellman et al., 1961). Before the addition of substrate, 10 μg of the protein sample was preincubated with the reaction medium described above for 10 min at 25 °C.

Acetylthiocholine hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2–3 min at 30-s intervals. Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of acetylthiocholine. The linearity of absorbance related to time and protein concentration was previously determined. Acetylcholinesterase activity was expressed as micromoles of thiocholine (SCh) released per hour per milligram of protein. Four different experiments were performed and the assays were run in triplicate.

2.6. Determination of protein concentration

Protein was measured using Coomassie Blue as the color reagent and bovine serum albumin as the standard (Bradford, 1976).

2.7. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Specific primers used were as follows: *entpd1* (DrNTPDase1F 5′CCCATGGC ACAGGCCGGTTG-3′ and DrNTPDase1R 5′GCAGTCT-CATGCCAGCCGTG-3′); *entpd2_mg* (DrNTPDase2_mgF 5′GG-AAGTGTTTGACTCGCCTTGCACG-3′ and DrNTPDase2_mgR 5′-CAGGACACAAGCCCTTCCGGATC-3′); *entpd2_mq* (DrNTPDase2_mqF 5′- CCAGCGGAT TTAGAGCACGCTG-3′ and DrNTPDase2_mqR 5′-GAAGAACGGCGGCACGCCAC-3′); *entpd2_mv* (DrNTPDase2_mvF 5′ GCTCATTTAGAGGACGCTGCTCGTG-3′ and DrNTPDase2_mvR 5′-GCAACGTTT TCGGCAGGCAGC-3′); *entpd3* (DrNTPDase3F 5′ TACT-TTCTTTGGACAGAGCAACCCTG-3′ and DrNTPDase3R 5′-AAGCATATA GCCCAGGGACCAGG-3′); *5′-nucleotidase* (DrCD73F 5′-ACCTCCGAG-GAGTGTCGC TTTCG-3′ and DrCD73R 5′-CCTTGTTGGGGACCAGCGGT-TC-3′); and *ache* (Forward 5′ CCAAAAGAATAGAGATGCCATGGACG-3′ and Reverse 5′TGTGATGTTAAGCAGACGAGGCAGG-3′). Optimal conditions for RT-PCR using these primers were determined as described previously (Rico et al., 2006; Appelbaum et al., 2007; Rosemberg et al., 2007). The *β-actin* primers (Forward 5′GTCCCT-GTACGCCTCTGGTCG-3′ and Reverse 5′-GCCGGACTCATCGTACTC-CTG-3′) were used according to Chen et al. (2004).

Immediately following *in vivo* treatments with 5 and 10 mg/l lithium chloride (described above), the animals were euthanized by decapitation and their brains were dissected from the cranial skull. For each sample, a pool of five zebrafish brains was used. Total RNA was isolated from zebrafish brain using the TRIzol reagent (Invitrogen) according to manufacturer's instructions. RNA was quantified by spectrophotometry and all samples were adjusted to 160 ng/μl. cDNA was synthesized using the SuperScript III First-Strand™ (Synthesis System for RT-PCR) Invitrogen Kit following supplier's instructions. One microliter of RT reaction mix was used as a template for each PCR reaction. PCR reactions for the *entpd2*, *entpd3*, *5′-nucleotidase*, and *β-actin* genes were performed in a total volume of 20 μl with a final concentration of 0.1 μM primers, 0.2 μM dNTPs, 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Invitrogen). The PCR conditions for NTPDase1 were as above, except that 1.5 mM $MgCl_2$ was used. PCR reactions for acetylcholinesterase were performed in a total volume of 25 μl, with a final concentration of 0.08 μM primers, 0.2 μM dNTPs, 2 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen). The following conditions were used for the PCR reactions: 1 min at 94 °C, 1 min at the appropriate annealing temperature (*entpd1*, *β-actin* and *5′-nucleotidase*: 54 °C; *entpd2* and *entpd3*: 64 °C; *ache*: 60 °C) and 1 min at 72 °C for 35 cycles. Post-extension at 72 °C was performed for 10 min. For each set of PCR reactions, a negative control was included. PCR products were analyzed on a 1.5% agarose gel containing ethidium bromide and visualized with ultraviolet light. The Low DNA Mass Ladder (Invitrogen) was used as a molecular marker and normalization was performed against the *β-actin* gene for quantification.

2.8. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and expressed as the mean ± SD of four different experiments ($n = 4$). Post hoc analysis using the Tukey multiple test range was performed, considering $P<0.05$ as significant.

3. Results

We tested the *in vivo* effects of three concentrations of lithium chloride (1, 5, and 10 mg/l, corresponding to 23, 118, and 236 μM, respectively) on ectonucleotidase and acetylcholinesterase activities in zebrafish brain. There were no significant changes in ATP hydrolysis at all lithium chloride concentrations tested (Fig. 1A). However, after 7 days, lithium chloride exposure inhibited ADP hydrolysis at 5 and 10 mg/l (27.6 and 29%, respectively, $P<0.05$) and AMP hydrolysis at 10 mg/l (30%, $P<0.05$) when compared to the control group (Fig. 1B and C). This same treatment decreased acetylcholinesterase activity from zebrafish brain homogenates at 10 mg/l (21.9%; $P<0.05$) (Fig. 1D).

The inhibition of ADP, AMP, and acetylthiocholine hydrolysis by lithium chloride exposure could be a consequence of transcriptional control and/or post-translational regulation. RT-PCR analyses were performed when kinetic alterations were observed. The results demonstrate that the *entpd*, *5′-nucleotidase* (Fig. 2A and C), and *ache* *m*RNA transcript levels (Fig. 2B and C) were unchanged after exposure to 5 or 10 mg/l lithium chloride.

To evaluate a possible direct effect of lithium chloride on ectonucleotidase and acetylcholinesterase activities, we have performed *in vitro* assays with lithium chloride concentrations ranging from 1 to 1000 μM. There were no significant changes to NTPDase and 5′-nucleotidase activities in zebrafish brain membranes in the presence of lithium chloride at all concentrations tested (Fig. 3A–C). In addition, acetylcholinesterase activity from zebrafish brain was also unaltered after lithium chloride exposure when compared to the control group (Fig. 3D).

4. Discussion

In the present study, we have shown that lithium chloride can alter *in vivo* ectonucleotidase and acetylcholinesterase activities in zebrafish brain. Lithium treatment inhibited ADP hydrolysis at 5 and 10 mg/l and AMP hydrolysis at 10 mg/l. Changes were not observed in ATP hydrolysis after *in vivo* exposure to lithium chloride. Interestingly, the exposure of zebrafish to 10 mg/l lithium chloride also inhibited acetylcholinesterase activity when compared to control group. Conversely, when directly added to the *in vitro* enzyme assays, it did not induce significant changes on ectonucleotidase and acetylcholinesterase activities. These results could be related to the fact that the *in vitro* experiments evaluate the direct effect of the drug on the enzyme without the influence of outside mechanisms, such as other cell signaling pathways. Indeed, the mechanism of lithium action may be related to the inhibition of inositol monophosphatase, which would affect the function of the phosphatidylinositol cycle (PI cycle)

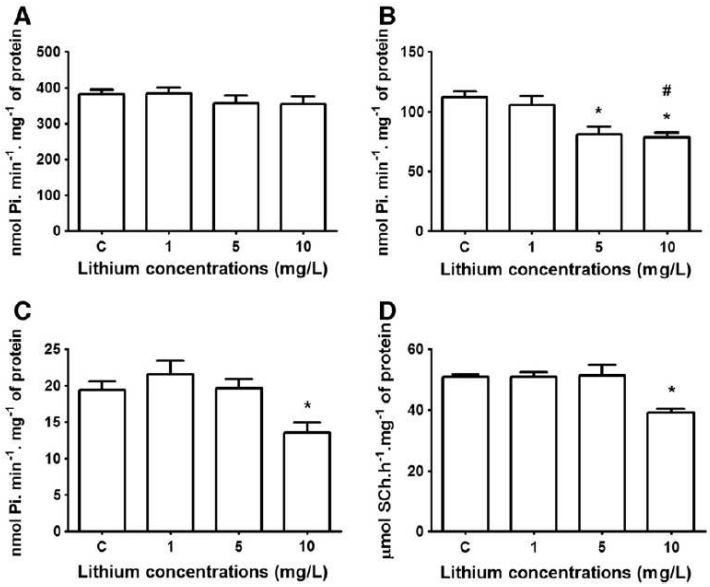

Fig. 1. *In vivo* effect of treatment with 1, 5, and 10 mg/l lithium chloride on NTPDase using ATP (A) or ADP (B) as substrates, 5′-nucleotidase (C) and acetylcholinesterase (D) activities in zebrafish brain. Data represent mean ± SEM of four different experiments ($n = 4$) performed in triplicate. *, difference when compared to the control group; #, difference when compared to the 1 mg/l lithium chloride-treated group. Data were analyzed statistically by one-way ANOVA followed by the post-hoc Tukey test; $P \leq 0.05$ was considered significant.

to cause accumulation of inositol phosphates and depletion of inositol (Shaldubina et al., 2001). Previous studies have shown that lithium decreased free inositol concentrations and increased inositol monophosphate (IP) concentrations in brain (Allison and Stewart, 1971). Therefore, our results indicate that the effect of lithium on ectonucleotidase and acetylcholinesterase activities is not related to a

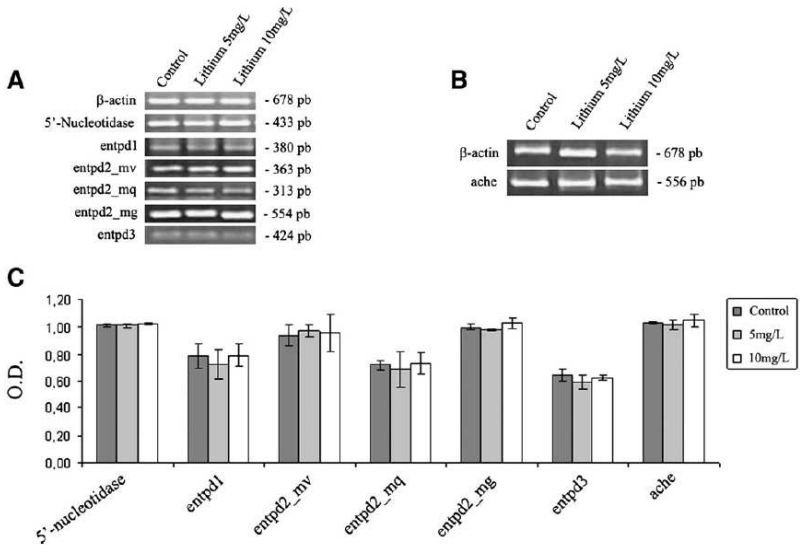

Fig. 2. Effect of treatment with 5 and 10 mg/l lithium chloride on ectonucleotidase and acetylcholinesterase mRNA transcripts. (A) A representative gel of *β-actin*, *entpd1*, *entpd2_mg*, *entpd2_mq*, *entpd2_mv*, *entpd3* and *5′-nucleotidase* mRNA expression in adult zebrafish. (B) A representative gel of acetylcholinesterase (*ache*) and *β-actin* mRNA expression in adult zebrafish brain. (C) Quantification using optical densitometry (O.D.) of the *entpd1*, *entpd2_mg*, *entpd2_mq*, *entpd2_mv*, *entpd3*, *5′-nucleotidase*, and *ache* genes versus *β-actin* (mean ± SD) of three independent experiments. The data were analyzed statistically by one-way ANOVA followed by the post-hoc Tukey test; $P < 0.05$ was considered significant.

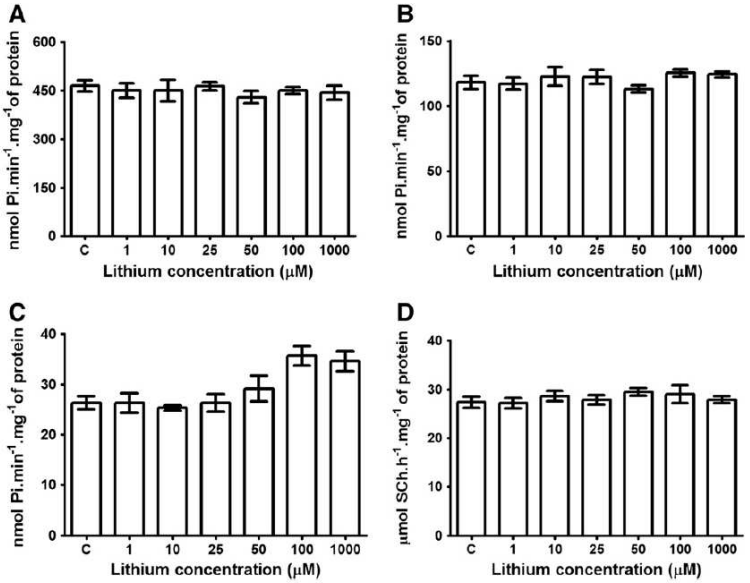

Fig. 3. *In vitro* effect of lithium chloride (1 to 1000 μM) on NTPDase using ATP (A) or ADP (B) as substrates, 5′-nucleotidase (C) and acetylcholinesterase (D) activities in zebrafish brain. Data represent mean ± SEM of four different experiments, each performed in triplicate.

direct action of this metal on the protein, but probably involves a post-transcriptional or post-translational modulation of these enzymatic activities.

The cholinergic system is one of the neurotransmitter systems implicated in the pathophysiologic mechanism of mood disorders (Shytle et al., 2002; Bertrand, 2005; Furey and Drevets, 2006). Acetylcholine is a neurotransmitter involved in essential brain functions, including memory and learning (Shaked et al., 2008). Lithium has been shown to modulate the levels of different neurotransmitters and could therefore improve learning, memory, cognition, and motor functions (Bhalla et al., 2010). Studies show that lithium may selectively interact with the cholinergic system (Williams and Jope, 1995; Bhalla et al., 2007). Lithium has been shown to potentiate seizures induced by pilocarpine, physostigmine, neostigmine and other cholinomimetics in the central nervous system, which may be prevented by either cholinergic antagonists or anticonvulsive drugs (Marinho et al., 1998; Chaudhary and Gupta, 2001). These findings indicate that lithium treatment stimulates cholinergic activity in certain brain regions, which may play a significant role on the therapeutic effect of lithium in neuropsychiatric disorders. Our results are in agreement with previous studies, as we observed a significant decrease in acetylcholine hydrolysis after lithium exposure. This finding reinforces the hypothesis that acetylcholine levels can be increased after lithium treatment, thereby modulating its effects on muscarinic receptors.

The roles of ATP as a neurotransmitter and adenosine as a neuromodulator have been studied extensively in the central and peripheral nervous systems. After ATP is released in the synaptic cleft, it can be hydrolyzed to ADP, AMP, and adenosine by ectonucleotidases, which is an important pathway for adenosine production (Zimmermann, 2006). Previous studies have shown hydrolysis of ATP and AMP was significantly increased in hippocampal synaptosomes of rats chronically treated with lithium, whereas no significant differences were observed in cortical synaptosomes (Wilot et al., 2004). In contrast, lithium did not affect the activity of these enzymes in *in vitro* studies (Barcellos et al., 1998). Acute and chronic lithium chloride exposure altered ATPase activities in several brain regions (McNulty et al., 1978). Studies have shown that chronic dietary lithium treatment appeared to reduce Na^+,K^+-ATPase activity in rat brain (Swann et al., 1980). In other studies, the activity of Na^+,K^+-ATPase is increased in membranes of intact synaptosomes in mouse brain after lithium treatment (Wood et al., 1989). Yildiz et al. (2005) have shown that lithium-induced alterations in nucleoside triphosphate levels in human brain caused a 25% reduction in Pi levels. Although lithium treatment induces controversial effects on ATP-metabolizing enzymes in brain, our findings have shown a significant inhibition of ADP and AMP hydrolysis, suggesting that lithium can exert a modulatory effect on ectonucleotidase activities and, consequently, on adenosine levels. Adenosine affects numerous physiological processes, including platelet aggregation, coronary vasodilation, lipolysis, and neuronal function in brain (Sebastião and Ribeiro, 2009). Studies have demonstrated neuroprotective actions of lithium against various insults in cultured cerebellar granule cells of rats and show that lithium protects against neuronal death caused by phenytoin and carbamazepine (Nonaka et al., 1998; Zhong et al., 2006). Therefore, the effect of lithium on this highly sophisticated pathway of ectonucleotidases may represent a tight control on adenosine levels, which can contribute to the neuroprotective effects of lithium.

Despite the neuroprotective actions described, lithium exposure can also induce toxic effects. Lithium has a profound effect on the development of diverse organisms (Klein and Melton, 1996). Most of the information on lithium toxicity related to aquatic organisms comes from studies on embryonic development (Selderslaghs et al., 2009). The irreversible neurologic lesions caused by lithium, particularly ataxia and dysarthria, are generally in the cerebellum (Kores and Lader, 1997). There is growing evidence that lithium can induce

chronic neurological sequelae. It has been suggested that lithium, cytokines, and neuroleptics synergize to disrupt calcium homeostasis in Purkinje cells and elicit calcium-mediated neurotoxicity (Grignon and Bruguerolle, 1996). Lithium toxicity may be life threatening or result in persistent cognitive and neurological impairment (Waring, 2006). Therefore, further studies evaluating chronic exposure to lithium in different doses will allow the investigation of the susceptibility of cholinergic and purinergic signaling as a target of neurotoxicological effects induced by this compound.

There are several mechanisms by which lithium could regulate acetylcholinesterase and NTPDase activity during *in vivo* experiments, including modifications at the transcriptional level and direct effects on the protein. In order to verify whether *ache*, *entpd* and *5′ nucleotidase* gene expression patterns were modulated when zebrafish were exposed to lithium chloride, we performed semi-quantitative RT-PCR experiments. Our results showed that *ache*, *entpd* and *5′ nucleotidase* mRNA transcript levels were unchanged in the lithium-treated group, suggesting that the change in the enzyme activities observed with lithium exposure was not directly related to changes in expression level.

In summary, our results demonstrate that purinergic and cholinergic systems are affected by lithium chloride exposure due to the inhibitory effect observed on ectonucleotidase and acetylcholinesterase activities in zebrafish brain. These findings may be related to an indirect effect promoted by lithium on NTPDase, 5′-nucleotidase, and acetylcholinesterase, since lithium did not significantly affect enzyme activity *in vitro*. These observations may represent a new mechanism underlying the neuroprotective and therapeutic effects of lithium. Furthermore, these findings contribute to a better understanding of lithium pharmacology and its interaction with purinergic and cholinergic neurotransmission.

Conflict of interest statement

Nothing declared.

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CAPÍTULO III

ARTIGO CIENTÍFICO

Antidepressants alter ectonucleotidase and adenosine deaminase activities in zebrafish *(Danio rerio)* brain.

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Antidepressants alter ectonucleotidase and adenosine deaminase activities in zebrafish *(Danio rerio)* brain

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Abstract

Clinical treatment of depression faces serious obstacles because the disease mechanism is not fully understood. Selective serotonin reuptake inhibitors and tricyclic antidepressants, such as fluoxetine and clomipramine, respectively, are commonly used in treatment for depression. Purines modulate the activity of diverse neurotransmitters involved in the pathophysiology of mood disorders, such as dopamine and serotonin. Furthermore, the involvement of purinergic system dysfunction in mood disorders has been described in diverse studies. In this study, we evaluated the *ex vivo* effects of fluoxetine, citalopram, and clomipramine on ectonucleotidase and ADA activities in zebrafish brain. Treatment with clomipramine showed an inhibition in the ecto-5'-nucleotidase acivity at the concentration of 5 μM when compared to the control group (26.87%; $P<0.05$). We also observed a significant inhibition in ADA activity after treatment with 5 and 10 μM clomipramine in zebrafish brain membranes (33.4% – 30.4%, respectively; $P<0.05$). However, treatments with fluoxetine and citalopram did not alter ectonucleotidase and ADA activities in the zebrafish brain. In conclusion, these results suggest that tricyclic antidepressants, such clomipramine, might modulate the extracellular nucleotide and nucleoside degradation, controlling the adenosine levels. Our findings may contribute to a better understanding of pharmacology of antidepressants and their interaction with the purinergic neurotransmission.

Keywords: antidepressants, adenosine deaminase, nucleoside triphosphate diphosphohydrolase, ecto-5’-nucletidase, zebrafish.

1. Introduction

Major depressive disorder is a widespread illness of great socioeconomic impact. According to the World Health Organization, it will be the second leading cause of disability in terms of burden disease in the future (Antonioli et al., 2012; Wang et al., 2012). Depression is a common psychiatric disorder characterized by a number of signs and symptoms which may include depressed mood, anhedonia, insomnia, anorexia, difficulty in concentration, and suicidal thought (Hashemi et al., 2012; Cavanagh et al., 2011).

Clinical treatment of depression faces serious obstacles because the disease mechanism is not fully understood (Haenisch et al., 2011). Tricyclic antidepressant (TCA) drugs have been employed widely as effective therapeutic agents for the treatment of affective disorder (Sanganahalli et al., 2000). The discovery of selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs) have changed important aspects of clinical treatment (Rosenzweig-Lipson et al., 2007; Gartlehner et al., 2011). Despite the beneficial effects induced by antidepressants, some adverse effects are induced by these drugs, especially related to memory and cognition. Memory deficit that has been observed differ widely between antidepressants due to their pharmacological properties (Naudon et al., 2007). Studies have shown that antidepressants with more selective actions on serotonergic neurotransmission, as SSRIs, induce less adverse effects, but appear not totally devoid of cognitive effects (Gorenstein et al., 2006).

Purines modulate the activity of diverse neurotransmitters involved in the pathophysiology of mood disorders, such as dopamine and serotonin (Burnstock et al., 2007). ATP and adenosine are signaling molecules involved in brain function with

regulatory effects on cognition, sleep, motor activity, memory, learning, and social interaction (Yacoubi et al., 2001). It has been proposed that extracellular ATP evokes responses through two general classes of extracellular receptors, the ionotropic P2X receptors and the metabotropic P2Y (for a review see Burnstock, 2006). ATP can be co-stored and co-released with other neurotransmitters: γ-aminobutyric acid (GABA), noradrenaline or glutamate (Abbracchio et al., 2009). After release, ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases (Robson et al., 2006). This group of enzymes includes the nucleoside triphosphate diphosphohydrolase (NTPDase) family that hydrolyzes both tri- and di-phosphonucleosides, and an ecto-5′-nucleotidase, which cleaves monophosphonucleosides to the respective nucleoside adenosine (Robson et al., 2006; Schetinger et al., 2007). Adenosine is a neuromodulator, which influences neuronal activity via P1 receptors, named A_1, A_{2A}, A_{2B} and A_3 (for a review, see Fredholm et al., 2011). The extracellular adenosine levels are controlled by the action of the ecto-adenosine deaminase (ADA), producing inosine or by nucleoside transporters (Latini & Pedata, 2001; Iwaki-Egawa et al., 2004).

The involvement of purinergic system dysfunction in mood disorders has been described in diverse studies (Machado-Vieira et al., 2010; Burnstock et al., 2011). Studies have shown that adenosine deaminase (ADA) levels increase in the depressive patients, and this increase persists after antidepressant treatment (Herken et al., 2007). An antidepressant effect of adenosine has been reported in mice, apparently involving adenosine A_1 and A_{2A} receptors (Burnstock, 2008). However, studies have demonstrated that adenosine A_{2A} receptor antagonists produce an antidepressant-like effect in animal models (Kaster et al., 2004).

As a model for use in neuroscience, the zebrafish (*Danio rerio*) is a small teleost widely used in biochemical studies (Rubinstein, 2006) and has been recently established as a very powerful model system for the genetic basis of brain development and diseases (Bandmann & Burton, 2010; Wong et al., 2010). Studies from our laboratory demonstrated the presence of NTPDases, ecto-5´-nucleotidase, and adenosine deaminase activities in zebrafish brain (Rico et al., 2003; Senger et al., 2004; Rosemberg et al., 2008).

Considering that purinergic signaling is involved in various pathological conditions including neuropsychiatric diseases, such as depression and that zebrafish may be an relevant vertebrate model system for numerous human diseases, the aim of this study was to verify *ex vivo* effects of different antidepressants, as fluoxetine, citalopram, and clomipramine on ectonucleotidases and ADA activities in zebrafish brain.

2. Methods

2.1. Animals

Adult zebrafish (*Danio rerio*; age around 2–3 months) of both sexes were obtained from commercial supplier (Delphis, RS, Brazil). All fish were acclimated to their new environment for at least 2 weeks in 50-L conditioned at 25±2 ◦C under natural light–dark photoperiod. They were used according to the National Institute of Health Guide for Care and Use of Laboratory Animals, being healthy and free of any signs of disease. The Ethics Committee of Pontifical Catholic University of the Rio Grande do Sul (PUCRS) approved the protocol under the number CEUA 10/00219.

2.2. Chemicals

Clomipramine (CAS No. 303-49-1), fluoxetine (CAS No. 54910-89-3), citalopram (CAS No. 59729-33-8), Trizma Base, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides (ATP, ADP and AMP), adenosine, calcium, magnesium chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phenol and sodium nitroprusside were purchased from Merck (Darmstadt, Germany). All reagents used were of analytical grade.

2.3. Treatments

Fish were kept in 1-L aquariums and exposed to water with three different concentrations of fluoxetine (1, 5, and 10 µM), clomipramine (1, 5, and 10 µM) and citalopram (70, 150 and 300 µM). The doses have been chosen according previous studies testing antidepressant drugs in zebrafish (Airhart et al., 2007; Sackerman et al., 2010). For the control group, the animals were exposed only to water in a test aquarium. Treated and control animals were maintained in the test aquarium for 1 h and, immediately after the exposure, the fish were euthanized and the brains were dissected. The drug solutions were changed for each experiment.

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 five brains. 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). The homogenates were centrifuged at 800 × g for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at 40 000 × g. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. For soluble ADA activity experiments, the supernatant was collected and kept on ice for enzyme assays. The pellets of membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers and centrifuged for 20 min at 40 000 × 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 Nucleotide hydrolysis assay

NTPDase and 5'-nucleotidase assays were performed as described previously (Rico et al, 2003; Senger et al, 2004). Zebrafish brain membranes (3 µg protein for NTPDase and 5 µg protein for 5'-nucleotidase) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the 5'-nucleotidase activity) at a 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 (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min by the addition of 200 µl

trichloroacetic acid at a final concentration of 5%. The samples were chilled on ice for 10 min and 1 ml of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added in order to determine the inorganic phosphate released (Pi) (Chan et al, 1986). After 20 min, the quantification of inorganic phosphate (Pi) released was determined spectrophotometrically at 630 nm. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity was expressed as nanomoles of Pi released per minute per milligram of protein. Four different experiments were performed and the assays were run in triplicate.

2.6. Adenosine deaminase assay

Adenosine deaminase activity was determined using a Berthelot reaction as previously reported (Weisman et al., 1988). 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 the assays with soluble and membrane fractions, respectively, in a final volume of 200 ml. The samples were preincubated for 10 min at 37 °C, and the reaction was initiated by the addition of adenosine to a final concentration of 1.5 mM. The reaction was stopped by the addition of 500 µl of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml) after incubation for 75 min (soluble fraction) or 120 min (membrane fraction). Controls with the addition of the enzyme preparation after mixing with the phenol-nitroprusside reagent were used to correct for non-enzymatic hydrolysis of substrates. The reaction mixtures were immediately mixed to 500 µl of alkaline-hypochlorite reagent (sodium

hypochlorite to 0.125% available chlorine, in 0.6MNaOH) 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. Specific activity was expressed as nmol of NH_3 min^{-1} mg^{-1} of protein.

2.7. Protein determination

Protein was measured using Coomassie Blue as color reagent (Bradford, 1976) and bovine serum albumin as a standard.

2.8 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), being expressed as means ± S.E.M of four different experiments (n = 4). A Tukey multiple test range as post-hoc was performed, considering $P < 0.05$ as significant.

3. Results

The experiments have been performed after a 1h-exposure to fluoxetine (1, 5, and 10 μM), clomipramine (1, 5, and 10 μM), or citalopram (70, 150, and 300 μM). We tested the *ex vivo* effect of these antidepressant drugs on ectonucleotidases and ADA activities in zebrafish brain. Our results have demonstrated that fluoxetine and citalopram treatments in all concentrations tested did not alter NTPDase and 5′-nucleotidase activities in zebrafish brain membranes. There were no significant effects of clomipramine on ATP and ADP hydrolysis in zebrafish brain membranes (Fig. 1A-E). However, clomipramine inhibited AMP hydrolysis at 5 μM when compared to the control group (26.9%; $P<0.05$) (Fig. 1F).

The effect of clomipramine was also observed on ADA activity in soluble and membrane fractions of zebrafish brain. In membrane fractions, inhibition was observed at the concentrations of 5 and 10 µM (33.4% – 30.4%, respectively; $P<0.05$) (Figure 2A). However, the soluble ADA activity was not altered by clomipramine exposure (Figure 2B). Fluoxetine and citalopram, at all concentrations tested, did not alter ADA activity in both membrane and soluble fractions of zebrafish brain (data not shown).

4. Discussion

Major depression is a multifactorial and complex disorder, where many theories have been put forward to account for depression, as well as antidepressant activity, but none of them is exhaustive. Furthermore, antidepressant pharmacotherapy is the most often used treatment for depression, but the exact mechanism of action underlying its therapeutic effect is not completely clear (Antonioli et al., 2012). In the present study, we verified whether antidepressant drugs alter ectonucleotidases and ADA pathway in zebrafish brain. Our results showed that fluoxetine and citalopram treatment in all concentrations tested did not alter ectonucleotidases and ADA activities. However, clomipramine treatment inhibited ecto-5´-nucleotidase activity at 5 µM and ADA activity at 5 and 10 µM in zebrafish brain membranes.

Adenosine acts as an extracellular signaling molecule influencing synaptic transmission and modulating the activity of the nervous system and is apparently involved in many neuropathological conditions (Ribeiro et al., 2003). Several studies have shown the involvement of adenosine in the pathophysiology of depression and in antidepressant action (Phillis, 1984; Berk et al., 2001; Gass et al., 2010). Stimulation of presynaptic adenosine A_1 receptors decreases the probability of neurotransmitter

release, whereas activation of presynaptic adenosine A_{2A} receptors enhances neurotransmitter release (Yawo and Chuhma, 1993; Lopes et al., 2002).

The involvement of adenosine A_1 and A_{2A} receptors in the modulation of depression has been confirmed in several studies (Okada et al., 1999; Berk et al., 2001; Lobato et al., 2008). Adenosine administration produces an antidepressant-like effect in mice, apparently mediated through an interaction with A_1 and A_{2A} receptors (Kaster et al., 2004). On the other hand, adenosine and its analogues have been shown to induce depressant-like action (El Yacoubi et al., 2003). El Yacoubi et al. (2001) have demonstrated an antidepressant-like effect in A_{2A} receptor knockout mice or A_{2A} receptor antagonist-treated rats in the tail suspension and forced swim tests. This difference probably depends on the applied dose and the differences in the animal model and/or procedures employed (Kaster et al., 2004). Either A_1 or A_{2A} recpetors are capable of forming heteromers with other G-protein receptors, such as dopamine, glutamate, and ATP receptors (Agnati et al., 2003). Cell-surface ADA needs to be anchored to the plasma membrane by means of specific receptors. Studies suggest that ADA exerts a control of the function of A_{2A} receptors homomers by a strong modification of their quaternary structure. Furthermore, the ADA-induced structural changes in the A_{2A} receptor molecule correlated with marked affinity modifications in the binding of both agonist and antagonist. Thus, the ADA-induced increase in the ligand affinities indicates that ADA behaved as a positive modulator of A_{2A} receptors (Gracia et al., 2011). Ecto-ADA is able to transmit signals when interacting with A1 receptor. In this way, it acts as a co-stimulatory molecule which facilitates a variety of specific signalling events in different cell types (Franco et al., 1997). Studies reported that ADA levels increase in the depressive patients, and this increase persists after the treatment with selective serotonin reuptake inhibitors (Herken et al., 2007). However,

Elgun et al. (1999) found decreased ADA activity in major depression, and there was an inverse correlation between ADA level and symptoms of major depression. We observed a significant decrease in ADA activity from membrane fractions, whereas we did not observed significant changes in soluble ADA activity. Such results suggest that clomipramine modulate the enzyme involved in the control of extracellular adenosine levels, indicating an increase in the concentration of this neuromodulator. However, we also observed an inhibition of AMP hydrolysis promoved by clomipramine. Therefore, these finding suggest a compensatory mechanism in order to maintain normal levels of adenosine, returning the adenosinergic signaling to the basal levels.

The involvement of adenosine in depression has also been supported by other indirect evidence showing that classical tricyclic antidepressants, such as nortriptiline, clomipramine or desipramine, can bind to adenosine receptors and reduce the activity of ectonucleotidases in cortical nerve terminals (Shen and Chen, 2009). Different studies have demonstrated the effect of antidepressant drugs in ATPase activities. Tricyclic antidepressant such as imipramine, amitriptyline, and nortriptyline inhibited Na^+, K^+-ATPase activity in synaptosomal membrane of rat brain (Sanganahalli et al., 2000). Pedrazza et al. (2007) have demonstrated that NTPDase activity from cerebral cortex and hippocampus of rats was decreased by the antidepressants sertraline and clomipramine after *in vitro* exposure. Furthermore, during chronic treatment, fluoxetine and nortriptyline changed NTPDase and ecto-5′-nucleotidase activities in cerebral cortex of rats (Pedrazza et al., 2008). In contrast, in acute treatment, ATP and ADP hydrolysis was decreased after administration of nortriptyline in hippocampus whereas only ADP hydrolysis was increased in cerebral cortex of rats (Pedrazza et al., 2008). Previous studies have shown that TCAs can alter ATPase activities due to the hydrophobicity of these drugs once the partitioning of the drugs into lipid bilayer affects

the membrane fluidity and consequently changing the membrane protein function and structure (Barcellos et al., 1998; Zanatta et al., 2001). Ecto-5´-nucleotidase is attached via a GPI (glycosylphosphatidylinositol) anchor to the extracellular membrane whereas NTPDases1, 2, 3 and 8 are firmly anchored to the membrane via two transmembrane domains. Thus, different ways of anchoring, are important for maintaining catalytic activity and substrate specificity (Grinthal and Guidotti, 2006; Sträter, 2006). The differences in membrane anchorage of these enzymes can be related to the different effects promoted by antidepressant drugs on NTPDase and ecto-5´-nucleotidase activities.

In conclusion, the findings presented in this study show that tricyclic drugs can affect the ecto-5'-nucleotidase and promote a decrease in adenosine deamination in zebrafish brain, suggesting that tricyclic antidepressants, such clomipramine, can modulate the extracellular adenosine levels. These observations may indicate another pharmacological mechanism of these antidepressants, which may be involved in the therapeutic effects or adverse effects induced by tricyclic drugs.

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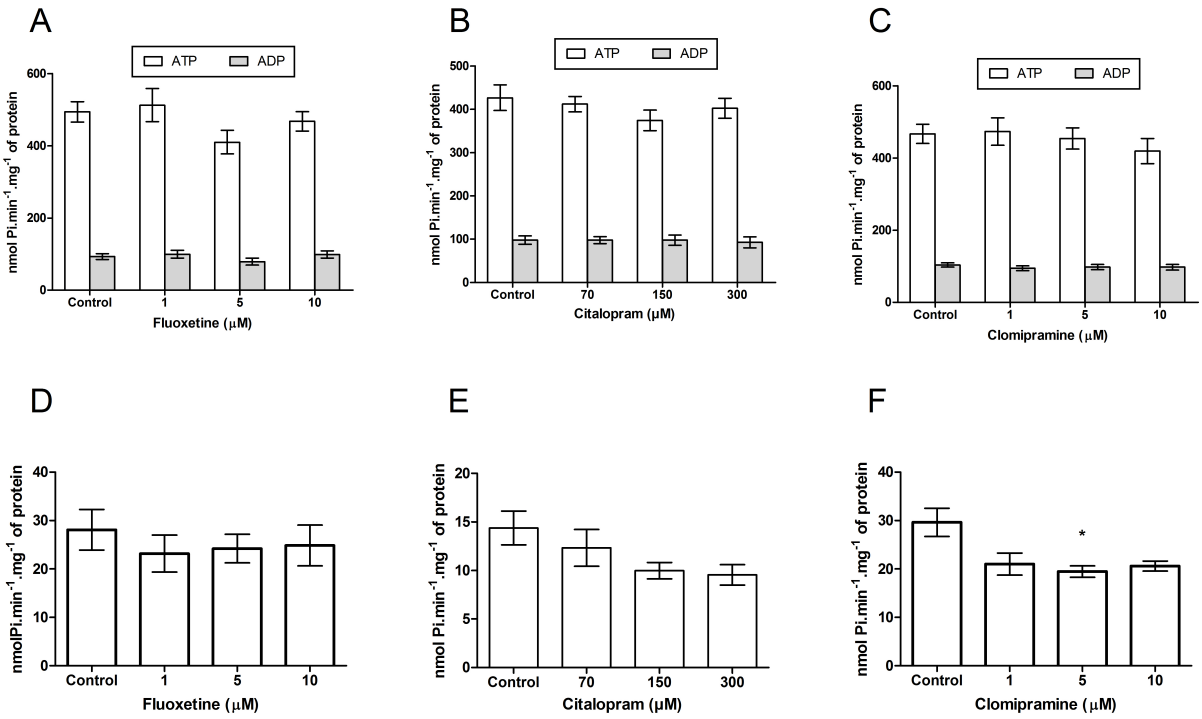
Figure Legends

Figure 1: Effect of fluoxetine (A), citalopram (B) and clomipramine (C) on NTPDase activities using ATP or ADP as substrates and effect of treatment with fluoxetine (D), citalopram (E) and clomipramine (F) on ecto-5´-nucleotidase activity in zebrafish brain. Data represent mean ± S.E.M of four different experiments (n=4) performed in triplicate. The symbol (*) represents a significant difference from control group (one-way ANOVA, followed by Tukey test as post hoc, $P \leq 0.05$). The specific enzyme activity is reported as nmol of Pi min^{-1} mg^{-1} of protein.

Figure 2: Effect of clomipramine on ADA activity from membrane (A) and soluble (B) fractions of zebrafish brain. Data represent mean ± S.E.M of four different experiments (n=4) performed in triplicate. The symbol (*) represents a significant difference from control group (one-way ANOVA, followed by Tukey test as post hoc, $P \leq 0.05$). The specific enzyme activity is reported as nmol of NH_3 min^{-1} mg^{-1} of protein.

Figure 1


A
ATP
ADP
nmol Pi.min-1.mg-1 of protein
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Fluoxetine (μM)
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nmol Pi.min-1.mg-1 of protein
500
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100
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Control
70
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Citalopram (μM)
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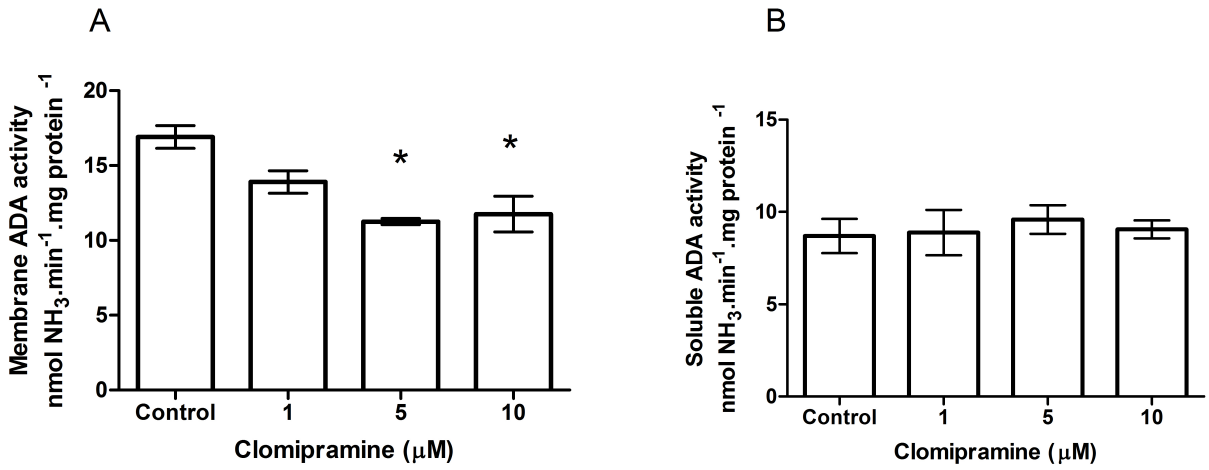
Figure 2


A
Membrane ADA activity
nmol NH3.min-1.mg protein -1
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Clomipramine (μM)
B
Soluble ADA activity
nmol NH3.min-1.mg protein -1
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Clomipramine (μM)

CAPÍTULO IV

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A depressão é um transtorno psiquiátrico comum, de grande impacto socioeconômico podendo ser caracterizada por uma série de sinais e sintomas que podem incluir humor deprimido, insônia e dificuldade de concentração (Wang et al., 2012; Hashemi et al., 2012). Além disso, apesar dos avanços importantes nas últimas décadas, o mecanismo da doença não é totalmente elucidado, o que dificulta o tratamento clínico da depressão (Haenisch et al., 2011). Os primeiros tratamentos para este transtorno psiquiátrico basearam-se na ação de fármacos IMAO e TCA (Dardennes et al., 1999). Após, surgiram novas classes de antidepressivos, como os ISRSs e os ISRNs que trouxeram benefícios no tratamento clínico da doença (Rosenzweig-Lipson et al., 2007). Pesquisas em animais e humanos permitem identificar uma série de anormalidades que compõem um modelo psicobiológico da fisiopatologia da depressão. As principais características estão relacionadas com a diminuição da neurotransmissão monoaminérgica (Krishnan & Nestler, 2008), baixas concentrações de BDNF (Piccinni et al., 2009; Domschke et al., 2010), citocinas elevadas, desregulação no eixo hipotálamo-pituitária-adrenal e susceptibilidade genética (Ruhe et al., 2007; Frodl et al., 2008; Palazidou et al., 2012). O desenvolvimento de novos fármacos antidepressivos tem sido baseado, principalmente no aumento da neurotransmissão monoaminérgica, apresentando uma melhora na eficácia terapêutica e diminuição dos efeitos adversos (Connolly & Thase, 2012). Desta maneira, estudos se tornam importantes a fim de melhorar a compreensão sobre as alterações causadas pela depressão e os tratamentos farmacológicos usados na clínica.

Neste estudo, nós trabalhamos com quatro fármacos antidepressivos, fluoxetina, citalopram, clomipramina e lítio, sendo este último, um fármaco bastante utilizado

clinicamente para tratar todas as fases do transtorno bipolar, incluindo a depressão aguda (Tkatcheva et al, 2007; Chang & Ha, 2011). Estes fármacos foram analisados sobre parâmetros bioquímicos em cérebro de peixe-zebra. Os modelos animais podem facilitar a compreensão de mecanismos biológicos do comportamento humano, além de serem essenciais para o desenvolvimento de perfis farmacológicos de novos fármacos. Recentemente, o peixe-zebra emergiu como um modelo complementar para o estudo das funções neurocomportamentais (Gerlai et al., 2009; Piato et al., 2011), sendo também, um modelo de escolha para elucidar o desenvolvimento e a função do circuito neuronal (Rinkwitz et al., 2011).

Os sistemas purinérgico e colinérgico são importantes vias de sinalização do SNC. Além disso, estes sistemas estão amplamente distribuídos e envolvidos em diversos mecanismos de controle neuronal (Zimmermann, 2008). Portanto no segundo capítulo deste trabalho, nós avaliamos o efeito *in vitro* e *ex vivo* do lítio (tratamento subcrônico de 7 dias) sobre a atividade e expressão gênica das NTPDases, ecto-5`-nucleotidase e acetilcolinesterase em membranas cerebrais de peixe-zebra. No tratamento *ex vivo*, nós testamos as concentrações de 1, 5 e 10 mg/L. A exposição ao lítio inibiu a hidrólise de ADP nas concentrações de 5 e 10mg/L (27,6 e 29% respectivamente) e inibiu a hidrólise de AMP na concentração de 10mg/L (30%) quando comparado ao grupo controle. Este mesmo tratamento diminuiu a atividade da AChE na concentração de 10mg/L (21,9%). Na análise do padrão de expressão não foram observadas alterações significativas nas concentrações testadas. No tratamento *in vitro*, testamos uma faixa de concentrações que varia de 1 a 1000µM. Não foram observadas alterações significativas na atividade das ectonucleotidases e AChE nas concentrações testadas. Nossos resultados mostraram que o tratamento com o lítio pode regular os

níveis de nucleotídeos e nucleosídeos extracelulares e os níveis de acetilcolina. O fato de não observarmos uma alteração nos testes *in vitro* pode estar relacionado com o efeito direto da droga sobre a enzima, sem a influência de mecanismos exteriores, tais como, outras vias de sinalização. O mecanismo de ação do lítio pode estar relacionado com a inibição da enzima inositol monofosfatase, que converte o inositol monofosfato em inositol, no qual poderia afetar as funções do ciclo fosfatidilinositol resultando em depleção do inositol livre (Shaldubina et al., 2001). Assim, nossos resultados indicam que o efeito do lítio observado sobre a atividade das ectonucleotidases e AChE não está relacionado somente com uma ação direta deste metal sobre a proteína, mas provavelmente envolve uma modulação pós-transcricional ou pós-traducional destas enzimas. Estudos mostram que o lítio pode interagir seletivamente com o sistema colinérgico (Bhalla et al., 2007). Além disso, o lítio pode potencializar convulsões induzidas por pilocarpina no SNC no qual podem ser prevenidas por antagonistas colinérgicos (Marinho et al., 1998; Chaudhary & Gupta, 2001). Esses achados indicam que o tratamento com lítio estimula atividade colinérgica em certas regiões do cérebro, que pode desempenhar um papel significativo sobre o efeito terapêutico de lítio em distúrbios neuropsiquiátricos. Em nosso estudo, observamos um decréscimo significativo na hidrólise da acetilcolina após exposição ao lítio. Este achado reforça a hipótese de que os níveis de acetilcolina podem estar aumentados após o tratamento com lítio, modulando os seus efeitos sobre os receptores muscarínicos. O lítio pode agir através de vários sistemas de sinalização, apresentando efeitos neuroprotetores contra uma variedade de insultos em neurônios cultivados em modelos animais portadores de doenças neurodegenerativas (Chakraborty et al, 2008; Yucel et al, 2008). Alguns estudos mostram que a exposição ao lítio pode alterar a atividade da ATPase em várias regiões cerebrais (McNulty et al., 1978). Contudo, estudos anteriores já haviam

demonstrado alterações na atividade da Na^+, K^+- ATPase em cérebros de ratos após tratamento com lítio (Swann et al., 1980; Wood et al., 1989). Os nossos resultados demonstraram uma inibição significativa na hidrólise de ADP e AMP, sugerindo que o lítio pode modular a atividade das ectonucleotidades e consequentemente os níveis de adenosina. Desta maneira, o efeito do lítio sobre a via das ectonucleotidases pode representar um controle sobre os níveis de adenosina, o que poderia estar contribuindo para os efeitos neuroprotetores do lítio.

No terceiro capítulo deste trabalho, nós continuamos analisando as alterações bioquímicas induzidas pelos fármacos antidepressivos no peixe-zebra. O nosso objetivo neste capítulo foi verificar o efeito do tratamento e*x vivo* agudo (1 hora) com antidepressivos, como fluoxetina, clomipramina e citalopram sobre a atividade das ectonucleotidases e ADA em cérebro de peixe-zebra. Nossos resultados mostraram que o tratamento com clomipramina inibiu a atividade da ecto-5´-nucleotidase na concentração de 5µM quando comparado ao grupo controle (26.9%). Na atividade da ADA também observamos uma inibição significativa no tratamento com clomipramina nas concentrações de 5 e 10 µM em frações de membrana de cérebro de peixe-zebra (33.4% e 30.4%, respectivamente). No entanto, o tratamento com fluoxetina e citalopram não alterou a atividade das ectonucleotidases e ADA no cérebro do peixe-zebra.

A adenosina age como um importante neuromodulador (Burnstock et al., 2011) e está envolvida na regulação de importantes mecanismos do SNC (Cunha et al., 2008), como estados de ansiedade e desordens psiquiátricas (Ruby et al., 2010; Asatryan et al., 2011). O receptor de adenosina A_{2A} está no centro de uma rede de neuromoduladores, afetando uma ampla gama de funções neuropsiquiátricas através da interação e

integração com vários sistemas de neurotransmissores, especialmente sistema dopaminérgico e glutamatérgico (Shen & Chen, 2009). Além disso, o envolvimento dos receptores de adenosina A_1 e A_{2A} na modulação da depressão têm sido confirmado através de vários estudos (Okada et al., 1999; Berk et al., 2001; Lobato et al., 2008). Evidências mostram o envolvimento da adenosina na depressão, uma vez que, antidepressivos tricíclicos clássicos, podem se ligar a receptores de adenosina e reduzir a atividade das ectonucleotidases em terminais nervosos (Shen & Chen, 2009). Em nosso estudo, mostramos que o tratamento com antidepressivos tricíclicos inibiu a hidrólise do nucleotídeo AMP e também promoveu a inibição da atividade da ADA, sugerindo a manutenção dos níveis normais de adenosina, a fim de manter a sinalização adenosinérgica nos níveis basais. Além disso, evidências mostram que os TCAs podem alterar a atividade das ATPases devido a hidrofobicidade destes fármacos, uma vez que a sua partição pela bicamada lipídica pode afetar a fluidez da membrana e consequentemente alterar a função e estrutura das proteínas de membrana (Barcellos et al., 1998; Zanatta et al., 2001). Em resumo, as alterações observadas neste estudo, permitem sugerir que, antidepressivos tricíclicos como a clomipramina, podem modular os níveis extracelulares de adenosina. Portanto, estas observações podem indicar um outro mecanismo farmacológico destes fármacos antidepressivos, o que permite melhorar a compreensão dos efeitos terapêuticos de fármacos antidepressivos tricíclicos.

Os resultados apresentados nesta Dissertação podem contribuir para uma melhor compreensão da farmacologia dos fármacos aqui estudados, bem como a sua interação com a neurotransmissão colinérgica e purinérgica. Portanto, estas informações podem representar um novo mecanismo subjacente aos efeitos neuroprotetores do lítio, bem como com relação aos efeitos terapêuticos dos fármacos antidepressivos.

Perspectivas

- Avaliar o efeito da exposição crônica aos antidepressivos, fluoxetina, clomipramina e citalopram sobre a atividade e padrão de expressão gênica das ectonucleotidades e adenosina desaminase em cérebro de peixe-zebra;

- Avaliar o efeito da exposição à reboxetina, um fármaco inibidor seletivo da recaptação de noradrenalina sobre a atividade e padrão de expressão gênica das ectonucleotidases e adenosina desaminase em cérebro de peixe-zebra.

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