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Nucleotidases e depressão: efeito de fármacos antidepressivos no catabolismo do ATP extracelular

Dissertação apresentada ao
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Resumo

A depressão é a manifestação mais comum dos distúrbios afetivos. É uma das doenças mais debilitadoras e causa problemas tanto para o indivíduo quanto para a sociedade e, se não for tratada, pode levar ao aumento da morbidade e mortalidade. O uso de fármacos antidepressivos é a base dos tratamentos para depressão. Os inibidores seletivos da recaptação de serotonina, como a fluoxetina e a sertralina, e os antidepressivos tricíclicos, nortriptilina e clomipramina, são constantemente utilizados no tratamento para a depressão. Evidências têm demonstrado o importante papel desempenhado pelo ATP e a adenosina no sistema nervoso central. O ATP pode ser armazenado e co-liberado juntamente com outros neurotransmissores, tais como a noradrenalina e a serotonina. O ATP extracelular pode ser hidrolisado até adenosina pela ação de um grupo de ecto-nucleotidases. Estas ecto-enzimas promovem a conversão enzimática de ATP, controlando os níveis deste nucleotídeo e do seu nucleosídeo adenosina. Dentro do grupo das ecto-nucleotidases, podemos destacar a família das NTPDases (Nucleosídeo trifosfato difosfoidrolases) e a ecto-5´-nucleotidase. Considerando que: (i) a adenosina exerce um importante papel neuromodulador, (ii) o ATP pode ser co-liberado com serotonina e noradrenalina e (iii) as ecto-nucleotidases representam a principal rota de formação de adenosina extracelular através do catabolismo do ATP, torna-se importante avaliar o efeito de fármacos antidepressivos sobre a via das ecto-nucleotidases. Fluoxetina, sertralina, nortriptilina e clomipramina foram testadas no tratamento *in vitro*, nas concentrações de 100, 250 e 500 μM no soro e em sinaptossomas de hipocampo e córtex cerebral de ratos. A hidrólise de ATP e ADP foram inibidas de maneira dose-dependente no tratamento *in vitro* realizado com os quatro fármacos em sinaptosomas de hipocampo e córtex cerebral de ratos, mas a hidrólise de AMP não foi alterada. O tratamento *in vitro* em soro de ratos não afetou nenhuma das atividades enzimáticas estudadas. Fluoxetina e nortriptilina foram testadas no tratamento *in vivo*, na dose de 10mg/Kg. O tratamento agudo (1 hora após a administração) com nortriptilina provocou uma inibição na hidrólise de ATP em soro de ratos. Entretanto, o tratamento crônico (14 dias) com ambas as drogas provocou uma inibição na atividade das NTPDases e da ecto-5´-nucleotidase em soro. O tratamento agudo com fluoxetina não alterou a atividade das enzimas em sinaptossomas de hipocampo e córtex cerebral. Entretanto, o tratamento agudo com nortriptilina levou a um aumento na hidrólise de ADP no córtex cerebral e induziu uma diminuição na hidrólise de ATP e ADP no hipocampo. O tratamento crônico com fluoxetina provocou uma inibição na hidrólise de ATP no hipocampo e no córtex cerebral e aumentou a hidrólise de ADP e AMP no córtex cerebral. Após tratamento crônico com nortriptilina, houve uma diminuição da hidrólise de ATP no hipocampo, mas foi observado um aumento nas atividades destas enzimas no córtex cerebral. A expressão gênica das NTPDases e ecto-5´-nucleotidase foi alterada após os tratamentos agudo e crônico com fluoxetina e nortriptilina em hipocampo e córtex cerebral de ratos. Os resultados demonstram que estes fármacos antidepressivos podem influenciar as enzimas envolvidas na formação do neuromodulador adenosina, sugerindo que o sistema purinérgico pode ser alvo dos efeitos neuroquímicos promovidos por estes fármacos.

Palavras chaves: Depressão, antidepressivos, ecto-nucleotidases, soro e sinaptossomas.

Abstract

Depression is the most common manifestation of affective disorders. It is one of the most disable diseases, and causes a significant burden to both the individual and the society and, if not treated, it can increase morbidity and mortality. The use of antidepressants drugs is the base of depression treatment. The selective serotonin reuptake inhibitors, such as fluoxetine and sertraline, and the tricyclic antidepressants, nortriptyline and clomipramine, are constantly used in the treatment for depression. Evidence has shown the important role performed by ATP and adenosine in the central nervous system. ATP can be stored and co-released with other neurotransmitters, such as noradrenaline and serotonin. Extracellular ATP can be hydrolyzed to adenosine by the action of ecto-nucleotidases. These ecto-enzymes promoted the enzymatic conversion of ATP, controlling the levels of this nucleotide and its nucleoside adenosine. Among the ecto-nucleotidases, we can highlight the NTPDases family and the ecto-5´-nucleotidase. Considering that: (i) adenosine exerts an important neuromodulatory role, (ii) ATP can be co-released with serotonin and noradrenaline, and (iii) the ecto-nucleotidases represent the main pathway for extracellular adenosine formation through ATP catabolism, become important to evaluate the effects of antidepressant drugs on the ecto-nucleotidases. Fluoxetine, sertraline, nortriptyline and clomipramine were tested for *in vitro* treatment, in the concentrations of 100, 250 and 500μM, in the blood serum and in hippocampal and cerebral cortex synaptosomes from rats. ATP and ADP hydrolysis were inhibited in a dose-dependent manner in the *in vitro* treatment with the four drugs in the hippocampal and cerebral cortex synaptosomes from rats, but AMP hydrolysis was not altered. The *in vitro* treatment did not affect any enzyme activities tested in rat blood serum. Fluoxetine and nortriptyline were used for the *in vivo* treatment in a dose of the 10mg/Kg. The acute treatment (1 hour after the administration) with nortriptyline promoted an inhibition on ATP hydrolysis in rat blood serum. However, the chronic treatment (14 dias) with both drugs caused an inhibition on NTPDases and ecto-5´-nucleotidasis activities in blood serum. Fluoxetine acute treatment did not alter the enzymes activities in synaptosomes of hippocampus and cerebral cortex. The acute treatment with nortriptyline induced an increase of ADP hydrolysis in cerebral cortex and a decrease of ATP and ADP hydrolysis in the hippocampus. The chronic treatment with fluoxetine promoted an inhibition of the ATP hydrolysis in the hippocampus and cerebral cortex and increased the ADP and AMP hydrolysis in the cerebral cortex. After chronic treatment with nortriptyline, there was a decrease of ATP hydrolysis in the hippocampus, but it was observed an increase of enzymes activities in cerebral cortex. The gene expressions of NTPDases and ecto-5´-nucleotidase were altered after acute and chronic treatments with fluoxetine and nortriptyline in hippocampus and cerebral cortex of rats. The findings demonstrated that these antidepressants drugs can affect the enzymes involved in the adenosine production, suggesting that the purinergic system can be a target to neurochemical effects promoted by these drugs.

Lista de Abreviaturas

ADP - adenosina 5'- difosfato

AMP - adenosina 5'- monofosfato

AMPc- adenosina 5'- monofosfato cíclico

ATP - adenosina 5'- trifosfato

BDNF – fator neurotrófico derivado do cérebro

Ca^{2+} - cálcio

CDP – citidina 5'- difosfato

CTP – citidina 5'- trifosfato

CREB – elemento de ligação de resposta ao AMP cíclico

GABA - ácido gama - aminobutírico

GDP – guanosina 5'- difosfato

GMP- guanosina 5'-monofosfato

GPI - glicosilfosfatidilinositol

IDP – inosina 5´-difosfato

IMAO - inibidores da monoaminoxidase

ISRNs - inibidores seletivos da recaptação de noradrenalina

ISRSs - inibidores seletivos da recaptação de serotonina

K^+ - potássio

MAPK - proteína quinase ativada por mitógenos

NA - noradrenalina

Na^+ - sódio

NMDA - *N*-metil-D-aspartato

NTPDase - nucleosídeo trifosfato difosfoidrolase

PKC - proteína quinase C

SNC - sistema nervoso central

TCA - antidepressivos tricíclicos

TTP – timidina 5´-trifosfato

UTP – uridina 5´-trifofosfato

UDP – uridina 5’- difosfato

UMP – uridina 5’-monofosfato

5-HT - serotonina

Sumário

1. INTRODUÇÃO

1.1 Depressão e antidepressivos

De acordo com a Organização Mundial da Saúde, a depressão é um problema de saúde que afeta cerca de 121 milhões de pessoas no mundo inteiro (Rosenzweig-Lipson et al., 2007). Ela constitui a manifestação mais comum dos distúrbios afetivos, podendo variar de uma condição muito discreta, até a depressão grave acompanhada de alucinações e delírios (Frazer, 1997). As desordens depressivas são condições crônicas que produzem sintomas emocionais e físicos (Delgado 2004; Mitchell, 2006). A depressão é uma das doenças mais debilitadoras, e causa problemas tanto para o indivíduo quanto para a sociedade, e se não for tratada pode levar ao aumento da morbidade e mortalidade (Sobocki et al., 2006). Embora a patofisiologia de depressão não seja bem clara, há grandes evidências de que anormalidades nos sistemas de neurotransmissão noradrenérgico e serotoninérgico estejam associadas a desordens depressivas (Fava, 2003).

Na última década, considerou-se que a neuroplasticidade e o estresse são fatores relacionados com a patofisiologia da depressão. Pelo fato dos antidepressivos apresentarem um tempo de ação tardio, é possível que a inibição da recaptação dos neutransmissores não seja suficiente para explicar mudanças a longo prazo. O aumento da neurogênese, a formação de fibras nervosas, novas sinapses e estabilização das antigas podem ser os fatores responsáveis por estas mudanças. A cascata celular AMPc-MAPk-CREB-BDNF pode ter um papel

significativo no mecanismo de reestruturação dendrítica, aumento de neurogênese hipocampal e sobrevivência de células nervosas no tratamento com antidepressivos (Duman RS., 2004; Angelucci et al., 2005; Arantes-Gonçalves & Coelho, 2006).

Existem evidências mostrando que desordens afetivas estão associadas com a disfunção nas rotas de transdução pós-sináptica de neurotransmissores e o tratamento crônico com fármacos clinicamente ativos, resultando na modificação adaptativa dessas vias (Moretti et al., 2003). Os primeiros tratamentos para depressão eram baseados nos fármacos inibidores da monoaminoxidase (IMAO) e antidepressivos tricíclicos (TCA). A descoberta dos inibidores seletivos da recaptação de serotonina (ISRSs) e dos inibidores seletivos da recaptação de noradrenalina (ISRNs) tem mudado aspectos importantes do tratamento clínico (Galeotti et al., 2002; Serra et al., 2006; Rosenzweig-Lipson et al., 2007).

Os sistemas serotoninérgico e noradrenérgico foram reconhecidos por terem um importante papel na etiologia da depressão. Fármacos que atuam nesses sistemas são amplamente utilizados no tratamento de desordens depressivas (Blier & Ward, 2003; Fava, 2003; Elhwueqi, 2004). A figura 1 mostra o mecanismo de liberação, re-captação e degradação de serotonina e noradrenalina. A maioria dos agentes antidepressivos utilizados na terapia de desordens afetivas tem em comum a capacidade de aumentar os níveis de monoaminas sinápticas. Este aumento é considerado o primeiro passo de uma série de adaptações ainda desconhecidas no cérebro, as quais são responsáveis pela eficácia a longo tempo (Carboni et al., 2006).

A fluoxetina e a sertralina são fármacos inibidores seletivos da recaptação de serotonina (5-HT) como mostra a figura 2a, que possuem poucos efeitos sobre outros neurotransmissores (Rossi et al., 2004; Bailly, 2006; Cecconi et al., 2006; Chen et al., 2007). A clomipramina e a nortriptilina são antidepressivos tricíclicos, que atuam com duplo papel, inibindo a recaptação de noradrenalina (NA) e/ou 5-HT, como mostra a figura 2b (Morishita & Aoki, 2002; Stoll et al., 2007; Bert et al., 2006 e Su et al., 2007). 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. Além da ação sobre as catecolaminas, estudos mostram outros efeitos para estes antidepressivos, tais como um aumento na atividade da PKC e a modulação de propriedades de ligação dos receptores NMDA e beta1-adrenérgicos corticais em modelos animais de depressão (Harkin et al., 2000; Giambalvo & Price, 2003).

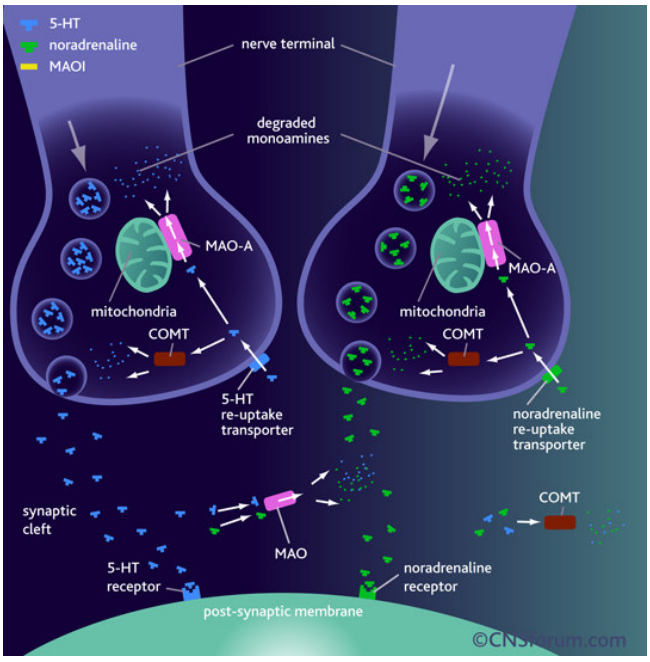

Figura 1 - Mecanismo normal de liberação, re-captação e degradação de serotonina e noradrenalina (http://www.cnsforum.com/imagebank/section/Antidepressants/default.aspx).

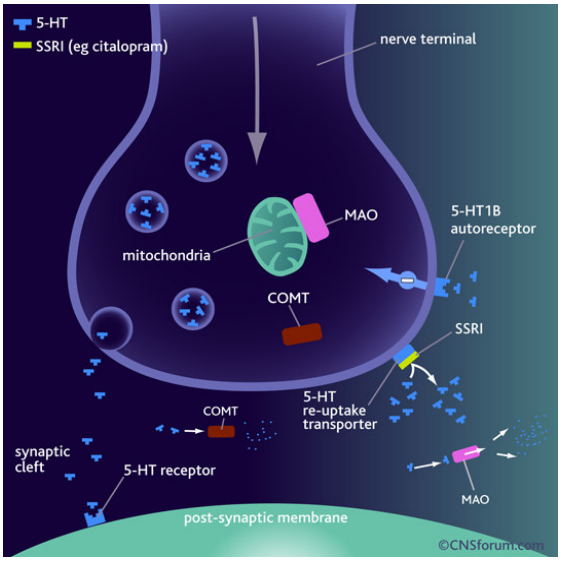

Figura 2- a- Mecanismo de ação da Fluoxetina e sertralina **b-** Mecanismo de ação da Nortriptilina e Clomipramina (http://www.cnsforum.com/imagebank/section/Antidepressants/default.aspx).

1.2 Sistema purinérgico

Diversas evidências têm demonstrado o importante papel desempenhado pelo ATP e a adenosina no sistema nervoso central (SNC) (Ralevic & Burnstock, 1998; Dunwiddie & Masino, 2001). O ATP pode ser armazenado e co-liberado juntamente com diversos outros neurotransmissores, tais como: acetilcolina, glutamato, norarenalina, serotonina e ácido γ-amino butirico (GABA) (Burnstock, 1999; Burnstock, 2004). Atualmente, se propõe que o ATP é uma molécula sinalizadora primitiva e ubíqua, a qual foi retida como um cotransmissor em quase todos os tipos celulares, desempenhando importantes papéis tanto em estados fisiológicos quanto patológicos (Chow et al., 1997; Burnstock, 2004; Burnstock, 2006). O ATP possui um papel no tônus vascular, na função cardíaca e no

transporte epitelial renal (Ralevic, 2000). Estudos demonstraram que em concentrações na faixa de milimolar o ATP inibe a agregação plaquetária via mecanismos competitivos e não competitivos, bem como baixas concentrações podem ser estimulatórias (Soslau & Youngprapakorn, 1997). Em relação ao ADP, sabe-se que ele induz alterações na forma e agregação das plaquetas. Diferentes estudos têm demonstrado o importante papel destes nucleotídeos nos processos de homeostase e na formação de trombos (Coade & Pearson, 1989; Piebar et al. 1991). Já o nucleosídeo adenosina, produzido pela degradação dos nucleotídeos, é uma estrutura hábil para atuar como vasodilatador e cardioprotetor (Frassetto et al., 1993; Soslau & Youngprapakorn, 1997).

Na fenda sináptica, receptores de superfície celular podem propagar sinais a partir de sua ligação com o ATP. Estes receptores são denominados purinoreceptores P2 e são divididos em duas subclasses, P2X e P2Y. A subclasse P2X consiste em receptores ionotrópicos, e a subclasse P2Y é constituída de receptores metabotrópicos (Ralevic & Burnstock, 1998; Ziganshin et al., 2002; Burnstock, 2006; Burnstock, 2007). Após sua liberação na fenda sináptica, o neurotransmissor ATP pode ser hidrolisado até adenosina, pela ação conjugada de um grupo de ecto-nucleotidases. Estas ecto-enzimas promovem a conversão enzimática de ATP, controlando os níveis deste nucleotídeo e do seu respectivo nucleosídeo adenosina na fenda sináptica (Zimmermann, 2001; Robson et al., 2006). Em SNC, dentro do grupo das ecto-nucleotidases, podemos destacar a família das NTPDases (nucleosídeo trifosfato difosfoidrolases), que são capazes de promover a hidrólise de nucleotídeos trifosfatados e difosfatados até nucleotídeos monofosfatados. Por sua vez, a hidrólise de nucleotídeos

monofosfatados aos seus respectivos nucleosídeos é catalisada por uma ecto-5´-nucleotidase (EC 3.1.3.5) (Zimmermann & Braun, 1999).

Estudos mostraram a presença de uma NTPDase solúvel em soro de ratos (Oses et al., 2004). Esta enzima atua junto com uma 5´-nucleotidase (EC 3.1.3.5, CD73), que hidrolisa o AMP até adenosina na circulação. Esta cascata enzimática regula a disponibilidade de ligantes (ATP, ADP, AMP e adenosina) para os receptores de nucleotídeos e nucleosídeos, conseqüentemente, a duração e o aumento da ativação dos receptores (Chen & Guidotti, 2001).

A família das NTPDases (Figura 3) constituem uma classe de enzimas que possuem as seguintes características: 1) um sítio de hidrólise de nucleotídeos, voltado para o espaço extracelular ou para o lúmen das organelas citoplasmáticas, (2) subunidade catalítica glicosilada, (3) atividade dependente de cátions divalentes (principalmente cálcio e/ou magnésio), (4) insensibilidade a inibidores específicos de ATPases do tipo P, F, V e (5) habilidade para hidrolisar uma ampla variedade de nucleotídeos púricos e pirimídicos trifosfatados e difosfatados (Plesner, 1995; Zimmermann et al., 1998; Goding, 2000; Robson et al., 2006). Até o momento, oito enzimas já foram descritas e caracterizadas dentro dessa família, a NTPDase1 (CD39, ATPDase, ecto-apirase) (Wang & Guidotti, 1996; Sevigny et al., 1997), a NTPDase2 (CD39L1, ecto-ATPase) (Vlajkovic et al., 1999; Kegel et al., 1997; Mateo et al., 1999; Heine et al., 1999), a NTPDase3 (CD39L3, HB6) (Chadwick et al., 1998), a NTPDase4 (UDPase, LALP70) (Wang & Guidotti, 1998; Biederbick et al., 2000), a NTPDase5 (CD39L4, ER-UDPase, PCPH) (Mulero et al., 1999; Paez et al., 2001), a NTPDase6 (CD39L2) (Yeung et al., 2000; Hicks-

Berger et al., 2000), a NTPDase7 (LALP1) (Shi et al., 2001) e a NTPDase8 (Bigonnesse al., 2004).

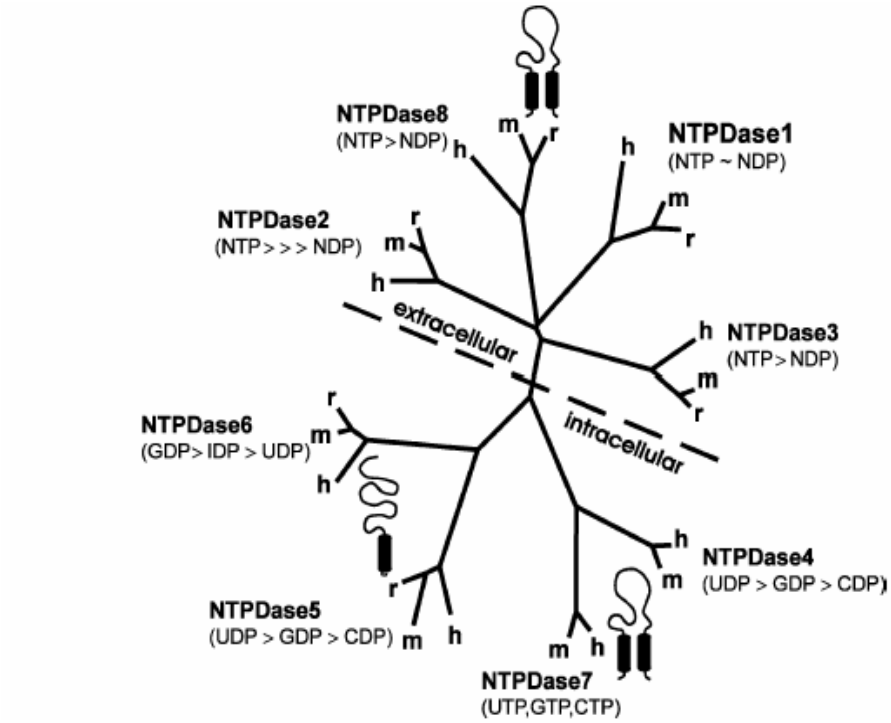

Figura 3 - Árvore filogenética da família das NTPDases de humanos (h), ratos (r) e camundongos (m) (Robson et al., 2006).

Em termos de hidrólise de nucleotídeos, a NTPDase1 hidrolisa ATP e ADP igualmente bem, sendo a proporção da hidrólise destes dois substratos de 1:1 (Heine et al., 1999). A enzima NTPDase2 hidrolisa 30 vezes mais ATP do que o ADP (Kirley et al., 1997). A NTPDase3 e a NTPDase8 preferem o ATP em relação ao ADP numa razão de hidrólise de aproximadamente 3:1 e 2:1, respectivamente (Chadwick et al., 1998; Bigonnesse al., 2004). Embora existam tais diferenças na especificidade pelos substratos, todos esses membros da família estão firmemente ligados à membrana plasmática via 2 domínios transmembrana e possuem uma região extracelular contendo o sítio ativo (Lavoie et al., 2004; Bigonnesse et al., 2004). A NTPDase4 tem sido localizada no aparelho de Golgi (UDPase, NTPDase4β) e em vacúolos lisossômicos/autofágicos (NTPDase4α). A

NTPDase4α tem uma alta preferência por UTP e TTP, enquanto que a NTPDase4β apresenta alta preferência por CTP e UDP. A função destas NTPDases ainda não é clara (Zimmermann, 2001). Como as outras NTPDases, a NTPDase5 e a NTPDase6 são ativadas por cátios divalentes, porém possuem uma preferência por nucleotídeos difosfatados. A NTPDase5 tem uma preferência na hidrólise de nucleotídeos na seguinte ordem: UDP>GDP = IDP>>ADP = CDP, enquanto que a NTPDase6 tem a seguinte preferência: GDP>IDP>>UDP = CDP>>ADP. Acredita-se que a NTPDase5 e a NTPDase6 participam das reações de glicosilação envolvidas nos processos de dobramento de glicoproteínas (Zimmermann, 2001). A NTPDase7 prefere nucleosídeos trifosfatados como substratos e está localizada em vesículas intracelulares (Zimmermann, 2001).

Com relação a ecto-5'-nucleotidase, trata-se de uma enzima ancorada à membrana plasmática por glicosilfosfatidilinositol (GPI). A ecto-5'-nucleotidase encontra-se presente na maioria dos tecidos e sua principal função é a hidrólise de nucleotídeos monofosfatados extracelulares, tais como AMP, GMP ou UMP, a seus respectivos nucleosídeos (Zimmermann, 1996; Zimmermann et al., 1998). Em SNC, a ecto-5'-nucleotidase está predominantemente associada a glia, mas várias evidências têm demonstrado esta atividade associada a neurônios (Zimmermann, 1996; Zimmermann et al., 1998). A ecto-5'-nucleotidase é transitoriamente expressa na superfície de células neuronais e nas sinapses durante o desenvolvimento sináptico (Schoen & Kreutzberg, 1994; Braun et al., 1995). As ecto-nucleotidases (Figure 4) desempenham uma função essencial na neurotransmissão purinérgica, controlando a disponibilidade e os níveis de

nucleotídeos e nucleosídeos extracelulares e, consequentemente a ativação dos purinoreceptores P_2 e P_1 (Zimmermann, 2001, Robson et al., 2006).

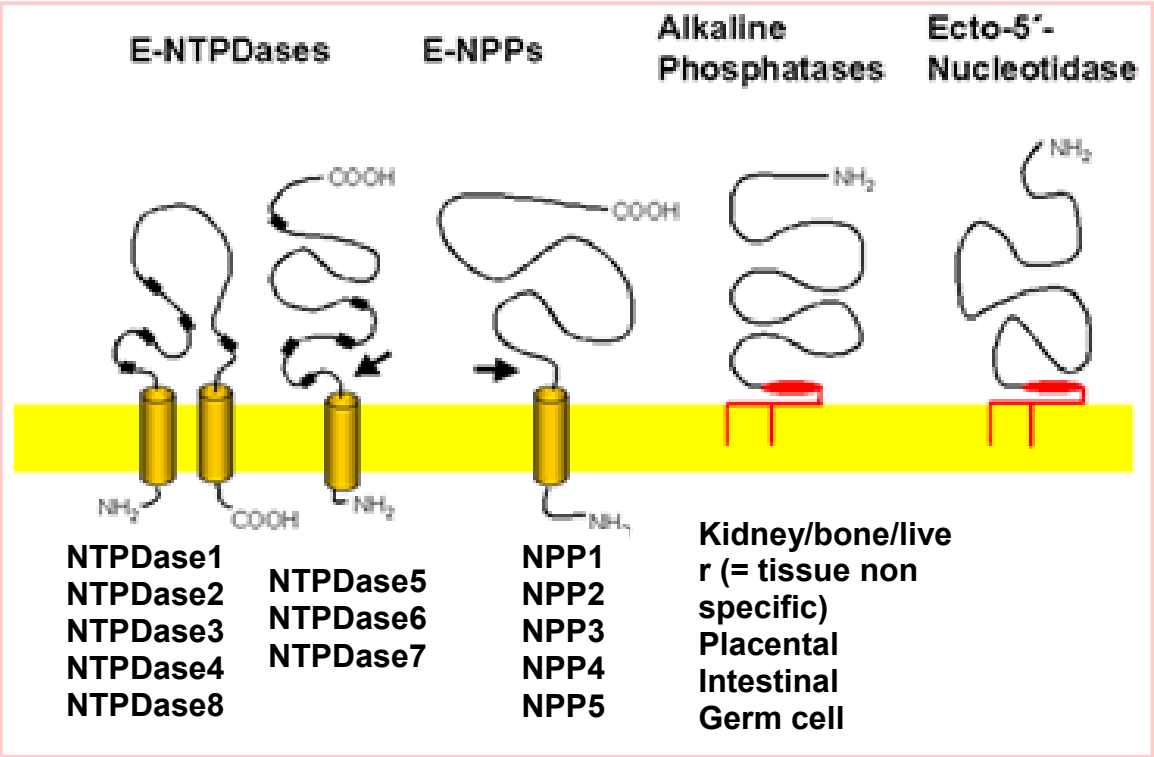

Figura 4: Topografia de membrana de ecto-nucleotidases. As NTPDases de 1 a 4 e a NTPDase 8 estão ligadas a membrana plasmática por dois domínios transmembrana, N e C-terminal. As NTPDases 5, 6 e 7 não possuem o domínio transmembrana C-terminal e podem ser clivadas ao próprio domínio N-terminal para formar uma proteína solúvel liberada (seta). Esta clivagem também pode ocorrer na família das E-NPPs (seta). A ecto-5`-nucleotidase está ancorada à membrana plasmática por uma molécula de glicosil fosfatidil inositol, a qual também pode sofrer clivagem, resultando em uma enzima solúvel. Os quadros escuros na seqüência das E-NTPDases representam as regiões conservadas da apirase. Adaptado de Zimmermann (2001).

Vários estudos apontam a via das ecto-nucleotidases como a principal via de fornecimento de adenosina extracelular (figura 5) (Brundege & Dunwiddie, 1997; Cunha, 2001; Dunwiddie & Masino, 2001).

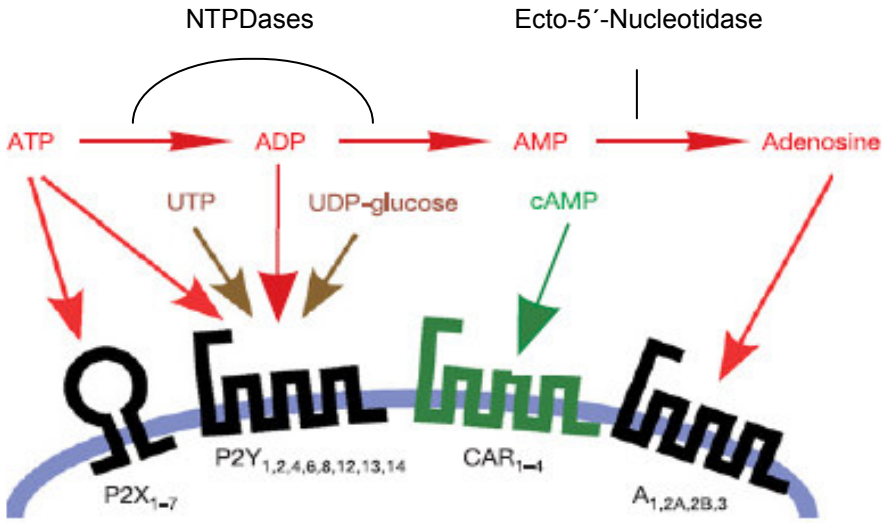

Figura 5 - Catabolismo extracelular dos nucleotídeos e potencial ativação dos receptores pelos nucleotídeos (receptores P2) e adenosina (receptores P1) (Khakh & North, 2006).

A adenosina, um importante neuromodulador, pode exercer seus efeitos através de uma classe de receptores denominados receptores P1, que se dividem em quatro subtipos: A_1, A_{2A}, A_{2B} e A_3 (Dunwiddie & Masino, 2001; Fredholm et al., 2001; Ribeiro et al., 2003). Os receptores A_1 são os receptores de adenosina mais abundantes no SNC, com alta expressão no córtex cerebral, hipocampo, cerebelo, tálamo, tronco cerebral e medula espinal (Fredholm et al., 2001). Já no sistema periférico este receptor é expresso nos vasos deferentes, testículos, tecido adiposo, estômago, rins, hipófise, adrenais, coração, aorta, fígado, olhos e bexiga (Ralevic & Burnstock, 1998). A ativação dos receptores adenosinérgicos do tipo A_1 promove efeitos inibitórios na neurotransmissão. A mais conhecida das vias de sinalização dos receptores A_1 é a inibição da enzima adenilato ciclase (EC 4.6.1.1) através da ativação da proteína G inibitória (G_i/G_0) (Freissmuth et al., 1991a; Freissmuth et al., 1991b; Munshi et al., 1991). Esta inibição proporciona a diminuição das concentrações do segundo mensageiro AMPc, inibindo as vias

dependentes desta molécula sinalizadora (Londos et al., 1980). Além disso, a hiperpolarização neuronal por ativação dos canais de K^+ pré-sinápticos e a inibição do influxo de cálcio parecem ser os outros mecanismos importantes de inibição da liberação de neurotransmissores por ativação dos receptores A_1(Pan et al., 1995; Scholz & Miller, 1996). Em células de cérebro de ratos, o acoplamento de receptores A_1 a canais de potássio K_{ATP} também tem sido demonstrado, o que está relacionado com redução da duração do potencial de ação, bem como a vasodilatação (Yaar et al., 2005). A fosfolipase C também tem sido descrita como componente do mecanismo de inibição exercido pelo receptor A_1 (Megson et al., 1995). Os receptores de adenosina do tipo A_2 estimulam a atividade da adenilato ciclase e, conseqüentemente, aumentam os níveis de AMP cíclico (Correia-de-Sá & Ribeiro, 1994; Kessey & Mogul, 1998). Os receptores A_2 são subdivididos em A_{2A} e A_{2B}, ligando a adenosina com alta e baixa afinidade, respectivamente (Bruns et al., 1986; Ralevic & Burnstock, 1998; Burnstock, 2007). Os receptores A_{2A} têm sua distribuição bastante restrita no SNC, ocorrendo basicamente no estriado, núcleo accumbens e tubérculo olfatório (Ongini & Fredholm, 1996). Sua ocorrência no sistema periférico inclui células do sistema imune, olhos, músculo esquelético, coração, útero, bexiga, plaquetas e células endoteliais (Dixon et al., 1996; Peterfreund et al., 1996). Os receptores A_{2B} possuem baixa expressão no SNC, nos pulmões, vasos deferentes e hipófise (Rees et al., 2003; Rosi et al., 2003; Gessi et al., 2005; Zhong et al., 2005). Uma alta expressão destes receptores é encontrada no intestino grosso e bexiga (Fredholm et al., 2001; Yaar et al., 2005).

Os receptores A_3 foram os últimos receptores adenosinérgicos descritos, sendo expressos de forma moderada no cerebelo e hipocampo e com baixa

expressão no restante do cérebro (Fredholm et al., 2001). Outros órgãos, tais como, testículos, fígado, útero, pulmões, rins, placenta, coração, jejuno, bexiga e o baço também expressam os receptores A_3 (Ralevic & Burnstock, 1998). A ativação dos receptores A_3 inibe a produção de AMPc, através da ação de uma proteína G inibitória (Ralevic & Burnstock, 1998). Além disso, a ativação da fosfolipase C também é descrita em cérebros de ratos após a ativação deste receptor (Brundege & Dunwiddie, 1997; Yaar et al., 2005). A adenosina também modula estados cognitivos e está associada com desordens afetivas e do humor, como ansiedade e depressão (Ledent, et al. 1997; Florio et al., 1998; Kaster et al. 2004).

Devido a este papel neuromodulador, a adenosina está envolvida na regulação de importantes mecanismos no SNC, como estados de ansiedade (Jain et al. 1995; El Yacoubi et al., 2000), agressividade (Ledent et al., 1997), sono (Porkka-Heiskanen, 1999), cognição e memória (Ribeiro et al., 2003). Além disso, este nucleosídeo apresenta especial importância nos estudos de patofisiologias, como na doença de Parkinson (Kanda et al. 1998; Fredduzzi et al., 2002) e na esquizofrenia (Lara et al., 2001).

1.3 Sistema purinérgico, nucleotidases e fármacos antidepressivos

Okada e colaboradores (1999) demonstraram que a neurotransmissão serotoninérgica 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, levariam a um aumento na liberação de serotonina no hipocampo. Estudos mostraram que antagonistas de receptores A_{2A} (SCH 58261,

ZM241385 e KW6002) de adenosina produziram um efeito antidepressivo em animais submetidos a modelos de depressão (El Yacoubi et al., 2001; El Yacoubi et al., 2003). Kaster e colaboradores (2004) mostraram que a administração de adenosina produz um efeito antidepressivo em camundongos submetidos ao teste do nado forçado e ao teste de suspensão pela cauda.

Antidepressivos tricíclicos (clomipramina, desipramina, imipramina e trimipramina) são inibidores da atividade Ca^{2+}-ATPase em membrana plasmática medida em eritrócitos. Clomipramina, o mais eficiente inibidor, não modificou a afinidade da ligação do ATP e o efeito inibitório observado deve estar relacionado à taxa de formação do intermediário fosforilado desta enzima (Plenge-Tellechea et al., 1999).

A imipramina e a desimipramina, em ensaios *in vitro* e *in vivo*, alteram a fluidez da membrana (Daniel et al., 1991), o que poderia levar a uma alteração na atividade de enzimas de membrana, como as ecto-nucleotidases. Barcellos et al. (1998) demonstraram que amitriptilina, desipramina e imipramina diminuíram a hidrólise de ATP e ADP em sinaptossomas de córtex cerebral de ratos em ensaios *in vitro*.

O efeito do tratamento crônico e da adição *in vitro* de fluoxetina e imipramina tem sido estudado sobre a atividade da Na^+,K^+-ATPase, um vez que esta enzima desempenha um papel importante na recaptação de noradrenalina e serotonina (Zanatta et al*.,* 2001). Sanganahalli et al. (2000) mostraram que amitriptilina e nortriptilina provocam uma maior inibição na atividade da Na^+,K^+-ATPase em relação a imipramina e desipramina em ensaios *in vitro* em sinaptossomas cerebrais de ratos. Estudos têm demonstrado que a administração

crônica de algumas drogas psicoativas usadas no tratamento do transtorno bipolar, como o haloperidol, a carbamazepina e o lítio, aumentam a atividade da Na^+,K^+-ATPase em cérebro de ratos (Wood et al., 1989). Fluoxetina inibe a F_0F_1-ATPase e o transporte de elétrons em mitocôndrias cerebrais de ratos por interação com a bicamada lipídica da membrana interna. Como conseqüência destes efeitos, a fluoxetina diminuiu a taxa de síntese de ATP e reduziu o potencial de fosforilação da mitocôndria (Curti et al., 1999).

Além disso, o efeito *in vitro* e a administração crônica de fluoxetina promoveram uma inibição na atividade da F_0F_1-ATPase em fatias de fígado de ratos, porém não houve alterações significativas nesta enzima após tratamento agudo com a droga (Souza et. al 1994). Interações de fármacos com a biomembrana influenciam na estrutura da bicamada, modulando processos que abrangem desde atividades de enzimas ligadas a membrana e ligação a receptores até a permeabilidade e transporte da membrana (Carfagna & Muhoberac, 1993). Antidepressivos tricíclicos (imipramina, desipramina, clomipramina, amitriptilina, fluoxetina) exibiram efeito inibitório sobre a captação de cálcio dependente de ATP pelo retículo endoplasmático em sinaptossomas lisados de córtex cerebral de ratos (Couture et al., 2001).

Portanto, o estudo da interação entre o sistema purinérgico e fármacos antidepressivos permitirá um maior entendimento sobre os efeitos neuroquímicos destes compostos em outros sistemas de neurotransmissão.

2. OBJETIVOS

Considerando que: (i) a adenosina exerce um importante papel neuromodulador sobre diversos sistemas de neurotransmissão, (ii) que o ATP pode ser co-liberado com a 5-HT e a NA (Burnstock, 2004) e (iii) as ecto-nucleotidases representam a principal rota de formação de adenosina extracelular através do catabolismo do ATP (Fredholm et al. 2001; Ribeiro et al. 2003), torna-se importante avaliar o efeito de fármacos antidepressivos sobre a via das ecto-nucleotidases em sinaptossomas de hipocampo e córtex cerebral de ratos.

2.1 Objetivos Específicos

Os objetivos específicos deste estudo são:

- Avaliar o efeito *in vitro* da fluoxetina, sertralina, clomipramina e nortriptilina sobre a hidrólise de ATP, ADP e AMP em sinaptossomas de hipocampo e córtex cerebral de ratos.
- Avaliar o efeito *in vitro* e *in vivo* (tratamentos agudo e crônico) da fluoxetina e nortriptilina sobre a hidrólise de ATP, ADP e AMP em soro de ratos.
- Avaliar o efeito do tratamento agudo e crônico com fluoxetina e nortriptilina sobre a hidrólise de ATP, ADP e AMP em sinaptossomas de hipocampo e córtex cerebral de ratos.
- Verificar a expressão gênica das NTPDases e da 5´-nucleotidase após os tratamentos com os fármacos antidepressivos.

3. APRESENTAÇÃO DOS ARTIGOS

Os resultados obtidos nesta dissertação originaram três artigos científicos:

1- Sertraline and clomipramine inhibit nucleotide catabolism in rat brain synaptosomes, o qual foi publicado no periódico ***Toxicology in vitro.***

2- Fluoxetine and nortriptyline affect NTPDase and 5'-nucleotidase activities in rat blood serum, o qual foi submetido ao periódico ***Life Sciences.***

3- Ecto-nucleotidases pathway is altered by differents treatment with fluoxetine and nortriptyline, o qual foi submetido ao periódico ***European Journal of Pharrmacology.***

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Sertraline and clomipramine inhibit nucleotide catabolism in rat brain synaptosomes

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Abstract

The effects of sertraline, a selective serotonin reuptake inhibitor, and clomipramine, a tricyclic antidepressant, were tested on ecto-nucleotidases from synaptosomes of cerebral cortex and hippocampus of rats. Sertraline and clomipramine (100–500 μM) inhibited NTPDase, but not ecto-5′-nucleotidase activity in both cerebral cortex and hippocampus. In cortical synaptosomes, sertraline inhibited both ATP and ADP hydrolysis in the concentrations tested. The inhibitory effect varied from 21% to 83% for ATP hydrolysis and 48% to 75% for ADP hydrolysis. The inhibition promoted by sertraline in hippocampal synaptosomes varied from 38% to 89% for ATP hydrolysis and 45% to 77% for ADP hydrolysis. A significant inhibition of cortical NTPDase activity by clomipramine was observed in the all concentrations tested (35–72% and 36–87% for ATP and ADP hydrolysis, respectively). Similar effects were observed in hippocampus (29–91% and 48–83% for ATP and ADP hydrolysis, respectively). There was no inhibitory effect of sertraline and clomipramine on AMP hydrolysis in cerebral cortex and hippocampus. Our results have shown that classical antidepressants inhibit the extracellular catabolism of ATP. Therefore, it is possible to suggest that changes induced by antidepressants on bilayer membrane could affect NTPDase activities and consequently, modulating ATP and adenosine levels in the synaptic cleft.

Keywords: Sertraline; Clomipramine; Ecto-nucleotidases; NTPDase; Ecto-5′-nucleotidase; Adenosine; Depression

1. Introduction

Depressive disorders are chronic conditions that produce both emotional and physical symptoms (Delgado, 2004). Although the pathophysiology of depression is still unknown, there is significant evidence for abnormalities of the norepinephrine (NE) and serotonin (5-HT) neurotransmitter systems in depressive disorders (Fava, 2003). These neurotransmitters can influence the neuroplasticity in the brain and both are involved in mediating the therapeutic effects of most currently available antidepressants (Delgado, 2004).

Depression is one of the most disabling diseases, and causes a significant burden to both the individual and the society. If not treated, it could lead to increased morbidity and mortality (Sobocki et al., 2006). In the last five decades, the psychopharmacology of depression has evolved rapidly, with the development of new antidepressants, showing therapeutic efficacy (Galeotti et al., 2002; Trivedi et al., 2004). The main function of antidepressants is to increase the extracellular neurotransmitter concentrations, inhibiting the metabolism and reuptake. They can also act in synaptic receptors (BezchlibnyK-Butler and Virani, 2004). Because antidepressants have a lag time on their action, it

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is possible that inhibition of neurotransmitter reuptake is not sufficient to explain long-term changes. These antidepressants include monoamine oxidase inhibitors, tricyclic compounds, selective 5-HT and NE reuptake inhibitors, as well as, some atypical drugs (Galeotti et al., 2002)

Clomipramine is a tricyclic antidepressant that acts with a dual role, inhibiting both norepinephrine and serotonin reuptake (Bert et al., 2006). In contrast, sertraline is a selective serotonin reuptake inhibitor (Bailly, 2006). Besides the main action on catecholamines, studies report other effects for these drugs, such as an increase of PKC activity and modulation of the binding properties of cortical NMDA and beta1-adrenergic receptors in an animal model of depression (Harkin et al., 2000; Giambalvo and Price, 2003). Catecholamines can be co-released with ATP, which is considered a neurotransmitter and neuromodulator in central nervous system (CNS) (Burnstock, 2004). Extracellular ATP evokes responses by two subclasses of P2 purinoreceptors, P2X and P2Y (Ralevic and Burnstock, 1998). It has been shown that P2X receptors are coupled to ligand-gated Ca^{2+}-permeable channels, whereas the P2Y receptors have been considered a G-protein-linked (Burnstock, 2004). The signaling actions induced by extracellular ATP are directly correlated to the activity of ecto-nucleotidases since these enzymes trigger enzymatic conversion of ATP to adenosine (Zimmermann, 2001; Robson et al., 2006). Ecto-nucleotidases comprise a group of ecto-enzymes involved in the control of nucleotide and nucleoside levels in the synaptic cleft, which includes NTPDase (nucleoside triphosphate diphosphohydrolase) family and ecto-5′-nucleotidase (Zimmermann, 2001). Four members of the NTPDase family are tightly bound to the plasma membrane via two transmembrane domains, and have a large extracellular region with an active site facing the extracellular side. NTPDase1, 3 and 8 slightly prefer ATP over ADP by a ratio of 1, 3 and 2, respectively. Meanwhile, NTPDase2 prefers triphosphonucleosides (Bigonnesse et al., 2004; Robson et al., 2006).

Adenosine, a product of ATP catabolism, can evoke its neuromodulatory effects by four subtypes of P_1-purinoreceptors named A_1, A_{2A}, A_{2B} and A_3 (Brundege and Dunwiddie, 1997; Cunha, 2001; Dunwiddie and Masino, 2001). Studies have shown that adenosine administration produces an antidepressant-like effect in the forced swimming test (FST) and in the tail suspension test through an interaction with A_1 and A_{2A} receptors (Kaster et al., 2004). Moreover, it has been shown that hippocampal serotonergic neurotransmission is modulated by hippocampal adenosine receptor subtypes (Okada et al., 1999).

Considering that (i) the adenosine is able to modulate 5-HT release and (ii) ecto-nucleotidases represent one of the most important sources of extracellular adenosine, the aim of this study was to evaluate the effect *in vitro* of sertraline and clomipramine on the ecto-nucleotidases in synaptosomes from cerebral cortex and hippocampus of rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (age 70–90 days; 220–280 g) from our breeding stock were housed four to a cage, with food and water *ad libitum*. The animal house temperature was kept between 22 °C and 23 °C with a 12 h light/dark cycle (lights on at 07:00). Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee (CEP 06/03016) of the Pontificia Universidade Católica do Rio Grande do Sul, Brazil.

2.2. Chemicals

Sertraline, clomipramine, Percoll, Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All reagents used were of analytical grade.

2.3. Synaptosomal preparation

The rats were killed by decapitation, and their cerebral cortex and hippocampus were dissected, homogenized in 10 and 5 volumes, respectively, in an ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.5. The synaptosomes were isolated as described previously (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 mL of the crude mitochondrial fraction was mixed with 4.0 mL of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15000*g* for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/mL. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

2.4. In vitro treatments

Antidepressants, sertraline and clomipramine, were added to reaction medium before the preincubation with the synaptosomes and maintained throughout the enzyme assays. Antidepressants were tested at final concentrations of 100, 250 and 500 μM.

2.5. Determination of ecto-nucleotidase activities

The reaction medium used to assay ATP and ADP hydrolysis was essentially as described previously (Battastini et al., 1991), and contained 5.0 mM KCl, 1.5 mM $CaCl_2$,

0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris–Hcl buffer, pH 8.0, in a final volume of 200 µl. The synaptosomal fraction (10–20 µg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was initiated by the addition of 1 mM ATP or ADP as substrate and stopped by the addition of 200 µl 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100 µl samples were taken to asses the released inorganic phosphate (Pi) (Chan et al., 1986).

The reaction medium used to assay 5′-nucleotidase activity contained 10 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.5 and 0.15 M sucrose to final volume of 200 µl (Heymann et al., 1984). The synaptosomal fraction (10–20 µg protein) was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of 1.0 mM AMP as substrate and stopped by the addition of 200 µl 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100 µl samples were taken to assess the released inorganic phosphate (Pi) (Chan et al., 1986).

In enzyme assays, incubation time and protein concentration were chosen in order to ensure the linearity of the reaction. Controls, with the addition of the enzyme preparation after the addition of trichloroacetic acid, were used to correct non-enzymatic hydrolysis of the substrates. All samples were run in triplicate.

2.6. Protein determination

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

2.7. Statistical analysis

Data were expressed as means ± SD and analyzed by one-way analysis of variance (ANOVA). A Tukey multiple range test considering $P \leqslant 0.05$ as significant followed the analysis.

3. Results

We evaluated the effect *in vitro* of antidepressant drugs on ATP, ADP and AMP hydrolysis from synaptosomes of cerebral cortex and hippocampus of rats. NTPDase activities were sensitive to antidepressants, clomipramine and sertraline, in cortical and hippocampal brain synaptosomes. In contrast, the ecto-5′-nucleotidase activity demonstrated no sensitivity to the concentrations tested.

In cortical synaptosomes, sertraline inhibited both ATPase and ADPase activities in all concentrations tested (100–500 µM). The inhibitory effect varied from 21% to 83% in ATP hydrolysis and from 48% to 75% for ADP hydrolysis (Fig. 1a). The inhibition promoted by sertraline in hippocampal synaptosomes varied from 38% to 89% for ATP hydrolysis and from 45% to 77% for ADP hydrolysis (Fig. 2a). No inhibitory effect of sertaline was observed in AMP hydrolysis in both hippocampus and cerebral cortex (Figs. 1b and 2b).

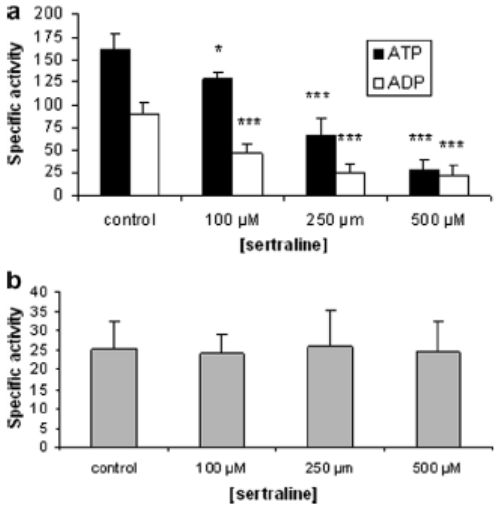

Fig. 1. *In vitro* effect of sertraline on cortical NTPDase (a) and ecto-5′nucleotidase (b) activity in rat brain. Bars represent the mean ± SD of three or more different experiments. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test (*$p \leqslant 0.05$, **$p \leqslant 0.01$, ***$p \leqslant 0.001$ when compared to the control).

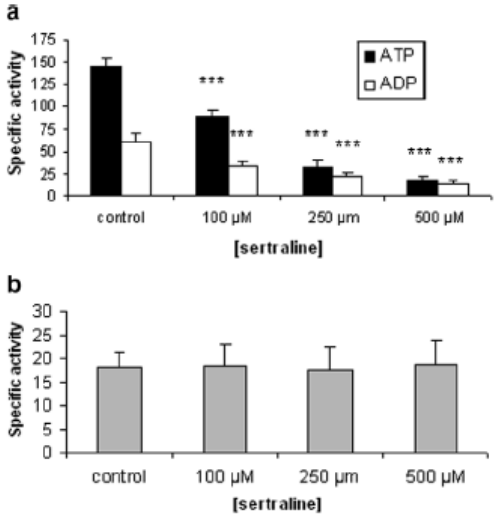

Fig. 2. *In vitro* effect of sertraline on hippocampal NTPDase (a) and ecto-5′nucleotidase (b) activity in rat brain. Bars represent the mean ± SD of three or more different experiments. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test (*$p \leqslant 0.05$, **$p \leqslant 0.01$, ***$p \leqslant 0.001$ when compared to the control).

The effect of clomipramine on nucleotide hydrolysis was also tested in cortical and hippocampal synaptosomes. A

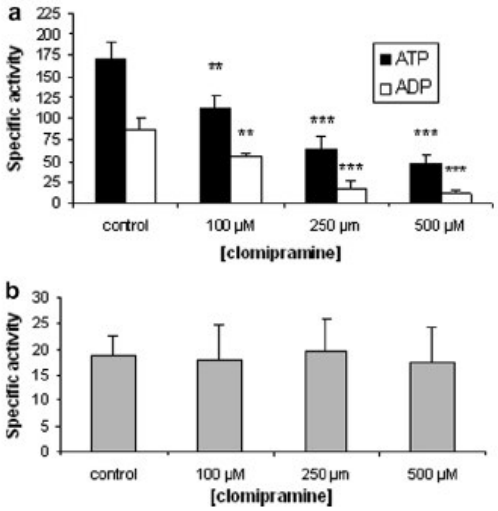

Fig. 3. *In vitro* effect of clomipramine on cortical NTPDase (a) and ecto-5′nucleotidase (b) activity in rat brain. Bars represent the mean ± SD of three or more different experiments. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test (*$p \leqslant 0.05$, **$p \leqslant 0.01$, ***$p \leqslant 0.001$ when compared to the control).

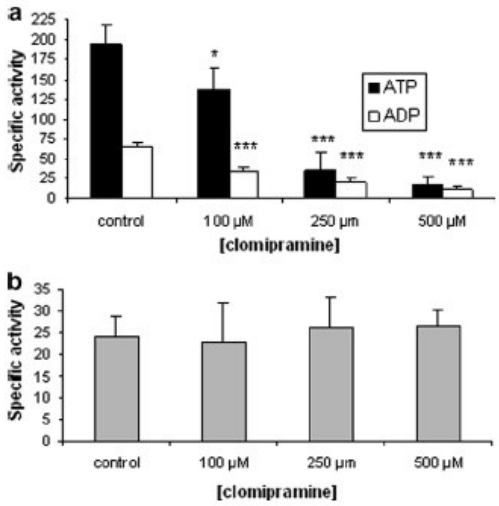

Fig. 4. *In vitro* effect of clomipramine on hippocampal NTPDase (a) and ecto-5′nucleotidase (b) activity in rat brain. Bars represent the mean ± SD of three or more different experiments. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test (*$p \leqslant 0.05$, **$p \leqslant 0.01$, ***$p \leqslant 0.001$ when compared to the control).

significant inhibition of cortical NTPDase activity was observed in the all concentrations tested (35–72% for ATP hydrolysis and 36–87% for ADP hydrolysis) (Fig. 3a). A similar decrease in ATP (29–91%) and ADP hydrolysis (48–83%) was observed in hippocampal synaptosomes (Fig. 4a). Clomipramine failed to inhibit AMP hydrolysis in both brain regions tested (Figs. 3b and 4b).

4. Discussion

The results of the present study demonstrated that NTPDase, but not ecto-5′-nucleotidase, activities from cerebral cortex and hippocampus are decreased by the antidepressants sertraline and clomipramine.

Different studies have demonstrated the effect of antidepressant drugs in ATPase activities such as Mg^{2+}-ATPase (Nag and Ghosh, 1973), F_0F_1-ATPase (Souza et al., 1994), and Na^+, K^+-ATPase (Zanatta et al., 2001). Sanganahalli et al. (2000) have demonstrated that tricyclic antidepressant (imipramine, desipramine, amitriptyline, and nortriptyline) inhibited Na^+, K^+-ATPase activity in synaptosomal membrane of rat brain. Furthermore, it has been shown that sertraline and clomipramine inhibit the 5-hydroxytryptamine transporter in primary cultures of rat and mouse cortical astrocytes (Bal et al., 1997). Studies have observed that the total uptake and lysosomal trapping of the antidepressants such as imipramine, amitriptyline, fluoxetine and sertraline were higher in the grey matter and neurones than in the white matter and astrocytes, respectively (Daniel et al., 2001). There is evidence that tricyclic drugs alter the structural organization of the lipid membranes. Furthermore, previous studies have shown that fluoxetine and imipramine can alter Na^+, K^+-ATPase activity due to the hydrophobicity of these drugs (Zanatta et al., 2001). Drug interaction with the biomembrane influences the bilayer structure, consequently, modulating processes which range from membrane-bound enzyme activity and receptor binding to membrane permeability and transport (Carfagna and Muhoberac, 1993). Barcellos et al. (1998) have demonstrated that imipramine and desipramine (antidepressants tryciclic) *in vitro* decreased ATP and ADP hydrolysis in synaptosomes from cerebral cortex of rats. The partitioning of the drugs into lipid bilayer affects the membrane fluidity consequently changing the membrane protein function and structure. NTPDases1, 2, 3 and 8 are firmly anchored to the membrane via two transmembrane domains that in the instance of NTPDase1 are important for maintaining catalytic activity and substrate specificity (Grinthal and Guidotti, 2006). Papanikolaou et al. (2005) have shown that removing cholesterol from membranes of NTPDase1-expressing cells reduces ATPase activity to the same extent as solubilization does. However, these authors have suggested that some aspects of cholesterol other than its effect on membrane fluidity is required for native transmembrane helix properties of the enzyme. In addition, several perturbing methods may change common mechanical feature other than fluidity. Lundbaek et al. (2004) have identified bilayer elasticity as a membrane property that is altered by a vari-

ety of structurally unrelated compounds. Changes in elasticity modify the energy required for local membrane deformation associated with a protein conformational change, modifying the total energy barrier between different transmembrane domain conformations. If the balance between stability and mobility is a key feature of the interplay between the transmembrane domains and the active site of NTPDase1, anything that changes bilayer elasticity might change NTPDase activity by altering this balance (Grinthal and Guidotti, 2006). Thus, changes in membrane bilayer environment promoted by the interaction with clomipramine and sertraline may be able to promote the inhibitory effect observed on NTPDase activity. In contrast, ecto-5′-nucleotidase was not altered by clomipramine and sertraline in the doses tested. Ecto-5′-nucleotidase is attached via a GPI (glycosylphosphatidylinositol) anchor to the extracellular membrane (Sträter, 2006). The different effects promoted by antidepressant drugs on NTPDase and ecto-5′-nucleotidase activities can be related to the differences in membrane anchorage of these enzymes.

Adenosine fulfills a double role (Cunha, 2001), acting both as a homeostatic transcellular messenger and as a neuromodulator, controlling neurotransmitter release and neuronal excitability (Fredholm et al., 2005). Studies have shown that the stimulatory effects of A_2 receptor and inhibitory effects of A_3 receptor on hippocampal extracellular 5-HT levels are masked or abolished by the inhibitory effects of A_1 receptor (Okada et al., 1999). The effects of four tricyclic antidepressants, nortriptyline, iprindole, clomipramine and desipramine on adenosine-evoked depressions of the firings of rat cerebral cortical neurones have been studied. Tricyclic antidepressants are potent inhibitors of neuronal uptake of adenosine, which may raise the endogenous adenosine levels (Phillis and Wu, 1982; Phillis, 1984). Moreover, clomipramine and desipramine elicited depressions, which were antagonized by caffeine, an adenosine antagonist (Phillis, 1984). Therefore, it is possible to suggest that changes induced by antidepressants on bilayer membrane could affect NTPDase activities, and consequently, could modulate ATP and adenosine levels in the synaptic cleft. Further studies are required to verify the *in vivo* effect of these drugs on ecto-nucleotidases activities.

In summary, we have shown that clomipramine and sertraline can modulate the ecto-nucleotidase pathway, an important source of extracellular adenosine. These results pointed out for another pharmacological mechanism of these drugs, which can influence their final effects.

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Fluoxetine and nortriptyline affect NTPDase and 5'-nucleotidase activities in rat blood serum

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Running title: Antidepressants affect nucleotide hydrolysis in rat blood serum.

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Abstract

Depression is a serious condition associated with considerable morbidity and mortality. Selective serotonin reuptake inhibitors and tricyclic antidepressants, such as fluoxetine and nortriptyline, respectively, were commonly used in treatment for depression. Selective serotonin reuptake inhibitors have been associated with increased risk of bleeding complications, possibly as a result of inhibition of platelet aggregation. ATP, ADP and adenosine are signaling molecules in the vascular system and nucleotidases activities are considered an important thromboregulatory system which functions in the maintenance of blood fluidity. Therefore, here we investigate the effect of *in vivo* (acute and chronic) and *in vitro* treatments with the antidepressant drugs on nucleotidases activities in rat blood serum. In acute treatment, nortriptyline decreased ATP hydrolysis (41%), but not altered ADP and AMP hydrolysis. In contrast, fluoxetine did not alter NTPDase and ecto-5′-nucleotidase activities. A significant inhibition of ATP, ADP, and AMP hydrolysis were observed in chronic treatment with fluoxetine (60%, 32%, and 42% for ATP, ADP, and AMP hydrolysis, respectively). Similar effects were shown in chronic treatment with nortriptyline (37%, 41%, and 30% for ATP, ADP, and AMP hydrolysis, respectively). In addition, there were no significant changes in NTPDase and ecto-5'-nucleotidase activities when fluoxetine and nortriptyline (100, 250, and 500μM) were tested *in vitro*. Our results have shown that fluoxetine and nortriptyline changed the extracellular catabolism of ATP, suggesting that homeostasis of vascular system can be altered by antidepressant treatments.

Keywords: Fluoxetine, nortriptyline, ecto-nucleotidases, NTPDase, ecto-5'-nucleotidase, depression, blood serum

1. Introduction

r nucleoside derivative
diverse biological and
data, 2001; Ralevic &
vascular tone, cardiac

Adenine nucleotides (ATP, ADP, and AMP) and the
adenosine are important signaling molecules that mediate
pathological processes (Agteresch et al. 1999; Latini and Pe
Burnstock, 2003). ATP has been suggested to play a role i

micromolar concentrations of ATP inhibit platelet aggregation by both competitive and non-competitive mechanisms, whereas lower concentrations can be stimulatory (Soslau & Youngprapakorn, 1997). ADP is a nucleotide known to induce changes in platelet shape and aggregation. Several studies have described the important role of these nucleotides in the process of homeostasis and thrombus formation (Coade & Pearson, 1989; Pieber et al. 1991). The nucleoside adenosine, produced by nucleotide degradation, is structure able to act as a vasodilator and cardioprotector (Frassetto et al., 1993; Soslau & Youngprapakorn, 1997).

The levels of extracellular nucleotides can be controlled by the action of ecto- and soluble nucleotidases, including enzymes of the E-NTPDase family as well 5′-nucleotidase (Zimmermann, 2001). Over the last few years, our group has demonstrated a soluble NTPDase activity in rat blood serum (Oses et al, 2004). This enzyme acts together with 5′-nucleotidase (EC 3.1.3.5, CD73), which hydrolyzes the monophosphonucleoside AMP to inorganic phosphate and adenosine in the circulation. This enzyme cascade regulates the availability of ligands (ATP, ADP, AMP, and adenosine) for both nucleotide and nucleoside receptors and, consequently, the duration and extent of receptors activation (Chen & Guidotti, 2001). Therefore, this cascade constituted by soluble NTPDase and 5′-nucleotidase is an enzyme pathway with a double function of removing a signal of ATP

and generating a second signal produced by adenosine (Böhmer et al. 2006). Adenosine also modulates cognitive states and is associated with affective and mood disorders, such as anxiety and depression (Ledent, et al. 1997; Florio et al., 1998; Kaster et al. 2004).

According to the World Health Organization, depression is a worldwide mental health problem affecting an estimated 121 million people. Moreover, depression is a multifaceted disease in terms of symptoms, co-morbidities and health complications. (Rosenzweig-Lipson et al., 2007). Over the last decades, the view that depression is related to a chemical imbalance in the brain has become widely accepted. The earliest treatments for depression were based upon the serendipitous discovery of monoamine oxidase inhibitors (MAOI) and tricyclic antidepressants (TCA), which eventually laid the foundation for further drug strategies. Indeed, the discovery of selective serotonin reuptake inhibitors (SSRIs) and, more recently, inhibitors of both serotonin and norepinephrine reuptake (SNRIs) has changed the face of clinical treatment (Galeotti et al., 2002; Serra et al., 2006; Rosenzweig-Lipson et al., 2007). Nortriptyline, a tricyclic antidepressant, is the leading drug in the treatment for depression. Many investigators have reported that administration of tricyclic antidepressants can result in inhibition of the presynaptic uptake of serotonin (5-HT) and/or noradrenaline (NA) (Morishita & Aoki, 2002). In contrast, fluoxetine, a selective inhibitor of serotonin re-uptake, has little effect on other neurotransmitters (Guze & Gitlin, 1994; Rossi et a., 2004).

Selective serotonin reuptake inhibitors have been associated with increased risk of bleeding complications, possibly as a result of inhibition of platelet aggregation (Monster et al., 2004). Case reports have described patients with various bleeding disorders treated with SSRIs, while observational studies have focused on upper gastrointestinal bleeding, intracranial bleeding, and bleeding during surgery (Meijer et al., 2004). Considering that

ADP is responsible for activation, recruitment and induction of platelet aggregation and the ratio nucleotides/nucleoside in the circulation could evoke responses in both central nervous system and circulatory system, we have here investigated the *in vivo* (acute and chronic) and *in vitro* effects of fluoxetine and nortriptyline on serum nucleotide hydrolysis.

2. Materials and methods

2.1. Chemicals

Fluoxetine, nortriptyline, nucleotides, Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium, and magnesium chloride were purchased from Sigma (USA). All other reagents used were of analytical grade.

2.2. Animals

Male Wistar rats (age around 90 days, with 260-320g) from our breeding stock were housed four to a cage, with food and water *ad libitum*. The animal house temperatures were kept between 22-23 °C with a 12-h ligth/dark cycle (lights on at 07:00). Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee (CEP 06/03016) of the Pontificia Universidade Católica do Rio Grande do Sul, Brazil.

2.3. Isolation of blood serum fraction

Blood was drawn after decapitation of the male Wistar rats, as described by Oses et al (2004). Blood samples were centrifuged in plastic tubes at 5000x *g* for 5 minutes at 20 °C. The serum samples were maintained in ice throughout the experiments.

2.4. *In vivo* treatments

2.4.1. Acute treatment: Animals received one single injection intraperitonially (ip) (10 mg/Kg) of fluoxetine and nortriptyline 1h before they were killed (Zanatta et al., 2001; Borelli et al., 2004; Ejsing & Linnet, 2005; Drapier, et al. 2006; Marx et al., 2006). Control animals received saline injections (0.9% NaCl) in the same volume as those applied to antidepressant-treated rats.

2.4.2. Chronic treatment: The antidepressant drugs were administered daily for 14 days (10 mg/Kg, ip) (Silva & Brandão, 2000; Zanatta et al., 2001; Borelli et al., 2004; Bonanno et al., 2005). Control animals received saline injections (0.9% NaCl) in the same volume as those applied to antidepressants-treated rats.

2.5. *In vitro* treatments

Antidepressants, fluoxetine or nortriptyline, were added to reaction medium before the preincubation with the rat blood serum and maintained throughout the enzyme assays. Antidepressants were tested at final concentrations of 100, 250 and 500 μM (Dhalla et al., 1980; Zanatta et al., 2001).

2.6. Measurement of ATP, ADP, and AMP hydrolysis

ATP, ADP, and AMP hydrolysis were determined using a modification of the method described by Yegutkin (1997). Briefly, as described by Oses et al. 2004, the

reaction mixture containing 3.0 mM ATP, ADP, or AMP as substrate, 112.5 mM Tris-HCl, pH 8.0, was incubated with 0.7 mg to 1.0 mg of serum protein at 37 ºC for 40 min in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of 10% TCA. The samples were chilled on ice and the amount of inorganic phosphate (Pi) released was measured as previously outlined (Chan et al 1986). Incubation times and protein concentrations were chosen to ensure the linearity of the reaction (Oses et al. 2004). Controls to correct for nonenzymatic hydrolysis were performed by adding the serum after the reaction. All samples were centrifuged at 5000x *g* for 5 minutes to eliminate the precipitate protein and 100 μL of supernatant was used for the colorimetric assay. All samples were assayed in duplicate (*in vitro* assays) and triplicate (*in vivo* assays). Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein.

2.7. Protein determination

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

2.8. Statistical analysis

Data were expressed by mean $\pm$ S.D and analyzed by one-way analysis of variance (ANOVA) followed by the Tukey multiple range test considering $P \leq 0.05$ as significant followed the analysis. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer.

2. Results

We evaluated the effect *in vivo* (acute and chronic treatments) and *in vitro* of antidepressant drugs on ATP, ADP and AMP hydrolysis from rat blood serum. NTPDase and ecto-5´-nucleotidase activities were sensitive to antidepressants, fluoxetine and nortriptyline, during chronic treatment. In contrast, in acute treatment only ATP hydrolysis was decreased after administration of nortriptyline. In addition, both drugs did not alter the enzyme activities in all doses tested for *in vitro* treatment.

In acute treatment, animals received a one single injection (10 mg/Kg; ip) of fluoxetine or nortriptyline and, after 1h, the animals were sacrificed. Fluoxetine did not alter ATP, ADP and AMP hydrolysis (Fig.1). However, nortriptyline only changed ATP hydrolysis (41%), when compared to control group (Fig.1).

The antidepressant drugs were administered daily for 14 days in dose of 10 mg/Kg in the chronic treatment. Significant inhibitions of ATP, ADP, and AMP hydrolysis were observed in treatment with fluoxetine (60%, 32%, and 42% for ATP, ADP, and AMP hydrolysis, respectively) (Fig.2). Similar inhibition was observed in chronic treatment with nortriptyline (37%, 41%, and 30% for ATP, ADP, and AMP hydrolysis, respectively) (Fig.2).

In addition, for *in vitro* treatment, antidepressants were tested at final concentrations of 100, 250 and 500 μM. The results have shown that fluoxetine and nortriptyline did not alter NTPDase (Fig.3A and 4A) and 5'-nucleotidase (Fig.3B and 4B) activities from rat blood serum in all concentrations tested (100, 250 and 500μL) when compared to control groups.

3. Discussion

In the present study, there was a significant inhibition of ATP hydrolysis after acute treatment with nortriptyline, but no changes were observed for ADP and AMP hydrolysis in rat blood serum. In contrast, NTPDase and ecto-5′-nucleotidase activities were decreased by chronic treatment with both drugs.

Several studies have found an increased risk of myocardial infarction among depressed patients (Schlienger and Meier, 2003). Tricyclic antidepressants are not recommended in patients with cardiovascular disease owing to their arrhythmic effects (Cohen, et al., 2000, Roose & Spatz, 1998; Roose et al., 1998). Selective serotonin reuptake inhibitors (SSRI), on the other hand, appear to lack the adverse cardiovascular effects of other antidepressants (Roose & Spatz, 1998; Roose et al., 1998). Moreover, SSRIs have been shown to inhibit platelet function both *in vitro* (Serebruany et al., 2001) and *in vivo* (Hergovich et al., 2000), and may thus lower the risk of myocardial infarction (Serebruany et al., 2001, Hergovich et al., 2000). Studies have described patients with various bleeding disorders treated with SSRIs, while observational studies have focused on upper gastrointestinal bleeding, intracranial bleeding, and bleeding during surgery (Meijer et al., 2004). The suggested mechanism underlying these adverse effects is that SSRIs limit uptake of blood serotonin by platelets. Since platelets are unable to synthesize serotonin, this leads to a lower concentration of serotonin within the platelets, and because one of the functions of serotonin within the platelets is to promote platelet aggregation, a decreased amount of serotonin in the platelets may increase the risk of abnormal bleeding (Hergovich et al., 2000).

Nucleotides have been shown to act in blood serum (Kaczmarek et al., 1996; Torres et al., 2002). Extracellularly, the nucleotide ATP has important vascular actions (Burnstock, 1990; Tamajusuko et al., 2006), can be released by cell lyse and/or cell death as well as exocytosis. The role of ATP in the vascular system as a vasoconstrictor is well established and the ADP nucleotide is demonstrated to induce changes in platelet shape and aggregation (Mills et al., 1996; Ralevic, 2000). Their biological effects are mainly determined by their rate of release in the extracellular medium, the activity of nucleotidases and their binding affinity to specific receptors. In elevated concentrations, it induces vasoconstriction of the vascular wall, and promotes its own-stimulated release from endothelial cells. On the other hand, ADP is potent platelet-recruiting factor inducing platelet aggregation via interaction of platelet $P2Y_{12}$ receptors (Gachet, 2001). Circulating soluble ecto-enzymes such as a nucleotidase may reduce the excess of the levels of these molecules and play an important role in maintaining normal physiology. Our laboratory has described a nucleotidase, in fact, a NTPDase activity in rat blood serum (Oses et al., 2004), that together with a 5´-nucleotidase reinforces the effect of nucleotides/nucleoside ratio in the circulation, modulating platelet aggregation and vascular response. Inhibition of nucleotidases may prolong the effect of nucleotides ATP, ADP and AMP at their respective receptors (Imai et al., 1999; Gendron, 2002). Therefore, changes in enzyme activities involved in the nucleotide levels in rat blood serum could contribute to abnormal bleeding observed for antidepressant therapy. Vascular disorders can be also associated to an unbalance in the ratio nucleotides/nucleoside in the circulation. Chronic treatment with antidepressant drugs was able to decrease the nucleotide hydrolysis in rat blood serum. This effect suggests a modulatory role of fluoxetine and nortriptyline on nucleotidase pathway and a possible consequence of the

decrease in the ATP, ADP and AMP hydrolysis is an increase of the levels of these nucleotides and a decrease in the levels of adenosine in the circulation.

We also evaluated the direct effect of antidepressants in ecto-nucleotidases. Previous studies from our laboratory have shown that NTPDase, but not ecto-5'-nucleotidase, activities from cerebral cortex and hippocampus are decreased by the antidepressants sertraline and clomipramine after *in vitro* exposure (Pedrazza et al., in press). It has been suggested that changes in membrane bilayer environment promoted by the interaction with clomipramine and sertraline may be able to promote the inhibitory effect observed on NTPDase activity. The different effects promoted by antidepressant drugs on NTPDase and ecto-5´-nucleotidase activities can be related to the differences in membrane anchorage of these enzymes. Here our results have shown that the antidepressant drugs are not able to alter enzyme activities in rat blood serum, probably because these enzymes are not attached to membrane, but present a soluble form.

In summary, our findings have shown that antidepressants alter nucleotide hydrolysis in rat blood serum, suggesting that homeostasis of vascular system can be influenced by these drugs. This alteration on the nucleotide pathway could be considered one of side effects promoted by the chronic treatment with antidepressant drugs, which could induce relevant actions on vascular system.

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Fig.1

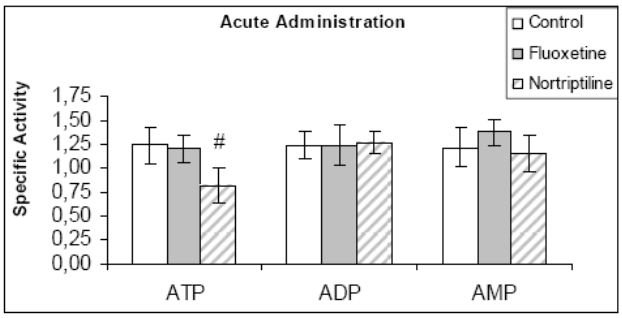
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Fig.2

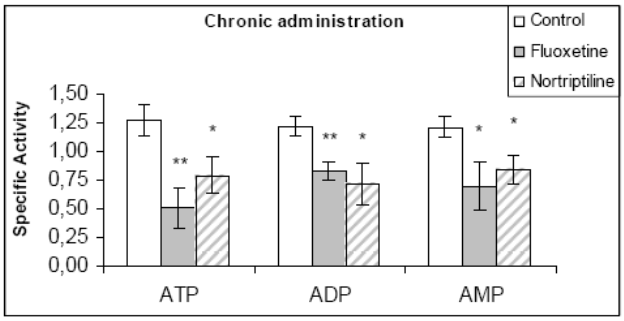
Chronic administration
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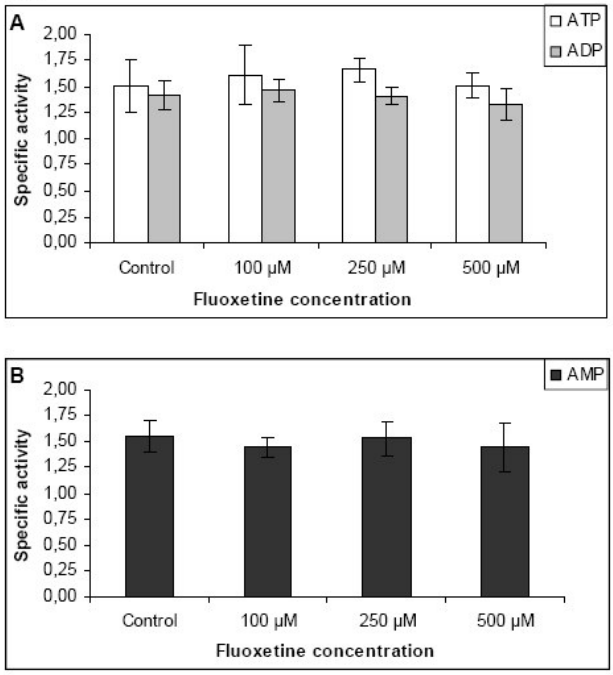
Fig.3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Fluoxetine concentration
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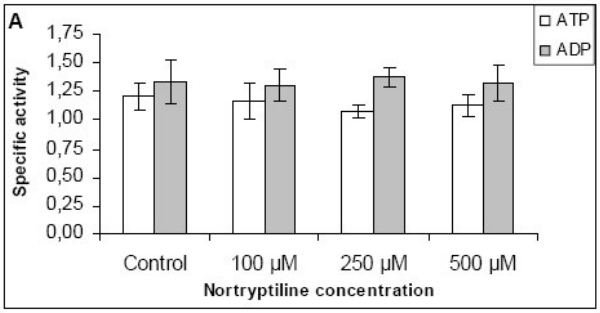
Fluoxetine concentration

Fig.4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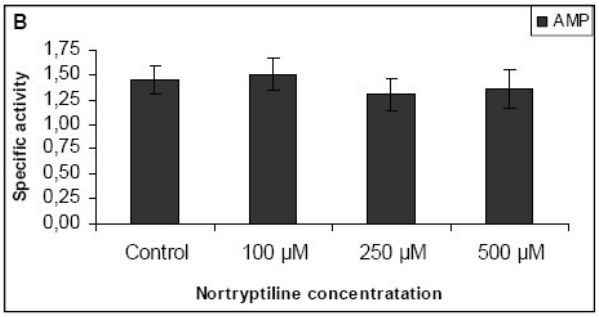
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Nortryptiline concentratation

Figure legends

Fig. 1: Effect of acute treatment with fluoxetine or nortriptyline on ATP, ADP and AMP-hydrolyzing enzyme activities in rat blood serum. Bars represent the mean ± S.D. of eight different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($^{\#}$ represents difference when compared with control and fluoxetine groups).

Fig. 2: Effect of chronic treatment with fluoxetine or nortriptyline on ATP, ADP and AMP-hydrolyzing enzymes activities in rat blood serum. Bars represent the mean ± S.D. of eight different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test (*$p \leq 0.01$, **$p \leq 0.001$ when compared to control group).

Fig. 3: *In vitro* effect of fluoxetine on NTPDase (A) and ecto-5'nucleotidase (B) activities in rat blood serum. Bars represent the mean ± S.D. of eight different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test.

Fig. 4: *In vitro* effect of nortriptyline on NTPDase (A) and ecto-5'nucleotidase (B) activities in rat blood serum. Bars represent the mean ± S.D. of eight different

experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test.

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Ecto-nucleotidase pathway is altered by different treatments with fluoxetine and nortriptyline

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Running title: Antidepressants affect nucleotide hydrolysis in CNS

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Abstract

Depression is one of the most disabling diseases and causes a significant burden to both individual and society. Selective serotonin reuptake inhibitors and tricyclic antidepressants, such as fluoxetine and nortriptyline, respectively, are commonly used in treatment for depression. These antidepressants were tested on cerebral cortex and hippocampal synaptosomes after acute and chronic *in vivo* and *in vitro* treatments. In chronic treatment, fluoxetine and nortriptyline decreased ATP hydrolysis (17,8% and 16,3%, respectively) in hippocampus. In cerebral cortex, nortriptyline increased ATP (32,3%), ADP (51,8%), and AMP (59,5%) hydrolysis. Whereas fluoxetine decreased ATP (25,5%) hydrolysis and increased ADP (80,1%) and AMP (33,3%) hydrolysis. Significant activation of ADP hydrolysis was observed in acute treatment with nortriptyline (49,8%) in cerebral cortex. However, in hippocampus, ATP (24,7%) and ADP (46,1%) hydrolysis were inhibited. Fluoxetine did not alter enzyme activities in acute treatment in both structures. In addition, there were significant changes in NTPDase activities when fluoxetine and nortriptyline (100, 250, and 500 μM) were tested *in vitro*. There was no inhibitory effect of fluoxetine and nortriptyline on AMP hydrolysis in cerebral cortex and hippocampus. The findings showed that these antidepressant drugs can affect the enzymes involved in the adenosine formation, suggesting that the purinergic system can be a target to neurochemical effects promoted by these drugs.

Keywords: Fluoxetine, nortriptyline, ecto-nucleotidases, NTPDases, ecto-5'-nucleotidase, depression, synaptosomes

1. Introduction

According to the World Health Organization, depression is a worldwide mental health problem affecting an estimated 121 million people. It is one of the most disabling diseases and causes a significant burden to both individual and society. If not treated, it could lead to increase morbidity and mortality (Sobocki et al., 2006). Moreover, depression is a multifaceted disease in terms of symptoms, co-morbidities and health complications and the treatment is difficult due to the heterogeneity of the disease (Rosenzweig-Lipson et al., 2007).

There is increasing evidence that affective disorders are associated with dysfunction of neurotransmitter postsynaptic transduction pathways and that chronic treatment with drugs results in adaptative modification of these pathways (Moretti et al., 2003). Over the last decades, the view that depression is related to a chemical imbalance in the brain has become widely accepted. The earliest treatments for depression were based upon the serendipitous discovery of monoamine oxidase inhibitors (MAOI) and tricyclic antidepressants (TCA), which eventually laid the foundation for further drug strategies. Indeed, the discovery of selective serotonin reuptake inhibitors (SSRIs) and, more recently, inhibitors of both serotonin and noradrenaline reuptake (SNRIs) has changed the face of clinical treatment (Galeotti et al., 2002; Serra et al., 2006; Rosenzweig-Lipson et al., 2007).

Most antidepressive agents effective in the therapy of mood disorders share the common feature of being able to increase the synaptic monoamine levels. This

increase is considered only the first step in a series of unknown adaptations in brain, which are responsible for the long-term efficacy (Carboni et al., 2006).

Nortriptyline, a tricyclic antidepressant, is the leading drug in the treatment for depression. Many investigators have reported that administration of tricyclic antidepressants can result in inhibition of the presynaptic uptake of serotonin (5-HT) and/or noradrenaline (NA) (Morishita & Aoki, 2002; Stoll & Gentile, 2006; Su et al., 2007). In contrast, fluoxetine, a selective inhibitor of serotonin reuptake, has little effect on other neurotransmitters (Rossi et al., 2004; Cecconi et al., 2007; Chen et al., 2007).

Serotonin and noradrenaline can be co-released with ATP, which is considered a neurotransmitter and neuromodulator in central nervous system (CNS) (Burnstock, 2004). Extracellular ATP evokes responses by two subclasses of P2 purinoreceptors, P2X and P2Y (Ralevic & Burnstock, 1998, Burnstock, 2007). It has been shown that P2X receptors are coupled to ligand-gated Ca^{2+}-permeable channels, whereas the P2Y receptors have been considered a G-protein-linked (Burnstock, 2004). The signaling actions induced by extracellular ATP are directly correlated to the activity of ecto-nucleotidases since these enzymes trigger enzymatic conversion of ATP to adenosine (Zimmermann, 2001; Robson et al., 2006). Ecto-nucleotidases comprise a group of ecto-enzymes involved in the control of nucleotide and nucleoside levels in the synaptic cleft, which includes NTPDase (nucleoside triphosphate diphosphohydrolase) family and ecto-5´-nucleotidase (Zimmermman, 2001). Four members of the NTPDase family are tightly bound to the plasma membrane via two transmembrane domains, and have a large extracellular region with an active site facing the extracellular side.

NTPDase1, 3 and 8 slightly prefer ATP over ADP by a ratio of 1, 3 and 2, respectively. Meanwhile, NTPDase2 prefers triphosphonucleosides (Bigonnesse et al., 2004; Robson et al., 2006). Adenosine, a product of ATP catabolism, can evoke its neuromodulatory effects by four subtypes of P_1-purinoreceptors named A_1, A_{2A}, A_{2B} and A_3 (Brundege & Dunwiddie, 1997; Cunha, 2001; Dunwiddie & Masino, 2001; Cunha, 2005). Studies have shown that adenosine modulates cognitive states and is associated with affective and mood disorders, such as anxiety and depression (Ledent, et al. 1997; Florio et al., 1998; Kaster et al. 2004). Moreover, it has been shown that hippocampal serotonergic neurotransmission is modulated by hippocampal adenosine receptor subtypes (Okada et al., 1999).

Considering that (i) adenosine is able to modulate 5-HT release, (ii) 5-HT and NA can be co-released with ATP and (iii) the action of ecto-nucleotidases represents one of the most important sources of extracellular adenosine, the aim of this study was to evaluate the effect *in vivo* and *in vitro* of fluoxetine and nortriptyline on the ecto-nucleotidases in synaptosomes from hippocampus and cerebral cortex of rats.

2. Materials and Methods

2.1. Chemicals

Fluoxetine, nortriptyline, nucleotides, Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium, and magnesium chloride were purchased from Sigma (USA). All other reagents used were of analytical grade.

2.2. Animals

Male Wistar rats (age around 90 days, with 260-320g) from our breeding stock were housed four to a cage, with food and water *ad libitum*. The animal house temperatures were kept between 22-23 ^{o}C with a 12-h ligth/dark cycle (lights on at 07:00). Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee (CEP 06/03016) of the Pontificia Universidade Católica do Rio Grande do Sul, Brazil.

2.3. *In vivo* treatments

2.3.1. Acute treatment: Animals received one single injection intraperitonially (i.p.) (10 mg/Kg) of fluoxetine or nortriptyline 1h before they were killed (Zanatta et al., 2001; Borelli et al., 2004; Ejsing & Linnet, 2005; Drapier, et al. 2006; Marx et al., 2006). Control animals received saline injections (0.9% NaCl) in the same volume as those applied to antidepressant-treated rats.

2.3.2. Chronic treatment: The antidepressant drugs were administered daily for 14 days (10 mg/Kg, i.p.) (Silva & Brandão, 2000; Zanatta et al., 2001; Borelli et al., 2004; Bonanno et al., 2005). Control animals received saline injections (0.9% NaCl) in the same volume as those applied to antidepressant-treated rats.

2.4. *In vitro* treatments

Antidepressants, fluoxetine or nortriptyline, were added to reaction medium before the preincubation with synaptosomal preparation and maintained throughout the enzyme assays. Antidepressants were tested at final concentrations of 100, 250 and 500 μM (Dhalla et al., 1980; Zanatta et al., 2001; Pedrazza et al. 2007).

2.5. Synaptosomal preparation

The rats were killed by decapitation, and their cerebral cortex and hippocampus were dissected, homogenized in 10 and 5 volumes, respectively, in an ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.5. The synaptosomes were isolated as described previously (Nagy & Delgado-Escueta, 1984). Briefly, 0.5 mL of the crude mitochondrial fraction was mixed 4.0 mL of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15,000x *g* for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/mL. The material was prepared fresh daily and maintained at 0-4ºC throughout preparation.

2.6. Determination of ecto-nucleotidase activities

The reaction medium used to assay ATP and ADP hydrolysis was essentially as described previously (Battastini et al., 1991), and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM TRIS-HCl buffer, pH 8.0, in a final volume of 200 µl. The synaptosomal fraction (10-20 µg protein) was added to the reaction mixture and preincubated for 10 min at 37˚C. The reaction was initiated by the addition of 1 mM ATP or ADP as substrate and stopped by the addition of 200 µl 10% trichloroacetic acid. The samples were chilled on ice for 10 minutes and 100 µl samples were taken to

assess the released inorganic phosphate (Pi) (Chan et al., 1986).The reaction medium used to assay 5'-nucleotidase activity contained 10 mM $MgCl_2$, 0.1 M TRIS-HCl, pH 7.5 and 0.15 M sucrose to final volume of 200 µl (Heymann et al., 1984). The synaptosomal fraction (10-20µg protein) was preincubated for 10 min at 37˚C. The reaction was initiated by the addition of 1.0 mM AMP as substrate and stopped by the addition of 200 µl 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100 µl samples were taken to assess the released inorganic phosphate (Pi) (Chan et al., 1986). In enzyme assays, incubation time and protein concentration were chosen in order to ensure the linearity of the reaction. Controls, with the addition of the enzyme preparation after the addition of trichloroacetic acid, were used to correct non-enzymatic hydrolysis of the substrates. All samples were assayed in duplicate (*in vitro* assays) and triplicate (*in vivo* assays). Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein.

2.7. Protein determination

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

2.8. Analysis of gene expression by semi-quantitative RT-PCR

The analysis of the expression of NTPDase 1, 2, 3 and ecto-5´-nucleotidase was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. After acute and chronic treatments, hippocampus and cerebral cortex of rats were isolated for total RNA extraction using Trizol reagent

(Invitrogen) in accordance with manufacture´s instructions. RNA purity was quantified spectrophotometrically and tested by eletrophoresis in a 1.0% agarose gel containing ethidium bromide. The cDNA species were synthesized with SuperScript^{TM} III First-Strand Synthesis SuperMix (Invitrogen) from 3 µg of total RNA following suppliers. RT reactions were performed for 50 min at 42°C. cDNA (1µL) was used as a template for PCR with specific primers for NTPDase1,2,3 and 5´-nucleotidase. β-actin was used for normatization as a constitutive gene. PCR reactions have a volume of 25 µL using a concentration of 0.4 µM of each primer indicated below and 200 µM and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. Conditions for all PCR were as follows: Initial 1 min denaturation step at 94°C, 1 min annealing step (NTPDase 1, 3 and 5´-nucleotidase: 65°C; NTPDase2: 66°C; β-actin: 58.5°C), 1 min extension step at 72°C for 35 cycles and a 10 min final extension a 72°C. The amplification products were: NTPDase1 – 543bp; NTPDase2 – 331bp; NTPDase3 – 267bp; 5´-nucleotidase – 403bp; β-actin – 210bp. The primers were described previously (Vuaden et al., 2007). For each set of PCR reactions, negative controls were included. Five microliters of the PCR reaction was analyzed on a 1% agarose gel, containing ethidium bromide and visualized with ultraviolet light.

2.9. Statistical analysis

Data were expressed by mean $\pm$ S.D and analyzed by one-way analysis of variance (ANOVA) followed by the Tukey multiple range test considering $P<0.05$ as significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer.

3. Results

We evaluated the effect *in vivo* (acute and chronic treatments) and *in vitro* of antidepressant drugs on ATP, ADP and AMP hydrolysis from cerebral cortex and hippocampus of rats.

In acute treatment with nortriptyline, ATP (24.7%) and ADP (46.1%) hydrolysis were inhibited in hippocampus (Fig.1A). However, a significant activation of ADP hydrolysis was observed in acute treatment with nortriptyline (49.8%) in cerebral cortex (Fig.2A). AMP hydrolysis was not affected by nortriptyline (Fig. 1B and Fig. 2B). Fluoxetine did not altered enzymes activities in acute treatment for both structures (Fig.1 A,B and Fig.2 A,B).

In chronic treatment, nortriptyline and fluoxetine decreased ATP hydrolysis (17.8% and 16.3%, respectively) (Fig.3A) in hippocampus whereas these drugs did not alter ADP and AMP hydrolysis in both structures (Fig. 3A,B). However, in cerebral cortex, these drugs promote different effects on nucleotide hydrolysis. Nortriptyline increased ATP, ADP and AMP hydrolysis (32.3%, 51.8% and 59.5%, respectively) (Fig.4A,B) and fluoxetine decreased ATP (25.5%) hydrolysis and increased ADP (80.1%) and AMP (33.3%) hydrolysis.

The effect *in vitro* of nortriptyline on nucleotide hydrolysis was also tested in cortical and hippocampal synaptosomes. A significant inhibition in ATP hydrolysis (21.1-74.5%) was observed in hippocampal synaptosomes for all concentrations tested, but ADP hydrolysis was inhibited only at 250 and 500 μM (39.8%-60.4%) (Fig.5A). A similar decrease of cortical NTPDase activity was observed in the all concentrations tested (37.5 - 73% for ATP hydrolysis and 41.4 - 80.3% for ADP

hydrolysis) (Fig.5C). Nortriptyline failed to inhibit AMP hydrolysis in both brain regions tested (Fig.5B,D).

In addition, for *in vitro* treatment, fluoxetine inhibit both ATPase and ADPase activities in all concentrations tested (100 to 500μM). The inhibition promoted by fluoxetine in hippocampal synaptosomes varied from 66.8% to 82.2% for ATP hydrolysis and from 54.7% to 68.3% for ADP hydrolysis (Fig.6A). The inhibitory effect in cortical synaptosomes varied from 34.7% to 86.9% in ATP hydrolysis and from 31.2% to 76.4% for ADP hydrolysis (Fig.6C). There was no observed effect of fluoxetine on AMP hydrolysis in both hippocampus and cerebral cortex (Fig.6B,D).

The effects promoted by antidepressant drugs could be a consequence of transcriptional control. We have evaluated the gene expression for NTPDase1, NTPDase2 e NTPDase3 and 5´-nucleotidase. The constitutive gene was normalized to β-actin expression to allow the comparison in different experimental conditions. The semi-quantitative RT-PCR analyses were performed when kinetic alterations had occurred. For this reason, NTPDases and 5'-nucleotidase were not analyzed after acute treatment with fluoxetine in hippocampus and cerebral cortex. The acute treatment with nortriptyline produced an increase in the NTPDase3 transcript levels in hippocampus (Fig. 7A, 7B). Interestingly, NTPDase1 and NTPDase 2 demonstrated an increase in the transcript levels in cerebral cortex (Fig. 8A, 8B).

The chronic treatment with nortriptyline promoted a decrease in NTPDase1, NTPDase2 and NTPDase3 transcript levels in hippocampus (Fig. 7C, 7D). In contrast, NTPDase1 and 5´-nucleotidase presented an increase of gene

expression for NTPDase1 and 5´-nucleotidase in cerebral cortex (Fig 8C, 8D). The chronic treatment with fluoxetine produced an enhancement for NTPDase1 and NTPDase3 transcripts levels in hippocampus (Fig. 7E, 7F) whereas NTPDase 1 and NTPDase2 were increased in cerebral cortex (Fig. 8E, 8F).

4. Discussion

NTPDase and ecto-5´-nucleotidase activities were sensitive to antidepressants, fluoxetine and nortriptyline, during chronic treatment in cerebral cortex. 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. In addition, for *in vitro* treatment, fluoxetine and nortriptyline inhibited the NTPDase activities in both structures tested. Furthermore, antidepressant drugs promoted changes in the transcript levels for NTPDase1, NTPDase2, NTPDase3 and 5´-nucleotidase.

Since these enzymes contribute to maintenance of physiological effects of extracellular ATP, ADP, AMP and adenosine, the influence of the enzymatic cascade involved in the control of these nucleotides and nucleosides have been proposed in several pathophysiological situations (Agteresch et al., 1999). Chronic treatment with antidepressant drugs was able to alter the nucleotide hydrolysis in cerebral cortex whereas only ATP hydrolysis was inhibited in hippocampus. This effect suggests a modulatory role of fluoxetine and nortriptyline on nucleotidase pathway in cerebral cortex, suggesting that the increase in the ATP, ADP and AMP hydrolysis could induce an increase in the levels of adenosine extracellular. However, fluoxetine and nortryptiline decrease ATP hydrolysis in hippocampus,

which could induce an increase of ATP levels and a delayed production of adenosine. Okada et al., 1999, clearly shows that hippocampal serotonergic neurotransmission is modulated by hippocampal adenosine receptor subtypes. Tricyclic antidepressants (clomipramine, desipramine, imipramine, and trimipramine) are moderately potent inhibitors of the plasma membrane Ca^{2+}-ATPase activity measured in erythrocyte ghosts. Clomipramine, as the more efficient inhibitor, does not modify the binding affinity of ATP and the inhibitory effect should be related to the rate of phosphorylated intermediate of the enzyme formation (Plenge-Tellechea et al., 1999). Furthermore, previous studies demonstrated that imipramine and fluoxetine altered Na^+,K^+-ATPase activity in synaptic plasma membranes from the cerebral cortex of rats after chronic treatment (Zanatta et al., 2001).Tricyclic antidepressants (imipramine, desipramine, clomipramine, amitriptyline, fluoxetine) exhibited inhibitory effect on ATP-dependent calcium uptake by the endoplasmatic reticulum of lysed synaptosomes from rat brain cortex (Couture et al., 2001). Tricyclic antidepressants are potent inhibitors of neuronal uptake of adenosine, which may raise the endogenous adenosine levels (Phillis & Wu, 1982; Phillis, 1984). Moreover, clomipramine and desipramine elicited depressions, which were antagonized by caffeine, an adenosine antagonist (Phillis, 1984). Recent findings have demonstrated that adenosine A_{2A} receptor antagonists produce an antidepressant-like effect in two models predictive of clinical antidepressant activity, the forced swimming test and the tail suspension test (El Yakoubi et al., 2001). Studies have shown that adenosine administration produces an antidepressant-like effect in the forced swimming test (FST) and in the tail suspension test, mediated through an

interaction with A1 and A2A receptors (Kaster et al., 2004). Therefore, changes in the ecto-nucleotidase pathway induced by antidepressant drugs could modulate the adenosine levels and, consequently, the neuromodulation promoted by this nucleoside in depressive patients treated with these drugs.

In order to verify the direct effect of antidepressant drugs on ecto-nucleotidase activities, *in vitro* assays were performed in synaptosomes from hippocampus and cerebral cortex of rats. All drugs promoted significant changes on NTPDase activities after in vitro exposure. Previous studies demonstrated that imipramine and fluoxetine decreased Na^+,K^+-ATPase activity in synaptic plasma membranes from the cerebral cortex of rats in a dose-dependent manner (Zanatta et al., 2001). Morever, our laboratory have shown that NTPDase, but not ecto-5'-nucleotidase, activities from cerebral cortex and hippocampus are decreased by the antidepressants sertraline and clomipramine after *in vitro* exposure (Pedrazza et al., 2007). Barcellos et al. (1998) have demonstrated that imipramine, desipramine and amitriptyline (antidepressants tryciclic) *in vitro* decreased ATP and ADP hydrolysis in synaptosomes from cerebral cortex of rats. It has been suggested that changes in membrane bilayer environment promoted by the interaction with antidepressant may be able to promote the inhibitory effect observed on NTPDase activity. The different effects promoted by antidepressant drugs on NTPDase and ecto-5´-nucleotidase activities can be related to the differences in membrane anchorage of these enzymes.

The kinetic effect promoted by antidepressant drugs could be a consequence of transcriptional control and/or post-translational mechanisms. For acute treatment, nortriptyline promoted an increase of ADP hydrolysis and a

simultaneous increase of NTPDases transcript levels. However, some changes on nucleotide hydrolysis promoted by chronic treatment with fluoxetine are not in agreement with the changes observed in the transcript levels for NTPDases and ecto-5´-nucleotidase. The transcription machinery is continuously controlled by a complex signaling system, creating a set of signals able to adjust gene expression profile of the cell. This signal transduction can be exerted by proteins, products of enzyme reactions or even toxins able to regulate transcription factors (Krishna et al., 2006). The phenomena known as positive feedback loop (Pomerening et al., 2003; 2005), which is situated at the interface of genetic and metabolic networks, could explain the concomitant decrease of ATP hydrolysis and the increase of NTPDase1, NTPDase2 and NTPDase3 mRNA levels after chronic fluoxetine treatment.

In summary, we have shown that fluoxetine and nortriptyline can affect the enzymes involved in the adenosine formation, suggesting that the purinergic system can be a target to neurochemical effects promoted by these drugs.

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Figure 1- Acute treatment in hippocampus

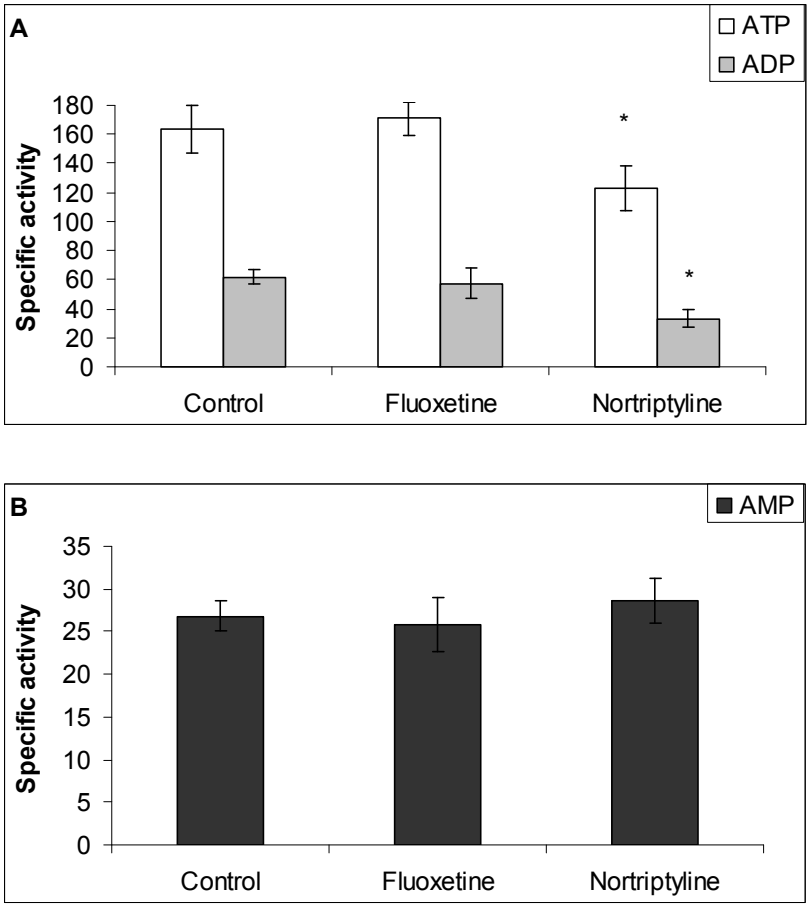
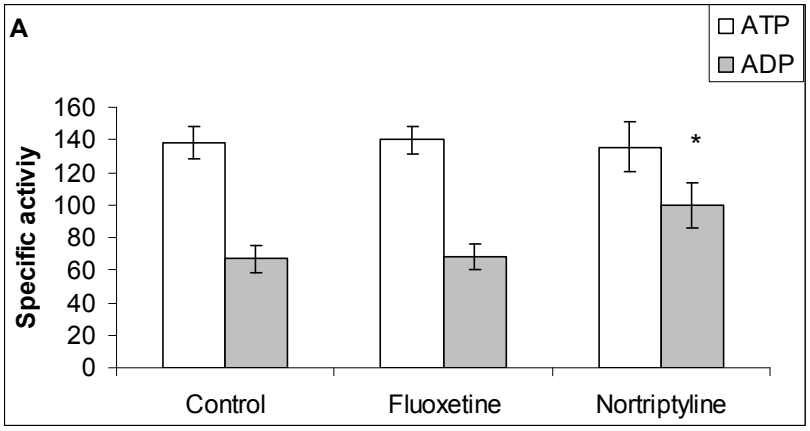


Figure 2- Acute treatment in cerebral cortex



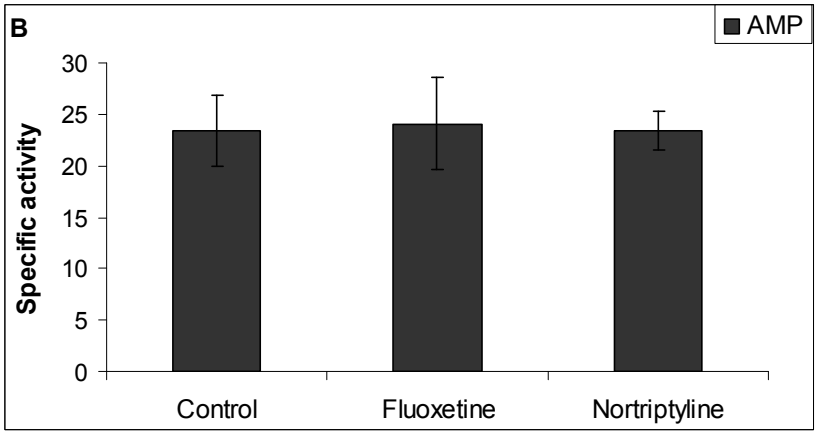


Figure 3- Chronic treatment in hippocampus

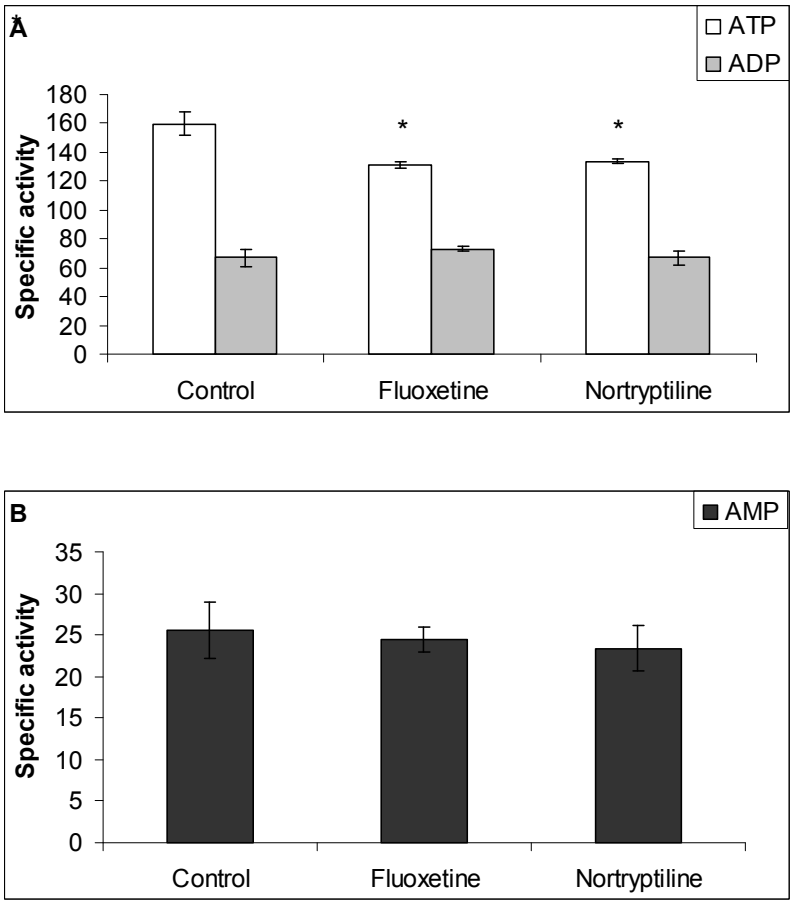


Figure 4 – Chronic treatment in cerebral cortex

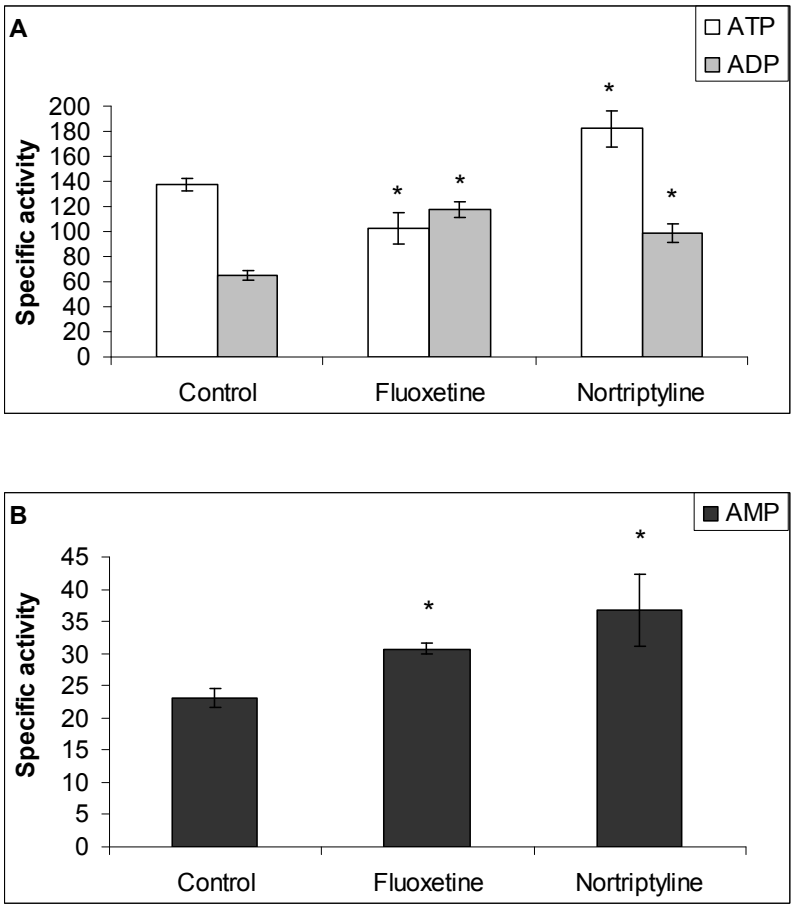


Figure 5- *In vitro* treatment with nortriptyline

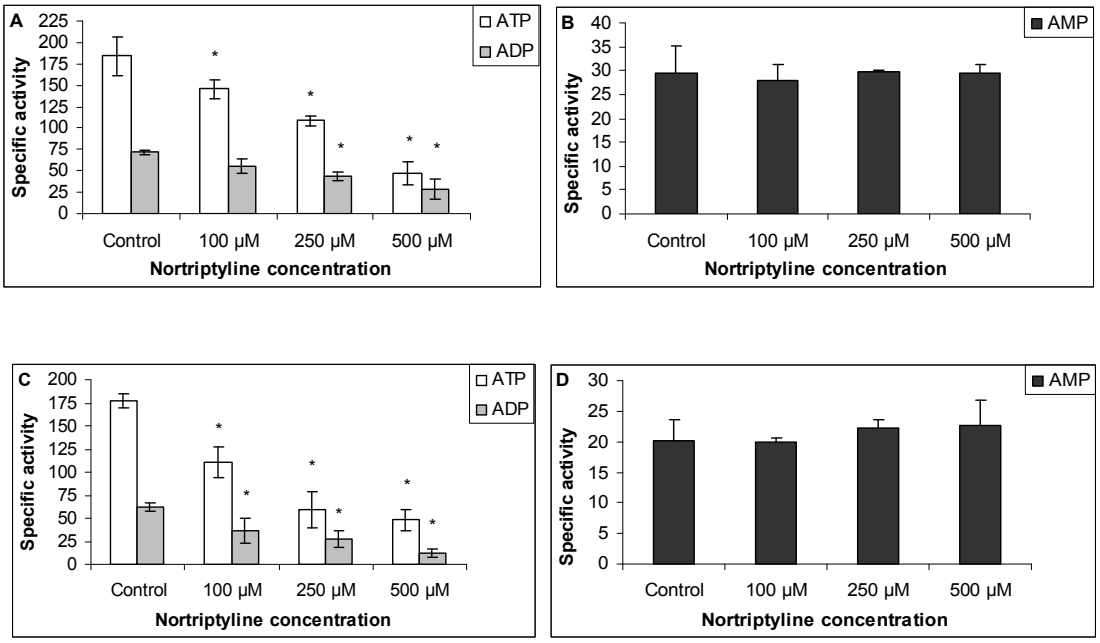


Figure 6- *In vitro* treatment with fluoxetine

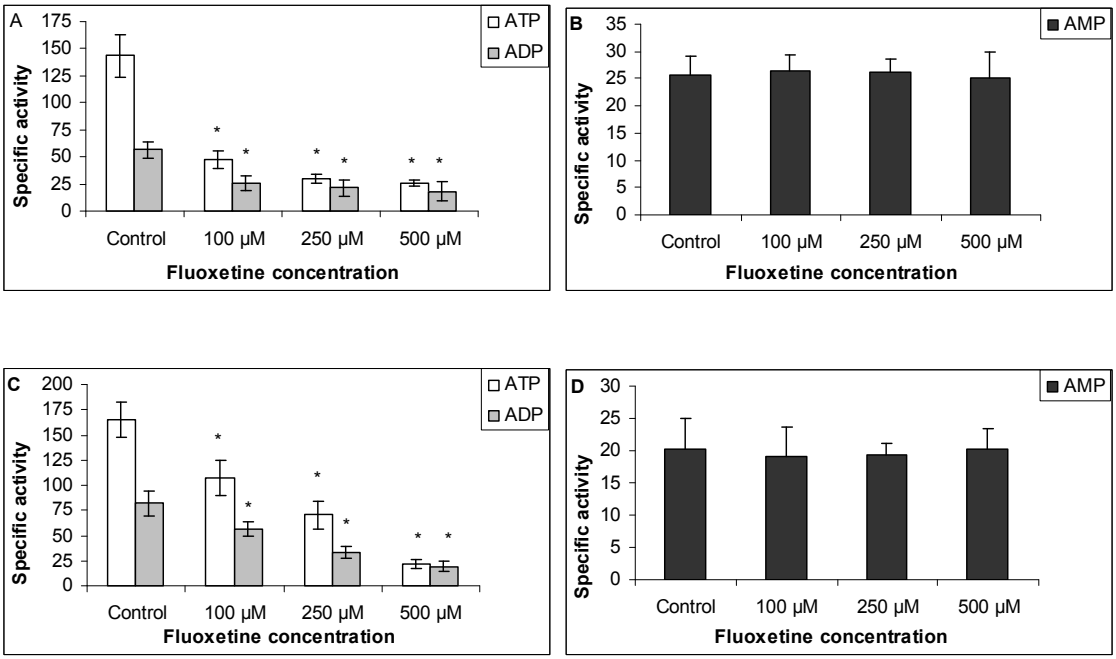


Figure 7 – Gene Expression after treatment with nortriptyline

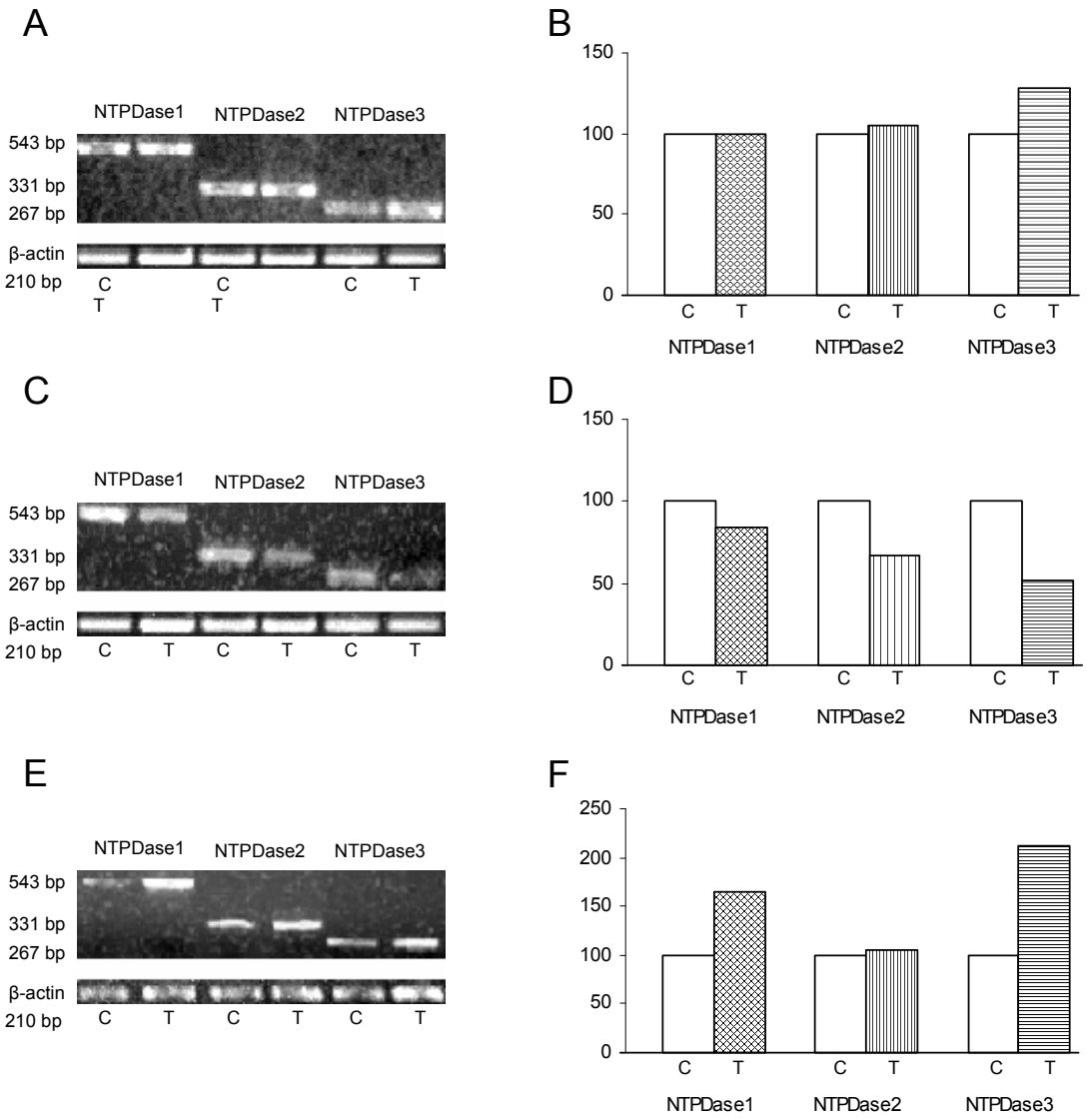


Figure 8- Gene Expression after treatment with fluoxetine

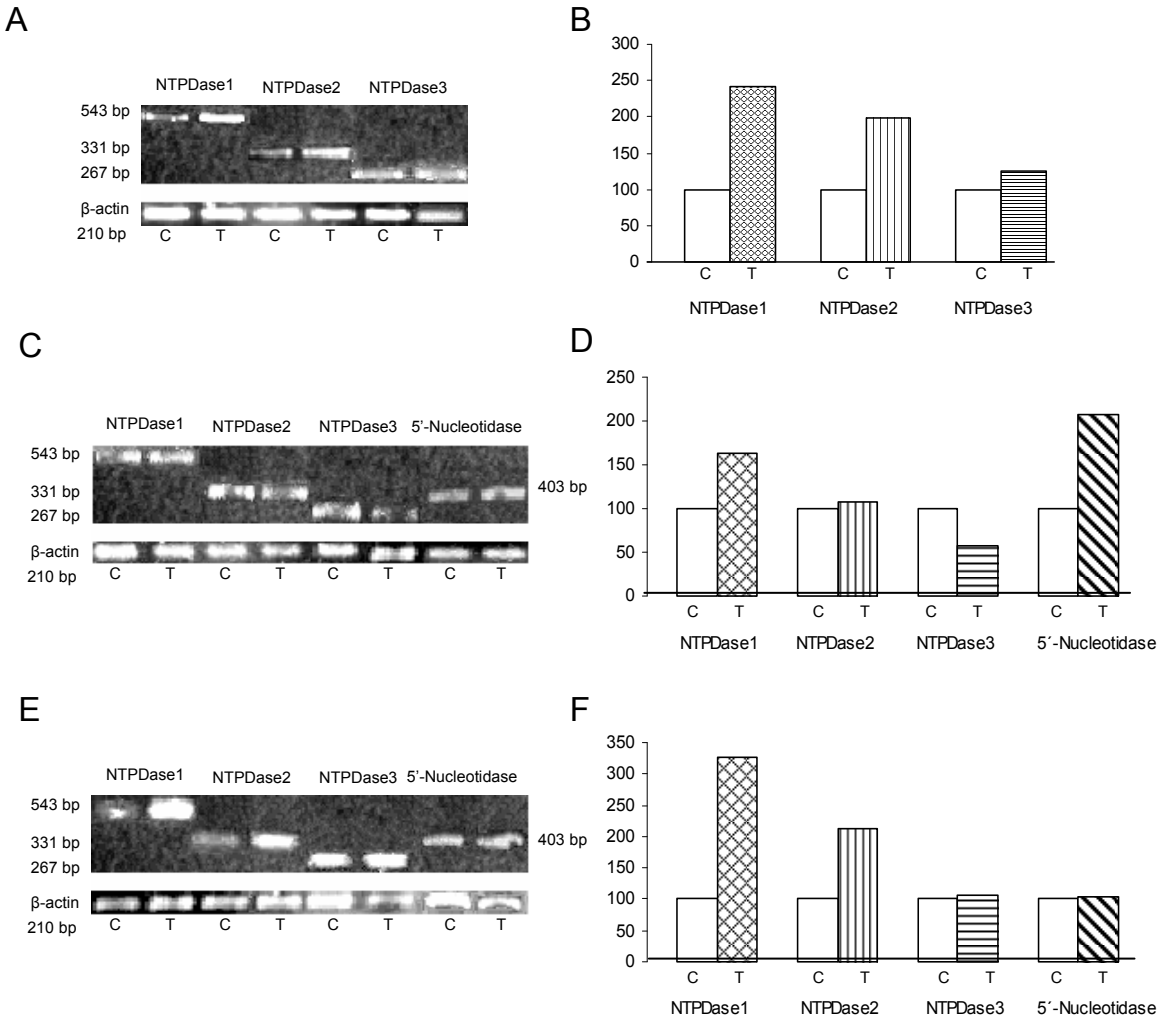


Figure legends

Fig. 1: Effect of acute treatment with fluoxetine or nortriptyline on ATP and ADP (1A) and AMP (1B) hydrolysis in hippocampus of rat. Bars represent the mean ± S.D. of five different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($P \leq 0.01$, when compared to control group).

Fig. 2: Effect of acute treatment with fluoxetine or nortriptyline on ATP and ADP (A) and AMP (B) hydrolysis in cerebral cortex of rats. Bars represent the mean ± S.D. of five different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($p \leq 0.01$, when compared to control group).

Fig. 3: Effect of chronic treatment with fluoxetine or nortriptyline on ATP and ADP(A) and AMP(B) hydrolysis in hippocampus of rat. Bars represent the mean ± S.D. of five different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($p \leq 0.01$, when compared to control group).

Fig. 4: Effect of chronic treatment with fluoxetine or nortriptyline on ATP and ADP (A) and AMP (B) hydrolysis in cerebral cortex of rats. Bars represent the mean ± S.D. of five different experiments. The specific enzyme activities are reported as

nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($p \leq 0.01$, when compared to control group).

Fig. 5: *In vitro* effect of fluoxetine on NTPDase (A,C) and ecto-5'nucleotidase (B,D) activities in hippocampus and cerebral cortex of rat, respectively. Bars represent the mean ± S.D. of five different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($p \leq 0.01$, when compared to control group).

Fig. 6: *In vitro* effect of nortriptiline on NTPDase (A,C) and ecto-5'nucleotidase (B,D) activities in hippocampus and cerebral cortex of rat, respectively. Bars represent the mean ± S.D. of five different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($p \leq 0.01$, when compared to control group).

Fig. 7: Gene expression patterns after acute treatment with nortriptyline (A and B) and chronic treatment with nortriptyline(C and D) and fluoxetine (E and F) in hippocampus of rats. Three independent experiments were performed, with entirely consistent results.

Fig. 8: Gene expression patterns after acute treatment with nortriptyline (A and B) and chronic treatment with nortriptyline(C and D) and fluoxetine (E and F) in

cerebral cortex of rats. Three independent experiments were performed, with entirely consistent results.

4. Discussão Geral

O ATP é uma importante molécula sinalizadora no espaço extracelular e desempenha importantes papéis em condições fisiológicas e patológicas. A degradação desta molécula é catalisada pelas ecto-nucleotidases, entre as quais se destacam a família das NTPDases e a ecto-5'-nucleotidase. Estas enzimas são muito importantes nas respostas mediadas pelos nucleotídeos, já que controlam os níveis extracelulares destas substâncias e a possível ativação dos receptores purinérgicos (Ralevic & Burnstock, 1998; Zimmermann, 2001).

Diferentes estudos têm demonstrado o efeito de fármacos antidepressivos sobre algumas ATPases, como a Mg^{2+}-ATPase (Nag and Ghosh, 1973), F_0F_1-ATPase (Souza et al., 1994), and Na^+, K^+-ATPase (Zanatta et al., 2001). Além disso, um estudo anterior demonstrou o efeito *in vitro* de alguns fármacos antidepressivos sobre as ecto-nucleotidases em sinaptossomas de córtex cerebral de ratos (Barcellos et al., 1998). Sanganahalli et al. (2000) demonstrou que antidepressivos tricíclicos (imipramina, desipramina, amitriptilina e nortriptilina) inibem a atividade da Na^+, K^+-ATPase em membranas sinaptossomais de cérebro de ratos.

No Capítulo 1, foi avaliado o efeito *in vitro* dos fármacos antidepressivos, sertralina e clomipramina, na hidrólise de ATP, ADP e AMP em sinaptossomas de hipocampo e córtex cerebral de ratos. Esses experimentos foram realizados com o objetivo de verificar se os fármacos antidepressivos tinham algum efeito direto sobre as atividades ectonucleotidásicas, uma vez que tal efeito poderia influenciar

os resultados dos experimentos *in vivo* que seriam posteriormente realizados. Os resultados demonstraram que tanto sertralina quanto clomipramina inibiram a hidrólise de ATP e ADP nas concentrações testadas em sinaptossomas de hipocampo e córtex cerebral de ratos, não provocando nenhuma alteração significativa na atividade da ecto-5′-nucleotidase. Há evidências que fármacos antidepressivos tricíclicos alteram a organização estrutural de membranas. Além disso, estudos prévios têm mostrado que fluoxetina e imipramina alteram a atividade da Na^+,K^+-ATPase devido a hidrofobicidade destes fármacos (Zanatta et al., 2001). Este efeito poderia estar acontecendo com as NTPDases, já que são enzimas de membrana como a Na^+, K^+-ATPase. A partição de fármacos pela bicamada lipídica afeta a fluidez da membrana, consequentemente mudando a função e estrutura das proteínas de membrana. NTPDases1, 2, 3 e 8 estão firmemente ancoradas na membrana via dois dominios transmembranas que, para a NTPDase1, são importantes para manter a atividade catalítica e a utilização do substrato específico (Grinthal and Guidotti, 2006).

O Capítulo 2 foi realizado com o intuito de verificar o efeito dos antidepressivos sobre a família das NTPDases e a ecto-5′-nucleotidase solúveis. No tratamento *in vitro,* os resultados mostraram que tanto fluoxetina quanto nortriptilina não tiveram nenhum efeito sobre as enzimas nas doses testadas, o que poderia ser explicado pelo fato de que elas não estão ancoradas em membranas. Este estudo mostrou que após o tratamento agudo com nortriptilina, a hidrólise de ATP foi inibida, mas não foi observada nenhuma alteração na hidrólise de ADP e AMP em soro de ratos. Entretanto, após o tratamento crônico com ambas as drogas, a hidrólise da ATP, ADP e AMP foram diminuídas. Diferentes

estudos têm mostrado um aumento do risco de infarto no miocárdio em pacientes depressivos (Schlienger & Méier, 2003). Além disso, já se sabe que não é recomendado o uso de antidepressivos tricíclicos em pacientes com problemas cardiovasculares, devido aos efeitos adversos dos TCAs (Roose & Spatz, 1998; Roose et al., 1998; Cohen et al., 2000). Extracelularmente, o nucleotídeo ATP possui importante ação vascular (Burnstock,1990; Tamajusuku et al., 2006), podendo ser liberado pela lise da célula e/ou morte celular e exocitose. O ATP no sistema vascular atua como vasoconstritor, já o ADP demonstrou ter a capacidade de alterar a agregação plaquetária (Ralevic, 2000). Além disso, diversos estudos mostram o efeito cardioprotetor da adenosina (Peart et al., 2007). Os resultados observados no capítulo 2 mostram que os antidepressivos testados alteram a hidrólise de nucleotídeos no soro de ratos, sugerindo que a homeostasia do sistema vascular pode ser influenciada por estas drogas. Esta alteração na rota de formação de adenosina pode ser considerada um dos efeitos adversos promovidos pelo tratamento crônico com fármacos antidepressivos, os quais podem induzir ações relevantes no sistema vascular.

O capítulo 3 mostra o efeito *in vivo* (tratamentos agudo e crônico) e *in vitro* da fluoxetina e da nortriptilina na hidrólise de ATP, ADP e AMP em hipocampo e córtex cerebral de ratos. Além disso, foi avaliada a expressão gênica das NTPDases1, NTPDase2, NTPDase3 e da ecto-5´-nucleotidase após os tratamentos *in vivo* que induziram alterações cinéticas. As atividades das NTPDases e da ecto-5´-nucleotidase foram alteradas após o tratamento crônico com os antidepressivos em córtex cerebral. Entretanto, no hipocampo, o tratamento crônico com ambas as drogas produziu somente uma diminuição na

hidrólise de ATP. Já o tratamento agudo com nortriptilina no córtex cerebral provocou um aumento na hidrólise de ADP e inibiu a hidrólise de ATP e ADP no hipocampo. A fluoxetina não alterou a hidrólise de ATP, ADP e AMP no tratamento agudo no córtex cerebral e no hipocampo. Além disso, ambas as drogas inibiram a atividade das NTPDases em todas as doses testadas durante o tratamento *in vitro* em ambas estruturas. Um efeito similar foi observado com sertralina e clomipramina, descrito no capítulo 1. A inibição das NTPDases poderia promover um aumento extracelular dos níveis de ATP e estimular a apoptose. Além disso, considerando que o ATP é um importante neurotransmissor excitatório no SNC (Di Ioro et al., 1998), a inibição da hidrólise do ATP poderia promover diversos processos relacionados com a excitabilidade cerebral. Sabe-se que as ecto-nucleotidases contribuem para a manutenção dos níveis extracelulares de ATP, ADP, AMP e adenosina e que diversas situações patofisiológicas podem influenciar esta cascata enzimática (Agteresch et al., 1999, Bonan et al., 2001). Assim, nossos resultados demonstrando o efeito inibitório promovido por estes fármacos sugerem um possível aumento nos níveis extracelulares de ATP e a conseqüente diminuição nos níveis de adenosina. Após os tratamentos agudo e crônico no hipocampo e o tratamento agudo no córtex cerebral não foram observadas alterações na reação de hidrólise do AMP até adenosina, mostrando que a ecto-5´-nucleotidase não foi sensível aos efeitos destes fármacos. Além das alterações cinéticas, os fármacos mostraram efeitos sobre os níveis de transcritos na maioria dos genes das NTPDases. As alterações provocadas pela nortriptilina na cinética das duas estruturas foram concomitantes com as mudanças nos níveis de transcritos na maioria dos genes das NTPDases e na ecto-5´-nucleotidase. Já

as mudanças nos níveis de transcritos para as NTPDases após o tratamento crônico com fluoxetina no hipocampo e no córtex cerebral foram contrárias às alterações encontradas na cinética do ATP. Estes efeitos podem ocorrer, pois o maquinário de transcrição é continuamente controlado por um complexo sistema de sinalização, criando um ajuste no perfil de expressão gênica na célula. Assim, esta transdução pode ser exercida por proteínas e produtos de reações enzimáticas capazes de regular os fatores de transcrição (Krishna et al., 2006). Este fenômeno é conhecido como “positive feedback loop” (Pomerening et al., 2003; 2005), na qual ocorre uma interface nas vias metabólica e gênica que poderia explicar a diminuição na atividade com o concomitante aumento na nos níveis de mRNA.

Portanto, estes resultados mostram as ações induzidas por fármacos antidepressivos de duas classes diferentes, os ISRS e os TCA, nas ecto-nucleotidases em soro e sinaptossomas de hipocampo e córtex cerebral de ratos. Esta investigação avaliou os efeitos destes antidepressivos em um outro sistema de neurotransmissão, o sistema purinérgico, que não é classicamente analisado na depressão. As alterações transcricionais e cinéticas observadas nestas enzimas após os tratamentos com os fármacos sugerem que o sistema purinérgico pode se tornar um interessante alvo para potenciais estudos farmacológicos relacionados à depressão.

5. CONCLUSÃO FINAL

Com os resultados apresentados nesta Dissertação, foi possível concluir que as NTPDases e a 5´-Nucleotidase são enzimas sensíveis à ação de diferentes fármacos antidepressivos tanto na sua atividade quanto no padrão de expressão. Essas alterações mostram que, além dos sistemas classicamente alterados pelos fármacos antidepressivos, o sistema purinérgico também é afetado. Portanto, nosso trabalho contribui para um melhor esclarecimento sobre os efeitos neuroquímicos dos fármacos antidepressivos e o papel destas enzimas nas respostas induzidas pelos tratamentos *in vitro* e *in vivo* (agudo e crônico) com fluoxetina, sertralina, nortriptilina e clomipramina no soro, hipocampo e córtex cerebral de ratos.

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7. ANEXOS

De: Life Sciences [mailto:lifesci@elsevier.com]
Enviada: sex 27/4/2007 03:16
Para: Carla Denise Bonan
Assunto: Submission Confirmation

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