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ENVOLVIMENTO DO SISTEMA PURINÉRGICO, LACTATO E GLICOSE EM INSULTOS DO SISTEMA NERVOSO CENTRAL

JEAN PIERRE OSES

ORIENTAÇÃO

LUIS VALMOR PORTELA

JOÃO JOSÉ FREITAS SARKIS

(*In Memoriam*)

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Ao Sarkis,

Um mestre, um amigo.

“El hombre debe transformase al mismo tiempo que la producción progressa; norealizaríamos una tarea adecuada si fuéramos tan sólo productores de artículos, de materias primas y no fuéramos al mismo tiempo productores de hombres”.

(Ernersto Che Guevara)

“None but those who have experienced them can conceive of the enticements of science. In other studies you go as far as others have gone before you, and there is nothing more to know; but in a scientific pursuit there is continual food for discovery and wonder.”

(Victor Frankenstein)

[do livro Frankenstein, the Modern Prometheus]

“Sometimes you gotta say "What the F--k", make your move. Joel, every now and then, saying "What the F--k", brings freedom. Freedom brings opportunity, opportunity brings freedom.”

(do filme movie Risky Business, 1983)

Agradecimentos

Ao final desta tese, agradeço a muitas pessoas, afinal de contas nunca fazemos nada sozinhos e sem a ajuda e a participação dos amigos e colegas esta tese com certeza não seria uma tese.

Aos mestres:

O Sarkis foi mais que um orientador foi um amigo, uma pessoa clara e sincerna na maneira de lidar com as coisas, sempre dando incentivo a seus alunos, sendo um exemplo não só profissional, mas também como pessoa.

O Roska, que sempre esteve ao lado, sendo um orientador, um amigo, como um irmão mais velho.

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PARTE I

Introdução & Objetivos

Resumo

O ATP pode influenciar diversos processos fisiológicos através dos receptores P1 e P2, controlando as concentrações de ATP e adenosina extracelulares. O ATP pode modular a liberação e/ou ligação de outros neurotransmissores. No SNC, a adenosina e guanosina atuam como importantes neuromoduladores com efeitos inibitórios sobre a atividade neuronal. A inativação da sinalização do ATP é mediada pela ação das ecto-nucleotidases. Além dos seus papéis fisiológicos, as ações extracelulares das purinas podem ser relevantes para a patogênese e a atenuação de doenças cerebrais agudas e crônicas. Epilepsia é uma doença neurológica crônica caracterizada por crises recorrentes que são expressas na forma de convulsões acompanhadas pela modificação dos circuitos límbicos e freqüentemente apresenta características neurodegenerativas. A doença de Parkinson é caracterizada por uma neurodegeneração progressiva na *substantia nigra pars compacta* com subseqüente redução no conteúdo de dopamina estriatal. Encefalopatia diabética é uma reconhecida complicação do diabetes não tratado, resultando em um comprometimento cognitivo acompanhado de uma modificação da função hipocampal. Nesta tese, nós observamos: i) na epilepsia, nós demonstramos um aumento nas atividades das ecto-nucleotidases e das nucleotidases solúveis em fatias hipocampais e líquor após o modelo do abrasamento com PTZ, com distinta influência nos níveis dos nucleotídeos, nucleosídeos e oxipurinas e também nos níveis de RNAm das NTPDases de hipocampo. No modelo agudo, houve um aumento nos níveis de glicose e lactato extracelulares. Além disso, houve uma diminuição na captação de [^{14}C]-2-deoxi-D-glicose e nos níveis de glicogênio hipocampais 10 minutos após a convulsão, retornando aos níveis normais 30 minutos após a convulsão; ii) na doença de Parkinson, nossos resultados reforçam a hipótese de que as mudanças no sistema adenosinérgico contribuem para a progressão da neurodegeneração na doença de Parkinson. Além disso, pela primeira vez, as mudanças no “sistema guanosinérgico” podem também ser mediadores na progressão desta doença; e iii) no diabetes, nosso estudo oferece evidências demonstrando que o sistema de sinalização do ATP está comprometido no hipocampo de ratos tratados com STZ, um modelo experimental de diabetes mellitus tipo 1. Estas modificações podem levar a alterações na modulação da neurotransmissão e gliotransmissão, podendo contribuir para um progressivo comprometimento cognitivo induzido pelo diabetes.

Abstract

ATP may influence several physiological processes P1 and P2 receptors-mediated by controlling extracellular concentrations of ATP and adenosine. ATP may modulate the release and/or influence other neurotransmitters either by acting through its own receptors or by altering the neurotransmitter receptors. In the CNS, adenosine and guanosine act both as important neuromodulators with major inhibitory effects on neuronal activity. The inactivation of ATP signaling is mediated by the action of ecto-nucleotidases. Besides their physiological roles, purine-mediated extracellular actions may be relevant to the pathogenesis and/or alleviation of acute and chronic brain disorders. Epilepsy is a chronic neurologic disorder characterized by recurrent seizures that have a behavioral expression in the form of convulsions accompanied by a modification of limbic circuits and the frequent presence of neurodegenerative features. Parkinson's disease is characterized by a progressive neurodegeneration in the *substantia nigra pars compacta* with a subsequent reduction in the striatal dopamine content. Diabetic encephalopathy is a recognized complication of untreated diabetes resulting in a progressive cognitive impairment accompanied by modification of hippocampal function. In this thesis, we observed: i) in epilepsy, we demonstrated an enhancement of ecto- and soluble nucleotidase activities in hippocampal brain slices and CSF after PTZ-kindling model, with distinct influence on the levels of nucleotides, nucleosides and oxypurines and also hippocampal NTPDases mRNA levels. Animals that presented tonic-clonic seizure had CSF glucose and lactate levels increased. Moreover, the hippocampal [^{14}C]-2-deoxy-D-glucose uptake and glycogen content decreases at 10 min after seizure and returned to control levels at 30 minutes; ii) in Parkinson's disease, our results reinforce the hypothesis that changes in the adenosinergic system contribute to the progression of neurodegeneration in Parkinson's disease and point to the possibility of using the ectonucleotidases as targets for therapy. In addition, this is the first time, in our knowledge, that changes in the "guanosinergic system" might also be considered as mediators of the progression of this disease; and iii) in diabetes, our study provides evidence showing that the ATP signaling system is compromised in the hippocampus of STZ-treated rats, an experimental model of type 1 diabetes mellitus. These modifications could lead to alterations in the modulation of neurotransmission and gliotransmission, which may contribute to the diabetes-induced progressive cognitive impairment.

Lista de Abreviaturas:

SNC – sistema nervoso central

P1 – receptores de adenosina

P2 – receptores de ATP

E-NTDPase – ecto-nucleotideo trifosfato difosfoidrolase

E-NPP –ecto-nucleotideo pirofosfato/fosfohidrolase

GABA – ácido γ-aminobutírico

EAAT – transportadores de aminoácidos excitatórios

NMDA-R – receptores N-metil-d-aspartato

AMPA-R – receptores ácido α-amino-3-hidroxi-5-metil-4-isoxazolpropionico

EPSP – potenciais pós-sinápitcos excitatórios

LDH – enzima lactato desidrogenase

MCT – transportadores de monocarboxilatos

TLE – epilepsia do lobo temporal

PTZ – pentilenotetrazol

DP – doença de Parkinson

6-OHDA – 6-hidróxidopamina

STZ – estreptozotocina

INTRODUÇÃO

SISTEMA PURINÉRGICO

As purinas desempenham uma ação ampla e específica na sinalização extracelular, regulando assim diversas funções em tecidos de vertebrados e invertebrados, desempenhando um papel chave no desenvolvimento, proliferação e diferenciação celular (Abbracchio, Ceruti *et al.*, 1996; Abbracchio e Burnstock, 1998). O ATP, sobretudo a adenosina desempenham uma ação neuromoduladora no sistema nervoso central (SNC). Há um aumento nos níveis extracelulares de ATP e de adenosina com o aumento da freqüência de disparo neuronal (Cunha, Correia-De-Sa *et al.*, 1996) em diversas regiões cerebrais em diferentes modelos experimentais de injúrias ao SNC (Wieraszko, Goldsmith *et al.*, 1989; Wieraszko e Seyfried, 1989b; 1989a; During e Spencer, 1992; Cunha, Vizi *et al.*, 1996; Fields e Stevens, 2000). Estas purinas têm a capacidade de controlar quer a freqüência de disparo neuronal quer a degeneração neuronal por ativação de receptores de membrana do sub-tipo P1 (seletivos para a adenosina: A_1, A_{2A}, A_{2B} e A_3, todos metabotrópicos) e do sub-tipo P2 (seletivos para nucleotídeos incluindo duas famílias com vários membros: P2Y metabotrópicos e P2X ionotrópicos) (Ralevic e Burnstock, 1998).

O ATP pode ser armazenado em vesículas sinápticas nos terminais nervosos, e co-liberado com diferentes neurotransmissores (Zimmermann, 1994). No entanto, o ATP pode não só ser liberado como resultado da ativação neuronal, como também de astrócitos ou de células de microglia (Cunha e Ribeiro, 2000). Isto está de acordo com as diferentes funções desempenhadas pelo ATP no SNC, nomeadamente de neurotransmissor, de neuromodulador, de mensageiro neurônio-glia e de mediador inflamatório (Fields e Stevens, 2000). Nestas múltiplas funções intervêm diferentes receptores (8 metabotrópicos:

P2Y1,2,4,6,11,12, 13 e 14; e vários ionotrópicos: P2X1-6 que se misturam em trímeros e P2X7), embora ainda permaneça por esclarecer a responsabilidade dos vários sub-tipos de receptores no controle das diferentes funções. Devido à capacidade dos receptores P2Y (em particular P2Y1) e dos receptores P2X7 controlarem a frequência de disparo neuronal (Panenka, Jijon *et al.*, 2001; Yoshioka, Saitoh *et al.*, 2001) e a degeneração neuronal (Le Feuvre, Brough *et al.*, 2002; Volonte, Amadio *et al.*, 2003), e de serem particularmente abundantes no hipocampo (Panenka, Jijon *et al.*, 2001; Yoshioka, Saitoh *et al.*, 2001). Estes receptores constituem-se como novos alvos potenciais para desenvolvimento de fármacos nas mais diversas situações que afetam o SNC (Rudolphi, Schubert *et al.*, 1992; Obrenovitch, 1996; Cavaliere, D'ambrosi *et al.*, 2001; Kharlamov, Jones *et al.*, 2002; Lorenzo Fernandez, 2002; Amadio, D'ambrosi *et al.*, 2005; Shinozaki, Koizumi *et al.*, 2005; Abbracchio e Verderio, 2006; Duarte, Proença *et al.*, 2006; Franke e Illes, 2006; Kim, Kwak *et al.*, 2006; Potucek, Crain *et al.*, 2006; Cavaliere, Amadio *et al.*, 2007). No entanto, só foi ainda documentado ocorrer um aumento da densidade de diversos receptores P2 em diveras situações patológicas (Vianna, Ferreira *et al.*, 2002), estando ainda por testar o possível impacto da manipulação farmacológica destes receptores na indução, evolução e conseqüências de diversas patologias em modelos animais.

Ao contrário do ATP e dos receptores P2, existe consideravelmente maior informação sobre o papel da adenosina no SNC e sobre o seu impacto frente a injúrias do SNC. A adenosina é um nucleosídeo que desempenha, sobretudo um papel neuromodulador no SNC, controlando, sobretudo a excitabilidade neuronal e a liberação de neurotransmissores excitatórios, embora também controle respostas de astrócitos e o fluxo sanguíneo cerebral (Fredholm, Chen, Cunha *et al.*, 2005; Fredholm, Chen, Masino *et al.*, 2005). Os efeitos da

adenosina no SNC são sobretudo de inibição da atividade neuronal por ativação do seu receptor mais abundante (o receptor inibitório do sub-tipo A_1). Consequentemente, a administração de agonistas de receptores A_1 diminui a duração e frequência dos disparos neuronais, enquanto o bloqueio destes receptores A_1 aumenta estes disparos, podendo ocasionar efeitos neurotóxicos (Dunwiddie e Diao, 1994; Cunha, Constantino *et al.*, 2001; Wu, Hu *et al.*, 2006). Por outro lado, a ativação dos receptores A_1 é também neuroprotetora (Abbracchio e Cattabeni, 1999; Calabresi, Picconi *et al.*, 2000; Von Arnim, Timmler *et al.*, 2000), diminuindo a lesão neuronal que se desenvolve como conseqüência do aumento de neurotransmissores excitatórios (Malva, Silva *et al.*, 2003; Pinheiro, Rodrigues *et al.*, 2003; Rebola, Pinheiro *et al.*, 2003). Embora este receptor A_1 continue a ser alvo de inovadoras e estratégias para desenho de novas estratégias neuroprotetoras (Schubert, Ogata *et al.*, 1997; Harkany, Hortobagyi *et al.*, 1999; Johansson, Halldner *et al.*, 2001; Frenguelli, Llaudet *et al.*, 2003; Hellweg, Von Arnim *et al.*, 2003; Scammell, Arrigoni *et al.*, 2003; Schwarzschild, Xu *et al.*, 2003; Boeck, Kroth *et al.*, 2005; Liu, Xie *et al.*, 2005; Andoh, Ishiwa *et al.*, 2006; Fedele, Li *et al.*, 2006; Xu, Zhang *et al.*, 2006), um outro receptor de adenosina, o receptor do sub-tipo A_{2A}, tem despertado particular interesse como potencial alvo molecular para o desenvolvimento de novos fármacos (Ongini, Adami *et al.*, 1997; Chen, Huang *et al.*, 1999; Chen, Xu *et al.*, 2001; Brambilla, Cottini *et al.*, 2003; Dall'igna, Porciuncula *et al.*, 2003; Schwarzschild, Xu *et al.*, 2003; Agnati, Leo *et al.*, 2004; Popoli, Minghetti *et al.*, 2004; Bove, Serrats *et al.*, 2005; Rebola, Canas *et al.*, 2005; Chen, Sonsalla *et al.*, 2007; Minghetti, Greco *et al.*, 2007; Popoli, Blum *et al.*, 2007). Com efeito, foi observado que o bloqueio deste receptor confere uma robusta neuroproteção. Em situação crônicas de neurodegeneração a densidade deste receptor aumenta dramaticamente em regiões do córtex límbico e córtex sensorial e motor em roedores sujeitos a processos

danosos ao SNC (Chen, Huang *et al.*, 1999; Chen, Xu *et al.*, 2001; Brambilla, Cottini *et al.*, 2003; Dall'igna, Porciuncula *et al.*, 2003; Schwarzschild, Xu *et al.*, 2003; Agnati, Leo *et al.*, 2004; Bove, Serrats *et al.*, 2005; Minghetti, Greco *et al.*, 2007) (Rebola, Oliveira *et al.*, 2002; Rebola, Sebastiao *et al.*, 2003; Rebola, Canas *et al.*, 2005; Rebola, Porciuncula *et al.*, 2005; Rebola, Rodrigues, Lopes *et al.*, 2005; Rebola, Rodrigues, Oliveira *et al.*, 2005).

O fato de antagonistas de receptores A_1 serem pró-excitotóxicos e antagonistas de receptores A_{2A} serem neuroprotetores indica que a adenosina endógena tem um importante papel no desenvolvimento dos processos neuroprotetores. A adenosina pode aparecer no meio extracelular por dois processos: i) sendo liberada diretamente sob a forma de adenosina, via transportador bi-direcional de nucleosídeos, ou ii) ser formada no espaço extracelular pela ação da via das ecto-nucleotidases, que convertem o ATP extracelular em adenosina (Cunha, 2001). Esta via das ecto-nucleotidases desempenha um papel crucial no funcionamento do sistema purinérgico. Com efeito, esta via tanto é responsável pela remoção do ATP do espaço extracelular, removendo assim uma molécula sinalizadora, como é responsável pela formação de adenosina, e formando assim outro tipo de molécula sinalizadora. Esta via das ecto-nucleotidases é constituída por uma concatenação de dois grandes grupos de enzimas: uma responsável pela remoção de ATP e a segunda responsável pela formação de adenosina (Zimmermann, Thurmer *et al.*, 2001; Robson, Sévigny *et al.*, 2006; Zimmermann, 2006). As ecto-enzimas responsáveis pela remoção do ATP extracelular pertencem, sobretudo a dois grandes grupos, a família das E-NTDPases (ecto-nucleotideo trifosfato difosfoidrolases) e a família das E-NPPs (ecto-nucleotideo pirofosfato/fosfohidrolases), ambas acabando por gerar AMP. Este AMP é depois convertido em adenosina por ação da ecto-5'-nucleotidase, podendo também esta reação ser

realizada por ecto-fosfatases alcalinas. Confirmando a importância desta via das ecto-nucleotidases para a modulação purinérgica no SNC está o elevado grau de controle enzimático desta via (Cunha, 2001), que permite controlar no espaço e no tempo quer o catabolismo de ATP quer a conseqüente ativação diferencial de receptores de adenosina do sub-tipo A_1 (Dunwiddie e Diao, 1994; Cunha, 1998) ou A2A (Cunha, Johansson *et al.*, 1996).

Assim como os derivados da adenina (ATP, ADP, AMP e adenosina), outras purinas têm apresentado efeito extra- e intracelulares. Diversos trabalhos têm indicado que os derivados da guanina (GTP, GDP, GMP e guanosina) possuem importantes ações tróficas, atuando como moléculas sinalizadoras frente a situações fisiológicas (Ciccarelli, Di Iorio *et al.*, 1999; Ciccarelli, Di Iorio *et al.*, 2000; Pietrangelo, Fioretti *et al.*, 2006; Deutsch, Rosse *et al.*, 2007; Schmidt, Lara *et al.*, 2007). Alguns destes efeitos dos derivados da guanina podem ser indiretamente mediados pelos derivados da adenosina (Ciccarelli, Di Iorio *et al.*, 1999; Ciccarelli, Di Iorio *et al.*, 2000). Basicamente, os estudos referentes aos derivados da guanina têm se dividido em: i) efeitos sobre a atividade glutamatérgica em condições fisiológicas e patológicas; ii) efeitos relacionados à memória e comportamento; e iii) efeitos tróficos nas células neurais (Pietrangelo, Fioretti *et al.*, 2006; Schmidt, Lara *et al.*, 2007). No SNC, as reservas dos derivados da guanina são aproximadamente de 2-3 vezes maiores quando comparados com os derivados da adenina (Deutsch, Rosse *et al.*, 2007).

O mecanismo de liberação dos derivados da guanina ainda é desconhecido, porém na literatura já foi descrito que tanto as células neuronais quanto gliais são capazes de armazenar em vesículas e liberar GTP, GDP, GMP e guanosina. Acredita-se que os astrócitos são os principais tipos celulares a liberar os derivados da guanina, uma vez que já

foi observado que em culturas de astrócitos, a quantidade de GTP, GDP, GMP e guanosina liberados é aproximadamente 3 vezes maior quando comparado com os derivados da adenina (Ciccarelli et al., 1999). Além disso, os níveis extracelulares de GTP, GDP, GMP e principalmente guanosina rapidamente aumentam no SNC frente a um insulto, tendendo a acumular e permanecer por mais tempo que os derivados da adenina (Frizzo, Antunes Soares *et al.*, 2003; Tasca, Santos *et al.*, 2004).

Os derivados da guanosina estão envolvidos na modulação da transmissão glutamatérgica, inibindo esta sinalização. Apesar de ser demonstrado que o GTP pode inibir a ligação de neurotransmissores através de receptores metabotrópicos e modular a atividade da adenilato ciclase, a ação modulatória dos derivados da guanina está dissociada da ligação à proteína G (Ciccarelli, Di Iorio *et al.*, 1999; Ciccarelli, Di Iorio *et al.*, 2000; Pietrangelo, Fioretti *et al.*, 2006; Deutsch, Rosse *et al.*, 2007; Schmidt, Lara *et al.*, 2007). Os derivados da guanina podem, possivelmente, atuar sobre a transmissão glutamatérgica através de sítios extracelulares da membrana plasmática (Ciccarelli, Di Iorio *et al.*, 1999; Ciccarelli, Di Iorio *et al.*, 2000; Pietrangelo, Fioretti *et al.*, 2006; Deutsch, Rosse *et al.*, 2007; Schmidt, Lara *et al.*, 2007). Estudos sobre os efeitos dos derivados da guanina sobre o sistema glutamatérgico feitos em sinaptossomas e culturas celulares (astrócitos e neurônios), demonstraram que os derivados da guanina foram capazes de antagonizar os efeitos do glutamato e seus análogos sem interferir na ligação do glutamato e seus derivados nos seus respectivos receptores (Malcon, Achaval *et al.*, 1997; Rubin, Medeiros *et al.*, 1997; Regner, Ramirez *et al.*, 1998; Tasca, Cardoso *et al.*, 1998; Frizzo, Lara *et al.*, 2001). Estes estudos demonstraram que os efeitos dos derivados da guanina não ocorrem diretamente sobre os receptores glutamatérgicos, corroborando com a hipótese da

participação de um possível receptor para os derivados da guanina. Além de desempenhar ações sobre os receptores glutamatérgicos, os derivados da guanina são capazes de aumentar a captação de glutamato em cultura de astrócitos e fatias cerebrais (Frizzo, Lara *et al.*, 2001; Frizzo, Lara *et al.*, 2002; Frizzo, Antunes Soares *et al.*, 2003; Frizzo, Schwalm *et al.*, 2005). Os efeitos exercidos pelos derivados da guanina na captação de glutamato não são afetados quando a adenosina é co-administrada (Schmidt, Lara *et al.*, 2007). Outros experimentos demonstraram que o dipiridamole, um inibidor do transporte de nucleosídeos, foi capaz de estimular a captação de glutamato, e tendo um efeito aditivo quando co-administrado com guanosina (Frizzo, Lara *et al.*, 2001).

Assim, uma vez que a sinapse excitatória no SNC tem o glutamato como principal neurotransmissor, a ação modulatória dos derivados da guanina na transmissão glutamatérgica pode ter um grande potencial na elucidação de um novo sistema modulatório e na abordagem de novas estratégias farmacológicas no tratamento de injúrias do SNC.

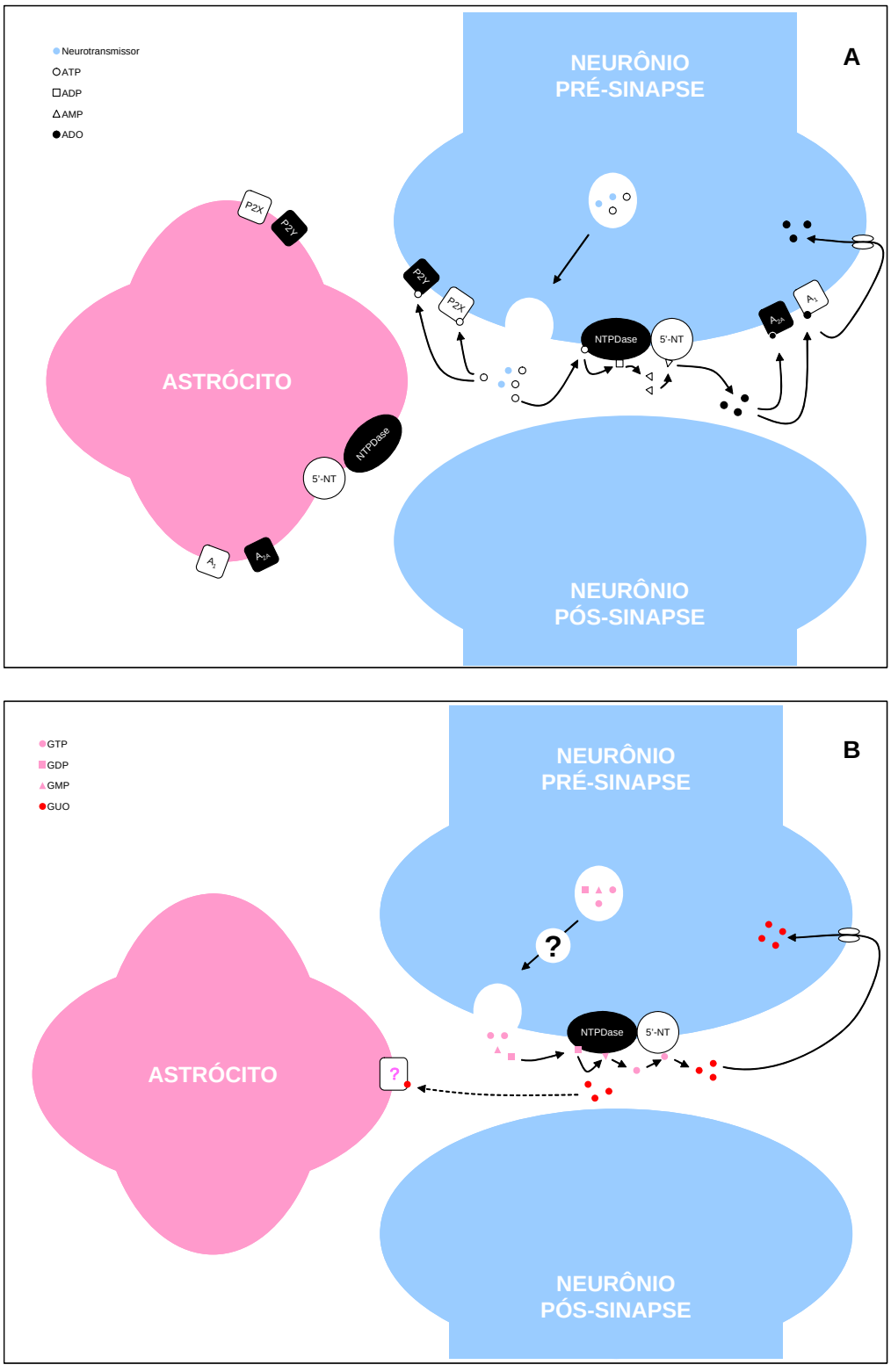

Figura 1: modelo esquemático do sistema purinérgico no SNC. (A) Sinalização e catabolismo dos derivados da adenina, (B) sinalização e catabolismo dos derivados da guanina.

SISTEMA GLUTAMATÉRGICO

O aminoácido glutamato é o neurotransmissor excitatório mais abundante no SNC. Diversos trabalhos têm demonstrado os efeitos excitatórios destes neurotransmissor e confirmado seu papel chave como neurotransmissor no tecido cerebral. O glutamato também pode participar como precursor do neurotrasmissor inibitório ácido γ-aminobutírico (GABA). Os neurônios glutamatérgicos são encontrados principalmente no córtex cerebral, projetando-se para diversas estruturas subcorticais (hipocampo, amígdala, substância negra, núcleo acúmbens, núcleo caudato dentre outras). Várias vias glutamatérgicas têm sido descritas no hipocampo e também projeções glutamatérgicas do hipocampo para outras estruturas (Olney, 1989a; 1989b; 1990; Olney, Zorumski *et al.*, 1990; Olney, 1994; Olney e Farber, 1995; Magistretti e Pellerin, 1996; Bar-Peled, Ben-Hur *et al.*, 1997; Magistretti e Pellerin, 1997; Olney, Wozniak *et al.*, 1997; , 1998; Magistretti e Pellerin, 1999; Magistretti, Pellerin *et al.*, 1999; Claudio, Ferchmin *et al.*, 2000; Deitmer, 2000; O'shea, 2002; Olney, 2002; Beretta, Begni *et al.*, 2003; Hayase, Yamamoto *et al.*, 2003; Maragakis, Dietrich *et al.*, 2004; Dunlop, 2006).

Uma vez que o glutamato não atravessa a barreira hemato-encefálica, é necessário um sistema altamente eficaz na síntese e reciclagem deste neurotransmissor. O glutamato é sintetizado nos terminais nervosos através do ciclo de Krebs pela atividade da enzima glutamato desidrogenase ou pela transaminação do α-cetoglutarato, e assim armazenado em vesículas sinápticas. Após ser liberado para o espaço extracelular, o glutamato é principalmente captado por um mecanismo específico através de transportadores de alta-afinidade localizados na membrana plasmática dos astrócitos adjacentes. Ao ser captado pelo astrócito, o gluatamato é metabolizado à glutamina através da ação da glutamina

sintetase, e assim liberado para subseqüente captação dos terminais neuronais, para ser transformada em glutamato (Olney, 1989b; , 1994; Stella, Pellerin *et al.*, 1995; Magistretti e Pellerin, 1996; , 1997; , 1999; Magistretti, Pellerin *et al.*, 1999).

Diversos transportadores específicos regulam a captação do glutamato no espaço extracelular para os neurônios ou astrócitos. A família destes transportadores é composta por quatro diferentes membros de transportadores de aminoácidos excitatórios (EAAT1-4, EAAT-1, EAAT-2 e EAAT-3). Estes transportadores são essenciais para o término do sinal excitatório e diminuição dos níveis extracelulares de glutamato, uma vez que níveis elevados de glutamato podem induzir um dano excitotóxico. Diversos trabalhos têm demonstrado que uma redução na atividade destes transportadores está associada à um aumento na neurotoxicidade (Bar-Peled, Ben-Hur *et al.*, 1997; Claudio, Ferchmin *et al.*, 2000; Deitmer, 2000; O'shea, 2002; Beretta, Begni *et al.*, 2003; Hayase, Yamamoto *et al.*, 2003; Maragakis, Dietrich *et al.*, 2004; Dunlop, 2006).

Na fenda sináptica, o glutamato liberado vai atuar em receptores pós-sinápticos específicos, estes receptores podem ser divididos em receptores ionotrópicos (receptores N-metil-d-aspartato (NMDA-R), receptores ácido α-amino-3-hidroxi-5-metil-4-isoxazolpropionico (AMPA-R) e receptores cainato) e receptores metabotrópicos (Grupo I (mGluR1 e mGluR5), Grupo II (mGluR2, mGluR3 e mGluR8) e Grupo III (mGluR4, mGluR6 e mGluR7)(Mcculloch, 1994; Westbrook, 1994; Chapman, 1998; Ozawa, Kamiya *et al.*, 1998; Parsons, Danysz *et al.*, 1998; Tzschentke, 2002; Riedel, Platt *et al.*, 2003).

METABOLISMO CEREBRAL

O cérebro apesar de representar somente 2% do peso corpóreo total, é responsável por aproximadamente 20% do consumo total de O_2, e 25% de glicose no organismo, ainda que apresente baixos níveis de armazenamento de glicogênio (Kristian, 2004). O metabolismo cerebral requer um fornecimento constante de glicose, sendo assim, os níveis de glicose sanguíneos são fortemente regulados (Alvarez-Buylla, Huberman *et al.*, 2003). O metabolismo da glicose no cérebro é similar aos outros tecidos e inclui as 3 principais vias metabólicas: glicólise, ciclo do ácido tricarboxílico e a via da pentose fosfato.

Astrócitos e células endoteliais vasculares contribuem para o metabolismo cerebral, assim como os neurônios. Entretanto, essas células, também desempenham um papel crucial no fluxo de substratos energéticos para os neurônios. Os astrócitos têm prolongamentos celulares que circundam os capilares cerebrais, chamados pés-terminais, responsáveis por formar a barreira para a entrada da glicose no parênquima cerebral (Deitmer, 2000). Em um primeiro momento, a glicose é metabolizada a piruvato e depois convertida à lactato, no astrócito. A enzima lactato desidrogenase (LDH), que converte de forma reversível piruvato à lactato, é encontrada na isoforma 5 (LDH-5) nos astrócitos, favorecendo a formação de lactato. Por outro lado, nos neurônios é encontrada a isoforma 1 da LDH (LDH-1), que cataliza a reação de lactato para piruvato (Bittar, Charnay *et al.*, 1996). Dessa forma, o lactato é formado e secretado principalmente pelos astrócitos, e os neurônios captam o lactato secretado, convertendo-o após, a piruvato, pela ação da LDH-1, e este piruvato entrará no Ciclo de Krebs.

Uma vez que o lactato tem baixa permeabilidade, diferentes isoformas de transportadores de monocarboxilatos (MCT) são encontrados em células endoteliais (MCT 1), astrócitos (MCT-1) e neurônios (MCT-2) (Abi-Saab et al., 2002). Estudos de afinidade,

têm indicado um K_M de 3 – 5 mM para os MCT-1, e valores próximos de 0,5 mM para os MCT-2. Estas diferenças de K_M entre os transportadores indicariam que os MCT-1 presentes nos astrócitos favoreceriam a secreção, e os MCT-2 neuronais captariam o lactato (Bouzier-Sore, Merle *et al.*, 2002).

Uma das principais funções dos astrócitos é a recaptação de aminoáciodos neurotransmissores, especialmente o glutamato. Diversos estudos têm demonstrado que o glutamato estimula a glicólise aeróbica em diversos tipos de células da glia (Pellerin e Magistretti, 1994; Poitry-Yamate, Poitry *et al.*, 1995; Takahashi, Cook *et al.*, 1995; Takahashi, Crane *et al.*, 1995; Takahashi, Driscoll *et al.*, 1995; Bittar, Charnay *et al.*, 1996; Poitry, Poitry-Yamate *et al.*, 2000), diferentemente de outras ações do glutamato, este estímulo é mediado pelos transportadores de glutamato e não pelos receptores glutamatérgicos (Pellerin e Magistretti, 1994; , 1996). Este glutamato re-captado é convertido a glutamina pela ação da glutamina sintetase, e esta glutamina, por sua vez, é liberada para o meio extracelular, onde é captada pelo neurônio para ser convertida à glutamato (Pellerin, 2003). Dentre estas e outras observações, permitiriam supor em um mecanismo astrocitário capaz sinalizar diante de um estímulo glutamatérgico, e assim ativar a glicólise para providenciar substrato energético ao neurônio (Pellerin, 2003).

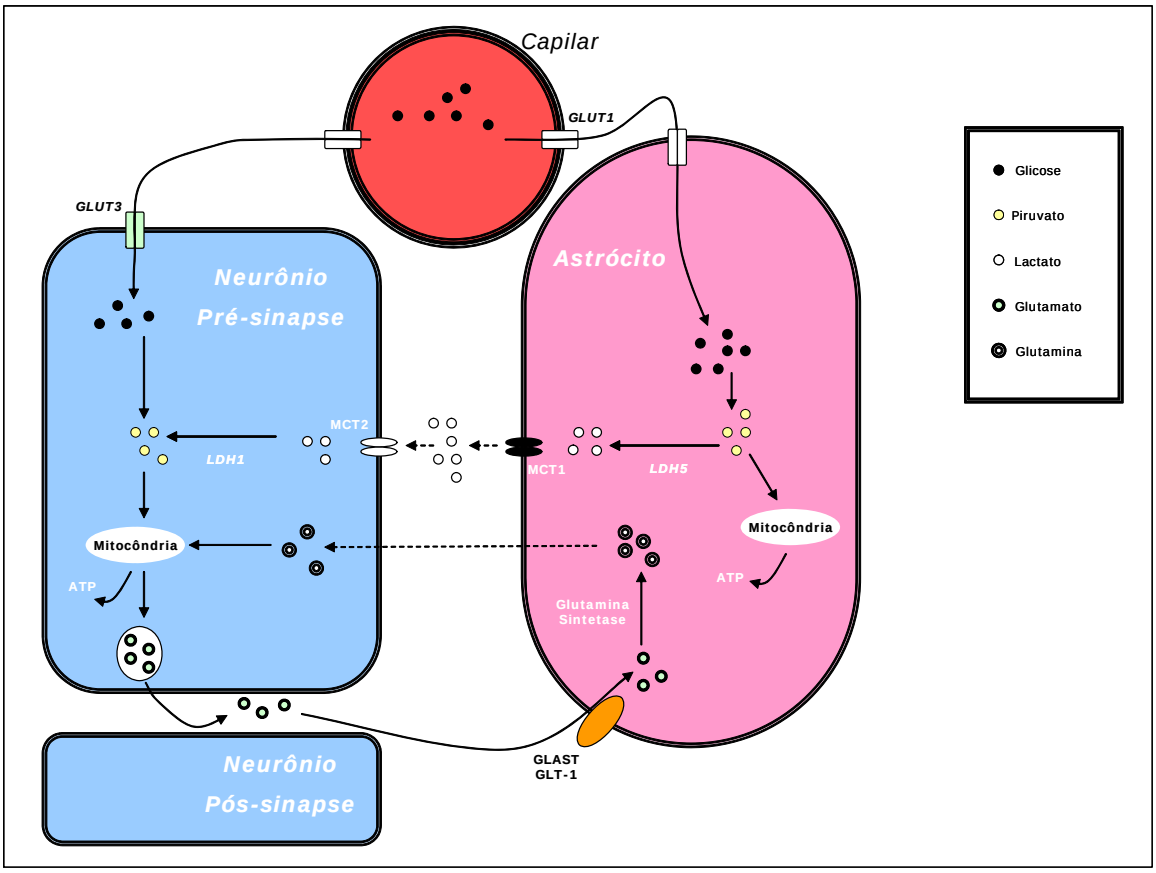

Figura 2: modelo esquemático do metabolismo do lactato no SNC. [Adaptado de (Pellerin, 2003)].

NEUROTOXICIDADE E NEUROPROTEÇÃO

Neurotransmissores excitatórios e inibitórios medeiam a transmissão sináptica e controlam a estabilidade e eficiência das conexões sinápticas. Entretanto, alterações no balanço, ativação e inativação destes dois tipos de neurotransmissores pode levar a eventos neurotóxicos e pode estar envolvido na patogênese de diversas doenças neurológicas (Olney, 1990; , 1994).

No SNC, o sistema purinérgico está envolvido na remodelagem após diferentes injúrias, incluindo epilepsias, doença de Parkinson, encefalopatia diabética (Abbracchio e Burnstock, 1998; Abbracchio e Cattabeni, 1999; Chen, Huang *et al.*, 1999; Cunha e Ribeiro, 2000; Chen, Xu *et al.*, 2001; Dall'igna, Porciuncula *et al.*, 2003; Duarte, Proenca *et al.*, 2006; Robson, Sévigny *et al.*, 2006; Zimmermann, 2006; Chen, Sonsalla *et al.*, 2007; Schmidt, Lara *et al.*, 2007). Após um insulto neuronal, diferentes células neurais liberam diferentes fatores tróficos. A liberação destes fatores, juntamente com o ATP pode estimular a astrogliose reativa, neurogênese e o desenvolvimento de neuritos, podendo estar associada aos processos neurotóxicos e/ou neuroprotetores (Olney, 1989b; , 1990; , 1994; Abbracchio e Burnstock, 1998; Abbracchio e Cattabeni, 1999; Chen, Huang *et al.*, 1999; Ciccarelli, Di Iorio *et al.*, 1999; Magistretti, Pellerin *et al.*, 1999; Calabresi, Picconi *et al.*, 2000; Ciccarelli, Di Iorio *et al.*, 2000; Cunha e Ribeiro, 2000; Deitmer, 2000; Fields e Stevens, 2000; Chen, Xu *et al.*, 2001; Dall'igna, Porciuncula *et al.*, 2003; Malva, Silva *et al.*, 2003; Pellerin, 2003; Kristian, 2004; Fredholm, Chen, Cunha *et al.*, 2005; Duarte, Proenca *et al.*, 2006; Chen, Sonsalla *et al.*, 2007). Algumas respostas ao ATP após a um insulto cerebral são neuroprotetoras, porém níveis elevados de ATP são neurotóxicos, podendo contribuir para a patofisiologia após estes insultos (Burnstock, 2006; , 2007). Além disso, a adenosina, também têm sido implicada com essa dupla função em diferentes doenças do SNC. Tanto o ATP quanto a adenosina, possuem efeitos neurotóxicos e neuroprotetores, dependendo dos níveis alcançados na espaço extracelular e do sub-tipo de receptores ativados (Chen, Huang *et al.*, 1999; Chen, Xu *et al.*, 2001; Fredholm, Chen, Cunha *et al.*, 2005; Fredholm, Chen, Masino *et al.*, 2005; Burnstock, 2006; , 2007; Chen, Sonsalla *et al.*, 2007). O ATP e adenosina inibem a liberação do glutamato, o ATP estimula a liberação do GABA, e a guanosina estimula a recaptação do glutamato (Cunha, 2003;

Fredholm, Cunha *et al.*, 2003; Malva, Silva *et al.*, 2003; Ferre, Borycz *et al.*, 2005; Fredholm, Chen, Cunha *et al.*, 2005; Fredholm, Chen, Masino *et al.*, 2005; Burnstock, 2006; , 2007; Ferre, Ciruela *et al.*, 2007). Diversos trabalhos têm demonstrado que em diferentes injúrias do SNC, os níveis de glutamato estão aumentados, tanto por um aumento na liberação deste neurotransmissor, como por uma diminuição na recaptação deste (Cunha, 2003; Fredholm, Cunha *et al.*, 2003; Malva, Silva *et al.*, 2003; Ferre, Borycz *et al.*, 2005; Fredholm, Chen, Cunha *et al.*, 2005; Fredholm, Chen, Masino *et al.*, 2005; Burnstock, 2006; , 2007; Ferre, Ciruela *et al.*, 2007). Com esta visão, agonistas e antagonistas de receptores purinérgicos (P1 e P2) têm sido propostos como possíveis fármacos neuroprotetores frente a injúrias do SNC (Burnstock, 2006; , 2007).

Nos processos sinápticos, tanto em situações fisiológicas como em situações patológicas, o metabolismo energético cerebral é extremamente relevante. Em situações patológicas, a atividade cerebral aumenta, consideráveis quantidades de lactato são formadas através da utilização anaeróbica da glicose nos neurônios (Fornai, Bassi *et al.*, 2000). Este lactato, produzido e secretado pelo astrócito, pode providenciar uma importante fonte energética para o metabolismo energético neuronal (Folbergrova, He *et al.*, 1999; Cornford, Shamsa *et al.*, 2002; Chow, Rooney *et al.*, 2005; Makoroff, Cecil *et al.*, 2005). Diversos trabalhos têm demonstrado o lactato como um importante agente neuroprotetor frente a diferentes injúrias, podendo ser uma importante ferramenta no tratamento e diagnóstico dessas doenças (Cassady, Phillis *et al.*, 2001; Langemann, Alessandri *et al.*, 2001; Mendelowitsch, Ritz *et al.*, 2001; Ros, Pecinska *et al.*, 2001; Schurr, Payne, Miller e Tseng, 2001; Schurr, Payne, Miller, Tseng *et al.*, 2001; Schurr, Payne, Tseng *et al.*, 2001; Ros, Jones *et al.*, 2002; Schurr, 2002a; 2002b; Yang, Sakurai *et al.*, 2003; Schurr, 2006).

EPILEPSIA E CONVULSÕES

O termo epilepsia refere-se a uma ampla categoria de sintomas complexos em torno de funções cerebrais desordenadas, decorrentes de uma anormalidade e hipersincronia da atividade neuronal, podendo ser secundárias a uma variedade de processos patológicos (Engel e Pedley, 2008), com uma prevalência na população é de aproximadamente de 1% (Chang e Lowenstein, 2003). Destes números, aproximadamente 70% dos pacientes epilépticos têm suas crises controladas através de drogas antiepilépticas. O restante dos pacientes constitui um grupo de alta morbidade, uma vez que suas crises epilépticas não são controladas através do uso de fármacos (Palmini, Gambardella *et al.*, 1995).

Convulsões caracterizando-se por um episódio estereotipado envolvendo alterações sensoriais, das funções autonômicas motoras e de consciência, devido a uma descarga elétrica anormal no SNC (Chang e Lowenstein, 2003). Convulsões representam uma disfunção neurológica em que os sintomas incluem manifestações sensoriais e motoras "positivas" e "negativas", sendo que a manifestação destes sintomas é dependente da região afetada. Em humanos, a recorrência espontânea de convulsões é o principal sintoma da epilepsia, no qual síndromes específicas podem ser definidas por diversos fatores clínicos (Engel e Pedley, 2008).

Anormalidades na neurotransmissão ocorrem através de um aumento na transmissão excitatória, diminuição da transmissão inibitória ou de ambos eventos ocorrendo concomitantemente (Loscher e Meldrum, 1984; Meldrum, 2000; Moldrich, Chapman *et al.*, 2003; Meldrum e Rogawski, 2007).

Diversos modelos de convulsões e epilepsias são descritos na literatura (Schwartzkroin, 1997; Naffah-Mazzacoratti, Arganaraz *et al.*, 1999; Naffah-Mazzacoratti, Funke *et al.*, 1999). Os modelos de convulsões, também chamados modelos agudos, são

aqueles em que o animal apresenta episódios convulsivos somente durante a ação do agente indutor. Estes agentes indutores podem ser químicos, no qual compostos são aplicados por via tópica ou injetável, interferindo no balanço neuroquímico, ou elétricos em que é aplicado um choque eletroconvulsivo no animal, alterando a atividade elétrica cerebral (Schwartzkroin, 1997). Os modelos crônicos de epilepsias são aqueles em que as crises epilépticas surgem em intervalos de tempo variados, sem um estímulo exógeno antes de cada crise convulsiva. A característica principal destes modelos é a apresentação de um fator incidente conhecido, capaz de induzir a epileptogênese, e após certa latência, ocorrem crises epilépticas espontâneas (Naffah-Mazzacoratti, Arganaraz *et al.*, 1999; Naffah-Mazzacoratti, Funke *et al.*, 1999). Destes modelos de convulsões e epilepsias, os mais utilizados estão o modelo pentilenotetrazol (PTZ) (convulsão), do ácido caínico (crônico) e o abrasamento ("*kindling*") (crônico), sendo que o do ácido caínico e PTZ mimetizam a epilepsia de lobo temporal (TLE), o tipo mais freqüente na população humana (Goddard, 1967; Turski, Cavalheiro *et al.*, 1983; Ben-Ari, 1985; Ricci, De Feo *et al.*, 1985; Turski, 2000).

No modelo do PTZ, um agente pró-convulsivante, é amplamente empregado em estudos de modelos convulsões. A dose geralmente utilizada da droga, capaz de produzir um episódio convulsivo generalizado, com uma mortalidade mínima, é aproximadamente de 60 mg/ Kg (Malhotra e Gupta, 1997). O mecanismo de ação do PTZ, na propriedade farmacológica desta droga é de interagir com os canais aniônicos dos receptores $GABA_A$, alterando as propriedades dos receptores $GABA_A$, e fazendo com que aumente a liberação de glutamato na amígdala e córtex pré-frontal e no líquido cefálo-raquidiano (Halonen, Pitkanen *et al.*, 1992; Rocha, Briones *et al.*, 1996; Walsh, Li *et al.*, 1999).

No modelo do "*kindling*", as aplicações de estímulos químicos ou elétricos inicialmente subconvulsivantes resultam em crises progressivamente mais intensas ao longo das subseqüentes estimulações (Morimoto, Fahnestock *et al.*, 2004). O estímulo inicial pode não demonstrar nenhuma alteração, mas à medida que estímulos subseqüentes são aplicados, surge a pós-descarga, desencadeando uma crise generalizada (Racine, 1978; Mcintyre e Racine, 1986; Mcintyre, Poulter *et al.*, 2002; Morimoto, Fahnestock *et al.*, 2004). Neste modelo, existem diferentes estágios de progressão das crises convulsivas através de características clínicas e comportamentais: estágio 1 – clônus facial; estágio 2 – movimentação de flexão e extensão da cabeça; estágio 3 – clônus de patas contra-laterais ao hemisfério estimulado; estágio 4 – respostas de orientação, onde o animal permanece de pé apenas sobre as patas traseiras ("*rearing*") e/ou queda; e estágio 5 – crises tônico/clônicas generalizadas. Uma vez atingido o estágio 5, os animais podem, dependendo do agente indutor utilizado, permanecer por um longo espaço de tempo, sem serem estimulados, vir a ter crises recorrentes.

O modelo do ácido caínico (um análogo do glutamato) é um dos modelos de indução química mais utilizada de TLE, e pode ser sistematicamente ou intracerebralmente injetado no animal e rapidamente produz convulsões agudas. Em roedores, esta droga induz convulsões agudas severas com subseqüente *status epilepticus* seguido de um período latente por diversas semanas. Este período de latência é seguido de crises convulsivas espontâneas recorrentes (Chang e Lowenstein, 2003; Morimoto, Fahnestock *et al.*, 2004; Engel e Pedley, 2008). Uma dose convulsiva de ácido caínico induz uma variedade de manifestações comportamentais, mimetizando alguns automatismos observado em humanos, além de outros fenômenos como perda de postura, "*scratchings*", e "*wet dog shakes*" seguidos de respostas de orientação, onde o animal permanece de pé apenas sobre

as patas traseiras (rearing), salivação profusa, e/ou queda (Racine, 1978; Mcintyre e Racine, 1986; Mcintyre, Poulter *et al.*, 2002; Morimoto, Fahnestock *et al.*, 2004; Engel e Pedley, 2008). O ácido caínico gera lesões similares as observadas em pacientes com esclerose mesial temporal. Estas lesões tipicamente incluem perda de interneurônios GABAérgicos no hilo dentato e morte de células piramidais nas regiões CA1 e CA3 do hipocampo (Racine, 1978; Mcintyre e Racine, 1986; Rocha, Briones *et al.*, 1996; Mcintyre, Poulter *et al.*, 2002; Hayase, Yamamoto *et al.*, 2003; Morimoto, Fahnestock *et al.*, 2004; Engel e Pedley, 2008). Outro efeito observado no modelo do ácido caínico, são as alterações nos circuitos neuronais induzidos pelas convulsões (Racine, 1978; Mcintyre e Racine, 1986; Rocha, Briones *et al.*, 1996; Mcintyre, Poulter *et al.*, 2002; Hayase, Yamamoto *et al.*, 2003; Morimoto, Fahnestock *et al.*, 2004; Engel e Pedley, 2008).

DOENÇA DE PARKINSON

A Doença de Parkinson (DP) é uma doença crônica e progressiva caracterizada por uma elevada e seletiva perda de neurônios dopaminérgicos nigroestriatais. As manifestações clínicas desta doença complexa incluem comprometimento motor envolvendo tremores, bradicinesia, instabilidade postural, dificuldades para caminhar e rigidez (Thomas e Beal, 2007)**.** Atualmente, o tratamento somente propicia uma melhoria nos sintomas e não apresentam eficácia em retardar o processo de morte dos neurônios atingidos. A maioria dos casos de DP é esporádica, entretanto, a descoberta de genes a raras formas familiares da doença (envolvendo α-sinucleina, pakinina, DJ-1, PINK-1 e LRRK2) e estudos em modelos animais permitiram compreender alguns mecanismos moleculares na DP e identificaram possíveis abordagens para a intervenção terapêutica. Estudos recentes

observaram disfunção mitocondrial, dano oxidativo, acúmulo e fosforilação anormal de proteínas e alterações na liberação de neurotransmissores e na ligação destes com seus respectivos receptores como tendo um papel chave nos mecanismos moleculares na patogênese da DP (Asanuma, Miyazaki *et al.*, 2003; Cookson, 2003; Jenner, 2003; Heales, Lam *et al.*, 2004; Mcgeer e Mcgeer, 2004; Cookson, 2005; Norris e Giasson, 2005; Watanabe, Himeda *et al.*, 2005; Nakabeppu, Tsuchimoto *et al.*, 2007; Quik, Bordia *et al.*, 2007; Quik, O'neill *et al.*, 2007; Rommelfanger e Weinshenker, 2007; Thomas e Beal, 2007).

Diferentes modelos animais têm sido empregados para mimetizar a DP, permitindo uma melhor compreensão de alguns mecanismos da doença e podendo ser aplicados para o desenvolvimento e refinamento terapêuticos (Bove, Prou *et al.*, 2005; Ferro, Bellissimo *et al.*, 2005; Willis e Robertson, 2005; Lane e Dunnett, 2007; Uthayathas, Karuppagounder *et al.*, 2007). Neurotoxinas como a 6-hidróxidopamina (6-OHDA) são amplamente utilizados como modelos animais da DP (Hattori e Sato, 2007). O mecanismo comum destes compostos é que eles afetam a mitocôndria dos neurônios da *substantia nigra* pela inibição do complexo I (Hattori e Sato, 2007).

O modelo de lesão da 6-OHDA promove uma depleção seletiva dos neurônios dopaminérgicos da via nigro-estriatal, área predominantemente afetada na DP. A depleção bilateral induzida por 6-OHDA, produz uma síndrome motora severa que os animais ficam incapazes de alimentar-se sem assistência (Zigmond, Acheson *et al.*, 1984; Zigmond e Stricker, 1984), entretanto a toxina é normalmente injetada unilateralmente em áreas específicas da via nigro-estriatal (Marshall, 1979; Siever, Cohen *et al.*, 1981; Zetterstrom, Brundin *et al.*, 1986; Zetterstrom, Herrera-Marschitz *et al.*, 1986). Um animal com lesão nigro-estriatal unilateral exibe um prejuízo postural ao lado ipsilateral, que é transformado

numa forte rotação ipsilateral após a injeção de uma droga estimulante como a anfetamina. A dopamina extracelular é aumentada no estriado intacto, criando um desequilíbrio na ativação motora, resultando em uma resposta rotacional. Os animais também podem vir a ter uma rotação ipsilateral após uma variedade de outros estímulos, embora os métodos farmacológicos de ativação são mais amplamente usados devido a sua consistência e reprodutibilidade (Lane, Cheetham *et al.*, 2006; Lane e Dunnett, 2007).

ENCEFALOPATIA DIABÉTICA

Diabetes mellitus é uma doença metabólica caracterizada por um prejuízo na homeostasia da glicose, causada por uma deficiência na produção ou ação da insulina. Condições diabéticas freqüentemente causam modificações centrais neuropáticas (diminuição cognitiva) acompanhadas por modificações da morfologia e plasticidade hipocampal (Gispen e Biessels, 2000; Trudeau, Gagnon *et al.*, 2004; Convit, 2005; Martinez-Tellez, Gomez-Villalobos Mde *et al.*, 2005; Gold, Dziobek *et al.*, 2007). As disfunções da plasticidade sináptica induzidas pelo diabetes são, provavelmente, devido a alterações na liberação de neurotransmissores e mudanças nas proteínas pré-sinápticas associadas com a liberação vesicular de neurotransmissores (Guyot, Diaz *et al.*, 2001; Yamamoto, Yamato *et al.*, 2004; Yamato, Misumi *et al.*, 2004; Duarte, Proenca *et al.*, 2006).

Agentes químicos, como a estreptozotocina (STZ) podem causar um dano seletivo nas células B produtoras de insulina do pâncreas, resultando em uma hiperglicemia. Estes compostos são importantes ferramentas para o desenvolvimento de modelos animais de complicações diabéticas. Modelos que usam o STZ para induzir diabetes tipo 1, têm demonstrado alterações similares as observadas em humanos (Gispen e Biessels, 2000;

Trudeau, Gagnon *et al.*, 2004; Convit, 2005; Martinez-Tellez, Gomez-Villalobos Mde *et al.*, 2005; Gold, Dziobek *et al.*, 2007).

OBJETIVOS

Essa tese tem como objetivo geral avaliar diferentes parâmetros bioquímicos do SNC, especialmente o sistema purinérgico, em modelos experimentas de convulsão e epilepsia, doença de Parkinson e Diabetes Mellitus.

Os objetivos específicos desta tese estão divididos em 5:

Sobre Epilepsia e Convulsões:

I. Avaliar alterações nas atividades e expressão das enzimas envolvidas no catabolismo dos derivados da adenina e guanina, bem como nos níveis extracelulares destas purinas em animais epilépticos;

II. Avaliar a densidade dos receptores de ATP (receptores P2x e P2Y) em animais epilépticos (resultados preliminares);

III. Avaliar diferentes parâmetros do metabolismo cerebral após uma crise convulsiva em ratos (artigo 2);

Sobre a Doença de Parkinson:

IV. Avaliar alterações nas atividades e expressão das enzimas envolvidas no catabolismo dos derivados da adenina e guanina em animais submetidos ao modelo da 6-OHDA (artigo 3);

Sobre Encefalopatia diabética:

V. Avaliar a liberação e o catabolismo do ATP, e a densidade dos receptores dos receptores de ATP (receptores P2x e P2Y) em animais submetidos ao modelo da estreptozotocina (artigo 4).

PARTE II

Artigos Científicos & Dados

CAPÍTULO I

PENTYLENETETRAZOL KINDLING ALTERS ADENINE AND GUANINE NUCLEOTIDE CATABOLISM IN RAT HIPPOCAMPAL SLICES AND CEREBROSPINAL FLUID

Jean Pierre Oses, Giordano Gubert Viola, Giana de Paula Cognato, Victor Hermes Cereser Júnior, Gisele Hansel, Ana Elisa Bohmer, Renata Leke, Alessandra Nejar Bruno, Carla Denise Bonan, Maurício Reis Bogo, Luis Valmor Portela, Diogo Onofre Souza, João José Freitas Sarkis.

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Pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid

Jean Pierre Oses[a,*], Giordano Gubert Viola[a], Giana de Paula Cognato[a], Victor Hermes Cereser Júnior[a], Gisele Hansel[a], Ana Elisa Böhmer[a], Renata Leke[a], Alessandra Nejar Bruno[a], Carla Denise Bonan[b], Maurício Reis Bogo[c], Luis Valmor Portela[a], Diogo Onofre Souza[a], João José Freitas Sarkis[a,*]

[a] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brazil*
[b] *Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Caixa Postal 1429, 90619-900 Porto Alegre, RS, Brazil*
[c] *Centro de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Caixa Postal 1429, 90619-900 Porto Alegre, RS, Brazil*

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KEYWORDS
PTZ-kindling;
Epilepsy;
Nucleotidase activities;
Nucleotidase expression;
Adenosine;
Guanosine

Summary Pentylenetetrazol (PTZ) is commonly used as a convulsant drug. The enhanced seizure susceptibility induced by kindling is probably attributable to plastic changes in the synaptic efficacy. Adenosine and guanosine act both as important neuromodulators and neuroprotectors with mostly inhibitory effects on neuronal activity. Adenosine and guanosine can be released *per se* or generated from released nucleotides (ATP, ADP, AMP, GTP, GDP, and GMP) that are metabolized and rapidly converted to adenosine and guanosine. The aim of this study was to evaluate nucleotide hydrolysis by ecto- and soluble nucleotidases (hippocampal slices and CSF, respectively) after PTZ-kindling (stages 3, 4, or 5 seizures) or saline treatment in rats. Additionally, the levels of purines in rat cerebrospinal fluid (CSF), as well as ecto-NTPDases (1, 2, 3, 5, 6 and 8) and ecto- 5′-nucleotidase expression were determined. Ecto-enzyme assays demonstrated that ATP, AMP, GDP, and GMP hydrolysis enhanced when compared with controls. In addition, there was an increase of ADP, GDP, and GMP hydrolysis by soluble nucleotidases in PTZ-kindling rats compared to control group. The HPLC analysis showed a marked increase in PTZ-kindled CSF concentrations of GTP, ADP, and uric acid, but GDP, AMP, and hypoxanthine

* Corresponding author. Tel.: +51 33085557; fax: +55 3308 5540.
E-mail address: jean.pierre.oses@gmail.com (J.P. Oses).

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concentrations were decreased. Such alterations indicate that the modulatory role of purines in CNS could be affected by PTZ-kindling. However, the physiological significance of these findings remains to be elucidated.

Introduction

Epilepsy is a common and chronic neurological disorder characterized by recurrent unprovoked seizures. Epileptic activity is based on broken excitatory-inhibitory balance in the brain tissue (Mody, 1999). It is a complex mechanism that leads to epileptic firing, and involves several transmitters and modulators in the brain. Epilepsy is a relatively common neurological condition, affecting about 1% of the population worldwide. There is a significant group of patients (20—30%) resistant to the currently available therapeutic agents (Morimoto et al., 2004). Chemical kindling is widely used as an experimental model of epilepsy and epileptogenesis. This phenomenon is characterized by progressive intensification of seizures activity after repeated administration of doses of different central nervous system (CNS) stimulants, including pentylenetetrazol (PTZ) (Racine, 1972). Pentylenetetrazol is commonly used as a convulsant drug, acting as a $GABA_A$ antagonist as well as by altering potassium permeability of the cell membrane via a voltage-dependent mechanism. Studies have shown that the mechanisms involved in PTZ kindling may include a decrease in central GABAergic function (Berman et al., 2000). The enhanced seizure susceptibility induced by kindling is probably attributable to plastic changes in the synaptic efficacy (Morimoto et al., 2004).

In the CNS, adenosine and guanosine act both as important neuromodulators with major inhibitory effects on neuronal activity. In the brain, the development and spread of seizures is thought to be prevented by a tonic anticonvulsant effect mediated by released adenosine via adenosine A_1 receptor (Dunwiddie and Masino, 2001; Avsar and Empson, 2004; Fedele et al., 2006; Pagonopoulou et al., 2006). Guanosine is another purine nucleoside that has only recently been shown to exert anticonvulsant properties and to increase glial glutamate uptake (Schmidt et al., 2000; Soares et al., 2004).

The nucleosides adenosine and guanosine can be released as well or generated from released nucleotides (ATP, ADP, AMP, GTP, GDP and GMP) that are metabolized and rapidly converted to adenosine and guanosine. There is evidence for the vesicular release of ATP and other nucleotides from nerves in CNS (Fields and Burnstock, 2006). After the release, ATP acts producing an excitation or an inhibition of neurotransmission (Malva et al., 2003). The inactivation of ATP signaling is mediated by the action of ecto-nucleotidases. In this group of enzymes, it has been proposed that NTPDases and a 5′-nucleotidase constitute an enzymatic cascade able to promote the hydrolysis of triphosphate and diphosphate nucleotides to the respective nucleosides. In rats, this hydrolysis can occur in the CNS (Battastini et al., 1991; Cruz Portela et al., 2002) by the action of the same set of enzymes. Eight different enzymes are described as members of the NTPDase family (Robson et al., 2006; Zimmermann, 2006). NTPDases1, 2, 3 and 8 are ecto-enzymes (E-NTPDases), with the catalytic site facing to extracellular space. On the other hand, the enzymes classified as NTPDase4, 5, 6 and 7 present intracellular localization. Among them, NTPDases5 and 6 could present secreted forms (Robson et al., 2006). Ecto- 5′-nucleotidase is a GPI-anchored enzyme which was also described in rat CNS (Sadasivudu et al., 1980; Braun et al., 1994). This ecto-enzyme was identified on mossy fibers that sprout after seizures in kainate-treated and kindled rats (Schoen et al., 1999). In previous studies, we have shown the presence of ecto-nucleotidase pathway in CNS and its involvement in several pathological conditions, including seizures and epilepsy (Battastini et al., 1991; Bruno et al., 2003; Bonan et al., 2000a,b; Cruz Portela et al., 2002; Oses et al., 2004).

The aim of this study was evaluate the different adenine and guanine nucleotide hydrolysis promoted by ecto- and soluble nucleotidases in rat CNS after PTZ-kindling treatment. Additionally, we determined the levels of the purines in rat cerebrospinal fluid (CSF), as well as ecto-NTPDases (1, 2, 3, 5, 6 and 8) and 5′-nucleotidase expression in hippocampi of these animals.

Material and methods

Chemicals

Reagents were obtained as follows: nucleotides, Malachite Green Base, Coomassie Brilliant Blue G, Trizma base and PTZ from Sigma (St. Louis, MO, USA); anesthetic sodium thiopental from Cristalia (São Paulo, SP, Brazil). All other reagents were of analytical grade.

Animals

Female Wistar rats (60—90 days old; 150—250 g) were used. Animals were kept on a 12 h light:12 h dark cycle (lights on at 07:00 a.m.) at constant temperature of $22 \pm 1\,^{\circ}C$. They were housed in plastic cages (five animals per cage) with water and food *ad libitum*. In all experiments, institutional protocols with animals were performed to minimize suffering and limit the number of animals sacrificed.

Pentylenetetrazol-kindling treatment

In the PTZ-kindling model, a subconvulsant dose of PTZ (35 mg/kg, i.p.) was administered every 48 h for 20 days (10 stimulations). All the injections were performed during the day, between 10:00 and 12:00 h. Animals were observed for 30 min after each injection, and seizure severity was graded according to the following scale: 1, facial clonus; 2, head nodding; 3, myoclonic jerks; 4, rearing/falling seizures; 5, running/bouncing seizures [adapted from Racine, 1972]. Seizure intensity increased with each administration until animals reached stages 3, 4 or 5 seizures, in which they are considered kindled. Animals without convulsive behavior or in stages 1 or 2 seizures at the end of treatment were not used. Behavioral response during the early stages of kindling is characterized as a freezing response during the evoked ictal discharge; and at the end of treatment, kindled animals was able to progress into a generalized seizure. Ten days after the last injection the animals were

used for experimental procedures. The control group was treated with saline using the same protocol of PTZ group.

CSF sampling

Ten days after the last injection rats were anesthetized with 40 mg/kg of sodium thiopental, i.p., and the cerebrospinal fluid (CSF) was drawn (60—80 μL per rat), by direct puncture of the *cisterna magna* with an insulin syringe (27 gauge × 1/2 in. length). It was performed only one CSF sampling from each rat in the morning, and the samples were pooled in a single tube and stored for 1 h on ice (CSF volume pooled was approximately 200 μL). Individual samples that presented visible blood contamination were discarded. Before performing the assays, the pool was centrifuged at 4500 × *g* at 5°C for 5 min, to obtain cell-free supernatants. The supernatant was added to the reaction medium, and the enzymatic assay carried out within 2 h of the sampling (Cruz Portela et al., 2002).

Hippocampal slices

The rats were killed by decapitation and the brains were rapidly removed into a bicarbonate-buffered salt solution with the following composition: 115 mM NaCl, 3.0 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose, 2.0 mM $CaCl_2$, pH 7.4, and gassed with 95% O_2 and 5% CO_2 mixture (incubation medium). The brains were cut longitudinally, their hippocampi dissected and slices transversely cut to 400 μm thick on a Mcllwain tissue chopper.

Assay for nucleotides hydrolysis by CSF

The reaction medium contained 2.0 mM $CaCl_2$, and 50 mM Tris—HCl buffer, pH 7.5, in a final volume of 200 μL. CSF (40 μL) was added to the reaction medium (4 μg of protein per tube), and pre-incubated for 10 min at 37°C. The reaction started by addition of ADP, GDP, AMP and GMP to a final concentration of 1.0 mM and stopped, after 300 min, by the addition of 200 μL of trichloroacetic acid 10%. The amount of released Pi was measured by the method described by Chan et al., 1986. Controls to correct for non-enzymatic hydrolysis were performed by adding CSF after stopping the reaction. Enzymatic activity was expressed as nmol of Pi/min/mg.

Assay for nucleotides hydrolysis by hippocampal slices

Two slices per tube (approx. 0.16 mg protein) were preincubated for 10 min at 37°C with 500 μL of the incubation medium (described above) and gassed directly with 95% O_2 and 5% CO_2. Adding ATP, ADP, AMP, GTP, GDP or GMP started the reaction, to a final concentration of 2.0 mM. The reaction was stopped by the addition of 100 μl of 10% trichloroacetic acid. Non-enzymatic Pi released from nucleotides into assay medium without slices and Pi released from slices without nucleotide were subtracted from total Pi released during incubation. All assays were performed in duplicate or triplicate. Pi was measured by the method of Chan et al., 1986 and the enzymatic activity was expressed as nmol of Pi/min/mg.

HPLC analysis of nucleotides in CSF

High-performance liquid chromatography (HPLC) was done to measure CSF concentrations of the following nucleotides and nucleosides: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (Ado), guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine (Guo), inosine monophosphate (IMP), inosine (Ino), hypoxanthine (Hypo), xanthine (Xan), and uric acid (UA). Analyses were performed with Shimadzu Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 μL loop, UV—vis detector. Separations were achieved on Discovery C18 250 mm × 4.6 mm, 5 μm particle size column (Supelco). The mobile phase flowed at a rate of 1.2 mL/min and column temperature was 24°C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, 100% at 12.30 min, 0% at 12.40 min. Samples of 5 μL were injected every 18 min into the injection valve loop. Absorbance was read at 254 nm. Concentrations were expressed as median in picomol/mg protein (Domanski et al., 2006).

Semi-quantitative RT-PCR

Total RNA from hippocampus was isolated with Trizol™ reagent (Invitrogen) in accordance with the manufacturer's instructions. The cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) from 3 μg of total RNA in a total volume of 20 μl with an oligo (dT) primer in accordance with the manufacturer's instructions. cDNA reactions were performed for 50 min at 42°C and stopped by boiling for 5 min. Two microliters of cDNA were used as a template for PCR with primers specific for E-NTPDases1, 2, 3, 5, 6, 8 and 5′-nucleotidase. As a control for cDNA synthesis, β-actin-PCR was performed. Two microliters of the cDNA were used for PCR in a total volume of 25 μl using a concentration of 0.4 μM of each primer indicated below, 200 μM of dNTPs and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer.

The PCR cycling conditions were as follows: initial 1 min denaturation step at 94°C, 1 min at 94°C, 1 min annealing step (NTPDase 1—3, 5, 6 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 final 10 min extention at 72°C. The amplification products were: NTPDase1, 543bp; NTPDase2, 331bp; NTPDase3, 267 bp; 5′-nucleotidase, 405bp; β-actin, 210 bp. Primers for NTPDase8 (394 bp) were also used in this study. Seven microliters of the PCR reaction was analyzed on a 1% agarose gel. The following set of primers were used for NTPDase1: 5′-GAT CAT CAC TGG GCA GGA GGA AGG-3′ and 5′-AAG ACA CCG TTG AAG GCA CAC TGG-3′; for NTPDase2: 5′-GCT GGG TGG GCC GGT GGA TAC G-3′ and 5′-ATT GAA GGC CCG GGG ACG CTG AC-3′; for NTPDase3: 5′-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3′ and 5′-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3′; for NTPDase5: 5′-TGG TGG TAA CCA AGA AGG GGA GAT GG-3′ and 5′-GCA GGT GAA AGG TGG CTC CCA AGG-3’; for NTPDase6: 5′-GGC CTC TAC GAG CTG TGT GCC AGC AG-3′ and 5′-TCA GTA CCT TGT CCC CGG GAA AAC C-3′; for NTPDase8: 5′-CCA CAC TGT CAC TGG CTT CCT TG-3′ and 5′-ACG AGG ATG TAT AGG CCT GAG G-3′; for 5′-nucleotidase (CD73): 5′-CCC GGG GGC CAC TAG CAC CTC A-3′ and 5′-GCC TGG ACC ACG GGA ACC TT-3′; for β-actin: 5′-TAT GCC AAC ACA GTG CTG TCT GG-3′ and 5′-TAC TCC TGC TTC CTG ATC CAC AT-3′.

Protein determination

Protein was determined by the Coomassie Blue method, according to Bradford using bovine serum albumin as standard (Bradford, 1976).

Statistical analysis

The data are represented as mean ± S.D. and the statistical analysis was performed with Student's *t*-test for NTPDase results. The data are shown as mean ± S.E. for HPLC results and the statistical analysis was performed using a non-parametric Mann—Whitney test. For all results, $P \leq 0.05$ was considered a significant difference between groups.

Results

PTZ-kindling produced a progressive increase in the seizure susceptibility of the treated rats. In the first injection the animals began with an average of 1.92 ± 0.26 in the kindling stage and after the 10th stimulation, the mean kindling stage was 4.45 ± 0.10. At the end of 20 days of treatment 11 animals reached stage 3, 51 stage 4 and 42 stage 5 of seizure ($N = 104$ animals) and were considered kindled. The convulsive behaviour was evaluated during 30 min each day after PTZ injection. It was observed that each animal experienced at least seven seizures during the treatment.

The study of the ecto-nucleotidase activities may be an interesting approach to understanding the hippocampal functional response in epileptic events. We determined the enzymatic activities for PTZ-kindled rats in stages 3, 4, and 5. These three stages were not different among them but were statistically different from the control group. For this reason PTZ-kindled rats were included in a single group namely kindling. In this investigation, hippocampal slices demonstrated an enhancement of ATP (151.00 ± 25.90 nmol Pi/min/mg), AMP (46.46 ± 5.90 nmol Pi/min/mg), GDP (4.43 ± 0.96 nmol Pi/min/mg), and GMP (4.3 ± 1.08 nmol Pi/min/mg) hydrolysis when compared with controls (115.43 ± 21.90; 29.41 ± 1.97; 2.10 ± 0.42 and 2.04 ± 0.50 nmol Pi/min/mg, for ATP; AMP; GDP; and GMP, respectively). For ADP and GTP hydrolysis, there were no significant differences between controls and PTZ-kindled animals (ADP: 66.53 ± 5.58 and 61.47 ± 8.60; GTP 8.73 ± 0.50 and 10.09 ± 1.98 nmol Pi/min/mg for control and PTZ-kindled animals, respectively) (Fig. 1).

CSF analysis is a basic tool to study a number of parameters in the CNS, including the ongoing changes in the brain during acute and chronic diseases. In this sense we evaluated the hydrolysis of monophosphate and diphosphate nucleotides by soluble nucleotidases present in CSF. Soluble nucleotidases from PTZ-kindled animals promoted an increase of ADP (9.59 ± 2.79 nmol Pi/min/mg), GDP (46.34 ± 2.93 nmol Pi/min/mg), and GMP (6.87 ± 3.07 nmol Pi/min/mg) hydrolysis when compared with controls (6.12 ± 2.07; 40.72 ± 3.39; and 3.81 ± 1.75 nmol Pi/min/mg for ADP, GDP and GMP, respectively). AMP hydrolysis did not change after kindling treatment (control: 6.05 ± 0.8; PTZ-kindled rat: 5.35 ± 1.6 nmol Pi/min/mg) (Fig. 2). Additionally, we evaluated the levels of purine derivatives present in CSF. CSF levels for the purines GTP, ADP and the oxypurine uric acid were increased in PTZ-kindled animals in comparison with control rats (Table 1, Fig. 3). Moreover, the

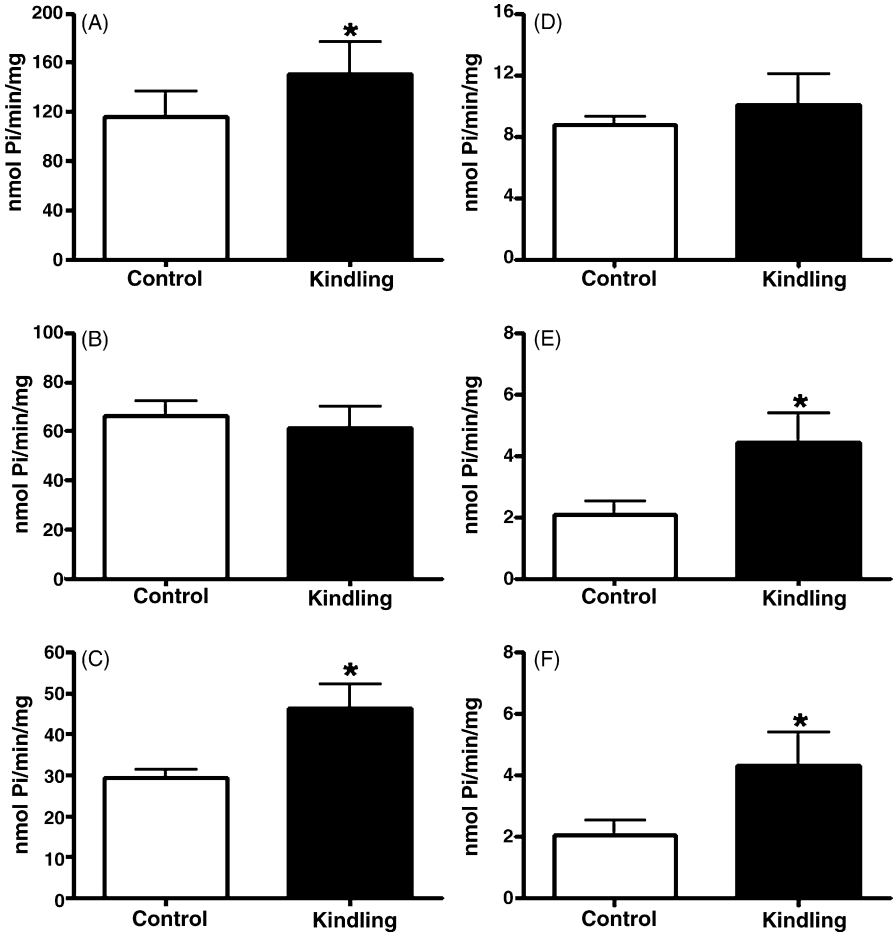

Figure 1 Effects of PTZ-kindling (stages 3, 4, or 5; black bars) when compared with control animals (white bars) on ATP (A), ADP (B), AMP (C), GTP (D), GDP (E) and GMP (F) hydrolysis in rat hippocampal slices 10 days after last injection. Bars represent mean $\pm$ S.D. of at least four animals. *PTZ-treated group significantly different from control group ($P \leq 0.05$, Student's t-test).

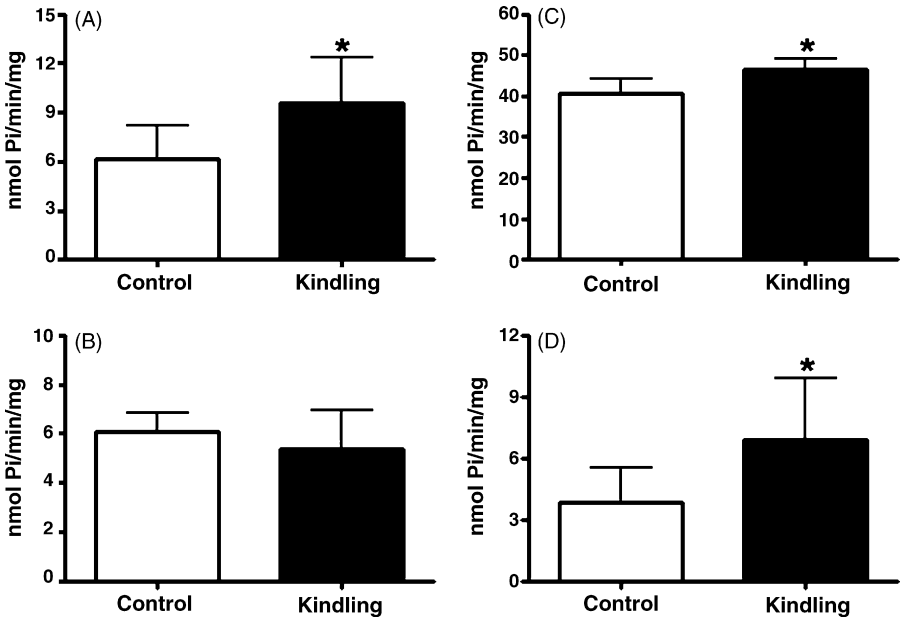

Figure 2 Effects of PTZ-kindling (stages 3, 4, or 5; black bars) when compared with control animals (white bars) on ADP (A), AMP (B), GDP (C) and GMP (D) hydrolysis in rat CSF 10 days after last injection. Bars represent mean ± S.D. of at least four animals. *PTZ-treated group significantly different from control group ($P \leq 0.05$, Student's t-test).

purines GMP, AMP and the oxypurine hypoxantine levels were decreased in the same animals when compared with control rats (Table 1, Fig. 3). PTZ-kindling treatment did not promote significant changes in the levels of other nucleosides analyzed (Table 1, Fig. 3).

Considering that the changes observed on ecto-nucleotidase activities could be promoted by transcriptional control, the relative expression of hippocampal ecto-enzymes (NTPDase1, 2, 3, 5, 6, 8, and 5′-nucleotidase) of control and PTZ-kindled rats has also been analyzed by semi-quantitative RT-PCR. We observed a significant decrease (50%) in the expression of NTPDase1 (Fig. 4A), but there were no changes in the transcript mRNA levels for the enzymes NTPDase2, 3, 5, 6 and 5′-nucleotidase in hippocampus from PTZ-kindled rats (Fig. 4B—F). NTPDase8 was not expressed in hippocampus of rats, which was in accordance to previous studies (data not show).

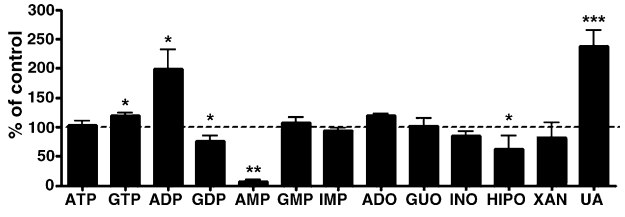

Figure 3 Relative nucleotides, nucleosides and oxypurines levels, when compared with control in rat CSF after PTZ-kindling (stages 3, 4, or 5). ATP, GTP, ADP, GDP, AMP, GMP, IMP, adenosine (ADO), guanosine (GUO), inosine (INO), xantine (XAN), hypoxantine (HIPO) and uric acid (UA) were measured 10 days after the last injection. Data represent mean ± S.E. ($n \geq 4$) *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$, as compared to control, using a non-parametric Mann—Whitney test.

Discussion

Previous studies have described ATP, ADP, and AMP hydrolysis by slices from the CNS of rats (Bruno et al., 2002). For ATP and ADP hydrolysis (Fig. 2A and B), our results suggests a possible participation of NTPDase3 or NTPDase8, since ATP hydrolysis is approximately two times higher than ADP hydrolysis (Fig. 1A and B) (Robson et al., 2006). We excluded the presence of NTPDase8, since this enzyme was not detected in rat hippocampus as previously described (Bigonnesse et al., 2004). Our results have shown that PTZ-kindling was able to alter ATP and AMP hydrolysis, but not ADP hydrolysis (Fig. 1A—C). ATP may influence several P1 and P2 receptors-mediated processes by controlling extracellular concentrations of ATP and adenosine (Bruno et al., 2002b). ATP may modulate the release and/or influence other neurotransmitters either by acting through its own receptors or by altering the neurotransmitter receptors (Fields and Burnstock, 2006). Several works have demonstrated an involvement of ATP hydrolysis in other animal models of seizures and epilepsies (Anderson et al., 1994; Fernandes et al., 1996; Nagy et al., 1997; Bruno et al., 2002a; Bruno et al., 2003; Nicolaidis et al., 2005; Oses et al., 2004). Adaptive plasticity in chronic epilepsy could involve nucleotide homeostasis, and this balance can be achieved through a delicate regulation of the amount of ATP released and of the rate of ATP hydrolysis by ecto-enzymes.

In addition, we evaluated the GTP, GDP, and GMP hydrolysis in PTZ-kindled slices, but just GDP and GMP hydrolysis was affected (Fig. 1D—F). The guanosine, generated by hydrolysis of these nucleotides, stimulates the glutamate uptake and protect against seizures induced by glutamatergic agents (Lara et al., 2001; Soares et al., 2004). Therefore, these results suggest an involvement of NTPDase5 and

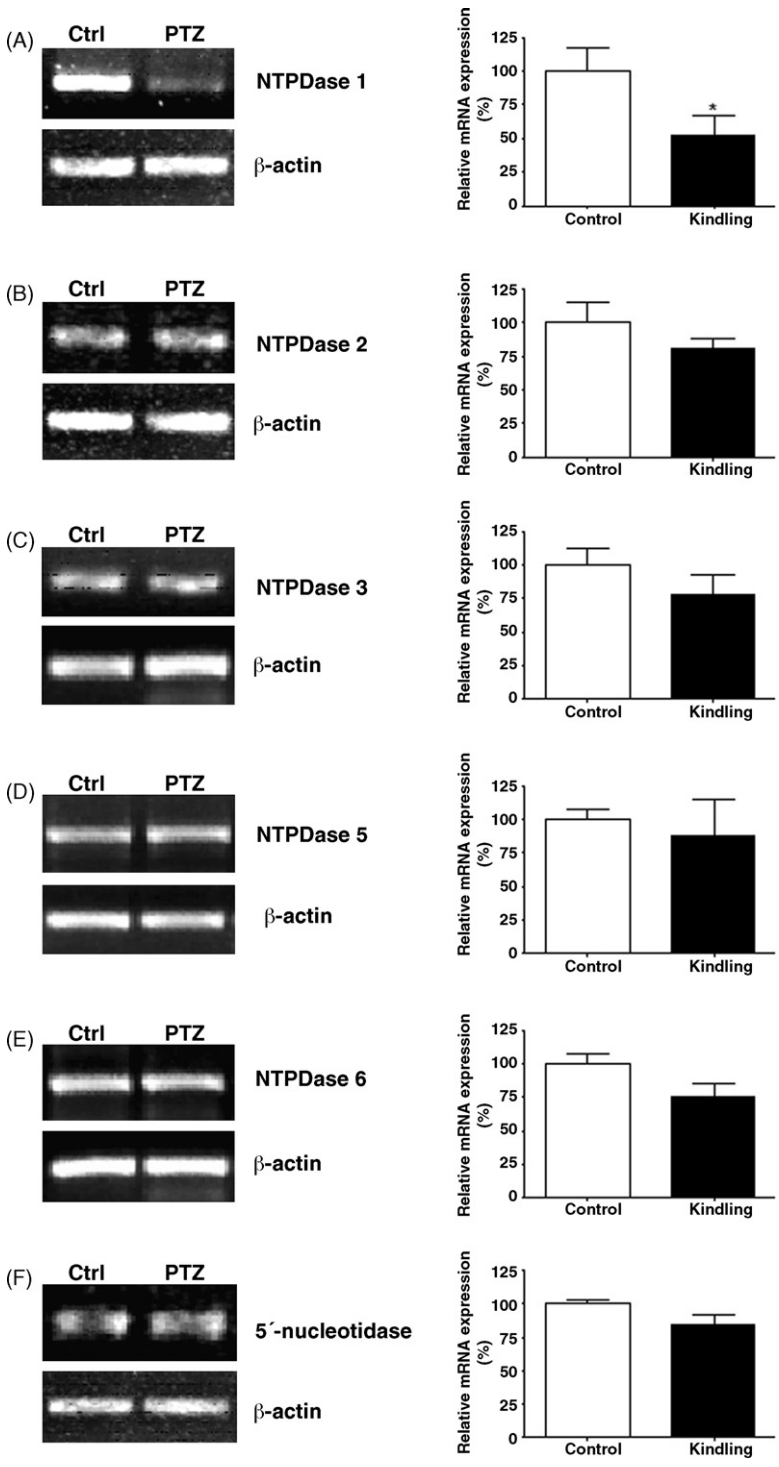

Figure 4 Representative semi-quantitative RT-PCR mRNA for NTPDase1, 2, 3, 5, 6 and 5′-nucleotidase from hippocampus of saline (Ctrl) and PTZ-kindled (stages 3, 4, or 5; PTZ) rats. The animals were sacrificed 10 days after the last injection and the samples were stored at −70 °C until PCR-analysis. The expression was evaluated for NTPDases to β-actin mRNA ratio. Three independent experiments were performed with entirely consistent results.

6, since these enzymes present a preference for guanine nucleotides.

The kinetic effects observed on adenine and guanine nucleotide hydrolysis after PTZ-kindling could be a consequence of transcriptional control. To assess the effects of PTZ-kindling on the expression of NTPDase1, 2, 3, 5, 6 and 5′-nucleotidase in the hippocampi of rats, we evaluated the mRNA levels of these enzymes with the use of RT-PCR. For NTPDase 2, 3, 5, 6 and 5′-nucleotidase, the results have shown that PTZ-kindling did not alter the enzyme expression (Fig. 4B—F), suggesting that the effects observed were not due to a decreased synthesis of these

Table 1 Nucleotides, nucleosides and oxypurines levels in control and treated rats CSF

Compound	Treatment	Compound level (picomol/μL) (mean ± S.E.)
ATP	Control	244 ± 24
	Kindling	252 ± 18
GTP	Control	0.97 ± 0.09
	Kindling	1.15 ± 0.05*
ADP	Control	317 ± 20
	Kindling	633 ± 104*
GDP	Control	257 ± 134
	Kindling	197 ± 22*
AMP	Control	150 ± 67
	Kindling	10 ± 4**
GMP	Control	172 ± 13
	Kindling	186 ± 25
IMP	Control	970 ± 43
	Kindling	912 ± 44
ADO	Control	851 ± 155
	Kindling	1016 ± 23
GUO	Control	263 ± 37
	Kindling	269 ± 34
INO	Control	870 ± 127
	Kindling	737 ± 73
HYPO	Control	436 ± 92
	Kindling	273 ± 98*
XAN	Control	1995 ± 614
	Kindling	1648 ± 504
URIC ACID	Control	1075 ± 140
	Kindling	2560 ± 291***

$^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and as compared to control, using a non-parametric Mann–Whitney test.

proteins. Despite the ATP and GDP hydrolysis increased in PTZ-kindled slices, the levels of the NTPDase1 mRNA have been decreased (Fig. 4A). The mechanism that could explain the up-regulation of NTPDase1 activity and at the same time down-regulation of transcriptional levels is known as negative feedback autoregulatory loop. This mechanism allows for genes that are not transcription factors to negatively regulate their own synthesis (Krishna et al., 2006). Several works have proposed putative mechanisms that involve a negative feedback control associated with long-term synaptic changes in epilepsies (Simonato, 1993; Qian et al., 1994; Vreugdenhil et al., 1999).

Other NTPDases members can contribute to extracellular nucleotide metabolism. Soluble NTPDases were described in CSF and there is an increased activity during seizures induced by PTZ (Cruz Portela et al., 2002; Oses et al., 2004). Thus, we evaluated the nucleotidase activities present in CSF, demonstrating an increase in ADP, GDP and GMP hydrolysis (Fig. 2A, C and D). The increment in hydrolysis of purine nucleotides after epileptic episode suggests that this CSF enzymatic system may have a biological relevance in the regulation of purine levels and also could be a putative marker of brain injuries (Bonan et al., 2000a,b; Bruno et al., 2002b and 2003; Oses et al., 2004).

The analysis of nucleotides, nucleosides and oxypurines levels in CSF by HPLC showed a marked increase in PTZ-kindled CSF concentrations of GTP, ADP and uric acid when compared with controls (Fig. 3). By the other hand, CSF concentrations of GDP, AMP and hypoxantine appear decreased (Fig. 3). Increase in ADP levels agree with the increment of ATPase activity (Fig. 1A), but not change ATP levels (Fig. 3). It could be explained by previous studies showing that the ATP levels increase followed by a concomitant enhancement of ATPase activity after electrical stimulation (Wieraszko and Seyfried, 1989). The marked decrease in AMP levels are in agreement with the data obtained for 5′-nucleotidase activities (Fig. 1C). It has been observed an increase of GTP levels and a decrease of GDP levels, which corroborate with results obtained in nucleotidase assays (Figs. 1 and 2). The lack of nucleosides accumulation could be the result of extracellular mechanisms that are responsible for the control of their concentrations, such as the nucleoside uptake by a specialized transport system. The enhanced uric acid and the decreased hypoxantine found in the CSF of these animals may not be solely due to increased hemato-encephalic barrier permeability, but may be the result of the catalysis by xanthine oxidase (Rodriguez-Nunez et al., 2003). Moreover, these results can be reflecting a glutamate-mediated excitotoxicity, since the uric acid levels were increased after neurological damage (Stover et al., 1997; Rodriguez-Nunez et al., 2003). Therefore, measurements of nucleotide and nucleoside levels can constitute an important contribution to the knowledge of the role of purines in epilepsy.

In summary, our results demonstrated an enhancement of ecto- and soluble nucleotidase activities in hippocampal brain slices and CSF after PTZ-kindling, with distinct influence on the levels of nucleotides, nucleosides and oxypurines and also hippocampal NTPDases mRNA levels. Such alterations indicate that the modulatory role of purines could be affected by PTZ-kindling. However, the physiological significance of these findings remains to be elucidated.

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CAPÍTULO II

MODIFICAÇÕES NA DENSIDADE DOS RECEPTORS P2X E P2Y NO MODELO DE EPILEPSIA DO ÁCIDO CAÍNICO.

Jean Pierre Oses, Ana Patricia Simões, Paula Margarida Canas, Ricardo Jorge Rodrigues, Catarina Rezende Oliveira, João José Freitas Sarkis, Luis Valmor Portela e Rodrigo Antunes Cunha.

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MODIFICAÇÕES NA DENSIDADE DOS RECEPTORS P2X E P2Y NO MODELO DE EPILEPSIA DO ÁCIDO CAÍNICO.

Jean Pierre Oses[1,2], Ana Patricia Simões[1], Paula Margarida Canas[1], Ricardo Jorge Rodrigues[1], Catarina Rezende Oliveira[1], João José Freitas Sarkis[2], Luis Valmor Portela[2] e Rodrigo Antunes Cunha[1].

[1] Centro de Neurociências de Coimbra, Instituto de Bioquímica, Faculdade de Medicina, Universidade de Coimbra, Coimbra, Portugal.

[2] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil.

INTRODUÇÃO

O termo *epilepsia* se refere a uma ampla categoria de sintomas complexos em torno de qualquer número de funções cerebrais desordenadas, decorrentes de uma anormalidade e hipersincronia da atividade neuronal, podendo ser secundárias a uma variedade de processos patológicos, não sendo esta uma doença específica, ou a uma síndrome simples (Engel e Pedley, 2008). A prevalência na população humana da epilepsia é aproximadamente de 1% (Palmini, Gambardella *et al.*, 1995), acreditando-se que, de toda a população mundial, 3% desenvolverão epilepsia em algum momento de sua vida (Chang e Lowenstein, 2003). Destes números, aproximadamente 70% dos pacientes epilépticos têm suas crises controladas através de medicamentos com drogas antiepilépticas. O restante dos pacientes constitui um grupo de alta morbidade, uma vez que suas crises epilépticas não são controladas através do uso de fármacos (Engel e Pedley, 2008). Esta desordem neurológica é caracterizada por crises recorrentes, com alterações comportamentais acompanhadas de modificações dos circuitos límbicos (Chang e Lowenstein, 2003; Engel e Pedley, 2008). A atividade convulsiva resulta de um desequilíbrio das transmissões excitatórias-inibitórias cerebrais, evidenciando o efeito dos amino ácidos excitatórios, principalmente o glutamato, nos efeitos neurotóxicos observados nas convulsões (Morimoto, Fahnestock *et al.*, 2004).

O Papel do ATP com um neurotransmissor têm sido demonstrado no sistema nervoso central (SNC) e periférico (Abbracchio e Burnstock, 1998; Abbracchio e Verderio, 2006; Burnstock, 2006; , 2007). No SNC, o ATP é armazenado em vesículas nos terminais nervosos, e co-liberado com noradrenalina, acetilcolina e serotonina (Abbracchio e Burnstock, 1998; Abbracchio e Verderio, 2006; Burnstock, 2006; , 2007). Esta liberação é realizada de uma forma Ca^{2+} -dependente (Phillis e Wu, 1981a; 1981b). As ações patofisiológicas mediadas pelo ATP são através do estímulo dos receptores purinérgicos

P2, classificados como P2X e P2Y (Burnstock, 2006; 2007). Receptores P2X são receptores ligados a canais iônicos (Na^+, K^+ e Ca^+), estando presentes nas formas homo-heteroméricas sobre a superfície celular (Burnstock, 2006; 2007). Sete diferentes subunidades dos receptores P2X têm sido clonados (P2X1-7). Receptores P2Y são receptores ligados a proteína-G, apresentando uma simples subunidade com sete domínios transmembrana (Burnstock, 2006; , 2007). Os receptores P2 são amplamente expressos em diferentes áreas do cérebro, incluindo o hipocampo (Burnstock, 2006; , 2007).

Estudos anteriores têm demonstrado que o ATP é liberado sobre o estímulo glutamatérgico, em particular em estímulos de alta freqüência como os observados em crises convulsivas (Wieraszko e Seyfried, 1989b; Cunha, Correia-De-Sa *et al.*, 1996). Além disso, recentemente foi demonstrado que os receptores P2X (P2X1-3) estão pré-sinapticamente localizados e controlam a liberação de glutamato dos terminais nervosos hipocampais (Rodrigues, Alfaro *et al.*, 2005). Assim, o objetivo deste trabalho é observar a possível modificação na densidade dos receptores P2Xe P2Y após um período convulsivo induzido pelo ácido caínico (cainato), um modelo animal de epilepsia de lobo temporal (Ben-Ari, 1985).

MATERIAL & MÉTODOS

Reagentes:

Os anticorpos para receptores P2X1 (resíduos 382-399, rato, diluição 1:5.000 de uma solução de estoque 0,6 mg/mL), receptores P2X3 (resíduos 383-397, rato, diluição 1:1.000 de uma solução estoque de 0,2 mg/mL), receptores P2X4 (resíduos 370-388, rato, diluição 1:500 de uma solução estoque 0,3 mg/mL), receptores P2X7 (resíduos 576-595,

rato, diluição 1:5.000 de uma solução estoque 0,3 mg/mL), receptores P2Y2 (resíduos 227-244, rato, diluição 1:5.000 de uma solução estoque de 0,6 mg/mL), receptores P2Y4 (resíduos 337-350, rato, diluição 1:1.000 de uma solução estoque de 0,3 mg/mL) foram obtidos da Alamone Laboratories (Jerusalém, Israel). Os anticorpos para os receptores P2X2 (resíduos 356-471, humano, diluição 1:500 de uma solução estoque 0,2 mg/mL), receptores P2X6 (resíduos 351-431, humano, diluição 1:200 de uma solução estoque 0,2 mg/mL), receptores P2Y1 (seqüência interna, humano, diluição 1:500 de ums solução estoque de 0,2 mg/mL), receptores P2Y6 (seqüência interna, humano, diluição 1:500 de uma solução estoque de 0,2 mg/mL) e os anticorpos secundários (anti-goat e anti rabbit IgG) foram obtidos da Santa Cruz Biotechnology (Santa Cruz, Califórnia, EUA). O cainato foi obtido da Tocris Cookson (Bristol, Reino Unido). Os demais reagentes foram obtidos da Sigma.

Animais:

Ratos Wistar machos (6-8 semanas, 140-160g, Harlan Ibérica, Barcelona, Espanha) foram utilizados nestes experimentos, todos os procedimentos foram feitos de acordo com as normas da União Européia para uso de animais experimentais.

Tratamento com Cainato:

Os animais foram divididos em dois grupos, o grupo controle (injetados com solução salina, i.p.) e o caínato (injetados com cainato 10 mg/ Kg, i.p.). Após a injeção, os animais eram observados por 2 horas e a severidade da convulsão foi avaliada de acordo com a escala descrita por Racine: 1) clônus facial; 2) movimentação de flexão e extensão

da cabeça; 3) clônus das patas contra-laterais ao hemisfério estimulado; resposta de orientação, onde o animal permanece de pé apenas sobre as patas traseiras ("*rearing*"), e /ou chacoalhar de cachorro molhado (do inglês "*wet dog shake*"); e 5) "*rearing*" seguido de queda, e convulsões tônico-clônicas generalizadas (Racine, 1978; Mcintyre, Poulter *et al.*, 2002). Todos os animais do grupo caínato, apresentaram um rápido período de convulsões severas (30 minutos após a injeção), que alcançaram os estágios 4-5 de acordo com a escala de Racine. Os animais foram sacrificados 24 horas, 7 dias, ou 30 dias após o tratamento.

Preparação das membranas sinaptossomais e totais:

Membranas totais ou sinaptossomas de hipocampo foram preparadas como descrito previamente (Rodrigues et al., 2005). Os hipocampos foram removidos e homogeneizados à 4°C em tampão sacarose-Hepes (sacarose 0,32 M, EDTA 1mM, Hepes 10 mM, albumina sérica bovina 1 mg/mL, pH 7,4). O homogenato resultante foi centrifugado à 3000 x *g* por 10 minutos à 4°C, o sobrenadante foi coletado e centrifugado à 14.000 x *g* por 12 à 4°C. O precipitado resultante foi ressuspendido em 1 mL de solução Percoll 45% (v/v) feito em solução Krebs-Hepes (NaCl 140 mM, KCl 5 mM, Hepes 10 mM, EDTA 1mM, glicose 5 mM, pH 7,4). Após uma centrifugação à 21.000 x *g* por 2 min à 4°C, a camada superficial (fração sinaptossomal) foi removida, lavada e ressuspendida em solução Krebs-Hepes. Para a obtenção de membranas totais, uma parte do sobrenadante da primeira centrifugação foi retirado, ressuspendido em Tris-HCl 50 mM e $MgCl_2$ 10 mM, centrifugado a 28.000 x *g* por 30 min à 4°C, e o precipitado resultante foi resuspendido em uma solução Krebs-Hepes. Uma alíquota de cada amostra

foi utilizada para quantificação de proteína usando o método do ácido bicinconínico (kit comercial da Pierce Biotecnologia, Rockford, EUA).

Análise por eletroforese e imunodetecção dos receptores P2:

A determinação da densidade dos receptores P2 técnica de *Western Blotting*, como previamente descrito por Rodrigues et al., 2005. Cada amostra foi diluída com 5 volumes de tampão SDS-PAGE contendo glicerol 30% (v/v), ditiotritol 0,6 M, sódio dodecil sulfato (SDS) 10% (v/v) e Tris-HCl pH 6,8, e aquecido à 95°C por 5 min. Estas amostras diluídas foram separadas por uma eletroforese em um gel SDS-poliacrilamida (gel de entrada com uma concentração de 4% e o gel de corrida de 7,5%) junto com padrões de peso molecular (BioRad, EUA). Após a separação das amostras por eletroforese, foi feita uma eletrotransferência das proteínas para uma membrana de polivinilideno difluorida (0,45 μm, Amersham Biosciences, Reino Unido). Depois da transferência, as amostras foram bloqueadas por 1 hora à temperatura ambiente com tampão Tris-salina (Tris 20 mM, NaCl 140 mM, pH 7,6) com 5% de leite, contendo Tween 20 0,1% (TBS-T). Após o bloqueio, as membranas são lavadas 3 vezes por 20 min em TBS-T sem leite, e incubadas à 4°C *overnigth* com os anticorpos primários para os receptores P2 diluídos em TBS-T. Após a incubação com os anticorpos primários, as membranas foram lavadas 3 vezes com TBS-T e incubadas com anticorpo secundário anti-rabitt ou anti-goat diluídos em TBS-T (1:10.000) por 90 min à temperatura ambiente. Ao término da incubação com os anticorpos secundários, as membranas eram lavadas em TBS-T por 20 min e incubadas

com substrato luminescente (Amersham Biosciences) e analisadas no sistema VersaDoc 3000 (Bio Rad).

Análise Estatística:

Os resultados estão apresentados como média ± erro padrão e a significância foi considerada para $P \leq 0,05$ utilizando ANOVA de uma via seguida do teste *pos-hoc* de Benferroni.

RESULTADOS

Uma vez que o ATP possui um papel como neurotransmissor e na sinalização neurônio-glia (Fields e Stevens, 2000), nós simultaneamente avaliamos as modificações na densidade nas membranas sinápticas e membranas totais após o modelo de epilepsia induzida por cainato.

Um dia após a convulsão, foi observada, em membranas sinápticas, um aumento na densidade dos receptores P2X1, P2Y1 e P2Y6 (25%, 57% e 143%, respectivamente), enquanto que nas membranas totais houve um aumento em P2X1, P2X2, P2X4, P2Y4 e P2Y6 (62%, 38%, 16%, 31% e 223%, respectivamente)(Fig. 1 e 2).

No grupo em que foi analisada a densidade dos receptores P2 7 dias após a convulsão, foi observado um aumento na densidade dos recptores P2Y4 de membranas

sinápticas e P2Y1 de membranas totais (102% e 32%, respectivamente), e uma diminuição em P2X1 de membranas sinápticas e P2Y6 de membranas totais (23% e 40%, respectivamente)(Fig. 1 e 2).

E, no grupo de 30 dias, foi observado, um aumento em P2X3, e P2Y6 de membranas sinápticas, P2X2, P2X4 de membranas totais (30%, 57%, 67% e 112%, respectivamente), enquanto houve uma diminuição na densidade dos receptores P2X2 de membranas sinápticas (33%) (Fig. 1 e 2).

DISCUSSÃO

O ATP é liberado para o espaço extracelular em condições fisiológicas, contribuindo para a sinalização celular, através da ação nos receptores P2 (Abbracchio e Burnstock, 1998; Burnstock, 2006). Entretanto, em situações como epilepsia, trauma, esquemia/hipóxia e inflamação, o ATP extracelular pode originar-se de células rompidas, astrócitos ativados, neurônios, microglia e células endoteliais (Abbracchio e Verderio, 2006; Burnstock, 2007). Neste trabalho, observamos alterações na densidade dos receptores P2 em hipocampo, tanto na sua localização no tipo celular (membranas sinápticas ou membranas totais), quanto em diferentes períodos após o processo convulsivo.

No hipocampo, a ativação dos receptores P2 pode estimular ou inibir a liberação do glutamato. Os receptores P2X1, P2X2/3 e P2X3 podem facilitar a liberação de glutamato, enquanto a ativação dos receptores P2Y1, P2Y2 e P2Y4 podem inibir a liberação do glutamato (Burnstock, 2007). Porém, altos níveis de ATP, tendem de uma maneira global, inibir a liberação de glutamato, e estimular a liberação de GABA, um

neutrotransmissor inibitório (Fields e Stevens, 2000; Abbracchio e Verderio, 2006). Nossos resultados demonstram que logo após uma crise epiléptica, não há alterações significativas em membranas sinápticas nos receptores P2X1-3, e há uma ativação dos receptores P2Y1 e P2Y4 de membranas sinápticas, esta ativação dos receptores P2Y, estariam inibindo a liberação de glutamato (Rodrigues, Alfaro *et al.*, 2005; Rodrigues, Almeida *et al.*, 2005).

É descrito na literatura que o hipocampo de animais com crises epilépticas crônicas apresentam respostas associadas com um aumento dos receptores P2X7 (Vianna et al., 2002). Aqui, nós não observamos nenhuma alteração na densidade destes receptores nos tempos analisados (Fig. 1 e 2). As alterações observadas nos outros receptores P2X, foram observadas em membranas totais, podendo sugerir que estas alterações podem estar ocorrendo nos astrócitos ou microglia, como uma resposta na tentativa de proteger os neurônios de um dano maior.

Em suma, estes resultados estão consistentes com a hipótese, em particular os receptores P2Y1, podem contribuir para um processo neuroprotetor na epilepsia, sendo necessário mais estudos para confirmar a função neuroprotetora deste receptor.

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Legendas das Figuras:

Figura 1: Efeito da convulsão induzida por ácido caínico na densidade dos receptores P2X de membranas sinápticas e membranas totais de hipocampo. (A) Membranas sinápticas, (B) membranas totais. *$P \leq 0,05$, **$P \leq 0,01$.

Figura 2: Efeito da convulsão induzida por ácido caínico na densidade dos receptores P2Y de membranas sinápticas e membranas totais de hipocampo. (A) Membranas sinápticas, (B) membranas totais. *$P \leq 0,05$, **$P \leq 0,01$.

Figura 1:

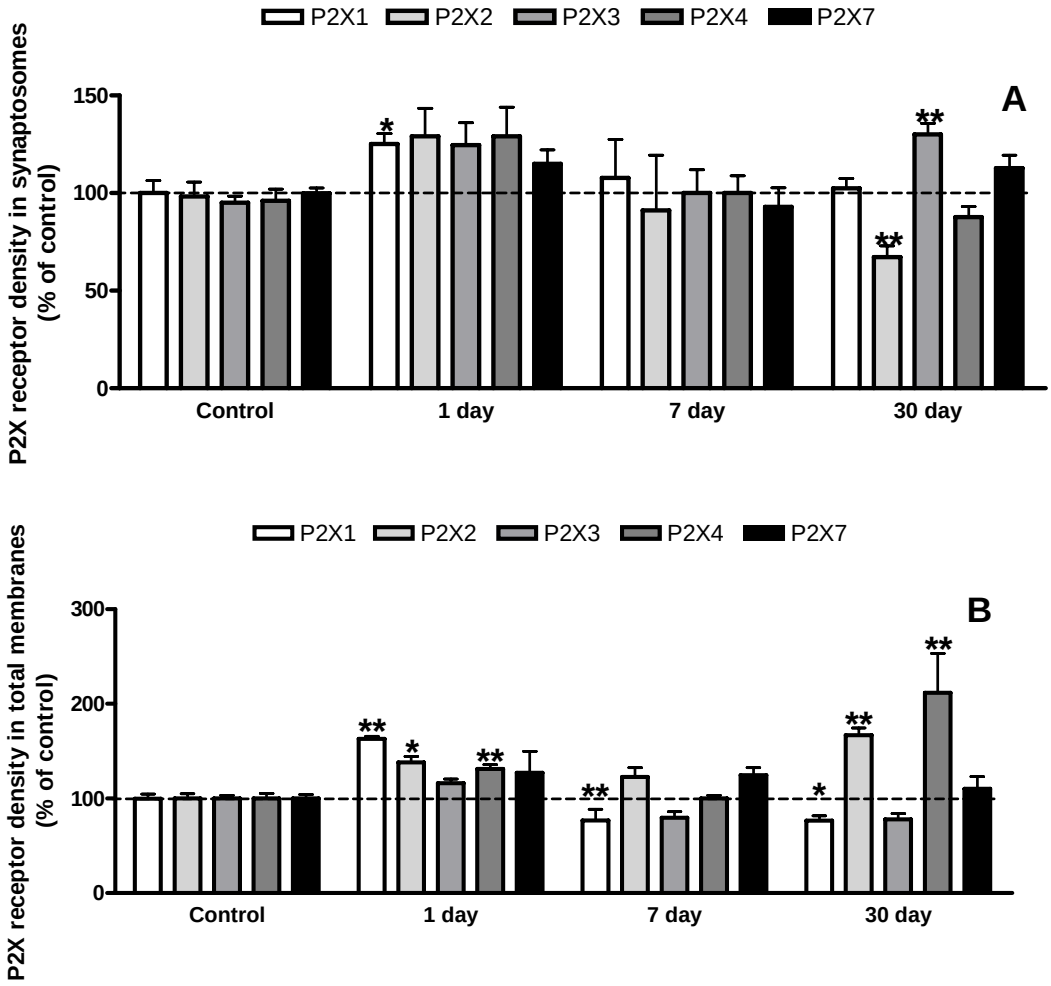
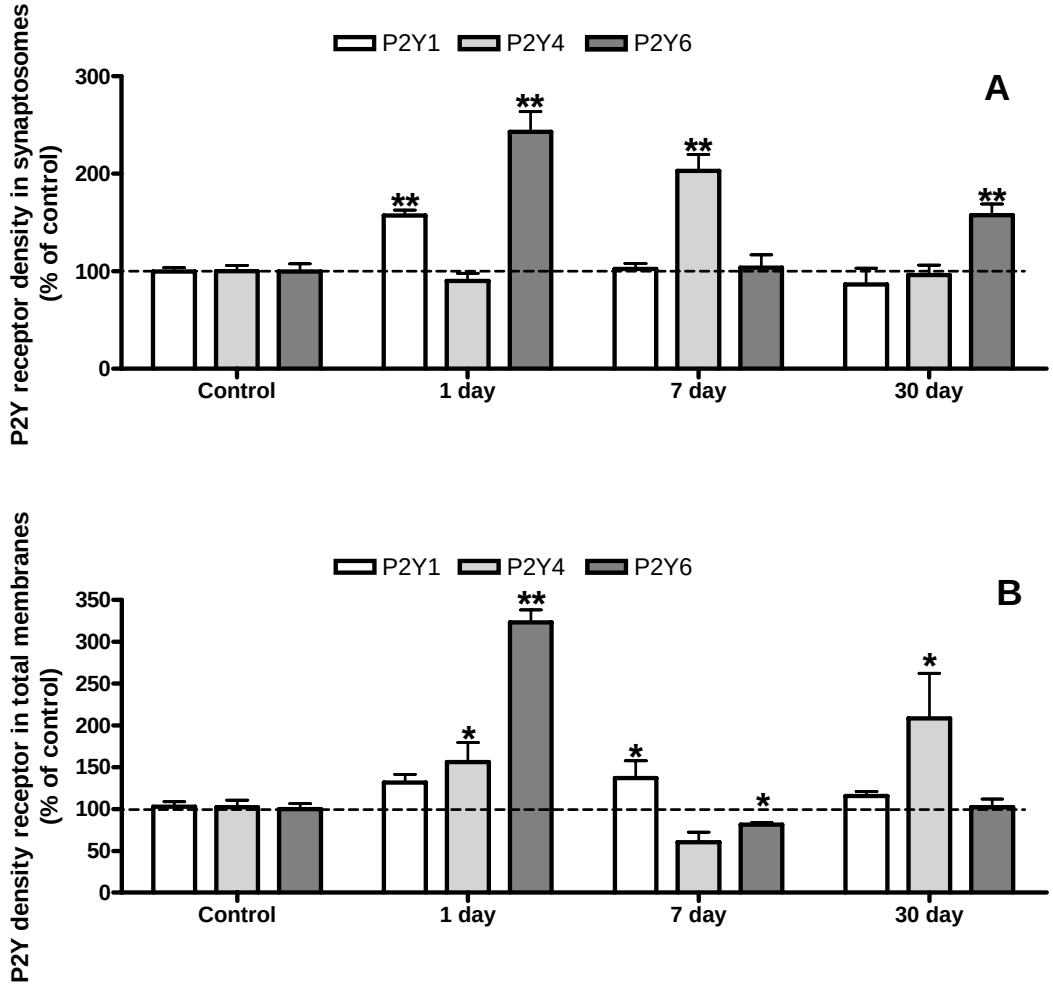

P2X1
P2X2
P2X3
P2X4
P2X7
P2X receptor density in synaptosomes (% of control)
150
100
50
0
A
Control
1 day
7 day
30 day
P2X receptor density in total membranes (% of control)
300
200
100
0
B

Figura 2:


P2Y1
P2Y4
P2Y6
A
P2Y receptor density in synaptosomes (% of control)
300
200
100
0
**
**
**
**
Control
1 day
7 day
30 day
B
P2Y density receptor in total membranes (% of control)
350
300
250
200
150
100
50
0
**
*
*
*
*
Control
1 day
7 day
30 day

CAPÍTULO III

PENTYLENTETRAZOL INDUCED SEIZURE INCREASES GLUCOSE AND LACTATE LEVELS IN CEREBROSPINAL FLUID OF RATS

Jean Pierre Oses, Alexandre Pastoris Müller, Giordano Gubert Viola, Ana Elisa Böhmer, Luiza Knorr, Júlia Dubois Moreira, Marcos Luiz Perry, João José Freitas Sarkis, Diogo Onofre Souza and Luis Valmor Cruz Portela*.

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Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul.

*Corresponding author:

Luis Valmor Cruz Portela, PhD

Departamento de Bioquímica

Instituto de Ciências Básicas da Saúde

Universidade Federal do Rio Grande do Sul

Email: roskaportela@gmail.com

Phone: +55 51 3308 5558

FAX: +55 51 3308 5540

Running tittle: Glucose and lactate in CSF from PTZ seizures

Key words: Glucose; Lactate; CSF, Seizure, PTZ

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ABSTRACT

Purpose: Seizures induce several morphologic and biochemical alterations in the brain. In experimental studies, pentylentetrazol (PTZ) has been used as an animal model to induce seizures. Generalized convulsive seizures cause excessive neuronal firing that place large metabolic demands on the central nervous system resulting in a great impact in glycogen, glucose/lactate utilization and availability as energy substrate. To assess the metabolic profile in the brain underwent to an excitotoxic condition we investigated in this study the influence of a single convulsive crisis induced by PTZ on CSF levels of glucose and lactate. Methods: CSF levels of glucose and lactate were measured and hipocampal [^{14}C]-2-deoxy-D-glucose uptake and glycogen content were also evaluated. Results: animals that presented tonic-clonic seizure had CSF glucose level increased at 10 minutes (96.25 ± 13.19 mg/dL) peaking at 60 minutes (113.03 ± 16.34 mg/dL) and returned to control levels at 24 hours (50.12 ± 12.81 mg/dL), while CSF lactate level also increased at 10 minutes (3.23 ± 1.57 mmol/L) but returned to control level at 360 minutes (1.58 ± 0.21 mmol/L). The [^{14}C]-2-deoxy-D-glucose uptake decreases 27% at 10 min after seizure and returned to control levels at 30 minutes. Similarly, there was a decrease of 37% in hipocampal glycogen content at 10 minutes, which returned to control levels at 30 minutes. Discussion: In summary, we showed that acute seizures induced by PTZ elicit a significant temporal increase in CSF glucose and lactate levels and a brief decrease in [^{14}C]-2-deoxy-D-glucose uptake and glycogen content.

Key words: Glucose; Lactate; CSF, Seizure, PTZ

INTRODUCTION

Seizures induce several morphologic and biochemical alterations in the brain, which may involve the glutamatergic system (Morimoto, Fahnestock *et al.*, 2004; Fellin e Haydon, 2005). Excessive stimulation of brain glutamate receptors by glutamate, leads to deleterious effects on neuronal and glial cells. This process, termed excitotoxicity, is probably a common pathway involved in various chronic and acute brain disorders including Parkinson disease, ischemia, epilepsies and seizures (Olney, 1990; Meldrum, 1993; Pellerin, 2003; Darbin, Risso *et al.*, 2005; Walling, Rigoulot *et al.*, 2007).

In experimental studies, pentylentetrazol (PTZ) has been used as a model to induce seizures by mechanisms that involve the inhibition of GABAergic system producing a massive depolarization and overstimulation of glutamatergic system (Piotrovsky, Garyaev *et al.*, 1991; Berman, Cappiello *et al.*, 2000). Generalized convulsive seizures cause excessive neuronal firing that place large metabolic demands on the central nervous system (CNS) resulting in a great impact in glycogen, glucose/lactate utilization and availability as energy substrate (Pellerin, 2003; Darbin, Risso *et al.*, 2005; Walling, Rigoulot *et al.*, 2007).

Some studies have examined specific parameters of glucose metabolism related to seizures as glycogen, endogenous glucose and/or lactate (Fornai, Bassi *et al.*, 2000; Darbin, Risso *et al.*, 2005; Walling, Rigoulot *et al.*, 2007). Glycogen, located primarily in astrocytes in the central nervous system (CNS), is the principal reservoir of energy substrates in the brain, moreover, brain glycogen is a fast-activated source of energy (Magistretti, Sorg *et al.*, 1993; Cruz e Dienel, 2002; Walling, Rigoulot *et al.*, 2007). Lactate, the end product of glycolysis in the absence of oxygen, plays an essential role in brain energy metabolism possibly because it works in the synaptic cleft linking astrocytes and neurons. Under physiological conditions, the brain energy demands are sufficiently to

satisfy the physiological requirements but in abnormal situations, such as during seizures, when metabolic brain activity rises, glucose utilization is supposed to engage the anaerobic pathway, generating considerable amounts of lactate (Fornai, Bassi *et al.*, 2000; Pellerin, 2003). During brain activation, neurons release glutamate, which is removed from the synaptic cleft by astrocytes (Pellerin e Magistretti, 1994). The energy needed for this process is derived from glycolysis in astrocytes, which in turn, release lactate in the cerebrospinal fluid (CSF) to be used by neurons (Tsacopoulos e Magistretti, 1996). Moreover, lactate has been shown under certain circumstances to have a neuroprotective effect and support neuronal activity. Similar confirmation of lactate utilization in vivo as well as putative neuroprotection in various excitotoxic models has been provided (Pellerin, 2003).

The central effects caused by seizures in vivo have not been fully determined by controversial issues regarding symptoms, and due to lack of precise and safe parameters for such evaluation. CSF analysis is a basic tool to study a number of parameters in the CNS, including brain metabolism and ongoing changes in pathological events (Cruz Portela, Oses *et al.*, 2002; Busnello, Leke *et al.*, 2006; Oses, Viola *et al.*, 2007). Accordingly, the assessment of CSF glucose and lactate level has been postulated to have a role in neurological disorders as prognostic markers as well as reliable (Pellerin, 2003; Chow, Rooney *et al.*, 2005; Makoroff, Cecil *et al.*, 2005). Extracellular lactate and glucose alterations in the brain after head injury measured by microdialysis (Goodman, Valadka *et al.*, 1999; Vespa, Mcarthur *et al.*, 2003).

To assess the metabolic profile in the brain underwent to an excitotoxic condition we investigated in this study the influence of a single convulsive crisis induced by PTZ on

CSF levels of glucose and lactate. Hipocampal [^{14}C]-2-deoxy-D-glucose uptake and glycogen content were also evaluated.

MATERIAL AND METHODS

Chemicals

Lactate and glucose kit was obtained from Katal Biotecnologica®(MG, Brazil). [^{14}C]-2-deoxy-D-glucose was obtained from Dupont NEN (Boston, MA, USA). All other reagents were of analytical grade.

Animals

At least 12 Wistar rats (200 - 300 g) were used. Animals were kept on a 12 h light: 12 h dark cycle (lights on at 07:00 a.m.) at constant temperature of 22 ± 1 ◦C. They were housed in plastic cages (five animals per cage) with water and food *ad libitum*. Procedures for the care and use of animals were adopted according to the regulations of Guide for the Care and Use of Laboratory Animals (National Research Council, USA).

PTZ treatment and cerebrospinal fluid (CSF) sampling

Rats received a single injection of PTZ (60 mg/kg, i.p., dissolved in saline), producing tonic-clonic seizures within approximately 2 min in around 60% of rats, which lasted around 1 min. Only rats with tonic-clonic seizures were used in experiments. Animals were anaesthetized with sodium thiopental (40 mg/kg i.p.) 10, 30, 60, 120 or 240 min after the onset of seizures, followed immediately by collection of a CSF sample (40–60µL per rat) by direct puncture of the *cisterna magna* with an insulin syringe (27 gauge ×

1/2 in. length). CSF samples for lactate, glucose were centrifuged at 5000 × *g* for 5 min and the supernatant was stored at -70 °C until analysis.

Measurements of CSF glucose and lactate levels

Glucose was assayed in CSF using a glucose oxidase methodology manufactured by Katal Biotecnologica, MG, Brazil. All samples and standard were carried out in duplicate in the same experiment. A calibration factor was determined using a standard of glucose (5.56 mmol/L).

Lactate was assayed in CSF using a lactate oxidase methodology manufactured by Katal Biotecnologica, MG, Brazil. All samples and standard were carried out in duplicate in the same experiment. A calibration factor was determined using a standard of lithium lactate (4.44 mmol/L).

[^{14}C]-2-deoxy-D-glucose uptake

The sample of hippocampus, around 50 mg, was added to flasks (11cm^3) containing 0.5 mL Krebs-Ringer bicarbonate buffer, pH 7.4, 0.2 μCi of [^{14}C]-2-deoxy-D-glucose and incubated at 35°C in a metabolic shaker for 30 min. To stop the reaction the flasks were inserted on ice and the tissue were washed with ice-cold incubation buffer. Hipocamppi were dissolved in 200μL of lysis buffer (NaOH 2N), and the incorporated radioactivity was measured by liquid scintillation spectrometry.

Measurement of hipocampal glycogen levels

The sample of hippocampus, around 200 mg, was added to 2.0 mL of KOH 30% and heated at 100 °C until full dissolution of tissue and, then cooled in cool water. Ethanol (2.0 mL) was added and the mixture was heated at 70 °C for 10 min. Next, the tubes were cooled on ice for 3 min and centrifuged at 3,000 rpm for 5 min. Supernatant was discarded and 0.2 mL of HCl 5 N and 3.8 mL of distilled water were added to the pellet. The glycogen content was determined by the Krisman method (Krisman, 1962). The sample (50 μL) was mixed with iodine reactive [I_2 0.01% and KI 0.1% in a saturated solution of $(NH_4)_2SO_4$] and the resultant absorbance was determined at 460 nm and compared with a glycogen standard.

Statistical analysis

The data are represented as mean ± S.D. and the statistical analysis was performed with One-Way ANOVA followed a post hoc Dunnett test. For all results, $P \leq 0.05$ was considered a significant difference between groups.

RESULTS

In controls CSF glucose and lactate levels were 68.08 ± 11.62 mg/dL and 1.17 ± 0.32 mmol/L, respectively. In animals that were injected with PTZ and did not present seizures, neither CSF glucose nor lactate levels showed a significantly statistical difference when compared to controls (data not showed). As showed in figure 1A and 1B animals that presented tonic-clonic seizure had a sudden increase in CSF glucose and lactate levels. However, glucose and lactate levels displayed a distinct time profile. CSF glucose level increased at 10 minutes (96.25 ± 13.19 mg/dL) peaking at 60 minutes (113.03 ± 16.34

mg/dL) and returned to control levels at 24 hours (50.12 ± 12.81 mg/dL) (Fig. 1A), while CSF lactate level also increased at 10 minutes (3.23 ± 1.57 mmol/L) but returned to control level at 360 minutes (1.58 ± 0.21 mmol/L) (Fig. 1B). In comparison to control group, the lactate/glucose ratio were increased 10 minutes after seizure (92%), decreases at 60 minutes, return increase peaking in 180 (118%) minutes only returning to control levels 24 hours after seizure (Fig. 2).

We also evaluated additional parameters associated to glucose/lactate homeostasis. We carried out experiments in hippocampus to assess [^{14}C]-2-deoxy-D-glucose uptake capacity, which involve glucose transporters. Also, glycogen level was evaluated (Fig. 3A and B). The [^{14}C]-2-deoxy-D-glucose uptake decreases 27% at 10 min after seizure and returned to control levels at 30 minutes (Fig. 3A, $P \leq 0.05$). Similarly, there was a decrease of 37% in hipocampal glycogen content at 10 minutes, which returned to control levels at 30 minutes (Fig. 3B, $P \leq 0.05$).

DISCUSSION

In the present study, extracellular glucose and lactate levels increased in CSF after seizures whereas glucose uptake and glycogen level decreased in hipocampal tissue. The impacts of increased brain glucose utilization by astrocytes in the course of generalized convulsive seizures appear to contribute decisively for neuronal survival (Pellerin, 2003). Glucose and glycogen are primary energy substrate for the adult mammalian brain. Moreover, several studies have postulated the existence of an astrocyte–neuron lactate shuttle in vivo whereby lactate could be supplied by astrocytes to neurons as a preferential

energy substrate, especially during period of high activity (Pellerin, 2003; Pellerin, Bouzier-Sore *et al.*, 2007).

Because the brain has very little energy reserve, a continuous vascular supply of glucose and oxygen is mandatory to sustain neuronal activity. Seizures can cause changes in brain glucose and lactate, which may have a neuroprotective role or, in some instances, serve as biochemical markers of brain injury/activity in both clinical and experimental studies (Schurr, Payne, Miller e Tseng, 2001; Cornford, Shamsa *et al.*, 2002; Chow, Rooney *et al.*, 2005; Busnello, Leke *et al.*, 2006; Walling, Rigoulot *et al.*, 2007). In agreement with previous proposals, lactate was neuroprotective in models of neurological disorders (Thurston, Hauhart *et al.*, 1983; Cassady, Phillis *et al.*, 2001; Mendelowitsch, Ritz *et al.*, 2001; Schurr, Payne, Miller e Tseng, 2001). In addition, CSF glucose and lactate levels presented a similar time profile at 10 min, albeit the elevation in CSF glucose (Fig. 1A) was more time persistent (Fig 1B). Also, the convulsive seizure impaired glucose transporter functionality only in a short period (at 10 min) (Fig. 3A). Moreover, the production of energy, which was not being compensated by increased consumption, could result in sustained high CSF glucose and lactate concentrations. Similar increases of glucose and lactate were observed in striatum during the recovery period following electroshock-induced seizure (Darbin, Risso *et al.*, 2005). Although the exact role of brain glycogen has not been clarified completely, its relationship to neuronal activity is clear, because glycogen levels in the brain respond rapidly to even the weakest of sensory stimuli, and inhibition of brain glycogen metabolism during periods of intense neuronal stimulation have dramatic effects on neural activity (Brown, Sickmann *et al.*, 2005; Walling, Rigoulot *et al.*, 2007).

The PTZ model triggers an imbalance in the glutamatergic and GABAergic neurotransmission. The maintenance of glutamatergic and GABAergic activity requires a continuous supply of energy since the exocytotic processes as well as high affinity glutamate and GABA uptake and subsequent metabolism of glutamate to glutamine are energy demanding processes (Schousboe, Bak *et al.*, 2007). Independent on the temporal CSF differences between lactate and glucose levels founded by our work, the importance of lactate for synaptic recovery after hypoxic conditions (as seizures) (Schurr, Payne *et al.*, 1997c; 1997a; 1997b) and the mandatory role of glucose, glycogen and lactate in the maintenance of neurotransmitter homeostasis have been well demonstrated (Schousboe, Bak *et al.*, 2007).

A number of molecules and proteins have been suggested as markers of neuronal and astrocytic injury/activity. These would represent a major step forward in the diagnostic and monitoring of patients. Thus, brain glucose concentration reflects the balance of the supply from the blood and neuronal utilization (Fellows, Boutelle *et al.*, 1992). A number of studies showed that lactate might be a good marker for ongoing pathophysiological processes in the brain (Staub, Graf *et al.*, 2000; Matsubara, Senda *et al.*, 2001; Kuo, Lin *et al.*, 2003; Sarrafzadeh, Haux *et al.*, 2003; Chan, Ng *et al.*, 2005; Frykholm, Hillered *et al.*, 2005). Furthermore, the increased CSF lactate reported in this study appears to be a marker of astrocytic response (glial activation) reflecting the increment of astrocyte participation during seizures. The use of sensitive, but unspecific parameters such as lactate and glucose could be used as neurochemical alarms, to rationalize analysis work. Only when alarm parameters become abnormal would other more specific substances be measured to assess the specific causes of alterations (Cabeca, Gomes *et al.*, 2001).

As pointed by Jakob Korf (Korf, 2006), CSF lactate is influenced by the rates influx and efflux across the blood brain barrier, lactate influx and efflux across the membranes of brain cells, lactate formation from glucose and glycogen, and also the size of extracellular space in brain and blood lactate concentration. The same factors also determine glucose and oxygen levels, with the obvious differences in transport capacity and diffusion capability. We assumed that the increased CSF lactate level reported here was solely related to brain acute convulsive crisis, albeit some aspects raised by Jacob Korf (2006) (Korf, 2006) needs to be take into account when interpreting the results.

In summary, we shown that acute seizures induced by PTZ elicit a significant temporal increase in CSF glucose and lactate levels and a brief decrease in [^{14}C]-2-deoxy-D-glucose uptake and glycogen content. Nevertheless, more studies allow us to determine the putative role of CSF lactate and glucose levels as brain markers of injury/activity in acute convulsive crisis.

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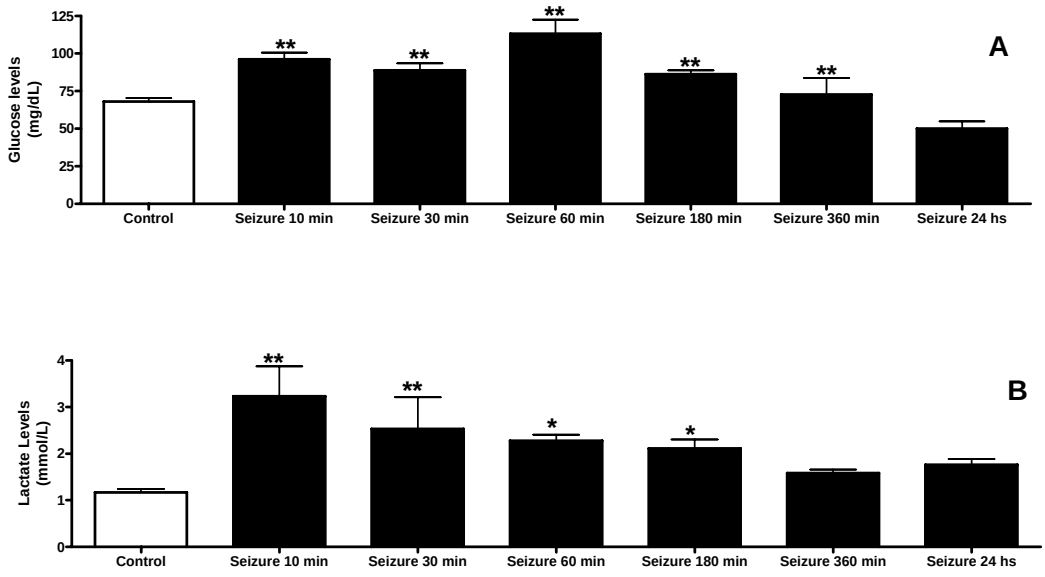
LEGENDS AND FIGURES

Fig. 1: CSF levels of glucose (A) and lactate (B) in control (white bars) and PTZ seizure animals (black bars) (10, 30, 60, 180, 360 min, and 24 h). The data represent means ± S.D. of at least 6 animals. **P*≤0.05 and ***P*≤0.01 when compared with controls according to ANOVA.

Fig. 2: Uptake of [^{14}C]-2-deoxy-D-glucose by hippocampal slices (A) and changes in hippocampal glycogen content (B) in control and after PTZ-seizure (black bars) (10 and 30 minutes). The data represent means ± S.D. of at least 6 animals. **P*≤0.05 and when compared with controls according to ANOVA.

Fig. 3: Lactate/glucose ratio in PTZ seizure animals (10, 30, 60, 180, 360 min, and 24 h). The data represent means ± S.D. of at least 6 animals. The line represent lactate glucose ratio in controls.

Figure 1:


A
Glucose levels (mg/dL)
125
100
75
50
25
0
**
**
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Control
Seizure 10 min
Seizure 30 min
Seizure 60 min
Seizure 180 min
Seizure 360 min
Seizure 24 hs
B
Lactate Levels (mmol/L)
4
3
2
1
0
**
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Control
Seizure 10 min
Seizure 30 min
Seizure 60 min
Seizure 180 min
Seizure 360 min
Seizure 24 hs

Figure 2:

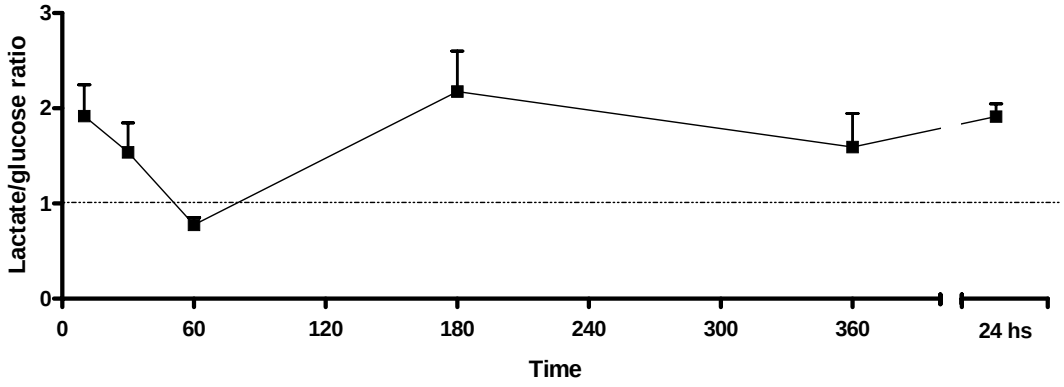
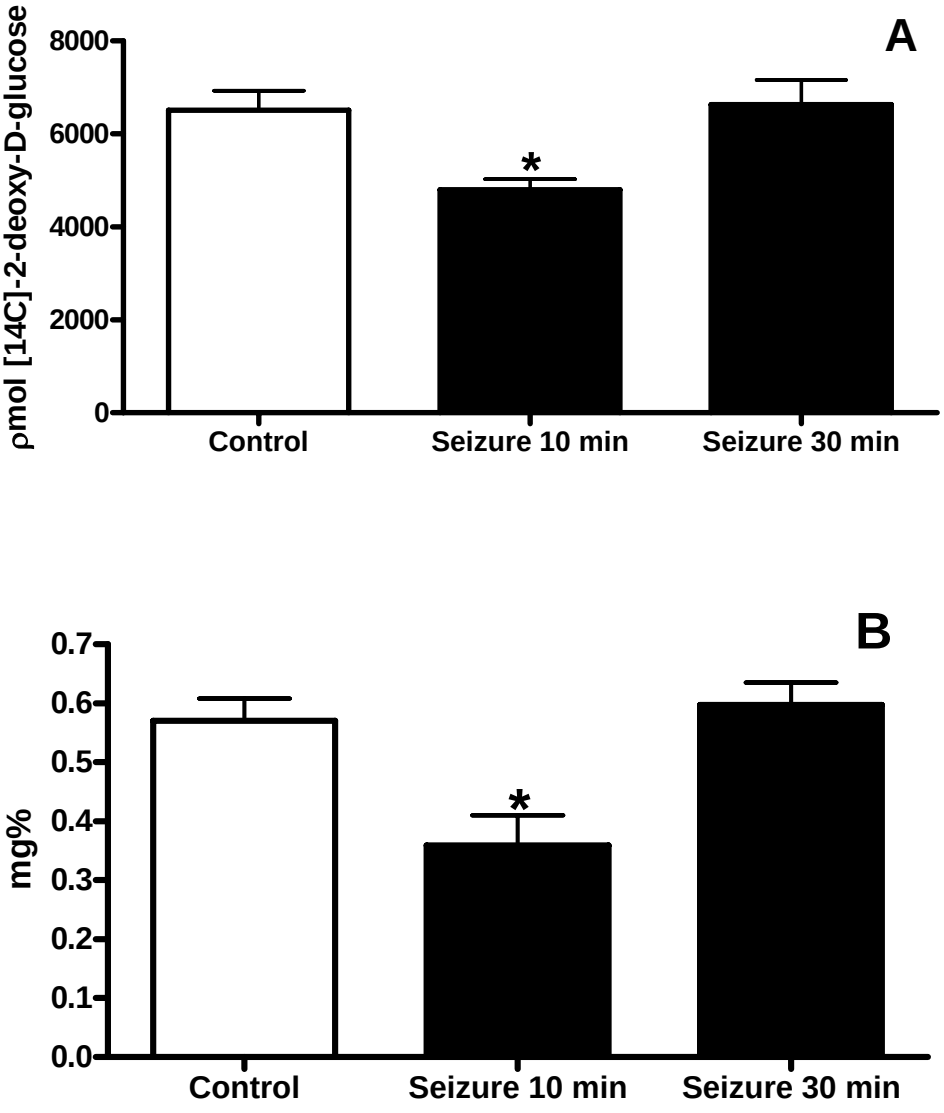


Figure 3:


A
ρmol [14C]-2-deoxy-D-glucose
8000
6000
4000
2000
0
*
Control
Seizure 10 min
Seizure 30 min
B
mg%
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
Seizure 10 min
Seizure 30 min

CAPÍTULO IV

THE HYDROLYSIS OF STRIATAL ADENINE- AND GUANINE-BASED PURINES IN A 6-HYDROXYDOPAMINE RAT MODEL OF PARKINSON´S DISEASE

Jean Pierre Oses, Cristiane Batassini, Daniela Pochmann, Ana Elisa Böhmer, Fernanda Cenci Vuaden, Roberta Bristot Silvestrin, Carla Denise Bonan, Maurício Reis Bogo, Diogo Onofre Souza, Luis Valmor Cruz Portela, João José de Freitas Sarkis and Tadeu Mello e Souza.

Artigo submetido ao periódico *Neuroscience Letters.*

2008

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THE HYDROLYSIS OF STRIATAL ADENINE- AND GUANINE-BASED PURINES IN A 6-HYDROXYDOPAMINE RAT MODEL OF PARKINSON´S DISEASE

Jean Pierre Oses[1,*], Cristiane Batassini[1], Daniela Pochmann[1], Ana Elisa Böhmer[1], Fernanda Cenci Vuaden[1], Roberta Bristot Silvestrin[1], Carla Denise Bonan[2], Maurício Reis Bogo[3], Diogo Onofre Souza[1], Luis Valmor Cruz Portela[1], João José de Freitas Sarkis[1] and Tadeu Mello e Souza[1].

[1]*Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brasil.* [2]*Laboratório de Neuroquímica e Psicofarmacologia e* [3]*Centro de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Caixa Postal 1429,90619-900 Porto Alegre, RS, Brasil.*

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*Corresponding Author

Jean Pierre Oses

Rua Ramiro Barcelos, 2600 – anexo

90035-003, Porto Alegre, Rio Grande do Sul, Brasil

jean.pierre.oses@gmail.com

Phone/Phax number: +55 51 33085556 / +55 51 33085540

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Abstract

Parkinson's disease (PD) is characterized by a progressive neurodegeneration in the *substantia nigra* with a subsequent reduction in the striatal dopamine content. Extracellular adenosine and ATP modulate the dopaminergic neurotransmission in the basal ganglia whereas guanosine has a protective role in the brain. The inactivation of ATP signaling and the production of adenosine and guanosine may be mediated by a variety of ectonucleotidases. Here we evaluated the extracellular nucleotide hydrolysis promoted by ectonucleotidases from striatal slices 4 weeks after a unilateral infusion with 6-OHDA into the medial forebrain bundle. 6-OHDA infusion increased ADP, AMP, and GTP hydrolysis by 15%, 25%, and 41%. In contrast, GDP hydrolysis was decreased by 60%. There was no change in the transcript mRNA levels for the enzymes tested. Therefore, it is likely that these enzymatic changes contribute to alleviate the decrease of the level of extracellular striatal adenosine in the model and to decrease that of guanosine. Since extracellular adenosine and dopamine have opposite physiological roles in striatopallidal synapses and extracellular guanosine might prevent neurotoxicity in the striatum, these changes might favor the progression of the neurodegenerative process due to the 6-OHDA infusion.

Introduction

Parkinson's disease (PD) is characterized by a progressive neurodegeneration in the *substantia nigra pars compacta* (SNpc) with a subsequent reduction in the striatal dopamine content. Its cause is not well understood and current therapies do not stop the neurodegenerative process and have several side effects (Deumens, Blokland *et al.*, 2002). The 6-hydroxydopamine (6-OHDA) administration into the rat's substantia nigra, medial forebrain bundle (MFB), or striatum leads to a depletion of dopamine in the basal ganglia (Hefti, Melamed *et al.*, 1980; Yuan, Sarre *et al.*, 2005), which confers to this animal model of PD a high construct and predictive value (Deumens, Blokland *et al.*, 2002).

Endogenous adenosine and ATP modulate the dopaminergic neurotransmission in the basal ganglia (Yang, Liu *et al.*, 2005; Wu, Hu *et al.*, 2006). P2 receptor agonists and adenosine A_{2A} receptor antagonists generally reduce the neurotoxic effects on dopaminergic neurons and enhance the motor activity in animal models of PD. Therefore, ATP and adenosine may have protective and deleterious effects, respectively, on dopaminergic neurons (Sorimachi, Yamagami *et al.*, 2002; Alcayaga, Retamal *et al.*, 2003; Newman, 2003), and the regulation of their extracellular levels might be relevant to the evolution of the neurodegenerative process in PD.

In rodents, guanosine administered intracerebroventricularly, intraperitonially, or orally protects against seizures induced by glutamatergic agents or against ischemia (Lara, Schmidt *et al.*, 2001; Frizzo, Lara *et al.*, 2002; De Oliveira, Horn *et al.*, 2004; Soares, Schmidt *et al.*, 2004; Schmidt, Lara *et al.*, 2007). Additionally, GDP hydrolysis promoted by ectonucleotidases presented in the rat cerebrospinal fluid is much higher than that of ADP, but only at very high concentrations of these nucleotides, a condition that occurs at neural insults, such as in seizures and in ischemia (Cruz Portela, Oses *et al.*, 2002). PD

might be another condition in which this is relevant. Therefore, studying the hydrolysis of guanine-based purines might be important in the understanding of the evolution of the neurodegenerative process seen in PD.

NTPDases and a 5'-nucleotidase constitute an enzymatic cascade able to promote the hydrolysis of triphosphate and diphosphate nucleotides to the respective nucleosides, such as adenosine and guanosine (Battastini, Da Rocha *et al.*, 1991; Cruz Portela, Oses *et al.*, 2002). Eight different enzymes are described as members of the NTPDase family. NTPDases 1, 2, and 3 are ecto-enzymes (E-NTPDases), since their catalytic site faces the extracellular space. In addition, NTPDases 5 and 6 may present secreted forms (Robson, Sévigny *et al.*, 2006; Zimmermann, 2006). Ecto- 5'-nucleotidase is a GPI-anchored enzyme that was also described in the rat central nervous system (Sadasivudu, Rao *et al.*, 1980). Therefore, the regulation of the extracellular level of adenine- and guanine-based purines is mediated by ectonucleotidases and, since the level of these molecules might be relevant to the neurodegenerative process in PD, it is important to quantify the activity of these enzymes in animal models of PD.

The aim of this study is to evaluate, 4 weeks after lesioning the SNpc with a unilateral infusion of 6-OHDA into the rat MFB, (i) the level of adenine- and guanine-based purine hydrolysis promoted by the ectonucleotidases from striatal slices ipsilateral to the lesion from these rats, and (ii) the expression level of these enzymes (5'-nucleotidase and ecto-NTPDases 1, 2, 3, 5, and 6).

Materials and methods

Chemicals

Nucleotides, Malachite Green Base, Coomassie Brilliant Blue G, Trizma base, and 6-OHDA were purchased from Sigma (St. Louis, MO, USA). The other reagents were of analytical grade.

Subjects

Adult male Wistar rats (290 – 350 g,, 3-4 months old) were obtained from the Central Animal House of the Department of Biochemistry, Federal University of Rio Grande do Sul, and kept on a 12-h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in a temperature-controlled (22°C) colony room. The animals were housed 5 by cage and had access to water and standard lab chow *ad libitum*. They were handled in accordance to the governmental guidelines and Brazilian Experimental Biology Societies Federation's recommendations.

6-OHDA treatment

The animals were anaesthetised with 40 mg/kg of sodium thiopental and placed in a stereotaxic apparatus. After that, they received two unilateral infusions of either vehicle (0.2% ascorbic acid-saline, Sham) or 6-OHDA (11.0 μg) into the right MFB (1st infusion: 0.5 μL/min, 2.5 μl, AP: -4.4 mm, LL: -1.8 mm, DV: –8.8 mm from the bregma; 2nd infusion: 3.0 μl, AP: –4.0 mm, LL: –1.6 mm, DV: –9.0 mm). For that, we used an injection needle attached to a 10-μL microsyringe (Hamilton, 701 N) and an infusion pump (Insight, Brazil). The needle was left in the brain for 4 minutes after each infusion to allow for drug diffusion. Rats received post-operative care until awake and were returned to their home cages. A second control group was used in which the animals were not submitted to the surgical procedure (Control). Two weeks after the surgical procedure, the animals were

challenged with 3 mg/kg of amphetamine (Sigma; i.p.). Immediately after this, they were placed in an 80 cm-diameter open field and were observed for 35 minutes. The number of ipsilateral and contralateral rotations was registered.

Two weeks after the amphetamine challenge, the rats were sacrificed by decapitation and the biochemical analyses were carried out. Different cohorts of animals were used either for the hydrolysis assays, immunohistochemistry, or the RT-PCR. In the 6-OHDA group, only animals that presented rotational behavior were included in these biochemical analyses.

Striatal slices

The brains were rapidly removed to a bicarbonate-buffered salt solution with the following composition: 115 mM NaCl, 3.0 mM KCl, 1.2 mM $MgSO_4$, 25mM $NaHCO_3$, 10 mM glucose, 2.0mM $CaCl_2$, pH 7.4, and gassed with 95% O_2 and 5% CO_2 mixture (incubation medium). Then, they were longitudinally cut, the striatum ipsilateral to the lesion dissected and slices transversely cut to a 400μm-thick on a McIlwain tissue chopper.

Assay for nucleotides hydrolysis

Two slices per tube from the striatum ipsilateral to the infusion site (approx. 0.16 mg protein) were preincubated for 10 min at 37 °C with 500 μL of the incubation medium (described above) and gassed directly with 95% O_2 and 5% CO_2. Adding ATP, ADP, AMP, GTP, GDP, or GMP started the reaction to a final concentration of 2.0 mM. The reaction was stopped by the addition of 100 μl of 10% trichloroacetic acid. The sum of nonenzymatic Pi released from nucleotides into the assay medium without slices and of Pi released from slices without nucleotide was subtracted from total Pi released during

incubation. All assays were performed in duplicate or triplicate. Pi was measured according to the literaturare (Chang e Lowenstein, 2003) and the enzymatic activity was expressed as nmol of Pi/min/mg.

Semi-quantitative RT-PCR

In accordance with the manufacturer's instructions, total RNA from the striatum ipsilateral to the infusion site was isolated with TRIzol^{TM} Reagent (Invitrogen) and the cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) from 3 μg of total RNA in a total volume of 20 μl with an oligo (dT) primer. cDNA reactions were performed for 50 min at 42°C and stopped by boiling for 5 min. Two microliters of cDNA were used as a template for PCR with primers specific for E-NTPDases1, 2, 3, 5, 6 and 5'-nucleotidase. As a control for cDNA synthesis, β-*actin*-PCR was performed. Two microliters of the cDNA were used for PCR in a total volume of 25 μl using a concentration of 0.4 μM of each primer indicated below, 200 μM of dNTPs and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer.

The PCR cycling conditions were as follows: initial 1-min denaturation step at 94 °C, 1-min at 94 °C, 1-min annealing step (NTPDase 1 - 3, 5, 6 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 final 10 min extention at 72 °C. The amplification products were: NTPDase1, 543bp; NTPDase2, 331bp; NTPDase3, 267 bp; NTPDase5, 1117bp; NTPDase6, 1264bp; 5'-nucleotidase, 405bp; β-actin, 210 bp. Seven microliters of the PCR reaction was analyzed on a 1% agarose gel containing gel Red. The Low DNA Mass Ladder (Invitrogen) was used as molecular marker. The relative abundance of each mRNA versus β-*actin* was determined

by densitometry using the freeware ImageJ 1.37 for Windows. The following set of primers were used for NTPDase1: 5'-GAT CAT CAC TGG GCA GGA GGA AGG-3' and 5'-AAG ACA CCG TTG AAG GCA CAC TGG-3'; for NTPDase2: 5'-GCT GGG TGG GCC GGT GGA TAC G-3' and 5'-ATT GAA GGC CCG GGG ACG CTG AC-3'; for NTPDase3: 5'-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3' and 5'-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3'; for NTPDase5: 5'-TGG TGG TAA CCA AGA AGG GGA GAT GG-3' and 5'-GCA GGT GAA AGG TGG CTC CCA AGG-3'; for NTPDase6: 5'-GGC CTC TAC GAG CTG TGT GCC AGC AG-3' and 5'-TCA GTA CCT TGT CCC CGG GAA AAC C-3'; for 5'-nucleotidase (CD73): 5'-CCC GGG GGC CAC TAG CAC CTC A-3' and 5'-GCC TGG ACC ACG GGA ACC TT-3'; for β-actin: 5'-TAT GCC AAC ACA GTG CTG TCT GG-3' and 5'-TAC TCC TGC TTC CTG ATC CAC AT-3'.

Protein determination

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

Tyrosine hydroxilase immunohistochemistry

Three rats were selected for tyrosine hydroxilase (TH) immunohistochemistry. Animals were anesthetized with a mixture of ketamine and xilazine and transcardially perfused with a cold 0,9% NaCl solution (150 mL) followed by cold 4% paraformaldehyde (150 mL) before the removal of their brain. The brains were further post-fixed overnight in a 4%-paraformaldehyde phosphate buffer saline (PBS) solution (pH 7.4) and then cryoprotected in a 30%-sucrose PBS solution until they were completely submerged. Coronal sections (45 μm) were obtained using a - 20°C cryostat (Leica Microsystems

GmbH). Midbrain sections were washed five times for 5 min each in PBS at room temperature, incubated with a rabbit anti–TH primary antibody (AB 152 1:750, Chemicon) at room temperature for 18h, washed five times for 5 min each in PBS and incubated with the secondary antibody (AlexaFluor 568 A11036, Molecular Probes, USA) at room temperature for 60 min. After another wash, sections received an antifading solution (Fluorsave, Calbiochem, USA) and coverslipped. Antibodies were diluted in PBS. Sections were selected according to their coronal section and observed under a fluorescence microscope. Then, their images were quantified using Scion Image for Windows program.

Statistical analysis

The data are shown as mean ± S.D. and the statistical analysis was performed using the one-way ANOVA test followed by the Dunnett post-hoc test. $P < 0.05$ indicated statistical difference.

Results

The effects of the unilateral infusion of 6-OHDA into the MFB on ATP, ADP, and AMP hydrolysis by ectonucleotidases from striatal slices of rats are shown in Fig.1. There was no difference among groups in ATP hydrolysis [ANOVA, $F(2,12) = 0.079$, $P = 0.925$]. However, there was a difference in ADP hydrolysis [ANOVA, $F(2,13) = 4.991$, $P = 0.025$], since this was increased in the animals lesioned with 6-OHDA (21.42 ± 2.34 nmol P_i/min/mg) relative to control and sham-operated animals (18.55 ± 1.21 and 17.68 ± 2.56 nmol Pi/min/mg, respectively; Dunnet post-hoc test, P = 0.024 and 0.049, respectively). In addition, there was a difference in AMP hydrolysis [ANOVA, $F(2,10) = 1.400$, $P = 0.291$], since this was increased in the animals lesioned with 6-OHDA (5.13 ± 1.37 nmol

Pi/min/mg) relative to control and sham-operated animals (4.10 ± 1.38 4.01 ± 0.58 nmol Pi/min/mg, respectively; Dunnet post-hoc test, P = 0.050 and 0.045, respectively).

The effects of the unilateral infusion of 6-OHDA into the MFB on GTP, GDP, and GMP hydrolysis by ectonucleotidases from striatal slices of rats are shown in Fig. 2. The animals submitted to 6-OHDA treatment presented a 41%-increase in GTP hydrolysis (16.60 ± 5.57 nmol Pi/min/mg) relative to control and sham-operated animals [11.72 ± 4.00 and 9.11 ± 3.63 nmol Pi/min/mg, respectively; ANOVA, $F(2,16) = 3.957$, $P = 0.040$, Dunnet post-hoc test, P = 0.036 and 0.048, respectively]. In contrast, GDP hydrolysis was decreased by 60% in the 6-OHDA group (0.83 ± 0.19 nmol Pi/min/mg) relative to control and sham-operated animals [2.13 ± 0.47 and 2.12 ± 0.81 nmol Pi/min/mg, respectively; ANOVA, $F(2,12) = 8.93$, $P = 0.004$; Dunnet post-hoc test, P = 0.006]. The infusion of 6-OHDA did not change GMP hydrolysis [ANOVA, $F(2,12) = 0.185$, $P = 0.833$].

There was no difference among groups in the transcript mRNA striatal levels of the enzymes 5′-nucleotidase and NTPDases 1, 2, 3, and 5 [ANOVA, $F(2,8) < 1.916$, $P > 0.22$), and of NTPDase 6, where there was a trend towards an effect [ANOVA, $F(2,8) = 3.517$, $P = 0.098$] (Fig. 3).

TH immunohistochemistry revealed a decrease between 98,3 and 99,1% in the levels of mesencephalic TH in the three rats analyzed, which indicates an extensive lesion of the nigrostriatal pathway (Fig. 4).

Discussion

Our results show that the infusion of 6-OHDA into the MFB in rats increases ADP, AMP, and GTP hydrolysis, whereas decreases GDP hydrolysis in biochemical assays using

slices from the striatum ipsilateral to the infusion procedure. In addition, no change was found in the transcription of the enzymes 5′-nucleotidase and NTPDases 1, 2, 3, 5, and 6.

Adenosine A_{2A} and D_2 receptors are coexpressed in GABAergic striatopallidal spiny neurons (Latini, Pazzagli *et al.*, 1996; Svenningsson, Le Moine *et al.*, 1999). In these cells adenosine increases and dopamine decreases the production of AMPc (Svenningsson, Le Moine *et al.*, 1999; Yabuuchi, Kuroiwa *et al.*, 2006). Therefore, these neurotransmitters have antagonistic effects and changes in the balance in their levels may cause the system to malfunction. This may explain the fact that adenosine A_{2A} receptor antagonists enhance the motor activity in animal models of PD and are candidates for PD therapy (Svenningsson, Le Moine *et al.*, 1999; Ferre, Popoli *et al.*, 2001). 6-OHDA infused into the MFB decreases the levels of adenosine by 35% when measured 15 days after the infusion procedure. However, this decrease was far less than that of dopamine, which might indicate that adenosine levels should be even lower to counterbalance the effect of dopamine depletion (Pinna, Corsi *et al.*, 2002). In the present work, we found that ADP and AMP hydrolysis is increased after a 6-OHDA infusion into the MFB. In an *in vitro* study, it was shown that high ADP concentrations may delay striatal adenosine production (James e Richardson, 1993). Therefore, the increase of both ADP and AMP hydrolysis mediated by ectonucleotidases that we observed might accelerate the production of adenosine and favor the imbalance between adenosine and dopamine levels, contributing to the evolution of the neurodegenerative process promoted by the 6-OHDA infusion.

Here we show that, in the 6-OHDA model, GTP hydrolysis increases, but much less than GDP hydrolysis decreases. In our knowledge, this is the first time that it is shown some alteration in this enzymatic cascade in the 6-OHDA model and, as a consequence, several questions arise. First of all, we may speculate that both effects combined results in

the decrease of guanosine production mediated by ectonucleotidases. Second, if we extrapolate the results from other models, such as seizures induced by glutamatergic agents (Lara, Schmidt *et al.*, 2001; Soares, Schmidt *et al.*, 2004) and ischemia (Frizzo, Lara *et al.*, 2002; Oliveira, Molz *et al.*, 2002; Thomazi, Godinho *et al.*, 2004; Oleskovicz, Martins *et al.*, 2008), where guanosine has a protective role, this putative decrease in the net production of guanosine might contribute to the evolution of the neurodegenerative process in the 6-OHDA model. However, we can not rule out the possibility that alterations in the level of other guanine-based purines may be relevant to the process. Third, we do not know the mechanisms underlying the differential change in GTP and GDP hydrolysis. Forth, the putative net effects of the 6-OHDA infusion on the hydrolysis of the adenine- and guanine-based purines are opposite. One possible explanation for these putative opposite net effects is the differential allosteric effect of each purine on the different ectonucleotidase isoenzymes. Further studies may clarify these issues.

It is worthy pointing out that the changes in hydrolysis found in the present work are due to changes in the activity rather than the expression of the ectonucleotidases, since no alteration of their mRNA levels was found.

In summary, our results point to an increase and decrease, respectively, of adenine- and guanine-based purine hydrolysis in the striatum of rats in a 6-OHDA PD`s model. Therefore, it is likely that these changes contribute to a lesser decrease in the striatal adenosine level and to diminish the extracellular guanosine level. One may speculate that these changes favor the progression of the neurodegenerative process due to the 6-OHDA infusion, since extracellular adenosine and dopamine have opposite physiological roles in striatopallidal synapses, and extracellular guanosine might prevent neurotoxicity in the striatum. Our results reinforce the hypothesis that changes in the adenosinergic system

contribute to the progression of neurodegeneration in PD and point to the possibility of using the ectonucleotidases as targets for therapy. In addition, this is the first time, in our knowledge, that changes in the "guanosinergic system" might also be considered as mediators of the progression of PD.

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Legends to Figures:

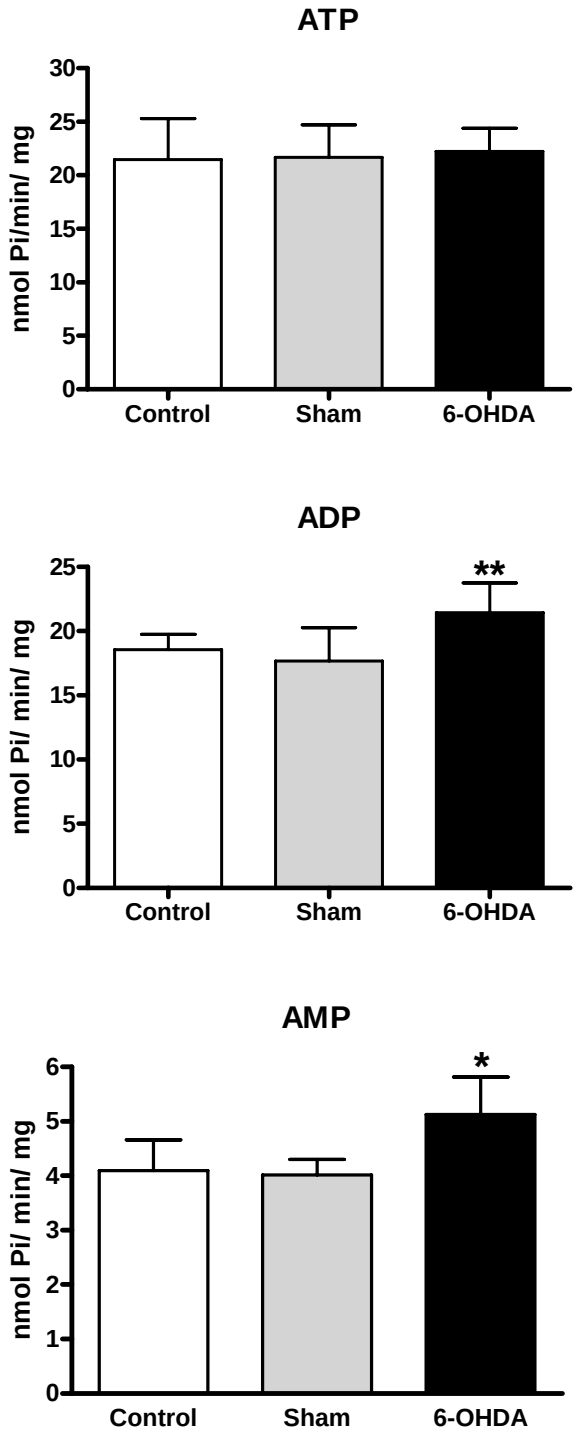
Figure 1: ATP, ADP, and AMP hydrolysis from striatum of saline (Ctrl), Sham and 6-OHDA-rats. Bars represent mean ± S.D. of at least three animals. Asterisk indicates difference from both control groups ($P \leq 0.05$, one way ANOVA followed the post hoc Dunnett test).

Figure 2: GTP, GDP, and GMP hydrolysis from striatum of saline (Ctrl), Sham and 6-OHDA-rats. Bars represent mean ± S.D. of at least three animals. Asterisk indicates difference from both control groups ($P \leq 0.05$, one way ANOVA followed the post hoc Dunnett test).

Figure 3: Representative semi-quantitative RT-PCR mRNA for NTPDase1, 2, 3, 5, 6 and 5'-nucleotidase from striatum of saline (Ctrl), Sham and 6-OHDA rats. The expression was evaluated for integrated density since the NTPDases to β-actin mRNA ratio was the same for all samples. Three independent experiments with entirely consistent results were performed.

Figure 4: Immunohistochemistry showing the decrease of the TH content in the substantia nigra ipsilateral to the MFB infused with 6-OHDA (right side).

Figure 1:


ATP
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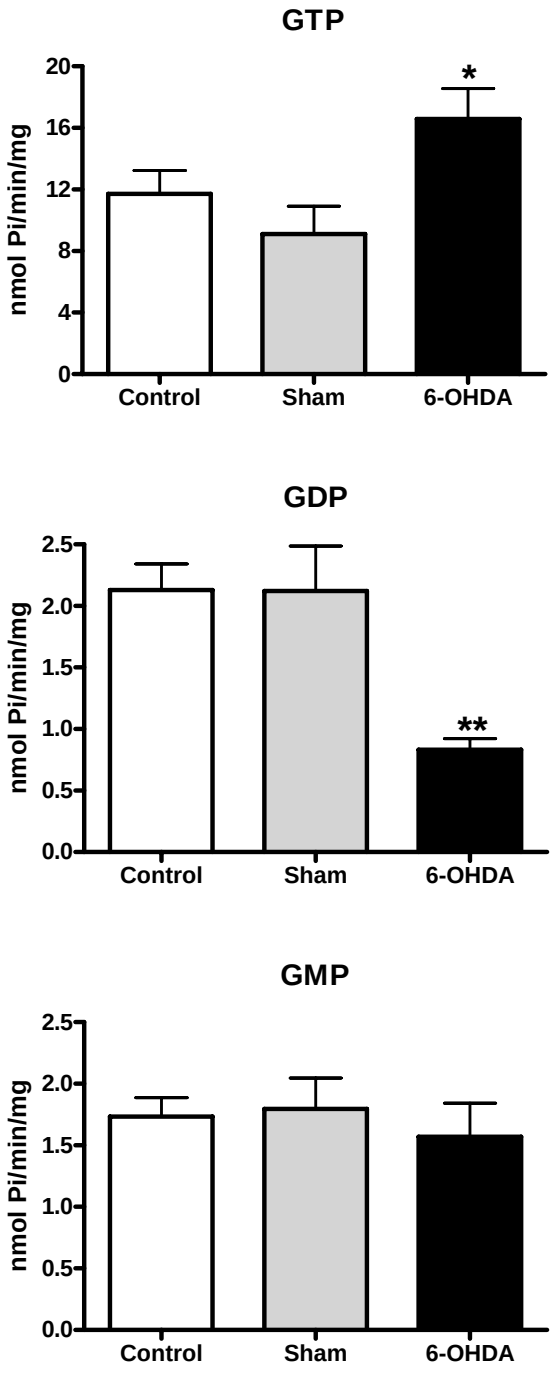
Control
Sham
6-OHDA

Figure 2: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20
16
12
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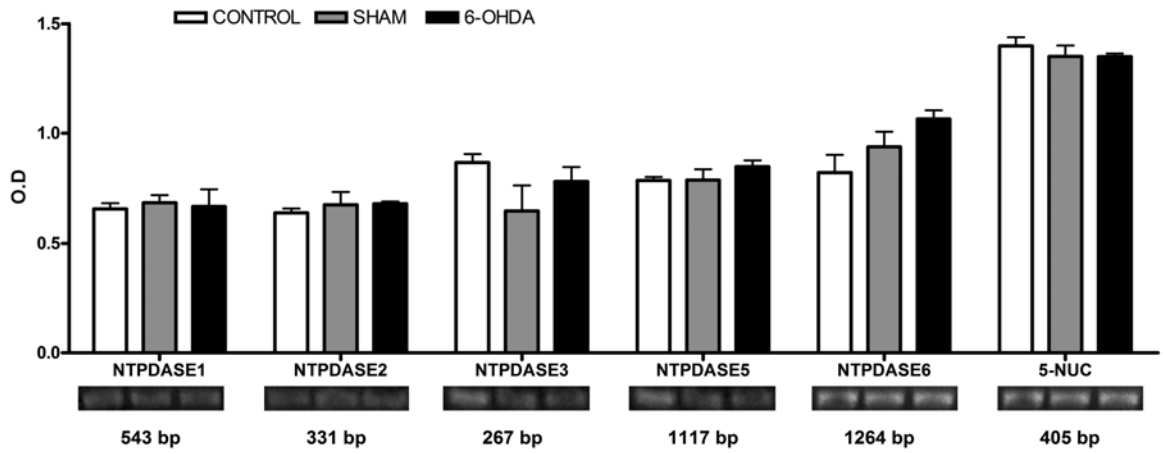
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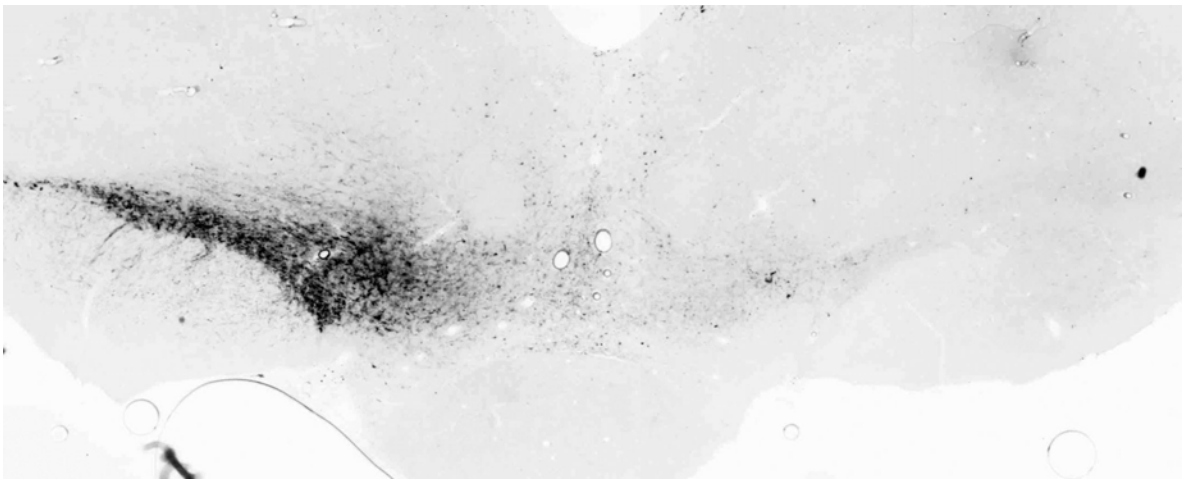

CAPÍTULO V

MODIFICATION OF PURINERGIC SIGNALING IN THE HIPPOCAMPUS OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

João Manuel Duarte, Jean Pierre Oses, Ricardo Jorge Rodrigues and Rodrigo Antunes Cunha

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MODIFICATION OF PURINERGIC SIGNALING IN THE HIPPOCAMPUS OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

J. M. N. DUARTE,* J. P. OSES, R. J. RODRIGUES AND R. A. CUNHA

Centre for Neurosciences of Coimbra, Faculty of Medicine, Institute of Biochemistry, University of Coimbra, 3004-504 Coimbra, Portugal

Abstract—Diabetic encephalopathy is a recognized complication of untreated diabetes resulting in a progressive cognitive impairment accompanied by modification of hippocampal function. The purinergic system is a promising novel target to control diabetic encephalopathy since it might simultaneously control hippocampal synaptic plasticity and glucose handling. We now tested whether streptozotocin-induced diabetes led to a modification of extracellular ATP homeostasis and density of membrane ATP (P2) receptors in the hippocampus, a brain structure involved in learning and memory. The extracellular levels of ATP, evaluated in the cerebrospinal fluid, were reduced by 60.4±17.0% in diabetic rats. Likewise, the evoked release of ATP as well as its extracellular catabolism was also decreased in hippocampal nerve terminals of diabetic rats by 52.8±10.9% and 38.7±6.5%, respectively. Western blot analysis showed that the density of several P2 receptors ($P2X_{3,5,7}$ and $P2Y_{2,6,11}$) was decreased in hippocampal nerve terminals. This indicates that the synaptic ATP signaling is globally depressed in diabetic rats, which may contribute for diabetes-associated decrease of synaptic plasticity. In contrast, the density of P2 receptors ($P2X_{1,2,5,6,7}$ and $P2Y_6$ but not $P2Y_2$) increased in whole hippocampal membranes, suggesting an adaptation of non-synaptic P2 receptors to sense decreased levels of extracellular ATP in diabetic rats, which might be aimed at preserving the non-synaptic purinergic signaling.

Key words: ATP, P2 receptors, ecto-nucleotidases, hippocampus, diabetes, streptozotocin.

Diabetes mellitus is a metabolic disease characterized by an impaired glucose homeostasis, which is caused by deficient production or action of insulin. Diabetic conditions often include central neuropathic modifications, namely decreased cognitive performance (e.g. Cox et al., 2005; Gispen and Biessels, 2000) accompanied by modifications of hippocampal morphology and plasticity (e.g. Convit et al., 2003; Trudeau et al., 2004). This diabetes-induced dysfunction of synaptic plasticity is likely to be due to a perturbed efficiency of the release of neurotransmitters, as gauged by the reduction of neurotransmitter release (Guyot et al., 2001; Yamato et al., 2004) and change in presynaptic proteins associated with vesicular release of neurotransmitters upon diabetes in cortical preparations (Duarte et al., 2006). Thus,one possible strategy to correct this diabetes-induced modification of synaptic efficiency might be to target presynaptic neuromodulation systems. One promising candidate is the ATP modulation system since it has the simultaneous potential to control neurotransmitter release (Rodrigues et al., 2005), synaptic plasticity (e.g. Pankratov et al., 2002), a purported neurophysiological trait of learning and memory (e.g. Lynch, 2004), and can also control glucose utilization (e.g. Solini et al., 2003) and insulin release (e.g. Léon et al., 2005).

ATP is released by most cells, namely from neurons in an exocytotic manner (North and Verkhratsky, 2006), and extracellular ATP regulates a variety of cellular processes through activation of ATP receptors (P2Rs), which include ionotropic ($P2X_{1-7}$) and metabotropic ($P2Y_{1-15}$) receptors (Abbracchio et al., 2006; Khakh and North, 2006). Extracellular ATP regulates key physiological functions such as neurotransmitter release (Rodrigues et al., 2005), synaptic plasticity phenomena (e.g. Almeida et al., 2003) and glucose homeostasis, namely through the modulation of insulin secretion (e.g. Léon et al., 2005), hepatic glucose metabolism and release (Buxton et al., 1986; Haussinger et al., 1987), and glucose transport in several cell types (Solini et al., 2003; Fischer et al., 1999; Kim et al., 2002). However, extracellular ATP is a double-edge sword signaling system since it is also a danger signal (di Virgilio, 2000), and P2R blockade was shown to afford neuroprotection against metabolic insults (Cavaliere et al., 2001a,b), ischemic conditions (e.g. Lammer et al., 2006) and glutamate toxicity (reviewed in Franke et al., 2006).

Previous studies have already indicated diabetes-induced changes of the efficiency of P2R in peripheral tissues. Thus, the ATP-driven modulation of glucose transport, proposed to rely on P2YR activation (Fischer et al., 1999; Kim et al., 2002), was found to be impaired in fibroblasts of type 2 diabetic individuals (Solini et al., 2003) and changes in pancreatic P2Rs were reported to occur in an experimental model of type 1 diabetes (Coutinho-Silva et al., 2003). Also, an enhanced $P2X_7R$ activity was associated with diabetes-induced vascular damage (Solini et al., 2004) and retinopathy (Sugiyama et al., 2004) and the $P2X_7R$ gene emerges as a candidate susceptibility gene for non-obese diabetes (Elliott and Higgins, 2004). This prompts the hypothesis that diabetes may also cause modifications of the purinergic system in the brain, which may lead to an impairment of the physiological actions operated

*Corresponding author. Tel: +351-239-820190; fax: +351-239-822776.
E-mail address: jmnduarte@portugalmail.pt (J. M. N. Duarte); URL: http://cnc.cj.uc.pt/lab_lef/.

Abbreviations: CSF, cerebrospinal fluid; HPLC, high performance liquid chromotography; LTP, long-term potentiation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; STZ, streptozotocin; TBS-T, Tris-buffered saline (Tris 20 mM, NaCl 140 mM, pH 7.6), containing 0.1% Tween 20.

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by ATP through the activation of P2Rs, such as long-term potentiation (LTP), that may underlie diabetes-induced cognitive impairment. The present study was designed to investigate if the purinergic signaling, namely extracellular ATP homeostasis and the density of different P2Rs, is altered in the hippocampus of streptozotocin (STZ) -treated rats, an animal model of type 1 diabetes, which displays learning deficits (e.g. Biessels et al., 1996).

EXPERIMENTAL PROCEDURES

Reagents

The antibodies against $P2X_1$ (generated against residues 382–399 of rat $P2X_1$ and used at a 1:500 dilution from a 0.6 mg/ml stock), $P2X_3$ (generated against residues 383–397 of rat $P2X_3$ and used at a 1:1000 dilution from a 0.2 mg/ml stock), $P2X_4$ (generated against residues 370–388 of rat $P2X_4$ and used at a 1:500 dilution from a 0.3 mg/ml stock), $P2X_7$ (generated against residues 576–595 of rat $P2X_7$ and used at a 1:5000 dilution from a 0.3 mg/ml stock), $P2Y_2$ (generated against residues 227–244 of rat $P2Y_2$ and used at a 1:500 dilution from a 0.6 mg/ml stock) and $P2Y_4$ receptors (generated against residues 337–350 of rat $P2Y_4$ and used at a 1:1000 dilution from a 0.3 mg/ml stock) were from Alomone Laboratories (Jerusalem, Israel); the antibodies against $P2X_2$ (generated against residues 356–471 of human $P2X_2$ and used at a 1:500 dilution from a 0.2 mg/ml stock), $P2X_5$ (generated against residues 356–455 of rat $P2X_5$ and used at a 1:200 dilution from a 0.2 mg/ml stock), $P2X_6$ (generated against residues 351–431 of human $P2X_6$ and used at a 1:200 dilution from a 0.2 mg/ml stock), $P2Y_1$ (generated against an internal sequence of human $P2Y_1$ and used at a 1:500 dilution from a 0.2 mg/ml stock) and $P2Y_6$ receptors (generated against an internal sequence of human $P2Y_6$ and used at a 1:500 dilution from a 0.2 mg/ml stock), as well as the anti-goat IgG secondary alkaline phosphatase-tagged antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-$P2Y_{11}$ receptor antibody (generated against the third cytoplasmic loop of human $P2Y_{11}$ and used at a 1:500 dilution from a 0.3 mg/ml stock) was from Zymed (Queluz, Portugal); anti-α-tubulin antibody was from Sigma (Sintra, Portugal); alkaline phosphatase–tagged anti-mouse IgG and anti-rabbit IgG secondary antibodies for Western blot were purchased from Amersham Biosciences (Carnaxide, Portugal). KH_2PO_4 was purchased from VWR International (Carnaxide, Portugal). STZ and all the other common use reagents were from Sigma.

Animals

Male Wistar rats (8-weeks old, 150–170 g, obtained from Harlan Ibérica, Barcelona, Spain) were used throughout study and were handled according with the EU guidelines for the use of experimental animals (86/609/EEC), the rats being anesthetized under halothane atmosphere before being killed by decapitation. The experiments were performed with special care for minimizing the number of animals used and their suffering.

We used a well-studied and validated model of type 1 diabetes mellitus, which is based on the administration of STZ (e.g. Rees and Alcolado, 2005). STZ (65 mg/kg, prepared in sodium citrate buffer 10 mM, pH 4.5) was administered by i.p. injection and induced sustained levels of blood glucose above 250 mg/dl after 3 days onwards (Duarte et al., 2006, 2007), as measured by the glucose oxidase method using a glucometer (Elite, Bayer, Portugal). The rats were maintained for 30 days with food and water *ad libitum* and all the analyses were carried out 30 days after STZ treatment. The control rats were age-matched untreated rats maintained in the same conditions. Table 1 summarizes body weight and glycemia of both control and diabetic rats. Since STZ is not known to cross the blood–brain barrier and has to be directly injected into the brain to cause direct effects in the brain parenchyma (e.g. Lester-Coll et al., 2006), it is assumed that the modifications caused by STZ mainly result from its ability to induce a type I diabetic state (e.g. Rees and Alcolado, 2005).

Cerebrospinal fluid (CSF) sampling and ATP quantification

Rats were anesthetized with sodium thiopental (40 mg/kg, i.p.) and the CSF was drawn (40–60 μl per rat) by direct puncture of the cisterna magna with a tuberculin syringe (27 gauge$\times$13 mm length), and immediately stored at -80 °C until ATP quantification. These samples and standard solutions of ATP (10^{-9} to 10^{-12} M) were placed in wells of a white 96-well microplate to determine ATP levels using the luciferin-luciferase luminometric assay (Cunha et al., 1996). Briefly, 50 μl of luciferin–luciferase solution (FLAAM kit from Sigma, resuspended in 5 ml) was added to 25 μl of sample (diluted 1/5) and the luminescence produced was quantified in an LMax II^{384} luminometer (Molecular Devices, Union City, USA).

Preparation of purified nerve terminals and hippocampal membranes

Membranes from the whole hippocampus or from Percoll-purified hippocampal synaptosomes were prepared as previously described (Duarte et al., 2006). Briefly, the two hippocampi from one rat were homogenized at 4 °C in sucrose–Hepes buffer (composition 0.32 M sucrose, 1 mM EDTA, 10 mM Hepes, 1 mg/ml bovine serum albumin, pH 7.4). The resulting homogenate was centrifuged at 3000$\times g$ for 10 min at 4 °C, the supernatant collected and centrifuged at 14,000$\times g$ for 12 min at 4 °C. The pellet was resuspended in 1 ml of a 45% (v/v) Percoll solution made up in Krebs–Hepes solution (composition in mM: 140 NaCl, 5 KCl, 10 Hepes, 1 EDTA, 5 glucose, pH 7.4). After centrifugation at 21,000$\times g$ for 2 min at 4 °C, the top layer (synaptosomal fraction) was removed, washed and resuspended in Krebs–Hepes solution. For total membrane preparation, a portion of the supernatant of the first centrifugation was taken, resuspended in a solution of 50 mM Tris and 10 mM $MgCl_2$ (pH 7.4), centrifuged at 28,000$\times g$

Table 1. Body weight and glycemia of the rats used in the experiments before and after the induction of diabetes

	Weight (g)		Glycemia (mg/dl)	
	Control	STZ-treated	Control	STZ-treated
Before treatment	254.7±13.2	245.1±8.0	93.0±6.1	100.5±4.3
3 days after treatment	n.d.	n.d.	108.5±7.8	471.6±24.6*,**
1 month after treatment	345.7±14.8**	225.0±5.6*	105.2±6.4	491.3±16.6*,**

n=18 for each condition; n.d., not determined.

* $P<0.01$, different from control.

** $P<0.01$, different from before treatment.

for 20 min at 4 °C, and the resulting pellet resuspended in a Krebs–Hepes solution. An aliquot of each membrane preparation was saved for protein quantification using the bicinchoninic acid method (kit from Pierce Biotechnology, Rockford, USA).

ATP release from hippocampal nerve terminals

The measurement of ATP release from synaptosomes was adapted from Cunha et al. (1996). Synaptosomes were resuspended in calcium containing Krebs–Hepes solution (composition in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 2 $CaCl_2$, 26 Hepes, 10 glucose, pH 7.4). Aliquots of 240 μl of synaptosomes (0.21–0.36 mg of protein) were placed in wells of a white 96-well microplate to which 50 μl of luciferin–luciferase solution (FLAAM kit from Sigma, prepared in 5 ml of water) were added. The mixture was placed in the luminometer at 37 °C and the electrical signal generated by the photomultiplier recorded. After obtaining a stable baseline, 10 μl of Krebs–Hepes solution with concentrated KCl (to attain a final concentration of 20 mM) was automatically injected and the plate shaken for 2 s. The measurement of the photomultiplier signal restarted after 4 s, and the variation in signal recorded was used to estimate the evoked release of ATP by interpolation in a calibration curve of ATP standards. We confirmed that mechanical stimulation of the nerve terminals also triggered an outflow of ATP, but this displayed a slower time course and had an amplitude considerably lower than the K^+-evoked release of ATP.

ATP catabolism in hippocampal nerve terminals

Synaptosomes (0.32–0.43 mg) were resuspended in 500 μl of Krebs–Hepes solution and incubated at 37 °C for at least 5 min of stabilization. At time zero, ATP (made up in Krebs–Hepes solution) was added to a final concentration of 10 μM and samples (75 μl) were collected every minute during the next 5 min of incubation. Each sample was spun down, and the supernatant immediately frozen in liquid N_2 and then at −80 °C until high performance liquid chromatography (HPLC) analysis (Cunha et al., 1998). Separation of adenine nucleotides was performed at room temperature using a reverse-phase column [LiChroCART 125×4 mm LiChrospher 100 RP-18 (5 μm) cartridge fitted into a ManuCART holder (Merck Darmstadt, Germany)], using a GOLD™ system (Beckman, UK) equipped with a UV detector set at 254 nm. The eluent was a 100 mM KH_2PO_4 solution with 1.2% methanol (pH 5.5) with a flow rate of 1.2 ml/min. The identification of the peaks was performed by comparison of relative retention times with standards and their quantification achieved by calculating the peak areas then converting to concentration values by calibration with known standards (0.1–10 μM). The activity of the ecto-enzymes responsible for the extracellular catabolism of ATP was defined as the rate of ATP degradation.

Western blot analysis

The determination of the density of P2 receptors was carried out by Western blot analysis, as previously described (Rodrigues et al., 2005). Briefly, each sample was diluted with five volumes of SDS-PAGE buffer containing 30% (v/v) glycerol, 0.6 M dithiothreitol, 10% (w/v) sodium dodecyl sulfate and 375 mM Tris–HCl pH 6.8, and boiled at 95 °C for 5 min. These diluted samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% separation gel with a 4% concentrating gel in the top) under reducing conditions, in two or three different protein concentrations, together with pre-stained molecular weight markers (Biorad, USA), and then electro-transferred to polyvinylidene difluoride membranes (0.45 μm, from Amersham Biosciences, UK). After blocking for 1 h at room temperature with 5% milk in Tris-buffered saline (Tris 20 mM, NaCl 140 mM, pH 7.6), containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight at 4 °C with the primary antibodies against P2 receptors. The selectivity of the tested antibodies against hippocampal P2 receptors has previously been validated (Rodrigues et al., 2005). After three 15 min washing periods with TBS-T containing 0.5% milk, the membranes were incubated with the alkaline phosphatase–conjugated anti-rabbit IgG or anti-goat IgG secondary antibody (dilution 1:10,000) in TBS-T containing 1% milk during 90 min at room temperature. After three 20 min-washes in TBS-T with 0.5% milk the membranes were incubated with enhanced chemi-fluorescent substrate (Amersham Biosciences) and then analyzed with a VersaDoc 3000 system (Biorad).

The membranes were then re-probed and tested for α-tubulin immunoreactivity to confirm that similar amounts of protein were applied to the gels (Duarte et al., 2006). Briefly, the membranes were incubated at room temperature for 30 min with 40% (v/v) methanol and 1 h with 0.1 M glycine buffer pH 2.3, and then blocked as previously described before incubation with an anti-α-tubulin antibody (dilution 1:10,000) for 2 h at room temperature. The membranes were then washed, incubated with an anti-mouse IgG alkaline phosphatase-conjugated secondary antibody and analyzed as described above.

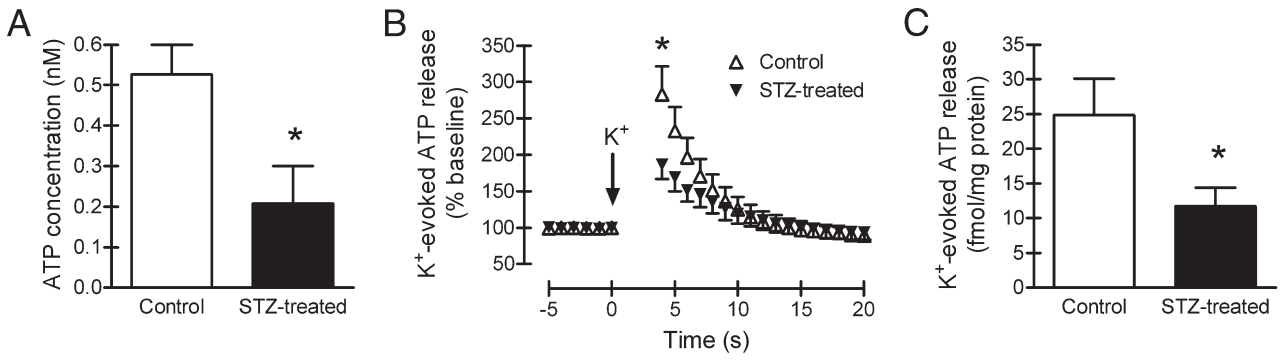

Fig. 1. Extracellular ATP levels are modified in the hippocampus of STZ-treated rats. (A) ATP concentration in the CSF, measured with a luminometric assay, was lower in diabetic (filled bars) than control (open bars) rats (n=7, * P<0.05). (B) The K^+-evoked release of ATP from hippocampal nerve terminals prepared from STZ-treated rats (filled symbols) was also lower when compared with controls (open symbols). In this experiment, KCl was added at time zero in a concentration of 20 mM, thus depolarizing the nerve terminals and triggering a vesicular release of ATP, which was quantified by luminometry. In (C) are presented the average data showing that the initial evoked release of ATP (measured 4 s after KCl addition) from hippocampal nerve terminals of diabetic (filled bars) was nearly half of that observed in hippocampal nerve terminals of control (open bars) rats (n=4, * P<0.05).

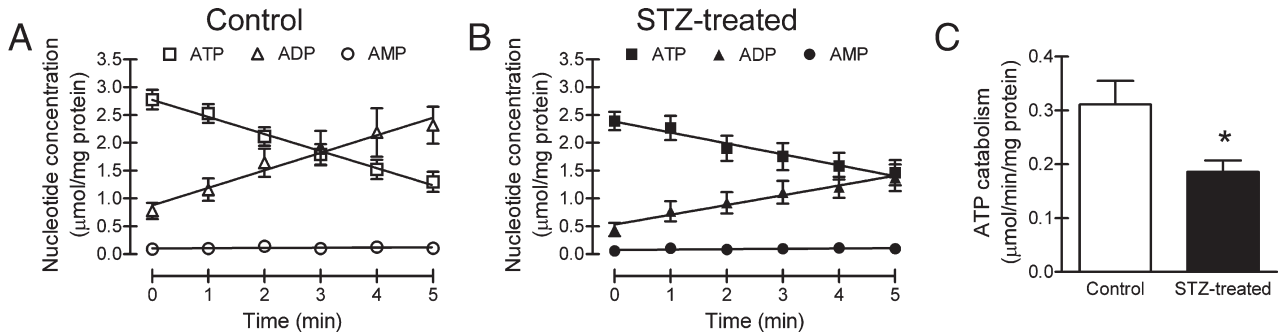

Fig. 2. Diabetes reduced extracellular ATP hydrolysis activity in rat hippocampal nerve terminals. ATP (10 μM) was added at zero time to rat hippocampal nerve terminals (87–145 μg protein) and samples were collected from the bath at 0, 1, 2, 3 and 5 min. Each collected sample was analyzed by HPLC to separate and quantify ATP and its metabolites, ADP and AMP. (A, B) The average time-course kinetics of ATP (square symbols) catabolism and consequent formation of ADP (triangle symbols) and AMP (circle symbols) hippocampal nerve terminals in nerve terminals from either control (A) or rats treated1 month before with STZ (B). The ordinates display the amount of each nucleotide in the bath at each time point, normalized by the amount of synaptosomal protein in the assay and each point is the mean±SEM of seven experiments. (C) The average rate of ATP catabolism (n=7, * P<0.05) found in control (open bars) and STZ-treated rats (filled bars). The initial rates were calculated by fitting the initial decrease of ATP concentration normalized by the amount of protein in the assay.

Statistical analysis

Results are presented as mean±S.E.M. values of n experiments, and significance was considered at P<0.05 using a Student's t-test.

RESULTS

Modification of extracellular ATP concentration and metabolism

We first evaluated if the diabetic rats presented abnormal extracellular ATP levels in the brain. As shown in Fig. 1A, 1 month after STZ-induction of diabetes, the concentration of ATP in the CSF was less than half of that in control rats. The synaptic levels of ATP were also decreased in diabetic rats, as gauged by the reduction of the K^+-induced evoked release of ATP from hippocampal nerve terminals (Fig. 1B, C).

We next investigated the rate of extracellular catabolism of ATP by following the consumption of ATP after its addition to a synaptosomal suspension (see Cunha, 2001). It was found that the rate of hydrolysis of extracellular ATP and the consequent formation of ADP was reduced in hippocampal nerve terminals derived from diabetic rats (Fig. 2A, B). This indicates that diabetes induces a reduction in the activity of membrane-bound ecto-enzymes involved in ATP catabolism (Fig. 2C). This global reduction of extracellular ATP homeostasis prompts the hypothesis that P2Rs in the brain of diabetic rats may face lower extracellular ATP levels than in control rats.

Modification of the density of P2 receptors in hippocampal membranes

Since ATP simultaneously fulfils a role as a synaptic modulator (North and Verkhratsky, 2006) and as a non-synaptic role as a neuron–glia messenger (Fields and Burnstock, 2006), we simultaneously evaluated if there was a modification of the density of P2Rs in synaptic membranes and in whole membranes of the hippocampus of diabetic rats. We use a Western blot analysis using antibodies that we were previously defined to be selective for hippocampal P2 receptors (Rodrigues et al., 2005). We always evaluated two or three different amounts of loaded protein (hippocampal nerve terminals or whole membranes) from the

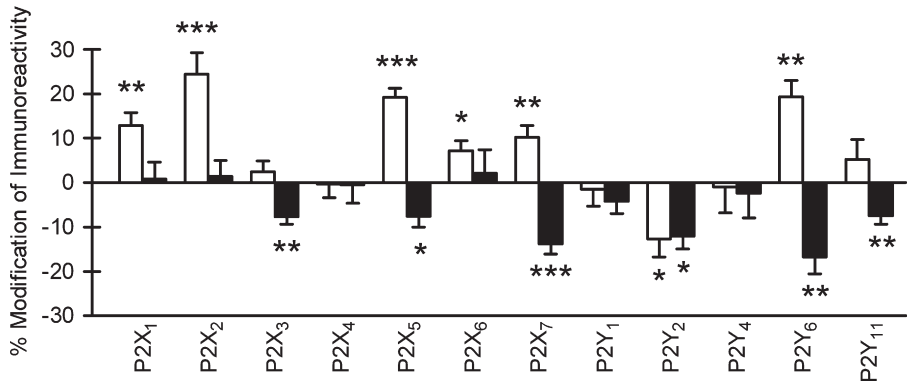

Fig. 3. STZ-induced diabetes causes opposite modification of the density of P2Rs in nerve terminals (filled bars) and in total membranes (open bars) prepared from the hippocampus. Thus, whereas P2Rs immunoreactivity tends to decrease in nerve terminal–enriched membranes, there is a global trend for the increase of P2Rs immunoreactivity in whole membranes of STZ-treated rats relative to controls, as evaluated by Western blot analysis (n=3–8, * P<0.05, ** P<0.01, *** P<0.001).

hippocampus of control and diabetic rats in each gel to simultaneously evaluate the sensitivity of the Western assay. This was attempted for all the P2XR subunits and most P2YRs and all results were then expressed as average percentage of modification (density found in diabetic relative to its respective control) and summarized in the Fig. 3.

In hippocampal nerve terminal membranes of diabetic rats 1 month after STZ-treatment, there was a global trend toward a decrease of the immunoreactivity of P2Rs. The

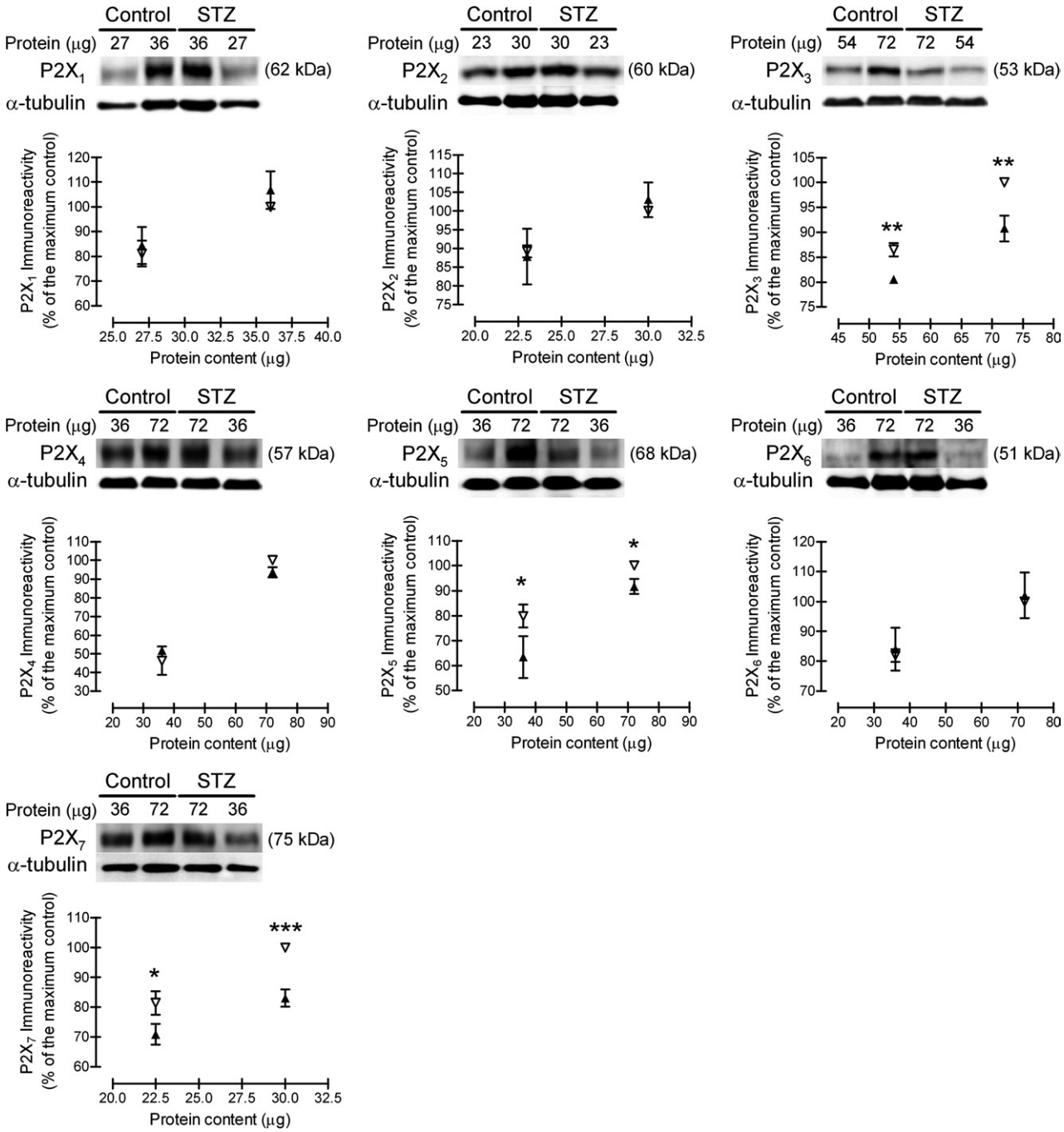

Fig. 4. Representative Western blots comparing the P2XR immunoreactivity in nerve terminal–enriched membranes from the hippocampus of control rats and from rats treated with STZ, applied in different quantities to the SDS-PAGE gel as indicated above each lane. A re-probing of the membranes with an anti-α-tubulin antibody, shown below each gel in each panel, confirmed that similar amounts of protein were added for each concentration of hippocampal membranes. The graphs below the gels show the average relative immunoreactivity of in synaptosomal membranes from control rats (open symbols) and from STZ-treated rats (filled symbols) according to the amount of protein applied to gel in different Western blot analysis using membranes from different groups of rats ($n=6$ for $P2X_{1,2,3,5,7}Rs$ and $n=5$ for $P2X_{4,6}Rs$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ comparing the immunoreactivity in the two groups of animals.

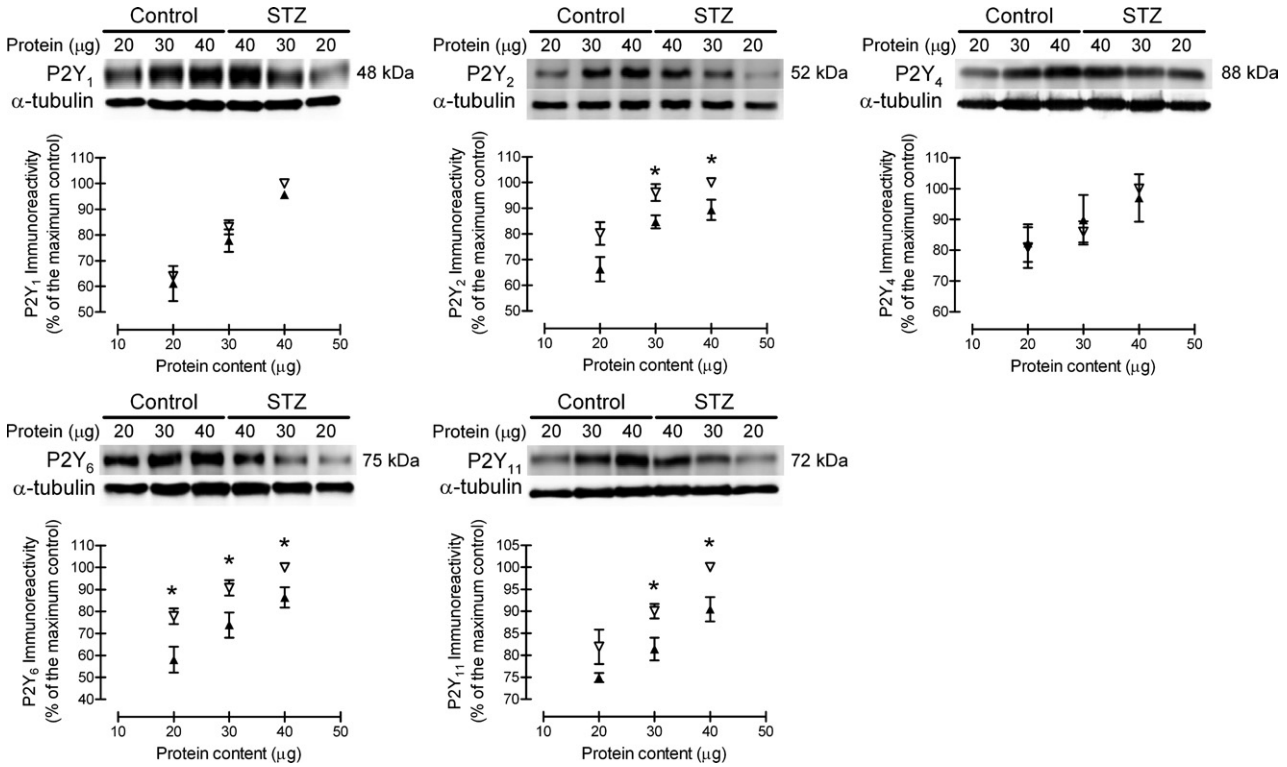

Fig. 5. Representative Western blots comparing the P2YR immunoreactivity in nerve terminal–enriched membranes from the hippocampus of control rats and from rats treated with STZ, applied in different quantities to the SDS-PAGE gel as indicated above each lane. A re-probing of the membranes with an anti-α-tubulin antibody, shown below each gel in each panel, confirmed that similar amounts of protein were added for each concentration of hippocampal membranes. The graphs below the gels show the average relative immunoreactivity of in synaptosomal membranes from control rats (open symbols) and from STZ-treated rats (filled symbols) according to the amount of protein applied to gel in different Western blot analysis using membranes from different groups of rats (n=3 for $P2Y_{1,11}$Rs and n=4 for $P2Y_{2,4,6}$Rs). * $P<0.05$ comparing the immunoreactivity in the two groups of animals.

density of the ionotropic receptors $P2X_3$, $P2X_5$ and $P2X_7$ was decreased by 7.8±1.6% (n=6, $P<0.005$), 7.5±2.4% (n=6, $P<0.03$) and 13.7±2.3% (n=6, $P<0.0001$), respectively (Fig. 4). Also the density of the metabotropic receptors $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ was reduced by 12.0±3.0% (n=4, $P<0.03$), 16.7±3.8% (n=4, $P<0.005$) and 7.5±1.8% (n=3, $P<0.005$), respectively (Fig. 5).

In contrast, in whole hippocampal membranes (which include both neurons and mainly glia) of diabetic rats 1 month after STZ-treatment, there was a global trend toward an increase of the immunoreactivity of P2Rs. In fact, as illustrated in Fig. 6, there was an increase of all ionotropic P2Rs (with the exception of $P2X_3$ and $P2X_4$). There was an increased density of $P2X_1$Rs (+12.8±3.0%, n=8, $P<0.005$), $P2X_2$Rs (+24.4±4.9%, n=8, $P<0.0001$), $P2X_5$Rs (+19.2±2.1%, n=5, $P<0.0001$), $P2X_6$Rs (+7.1±2.4%, n=5, $P<0.03$) and $P2X_7$Rs (+10.2±2.6%, n=6, $P<0.001$) in whole membranes derived from rats 1 month after STZ treatment. With respect to metabotropic P2YRs in whole hippocampal membranes of diabetic hippocampus, we found that the density of $P2Y_6$R was 19.3±3.7% larger than control (n=3, $P<0.001$). In contrast, the density of the other P2Yrs was not significantly modified ($P>0.05$) compared with whole membranes from control rats, except for $P2Y_2$Rs, whose density was decreased by 14.1±4.7% (n=7, $P<0.03$) in diabetic rats (Fig. 7).

DISCUSSION

The main conclusion of this study is that there is a deregulation of P2 receptor-mediated signaling in the hippocampus of STZ-induced type 1 diabetic rats. We found that there was a decrease in the CSF levels of ATP in diabetic rats, together with a decrease of the evoked release of ATP in hippocampal nerve terminals, suggesting that P2Rs may be facing lower concentration of extracellular ATP. Also the extracellular metabolism of ATP is reduced in nerve terminals from the diabetic hippocampus, possibly due to decreased activity of ecto-nucleotidases from the ecto-ATPase family (Zimmermann, 2000). Interestingly, we found that there was an asymmetric global modification of the density of P2 receptors (P2Rs) in synapses and outside synapses in the hippocampus, in accordance with the double role of ATP as a synaptic modulator (North and Verkhratsky, 2006; Cunha and Ribeiro, 2000) and as an extra-synaptic neuron–glia messenger (Fields and Burnstock, 2006). In fact, in nerve terminal membranes, there was a global decrease of the density of P2Rs, whereas in whole hippocampal membranes there was a global trend for an increased density of P2Rs.

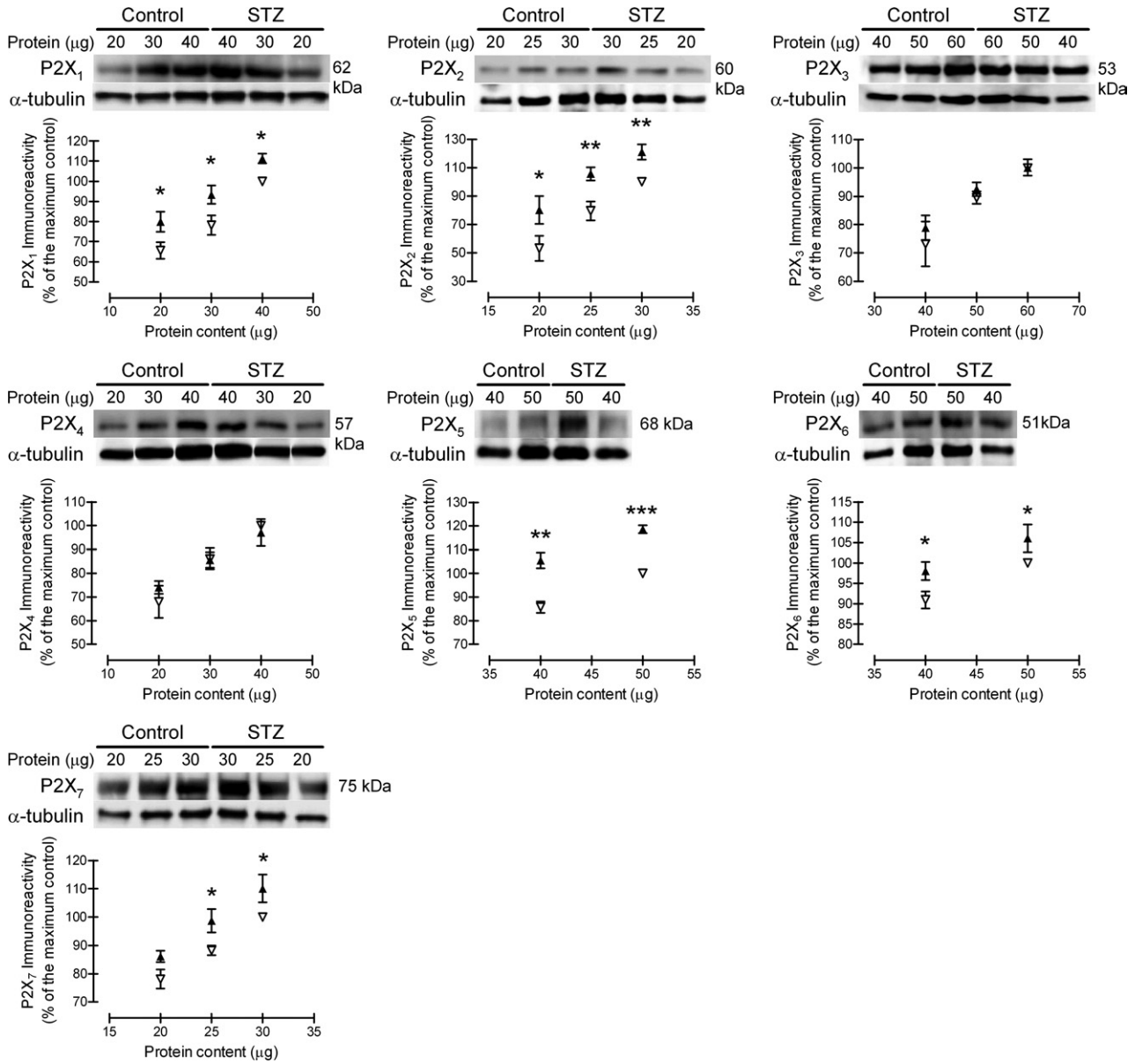

Fig. 6. Representative Western blots comparing the P2XR immunoreactivity in total membranes from the hippocampus of control rats and from rats treated with STZ, applied in different quantities to the SDS-PAGE gel as indicated above each lane. A re-probing of the membranes with an anti-α-tubulin antibody, shown below each gel in each panel, confirmed that similar amounts of protein were added for each concentration of hippocampal membranes. The graphs below the gels show the average relative immunoreactivity of in total hippocampal membranes from control rats (open symbols) and from STZ-treated rats (filled symbols) according to the amount of protein applied to gel in different Western blot analysis using membranes from different groups of rats (n=8 for $P2X_{1,2,3}$Rs, n=7 for $P2X_4$R, n=5 for $P2X_{5,6}$Rs and n=6 for $P2X_7$R). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ comparing the immunoreactivity in the two groups of animals.

It is well established that the development of a diabetic condition is accompanied by the increased incidence of neurological complications, in particular cognitive dysfunction (Cox et al., 2005; Gispen and Biessels, 2000). The hippocampus is a brain region with a key role in the implementation of mnemonic traits, and deficits in hippocampal function prime cognitive dysfunction (Squire et al., 2004). In particular, deficits in hippocampal synaptic plasticity phenomena, namely of LTP, are considered a neurophysiological trait of memory dysfunction (Lynch, 2004). Accordingly, there is a parallel deficit of the induction and maintenance of LTP as well as of the performance in memory-related tasks in STZ-induced diabetic rats (Biessels et al., 1996). Interestingly, extracellular ATP, which is released in a frequency-dependent manner (Cunha et al., 1996), plays a role in the development of LTP-like changes of synaptic efficiency through the activation of P2Rs (e.g. Pankratov et al., 2002; Almeida et al., 2003). This might be due to combined effects of different P2Rs acting both presynaptically to control the release of glutamate (Rodrigues et al., 2005) and postsynaptically to facilitate the activation of ionotropic glutamate receptors, namely

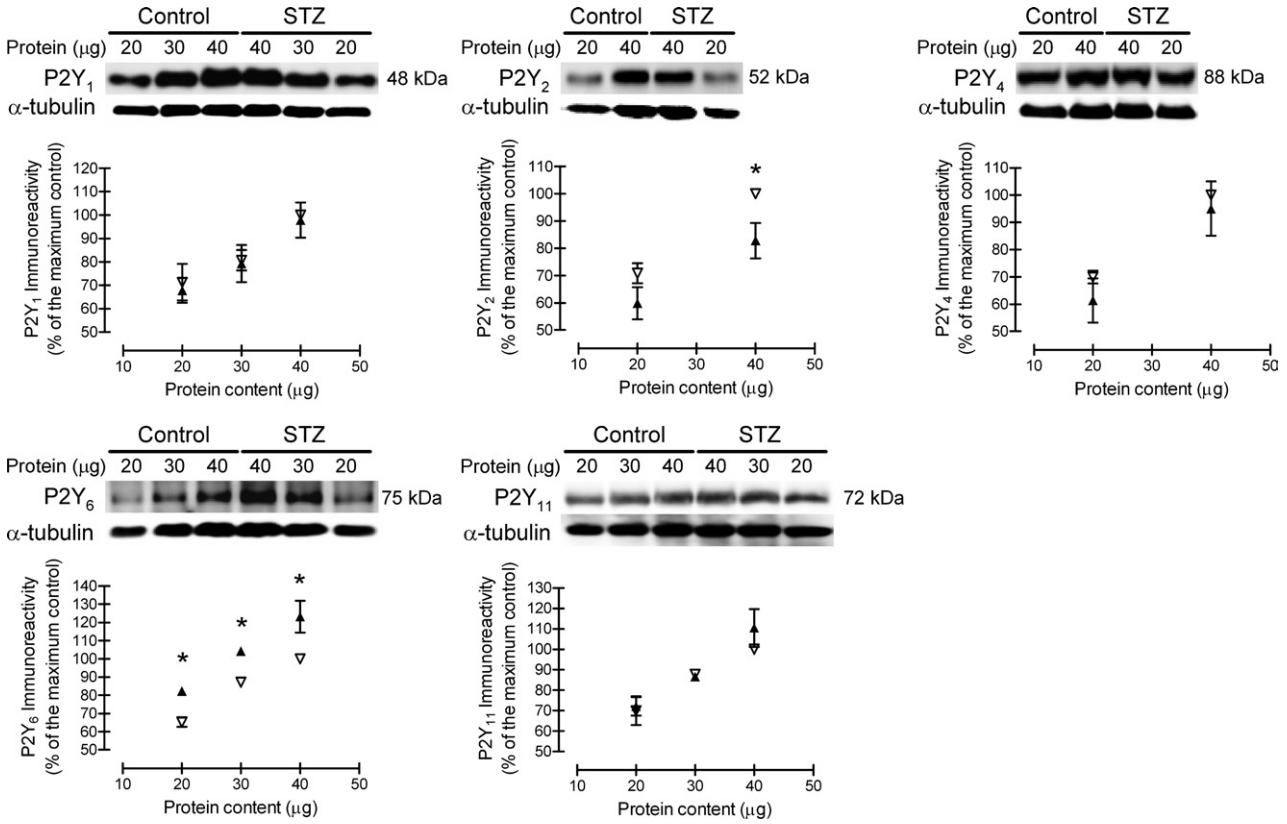

Fig. 7. Representative Western blots comparing the P2YR immunoreactivity in total membranes from the hippocampus of control rats and from rats treated with STZ, applied in different quantities to the SDS-PAGE gel as indicated above each lane. A re-probing of the membranes with an anti-α-tubulin antibody, shown below each gel in each panel, confirmed that similar amounts of protein were added for each concentration of hippocampal membranes. The graphs below the gels show the average relative immunoreactivity of in total hippocampal membranes from control rats (open symbols) and from STZ-treated rats (filled symbols) according to the amount of protein applied to gel in different Western blot analysis using membranes from different groups of rats ($n=8$ for $P2Y_1R$, $n=7$ for $P2Y_2R$, $n=5$ for $P2Y_4R$, $n=3$ for $P2Y_6R$ and $n=4$ for $P2Y_{11}R$). * $P<0.05$ comparing the immunoreactivity in the two groups of animals.

NMDA receptors (Kloda et al., 2004; Ortinau et al., 2003). Thus, the currently observed lower release of ATP together with the global down-regulation of synaptic P2Rs in the hippocampus of diabetic rats raises the hypothesis that the deficit in synaptic ATP signaling may contribute to the memory dysfunction observed in diabetes.

This down-regulation of ATP signaling in hippocampal synapses may also be an adaptive response to preserve nerve terminals. In fact, type 1 diabetes is associated and constitutes a risk factor for neurological conditions associated with dysfunction of synaptic transmission, which can eventually lead to idiopathic generalized seizures (e.g. McCorry et al., 2006). Convulsive episodes are precipitated by hypoglycemic episodes (see Jones and Davis, 2003) and are effective triggers for synaptic and neuronal damage (Pitkänen and Sutula, 2002). These hypoglycemic episodes cause an acute release of ATP (Juranyi et al., 1999), which plays a key role in triggering (e.g. Cavaliere et al., 2001a) and controlling the recovery (Aihara et al., 2002) of hypoglycemia-induced neuronal damage. Therefore, the decrease of synaptic ATP signaling can be viewed as an adaptive response to compensate for increased risk of P2R-mediated neurotoxicity in type 1 diabetes.

In contrast to what occurs in hippocampal synapses, we observed that there was a trend for an increase of the density of P2Rs in whole hippocampal membranes, which are mainly derived from extra-synaptic membranes given that synapses only represent <2% of hippocampal volume (Rusakov et al., 1998). Apart from its synaptic role in the control of synaptic transmission and plasticity, extracellular ATP also fulfils important signaling roles outside synapses, mainly in the communication between neurons and glia, which may also contribute for non-synaptic-mediated neuromodulation (reviewed in Fields and Burnstock, 2006). In astrocytes, which are the most abundant cellular elements in the brain, there is evidence that P2Rs can contribute to astrogliosis (Abbracchio and Verderio, 2006; Neary and Kang, 2005) and P2Rs actually protect astrocytes from damage (Shinozaki et al., 2005). This is particularly relevant in diabetic conditions since astrocytes are expected to be the main cellular element able to metabolize and resolve the increased extracellular levels of glucose (Pellerin and Magistretti, 2004). Hence, it seems logical to expect maintenance of this astrocytic ATP signaling in a diabetic condition and the increased P2R density in whole membranes may be an adaptive response to compensate for

the decreased ATP levels faced by these extra-synaptic P2Rs. Since it has been reported that there is an astrogliosis in type 1 diabetes (Saravia et al., 2002), it will be interesting to test if there will be an increased P2R-mediated signaling in astrocytes in the diabetic brain and whether this may be related to an increased ability to handle extracellular glucose.

CONCLUSION

In conclusion, the present study provides evidence showing that the ATP signaling system is compromised in the hippocampus of STZ-treated rats, an experimental model of type 1 diabetes mellitus. These modifications could lead to alterations in the modulation of neurotransmission and gliotransmission, which may contribute to the diabetes-induced progressive cognitive impairment, although the direct impact of such alterations on both neuronal and glial functions remains to be determined. In particular, the diversity of P2Rs and the currently observed different modification of the density of different receptors in this model of type 1 diabetes open the real possibility of selectively manipulating beneficial responses operated by particular P2Rs without exacerbating noxious responses mediated by other P2Rs.

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PARTE III
Discussão

DISCUSSÃO

Os resultados apresentados nesta tese demonstram que o sistema purinérgico (receptores P2, atividades das nucleotidases e níveis de purinas) e os níveis extracelulares de lactato e glicose cerebral estão alterados após diferentes situações patológicas (epilepsias e convulsões, doença de Parkinson e encefalopatia diabética).

Anteriormente, nosso laboratório já havia demonstrado alterações nas atividades das ecto-nucleotidases de sinaptossomas de animais epilépticos (Bonan, Amaral *et al.*, 2000; Bonan, Walz *et al.*, 2000). Diferentemente dos trabalhos anteriores, avaliamos as atividades das nucleotidases (solúveis e ecto-nucleotidases) na hidrólise dos derivados da adenina (ATP, ADP e AMP) e guanina (GTP, GDP e GMP), os níveis de purinas e seus derivados no líquor e a expressão das E-NTPDases de animais submetidos aos modelo do PTZ-kindling.

A hidrólise dos nucleotídeos promovida pela ação das nucleotidases, apresentam-se aumentadas no modelo do "PTZ-*kindling*". Entretanto, o aumento observado nos diferentes nucleotídeos não foi paralelo, favorecendo a idéia de que diferentes nucleotidases estão envolvidas neste processo. Nas ecto-nucleotidases, nossos resultados sugerem a participação de uma E-NTPDase 3, uma vez que a hidrólize do ATP foi alterada, enquanto nenhuma alteração foi observada na hidróise do ADP. O ATP, quando liberado na fenda sináptica, vai atuar em diferentes receptores P2, controlando a liberação de neurotransmissores e a sinalização neurônio-astrócito (Fields e Burnstock, 2006; Burnstock, 2007). Durante o processo convulsivo, o aumento do disparo neuronal faz com que aumente os níveis extracelulares de ATP e adenosina (Wieraszko e Seyfried, 1989b; During e Spencer, 1992; Cunha, Correia-De-Sa *et al.*, 1996). O ATP e outros nucleotídeos

podem ser rapidamente hidrolizados até adenosina e outros nucleosídeos na sinapse, que por sua vez vão ativar receptores pré- ou pós-sinápticos (Robson, Sévigny *et al.*, 2006).

A adenosina possui efeitos anticonvulsivantes em diferentes modelos animais de epilepsia (Dunwiddie, 1999). A administração de agonistas de adenosina tem efeitos anticonvulsivantes, sendo que no hipocampo estes efeitos são mediados pelos receptores A_1 (Dunwiddie, 1999; Ribeiro, Sebastiao *et al.*, 2002; Fredholm, 2003; Boison, 2005; Pagonopoulou, Efthimiadou *et al.*, 2006; Boison, 2007). Diversos estudos têm demonstrado uma redução dos receptores A_1 no tecido epiléptico (Dunwiddie, 1999; Ribeiro, Sebastiao *et al.*, 2002; Fredholm, 2003; Boison, 2005; Pagonopoulou, Efthimiadou *et al.*, 2006; Boison, 2007). Entretanto, não somente a adenosina tem sido demonstrada como um agente anticonlvusivante (Schmidt, Lara *et al.*, 2007). Estudos recentes do nosso laboratório demonstraram que a guanosina também possui propriedades anticonvulsivantes (Schmidt, Lara *et al.*, 2000; Lara, Schmidt *et al.*, 2001; Vinade, Schmidt *et al.*, 2003; De Oliveira, Horn *et al.*, 2004; Oses, Leke *et al.*, 2004; Soares, Schmidt *et al.*, 2004; Schmidt, Avila *et al.*, 2005; Tavares, Schmidt *et al.*, 2005; Tavares, Schmidt *et al.*, 2008).

O mecanismo de ação dos derivados da guanina não são bem compreendidos, estudos demostraram que o GDP capaz de inibir a ligação do ácido caínico no seu receptor (Souza e Ramirez, 1991) e, a guanosina foi capaz de aumentar a captação de glutamato em astrócitos e fatias cerebrais (Frizzo, Lara *et al.*, 2001; Frizzo, Lara *et al.*, 2002; Frizzo, Antunes Soares *et al.*, 2003; Thomazi, Godinho *et al.*, 2004). GMP e GTP foram capazes de estimular a captação do glutamato como a guanosina, entretando, não foi observado o efeito aditivo quando todos nucleotídeos foram incubados juntos (Frizzo, Antunes Soares *et al.*, 2003). Quando um análogo rígido do GTP foi incubado, ou o GMP quando pré-incubado com um inibidor da enzima 5'-nucleotidase, o α-β-metileno-ATP (AOPCP), não

foram capazes de aumentar a captação de glutamato (Frizzo, Antunes Soares *et al.*, 2003; Soares, Schmidt *et al.*, 2004). A captação astrocitária de glutamato é um importante mecanismo para cessar o estímulo glutamatérgico, sendo a captação do glutamato é um importante processo na regulação da neurotransmissão glutamatérgica, especialmente sobre condições excitotóxicas (Schmidt, Lara *et al.*, 2007).

A análise dos níveis de purinas e seus derivados demonstraram alterações nas concentrações de diversos compostos. Estas alterações estão consistentes com os resultados obtidos com a análise cinética das enzimas, e com outros dados da literatura (Wieraszko e Seyfried, 1989b; Dunwiddie e Diao, 1994; Cunha, Johansson *et al.*, 1996; Stover, Lowitzsch *et al.*, 1997; Dunwiddie, 1999; Bonan, Amaral *et al.*, 2000; Bonan, Walz *et al.*, 2000; Rodriguez-Nunez, Cid *et al.*, 2003; Boison, 2005).

Diversos estudos têm demonstrado que o ATP é capaz de induzir convulsões (Ciccarelli, Di Iorio *et al.*, 1994; Fields e Stevens, 2000; Cataldi, Lariccia *et al.*, 2005; Burnstock, 2007). As descargas epilépticas na região CA3 do hipocampo de ratos podem ser moduladas pelos nucleotídeos da adenina, acredita-se que esta modulação é exercida pelos receptores P2X (Ross, Brodie *et al.*, 1998). A distribuição dos receptores P2X no SNC é ampla, e existem receptores P2X2, P2X4 e P2X6 no córtex pirifome, uma área intimamente relacionada com a epilepsia, sugerindo que os receptores P2X podem ser um candidato em potencial para o desenvolvimento de drogas antiepilépticas.

O hipocampo de ratos epilépticos apresenta respostas anormais relacionadas ao ATP, como o aumento da expressão dos receptores P2X7 (Vianna, Ferreira *et al.*, 2002). As alterações nos receptores P2 em hipocampo dos ratos submetidos ao modelo do ácido caínico podem estar associados com o desenvolvimento de convulsões e/ou com a neurodegeneração durante a epilepsia.

Outras alterações que observamos, foi um aumento nos níveis extracelulares de glicose e lactato, e uma diminuição na captação de glicose e níveis de glicogênio hipocampais. Recentemente, tem sido descrito que a utilização da glicose cerebral contribui para a sobrevivência neuronal (Pellerin, 2003). Diversos estudos têm postulado a existência de um transporte de lactato para os neurônios como um substrato energético preferencial (Pellerin, 2003). Convulsões podem causar mudanças nos níveis de glicose e lactato cerebral, podendo, este fornecimento de lactato ser uma estratégia neuroprotetora, ou em algumas situações, refletir situações de injúria neuronal (Schurr, Payne, Miller e Tseng, 2001; Schurr, 2002b).

A manutenção da atividade glutamatérgica requer um contínuo fornecimento de energético, e em situações de excitoxicidade, a demanda de energia aumenta (Schousboe, Bak *et al.*, 2007). O aumento dos níveis de glicose e lactato observados nestes trabalho, independente das diferenças temporais entre os níveis de ambos, ajuda a demonstrar a importância do lactato na recuperação da atividade sináptica após condições de hipóxia (Schwartzkroin, 1997).

Diversos estudos têm demonstrado o envolvimento do hipocampo na epilepsia. O interesse no hipocampo dá-se pelo fato de que ele é altamente reativo a uma variedade de tratamentos. Em modelos animais, o hipocampo tem um baixo limiar convulsivo quando comparada a outras estruturas. As repetidas descargas hipersincrônicas induzidas torna o hipocampo altamente suscetível ao dano neuronal e reorganização dos circuitos cerebrais. Virtualmente, qualquer elevação substancial de atividade nesta estrutura inicia diversas mudanças nas células, circuitos e funções do hipocampo (Morimoto, Fahnestock *et al.*, 2004).

Um estudo com eletroencéfalograma (EEG) e ressonância magnética funcional (*f*MRI) em ratos epilépticos demonstrou diferenças na distribuição dos sinais dependentes dos níveis de oxigênio (BOLD) no hipocampo, dentre outras estruturas (Van Camp, D'hooge *et al.*, 2003). Estas áreas sensíveis ao PTZ são candidatos em potencial para contribuir para o início de convulsões generalizadas. Esta rápida e robusta mudança no BOLD aparece como tendo um papel proeminente no início da atividade convulsiva (Brevard, Kulkarni *et al.*, 2006).

A estrutura que tem sido mais fortemente implicada na TLE é o hipocampo. Em 1998, Nagy e Esiri demonstraram que a expressão da proteína ciclina está alterada nos neurônios hipocampais na TLE, sugerindo um ciclo celular aberrante pode ser ativado em neurônios hipocampais em processos de morte (Nagy e Esiri, 1998). Além disso, já foi demonstrado que na epilepsia há perda neuronal no hipocampo (Pohle, Becker *et al.*, 1997; Franke e Kittner, 2001; Pavlova, Yakovlev *et al.*, 2004; Pavlova, Yakovlev *et al.*, 2006).

Os receptores de adenosina A_{2A} e de dopamina D_2 são co-expressos nos neurônios GABAérgicos estriopalidais (Latini, Pazzagli *et al.*, 1996; Svenningsson, Le Moine *et al.*, 1999). Estes neurotransmissores têm efeitos antagônicos e alterações no balanço dos níveis extracelulares, e podem causar malfuncionamento do SNC. Estas alterações podem explicar os efeitos causados pela adenosina e seus agonistas e antagonistas em diversos modelos animais da DP (Latini, Pazzagli *et al.*, 1996; Fedorow, Tribl *et al.*, 2005; Ferre, Ciruela *et al.*, 2007). No modelo experimental da 6-OHDA foi observado uma diminuição nos níveis de adenosina, sendo que este efeito foi menor que o observado nos níveis de dopamina, indicando que a adenosina pode ter um menor contrabalanço que a dopamina (Pinna, Corsi *et al.*, 2002).

As hidrólises dos nucleotídeos derivados da adenina e guanina apresentaram-se modificado no modelo da 6-OHDA. Um estudo anterior demonstrou que altas concentrações de ADP *in vitro* podem diminuir a produção de adenosina (James e Richardson, 1993). Entretanto, um aumento na atividade das ecto-nucleotidases, principamente na hidrólise dos derivados da adenina, poderia mediar a produção de adenosina acelerando o processo neurodegenerativo promovido pelo modelo da 6-OHDA. Embora a hidrólise do GTP apresenta-se aumentada no estriado destes animais, a hidrólize do GDP foi diminuída nestes mesmos animais, podendo não haver nenhuma alteração nos níveis de guanosina extracelulares. Estas diferenças dos efeitos na hidrólise destes nucleotídeos podem ser devido a diferentes efeitos nas diferentes isoformas das E-NTPDases. Entretanto, quando foi avaliada uma possível alteração na expressão destas enzimas, nenhuma alteração significativa foi observada.

Uma vez que a guanosina pode ter efeitos neuroprotetores contra insultos glutamatérgicos, especulamos que estes níveis de guanosina não podem ser efetivos a fim de evitar o dano causado neste modelo de DP. Entretanto, estudos mais detalhados são necessários para avaliar melhor o envolvimento/participação dos derivados da guanina na DP.

Estudos em modelos animais da DP demonstraram que a modulação da neurotransmissão adenosinérgica pode ser de interesse para o tratamento da DP. Estudos com o modelo da 6-OHDA demonstraram que antagonistas da adenosina induziram rotações contralaterais quando administradas sozinhas, e aumentaram este comportamento quando a rotação foi induzida com agonistas da dopamina (Carta, Pinna *et al.*, 2003). A ativação dos receptores A_{2A} no complexo A_{2A}/D_2 leva a uma alteração conformacional diminuindo a ligação de D_2. Basedo no antagonismo funcional entre os receptores de

adenosina e dopamina, o bloqueio dos receptores A_{2A} com antagonistas leva a um aumento da sinalização pelos receptores D_2. Isto pode permitir que novas estratégias farmacológicas possam ser utilizadas no tratamento da DP. A adenosina é provavelmente derivada do ATP liberado como neurotransmissor através da ação das ecto-nucleotidases (Kase, 2001), ou liberada através da ruptura de células, assim, ativando os receptores P2X7 das células vizinhas, levando a um aumento do volume necrótico que tem sido proposto como um mecanismo celular na patogênese da DP (Jun, Kim *et al.*, 2007). Uma vez que a formação da adenosina e da guanosina é diretamente ligada à atividade das ecto-nucleotidases, é interessante especular que possíveis intervenções farmacológicas nestas enzimas possam vir a ser uma ferramenta importante no diagnóstico e tratamento da DP.

Estudos anteriores têm indicado que mudanças na eficiência dos receptores P2 no diabetes (Ralevic, Belai *et al.*, 1993; , 1995; Sugiyama, Kobayashi *et al.*, 2004; Sugiyama, Oku *et al.*, 2006). Assim, a hipótese de que o diabetes pode causar modificações no sistema purinérgico no cérebro, bem como pode levar a um prejuízo das funções fisiológicas desempenhadas pelo ATP.

Neste estudo encontramos uma diminuição na liberação e dos níveis extracelulares de ATP, diminuição no catabolismo do ATP extracelular no hipocampo. Além disso, uma modificação assimétrica da densidade dos receptores P2 no hipocampo destes animais. Estes resultados estão de acordo com os dados da literatura. O ATP tem sido proposto como tendo um papel duplo no SNC, podendo atuar como um modulador sináptico e como um mensageiro neurônio-astrócito extra-sináptico (Cunha e Ribeiro, 2000; Fields e Stevens, 2000; North e Verkhratsky, 2006). Em membranas sinápticas houve uma tendência a uma diminuição na densidade dos receptores P2, enquanto houve uma tendência a um aumento na densidade destes receptores em membranas totais de hipocampo.

No diabetes, é bem estabelecido que há um aumento da incidência de complicações neurológicas (Gispen e Biessels, 2000; Cox, Kovatchev *et al.*, 2005). O hipocampo desempenha um papel chave nas funções cognitivas, em particular na LTP (Lynch, 2004). O ATP extracelular desempenha um papel no desenvolvimento da LTP através da ativação dos receptores P2 (Pankratov, Lalo *et al.*, 2002; Almeida, Rodrigues *et al.*, 2003). A liberação do ATP e a densidade dos receptores P2, nos terminais sinápticos, diminuídos corrobora com a hipótese de um deficiência na sinalização pode contribuir para as disfunções da memória observadas no diabetes. Outra hipótese para essa diminuição na sinalização do ATP seria uma resposta adaptativa para preservar os terminais sinápticos. Crises convulsivas são precipitadas por episódios hipoglicêmicos (Jones e Davis, 2003). Estes episódios hipoglicêmicos causam uma liberação aguda de ATP, que desempenha um papel-chave no início e controle da recuperação do dano neuronal induzido pela hipoglicemia (Cavaliere, D'ambrosi *et al.*, 2001; Jones e Davis, 2003).

Ao contrário dos receptores P2 nas membranes sinápticas, houve um aumento na densidade destes receptores P2 localizados nas membranas totais. O ATP extracelular também desempenha um importante papel na comunicação neurônio-astrócito (Fields e Stevens, 2000). Nos astrócitos, os receptores P2 contribuem para a astrogliose reativa (Neary e Kang, 2005; Abbracchio e Verderio, 2006). No diabetes, os astrócitos desempenham um papel importante aumentando os níveis de glicose (Pellerin e Magistretti, 2004). Este aumento na densidade dos receptores P2 de membranas totais pode ser uma resposta adaptativa para a diminuição do ATP extracelular.

Estas alterações no sistema purinérgico no diabetes podem levar a alterações na neurotransmissão e interação neurônio-astrócito, podendo contribuir para os prejuízos cognitivos observados nesta doença.

CONSIDERAÇÕES FINAIS

Os resultados, de uma maneira geral, estão relacionados com os processos de neurotoxicidade, podem servir como possíveis ferramentas do diagnóstico e tratamento nos processos de injúria do SNC.

Sobre o sistema purinérgico:

- As atividades das E-NTPDases estão alteradas nos modelos de epilepsia, confirmando os dados obtidos na literatura e reforçando a hipótese de que estas enzimas têm um papel importante nos processos de neuroproteção;

- As mudanças observadas nos níveis de purinas e seus derivados demonstram que estas alterações podem estar refletindo, em parte, as mudanças ocorridas nas atividades das E-NTPDases, confirmando com dados anteriores da literatura que demonstram alterações nos níveis de purinas extracelulares em diversas situações patológicas;

- Embora a expressão da E-NTPDase1 esteja diminuída em animais submetidos ao modelo do PTZ-*kindling*, sugerindo que os efeitos observados nas atividades enzimáticas não são devido a diminuição na síntese desta proteína;

- Nos animais submetidos ao modelo do ácido caínico, as densidades dos receptores P2 em hipocampo estão alteradas. Houve diferentes alterações para diferentes receptores analisados. Estes dados reforçam a hipótese destes receptores

desempenharem um papel importante nos processos de neurotransmissão e neuroproteção.

- Crises convulsivas agudas promovem um aumento nos níveis extracelulares de glicose e lactato, e uma diminuição na captação de glicose e nos níveis de glicogênio hipocampais. Embora na literatura existam dados controversos, estes resultados confirmam com um possível papel neuroprotetor do lactato.

Sobre a Doença de Parkinson:

- As atividades das E-NTPDases estão alteradas nos modelo da DP, confirmando os dados obtidos na literatura e reforçando a hipótese de que estas enzimas têm um papel importante nos processos de neuroproteção;

- No modelo da DP, nenhuma alteração na expressão foi observada, sugerindo que os efeitos observados nas atividades enzimáticas não são devido a diminuição na síntese destas proteínas

Sobre a Encefalopatia diabética:

- A liberação de ATP está diminuída nos terminais nervosos de animais diabéticos. Esta diminuição pode levar a modificações na modulação da neurotransmissão e sinalização neurônio-astrócito.

- Diminuição no catabolismo extracelular do ATP em hipocampo de ratos submetidos ao modelo do STZ. Possivelmente devido a uma diminuição na atividade das ecto-nucleotidases, confirmando os dados obtidos na literatura e reforçando a hipótese de que estas enzimas têm um papel importante nos processos de neuroproteção;

- Nos animais submetidos ao modelo do STZ apresentaram na densidade dos receptores P2 de hipocampo alteradas. Neste moldelo, houve uma tendência para a diminuição da densidade dos receptores P2 localizados nas membranas sinaptossomais, e um aumento na densidade destes receptores nas membranas totais de hipocampo. Estes dados reforçam a hipótese destes receptores desempenharem um papel importante nos processos de neurotransmissão e neuroproteção.

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