UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

SISTEMA PURINÉRGICO: EXPRESSÃO ONTOGENÉTICA E LOCALIZAÇÃO SINÁPTICA DAS ECTONUCLEOTIDASES E SEU ENVOLVIMENTO NO DÉFICIT COGNITIVO CAUSADO POR CONVULSÃO NEONATAL

GIANA DE PAULA COGNATO

Orientadora:
Profa. Dra. CARLA DENISE BONAN

Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas – Bioquímica da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção de título de Doutor em Bioquímica

PORTO ALEGRE
2009

À minha mãe,
por todo o apoio, carinho e compreensão que recebi não só no período de minha tese, mas durante toda a vida.

Ao meu pai,
Por seu apoio, carinho e exemplo intelectual, meu principal incentivo ao estudo.

AGRADECIMENTOS

À minha querida amiga e orientadora Carla, por ter me iniciado no mundo da pesquisa, pelo ótimo convívio, pelos conselhos profissionais e incentivo, pelas gargalhadas, por sempre me exigir mais, pela confiança, e por ser meu maior exemplo profissional durante todos esses anos.

Ao professor Rodrigo Cunha, por me receber tão bem em seu laboratório, por me mostrar um outro ponto de vista com relação à ciência, pela confiança em mim depositada e por todas as alegres e inspiradoras conversas científicas no corredor do CNC.

À querida amiga Lisiane, por ter feito o convênio CAPES-GRICES acontecer, pela indicação à bolsa de doutorado sanduíche por esse convênio, pela confiança, por ser um dos meus fortes exemplos de mulheres na ciência e pela disponibilidade e ajuda em todas as situações que precisei.

Ao querido professor Diogo, por ser um importante exemplo de amor à ciência e à vida, por seu entusiasmo contagiante, pela confiança em mim depositada ao me aceitar como primeira bolsista de doutorado sanduíche pelo convênio CAPES-GRICES e pela oportunidade de estágio pós-doutoral a mim cedida após o término desta tese.

Ao professor Sarkis *(in memorian)*, pela oportunidade de realizar meus experimentos em seu laboratório, pela confiança e pelas engraçadas conversas na sala do café.

À professora Ana Battastini, pela ajuda, incentivo e paciência, pelo agradável convívio e por tolerar minhas “invasões” em seu laboratório.

A todos os colegas dos laboratórios 22, 24, 26 e 28 do Departamento de Bioquímica da UFRGS, em especial à Fernanda Vuaden, por sua disponibilidade e responsabilidade em me ajudar com meus experimentos de última hora, pela paciência e por nossa amizade.

A todos os colegas e professores do Laboratório de Neuroquímica e Psicofarmacologia da PUCRS, em especial: ao professor Renato, pelo exemplo de dedicação ao estudo e bom humor; à Rosane, por nossa amizade, pelas animadas conversas regadas a café e pelo exemplo profissional e pessoal.

Aos colegas e professores do laboratório de Biologia Genômica e Molecular, em especial: ao professor Maurício, pelo grande entusiasmo científico e pelo incentivo e confiança em mim depositados; à técnica Cladinara, por sua indispensável ajuda e disponibilidade para esclarecimento de dúvidas e por seu senso crítico e exigência.

Aos colegas e funcionários do Centro de Neurociências da Universidade de Coimbra (CNC) em especial: à Paula Canas, Patrícia Simões e Carla Silva, pela ajuda e ensinamentos; ao querido amigo Attila Köfalvi, pelo incentivo e interessantes conversas científicas; ao Pedro Garção, pela indispensável ajuda em meus experimentos finais em Coimbra; e às minhas queridas amigas, gajinhas e colegas, Manuela e Bibi, pela ajuda no laboratório, pela tolerância e paciência e por tornarem minha estadia em Portugal um pouco mais brasileira.

A minha família, por todo o apoio, compreensão, amor e carinho que recebi mesmo longe e por entenderem minha ausência.

Ao meu namorado Stefano, por sempre acreditar em mim, por me acalmar em momentos difíceis, por sua disponibilidade em me ajudar e principalmente, pelo esforço em compreender e tolerar a minha ausência durante tanto tempo.

Às equipes dos biotérios da UFRGS e do CNC, que foram essenciais na realização de todos os trabalhos desta tese.

À secretária do PPG-Bioquímica Cléia Bueno, pos todas as dúvidas esclarecidas, pela enorme paciência e por toda ajuda prestada durante todo o período dessa tese.

Ao CNPq e ao convênio CAPES-GRICES pelas bolsas de doutorado concedidas e aos demais órgãos que apóiam a pesquisa em nosso país.

E finalmente a todos que não foram citados aqui, tanto do departamento de Bioquímica da UFRGS quanto aos amigos de meu convívio, que de uma forma ou de outra, também prestaram a sua valiosa contribuição para a concretização deste trabalho.

SUMÁRIO

RESUMO

O ATP extracelular modula a atividade sináptica do SNC através de receptores purinérgicos do tipo P_1 e P_2. Os purinoreceptores do tipo P_1 (A_1, A_{2A}, A_{2B} e A_3) são mais eficientemente ativados por adenosina, enquanto que os purinoreceptores P_2 são ativados por ATP. Os níveis da ATP e adenosina na fenda sináptica são controlados pela ação de uma cascata de enzimas chamadas ectonucleotidases. Dentre outros membros, essa família de enzimas é composta pelas ectonucleosídeo trifosfato difosfoidrolases (E-NTPDases), ectonucleotídeo pirofosfatase/fosfodiesterases (E-NPPs) e pela Ecto-5'-nucleotidase. Todos esses membros possuem funções específicas em uma variedade de situações fisiológicas, como por exemplo, o desenvolvimento cerebral e a cognição, ou parecem exercer algum papel na patofisiologia de doenças do SNC, tais como a epilepsia. Portanto, no presente estudo foi investigado (1) o mapeamento ontogenético das E-NPPs em diferentes regiões cerebrais, (2)a localização das E-NTPDases e E-5'-nucleotidase a nível sináptico e subsináptico em hipocampo e estriado de ratos, (3)a participação das E-NTPDases e E-5'-nucleotidase nos mecanismos neuroquímicos envolvidos no déficit cognitivo causado por convulsão neonatal em ratos adultos e (4) se o tratamento crônico com antagonistas não seletivos (cafeína) ou seletivos (KW6002) dos receptores A_{2A} pode previnir os déficits de memória causados por convulsão neonatal em ratos adultos. Nossos resultados mostram uma ampla expressão relativa das E-NPPs durante todo o período de desenvolvimento cerebral. Além disso, as E-NTPDases e a E-5'-nucleotidase estão predominantemente localizadas nas sinapses, possuindo diferentes localizações pré e pós- sinápticas e diferentes associações glutamatérgicas e GABAérgicas. Nossos resultados também evidenciam que a hidrólise de ATP se encontra aumentada em ratos adultos que apresentaram déficit cognitivo após convulsão neonatal e que o consumo crônico de antagonistas dos receptores A_{2A} (cafeína e KW6002) pode previnir o déficit cognitivo causado por crises epilépticas no período neonatal. Em resumo, essa tese mostra pela primeira vez a localização subsináptica das ectonucleotidases e correlaciona o sistema purinérgico com a maturação cerebral e com os déficits cognitivos induzidos por convulsão neonatal. Além disso, nossos resultados evidenciam que o consumo crônico de cafeína pode ser um provável agente profilático na prevenção de disfunções de memória na vida adulta associadas às crises convulsivas neonatais.

Palavras-chave: ectonucleotidases, adenosina, epilepsia, convulsões, cafeína, cognição.

ABSTRACT

Extracellular ATP modulates the synaptic activity through P_1 and P_2 purinoceptors. Adenosine activates preferentially P_1 receptors (A_1, A_{2A}, A_{2B}, and A_3) and ATP activates preferentially P_2. The levels of ATP and adenosine in the synaptic cleft are controlled by the action of an enzyme cascade named ectonucleotidases. Besides other members, this enzyme family is constituted by ectonucleoside triphosphate diphosphohydrolase (E-NTPDase), ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) and Ecto-5'-nucleotidase. All these members have specific physiological functions in brain development and cognition or seem to have some role in the patophysiology of brain disorders, such as epilepsy. Therefore, in the present study, we have investigated (1) the expression mapping of E-NPPs in different brain regions during development, (2) the synaptic and subsynaptic localization of E-NTPDases and E-5'-nucleotidase in hippocampus and striatum of rats, (3) the participation of E-NTPDases and E-5'-nucleotidase in the neurochemical mechanisms of memory dysfunction induced by neonatal seizure in adult rats, and (4) if the chronic intake of nonselective (caffeine) or selective (KW-6002) antagonists of A_{2A} could prevent the cognitive impairment caused by convulsion in early life. Our results showed a wild relative expression of E-NPPs during brain development. In addition, E-NTPDases and E-5'-nucleotidase are predominantly localized in synapses, with different pre and post-localization and different glutamatergic and GABAergic associations. Our results also showed an enhanced ATP hydrolysis in adult rats with memory impairment induced by neonatal convulsion and a prevention of this kind of memory dysfunction by the chronic intake of A_{2A} receptors antagonists (caffeine and KW-6002). In summary, this study showed the first evidence of subsynaptic localization of ectonucleotidases and correlates the purinergic system with brain maturation and with the memory impairment induced by one single seizure in early life. Besides, our results pointed to the chronic intake of caffeine as a possible prophylactic agent for the prevention of cognitive dysfunction in adulthood induced by a single seizure in the neonatal period.

Key words: ectonucleotidases, adenosine, epilepsy, seizures, caffeíne, cognition

ÍNDICE DE FIGURAS e TABELAS

INTRODUÇÃO

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

CAPÍTULO 4

LISTA DE ABREVIATURAS

ACR – regiões conservadas da apirase

ADP – adenosine 5’-difosfato

AMP – adenosina 5’-monofosfato

ATP – adenosina 5’-trifosfato

CD39 - antígeno de ativação celular linfóide/NTPDase1

CD73 – proteína de superfície de linfócitos/ecto-5’-nucleotidase

GABA - γ-ácido aminobutírico

GPI – glicosil-fosfatidilinositol

KA – ácido caínico

KW6002 - 8-((*E*)-2-(3,4-dimetoxifenil)etenil)-1,3-dietil-7-metil-3,7-dihidro-1*H*-purina-2,6-diona

LTP – potenciação de longa duração

NMDA – N-metil-D-aspartato

NTPDase – nucleosídeo trifosfato difosfoidrolase

PKC – proteína quinase C

PTZ - Pentilenotetrazol

SE – *Status epilepticus*

SNC – sistema nervoso central

Outras abreviaturas encontram-se detalhadas no texto.

I. INTRODUÇÃO

I.1. SISTEMA PURINÉRGICO

I.1.1. ATP

A hipótese da existência de um sistema de transmissão purinérgica foi descrita pela primeira vez em 1972 por Geoffrey Burnstock. Nessa hipótese, os nucleotídeos da adenina possuem o papel de neurotransmissores, sendo o ATP considerado a principal molécula sinalizadora (BURNSTOCK, 1972). Desde então, diversos estudos vêm sendo realizados com o intuito de desvendar as funções fisiológicas do sistema purinérgico, bem como o seu papel em inúmeras patologias.

Como neurotransmissor, o ATP é liberado para o meio extracelular através de vesículas pré-sinápticas dependentes de cálcio (PHILLIS & WU, 1981) e é principalmente coliberado com outros neurotransmissores, como acetilcolina (RICHARDSON & BROWN, 1987), noradrenalina (RATHBONE et al., 1999) e serotonina (POTTER & WHITE, 1980). Ao ser liberado na fenda sináptica, este nucleotídeo pode modular a atividade sináptica através dos receptores purinérgicos ou purinoreceptores divididos em dois grandes grupos: P_1 e P_2. Os purinoreceptores do tipo P_1 são mais eficientemente ativados por adenosina, enquanto que os purinoreceptores P_2 são ativados por ATP (RALEVIC & BURNSTOCK, 1998; ABBRACCHIO et al., 2006).

Os purinoreceptores do tipo P_2 são divididos em duas subclasses: P_2X e P_2Y. A subclasse P_2X possui sete subtipos clonados e caracterizados farmacologicamente (P_2X_{1-7}) e todos eles atuam como receptores ionotrópicos. A família P_2Y é constituída por oito receptores metabotrópicos ($P_2Y_{1,2,4,6,11-14}$), acoplados a proteína G (RALEVIC & BURNSTOCK, 1998; ABBRACCHIO et al., 2006).

Em sistema nervoso, estudos demonstraram que a maturação dos receptores P2 ocorre na segunda semana de vida pós-natal (XIANG & BURNSTOCK, 2005a; CASEL et al., 2005; WIRKNER et al., 1998). Entretanto, a ação do ATP através dos receptores P_2X e P_2Y já se dá em estágios iniciais do desenvolvimento e contribui diretamente para a modulação da atividade do circuito hipocampal do rato (SAFIULINA et al., 2005). Recentemente, a sinalização purinérgica foi relacionada com o sistema nervoso em desenvolvimento, evidenciando diversas funções necessárias para a maturação cerebral, tais como proliferação de células progenitoras, migração celular, interações e diferenciações de neurônios e glia e a formação da rede sináptica (ZMMERMANN, 2006b).

Evidências indicam que o ATP pode exercer algum papel na plasticidade sináptica relacionada à memória e aprendizado (ver WIERASZKO, 1996). A aplicação de 10 μM de ATP por 10 minutos aumentou lentamente a transmissão sináptica em neurônios da região CA1 do hipocampo, conduzindo à potenciação de longa duração (LTP) (FUJII et al., 1995). Além disso, a fosforilação dos receptores NMDA parece ser necessária no mecanismo da LTP induzida por ATP (FUJII, 2004). Outro estudo demonstrou que os canais de potássio sensíveis a ATP estão envolvidos no aprendizado e memória, uma vez que a diazoxida (substância capaz de abrir esses canais) provoca um forte prejuízo na memória contextual, enquanto que o bloqueador talbutamina reverte esses efeitos (BETOURNE et al., 2009).

Em condições patológicas como trauma, isquemia/hipóxia e inflamação, o ATP extracelular é originado das células danificadas, astrócitos ativados, neurônios, microglia e células endoteliais (DUBYAK & el-MOATASSIM, 1993; JAMES & BUTT, 2002). Na doença de Alzheimer, o bloqueio dos receptores P_2X_7 exerceu neuroproteção, possivelmente através da inibição da reação inflamatória (RYU &

McLARNON, 2008). Após injúria por trauma, foi observado uma colocalização do receptor P_2Y_1 com o transportador de glutamato vesicular 3 (vGLUT3) nas áreas de lesão tecidual, indicando que este receptor pode ter algum papel patofisiológico nessa situação (FRANKE et al., 2006).

Após a liberação no meio extracelular e a ativação dos receptores específicos, os nucleotídeos da adenina podem ser metabolizados pela ação de ecto-enzimas que fazem a conversão destes nucleotídeos até adenosina (ZIMMERMANN, 1996; ZIMMERMANN, 2006a). Nosso laboratório tem estudado a degradação do ATP extracelular através de um conjunto de enzimas denominadas ectonucleotidases, tais como as Ecto-NTPDases (nucleosídeo trifosfato difosfoidrolases), as Ecto-NPPs (ectonucleosídeo pirofosfatase/fosfodiesterases) e a Ecto-5'-nucleotidase (BATTASTINI et al., 1991, 1995; WINK et al., 2000; COGNATO et al., 2005; OSES et al, 2004; FÜRSTENAU et al., 2008). A hidrólise extracelular de ATP por essa via resulta na formação de ADP, AMP e adenosina. Essa degradação pode inativar a sinalização do ATP via receptores P_2, contribuindo para a sinalização mediada pela adenosina através dos receptores P_1.

I.1.2. Adenosina

A adenosina é um nucleosídeo onipresente, sendo liberado por aparentemente todos os tipos celulares, incluindo neurônios e glia (NEWBY, 1981). Este nucleosídeo não é acumulado em vesículas nem liberado como os neurotransmissores clássicos (BRUNDEGE & DUNWIDDIE, 1997). A adenosina é liberada no espaço extracelular através de transportadores específicos, que ainda podem re-captar a adenosina para o espaço intracelular, dependendo do gradiente de concentração (GU et al., 1995). Além disso, a presença de adenosina na fenda sináptica também depende do

catabolismo extracelular do ATP promovido pela via das ectonucleotidases (DUNWIDDIE & MASIMO 2001). Esse nucleosídeo atua como um importante neuromodulador, controlando a atividade do sistema nervoso a nível pré-sinaptico, através da facilitação ou inibição da liberação dos neurotransmissores, e a nível pós-sináptico, através da hiperpolarização ou despolarização dos neurônios (WETHERINGTON & LAMBERT, 2002). Estes efeitos são mediados pelos receptores P1, subdivididos em A_1, A_{2A}, A_{2B} e A_3 (RALEVIC & BURNSTOCK 1998; FREDHOLM et al. 2001; YAAR et al., 2005). É interessante observar que a adenosina formada a partir do catabolismo dos nucleotídeos de adenina (ATP) parece agir preferencialmente nos receptores A_{2A}, enquanto que a adenosina liberada através do transportador de nucleosídeo específico parece agir preferencialmente nos receptores A_1 (CUNHA et al., 1996). Seu metabolismo ocorre através da desaminação até inosina pela ação da enzima adenosina deaminase (ADA), ou através de fosforilação até 5'-AMP realizada pela enzima adenosina quinase (ADK) (BRUNDEGE & DUNWIDIE, 1997).

A localização dos receptores de adenosina no SNC está ilustrada na figura 1 (adaptada de RIBEIRO et al., 2003).

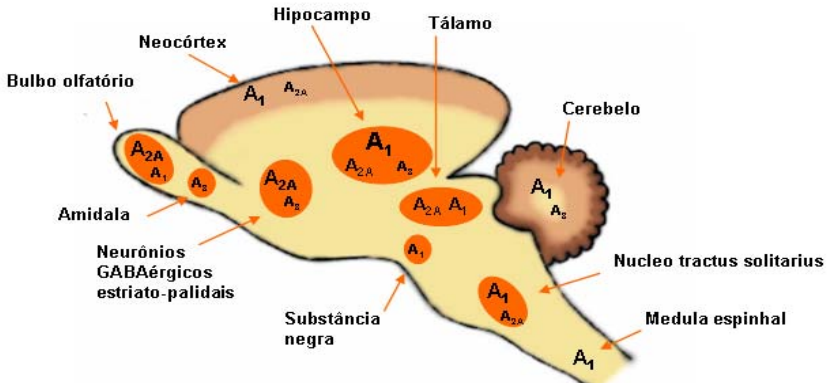


Figura 1: Distribuição dos receptores A_1, A_{2A} e A_3 nas principais regiões do SNC , onde a adenosina pode influenciar disfunções cerebrais e patologias. Altos níveis de expressão estão indicados por letras maiores (figura adaptada de RIBEIRO et al., 2003).

Os receptores A_1 estão localizados em praticamente todo o cérebro, sendo mais abundantes no córtex, cerebelo, hipocampo, corno dorsal e medula espinhal. Os receptores A_{2A} são altamente expressos nos neurônios GABAérgicos estriato-palidais e no bulbo olfatório, sendo pouco expressos no restante do cérebro (SEBASTIÃO & RIBEIRO, 1996). Os receptores A_{2B} possuem baixos níveis de expressão no cérebro e os A_3 possuem aparentemente um nível de expressão intermediário no cerebelo e hipocampo (FREDHOLM et al., 2001). Os agonistas e antagonistas seletivos destes receptores, bem como o acoplamento a proteínas G e seus respectivos valores de afinidade estão descritos na tabela 1.

Tabela 1: Características dos receptores de adenosina: agonistas e antagonistas seletivos, tipo de acoplamento a proteína G e afinidades por adenosina

Receptor	**A_1**	**A_{2A}**	**A_{2B}**	**A_3**
Agonistas seletivos (FREDHOLM et al., 2003)	CPA, CCPA, CHA	CGS 21680 HE-NECA CV-1808 CV-1674 ATL146c	Não há	CI-IB-MECA
Antagonistas seletivos (FREDHOLM et al., 2003)	DPCPX 8-CPT WRC0571	SCH 58261 KW 6002 (HOCKEMEYER et al., 2004)	MRS1754 EBBPOPX	MRS 1220 MRE 3008-F20 MRS 1191 MRS 1523
Acoplamento à proteína G (LINDEN, 2001)	G_i G_0	G_s	G_s	G_i
Afinidade por Adenosina (DUNWIDDIE & MASINO, 2001)	~70nM	~150nM	~5100nM	~6500nM

A adenosina está envolvida em diversas funções fisiológicas, tais como ansiedade, regulação do sono e cognição. Ratos “knock-out” para o receptor A_1 mostraram um aumento no comportamento relacionado com a ansiedade (JOHANSSON et al., 2001). Na regulação do sono, foi demonstrado que a adenosina

participa do re-estabelecimento do ciclo circadiano (ANTLE et al., 2001). A adenosina também pode modular o comportamento em vários paradigmas de aprendizado e memória, já que os fenômenos de plasticidade sináptica, tais como LTP e LTD, podem ser modulados via receptores A_1 (de MENDONÇA & RIBEIRO, 1997; PEREIRA et al.,2002). A figura 2 exemplifica as principais características da sinalização por adenosina no SNC (figura e legenda adaptadas de BENARROCH, 2008).

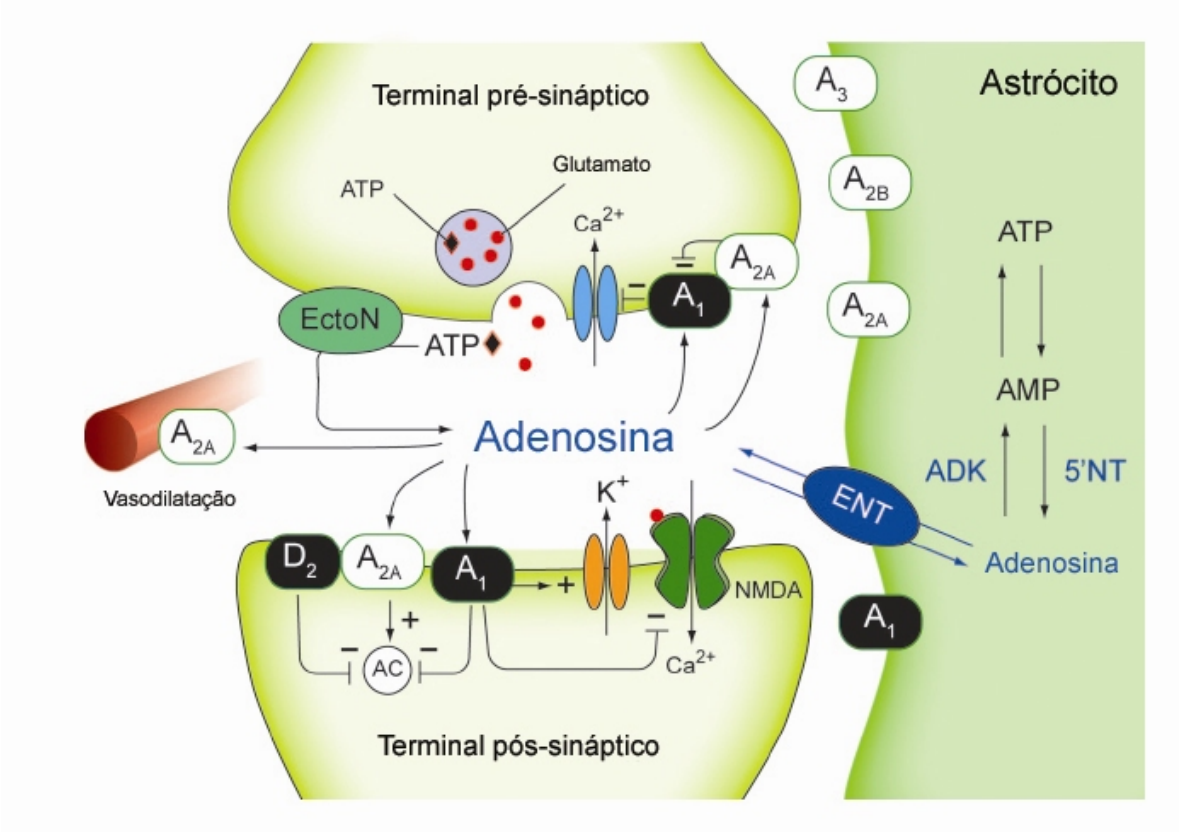


Figura 2: As duas fontes de adenosina extracelular são o seu transporte do compartimento intracelular via transportador equilibrativo de nucleosídeo (ENT) e a conversão de ATP até adenosina pela ação das ectonucleotidases (ectoN). Os receptores A_1 e A_{2A} medeiam a maioria dos efeitos fisiológicos da adenosina no cérebro. Os receptores A_1 inibem a adenilato ciclase (AC), aumentam a condutância de potássio (K^+) e inibem os canais présinápticos de cálcio (CA^{2+}). Estes receptores também inibem a liberação de glutamato e respostas pós-sinápticas mediadas pelos receptores *N*-metill-D-aspartate (NMDA). Os receptores A_{2A} estimulam a AC e podem formar complexos heteroméricos com receptores D2 antagonizando funcionalmente os efeitos da dopamina nos neurônios de estriado, ou com os receptores A_1 pré-sinápticos. Os receptores A_{2A} présinápticos inibem o a liberação de glutamato via A_1. Os receptores A_{2A} também se encontram nos vasos sanguíneos e mediam a vasodilatação no cérebro e periferia. ADK= adenosina quinase; 5'NT= 5'-nucleotidase.

Muitos estudos têm sido realizados utilizando antagonistas dos receptores P1 com o intuito de analisar a patofisiologia de doenças no SNC. A cafeína, uma substância psicotrópica extremamente consumida mundialmente, é capaz de bloquear não seletivamente os receptores de adenosina. Existem evidências de que o consumo crônico de cafeína durante a vida possa prevenir os déficits cognitivos associados ao envelhecimento (JOHNSON-KOZLOW et al., 2002). O consumo de cafeína também tem sido inversamente associado com a doença de Alzheimer (MAIA & de MENDONÇA, 2002). Na doença de Parkinson, o antagonismo do receptor A_{2A} é capaz de atenuar a rigidez muscular típica desta patologia e potencializar o efeito da $_{L}$-DOPA (WARDAS et al., 2001). Recentemente, o KW-6002, um potente antagonista seletivo dos receptores A_{2A} de adenosina, tem sido utilizado no sentido de elucidar o papel dos receptores A_{2A} em patologias do SNC. Foi demonstrado que o KW-6002 pode melhorar a disfunção motora em camundongos submetidos ao modelo animal de catalepsia (SHIOZAKI et al., 1999) e em ratos tratados com 6-hidroxydopamina (6-OHDA) (KOGA et al., 2000). Além disso, o bloqueio dos receptores A_{2A} pela administração de KW-6002, foi capaz de prevenir a disfunção de memória em ratos tratados com o peptídeo β-amilóide (CUNHA et al., 2008).

I.1.3. Ectonucleotidases

Há anos atrás, muitos membros da família das ectonucleotidases foram descobertos, clonados e funcionalmente caracterizados através de ferramentas bioquímicas. Atualmente, a família das ectonucleotidases é composta pelas ectonucleosídeo trifosfato difosfoidrolase (E-NTPDase), ectonucleotídeo pirofosfatase/fosfodiesterase (E-NPP), Ecto-fosfatases Alcalinas e a Ecto-5'-nucleotidase (ZIMMERMANN, 2006a). Essas enzimas possuem características em

comum, tais como (1) sítio de hidrólise voltado para o meio extracelular, (2) todas são proteínas de membranas com isoformas intracelulares clivadas e solúveis, (3) suas atividades de hidrólise são dependentes de cátions divalentes, (4) são ativas em pH alcalino e (5) possuem o K_M na faixa de micromolar (ZIMMERMANN, 2001). A figura 3 ilustra algumas das ectonucleotidases (adaptada de www.crri.ca/images/figure_sevigny_e.gif).

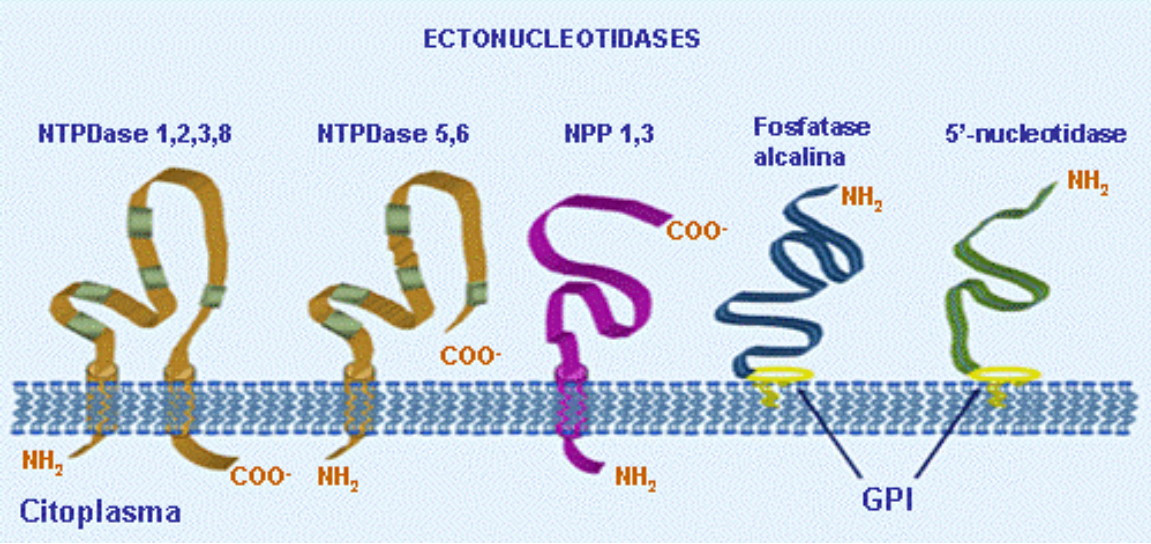


Figura 3:
Topografia de membrana das ectonucleotidases. (adaptada de www.crri.ca/images/figure_sevigny_e.gif).

I.1.3.1. NTPDases

As NTPDases (nucleosídeo trifosfato difosfoidrolases), previamente denominadas ecto-ATPase, ATP difosfoidrolase, apirase, ATPDase, são enzimas capazes de hidrolisar nucleosídeos tri e difosfatados em seus respectivos nucleosídeos monofosfatados e fosfato inorgânico. Nesta hidrólise, ocorre a liberação de 2 mols de Pi (fosfato inorgânico) por mol de nucleotídeo trifosfatado e 1 mol de Pi por mol de nucleotídeo difosfatado (PLESNER, 1995).

Além das características comuns ao grupo das ectonucleotidases, as NTPDases apresentam as seguintes características: (1) uma subunidade catalítica

glicosilada, (2) insensibilidade a inibidores específicos de ATPases do tipo P, F, V e (3) habilidade para hidrolisar uma ampla variedade de nucleotídeos púricos e pirimídicos tri e difosfatados (PLESNER, 1995; ZIMMERMANN et al., 1998). Em mamíferos, oito genes já foram identificados, clonados e suas respectivas enzimas foram expressas funcionalmente (ZIMMERMANN, 2001). Apenas as E-NTPDases 1, 2, 3 e 8 são enzimas tipicamente localizadas na superfície extracelular (BIGONNESSE et al., 2004) enquanto que as NTPDases 4-7 são enzimas intracelulares (VORHOFF et al., 2005). Todos os membros da família das NTPDases compartilham de 5 regiões idênticas chamadas regiões conservadas da apirase ("ACR I-V apyrase conserved regions") (HANDA & GUIDOTTI, 1996; ZIMMERMANN, 2001).

As E-NTPDases 1-3 e 8 possuem uma distribuição sobreposta apesar de não terem sido identificadas em células idênticas (BIGONNESSE et al., 2004). A E-NTPDase 1, correspondente ao antígeno de linfócitos ativados CD39, pode ser expressa nas células do sistema imaturo. No SNC, está localizada na superfície endotelial e das células musculares lisas dos vasos e está fortemente expressa na microglia (BRAUN et al., 2000). A E-NTPDase 2 está associada com as células progenitoras neurais no cérebro de roedores adultos (BRAUN et al., 2003; SHUKLA et al., 2005). Essa enzima também foi localizada em astrócitos em cultura (WINK et al., 2006). A E-NTPDase 3 é altamente expressa no cérebro (LAVOIE et al., 2004; VORHOFF et al., 2005) e estudos de imunohistoquímica mostraram que essa enzima está associada fortemente a neurônios positivos para hipocretina-1/ orexina-A (BELCHER et al., 2006). A NTPDase 4 tem sido localizada no aparelho de Golgi (UDPase, NTPDase 4β) e em vacúolos lisossômicos/ autofágicos (NTPDase 4α). As NTPDases 5 e 6 precem estar envolvidas nos processos de dobramento de

glicoproteínas e possuem uma preferência de hidrólise por nucleotídeos difosfatados (ZIMMERMANN, 2001). A NTPDase 7 (LALP1) prefere nucleosídeos trifosfatados como substratos e está localizada em vesículas intracelulares (ZIMMERMANN, 2001). A expressão da E-NTPDase 8 no cérebro é muito baixa ou ausente (BIGONNESSE et al., 2004), sendo localizada no fígado, rim e jejuno. A Figura 4 mostra a árvore filogenética e preferência do substrato das NTPDases (adaptada de ROBSON et al., 2006).

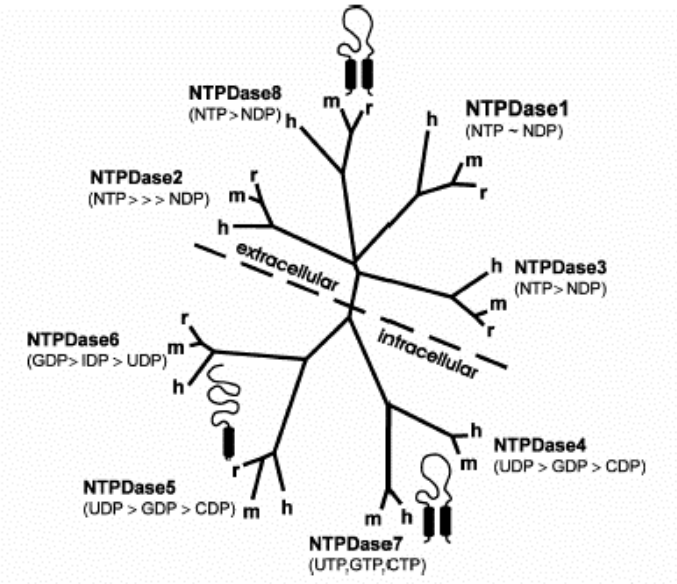


Figura 4: Possível árvore filogenética da família das E-NTPDases (NTPDase 1 a 8) de ratos (r), humanos (h) e camundongos (m). O comprimento das linhas indica as diferenças entre as proteínas. O gráfico mostra claramente uma separação entre as NTPDases localizadas na superfície celular (no topo) e as NTPDases intracelulares (abaixo). Além disso, a preferência pelo substrato de cada NTPDase está indicada entre parênteses. Figura adaptada de Robson e colaboradores (2006).

As diferenças na preferência pelos substratos e o padrão de formação dos produtos da hidrólise promovida pelas E-NTPDases 1-3 são de extrema importância para a regulação da sinalização purinérgica no SNC (ZIMMERMANN, 2006a). Todas hidrolisam nucleosídeos trifosfatados, incluindo ATP e UDP fisiologicamente ativos.

Entretanto, as razões de velocidade de hidrólise para nucleosídeos difosfatados variam consideravelmente. As razões de hidrólise (entre parênteses) para ATP e ADP como substrato para cada enzima são as seguintes: E-NTPDase 1 (~1.3), E-NTPDase 2 (~20 a 30) e E-NTPDase 3 (~4) (KUKULSKI et al., 2005).

Com a descoberta de várias classes de purinoceptores e com o ATP consolidado como o principal neurotransmissor purinérgico (ver BURNSTOCK, 2007), muitos trabalhos vêm estudando as ectonucleotidases no SNC (BATTASTINI et al., 1991, 1995; TODOROV et al., 1997; BIGONNESSE et al., 2004; BJELOBABA et al., 2006; LANGER et al., 2008). Estas enzimas podem ter uma importância crucial na inativação da ação neurotransmissora do ATP, que pode ser direta, hidrolisando o ATP em ADP e/ou AMP, ou ser indireta, tal como a conversão de um agonista (ATP) em um composto modulador (adenosina) que pode subseqüentemente ativar ou inibir outros receptores ou enzimas associadas ao sistema (ZIMMERMANN, 1996; SMITH & KIRLEY, 1998; RATHBONE et al., 1999).

Alguns estudos sobre as atividades nucleotidásicas têm sido relacionados com a ontogenia. Em 1990, MÜLLER e colaboradores demonstraram que, em sinaptossomas de córtex cerebral de ratos, as atividades de hidrólise de ATP e ADP da ecto-apirase aumentam gradualmente com a idade do animal. Outro estudo demonstrou que, em frações de membrana sinápticas de hipocampo e núcleo caudal, a hidrólise de ATP teve a sua maior ativação em 30 dias de vida (período importante na formação das sinapses funcionais) e em 365 dias (período de perda neuronal) (BANJAC et al., 2001). Além disso, foi observado um aumento na hidrólise de ATP e ADP em sinaptossomas de hipocampo e córtex cerebral de ratos fêmeas e machos com 14 a 16 dias de vida (COGNATO et al., 2005), em que ocorre intensa sinaptogênese e aumento das atividades de várias enzimas envolvidas no

metabolismo de neurotransmissores e funções neuronais (FIDLER e al., 1987). Estes estudos evidenciam que as ectonucleotidases podem exercer um papel importante nos processos de desenvolvimento cerebral e no envelhecimento.

I.1.3.2. E-NPPs

Os membros da família das E-NPPs (ectonucleotídeo pirofosfatase/fosfodiesterases) representam proteínas conservadas, as quais são capazes de hidrolisar ligações 5'-fosfodiésteres em nucleotídeos e seus derivados, resultando na liberação de nucleosídeos monofosfatados (GIJSBERS et al., 2001; GODING et al., 2003; CIMPEAN et al., 2004). O genoma dos mamíferos contém pelo menos 7 tipos distintos de genes que codificam as E-NPPs, mas apenas 3 (E-NPP1-3) mostram um intervalo de 40 a 50% de similaridade a nível proteico. Esses 3 membros são classificados como glicoproteínas transmembrana do tipo II, com um domínio intracelular N-terminal, um único domínio transmembrana e um grande domínio voltado para o meio extracelular (CIMPEAN et al., 2004). Estudos prévios demonstraram que esse domínio extracellular é formado por dois componentes homodimerizados (como somatomedina B), um domínío catalítico, uma sequência parecida com nuclease e uma parte C-terminal, a qual ainda não tem uma função definida (GIJSBERS et al; 2003; SAKAGAMI et al., 2005).

Evidências sugerem que as E-NPPs possuem múltiplos e extensos papéis fisiológicos, incluindo reciclagem de nucleotídeos, modulação da sinalização purinérgica, regulação dos níveis extracelulares de pirofosfato, estimulação da mobilidade celular, e possíveis papéis na regulação dos receptores de insulina e atividade das ecto-kinases (ver GODING et al., 2003). Até o presente momento, três

E-NPPs (E-NPP1, E-NPP2 e E-NPP3) foram localizadas no SNC. Estudos mostraram que a E-NPP1 é expressa nos capilares do cérebro (HARAHAP & GODING, 1988) e em glioma C6 de rato (GROBBEN et al., 1999; CLAES et al., 2001), embora não tenha sido detectada em neurônios ou glia (GODING et al, 2003). A E-NPP2 (também conhecida como autotaxina) tem sido relacionada com os estágios intermediários da diferenciação dos oligodendrócitos e na formação de mielina de ratos (FUSS et al., 1997). Além disso, essa enzima pode produzir o ácido lisofosfatídico, uma importante molécula na maturação cerebral. No cérebro em desenvolvimento, a E-NPP3 é expressa nos astrócitos imaturos (BLASS-KAMPMANN et al., 1997). Além disso, E-NPP2 e E-NPP3 são expressas no plexo coróide das células epiteliais (FUSS et al., 1997) e possivelmente, a modulação da sinalização purinérgica também exercida por essas enzimas pode ser capaz de contribuir para a secreção do fluido cérebro-espinhal (XIANG & BURNSTOCK., 2005b).

As E-NPPs também têm sido implicadas em várias situações patológicas. Por exemplo, foi demonstrado que se a atividade enzimática da E-NPP1 estiver inibida em células C6 de glioma de ratos, o ATP se torna um estimulador do crescimento nestas células (CLAES et al., 2001). Outro estudo mostrou que um potente inibidor da E-NPP1 (ácido piridoxalfosfato-6-azofenil-2V, 4V-dissulfônico) foi capaz de abolir a apoptose induzida por ATP em uma linhagem de neuroblastoma de camundongos (SCHRIER et al., 2002). Além disso, um aumento na quantidade da E-NPP1 em tecido adiposo e músculo esquelético tem sido correlacionado com reduções na sensibilidade à insulina pelos seus receptores e na atividade da tirosina quinase associada aos receptores de insulina (FRITTITTA et al., 1998). Com relação a E-NPP2, foi demonstrado que esta enzima é capaz de aumentar a capacidade invasiva e o potencial de metástase de células NIH3T3 ras-transformadas. Além disso, a E-

NPP2 estimula a formação de vasos em tumores provenientes destas células e também de células endoteliais humanas, indicando que esta enzima possui propriedades angiogênicas (NAM et al., 2000, 2001). O papel da E-NPP3 no desenvolvimento e nas transformações tumorais também vêm sendo estudado. Foi demonstrado que a expressão da E-NPP3 induz alterações morfológicas, síntese de proteínas relacionadas com a diferenciação e aumento das propriedades invasivas em fibroblastos e gliomas (DEISSLER et al., 1999)

I.1.2.3. Ecto-5'-nucleotidase

A E-5'-nucleotidase foi descrita pela primeira vez há cerca de 70 anos por sua atividade em coração e músculo esquelético (AIRAS et al., 1995). Previamente, foi evidenciado que esta enzima possuía um papel de cosinalização na proliferação dos linfócitos T (ZIMMERMANN, 1992). AIRAS e colaboradores (1995) concluíram que a proteína 2 de adesão vascular dos linfócitos e a CD73 (E-5'-nucleotidase) eram a mesma glicoproteína.

A E-5'-nucleotidase (CD73, EC 3.1.3.5) é uma enzima ancorada à membrana plasmática por glicosil-fosfatidilinositol (GPI) com peso molecular aparente de 62 a 74 kDa e que possui forma estrutural de dímero com pontes dissulfeto entre as cadeias (ZIMMERMANN 1992, 1996, 2001). O ancoramento por GPI pode ser clivado pela ação de uma fosfolipase C específica, dando origem a formas solúveis desta enzima (ZIMMERMANN, 1992). Enquanto a forma solúvel da 5'-nucleotidase controla os níveis intracelulares de nucleosídeos monofosfatados, a E-5'-nucleotidase localizada na superfície extracelular tem um importante papel na cascata que hidrolisa o ATP até adenosina.

Esta enzima tem sido encontrada em bactérias, tecidos de vertebrados e plantas e sua principal função é a hidrólise de nucleosídeos monofosfatados extracelulares, tais como AMP, GMP ou UMP, aos seus respectivos nucleosídeos, sendo o AMP o nucleotídeo hidrolisado com maior eficiência com valores de K_M na faixa de micromolar (ZIMMERMANN, 1996). O ATP e o ADP são potentes inibidores competitivos da 5'-nucleotidase com valores de K_i também na faixa de micromolar (ZIMMERMANN, 1996). O produto final da atividade da 5'-nucleotidase é a adenosina, que age em receptores P1 (GODING et al., 2003).

No SNC, a E-5'-nucleotidase está predominantemente associada à glia, mas várias evidências têm demonstrado que esta atividade também está associada a neurônios (ZIMMERMANN, 1996; ZIMMERMANN et al., 1998). A E-5'-nucleotidase é transitoriamente expressa na superfície de células neuronais e nas sinapses durante o desenvolvimento sináptico (SCHON & KREUTZBERG, 1994; BRAUN et al., 1995). Foi demonstrado que, em várias regiões cerebrais, a atividade desta enzima se mostra crescente à medida que o animal envelhece (FUCHS, 1991). Além disso, a E-5'-nucleotidase pode desempenhar uma função significativa na neurogênese e na formação de contatos celulares na fenda sináptica durante o desenvolvimento (ZIMMERMANN, 2006b). A E-5'-nucleotidase também desempenha um papel importante na diferenciação neurítica e na sobrevivência de células PC12 e células granulares de cerebelo de rato em cultura (HEILBRONN et al., 1995; HEILBRONN & ZIMMERMANN, 1995). Sua atividade encontra-se aumentada em astrócitos, células microgliais (BRAUN et al., 1997) e em sinaptossomas de hipocampo após isquemia focal e reperfusão (SCHETINGER et al., 1998). Além disso, estudos têm demonstrado que a E--5'-nucleotidases/CD73 tem características de uma molécula de adesão (AIRAS et al., 1995).

I.2. EPILEPSIA

Epilepsia é o nome da desordem cerebral que é caracterizada predominantemente por interrupções recorrentes e imprevisíveis da função cerebral normal, as chamadas crises epilépicas. Esta patologia não é uma única doença, mas sim um conjunto de desordens variadas que refletem uma pronunciada disfunção cerebral, podendo ser provocada por muitas e diferentes razões (FISCHER et al., 2005). As crises epilépticas são definidas por ocorrências de sinais transitórios ou sintomas causados por uma atividade cerebral anormal, excessiva e hipersincrônica (FISCHER et al., 2005). Os números de prevalência dessa patologia são altos, podendo chegar de 1% a 3%, sendo que 10% da população mundial deverá ter uma ou mais crises epilépticas em algum período de suas vidas (HAUSER et al., 1996).

O aumento da suscetibilidade do tecido nervoso às crises epilépticas tem sido ligado a uma anormalidade na neurotransmissão do SNC, através de um aumento na transmissão excitatória, ou por uma diminuição na transmissão inibitória, ou por ambos os mecanismos. Em consequência, a transdução de sinal tem sido investigada, uma vez que a neurotransmissão inadequada induz modificações no metabolismo da célula neuronal (NAFFAH-MAZACORATTI, 1998).

Para o estudo da epilepsia, abordagens clínicas e experimentais têm sido feitas.Entre os modelos experimentais, os modelos animais de epilepsia crônicos se caracterizam por crises recorrentes durante e após a vigência do agente indutor da epilepsia. Entre estes modelos, podem ser citados modelo da pilocarpina e o abrasamento/kindling químico com pentilenotetrazol (PTZ) ou ácido caínico (KA). Além dos modelos crônicos de epilepsia, existem os modelos agudos de convulsão onde o animal submetido ao modelo em questão apresenta crises convulsivas apenas durante a vigência do agente convulsivante. Alguns exemplos deste modelo são a

estimulação elétrica in vivo, o eletrochoque, o uso de inibidores de aminoácidos inibitórios como bicuculina, picrotoxina e estricnina ou os modelos agudos de PTZ ou de KA.

O conhecimento dos processos pelos quais a epilepsia é gerada (epileptogênese) são incompletos e muitos estudos têm abordado este tópico no intuito de esclarecer melhor este fenômeno. No caso de epilepsia adquirida, a epileptogênese é classicamente dividida em três fases: (1) o evento inicial (causado por uma mutação de um gene importante, ou talvez por malformação cerebral), (2) o período silencioso, onde a epileptogênese ocorre e, finalmente, (3) as crises epilépticas espontâneas e recorrentes. Em modelos de epilepsia adquirida no cérebro maduro, o evento inicial é tipicamente caracterizado por crises epilépticas/*status epilepticus* (SE), trauma ou derrame cerebral. Estes eventos provocam morte celular, com uma formação associada de novas sinapses nos neurônios sobreviventes e uma reorganização funcional do circuito normal anterior em um circuito "epiléptico" (SLOVITER, 1994; PITKANEN & SUTULA, 2002). Estes episódios precedem as crises recorrentes e, de fato, são necessários para o desenvolvimento da epilepsia (COULTER & De LORENZO, 1999; MATHERN et al., 2002; PITKANEN & SUTULA, 2002; NADLER, 2003). Além disso, foi sugerido que a epileptogênese no cérebro imaturo, incluindo as duas primeiras semanas neonatais de roedores, possui mecanismos similares aos de cérebro adulto. Entretanto, a maioria dos estudos da última década mostraram diferenças entre os cuicuitos cerebrais adultos e em desenvolvimento após SE (BARAM et al., 2002, 2003)

Vários estudos têm reportado que a epilepsia ou crises convulsivas ocorrem mais freqüentemente no período neonatal e infância do que em qualquer outro período da vida (HAUSER & HERSDORFFER, 1990; HOLMES, 1997). No geral,

existe uma prevalência de 0,5% em crianças, excluindo crises epilépticas causadas por febre (LAGAE, 2008). Uma modificação anormal da descarga neuronal, tal como ocorre em convulsões, causa poucos danos funcionais e morfológicos de imediato (STAFSTROM et al., 1992; KOH et al., 1999; HAAS et al., 2001). Entretanto, conseqüências posteriores na reatividade cerebral na vida adulta podem ocorrer (STAFSTROM, 2002; HOLMES et al., 2005; DUBÉ et al., 2007). Vários estudos têm mostrado que uma das conseqüências de crises convulsivas neonatais é o déficit cognitivo na vida adulta, principalmente na memória visual. Por exemplo, a administração de KA em ratos de 5 a 10 dias de vida (PN5-10) levou ao prejuízo da memória espacial de curta duração no labirinto radial, a um aprendizado espacial e evocação de memória deficientes no labirinto de Morris e um alto grau de ansiedade no labirinto em cruz elevada (SAYIN et al., 2004). Um deficit de memória visual na vida adulta também foi observado por LYNCH e colaboradores (2000) após a administração de KA neonatal. A administração diária de pentilenotetrazol (PTZ) em ratos jovens (PN10 a PN14) também foi capaz de causar déficits na memória espacial no labirinto de Morris em ratos com 35-40 e 60-65 dias de vida (HUANG et aul., 2002). Os mecanismos que fazem parte dos prejuízos de memória na vida adulta causados por convulsões neonatais ainda são pouco entendidos (HOLMES, 2005; DUBÉ et al., 2007).

I.3. O SISTEMA PURINÉRGICO E A EPILEPSIA

Através da evolução dos estudos sobre o papel da adenosina no sistema nervoso central, evidências levaram à hipótese de seu envolvimento na epilepsia, sendo apontada como anticonvulsivante endógeno (DRAGUNOW, 1986; DRAGUNOW, 1988). Foi demonstrado que durante a epilepsia, as concentrações de

adenosina aumentam rapidamente a níveis micromolares, sendo suficiente para ativar todos os receptores de adenosina (PEDATA et al., 2001).

Muitos estudos têm descrito mudanças na densidade dos receptores de adenosina em vários modelos de epilepsia de roedores e em tecidos epilépticos humanos. Essas mudanças não são uniformes, mas parecem ser diferentes devido ao tempo de tratamento, bem como à droga convulsivante utilizada. Em roedores, um aumento na densidade dos receptores A_1 foi observado após a administração aguda de pentilenotetrazol (PTZ) em camundongos (ANGELATOU et al., 1991; PAGONOPOULOU et al., 1993; VANORE et al., 2001; TCHEKALAROVA et al., 2005). Entretanto, existem estudos mostrando que os níveis dos receptores A_1 em córtex cerebral se mostram diminuídos a longo prazo após a indução de kindling por KA, enquanto que os receptores A_{2A} se mostraram aumentados (REBOLA et al., 2005).

Além disso, estudos também têm descrito mudanças na densidade dos receptores de adenosina em tecidos epilépticos humanos. Foi observado um aumento no número de receptores A_1 em neocórtex de pacientes com epilepsia de lobo temporal (ANGELATOU et al., 1993). Entretanto, existem estudos que evidenciaram resultados contrários tendo uma diminuição da densidade destes demostrada (GLASS et al., 1996).

Estes dados suportam a idéia de que a resposta do sistema adenosinérgico às convulsões não é uniforme, mas depende da duração do processo de abrasamento/kindling bem como do método ou do agente convulsivante utilizado. Além disso, estes estudos indicam que o mecanismo de ação destas drogas pode ser um parâmetro a ser levado em consideração na tentativa de compreender o modo

pelo qual a adenosina endógena atua como anticonvulsivante (PAGONOPOULOU et al., 2006).

As atividades nucleotidásicas também vêm sendo relacionadas com a epilepsia. É interessante ressaltar que o gene para CD39 (E-NTPDase 1) está co-localizados com o gene envolvido na epilepsia parcial humana (OTTMAN et al., 1995) sugerindo o envolvimento do sistema purinérgico com esse estado patológico. Nosso laboratório tem investigado essa relação entre as nucleotidases e epilepsia extensivamente. Estudos demonstraram que as atividades das E-NTPDases e da E-5'-nucleotidase estão significativamente aumentadas durante as fases silenciosa e crônica dos modelos animais de epilepsia induzidos por pilocarpina e KA (BONAN et al. 2000a) e do modelo de kindling induzido por pentilenotetrazol (BONAN et al. 2000b). O aumento na hidrólise de ATP, ADP e AMP induzido pelo modelo da pilocarpina em ratos adultos evidenciado por BONAN e colaboradores (2000a) não foi observado em animais jovens (COGNATO et al., 2005). BRUNO e colaboradores (2002) observaram um aumento significativo na hidrólise de ATP, ADP e AMP em soro de ratos 24 horas após uma única injeção de PTZ. Resultados similares foram observados quando os animais foram submetidos ao "kindling" com PTZ (BRUNO et al., 2003). Mais recentemente, OSES e colaboradores (2007) sugeriram que as atividades das nucleotidases solúveis de líquido cérebro-espinhal podem ser utilizadas como marcadores bioquímicos da injúria neuronal causada por crises epilépticas agudas, uma vez que essas enzimas tiveram suas atividades aumentadas 10 minutos após crise convulsiva induzida por PTZ.

A investigação da distribuição da E-5'-nucleotidase em pacientes com epilepsia de lobo temporal demonstrou que a enzima encontra-se significativamente aumentada no giro denteado e nas terminações das fibras musgosas nas regiões

CA4 e CA3, quando comparada com a atividade em hipocampo de humanos normais (LIE et al., 1999). Também foi detectado um aumento desta enzima nas sinapses de fibras musgosas de giro denteado de ratos após a injeção sistêmica de KA e indução de kindling elétrico (SCHOEN et al., 1999). Além disso, a atividade da E-5'-nucleotidase durante o período silencioso e crônico do modelo de epilepsia da pilocarpina pode bloquear as crises espontâneas através da produção da adenosina (VIANNA et al., 2005).

II. OBJETIVOS

Sabendo que os níveis sinápticos de ATP, o principal neurotransmissor do sistema purinérgico, e de adenosina, um importante e onipresente neuromodulador, são controlados pelas ectonucleotidases e que estes derivados da adenina podem influenciar a atividade sináptica através de receptores específicos (P1 e P2) em situações fisiológicas, tais como a maturação cerebral e a cognição, ou eventos patológicos, como a epilepsia, os objetivos desta tese foram:

1. Mapear a expressão ontogenética das NPPs em diferentes regiões cerebrais de ratos.
2. Localizar as E-NTPDases e E-5'-nucleotidase a nível sináptico e subsináptico em hipocampo e estriado de ratos.
3. Estudar a participação das E-NTPDases e E-5'-nucleotidase nos mecanismos neuroquímicos envolvidos no déficit cognitivo causado por convulsão neonatal em ratos adultos
4. Avaliar se o tratamento crônico com cafeína ou KW-6002 pode previnir os déficits de memória causados por convulsão neonatal em ratos adultos.

III. ARTIGOS CIENTÍFICOS

III.1. CAPÍTULO 1 - COGNATO, G. de P.; CZEPIELEWSKI, R.S.; SARKIS, J.J.; BOGO, M.R.; BONAN, C.D. Expression mapping of nucleotide pyrophosphatase/phosphodiesterase 1-3 (E-NPP1-3) in different brain structures during rat development. Artigo publicado no periódico International Journal of Developmental Neuroscience, 26 (6): 593-598.

ELSEVIER

Int. J. Devl Neuroscience 26 (2008) 593–598

INTERNATIONAL JOURNAL of DEVELOPMENTAL NEUROSCIENCE

www.elsevier.com/locate/ijdevneu

Expression mapping of ectonucleotide pyrophosphatase/phosphodiesterase 1-3 (E-NPP1–3) in different brain structures during rat development

Giana de Paula Cognato [a], Rafael Sanguinetti Czepielewski [b], João José Freitas Sarkis [a], Maurício Reis Bogo [c,*], Carla Denise Bonan [b,*]

[a] *Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, 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, Porto Alegre, RS, Brazil*
[c] *Laboratório de Biologia Genômica e Molecular, Departamento de Biodiversidade, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil*

Received 14 January 2008; received in revised form 25 April 2008; accepted 3 May 2008

Abstract

Ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs) are membrane-bound ecto-enzymes involved in the modulation of purinergic signaling. Important physiological roles related to brain development have been associated to purinergic neurotransmission. NPP1, two splice isoforms of NPP2, and NPP3 have already been identified in adult rat brain. However, there are no studies evaluating the mRNA expression of these NPP members during the brain development. The effort of the present study was to map NPP gene expression pattern in olfactory bulb, hippocampus, cerebral cortex, striatum, and cerebellum at crucial ages for rat development (7, 14, 21, 60, and 150 days old) by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) strategy. Our results demonstrated an increase in the relative expression of NPP1 throughout the aging in all structures analyzed, except in hippocampus, where the higher expression has been detected in 14 days old rats. Both NPP2 isoforms have shown a similar pattern of expression among all structures. The relative expression of NPP3 decreased during the aging mainly on cerebellum, hippocampus, and olfactory bulb. Altogether, the different patterns of NPP gene expression during rat brain development reinforce the idea that each enzyme may play a distinct role on modulating the purinergic signaling throughout aging.

Keywords: Developing brain; E-NPP; LPA; Autotoxin; Purinergic signaling; ATP

1. Introduction

The inactivation of extracellular nucleotides signaling represents a crucial control of purine-mediated functions in the nervous system. Nucleotides are released from glial cells or neurons upon activation and can act in two types of purinergic receptors: P2X (ligand-gated ionic channels receptors) and P2Y (G-protein coupled receptors) (Ralevic and Burnstock, 1998). Once released, ATP can exert a variety of physiological responses, including a trophic factor function during development (Burnstock, 2007). This nucleotide is sequentially degraded by ecto-nucleotidases (Robson et al., 2006), resulting in the formation of adenosine as a principle metabolite (Todorov et al., 1997). This nucleoside exerts its neuromodulatory effects through its own receptor subtypes (P1) (Fredholm et al., 1994) or serve as a salvage product of cellular purine metabolism.

Several members of ecto-nucleotidase families can contribute to extracellular nucleotide metabolism. The currently known ecto-nucleotidases include the ectonucleotide pyrophosphatase/phosphodiesterase family (E-NPP), ectonucleoside triphosphate diphosphohydrolase family (E-NTPDase), 5′-nucleotidase, and alkaline phosphatases (Zimmermann, 2006a).

The E-NPP members represent a family of ubiquitous and conserved proteins that are expressed as transmembrane ecto-enzymes, which are able to hydrolyze 5′-phosphodiester bounds in nucleotides, resulting in the release of 5′-nucleotide monophosphates (Bollen et al., 2000; Goding et al., 2003; Cimpean et al., 2004). Mammalian genomes contain at least seven distinct NPP-encoding genes, but only three NPPs

* Corresponding authors at: Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga 6681, Prédio 12D, Sala 340, Caixa Postal 1429, 90619-900 Porto Alegre, RS, Brazil.
Tel.: +55 51 3320 3545x4158; fax: +55 51 3320 3568.
E-mail addresses: mbogo@pucrs.br (M.R. Bogo), cbonan@pucrs.br (C.D. Bonan).

0736-5748/$34.00
doi:10.1016/j.ijdevneu.2008.05.001

(NPP1–3) show a range of 40–50% similarities at the protein level. These members are classified as type II transmembrane glycoproteins, with an intracellular N-terminal domain, a single transmembrane domain and a large domain faced to extracellular space (Cimpean et al., 2004). Previous studies demonstrated that this extracellular domain is consisted by two somatomedin-B-like homodimerization motifs, a catalytic domain, a ''nuclease-like'' sequence and a putative C-terminal ''EF-hand'' motif, which still has an uncertain role (Gijsbers et al., 2003; Sakagami et al., 2005). Although molecular and structural enzyme properties of all ecto-nucleotidase members have been investigated, the functional role of E-NPPs and their distribution in brain are still controversial.

Three NPP members (NPP1–3) have been localized in the central nervous system. Studies have shown that NPP1 is expressed in the brain capillaries (Harahap and Goding, 1988) and in rat C6 glioma (Grobben et al., 1999; Claes et al., 2001) whereas it is not detected in neurons or glia (Goding et al., 2003). NPP2 (also known as autotaxin) and NPP3 are expressed in the choroid-plexus epithelial cells (Fuss et al., 1997) and, possibly, the modulation of purinergic signaling is able to contribute to the secretion of cerebral spinal fluid (CSF) (Xiang and Burnstock, 2005). NPP2 has been correlated with intermediate stages of rat brain oligodendrocyte differentiation and myelin formation (Fuss et al., 1997). Moreover, this enzyme can produce lysophosphatidic acid (LPA), an important molecule to cerebral maturation. In the developing brain, NPP3 is expressed in immature astrocytes (Blass-Kampmann et al., 1997). Considering that NPP1–3 act in synergy to modulate purinergic signaling and also that this regulation is essential to the brain development, it became important to investigate the expression profile of NPPs during the rat cerebral maturation. Thus, our aim was to map the gene expression pattern of NPP1–3 members in rat brain regions to verify whether these genes can contribute differently for the control of nucleotide-mediated signaling during development.

2. Experimental procedures

2.1. Chemicals

Trizol reagent, SuperScript™ III First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) kit, and Taq DNA polymerase were purchased from Invitrogen. All other reagents were of analytical grade.

2.2. Animals

Male Wistar rats of different ages (7, 14, 21, 60, and 150 days) from our breeding stock were used in all experiments ($n = 3$ per age). The animals had access to water and food *ad libitum* and were kept on a 12-h light/dark cycle (lights on at 7:00 am) at a temperature of 23 ± 1 °C. Adult rats (60 and 150 day-old-rats) received rat chow (Nutrilab1, Brazil) and water *ad libitum*. The younger rats (7, 14, and 21 days old) were kept with their dams until the experimental procedure. The care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

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

The expression analysis of NPP1, NPP2, and NPP3 was carried out by a semi-quantitative RT-PCR assay. Naive rats (aged 7, 14, 21, 60, and 150 days; $n = 3$ per age) were sacrificed by decapitation, the brains were removed and placed into ice-cold RNAse free water. Olfactory bulb, hippocampus, striatum, cerebellum, and cerebral cortex of both hemispheres were dissected and immediately frozen with liquid nitrogen for storage in −80 °C freezer. The total RNA extraction of all brain structures was performed using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer instructions. The cDNA species were synthesized with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) from 1 μg of total RNA and oligo (dT) primer following the suppliers. RT reactions were performed for 50 min at 50 °C. cDNA (1 μL) was used as a template for PCR with specific primers for NPP1, NPP2, and NPP3. The NPP1 and β-actin primers were described previously (Vollmayer et al., 2001; Cognato et al., 2007). DNA sequences encoding to NPP1–3 were retrieved from GenBank database and aligned using ClustalX program. Regions with low scores of similarity among the sequences were used for searching specific primers, which were designed using the program Oligos 9.6. NPP2 splice isoforms were identified using primers localized upstream and downstream of the splice junction resulting in two different PCR products. NPP2 isoform 1 PCR product had a fragment length of 587 bp (with the intron) and NPP2 isoform 2 PCR product had 512 bp (without the intron). In order to confirm the primer specificity, each primer was blasted against rat genome and it was able to recognize only its specific target sequence. Thus, the strategy adopted to construct the primers did not allow cross-amplification. The primer sequences, GenBank accession numbers and PCR products of all NPP members analyzed and β-actin are shown in Table 1. PCR reactions were performed (total volume of 25 μL) using a concentration of 0.4 μM of each primer and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. The conditions for all PCRs were carried out as follows: initial 1 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step (NPP1: 60 °C; NPP2: 67 °C; NPP3: 65 °C; β-actin: 58.5 °C), 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C.

Table 1
GenBank accession number, primers sequences, and PCR products of NPP1–3 and β-actin

Protein	GenBank accession number	Primers sequences	PCR product
NPP1[a]	NP_445987	F 5′-GAATTCTTGAGTGGCTACAGCTTCCTA-3′ R 5′-CTCTAGAAATGCTGGGTTTGGCTCCCGGCA-3′	410 bp
NPP2 (1)[b] NPP2 (2)	Q64610	F 5′-CCATGCCAGACGAAGTCAGCCGACC-3′ R 5′-CCAAACACGTTTGAAGGCGGGGTAC-3′	587 bp, 512 bp
NPP3	AAH97326	F 5′-GAGAAGACAAATTTGCCATTTGGGAGG-3′ R 5′-TCTCATTATTTCCTTTGATTGCGGGAG-3′	301 bp
β-Actin	NP_112406	F 5′-TATGCCAACACAGTGCTGTCTGG-3′ R 5′-TACTCCTGCTTCCTGATCCACAT-3′	210 bp

[a] Primer sequences were obtained from Vollmayer et al., 2001.
[b] Represent the splice isoform of NPP2.

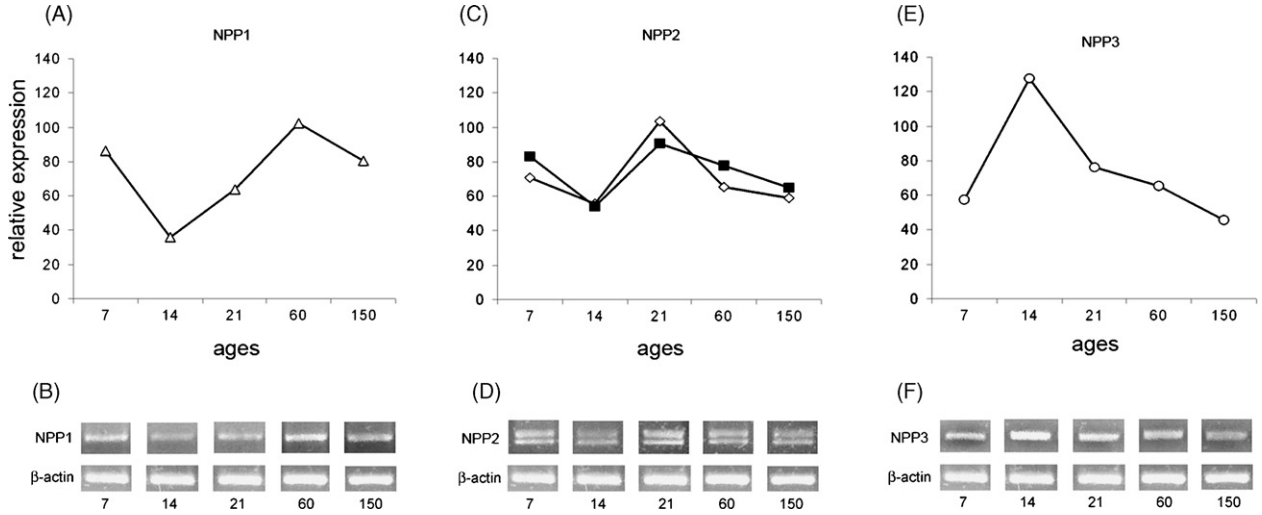


Fig. 1. Gene expression patterns of NPP1 (A and B), NPP2 isoforms 1 (◇) and 2 (■) (C and D), and NPP3 (E and F) on olfactory bulb of rats at post-natal day 7, 14, 21, 60, and 150. At least three independent experiments (PCRs) were performed, with entirely consistent results.

The fragments length of PCR reactions was confirmed with Low DNA Mass Ladder (Invitrogen, USA) and β-actin was carried out as an internal standard. The relative abundance of each mRNA versus β-actin was determined in the brain structures studied by densitometry using the freeware ImageJ 1.37 for Windows.

3. Results

3.1. NPP1 relative expression

It was observed an age-dependent increase in the relative expression of NPP1 in all brain structures analyzed here, except in hippocampus, where the higher relative expression was found in 14-day-old rats (62.5%) (Fig. 2A and B). The relative expression of NPP1 in olfactory bulb has begun in 7-day-old rats (86.5%) followed by a dramatic reduction in 14-day-old rats (36.2%). The relative expression of NPP1 in olfactory bulb has returned to high levels in 60-day-old rats (102.6%, Fig. 1A and B). In cerebral cortex, NPP1 presented the highest relative expression in 150-day-old rats (65.3%) and the lowest in 21-day-old rats (37%, Fig. 3A and B). A strong increase in the relative expression of NPP1 was observed in striatum (from 16% in 7-day-old rats to 71% in 60-day-old rats, Fig. 4A and B) and cerebellum (from 30% in 7-day-old rats to 92.5% in 60-day-old rats, Fig. 5A and B).

3.2. NPP2 relative expression

Using the primers designed to discriminate both splice isoforms of NPP2 (Table 1), it was possible to detect the relative expression of each enzyme in all brain regions and ages analyzed here. The expression pattern of NPP2 splice isoforms was similar in the course of the rat aging. The relative expressions of NPP2 splice isoforms in olfactory bulb of 14-day-old rats were 55.6% (isoform 1) and 54.3% (isoform 2). The relative expression of both isoforms of NPP2 in olfactory bulb of 21-day-old rats presented a higher expression (90.6% and 103.6% for isoforms 1 and 2, respectively, Fig. 1C and D).

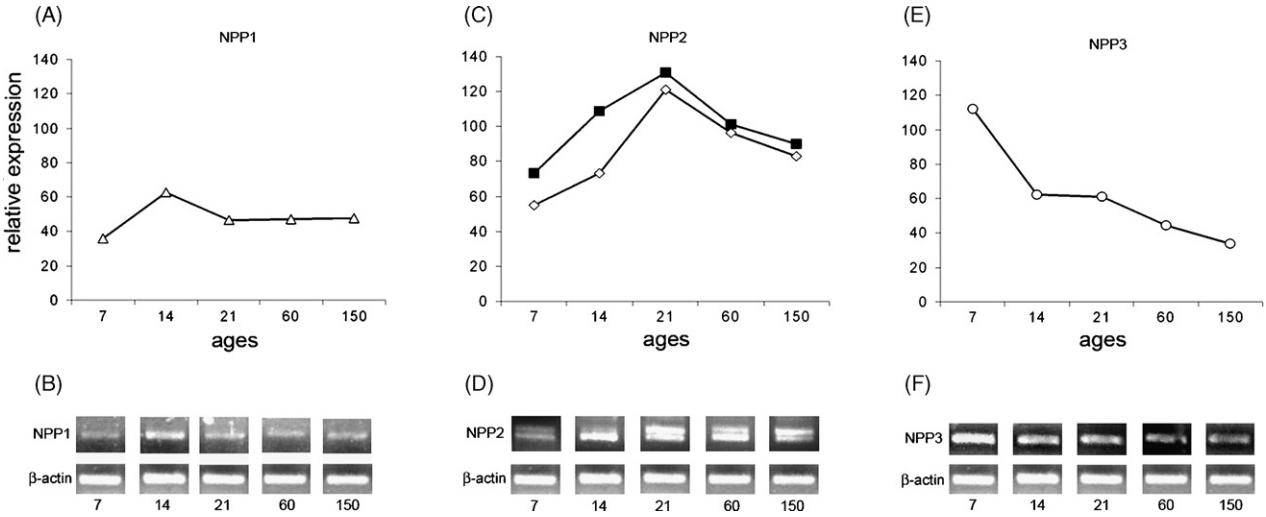


Fig. 2. Gene expression patterns of NPP1 (A and B), NPP2 isoforms 1 (◇) and 2 (■) (C and D), and NPP3 (E and F) on hippocampus of rats at post-natal day 7, 14, 21, 60, and 150. At least three independent experiments (PCRs) were performed, with entirely consistent results.

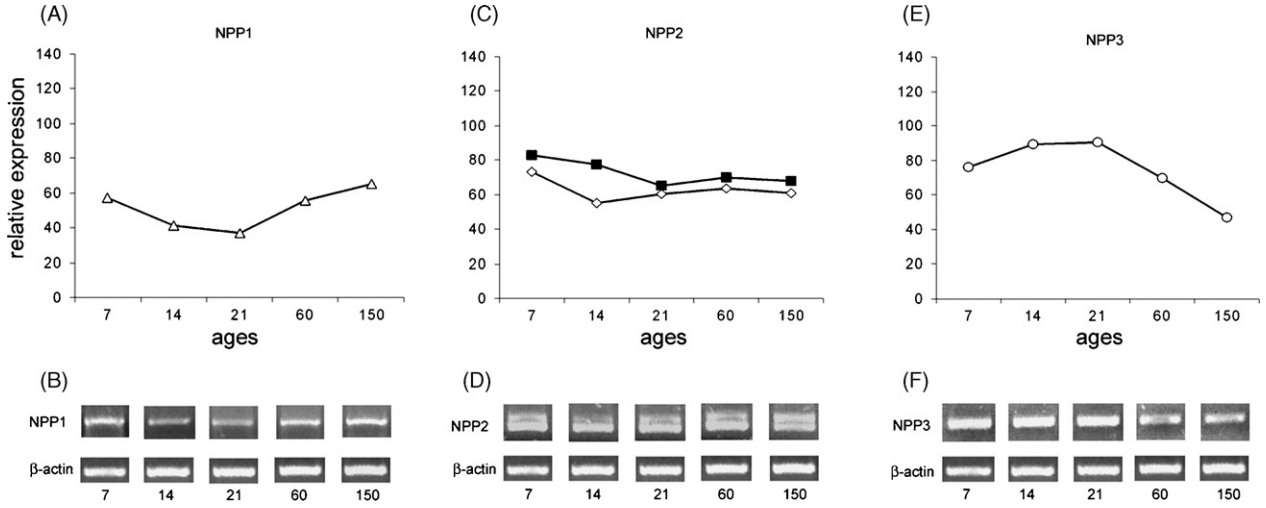


Fig. 3. Gene expression patterns of NPP1 (A and B), NPP2 isoforms 1 (◇) and 2 (■) (C and D), and NPP3 (E and F) on cerebral cortex of rats at post-natal day 7, 14, 21, 60, and 150. At least three independent experiments (PCRs) were performed, with entirely consistent results.

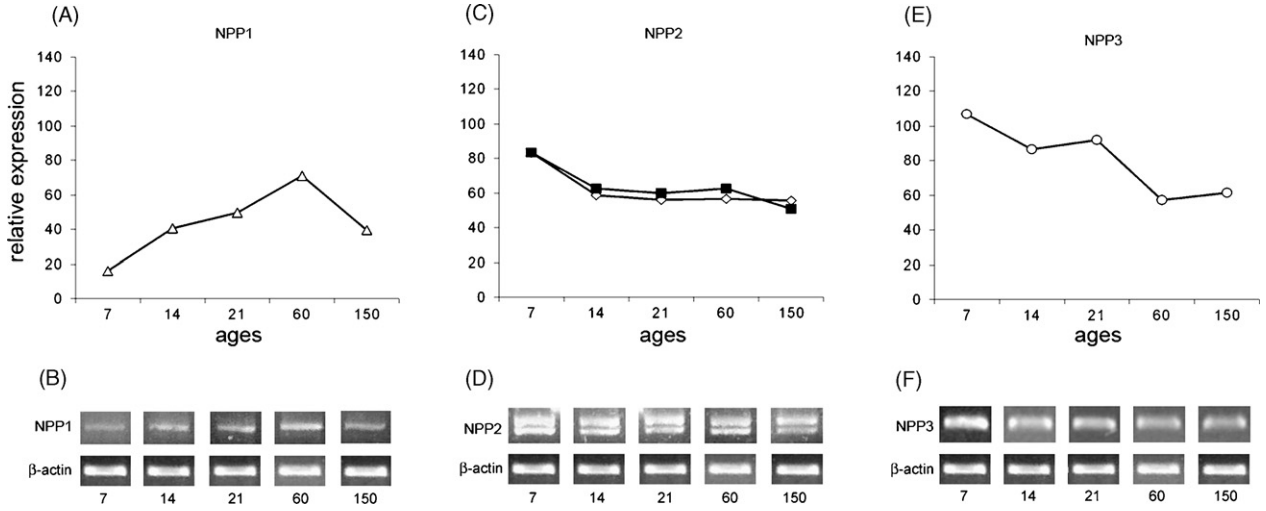


Fig. 4. Gene expression patterns of NPP1 (A and B) NPP2 isoforms 1 (◇) and 2 (■) (C and D) and NPP3 (E and F) on striatum of rats at post-natal day 7, 14, 21, 60, and 150. At least three independent experiments (PCRs) were performed, with entirely consistent results.

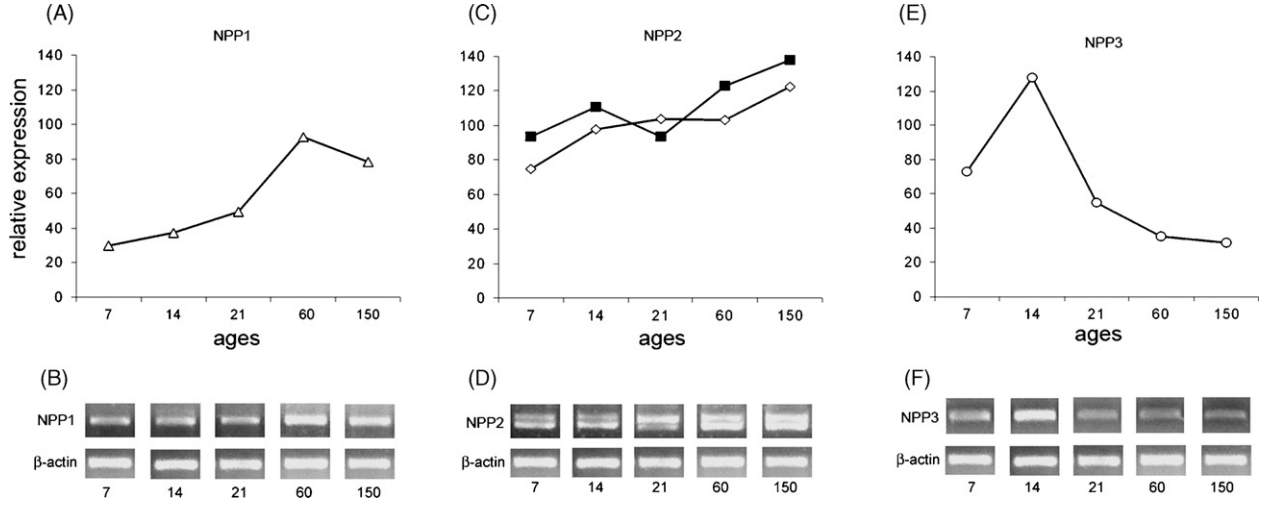


Fig. 5. Gene expression patterns of NPP1 (A and B) NPP2 isoforms 1 (◇) and 2 (■) (C and D) and NPP3 (E and F) on cerebellum of rats at post-natal day 7, 14, 21, 60, and 150. At least three independent experiments (PCRs) were performed, with entirely consistent results.

In hippocampus, the relative expression of both isoforms has also been highest in 21-day-old rats (121% and 131% for isoforms 1 and 2, respectively) when compared to all other ages tested, mainly in 7-day-old rats (54.6% and 73.3% for isoforms 1 and 2, respectively, Fig. 2C and D). The NPP2 isoforms presented a constant pattern of expression in cerebral cortex and striatum, remaining in the range of 60% for NPP2 isoform 1 and 70% for NPP2 isoform 2 in both brain regions (Figs. 3 and 4C and D). In cerebellum, NPP2 isoforms show an interesting expression pattern, which differs from the profiles presented by other brain regions. The relative expression has increased from 74% (NPP2 isoform 1) and 93% (NPP2 isoform 2) in 7-day-old rats to 122.3% (NPP2 isoform 1) and 138% (NPP2 isoform 2) in 150-day-old rats (Fig. 5C and D).

3.3. NPP3 relative expression

In all brain regions analyzed, it has been found a decrease in relative expression of NPP3 through rat aging. In hippocampus and striatum, the relative expression of NPP3 in 7-day-old rats has declined from 112% and 107% to 34% and 61% in rats with 150-day-old, respectively (Figs. 2 and 4E and F). This expression pattern was also observed in olfactory bulb and cerebellum, in which the relative expression in 14-day-old rats (127.6% and 127.5%, respectively) has diminished in relation to 150-day-old rats (45.75% and 31.6%, respectively, Figs. 1 and 5E and F). In cerebral cortex, the first three ages evaluated here (7, 14, and 21 days) have presented a similar relative expression of NPP3 (76%, 89.6%, and 90.6%, respectively) whereas 60- and 150-day-old rats have shown a diminished expression pattern (69% and 47%, respectively, Fig. 3E and F).

4. Discussion

The distribution of NPP1 in the adult rat brain has already been evaluated. Bjelobaba et al. (2006) have observed a widespread distribution of NPP1 in the rat forebrain, mainly in hippocampus and cerebral cortex. The results presented here show that NPP1 relative expression is long-standing for these brain regions during the lifetime. Another study revealed NPP1 has been expressed in the capillaries of the brain (Harahap and Goding, 1988). It was previously described that CNS is mainly vascularized by angiogenesis, the sprouting of capillaries from preexisting vessels, during the development (Marin-Padilla, 1985) whereas it is almost absent in adult tissues (Plate et al., 1994). In accordance to these studies, the relative expression of NPP1 in olfactory bulb, striatum and cerebellum increased until 60 days old and then decreased at 150 days old.

It has been suggested endogenous release of ATP starts to enhance the synaptic activity in Purkinje neurons by the end of the second post-natal week (Casel et al., 2005). The increase in relative expression of NPP1 and NPP2 through aging may reflect that these enzymes act in synergy hydrolyzing ATP during brain developing. Moreover, NPP2 also can produce LPA, a lipid mediator with a wide variety of biological functions, including important actions for brain development, such as cortical neurogenesis and pattern formation (Kingsbury et al., 2003). LPA is also implicated in the vascular development of immature brain. van Meeteren et al. (2006) have shown that NPP2-deficient mice embryos die at embryonic day 9 with severely impaired vessel formation. Since NPP2 is the ecto-nucleotidase responsible for modulating LPA levels through the hydrolysis of lysophosphatidylcholine (Tokumura et al., 2002), the presence of these enzymes during brain maturation is at least essential.

It has been hypothesized that NPP3 may represent an important factor in the process of glial cell proliferation which is accompanied by migration of glial precursors (Zimmermann, 2006b). In fact, it was reported NPP3 has been only detected on immature astrocytes (Blass-Kampmann et al., 1997). Our results support this data since we have observed a decreased relative expression of NPP3 during brain maturation in all areas investigated. Interestingly, mRNA expression of NPP3 in hippocampus and striatum begins to decline from the age of 7 days old. This pattern differs from cerebellum and olfactory bulb, since the relative expression of NPP3 in those brain areas starts to diminish from the age of 14 days old. These results indicate that NPP3 is differently altered during the brain development, which could influence the process of glial cell proliferation.

In summary, the present study demonstrated a widespread mRNA relative expression for NPPs, which are capable of hydrolyzing important molecules for proper maturation of the brain (for example, ATP and LPA). Altogether, the different patterns of NPP gene expression during rat brain development reinforce the idea that each enzyme may play a distinct role on modulating the purinergic signaling throughout aging.

Acknowledgements

The authors thank laboratory members for helpful comments on the manuscript. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and by the FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" 01.06.0842-00.

References

Bjelobaba, I., Nedeljkovic, N., Subasic, S., Lavrnja, I., Pekovic, S., Stojkov, D., Rakic, L., Stojiljkovic, M., 2006. Immunolocalization of ecto-nucleotide pyrophosphatase/ phosphodiesterase 1 (NPP1) in the rat forebrain. Brain Res. 1120, 54–63.

Blass-Kampmann, S., Kindler-Rohrborn, A., Deissler, H., D'Urso, D., Rajewsky, M.F., 1997. In vitro differentiation of neural progenitor cells from prenatal rat brain: common cell surface glycoprotein on three glial cell subsets. J. Neurosci. Res. 48, 95–111.

Bollen, M., Gijsbers, R., Ceulemans, H., Stalmans, W., Stefan, C., 2000. Nucleotide pyrophosphatases/phosphodiesterases on the move. Crit. Rev. Biochem. Mol. Biol. 35, 393–432.

Burnstock, G., 2007. Physiology and pathophysiology of purinergic neurotransmission. Physiol. Rev. 87, 659–797.

Casel, D., Brockhaus, J., Deitmer, J.W., 2005. Enhancement of spontaneous synaptic activity in rat Purkinje neurones by ATP during development. J. Physiol. 568, 111–122.

Cimpean, A., Stefan, C., Gijsbers, R., Stalmans, W., Bollen, M., 2004. Substrate-specifying determinants of the nucleotide pyrophosphatases/phosphodiesterases NPP1 and NPP2. Biochem. J. 381, 71–77.

Claes, P., Van Kolen, K., Roymans, D., Blero, D., Vissenberg, K., Erneux, C., Verbelen, J.P., Esmans, E.L., Slegers, H., 2001. P2Y(AC)(−)-receptor agonists enhance the proliferation of rat C6 glioma cells through activation of the p42/44 mitogen-activated protein kinase. Br. J. Pharmacol. 134, 402–408.

Cognato, G.de P., Bruno, A.N., da Silva, R.S., Bogo, M.R., Sarkis, J.J., Bonan, C.D., 2007. Antiepileptic drugs prevent changes induced by pilocarpine model of epilepsy in brain ecto-nucleotidases. Neurochem. Res. 32, 1046–1055.

Fredholm, B.B., Abbrachio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P., Williams, P., 1994. Nomencalture and classification of purino receptors. Pharmacol. Rev. 46, 143–156.

Fuss, B., Baba, H., Phan, T., Tuohy, V.K., Macklin, W.B., 1997. Phosphodiesterase I, a novel adhesion molecule and/or cytokine involved in oligodendrocyte function. J. Neurosci. 17, 9095–9103.

Gijsbers, R., Aoki, J., Arai, H., Bollen, M., 2003. The hydrolysis of lysophospholipids and nucleotides by autotaxin (NPP2) involves a single catalytic site. FEBS Lett. 538, 60–64.

Goding, J.W., Grobben, B., Slegers, H., 2003. Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. Biochim. Biophys. Acta 1638, 1–19.

Grobben, B., Anciaux, K., Roymans, D., Stefan, C., Bollen, M., Esmans, E.L., Slegers, H., 1999. An ecto-nucleotide pyrophosphatase is one of the main enzymes involved in the extracellular metabolism of ATP in rat C6 glioma. J. Neurochem. 72, 826–834.

Harahap, A.R., Goding, J.W., 1988. Distribution of the murine plasma cell antigen PC-1 in non-lymphoid tissues. J. Immunol. 141, 2317–2320.

Kingsbury, M.A., Rehen, S.K., Contos, J.J., Higgins, C.M., Chun, J., 2003. Non-proliferative effects of lysophosphatidic acid enhance cortical growth and folding. Nat. Neurosci. 12, 1292–1299.

Marin-Padilla, M., 1985. Early vascularization of the embryonic cerebral cortex: Golgi and electron microscopic studies. J. Comp. Neurol. 241, 237–249.

Plate, K.H., Breier, G., Risau, W., 1994. Molecular mechanisms of developmental and tumor angiogenesis. Brain Pathol. 4, 207–218.

Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. Pharmacol. Rev. 50, 413–492.

Robson, S., Sévigny, J., Zimmermann, H., 2006. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2, 409–430.

Sakagami, H., Aoki, J., Natori, Y., Nishikawa, K., Kakehi, Y., Natori, Y., Arai, H., 2005. Biochemical and molecular characterization of a novel choline-specific glycerophosphodiester phosphodiesterase belonging to the nucleotide pyrophosphatase/phosphodiesterase family. J. Biol. Chem. 280, 23084–23093.

Todorov, L.D., Mihaylova-Todorova, S., Westfall, T.D., Sneddon, P., Kennedy, C., Bjur, R.A., Westfall, D.P., 1997. Evaluation of a method for controlling molecular scaffold diversity in de novo ligand design. J. Comput. Aid. Mol. Des. 11, 175–192.

Tokumura, A., Majima, E., Kariya, Y., Tominaga, K., Kogure, K., Yasuda, K., Fukuzawa, K., 2002. Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. J. Biol. Chem. 277, 39436–39442.

van Meeteren, L.A., Ruurs, P., Stortelers, C., Bouwman, P., van Rooijen, M.A., Pradere, J.P., Pettit, T.R., Wakelam, M.J., Saulnier-Blache, J.S., Mummery, C.L., Moolenaar, W.H., Jonkers, J., 2006. Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. Mol. Cell. Biol. 26, 5015–5022.

Vollmayer, P., Koch, M., Braun, N., Heine, P., Servos, J., Israr, E., Kegel, B., Zimmermann, H., 2001. Multiple ecto-nucleotidases in PC12 cells: identification and cellular distribution after heterologous expression. J. Neurochem. 78, 1019–1028.

Xiang, Z., Burnstock, G., 2005. Expression of P2X receptors in rat choroids plexus. Neuroreport 16, 903–907.

Zimmermann, H., 2006a. Ectonucleotidases in the nervous system. Novartis Found Symp. 276, 113–128.

Zimmermann, H., 2006b. Nucleotide signaling in nervous system development. Eur. J. Physiol. 452, 573–588.

III. ARTIGOS CIENTÍFICOS

III.2. CAPÍTULO 2 - COGNATO, G.P.; GHISLENI, G.C.; RODRIGUES, R.J.; PORCIÚNCULA, L.O.; SOUZA, D.O; AGOSTINHO, P.M.; BONAN, C.D; CUNHA, R.A. Synaptic and subsynaptic localization of NTPDases 1, 2, 3, and ecto-5'-nucleotidase in the rat hippocampus and striatum. Artigo a ser submetido para o periódico Neuroscience

SYNAPTIC AND SUBSYNAPTIC LOCALIZATION OF NTPDases 1, 2, 3, AND ECTO-5'-NUCLEOTIDASE IN THE RAT HIPPOCAMPUS AND STRIATUM

Giana P. Cognato[1,2,3], Gabriele C. Ghisleni[1,3], Ricardo J. Rodrigues[1] Lisiane O. Porciúncula[2], Diogo O. Souza[2], Paula M. Agostinho[1], Carla D. Bonan[3], Rodrigo A. Cunha[1,*]

[1]Center for Neuroscience of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, Portugal;

[2]Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[3]Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, PUC/RS, Porto Alegre, RS, Brazil

Corresponding Author: Rodrigo A. Cunha, Center for Neuroscience of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal; e-mail: cunharod@gmail.com; Website: http://cnc.cj.uc.pt/lab_lef/

ABSTRACT

Purinergic signalling in the brain involves ATP and adenosine, both released from neurons and glia and acting through P2 and P1 receptors, respectively. Ecto-nucleotidases play a key role in controlling purinergic signalling since they hydrolyse ATP and can generate adenosine. Although purinergic control of synaptic transmission and plasticity mainly occurs through activation of synaptic receptors, it is not known which ecto-nucleotidases are present at synapses. We now used previously validated antibodies against different ecto-nucleotidases (NTPDase1, 2, 3, and ecto-5'-nucleotidase) to determine their synaptic and sub-synaptic localization in the rat hippocampus and striatum. We found that all ecto-nucleotidases were present in hippocampal and striatal synapses and most were more abundant in synapses than in extra-synaptic fractions. In the hippocampus, NTPDase1 and 5'-nucleotidase were evenly distributed pre- and post-synaptically whereas NTPDase2 and 3 were more abundant post-synaptically. In the striatum, all ecto-nucleotidases were distributed evenly pre- and post-synaptically with the exception of NTPDase3 that was more abundant post-synaptically. However, the set-up of ecto-nucleotidases was different in glutamatergic and GABAergic terminals in both regions; in fact, NTPDase1 was more abundant in GABAergic terminals, whereas NTPDase3 and ecto-5'-nucleotidase were more abundant in glutamatergic terminals and NTPDase2 was evenly distributed in both types of terminals. Overall, these results provide a direct demonstration of the presence and abundance of NTPDase1, 2, 3, and ecto-5'-nucleotidase in central synapses and provide initial evidence that the degradation of ATP and the formation of extracellular adenosine may be differently organised at inhibitory and excitatory synapses.

Key words: NTPDase, ecto-5'-nucleotidase, hippocampus, striatum, nerve terminals, synapse

INTRODUCTION

Signalling via extracellular nucleotides has been recognized for over a decade as an important intercellular signalling mechanism in the brain and in multiple other tissues (Burnstock, 2007). Nucleotides, such as ATP, can be released from nerve cells and glial cells where they exert their function via ionotropic (P2X) or metabotropic (P2Y) receptors (Burnstock, 2007). Thus, ATP is an important gliotransmitter and controller of neuroinflammation (Inoue et al., 2007), but also plays a role as a neuromodulator controlling synaptic transmission and plasticity (Cunha and Ribeiro, 2000) through activation of synaptic P2 receptors (Rodrigues et al., 2005). ATP signalling is terminated by its extracellular catabolism through a series of ecto-nucleotidases, which can generate adenosine (Zimmermann, 2006). Adenosine acts a synaptic neuromodulator defining the salience of information in neuronal circuits through a combined activation of inhibitory A_1 and facilitatory A_{2A} receptors (Cunha, 2008), both of which are mainly located in synapses (Rebola et al., 2003, 2005).

Although ecto-nucleotidases play a key role in controlling the availability of ATP and adenosine to activate their receptors, their cellular localization in the brain is only beginning to be unravelled (Langer et al., 2008). The family of ecto-nucleoside triphosphate diphosphohydrolase (NTPDase), which can hydrolyze extracellular nucleoside tri- and diphosphates, and ecto-5'-nucleotidase, which converts nucleoside monophosphates into adenosine (Zimmermann, 2006) have already been identified in several brain structures (Langer et al., 2008; Bjelobaba et al., 2007; Belcher et al., 2003). Amongst NTPDases, extracellular ATP in the brain can be catabolised by NTPDase1, 2 or 3, since NTPDases4, 5, 6 are intracellular and NTPDase8 is not expressed in the brain (Bigonnesse et al., 2004). NTPDase1 has been reported to have a widespread distribution in the rat brain (Wang and Guidotti, 1998;) with great abundance in microglia (Braun et al., 2000), NTPDase2 is most abundantly located in the subventricular zone and rostral migratory stream (Braun et al., 2003) and seems to be the main NTPDase present in astrocytes (Wink et al., 2006) whereas NTPDase3 displays a predominant neuronal localization (Belcher et al., 2006). Finally, ecto-5'-nucleotidase was argued to be mostly located in glial cells (Kreutzberg et al., 1986), although it is also present in neurons (Bernstein et al., 1978; Zimmermann et al., 1993; Cunha et al., 2000).

In accordance with the importance of ATP and adenosine signalling in synapses, there is previous evidence that ecto-nucleotidase activities are present in synapses (e.g. Nagy et al., 1986; Battastini et al., 1991; Cunha et al., 1992; James and Richardson, 1993; Nedeljkoovic et al., 2003;

Kukulski et al., 2004; Cognato et al., 2007), where their role is expected to be most important (Cunha, 2001) since these synaptic ecto-nucleotidases are organised in a highly efficient pathway coupling the degradation of ATP to the formation of extracellular adenosine within milliseconds (Dunwiddie et al., 1997; Cunha et al., 1998). However, the molecular identity of the ecto-nucleotidases present in synapses is currently unknown.

Thus, we now investigated the synaptic and sub-synaptic localization of NTPDases1, 2 and 3 and of ecto-5'-nucleotidase in rat hippocampal and striatal synapses, using a series of previously validated antibodies. The results revealed that all ecto-nucleotidases are located in synapses and most are actually concentrated at the synapse rather than in extra-synaptic fractions. Furthermore, it was found that these ecto-nucleotidases also have a different pre- and post-synaptic localization and are differently associated with glutamatergic and GABAergic synapses.

MATERIAL AND METHODS

Animals

Male adult Wistar rats with 8-10 weeks, weighing 160-220 g, were used throughout this study. They were maintained in our own animal facilities under controlled environment (23±2°C, 12 h-light/dark cycle, free access to food and water). Experiments were carried out according to the local guidelines on the ethical use of experimental animals, based on the EU guidelines for use of experimental animals (86/609/EEC) with care to minimize the number of animals used and their suffering.

Preparation of total and synaptosomal membranes

Striatal or hippocampal total membranes or synaptosomal membranes were prepared as previously described (Rebola et al., 2003, 2005). Hippocampi and striata from one rat were homogenized at 4°C in sucrose solution (0.32 M) containing 50 mM Tris–HCl, 2 mM EGTA, and 1 mM dithiothreitol, pH 7.6, centrifuged at 3,000 x *g* for 10 min at 4 °C, the supernatants collected and centrifuged at 14,000 x *g* for 20 min at 4 °C. The pellets constitute the total membrane fraction and were divided into two aliquots: one was resuspended in SDS-PAGE buffer (see below) for Western blot analysis; the other was resuspended in 1 ml of a 45% (v/v) Percoll solution made up in a Krebs solution (composition 140 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM EDTA, 10 mM glucose, pH

7.4). After centrifugation at 14,000 x *g* for 2 min at 4 °C, the top layer was removed (synaptosomal fraction), washed in 2 ml Krebs solution and centrifuged at 14,000 x *g* for 2 min at 4°C. The pellet corresponds to nerve terminals and was resuspended in the SDS-PAGE buffer (see below) for Western blot analysis. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce).

Sub-synaptic fractionation

The separation of the presynaptic active zone, postsynaptic density, and non-synaptic fractions from striatal or hippocampi nerve terminals was carried out as previously described (Rebola et al., 2003, 2005; Rodrigues et al., 2005), based on a method developed by Phillips and co-workers (2001). Briefly, synaptosomes were diluted 1:10 in cold 0.1 mM $CaCl_2$ and an equal volume of 2x solubilization buffer (2% Triton x-100, 40 mM Tris, pH 6.0) was added to the suspension. The membranes were incubated for 30 min on ice with mild agitation and the insoluble material (synaptic junction) pelleted (40,000 x *g* for 30 min 4 °C). The supernatant (extrasynaptic fraction) was decanted and proteins precipitated with six volumes of acetone at -20 °C and recovered by centrifugation (18,000 x *g* for 30 min at -15 °C). The synaptic junctions pellet was washed in a solubilization buffer (pH 6.0) and resuspended in 10 volumes of a second solubilization buffer (1% Triton X-100, 20 mM Tris but at pH 8.0). This increase in pH allows the dissociation of the extracellular matrix that maintains the presynaptic active zone tightly bound to the postsynaptic density (see Phillips et al., 2001). Hence, the active zone is solubilized whereas the postsynaptic density is essentially preserved because the amount of detergent is not enough for its solubilization (Phillips et al., 2001). After incubation for 30 min on ice with mild agitation, the mixture was centrifuged and the supernatant (presynaptic fraction) processed as described for the extracellular fraction. The pellets from the supernatants and the final insoluble pellet (postsynaptic fraction) were solubilized in the SDS-PAGE buffer (see below) for Western blot analysis and the protein concentration determined by the bicinchoninic acid protein assay (Pierce). As previously reported (Rebola et al., 2003, 2005; Rodrigues et al., 2005), this fractionation procedure allows an effective separation (over 90% efficiency) of markers of presynaptic (e.g. SNAP 25), postsynaptic (e.g. PSD-95) and non-synaptic (e.g. synaptophysin) fractions, and can be used to access the sub-synaptic distribution of proteins of interest by Western blot analysis.

Western blot analysis

The analysis of the enrichment of NTPDase1, 2, 3, and ecto-5'-nucleotidase in synapses was assessed by comparing their immunoreactivities in total and synaptosomal membranes, whereas the sub-synaptic distribution of ecto-nucleotidases was assessed by comparing their immunoreactivities in the different sub-synaptic fractions. This was carried out by Western blot analysis using antibodies against the different ecto-nucleotidases, which selectivity has been extensively described in previous studies (Sevigny et al., 2002; Koszalka et al., 2004;Kishore et al., 2005; Vekaria et al., 2005). For each of the antibodies, we defined their sensitivities using different dilutions and different amounts of loaded proteins for each of the membrane fractions analysed.

Western blot analysis was carried out as previously described (Rebola et al., 2003, 2005; Rodrigues et al., 2005). Briefly, after determining the amount of protein, each sample was diluted in two volumes SDS-PAGE buffer (final concentration of 2 μg/μl) containing 8 M urea, 2% (w/v) sodium dodecyl sulphate, and 375 mM Tris–HCl pH 6.8 and incubated for 10 min at 98 °C. The samples and the pre-stained molecular weight markers (Amersham) were separated by SDS-PAGE (7.5% with a 4% concentrating gel) and electro-transferred to polyvinylidene difluoride membranes (0.45 μm, from Amersham). After blocking for 1 h at room temperature with 3% bovine serum albumine (BSA) in Tris-buffered saline, pH 7.6 containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight at 4 °C with either guinea-pig anti-mouse NTPDase1 (mN1-2c-I5), rabbit anti-mouse NTPDase2 (mN2-36I-I6), rabbit anti-rat NTPDase3 (mN3-1C-I4) or rabbit anti-5'-nucleotidase (all supplied by Jean Sévigny, Univ.Laval, Canada and used at a 1:1,000 dilution). After three washing periods for 15 min with TBS-T, the membranes were incubated with the alkaline phosphatase-conjugated anti-guinea pig or anti-rabbit secondary antibodies (1:2,000 dilution, from Amersham) in TBS-T containing 3% BSA during 90 min at room temperature. After three 15 min-washes in TBS-T, the membranes were incubated with enhanced chemi-fluorescence during 30 sec and then analyzed with a VersaDoc 3000 (Biorad). The membranes were then stripped (incubation for 1 h with 0.1 M glycine, pH 7.2) and re-probed for β-actin immunoreactivity (anti-β-actin antibody, 1:10,000, from Sigma) to confirm that similar amounts of protein were applied to the gels (only for total and synaptosomal membranes).

Immunocytochemical analysis

The double labelling immunocytochemical analysis of hippocampal and striatal nerve terminals to quantify the localization of ecto-nucleotidases in glutamatergic and GABAergic terminals was performed essentially as previously described (Rebola et al., 2005; Rodrigues et al., 2005). Nerve terminals from the rat hippocampus were purified through a discontinuous Percoll gradient (see Rebola et al., 2005; Rodrigues et al., 2005) and placed onto coverslips previously coated with poly-l-lysine, fixed with 4% paraformaldehyde for 15 min and washed twice with PBS medium (140 mM NaCl, 3 mM KCl, 20 mM NaH_2PO_4, 15 mM KH_2PO_4, pH 7.4). The synaptosomes were permeabilized in PBS with 0.2% Triton X-100 for 10 min, blocked for 1 h in PBS with 3% BSA and 5% rat serum and washed twice with PBS. Synaptosomes were then incubated with either guinea-pig anti-mouse NTPDase1 (mN1-2c-I5), rabbit anti-mouse NTPDase2 (mN2-36I-I6), rabbit anti-rat NTPDase3 (mN3-1C-I4) or rabbit anti-5'-nucleotidase (all supplied by Jean Sévigny, Univ.Laval, Canada and used at a 1:200 dilution) and either chicken anti-vesicular GABA transporters (anti-vGAT, 1:1,000) or chicken anti-vesicular glutamate transporters type 1 (anti-vGluT1, 1:5,000; Alpha Diagnostic) for 1 h at room temperature. After washing with 3% BSA, synaptosomes were incubated for 1 h at room temperature with the appropriate secondary antibodies, namely goat anti-chicken IgG (labelled with AlexaFluor-488 green), goat anti-rabbit and goat anti-guinea pig IgG (both labelled with AlexaFluor-598 red, all 1:200, from Invitrogen). We confirmed that none of the secondary antibodies produced any signal in preparations to which the addition of the corresponding primary antibody was omitted. We also confirmed that the individual signals in double-labelled fields were not enhanced over the signals under single-labelling conditions. After washing and mounting on slides with Prolong Antifade, the preparations were visualized in a Zeiss Axiovert 200 inverted fluorescence microscope equipped with a cooled CCD camera and analyzed with MetaFluor 4.0 software. Each coverslip (three to four per experiment) was analyzed by counting three different fields and in each field a minimum of 50 individualized elements. The values are presented as the percentage of the total number of glutamatergic or GABAergic terminals (i.e. immunopositive for the vGluT or vGAT, respectively) that were labelled with each of the ecto-nucleotidases and are displayed as mean±S.E.M. of n experiments (i.e. in preparation obtained from different rats).

Statistics

Data are represented by mean ± S.E.M. values of n experiments. Significance was considered at $P<0.05$ using the paired Student's *t* test for experiments of subcellular localization, the Student's *t* test for immunocytochemistry experiments and the one way ANOVA followed by Newman-Keuls multiple comparison test for experiments of subsynaptic fractions.

RESULTS

Synaptic localization of NTPDase1, 2, 3 and ecto-5'-nucleotidase in hippocampus and striatum

To investigate the synaptic localization of NTPDase1, 2, 3, and ecto-5'-nucleotidase, we started testing if there was an enrichment of these ecto-nucleotidases in nerve terminals in either hippocampus or striatum. Thus, we compared the immunoreactivity of these ecto-nucleotidases in total membranes and in purified synaptosomal membranes in each of these brain regions.

As illustrated in the representative Western blots of hippocampal membranes (Figure 1A), the antibodies used recognised proteins with the expected molecular weight of NTPDase1 (75 kDa with an additional band at 55 kDa), NTPDase2 (72 kDa with an additional faint band at 40 kDa), NTPDase3 (single 78 kDa band) and ecto-5'-nucleotidase (65 kDa with additional bands at 60 kDa and 52 kDa). Furthermore, all ecto-nucleotidases were found in synaptosomal membranes, indicating that all ecto-nucleotidases are located at or near synapses. Figure 1B shows that NTPDase1 immunoreactivity was enriched (1.62±0.28 fold, n=4) in synaptosomal compared to total membranes, the same occurring with NTPDase3 (2.05±0.28 fold, n=4), whereas the density of NTPDase2 (1.11±0.06 fold, n=3) and ecto-5'-nucleotidase (0.94±0.20 fold, n=5) was similar in synaptosomal and total membranes.

As in the hippocampus, all the ecto-nucleotidases were also present in striatal nerve terminals, but only some were found to be enriched in synaptosomal compared to total membranes of the rat striatum. As shown in Figure 1C, the immunoreactivity of NTPDase1 (2.26±0.48 fold, n=4), NTPDase3 (2.30±0.38 fold, n=4) and ecto-5'-nucleotidase (1.77±0.39 fold, n=4) was larger in synaptosomal membranes compared to total membranes, whereas NTPDase2 was present but not enriched (1.11±0.21 fold, n=3) in synaptosomal membranes from the rat striatum.

Sub-synaptic lacalization of NTPDase1, 2, 3 and ecto-5'-nucleotidase in striatum and hippocampus

Knowing that NTPDase1, 2, 3, and 5'-nucleotidase were located in nerve terminals, we next attempted to define the sub-synaptic localization of these ecto-nucleotidases. We took advantage of a fractionation method that allows the separation of the pre-synaptic and post-synaptic components of the active zone with over 90% efficiency (Phillips et al., 2001).We now confirmed the quality of the separation by showing that a marker of the post-synaptic density (PSD-95) was nearly restricted to the post-synaptic fraction, a marker of the pre-synaptic active zone, SNAP-25 was not present in the post-synaptic density and a marker of extra-synaptic fraction synaptophysin (a protein located in synaptic vesicles) was nearly restricted to the extra-synaptic fraction (data not shown).

In the hippocampus (n=3), we found that NTPDase1 and ecto-5'-nucleotidase were evenly distributed pre- and post-synaptically and displayed a lower density in peri-synaptic regions whereas NTPDase2 and 3 were mostly located post-synaptically (Fig. 2A). A similar sub-synaptic distribution was found in the striatum (n=3), where NTPDase 1 and ecto-5'-nucleotidase were also evenly distributed pre- and post-synaptically and displayed a lower density in peri-synaptic regions whereas NTPDase3 was mostly located post-synaptically (Fig. 2B); however, NTPDase2 displayed a different sub-synaptic distribution since it was evenly distributed pre- post- and peri-synaptically in the striatum (Fig. 2B).

Different association of NTPDase1, 2, 3, and ecto-5'-nucleotidase with glutamatergic and GABAergic terminals in striatum and hippocampus

We then investigated if the different ecto-nucleotidases were differently associated with the two main types of nerve terminals in the hippocampus and striatum, namely inhibitory GABAergic nerve terminals and excitatory glutamatergic nerve terminals. This was assessed by investigating the double labelling of hippocampal or striatal nerve terminals with either NTPDase1, 2, 3, or 5'-nucleotidase together with either vesicular glutamate transporter type 1 (vGluT1, a marker of glutamatergic nerve terminals) or vesicular GABA transporter (vGAT, a marker of GABAergic nerve terminals).

As shown in the representative experiment displayed in Figure 3A, there was a striking segregation of NTPDase1 and 3 to GABAergic and glutamatergic terminals of the rat hippocampus. In fact, NTPDase1 immunoreactivity was mostly associated with vGAT-, but not with vGluT1-labelled terminals (left panels of Fig. 3A) whereas NTPDase3 immunoreactivity was mostly associated with

vGlut1, but not with vGAT-labelled hippocampal nerve terminals (right panels of Fig. 3A). We also found that ecto-5'-nucleotidase was more abundant in glutamatergic than GABAergic terminals, whereas NTPDase2 was associated with a smaller number of either glutamatergic or GABAergic terminals from the rat hippocampus (Fig. 3B). This segregation of ecto-nucleotidases in different nerve terminals was also observed in the striatum (Fig. 3C). Thus, NTPDase1 was mostly associated with GABAergic terminals whereas NTPDase3 and ecto-5'-nucleotidase were mostly associated with glutamatergic terminals of the rat striatum (Fig. 3C). Also, as observed in the hippocampus, NTPDase2 was associated with a smaller number of either glutamatergic or GABAergic terminals from the rat hippocampus (Fig. 3C).

DISCUSSION

The present study provides the first demonstration for the presence of different molecularly identified ecto-nucleotidases in hippocampal and striatal synapses. It shows that all plasma-membrane ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) expressed in the brain, namely NTPDase1, 2, and 3 (since NTPDase8 is not expressed in the brain, see Bigonnesse et al., 2004) are present at these synapses, albeit with different levels of enrichment. In fact, we found that the density of NTPDase1 and 3 is more abundant in synapses than in total membranes whereas NTPDase2 is also found in synapses but with a density similar to that found in total membranes of the hippocampus and striatum. Finally, we found that ecto-5'-nucleotidase is also present in synapses, thus showing all the enzymes required for the conversion of ATP into adenosine can be found in hippocampal and striatal synapses.

Previous studies exploring the distribution of ecto-nucleotidases in brain tissue have largely been unable to identify which ecto-nucleotidases were present at synapses (e.g. Belcher et al., 2006; Bjelobaba et al., 2007; Langer et al., 2008). This is largely due to the fact that epitopes present at synapses have low accessibility to antibodies. This is best exemplified by the conclusion from immunhistochemical studies that inhibitory A_1 receptors were mostly present in axons rather than in nerve terminals (Swanson et al., 1995), which is in contradiction with autoradiography studies using a radio-labelled A_1 receptor ligand (e.g. Tetzlaff et al., 1987) and with the wealth of evidence showing that inhibitory A_1 receptors mostly act pre- and post-synaptically to control synaptic transmission (reviewed in Fredholm et al., 2005). Only when using fractionated nerve terminals, following a

strategy similar to that used in the present study, it was possible to show that A_1 receptors are effectively enriched in nerve terminals (Rebola et al., 2003). Likewise, immunohistochemical studies have established the dogma that facilitatory A_{2A} receptors were largely only present in striatal medium spiny (e.g. Rosin et al., 1998); again, only the use of fractionated nerve terminals allowed showing that these A_{2A} receptors can be located in hippocampal synapses (Rebola et al., 2005), where they play a key role in controlling synaptic plasticity (Rebola et al., 2008). The present study, applying the use fractionated nerve terminals, also identified some key differences in the synaptic distribution of ecto-nucleotidases that were not previously appreciated on the basis of immunohistochemical studies. Thus, we now observed that NTPDase3 displayed a robust enrichment in synapses of the rat hippocampus and striatum, which is in contrast to the previously observed low density of NTPDase3 outside the mid- and hindbrain (Belcher et al., 2006). Likewise, this study also identified NTPDase1 as a major synaptically-located ecto-nucleotidase whereas previous studies largely identified the presence of this enzyme in fiber tracts (Bjelobaba et al., 2007). Interestingly, enzyme histochemical staining in brain sections identified an intense ATP degradation mostly in the neuropil that was abrogated in NTPDase1 knockouts (Langer et al., 2008), showing that histological techniques that are expected to be less prone to accessibility problems indeed identified NTPDase1 in synapses. Likewise, the use of fractionated nerve terminals confirmed previous studies showing that ecto-5'-nucleotidase could also be located in synapses (Bernstein et al., 1978; Zimmermann et al., 1993; Cunha et al., 2000) and now showed that the density of ecto-5'-nucleotidase in synapses is at least as intense as in glial cells where it was proposed to be mostly located (Kreutzberg et al., 1986).

The present study also revealed a particular association of different ecto-nucleotidases with different types of nerve terminals both in the hippocampus and striatum. In fact, it was now observed that NTPDase1 was mostly associated with GABAergic nerve terminals whereas NTPDase3 and ecto-5'-nucleotidase were mostly associated with glutamatergic nerve terminals. This scenario is in remarkable agreement with the current knowledge of purinergic modulation of glutamatergic and GABAergic synapses. In fact, it has been reported that ATP affects glutamatergic synapses through P2 receptors to control the release of neurotransmitters (e.g. Khakh and Henderson, 1998; Rodrigues et al., 2005; León et al., 2008) and the control of synaptic plasticity (e.g. Pankratov et al., 2002; Almeida et al., 2003; Sim et al., 2006), a signalling expected to be terminated by a synaptically-located NTPDase. However, glutamatergic synapses are also under a tight control by adenosine and

it has been shown that some of this adenosine is locally generated by the activity of ecto-nucleotidases (Cunha et al., 1996; Rebola et al., 2008). In fact, the extracellular formation of adenosine from ATP displays channelling properties (Cunha et al., 1998), occurring within milliseconds (Dunwiddie et al., 1997). Therefore, functional results made it expectable that both an NTPDase as well as ecto-5'-nucleotidase should be present in glutamatergic synapses. In contrast, GABAergic synapses are known to be controlled by ATP acting through P2 receptors (Hugel and Schlichter, 2000; Gómez-Villafuertes et al., 2001; Donato et al., 2008; Xiao et al., 2008), but there is no clear evidence for a modulation by adenosine in GABAergic synapses (reviewed in Fredholm et al., 2005). Thus, in GABAergic synapses one would expect to find an NTPDase able to degrade ATP and ADP (both P2 receptor ligands) whereas one would not foresee the need for an enzymatic activity devoted to the controlled local formation of adenosine. In accordance with this scenario, we indeed found that GABAergic synapses are mostly endowed with NTPDase1, whereas glutamatergic synapses posses both NTPDase3 and ecto-5'-nucleotidase.

The presently observed enrichment of NTPDases and ecto-5'-nucleotidease in hippocampal and striatal synapses further reinforces the importance of the localised purinergic signalling in synapses. In fact, there are several studies showing the importance of extracellular ATP in the control of synaptic plasticity, either through the activation of P2 receptors (e.g. Pankratov et al., 2002; Almeida et al., 2003; Sim et al., 2006) or through the action of ecto-protein kinases (e.g. Fujji et al., 2002; Erlich et al., 1996). In both situations, synaptic ecto-nucleotidases should determine the timing of this ATP signalling system. In parallel, ATP-derived adenosine formation is expected to occur in a confined manner in activated synapses to direct the activation of adenosine A_{2A} receptors that sustains some forms of synaptic plasticity (reviewed in Cunha, 2008) both in the hippocampus (Rebola et al., 2008) as well as in the striatum (D'Alcantara et al., 2001). Thus, this synaptic ecto-nucleotidase pathway controls both ATP signalling and the timing of adenosine signalling within a synapse. The importance of these synaptic ecto-nucleotidases is further emphasised by numerous observations showing that there the activities of synaptic ecto-nucleotidases are modified both upon physiological adaptation (e.g. Cunha et al., 2001; Pereira et al., 2002; Pedrazza et al., 2007) and upon pathological conditions (e.g. Schetinger et al., 1998; Bonan et al., 2000; Bruno et al., 2005; Cognato et al., 2007; Duarte et al., 2007). Thus, the present identification of the molecular nature of the ecto-nucleotidases present in hippocampal and striatal synapses paves the way for a more detail

understanding of the physio-pathological role of these ecto-nucleotidases in the control of purinergic signalling.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net, #01.06.0842-00) and Fundação para a Ciência e Tecnologia (PTDC/SAU-NEU/74318/2006) and was made possible by a joint Portuguese-Brazilian grant (CAPES-GRICES). We thank Pedro Garção for his help in preparing some of the sub-synaptic fractions and coverslips for immunocytochemical analysis.

References

Almeida T, Rodrigues RJ, de Mendonça A, Ribeiro JA, Cunha RA (2003) Purinergic P_2 receptors trigger adenosine release leading to adenosine A_{2A} receptor activation and facilitation of long-term potentiation in rat hippocampal slices. Neuroscience 122: 111-121.

Battastini AMO, Rocha JBT, Barcellos CK, Dias RD, Sarkis JJF (1991) Characterization of an ATP diphosphohydrolase (EC 3.6.1.5) in synaptosomes from cerebral cortex of adult rats. Neurochem Res 16: 1303-1310.

Belcher SM, Zsarnovszky A, Crawford PA, Hemani H, Spurling L, Kirley TL (2006) Immunolocalization of ecto-nucleoside triphosphate diphosphohydrolase 3 in rat brain: implications for modulation of multiple homeostatic systems including feeding and sleep-wake behaviors. Neuroscience 137: 1331-1346.

Bernstein H.-G., Weiß J., Luppa H. (1978) Cytochemical investigations on the localization of 5'-nucleotidase in the rat hippocampus with special reference to synaptic regions. Histochemistry 55: 261-267.

Bigonnesse F, Lévesque SA, Kukulski F, Lecka J, Robson SC, Fernandes MJ, Sévigny J (2004) Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-8. Biochemistry 43: 5511-5519.

Bjelobaba I, Stojiljkovic M, Pekovic S, Dacic S, Lavrnja I, Stojkov D, Rakic L, Nedeljkovic N (2007) Immunohistological determination of ecto-nucleoside triphosphate diphosphohydrolase1 (NTPDase1) and 5′-nucleotidase in rat hippocampus reveals overlapping distribution. Cell Mol Neurobiol 27: 731-743.

Bonan CD, Walz R, Pereira GS, Worm PV, Battastini AM, Cavalheiro EA, Izquierdo I, Sarkis JJ (2000) Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy. Epilepsy Res 39: 229-238.

Braun N, Sévigny J, Robson SC, Enjyoji K, Guckelberger O, Hammer K, Di Virgilio F, Zimmermann H (2000) Assignment of ecto-nucleoside triphosphate diphosphohydrolase-1/cd39 expression to microglia and vasculature of the brain. Eur J Neurosci 12:4357-4366.

Braun N, Sévigny J, Mishra SK, Robson SC, Barth SW, Gerstberger R, Hammer K, Zimmermann H (2003) Expression of the ecto-ATPase NTPDase2 in the germinal zones of the developing and adult rat brain. Eur J Neurosci 17:1355-1364.

Bruno AN, Diniz GP, Ricachenevsky FK, Pochmann D, Bonan CD, Barreto-Chaves ML, Sarkis JJ (2005) Hypo-and hyperthyroidism affect the ATP, ADP and AMP hydrolysis in rat hippocampal and cortical slices. Neurosci Res 52: 61-68.

Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 87: 659-797.

Chen W, Wieraszko A, Hogan MV, Yang HA, Kornecki E, Ehrlich YH (1996) Surface protein phosphorylation by ecto-protein kinase is required for the maintenance of hippocampal long-term potentiation. Proc Natl Acad Sci USA 93: 8688-8693.

Cognato Gde P, Bruno AN, da Silva RS, Bogo MR, Sarkis JJ, Bonan CD (2007) Antiepileptic drugs prevent changes induced by pilocarpine model of epilepsy in brain ecto-nucleotidases. Neurochem Res 32:1046-1055.

Cunha RA (2001) Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals. Neurochem Res 26: 979-991.

Cunha RA (2008) Different cellular sources and different roles of adenosine: A_1 receptor-mediated inhibition through astrocytic-driven volume transmission and synapse-restricted A_{2A} receptor-mediated facilitation of plasticity. Neurochem Int 52: 65-72.

Cunha RA, Ribeiro JA (2000) ATP as a presynaptic modulator. Life Sci 68: 119-137.

Cunha RA, Sebastião AM, Ribeiro JA (1992) Ecto-5'-nucleotidase is associated with cholinergic nerve terminals in the hippocampus but not in the cerebral cortex of the rat. J Neurochem 59: 657-666.

Cunha RA, Correia-de-Sá P, Sebastião AM, Ribeiro JA (1996) Preferential activation of excitatory adenosine receptors at rat hippocampal and neuromuscular synapses by adenosine formed from released adenine nucleotides. Br J Pharmacol 119: 253-260.

Cunha RA, Sebastião AM, Ribeiro JA (1998) Inhibition by ATP of hippocampal synaptic transmission requires localized extracellular catabolism by ecto-nucleotidases into adenosine and channeling to adenosine A_1 receptors. J Neurosci 18: 1987-1995.

Cunha RA, Brendel P, Zimmermann H, Ribeiro JA (2000) Immunologically distinct isoforms of ecto-5'-nucleotidase in nerve terminals of different areas of the rat hippocampus. J Neurochem 74: 334-338.

Cunha RA, Almeida T, Ribeiro JA (2001) Parallel modification of adenosine extracellular metabolism and modulatory action in the hippocampus of aged rats. J Neurochem 76: 372-382.

d'Alcantara P, Ledent C, Swillens S, Schiffmann SN (2001) Inactivation of adenosine A_{2A} receptor impairs long term potentiation in the accumbens nucleus without altering basal synaptic transmission. Neuroscience 107: 455-464.

Donato R, Rodrigues RJ, Takahashi M, Tsai MC, Soto D, Miyagi K, Villafuertes RG, Cunha RA, Edwards FA (2008) GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx. Cell Calcium 44: 521-532.

Duarte JM, Oses JP, Rodrigues RJ, Cunha RA (2007) Modification of purinergic signaling in the hippocampus of streptozotocin-induced diabetic rats. Neuroscience 149: 382-391.

Dunwiddie TV, Diao L, Proctor WR (1997) Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. J Neurosci 17:7673-7682.

Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM (2005) Adenosine and brain function. Int Rev Neurobiol 63: 191-270.

Fujii S, Kato H, Kuroda Y (2002) Cooperativity between extracellular adenosine 5'-triphosphate and activation of N-methyl-D-aspartate receptors in long-term potentiation induction in hippocampal CA1 neurons. Neuroscience 113: 617-628.

Gómez-Villafuertes R, Gualix J, Miras-Portugal MT (2001) Single GABAergic synaptic terminals from rat midbrain exhibit functional P2X and dinucleotide receptors, able to induce GABA secretion. J Neurochem 77: 84-93.

Hugel S, Schlichter R (2000) Presynaptic P2X receptors facilitate inhibitory GABAergic transmission between cultured rat spinal cord dorsal horn neurons. J Neurosci 20: 2121-2130.

Inoue K, Koizumi S, Tsuda M (2007) The role of nucleotides in the neuron-glia communication responsible for the brain functions. J Neurochem 102: 1447-1458.

James S, Richardson PJ (1993) Production of adenosine from extracellular ATP at the striatal cholinergic synapse. J Neurochem 60: 219–227.

Khakh BS, Henderson G (1998) ATP receptor-mediated enhancement of fast excitatory neurotransmitter release in the brain. Mol Pharmacol 54: 372-378.

Kishore BK, Isaac J, Fausther M, Tripp SR, Shi H, Gill PS, Braun N, Zimmermann H, Sévigny J, Robson SC (2005) Expression of NTPDase1 and NTPDase2 in murine kidney: relevance to regulation of P2 receptor signaling. Am J Physiol 288: F1032-F1043.

Koszalka P, Ozüyaman B, Huo Y, Zernecke A, Flögel U, Braun N, Buchheiser A, Decking UK, Smith ML, Sévigny J, Gear A, Weber AA, Molojavyi A, Ding Z, Weber C, Ley K, Zimmermann H, Gödecke A, Schrader J (2004) Targeted disruption of cd73/ecto-5'-nucleotidase alters thromboregulation and augments vascular inflammatory response. Circ Res 95: 814-821.

Kreutzberg GW, Heymann D, Reddington M (1986) 5'-Nucleotidase in the nervous system. In 'Cellular Biology of Ectoenzymes' (Kreutzberg GW, Reddington M, Zimmermann H, Eds), pp.147-164, Springer Verlag, Berlin.

Kukulski F, Sévigny J, Komoszyński M (2004) Comparative hydrolysis of extracellular adenine nucleotides and adenosine in synaptic membranes from porcine brain cortex, hippocampus, cerebellum and medulla oblongata. Brain Res 1030: 49-56.

Langer D, Hammer K, Koszalka P, Schrader J, Robson S, Zimmermann H (2008) Distribution of ectonucleotidases in the rodent brain revisited. Cell Tissue Res 334: 199-217.

León D, Sánchez-Nogueiro J, Marín-García P, Miras-Portugal MA (2008) Glutamate release and synapsin-I phosphorylation induced by P2X7 receptors activation in cerebellar granule neurons. Neurochem Int 52: 1148-1159.

Nagy AK, Shuster TA, Delgado-Escueta AV (1986) Ecto-ATPase of mammalian synaptosomes: identification and enzymic characterization. J Neurochem 47: 976-986

Nedeljkovic N, Banjac A, Horvat A, Stojiljkovic M, Nikezic G (2003) Ecto-ATPase and ecto-ATP-diphosphohydrolase are co-localized in rat hippocampal and caudate nucleus synaptic plasma membranes. Physiol Res 52: 797-804.

Pankratov YV, Lalo UV, Krishtal OA (2002) Role for P2X receptors in long-term potentiation. J Neurosci 22: 8363-8369.

Pedrazza EL, Riboldi GP, Pereira GS, Izquierdo I, Bonan CD (2007) Habituation to an open field alters ecto-nucleotidase activities in rat hippocampal synaptosomes. Neurosci Lett 413: 21-24.

Pereira GS, Mello e Souza T, Battastini AM, Izquierdo I, Sarkis JJ, Bonan CD (2002) Effects of inhibitory avoidance training and/or isolated foot-shock on ectonucleotidase activities in synaptosomes of the anterior and posterior cingulate cortex and the medial precentral area of adult rats. Behav Brain Res 128: 121-127.

Phillips GR, Huang JK, Wang Y, Tanaka H, Shapiro L, Zhang W, Shan WS, Arndt K, Frank M, Gordon RE, Gawinowicz MA, Zhao Y, Colman DR (2001) The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. Neuron 32: 63-77.

Rebola N, Pinheiro PC, Oliveira CR, Malva JO, Cunha RA (2003) Subcellular localization of adenosine A_1 receptors in nerve terminals and synapses of the rat hippocampus. Brain Res 987: 49-58.

Rebola N, Canas PM, Oliveira CR, Cunha RA (2005) Different synaptic and subsynaptic localization of adenosine A_{2A} receptors in the hippocampus and striatum of the rat. Neuroscience 132: 893-903.

Rebola N, Lujan R, Cunha RA, Mulle C (2008) Adenosine A_{2A} receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses. Neuron 57: 121-134.

Rosin DL, Robeva A, Woodard RL, Guyenet PG, Linden J (1998) Immunohistochemical localization of adenosine A_{2A} receptors in the rat central nervous system. J Comp Neurol 401: 163-186.

Rodrigues RJ, Almeida T, Richardson PJ, Oliveira CR, Cunha RA (2005) Dual presynaptic control by ATP of glutamate release via facilitatory P2X1, P2X2/3 and P2X3, and inhibitory P2Y1, P2Y2 and/or P2Y4 receptors in the rat hippocampus. J Neurosci 27: 6286-6295.

Schetinger MR, Bonan CD, Schierholt RC, Webber A, Arteni N, Emanuelli T, Dias RD, Freitas Sarkis JJ, Netto CA (1998) Nucleotide hydrolysis in rats submitted to global cerebral ischemia: A possible link between preconditioning and adenosine production. J Stroke Cerebrovasc Dis 7: 281-286.

Sévigny J, Sundberg C, Braun N, Guckelberger O, Csizmadia E, Qawi I, Imai M, Zimmermann H, Robson SC (2002) Differential catalytic properties and vascular topography of murine nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) and NTPDase2 have implications for thromboregulation. Blood 99: 2801-2809.

Sim JA, Chaumont S, Jo J, Ulmann L, Young MT, Cho K, Buell G, North RA, Rassendren F (2006) Altered hippocampal synaptic potentiation in P2X4 knock-out mice. J Neurosci 26: 9006-9009.

Swanson TH, Drazba JA, Rivkees SA (1995) Adenosine A1 receptors are located predominantly on axons in the rat hippocampal formation. J Comp Neurol 363: 517-531.

Tetzlaff W, Schubert GW, Kreutzberg GW (1987) Synaptic and extrasynaptic localization of adenosine binding sites in the rat hippocampus. Neuroscience 21: 869–875.

Vekaria RM, Shirley DG, Sévigny J, Unwin RJ (2006) Immunolocalization of ectonucleotidases along the rat nephron. Am J Physiol 290: F550-F5660.

Wang TF, Guidotti G (1998) Widespread expression of ecto-apyrase (CD39) in the central nervous system. Brain Res 790: 318-322.

Wink MR, Braganhol E, Tamajusuku AS, Lenz G, Zerbini LF, Libermann TA, Sévigny J, Battastini AM, Robson SC (2006) Nucleoside triphosphate diphosphohydrolase-2 (NTPDase2/CD39L1) is the dominant ectonucleotidase expressed by rat astrocytes. Neuroscience 138:421-432.

Xiao C, Zhou C, Li K, Davies DL, Ye JH (2008) Purinergic type 2 receptors at GABAergic synapses on ventral tegmental area dopamine neurons are targets for ethanol action. J Pharmacol Exp Ther 327: 196-205.

Zimmermann H (2006) Ectonucleotidases in the nervous system. Novartis Found Symp 276: 113-128.

Zimmermann H, Vogel M, Laube U (1993) Hippocampal localization of 5'-nucleotidase as revealed by immunocytochemistry. Neuroscience 55: 105-112.

Fig. 1- Comparison of the density of NTPDase1, 2 and 3 and ecto-5'-nucleotidase in synaptosomal membranes and total membranes from the rat hippocampus and striatum. Panel A shows representative Western blots comparing the immunoreactivity of the different ecto-nucleotidases in synaptosomal membranes (S) and total membranes (T) from the rat hippocampus of the same rat (30 μg protein loaded in each lane). Panel B and C displays the relative average immunoreactivity of NTPDase 1 (N1), NTPDase2 (N2), NTPDase3 (N3), and ecto-5'-nucleotidase (5'-N) in synaptosomal membranes (crossed bars) compared to total membranes (open bars, taken as 100%) of the rat hippocampus (B) and striatum (C). The results are mean±SEM of 3-5 experiments. * $P<0.05$ versus total membranes (paired Student's t test).

Fig. 2- Subsynaptic distribution of NTPDase 1 (N1), NTPDase2 (N2), NTPDase3 (N3), and ecto-5'-nucleotidase (5'-N) in the hippocampus (A) and striatum (B). Each panel displays the average immunoreactivity of the different ecto-nucleotidases in a fraction enriched in the presynaptic active zone (PRE, black bars), in the postsynaptic density (POST, grey bars), in nerve terminals outside the active zone (EXTRA, open bars). These fractions were obtained by pH fractionation, after solubilization, of purified hippocampal or striatal nerve terminals, as described previously (Phillips et al., 2001), allowing an over 90% efficiency of separation of a presynaptic marker (SNAP-25), a post-synaptic marker (PSD-95) and an extra-synaptic marker (synaptophysin) in these fractions (data not shown). Data are mean±SEM of 3 experiments. * $P<0.05$ between the indicated bars (ANOVA followed by Newman-Keuls multiple comparison test).

Fig. 3- Co-localization of NTPDase 1 (N1), NTPDase2 (N2), NTPDase3 (N3), and ecto-5'-nucleotidase (5'-N) in glutamatergic and GABAergic nerve terminals of the rat hippocampus and striatum. Panel A exemplifies the double immunostaining performed, showing the co-localization of NTPDase1 with GABAergic (vGAT-positive) but not glutamatergic (vGluT1/2-positive) terminals (left panels) and the co-localization of NTPDase3 with glutamatergic (vGluT1/2-positive) but not GABAergic (vGAT-positive) terminals (right panels). Panels B and C display the fraction of glutamatergic (filled bars) and GABAergic (open bars) terminals immunoreactive for the different ecto-nucleotidases in hippocampal (B) and striatal (C) nerve terminals. The data are mean±SEM of 3-5 experiments. * $P<0.05$ between the indicated bars (Student's t test).

FIGURE 1

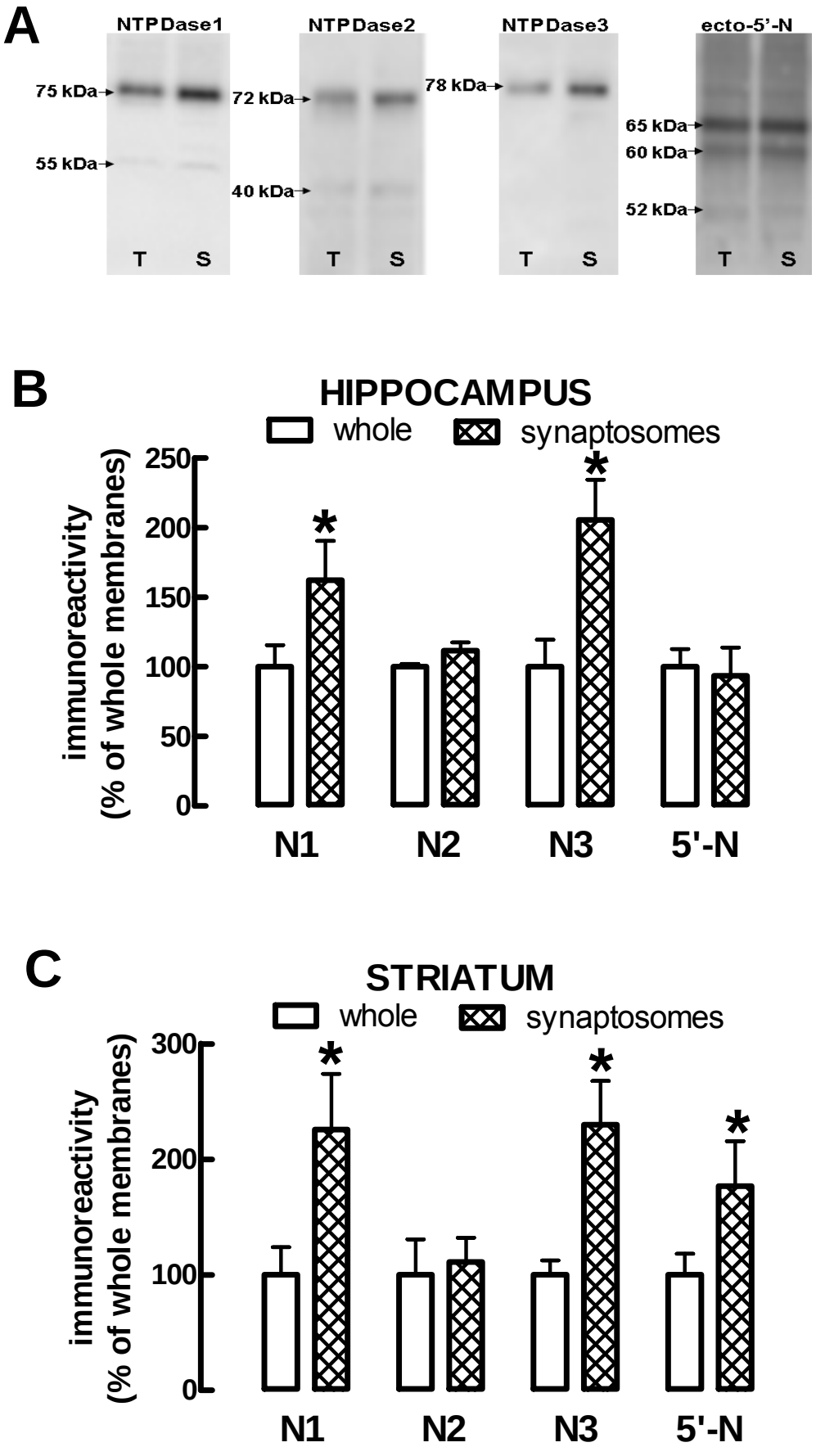

A
NTPDase1
NTPDase2
NTPDase3
ecto-5'-N
75 kDa
55 kDa
72 kDa
40 kDa
78 kDa
65 kDa
60 kDa
52 kDa
T
S
B
HIPPOCAMPUS
whole
synaptosomes
immunoreactivity
(% of whole membranes)
250
200
150
100
50
0
N1
N2
N3
5'-N
C
STRIATUM
whole
synaptosomes
immunoreactivity
(% of whole membranes)
300
200
100
0
N1
N2
N3
5'-N

FIGURE 2

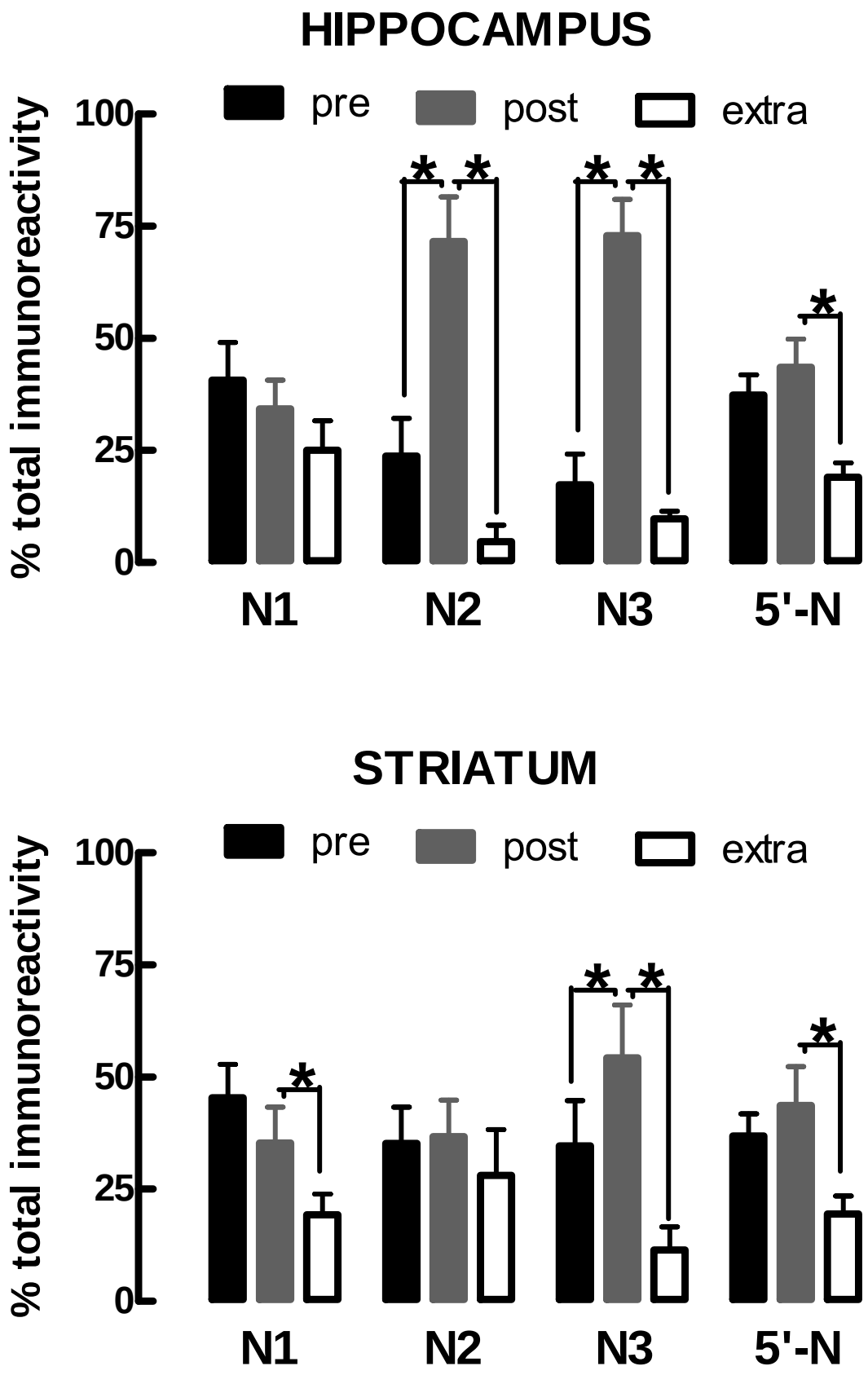
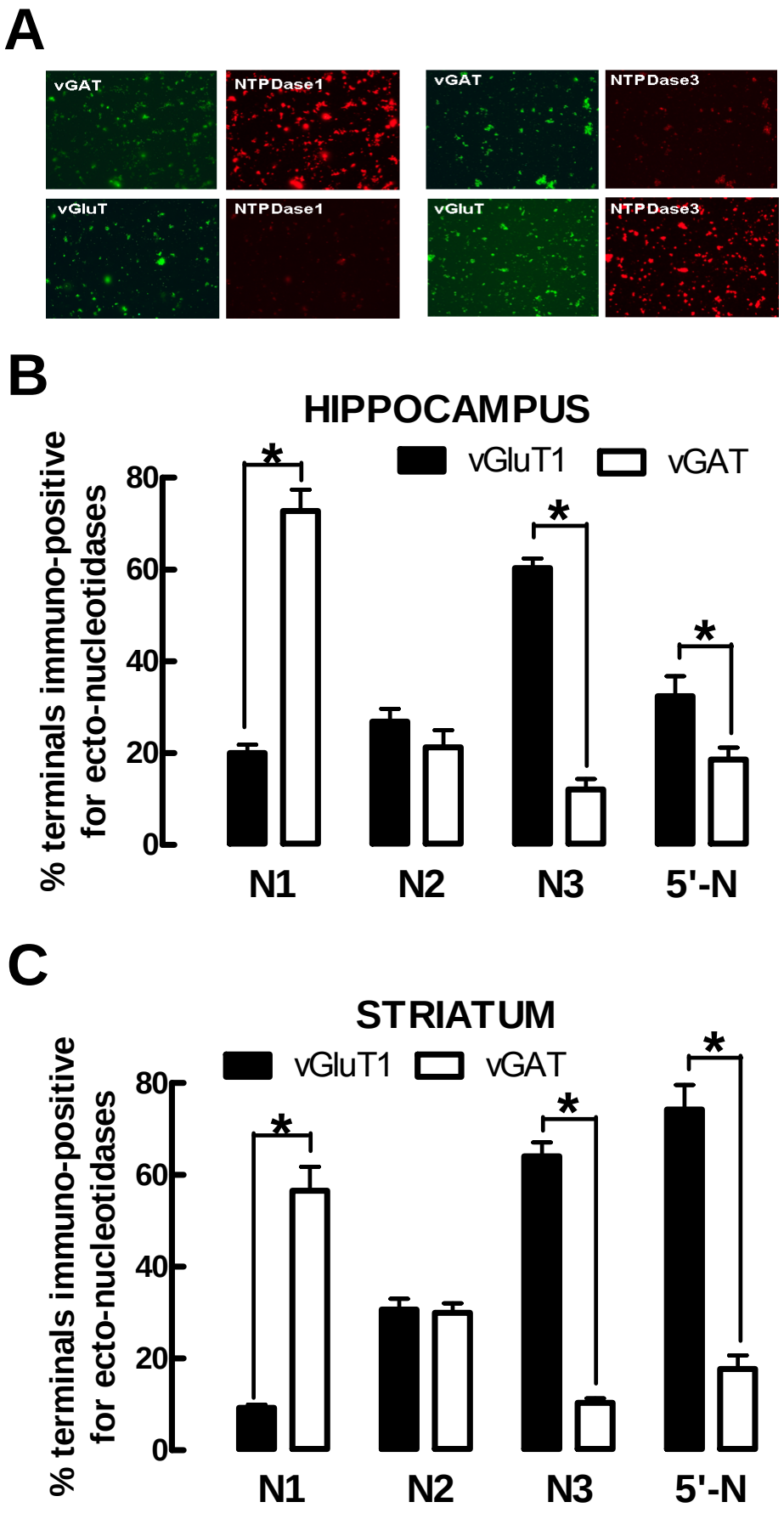

HIPPOCAMPUS
pre
post
extra
% total immunoreactivity
100
75
50
25
0
N1
N2
N3
5'-N
STRIATUM
pre
post
extra
% total immunoreactivity
100
75
50
25
0
N1
N2
N3
5'-N

FIGURE 3


A
vGAT
NTPDase1
vGAT
NTPDase3
vGluT
NTPDase1
vGluT
NTPDase3
B
HIPPOCAMPUS
vGluT1
vGAT
% terminals immuno-positive for ecto-nucleotidases
0
20
40
60
80
*
*
*
N1
N2
N3
5'-N
C
STRIATUM
vGluT1
vGAT
% terminals immuno-positive for ecto-nucleotidases
0
20
40
60
80
*
*
*
N1
N2
N3
5'-N

III. ARTIGOS CIENTÍFICOS

III.3. CAPÍTULO 3 - COGNATO, G.P.; VUADEN, F.C.; SAVIO, L.E.B.; BOGO, M.R.; BONAN, C.D. NTPDases role in the patophysiology of cognitive impairment induced by seizure in early age. Artigo a ser submetido para o periódico International Journal of Developmental Neuroscience.

NTPDases ROLE IN THE PATOPHYSIOLOGY OF COGNITIVE IMPAIRMENT INDUCED BY SEIZURE IN EARLY AGE.

Giana de Paula Cognato[1], Fernanda Cenci Vuaden[1], Luiz Eduardo Baggio Sávio[2], Maurício Reis Bogo[2], Carla Denise Bonan[2#]

[1]Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[2]Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, PUC/RS, Porto Alegre, RS, Brazil

Corresponding authors at: Faculdade de Biociências, Ponticífia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga 6681, Prédio 12D, Sala 340, Caixa Postal 1429, 90619-900 Porto Alegre, RS, Brazil.

Tel: + 55 51 3320 3545x4158; fax: + 55 51 3320 3568.

E-mail address: cbonan@pucrs.br

Abstract

Seizures occur more frequently in the neonatal period and early childhood than any other time in life and there is a high risk of developing cognition and behaviour problems later in life. many data have shown that seizures in young animals lead to later cognitive deficits. There are several evidence that long-term potentiation (LTP) and long-term depression (LTD) could contribute to the neural basis for learning and memory mechanism and could be modulated by ATP or adenosine. ATP may be hydrolyzed to its respective nucleoside adenosine by a cascade of cell-surface-bound enzymes, such as E-NTPDases (ecto-nucleoside triphosphate diphosphohydrolases) and ecto-5´-nucleotidase (Zimmermann, 2001). Thus, we have investigated if hippocampal ectonucleotidase activities are altered at different time periods after one episode of seizure induced by KA in 7 day-old rats. We also have evaluated if 90 day-old rats previously submitted to seizure induced by KA at 7 days of age presented cognitive impairment in Y-maze behaviour task. Our results have shown memory impairment of adult rats (PN90) previously submitted to one single seizure episode in neonatal period (PN7), which is accompanied by an increased ATP hydrolysis in hippocampal synaptosomes. Furthermore, the present results have shown that the relative expression of NTPDases responsible for ATP hydrolysis was not altered by the KA administration in early age. Since we have found an augmented hydrolysis of ATP and this nucleotide seems to be important to LTP induction, we could assume that impairment of memory and learning observed in adult rats which have experience a convulsive episode in postnatal period may be a consequence of the increased ATP hydrolysis activity.These findings correlate the purinergic signaling to the cognitive deficits induced by neonatal seizures and contribute to a better understanding about the mechanisms of seizure-induced memory dysfunction.

Keywords: ATP, LTP, early seizure, NTPDase, 5’-nucleotidase

INTRODUCTION

Seizures occur more frequently in the neonatal period and early childhood than any other time in life (Holmes, 1997). Furthermore, there is general agreement that epilepsy in childhood carries a significant risk for a variety of problems involving cognition and behaviour. To support this idea, many data have shown that prolonged or frequent seizures in young animals lead to later cognitive deficits, which can often be subtle (Holmes, 2004; Cílio et al, 2003; Huang et al, 2002). In fact, one single seizure induced by Kainic acid (KA) in rats at 7 days of age was able to induce impairment on spatial memory in radial and water mazes in adulthood (Sayin et al, 2004). Despite of several studies on this matter, the mechanism responsible for these seizure-related cognitive deficits in the developing brain are not clearly defined.

There is several evidence that long-term potentiation (LTP) and long-term depression (LTD) could contribute to the neural basis for learning and memory mechanism (see Cooke and Bliss, 2006). These synaptic plasticity phenomena could be modulated by ATP or adenosine. Application of extracellular ATP in CA1 neurons of hippocampus was able to induce LTP after 10 minutes (Fujii, 2004). Meanwhile, adenosine could attenuate both LTP and LTD through adenosine A_1 receptors in the same hippocampus CA1 neurons (Mendonça and Ribeiro, 1997). Furthermore, the neuromodulatory effects of this nucleoside remain persistent after the LTP and LTD (Mendonça et al, 2002).

ATP may be hydrolyzed to its respective nucleoside adenosine by a cascade of cell-surface-bound enzymes, such as E-NTPDases (ecto-nucleoside triphosphate diphosphohydrolases) and ecto-5´-nucleotidase (Zimmermann, 2001). Over the years, our laboratory has studied the involvement of ectonucleotidases in epilepsy. An significant increase in nucleotide hydrolysis have been demonstrated in hippocampal synaptosomes of rats submitted to pilocarpine and KA models of epilepsy (Bonan et al, 2000a) as well as kindling induced by Pentylenetetrazole (PTZ) (Bonan et al, 2000b). However, the increase of ATP, ADP, and AMP hydrolysis observed in adult animals was not observed in young rats (Cognato et al, 2005). The enhancement of nucleotide hydrolysis has been also observed in blood serum (Bruno et al., 2003) and cerebrospinal fluid (Oses et al, 2007) of rats submitted to administration of PTZ Thus, in the present work, we have investigated if hippocampal ectonucleotidase activities are altered at different time periods after one episode of seizure induced by KA in 7 day-old rats. The times analysed correspond to the maturation period of

the brain (7, 14 and 21 days of age) and in the adult brain (90 days of age). We also have evaluated if 90 day-old rats previously submitted to seizure induced by KA at 7 days of age presented cognitive impairment in Y-maze behaviour task.

MATERIAL AND METHODS

Reagents

Nucleotides (ATP, ADP and AMP) , Percoll, Trizma base, Malachite Green Base, Coomassie Brilliant Blue G, EDTA, HEPES, and Kainic Acid were purchased from Sigma, St. Louis, MO, USA. Trizol reagent, SuperScript™ III First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) kit, and Taq DNA polymerase were purchased from Invitrogen. All other reagents were of analytical grade.

Animal

Seven day-old male Wistar rats were used in the initial phase of this study. Animals had acces to food and water *ad libitum* and were housed in plastic cages with lights on from 7:00 to 19:00 at room temperature of 23 ± 1°C. To avoid interlitter variations, different treatment groups (saline and KA) as well as the age of analyses of each group (PN7, PN14, PN21, and PN90) were represented in each litter. During the induction of seizure, all pups were transiently separated from their mothers. Procedures for the care and use of animals were adopted according to the regulations of Brazilian College of Animal Experimentation (COBEA), based on the Guide for the Care and Use of Laboratory Animals)National Research Council).

Induction of seizure

Seizure was induced by the systemic administration of KA on postnatal day 7 (PN7). KA was dissolved in saline and administered intraperitoneally (1ml/Kg) to male rats at a dosage of 2 mg/kg. According to previous studies, this dosage reliably induces eletrographic and behaviour seizures (Stafstrom et al, 1992; Lynch et al, 2000). Male littermates, injected with 0.9% (wt/vol) NaCl, served as controls. Seizures were observed and their characteristics noted over a 3-4 hours period. After that, animals were returned to their dams and were allowed to reach the ages used in the analysis of

nucleotide hydrolysis (PN14, PN21, and PN90). Nucleotide hydrolysis of PN7 animals were analysed 12 hours after the injection of KA. Adult animals (PN90) were also evaluated in behaviour tests.

Behavioural Tasks

In order to verify if KA seizures induced at PN7 could lead to later cognitive impairment, we have performed behaviour tasks in adult rats (PN90) previously submitted to seizures in early childhood. Open-field task was applied on PN90. On the next day, rats were submitted to elevated plus maze task. Finally, on PN93, rats performed a memory task in a Y-maze apparatus with an inter-trial interval (ITI) of 2 min. One week later, rats (PN100) were submitted to a Y-maze task with 2 hours of ITI. All behavioural experiments were performed between 9:00 a.m. and 4:00 p.m. (light phase)

Open-field and elevated plus maze tasks

Locomotor activity and exploratory behaviour were monitored by using an open-field apparatus which consisted in a 50×50 cm arena, divided into 4 squares of 25 cm. The proposal of this test was to determine whether the control and experimental groups differed in baseline locomotor activity. The exploratory behaviour of the rats was evaluated by counting the total number of line crossings over a period of 5 min without prior habituation to the arena. In parallel, the number of rearings was also counted.

Evaluation of the anxiety was also tested by using the elevated plus maze. The apparatus consisted in two open arms 50 x 10 cm at right angles to two covered (closed) arms, 50 x 40 x 10 cm. The maze was elevated to a height of 50 cm above the ground, forming an aversive stimulus to animals in the open arms. The animal was placed in the center of the maze with its head facing a closed arm. Rats were tested for 5 min. Entry into a particular arm was defined as the placement of all four feet into the arm. The relative time spent in open versus close arms is a measure of anxiety with anxious rats preferring closed arms.

Y-maze task

The Y-maze is a simple two-trial recognition test for measuring spatial recognition memory in rodents. Y-maze apparatus consisted of three arms (10 cm of width, 50 cm of length and, 20 cm of height) with an angle of 120° between each arm. The three identical arms were randomly designated: start

arm, in which the rat started to explore (always open), novel arm, which was blocked during the 1st trial, but open during the 2nd trial, and other arm (always open). The maze was placed in a separate room with a red light. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed next to the walls of the maze. The Y-maze test consisted of two trials separated by an inter-trial interval (ITI) to assess response to novelty (2 min ITI) and spatial recognition memory (2 h ITI). The first trial (training) had 5 min duration and allowed the rat to explore only two arms (start arm and other arm) of the maze, with the third arm (novel arm) blocked. For the second trial (after ITI), the rat was placed back in the maze in the same starting arm, with free access to all three arms for 5 min. The number of entries and time spent in each arm were analyzed. Data were expressed as percentage of total time spent in arms as well as percentage of total numbers of entries in each arm with S.E.M. of each group.

Subcellular fraction

Animals previously submitted to KA-seizures at 7 days of age were euthanized by decapitation at different ages (PN7, PN14, PN21, and PN90). Their brains were removed and placed in ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, pH 7.5 and 0.1 mM EDTA) and were cut longitudinally. Total hippocampi of both hemispheres were immediately dissected on ice. The total hippocampi were gently homogenized in 5 volumes of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as previously described (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 ml of crude mitochondrial fraction was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15,000 x g for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

Enzyme assays

The reaction medium used to assay the ATP and ADP hydrolysis was essentially as described previously (Battastini et al., 1991). The reaction medium contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1

mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 ml. The synaptosome preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 8C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and the reaction was stopped by the addition of 200 μl 10% trichloroacetic acid. The released inorganic phosphoate (Pi) was measured as previously described (Chan et al., 1986). The reaction medium used to assay the 5'-nucleotidase activity (AMP hydrolysis) contained 10 mM $MgCl_2$, 0.1 M Tris–HCl, pH7.0 and 0.15 M sucrose in a final volume of 200 μl (Heymann et al., 1984). The synaptosome preparation (10–20 μg protein) was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and was stopped by the addition of 200 μl 10% trichloroacetic acid; the released inorganic phosphate (Pi) was measured as previously described (Chan et al., 1986). In all enzyme assays, incubation times and protein concentration were chosen in order to ensure the linearity of the reactions (Battastini et al., 1991; Heymann et al., 1984). Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct non-enzymatic hydrolysis of the substrates. All samples were run in triplicate.

Protein determination

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

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

The expression analysis of NTPDase 1-3 was carried out by a semi-quantitative RT-PCR assay. Rats at PN90 (saline n=4, KA n=5) were euthanized by decapitation, the brains were removed and placed into ice-cold RNAse free water. Hippocampus of both hemispheres were dissected and immediately frozen with liquid nitrogen for storage in - 80°C freezer. The total RNA extraction was performed using Trizol reagent in accordance with the manufacturer instructions. The cDNA species were synthesized with SuperScript SuperScript™ III First-Strand Synthesis System for RT-PCR from 1 μg of total RNA and oligo (dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 50°C. cDNA (1 μL) was used as a template for PCR with specific primers for NTPDase 1, 2, 3, and 5'-nucleotidase. Rat DNA sequences encoding to NTPDase1 (NM_022587.1), NTPDase2 (O35795),

NTPDase3 (NM_178106) was retrieved from GenBank database and aligned using ClustalX program. Regions with low scores of similarity among sequences were used to construct specific primers using Oligos 9.6 program. Each primer was blasted against rat genome in order to confirm its specificity. The strategy adopted to design the primers does not allow cross-amplification. Specific primers were also constructed to 5'-nucleotidase (NM_021576) and β-actin (NM_031144). β-actin-PCR was performed as a control for cDNA synthesis. PCR reactions had a volume of 25 μL using a concentration of 0.4 μM of each primer indicated below, 200 μM dNTP, 2mM $MgCl_2$ and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer.

Conditions for all PCRs were as follow: Initial 1 min denaturation step at 94°C, 1 min annealing step (NTPDase1 and 3: 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 extension at 72°C. The PCR products were: NTPDase1 - 543bp; NTPDase2 - 331bp; NTPDase3 - 267bp, β-actin – 210bp. PCR products were submitted to electrophoresis using a 1% agarose gel. Bands intensities were analyzed by Kodak 1D v.3.5.4 software. The following set of primers were used: RnNTPDase1F: 5'-GAT CAT CAC TGG GCA GGA GGA AGG-3' and RnNTPDase1R 5'-AAG ACA CCG TTG AAG GCA CAC TGG-3'; RnNTPDase2F: 5'-GCT GGG TGG GCC GGT GGA TAC G-3' and RnNTPDase2R 5'-ATT GAA GGC CCG GGG ACG CTG AC-3'; RnNTPDase3F: 5'-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3' and RnNTPDase3R 5'-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3'; and for β-actin: RnβactinF 5'-TAT GCC AAC ACA GTG CTG TCT GG-3' and RnβactinR 5'-TAC TCC TGC TTC CTG ATC CAC AT-3'.

The relative abundance of each mRNA versus β-actin was determined by densitometry using the freeware ImageJ 1.37 for Windows. Each experiment was repeated four times using RNA isolated from independent extractions. The expression analysis was performed in replicate and representative findings were shown.

RESULTS

Behavioral changes after KA injection

Status epilepticus (SE) developed in all KA-treated animals. PN7 rats initially became immobile with loss of limb tone and ataxia. Then, they exhibited intermittent hyperactivity with rhythmic "bicycling" movements of all extremities, opisthotonic arching, and tonic limb extension.

Previous studies with implanted hippocampal electrodes showed that such clinical signs represent electrographic ictal activity (Lynch et al, 2000)

Y-maze task

Initially, rats previously submitted to KA-seizures at PN7 were allowed to reach adulthood and the response to novelty (at PN93) and spatial memory (at PN100) were tested in to Y-maze task (Fig 1). A decrease ($P<0.0001$) in the time spent in the novel arm (28.3 ± 1.1) was observed in rats submitted to KA seizures at PN7 when compared to control group (45.88 ± 2.4; Fig 1A) after the ITI of 2 min. KA-treated rats also presented a diminished number of entrances in novel arm (33.6 ± 0.9) when compared to control group (42.0 ± 0.7; $P<0.0001$; Fig 1B;) after the ITI of 2 min. Spatial memory (2 hours ITI) of early seizures rats also revealed impairment in Y-maze test. Time spent in novel arm was reduced for rats submitted to KA seizures at neonatal period (26.6 ± 1.3) when compared with control groups that did not received KA (44.13 ± 3.3; $P<0.0001$; Fig 1C) after ITI of 2 hours. Finally, KA-treated rats also presented a diminished number of entrances in novel arm (33.3 ± 0.7) when compared to control animals (41.5 ± 1.3; $P<0.0001$; Fig 1D). It is important to mention that no alterations in locomotor activity (Fig 2A) nor anxiety profile (Fig 2B and C) were observed in animals submitted to KA seizures in neonatal period.

Nucleotide Hydrolysis

The extracellular hydrolysis of ATP, ADP, and AMP was evaluated at different time points in hippocampal synaptosomes after one single seizure episode induced by KA in PN7 rat pups.

ATP hydrolysis

Twelve hours after the seizure (PN7), ATP hydrolysis from KA group (92.7 ± 2.6 nmol $Pi.min^{-1}.mg^{-1}$ protein) was not significantly different in relation to control group (88.2 ± 6.6 nmol $Pi.min^{-1}.mg^{-1}$ protein; $P > 0.05$; Fig 3A). ATP hydrolysis from KA groups at PN14 (113 ± 15.6 nmol $Pi.min^{-1}.mg^{-1}$ protein) and PN21 (128.5 ± 32.8 nmol $Pi.min^{-1}.mg^{-1}$ protein) also were not significantly different when compared to the respective control groups (PN14 - 111.6 ± 16.61 nmol $Pi.min^{-1}.mg^{-1}$ protein and PN21 – 101.7 ± 17.3 nmol $Pi.min^{-1}.mg^{-1}$ protein; $P > 0.05$; Fig 3A). However, ATP hydrolysis in PN90 rats previously submitted to a convulsive episode at PN7 was increased (234.2 ± 44.6 nmol $Pi.min^{-}$

^{1}.mg^{-1} protein) when compared to the respective control group (160.2 ± 34.0 nmol Pi.min^{-1}.mg^{-1} protein ; P < 0.05; Fig 3A).

ADP hydrolysis

ADP hydrolysis from PN7 rats formerly treated with KA (43.4 ± 5.9 nmol Pi.min^{-1}.mg^{-1} protein) was not significantly different in relation to control group (36.6 ± 1.1 nmol Pi.min^{-1}.mg^{-1} protein; P > 0.05; Fig 3B). Likewise, PN14 rats submitted to KA at PN7 presented an ADP hydrolysis (45.4 ± 2.97 nmol Pi.min^{-1}.mg^{-1} protein) statistically similar to the control group (50.4 ± 4.9 nmol Pi.min^{-1}.mg^{-1} protein ; P > 0.05; Fig 3B). The ADP hydrolysis from KA group at PN21 (71.3 ± 7.3 nmol Pi.min^{-1}.mg^{-1} protein) was also not significantly different from ADP hydrolysis in the respective control groups (63.72 ± 23.4 nmol Pi.min^{-1}.mg^{-1} protein ; P > 0.05; Fig 3B). PN90 rats previously submitted to a convulsive episode at PN7 showed an ADP hydrolysis (68.5 ± 7.4) statistically similar to the one observed in the respective control group (56.5 ± 9.5 nmol Pi.min^{-1}.mg^{-1} protein ; P < 0.05; Fig 3B).

AMP hydrolysis

AMP hydrolysis from KA group at PN7 (8.2 ± 1.8 nmol Pi.min^{-1}.mg^{-1} protein) was not significantly different in relation to control group (5.25 ± 0.7 nmol Pi.min^{-1}.mg^{-1} protein ; P > 0.05; Fig 3C). The AMP hydrolysis of KA groups at PN14 (8.3 ± 1.4 nmol Pi.min^{-1}.mg^{-1} protein), and PN21 (21.48 ± 3.2 nmol Pi.min^{-1}.mg^{-1} protein) were also not significantly different when compared to respective the respective control groups (PN14 – 8.7 ± 1.5 nmol Pi.min^{-1}.mg^{-1} protein and PN21 – 18.9 ± 3.7 nmol Pi.min^{-1}.mg^{-1} protein; P > 0.05; Fig 3C). Likewise, AMP hydrolysis at PN90 of rats previously treated with KA at PN7 (34.7 ± 6.6 nmol Pi.min^{-1}.mg^{-1} protein) was statistically similar to the respective control group (38.35 ± 7.9 nmol Pi.min^{-1}.mg^{-1} protein; P < 0.05; Fig 3C).

Relative expression of NTPDases at PN90

Since only f ATP hydrolysis at PN90 rats was altered by the previous convulsive episode at PN7, we have evaluated the relative gene expression pattern of ectonucleotidases responsible for ATP hydrolysis: NTPDase1, NTPDase2, and NTPDase3. NTPDase1 relative expression analyzed at PN90 rats previously submitted to a single seizure in neonatal period (71 ± 7% of relative expression) was

not significantly different from NTPDase1 relative expression observed in control group (72 ± 5% of relative expression; P > 0.05; Fig 4AB). NTPDase2 relative expression of PN90 KA group (88 ± 6% of relative expression) also was not significantly different from NTPDase2 relative expression in control group (79 ± 6% of relative expression; P > 0.05; Fig 4AB). Likewise, the relative expression of NTPDase3 of PN90 rats treated with KA at PN7 (49 ± 9% of relative expression) was statistically similar to the one found in the control group (53 ± 7% of relative expression; P > 0.05; Fig 4AB).

DISCUSSION

The present study has shown memory impairment of adult rats (PN90) previously submitted to one single seizure episode in neonatal period (PN7), which is accompanied by an increased ATP hydrolysis in hippocampal synaptosomes. Furthermore, the present results have shown that the relative expression of NTPDases responsible for ATP hydrolysis was not altered by the KA administration in early age.

Previous studies had already reported that a single administration of KA at PN5-PN10 can lead to spatial memory impairment later in life (Lynch et al., 2000; Sayin et al., 2004; Cornejo et al., 2007). Similarly, induction of convulsive activity with different agents in immature rodents also triggers visuo-spatial memory dysfunction later in life (De Rogalski-Landrot et al., 2001; Lee et al., 2001; Huang et al., 2002; Cilio et al., 2003; Bo et al., 2004). Some of these studies confirmed that KA-induced neonatal convulsions-associated memory deficits are present at PN90 and later (Lynch et al., 2000; Sayin et al., 2004). However, others studies reported memory deficits early in adulthood (PN60) albeit they were rather marginal and only observed in some tasks (Cornejo et al., 2007). The present results have shown that the spatial memory deficits were observed at PN90 and corroborate with the data reported in literature. Furthermore, there was no modification of exploratory and locomotor activities (see also Lynch et al., 2000; Sayin et al., 2004) as well as no modification of anxiety-like behaviour in the elevated-plus maze test, parameters which could influence in the Y-maze task. Our anxiety results are in discordance with previous report (Sayin et al., 2004) which has shown a greater degree of anxiety of adult rats treated with KA in early age. This discrepancy might be related to the use of different rat strains (which were not specified in Sayin et al., 2004).

Once we have verify the cognitive deficits in adulthood induced by one single seizure episode in neonatal period and since ATP and adenosine could modulate the plastic phenomena (LTP and LTD)

involved in learning and memory mechanisms, we have tested if enzymes responsible for the availability of ATP and adenosine in the synaptic cleft are changed by previous KA treatment. The time points chosen for the analysis corresponds to the maturation periods of the brain (PN7, PN14, and PN21) and the adult age which presented the cognitive deficits after the neonatal seizure (PN90). Our results have demonstrated there were no significantly changes in nucleotide hydrolysis in earlier times after the seizure (PN7, PN14, and PN21). In fact, several studies have discussed that immature brain is less vulnerable to morphological and physiological alterations after status epilepticus when compared to mature brain (Haut et al., 2004; Cilio et al., 2003; Haas et al., 2001), although changes in behaviour and brain connectivity could be detected in adulthood (Holmes, 2004). The unaffected hydrolysis of nucleotides observed in the present study is in agreement with a previous study which revealed that ATP, ADP, and AMP hydrolysis were not changed in young rats exposed to pilocarpine model of epilepsy (de Paula Cognato et al., 2005). Our results also demonstrate that the ATP and ADP hydrolysis of control group have increased from PN7 to PN90. Previous study have demonstrated that, in synaptosomal preparations of rat cerebral cortex, ATP and ADP hydrolysis increased significantly from birth until the second postnatal week (Müller et al., 1990). Furthermore, the ecto-5'-nucleotidase is expressed at the surface of developing nervous cells and is regarded as a marker of neural development (Braun and Zimmermann, 1998). Also, it was reported that this enzyme activity increased in aging rat brain (Fuchs et al., 1991). Our results have are in agreement with this study, since we have observed an increase in AMP hydrolysis of control rats from PN7 until PN90.

The present results also have shown an increased ATP hydrolysis in hippocampal synaptosomes of PN90 rats, which were formerly submitted to KA seizure at PN7. Previous studies have already observed enhanced ectonucleotidase activities in CNS after adult animals were submitted to different models of epilepsy (Bonan et al., 2000a, 2000b; de Paula Cognato et al., 2005; Cognato et al., 2007; Oses et al., 2007). However, this is the first study demonstrating an increased nucleotide hydrolysis following a long period after the induction of one single seizure in early age. Despite of short convulsive periods early in life not lead to evident morphological modifications (Stafstrom et al., 2002; Holmes, 2005), it has been reported that, the neural activity during development not only determinates the organization of neural circuits but may also influence the capacity for circuit plasticity later in life (Abraham and Bear, 1996; Feldman and Knudsen, 1998). Once ATP seems to be related to LTP and synaptic plasticity induction (Wieraszko, 1996; Fujji et al., 2004), the increased

hydrolysis of this nucleotide observed in memory impaired adult rats could be one of several brain mechanisms altered by early seizure. In fact, Lynch and co-workers (2000) have observed that PN90 rats formerly submitted to KA-induced seizures in early age (PN7) presented impairment in hippocampal LTP induction. Since we have found an augmented hydrolysis of ATP and this nucleotide seems to be important to LTP induction, we could assume that impairment of memory and learning observed in adult rats which have experience a convulsive episode in postnatal period may be a consequence of the increased ATP hydrolysis activitiy. Interestingly, it was reported a decrease in nucleotide hydrolysis of hippocampal synaptosomes of rats immediately euthanized after the training session of the inhibitory avoidance task (Bonan e al., 1998). This finding assumes that the presence of ATP in the synaptic cleft may be required to the mechanisms of memory retention and highlight the opposite situation of our data, in which ATP hydrolysis is enhanced and a memory impairment is observed.

We also have observed that enhanced ATP hydrolysis was not accompanied by change in relative expression of the NTPDase 1-3, suggesting that the effect observed was not due to an increased synthesis of these proteins. Previous study has also reported that nucleotide hydrolysis changed by seizures was not accompanied by an increase of relative expression in ectonucleotidases. Oses and co-workers (2007) have found an increased AMP and GMP hydrolysis in hippocampal slices of rats submitted to kindling by pentylenetetrazole 10 days after the last injection with no significant changes in the relative expression of 5'-nucleotidase.

In conclusion, the present study shows that a convulsive period early in life causes a selective impairment of memory performance later in adulthood, which is accompanied by an enhancement of ATP hydrolysis in hippocampal tissue. These findings correlate the purinergic signaling to the cognitive deficits induced by neonatal seizures and contribute to a better understanding about the mechanisms of seizure-induced memory dysfunction.

Acknowledgements

This research was supported by grants from CAPES, FAPERGS and CNPq.

References

Abraham, W.C., and Bear, M.F. (1996). Metapladticity: the plasticity of synaptic plasticity. Trends Neurosci, 19:126-30.

Battastini, A.M., da Rocha, J.B., Barcellos, C.K., Dias, R.D., Sarkis, J.J. (1991). Characterization of an ATP diphosphohydrolase (EC 3.6.1.5) in synaptosomes from cerebral cortex of adult rats. Neurochem Res. 12:1303-10.

Bo, T., Jiang, Y., Cao, H., Wang, J., Wu, X. (2004). Long-term effects of seizures in neonatal rats on spatial learning ability and N-methyl-D-aspartate receptor expression in the brain. Dev Brain Res, 152, 137-142.

Bonan, C.D, Dias, M.M., Battastini, A.M., Dias, R.D., Sarkis, J.J. (1998). Inhibitory avoidance learning inhibits ectonucleotidases activities in hippocampal synaptosomes of adult rats. Neurochem Res, 23:977-82.

Bonan, C.D., Walz, R., Pereira, G.S., Worm, P.V., Battastini, A.M., Cavalheiro, E.A., Izquierdo, I., Sarkis, J.J. (2000a). Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy. Epilepsy Res, 39:229-38.

Bonan, C.D., Amaral, O.B., Rockenbach, I.C., Walz, R., Battastini, A.M., Izquierdo, I., Sarkis, J.J. (2000b). Altered ATP hydrolysis induced by pentylenetetrazol kindling in rat brain synaptosomes. Neurochem Res, 25:775-9.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal of Biochemistry, 72: 248-254.

Braun, N., and Zimmermann, H. (1998). Association of ecto-5'-nucleotidase with specific cell types in the adult and developing rat olfactory organ. J Comp Neurol, 393: 528-37.

Bruno, A.N., Diniz, G.P., Ricachenevsky, F.K., Pochmann, D., Bonan, C.D., Barreto-Chaves, M.L., Sarkis, J.J. (2005). Hypo-and hyperthyroidism affect the ATP, ADP and AMP hydrolysis in rat hippocampal and cortical slices. Neurosci Res, 52: 61-68.

Chan, K.; Delfert, D.; Junges, K.D. (1986). A direct colorimetric assay for Ca^{2+}-ATPase activity. Anal Biochem, 157: 375-380.

Cognato, G.P., Bruno, A.N., Vuaden, F.C., Sarkis, J.J., Bonan, C.D. (2005). Ontogenetic profile of ectonucleotidase activities from brain synaptosomes of pilocarpine-treated rats. Int J Dev Neurosci, 23:703-9.

Cognato, G. de P., Bruno, A.N., da Silva, R.S., Bogo, M.R., Sarkis, J.J., Bonan, C.D. (2007). Antiepileptic drugs prevent changes induced by pilocarpine model of epilepsy in brain ecto-nucleotidases Neurochem Res, 32:1046-55.

Cilio, M.R., Sogawa, Y., Cha, B.H., Liu, X., Huang, L.T., Holmes, G.L. (2003). Long-term effects of status epilepticus in the immature brain are specific for age and model. Epilepsia 44: 518-528.

Cornejo, B.J., Mesches, M.H., Coultrap, S., Browning, M.D., Benke, T.A. (2007). A single episode of neonatal seizures permanently alters glutamatergic synapses. Ann Neurol, 61: 411-426.

Cooke, S.F., Bliss, T.V. (2006). Plasticity in the human central nervous system. Brain, 129:1659-73.

De Mendonça, A. and Ribeiro, J.A. (1997). Adenosine and neuronal plasticity. Life Sci, 60:245-51.

De Mendonça A, Costenla AR, Ribeiro JA. (2002). Persistence of the neuromodulatory effects of adenosine on synaptic transmission after long-term potentiation and long-term depression. Brain Res, 932:56-60.

De Rogalski-Landrot, I., Minokoshi, M., Silveira, D.C., Cha, B.H., Holmes, G.L. (2001), Recurrent neonatal seizures: relationship of pathology to the electroencephalogram and cognition. Dev Brain Res 129: 27-38.

Feldman, D.E., and Knudsen, e.i. (1998). Experience dependent plasticity and the maturation of glutamatergic synapses. Neuron, 20: 1067-71.

Fuchs, J.L. (1991). 5'-nucleotidase activity increases in aging rat brain. Neurobiol Aging, 12:523-30.

Fujii, S. (2004). ATP- and Adenosine-Mediated Signaling in the Central Nervous System: The Role of Extracellular ATP in Hippocampal Long-Term Potentiation. J Pharmacol Sci, 94:103 – 106.

Haas, K.Z., Sperber, E.F., Opanashuk, L.A., Stanton, P.K., Moshé, S.L. (2001). Resistance of immature hippocampus to morphologic and physiologic alterations following status epilepticus or kindling. Hippocampus 11: 615-625.

Haut, S.R., Velisko´va, J., Moshe´, S.L. (2004). Susceptibility of immature and adult brains to seizure effects. Lancet Neurobiol, 3:608–617.

Heymann, D., Reddington, M., Kreutzberg, G.W. (1984). Subcellular localization of 50-nucleotidase in rat brain. J. Neurochem, 43: 971–978.

Holmes, G.L. (1997). Epilepsy in the developing brain: lesson from the laboratory and clinic. Epilepsia, 38: 12-30.

Holmes, G.L. (2004). Effects of early seizures on later behavior and epileptogenicity. Ment Retard Dev Disabil Res Rev, 10: 101-5.

Holmes, G.L. (2005). Effects of seizures on brain development: lessons from the laboratory. Pediatr Neurol 33: 1-11

Huang, L.T., Yang, S.N., Liou, C.W., Hung, P.L., Lai, M.C., Wang, C.L., Wang, T.J. (2002). Pentylenetetrazol-induced recurrent seizures in rat pups: time course on spatial learning and long-term effects. Epilepsia 43: 567-573.

Lee, C.L., Hannay, J., Hrachovy, R., Rashid, S., Antalffy, B., Swann, J.W. (2001), Spatial learning deficits without hippocampal neuronal loss in a model of early-onset epilepsy. Neuroscience, 107: 71-84.

Lynch, M., Sayin, U., Bownds, J., Janumpalli, S., Sutula, T. (2000). Long-term consequences of early postnatal seizures on hippocampal learning and plasticity. Eur J Neurosci, 7: 2252-2264.

Müller, J., Rocha, J.B., Battastini, A.M., Sarkis, J.J., Dias, R.D. (1990). Ontogeny of ATP and ADP hydrolysis by cerebral cortex synaptosomes from rats. Braz. J. Med. Biol. Res. 23, 935–939.

Nagy, A., and Delgado-Escueta, A.V. (1984). Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient (Percoll). J. Neurochem, 43:1114–1123.

Oses, J.P., Viola, G.G., de Paula Cognato, G., Júnior, V.H., Hansel, G., Böhmer, A.E., Leke, R., Bruno, A.N., Bonan, C.D., Bogo, M.R., Portela, L.V., Souza, D.O., Sarkis, J.J. (2007). Pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid. Epilepsy Res, 75:104-11.

Sayin, U., Sutula, T.P., Stafstrom, C.E. (2004), Seizures in the developing brain cause adverse long-term effects on spatial learning and anxiety. Epilepsia, 45: 1539-1548.

Stafstrom, C.E., Thompson, J.L., Holmes, G.L. (1992). Kainic acid seizures in the developing brain: status epilepticus and spontaneous recurrent seizures. Dev Brain Res, 65: 227-236.

Stafstrom, C.E., Chronopoulos, A., Thurber, S., Thompson, J.L., Holmes, G.L. (1993). Age-dependent cognitive and behavioral deficits after kainic acid seizures. Epilepsia, 34: 420-432.

Stafstrom, C.E. (2002). Assessing the behavioral and cognitive effects of seizures on the developing brain. Prog Brain Res, 135: 377-390

Zimmermann, H. (2001). Ectonucleotidases: some developments and a note on nomenclature. Drug Developmental Res, 52: 44-56.

Wieraszko, A. (1996). Extracellular ATP as a neurotransmitter: its role in synaptic plasticity in the hippocampus. Acta Neurobiol 56:637-648.

Legend to figures

Figure 1 Rats suffering from a kainate-induced convulsive period early in childhood display selective memory impairment in adulthood. Pups with 7 days of age received an i.p. injection of either saline (open bars) or kainate (2 mg/kg, black bars), which caused a period of convulsions. Rats were then behaviourally analysed at P90-100. Data represent the percentage of total entrances (B with 2 min ITI; D with 2 hours ITI) and percentage of time spent in each arm (A with 2 min ITI; C with 2 hours ITI). Bars represent the mean ± S.E.M. of 8 (saline) and 9 (KA) animals. Novel arm was analyzed individually using the *student t test* with $p < 0.05$ *(*)* and $p < 0.005$ *(***).*

Figure 2 Rats suffering from a kainate-induced convulsive period early in childhood display no alteration sin locomotor and anxiety behaviours. Locomotion was evaluated in an open field arena (A) by counting the number of crossings and rearings of the rats during 5 min. The degree of anxiety was determined by their preference for the closed and open arms of an elevated-plus maze (B, C). Data represent the means ± SEM of 8 (saline) and 9 (KA) animals; * P<0.05 using a Student's *t* test.

Figure 3 Nucleotide hydrolysis of hippocampal synaptosomes of rats at PN90 previously submitted to early seizure at PN7. Data represent the mean ± SD of ATP (A), ADP (B), and AMP (C) hydrolysis of at least 5 animals per group. * P<0.05 using a Student's *t* test

Figure 4 Relative expression of hippocampal ectonucleotidases of rats at PN90 previously submitted to early seizure at PN7. Data represent the relative expression of NTPDase 1, NTPDase 2, NTPDase 3 and 5'-nucleotidase (A) with the respective representative images (B). At least three independent experiments (PCRs) were performed, with entirely consistent results.

FIGURE 1

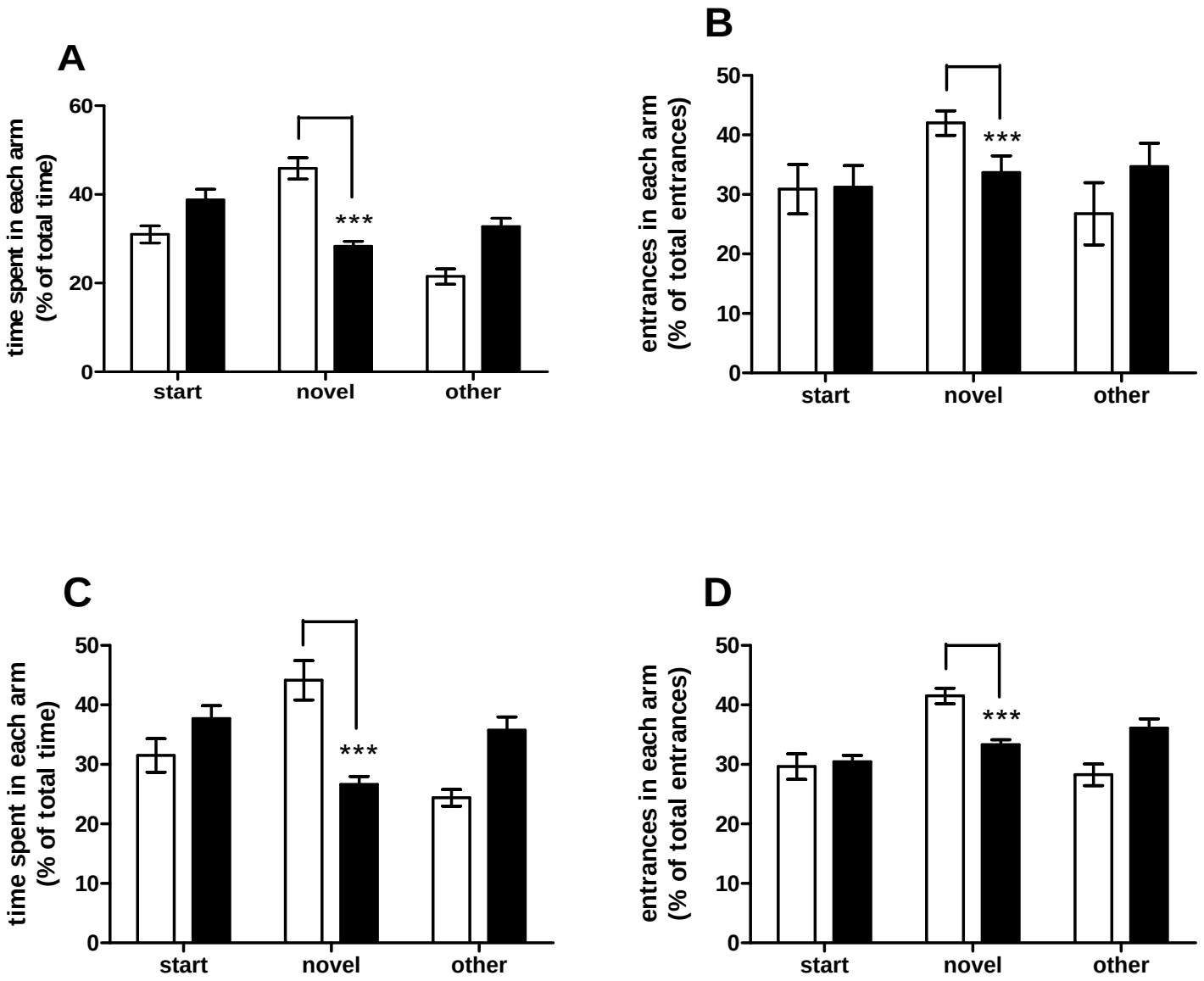

A
time spent in each arm
(% of total time)
60
40
20
0
***
start
novel
other
B
entrances in each arm
(% of total entrances)
50
40
30
20
10
0
***
start
novel
other
C
time spent in each arm
(% of total time)
50
40
30
20
10
0
***
start
novel
other
D
entrances in each arm
(% of total entrances)
50
40
30
20
10
0
***
start
novel
other

FIGURE 2

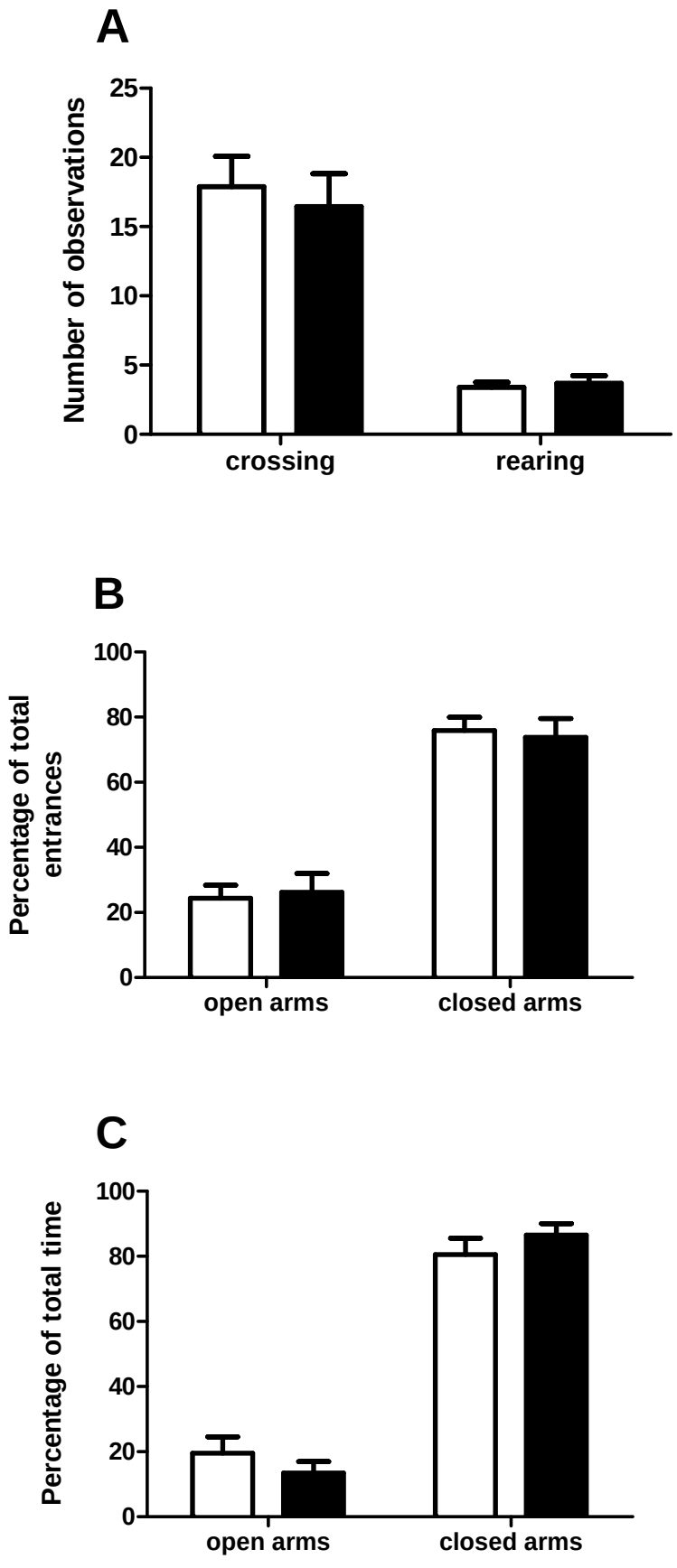

A
Number of observations
25
20
15
10
5
0
crossing
rearing
B
Percentage of total entrances
100
80
60
40
20
0
open arms
closed arms
C
Percentage of total time
100
80
60
40
20
0
open arms
closed arms

FIGURE 3

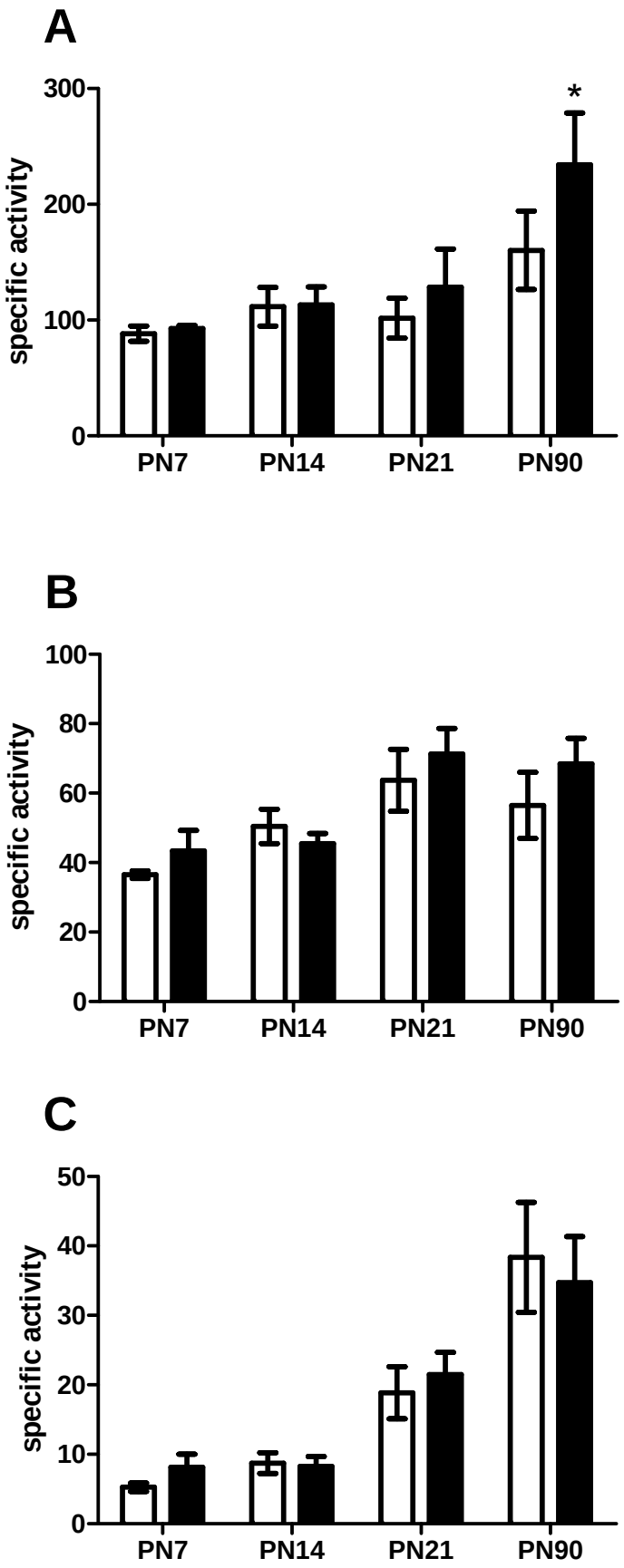
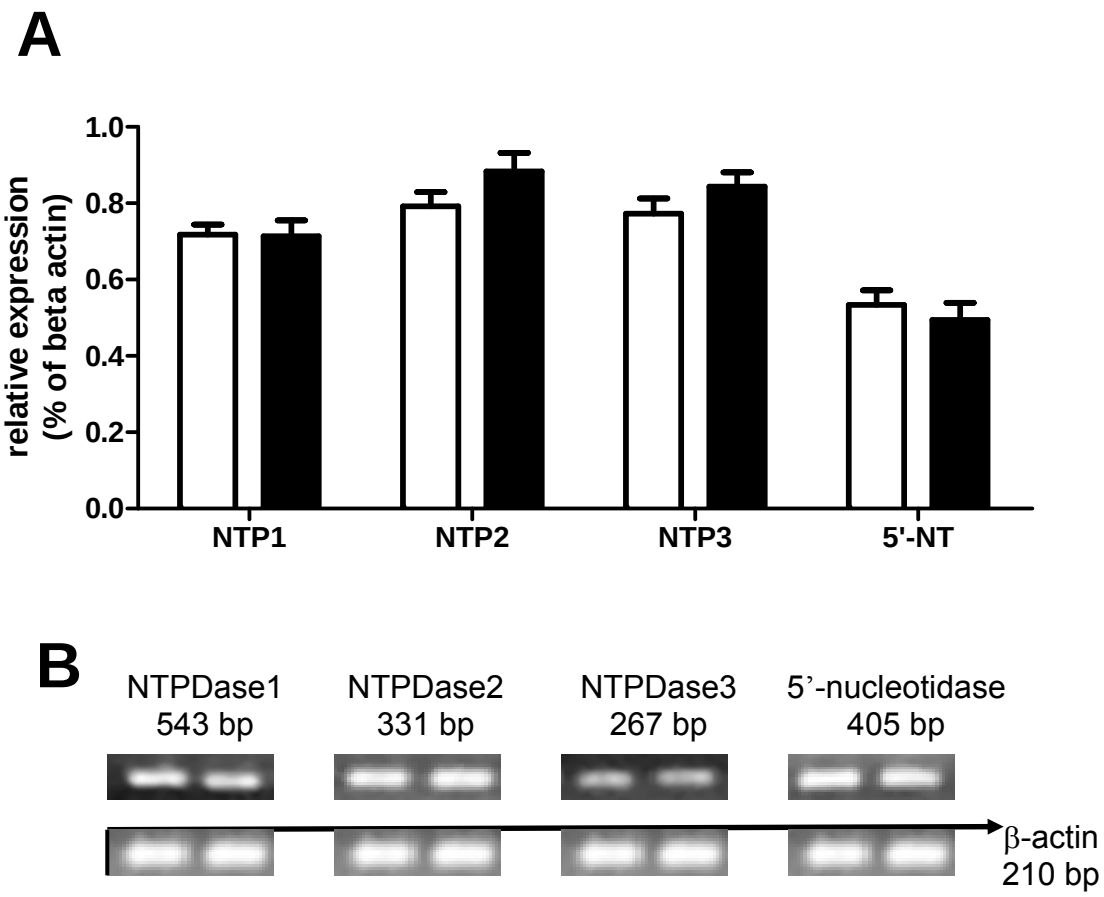

A
300
200
100
0
specific activity
*
PN7
PN14
PN21
PN90
B
100
80
60
40
20
0
specific activity
PN7
PN14
PN21
PN90
C
50
40
30
20
10
0
specific activity
PN7
PN14
PN21
PN90

FIGURE 4


A
relative expression
(% of beta actin)
1.0
0.8
0.6
0.4
0.2
0.0
NTP1
NTP2
NTP3
5'-NT
B
NTPDase1
543 bp
NTPDase2
331 bp
NTPDase3
267 bp
5'-nucleotidase
405 bp
β-actin
210 bp

III. ARTIGOS CIENTÍFICOS

III.4. CAPÍTULO 4 - COGNATO, G.P.; AGOSTINHO, P.M.; HOCKEMEYER, J.; MÜLLER, C.E.; BONAN, C.D.; PORCIÚNCULA, L.O.; SOUZA, D.O.; CUNHA, R.A. Caffeine and adenosine A_{2A} receptor antagonists prevent memory impairment and synaptotoxicity in adults triggered by a convulsive episode in early life. Artigo a ser submetido para o periódico Neuropharmacology

Caffeine and adenosine A_{2A} receptor antagonists prevent memory impairment and synaptotoxicity in adults triggered by a convulsive episode in early life

Giana P. Cognato[1,2], Paula M. Agostinho[1], Jörg Hockemeyer[3], Christa E. Müller[3], Carla D. Bonan[4], Lisiane O. Porciúncula[2], Diogo O. Souza[2], Rodrigo A. Cunha[1,*]

[1]Center for Neurosciences of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, Portugal, [2]Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, [3]Pharmazeutische Chemie I, Pharmazeutisches Institut, University of Bonn, Germany. [4]Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, PUC/RS, Porto Alegre, RS, Brazil

Running title: Caffeine prevents convulsions-induced memory dysfunction

Corresponding Author: Rodrigo A. Cunha, Center for Neuroscience of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal; e-mail: cunharod@gmail.com; Website: http://cnc.cj.uc.pt/lab_lef/

ABSTRACT

Seizures early in life cause long term behavioural modifications, namely long term memory deficits in experimental animals. Since caffeine and adenosine A_{2A} receptor ($A_{2A}R$) antagonists prevent memory deficits in adults, we now tested if they also prevented the long term memory deficits caused by a convulsive period early in life. Administration of kainate (KA, 2 mg/kg) to 7 days old (P7) rats caused a single period of self-extinguishable convulsions which lead to a poorer memory performance in the Y-maze when rats were 90 days old (P90), without modification of locomotion or anxiety-like behaviour in the elevated-plus maze. The hippocampus of these adult rats treated with kainate at P7 displayed a lower density of synaptic proteins such as SNAP25 and syntaxin (but not synaptophysin), as well as vesicular glutamate transporters type 1 (but not vesicular GABA transporters), with no changes in PSD95, NMDA receptor subunits (NR1, NR2A, NR2B) or AMPA receptor subunits (GluR1, GluR2) compared to controls. Caffeine (1 g/l) or the $A_{2A}R$ antagonist, KW6002 (3 mg/kg) applied in the drinking water from P21 onwards, prevented these memory deficits in P90 rats treated with KA at P7, as well as the accompanying synaptotoxicity. These results show that a single convulsive episode in early childhood causes a delayed memory deficit in adulthood accompanied by a glutamatergic synaptotoxicity that was prevented by caffeine or adenosine $A_{2A}R$ antagonists.

Keywords: A_{2A} adenosine receptor; caffeine, convulsions, memory, synaptotoxicity, astrogliosis

INTRODUCTION

During development, the brain undergoes marked plastic changes which format its wiring and determine its potential for future experience-dependent plastic changes, i.e. the developing brain expresses a marked metaplasticity (e.g. Abraham & Bear, 1996). An abnormal modification of firing of the developing brain, such as that occurring during a convulsive period, causes minor immediate morphological or functional modifications (e.g. Stafstrom et al., 1992; Koh et al., 1999; Haas et al., 2001) but has potential detrimental consequences for brain reactivity later in life (reviewed in Stafstrom, 2002; Holmes et al., 2005; Dubé et al., 2007). In humans, it is still debatable if single convulsive episodes early in childhood cause memory deficits later in life (see Shinnar and Hauser, 2002; Vingerhoets, 2006), although studies have concentrated in adolescents and have not carried out long follow-ups up to adulthood (e.g. Dikmen et al., 1975; Chang et al., 2001). In fact, in animal models, it has been observed that the experimental induction of a single convulsive period early in childhood causes a delayed memory deficit in adulthood (e.g. Stafstrom et al., 1993; Lynch et al., 2000; Lee et al., 2001; Huang et al., 2002; Rutten et al., 2002; Bo et al., 2004; Sayin et al., 2004). For instance, the administration of kainate, which models temporal lobe epilepsy in adult rats (Ben-Ari, 1985), causes minor immediate morphological or functional modifications in pups (e.g. Stafstrom et al., 1992; Koh et al., 1999; Haas et al., 2001), but triggers impairments of spatial memory learning when animals are 80-90 days old (Stafstrom et al., 1993; Lynch et al., 2000; Sayin et al., 2004). The mechanisms underlying this metaplastic ability of single convulsive episodes to affect performance later in life are poorly understood (see Holmes, 2005; Dubé et al., 2007). But more important than understanding the mechanisms of convulsions-induced delayed memory deficits is the possibility of devising novel strategies to preserve memory function, which may also offer some additional insights into key mechanistic processes.

One emerging candidate to manage memory impairment under different noxious conditions, in particular upon neurodegenerative diseases, is caffeine. Caffeine is the most widely consumed psychoactive substance (Fredholm et al., 1999), and alleviates cognitive impairment in both humans and animals (reviewed in Cunha, 2008; Takahashi et al., 2008), namely upon ageing (Prediger et al., 2005; Ritchie et al., 2007; Costa et al., 2008) or upon Alzheimer's disease (Maia and de Mendonça, 2002; Dall'Igna et al., 2003, 2007; Arendash et al., 2006; Eskelinen et al., 2009). Furthermore, caffeine also affords protection upon CNS injury (Cunha, 2005; Chen et al., 2007). These

neuroprotective effects of caffeine seem to be mediated by adenosine A_{2A} receptor blockade, since the prevention by caffeine of cognitive deficits and of neurodegeneration are mimicked by antagonists of A_{2A} but not A_1 receptors (e.g. Dall'Igna et al., 2003, 2007; Prediger et al., 2005). In particular, prolonged caffeine consumption (Georgiev et al., 1993; Johansson et al., 1996; Rigoulot et al., 2003; André et al., 2007; El Yacoubi et al., 2008) or adenosine A_{2A} receptor blockade (El Yacoubi et al., 2008) abrogate brain damage in experimental models of epilepsy in adult animals.

Thus, we now tested if chronic treatment with caffeine or with a selective A_{2A} receptor antagonist could prevent the memory deficits found in adult animals that suffered an episode of convulsions early in their childhood. Since memory deficits in different neuropsychiatric conditions are accompanied by dysfunction and loss of synapses (e.g. Coleman et al., 2004; Silva et al., 2007), we also investigated if a convulsive period early in childhood caused a modification of synaptic markers in adulthood.

MATERIALS AND METHODS

Animals

All studies were conducted in male Wistar rats obtained from Charles-River (Barcelona, Spain), which were maintained in our own animal facilities under controlled environment (23±2°C, 12 h-light/dark cycle, free access to food and water). Experiments were carried out in accordance with the principles and procedures outlined in the EU guidelines for use of experimental animals (86/609/EEC) with care to minimize the number of animals used and their suffering.

Kainate-induced convulsion and drug administration

Young rat pups (7 days old) were separated from their dams and weighted. A single seizure episode was induced by an intra-peritoneal (ip) administration of 2 mg/kg kainic acid (KA) (Strafstrom et al., 1992; Sayin et al., 2004) prepared in saline solution (0.9% w/v NaCl). Convulsive behaviour was observed 10 min after KA administration and consisted of hyperactive "bicycling" movements of all extremities with opisthonic arching of the back and tonic limbic extension, as previously described (Lynch et al., 2000). Control littermates were injected with saline solution. After the spontaneous termination of convulsions, which occurred approximately 3 h after their onset, KA-injected rats as well as their control littermates were returned to their dams for normal care.

Caffeine was added to the drinking water (final concentration of 1 g/L) and was introduced when the rats (KA- or saline-treated) reached the age of 21 days. Caffeine was continuously supplied until the rats were sacrificed at 100 days of age. Animals were placed in pairs in plastic cages and the volume of caffeine consumption was measured. Non-treated rats (KA- or saline-treated) drank normal water. Weight and water/caffeine consumption were monitored during the treatment. For measurement of the serum concentration of caffeine, blood samples (100 μl) were allowed clotting, and the serum was separated by centrifugation. Serum samples from each rat were added to equal volume of methanol-acetone (4:1), mixed for 15 minutes, centrifuged at 3,000 *g* for 15 minutes, and the supernatant saved for caffeine quantification. Samples (20 μl) were separated at room temperature using a reverse-phase column [LiChroCART 125 x 4 mm LiChrospher 100 RP-18 (5 μm) cartridge fitted into a ManuCART holder (Merck Darmstadt, Germany)], using a Gilson system equipped with a UV detector set at 274 nm (the maximum peak in the absorption spectra of caffeine). The eluent was 40% (v/v) methanol with a flow rate of 0.8 mL/min. The identification of the caffeine peak was performed by comparison of relative retention time with standard samples prepared in water-methanol-acetone (5:4:1) and its quantification achieved by calculating the peak areas then converted to concentration values by calibration with known standards (1-100 μM). We observed that the average caffeine intake was similar in both KA- and saline-treated rats through the treatment period, and achieved similar serum caffeine concentrations (21.4±2.8 μM, n=6) at the time of sacrifice.

The chronic intake of the selective A_{2A} receptor antagonist, KW6002, was also begun when rats (KA- or saline-treated) reached the age of 21 days. Animals were placed individually in plastic cages and weighted 3 times per week. Everyday, a suspension of 1 mg/L KW 6002 in a vehicle solution (composed by 0.9% saline and 0.4% methylcellulose) was prepared and the adequate volume of the suspension was added to the drinking water to obtain the desired dose of KW6002 (3 mg/kg). Non-treated rats (KA- or saline-treated) drank water supplemented with the vehicle solution.

Behavioural analysis

The open-field test was carried out when rats were 28, 58, and 88 days old. On the subsequent days (29, 59, and 89 days old) rats were submitted to the elevated-plus maze task. Finally, on days 30, 60, and 90, rats performed a memory task in a Y-maze apparatus with an inter-trial interval (ITI) of

2 min. One week later, rats were submitted to a Y-maze task with 2 hours of ITI. All behavioural experiments were conducted between 9:00 a.m. and 4:00 p.m. (light phase).

Open-field and elevated-plus maze tasks: Locomotor activity and exploratory behaviour were monitored using an open-field apparatus, as previously described (Cunha et al., 2008), to determine whether the different experimental groups differed in baseline locomotor activity. The exploratory behaviour of the rats was evaluated a 50×50 cm arena, divided into 4 squares of 25 cm, by counting the total number of line crossings over a period of 5 min without prior habituation to the arena. In parallel, the number of rearings was also counted. Evaluation of the anxiety status was carried out using the elevated-plus maze where the relative time spent in close versus open arms is a measure of anxiety (Dawson and Tricklebank, 1995). The apparatus consisted of two open arms 50 x 10 cm at right angles to two covered (closed) arms. The maze was elevated to a height of 50 cm above the ground, forming an aversive stimulus to animals in the open arms. The animal was placed in the centre of the maze with its head facing a closed arm and allowed to explore the maze for 5 min. Entry into a particular arm was defined as the placement of all four feet into the arm.

Y-maze task: The Y-maze is a simple two-trial recognition test for measuring spatial recognition memory in rodents, although it does not allow isolating memory performance (reviewed in Hughes, 2004). Y-maze apparatus consisted of three arms (8 cm of width, 30 cm of length and, 15 cm of height) with an angle of 120° between each arm. The maze was placed in a separate room with a red light. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed next to the walls of the maze. The three identical arms were randomly designated: start arm, in which the rat started to explore (always open), novel arm, which was blocked during the 1st trial, but open during the 2nd trial, and other arm (always open). The Y-maze test consisted of two trials separated by an inter-trial interval (ITI) to assess response to novelty (2 min ITI) and spatial recognition memory (2 h ITI) (Dellu et al., 1997). The first trial (training) had 5 min duration and the rat was allowed to explore only two arms (start arm and other arm) of the maze, with the third arm (novel arm) closed. For the second trial (after ITI), the rat was placed back in the maze in the same starting arm, with free access to all three arms for 5 min. The number of entries and time spent in each arm were analyzed. Data were also expressed as percentage of total time spent in arms during the total 5 min.

Western blot analysis

After all behaviour tests, the rats were deeply anesthetized under halothane atmosphere before being killed by decapitation for the preparation of Percoll-purified hippocampal synaptosomes as previously described (e.g. Rebola et al., 2005). Briefly, the hippocampi from rats were homogenized at 4°C in sucrose solution (0.32 M) containing 50 mM Tris–HCl, 2 mM EGTA and 1 mM dithiothreitol, pH 7.6. The resulting homogenates were centrifuged at 3,000 x *g* for 10 min at 4 °C, the supernatants collected and centrifuged at 14,000 x *g* for 20 min at 4 °C. The pellets were resuspended in 1 ml of a 45% (v/v) Percoll solution made up in a Krebs solution (composition 140 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM EDTA, 10 mM glucose, pH 7.4). After centrifugation at 14,000 x *g* for 2 min at 4 °C, the top layer was removed (synaptosomal fraction), and washed in 2 ml Krebs solution. This mixture was centrifuged at 14,000 x *g* for 2 min at 4 °C. The pellet corresponded to the nerve terminal membranes and was resuspended in the SDS-PAGE buffer (see below) for Western blot analysis.

Western blot analysis was carried out in synaptosomal membranes of the hippocampus, as previously described (e.g. Rebola et al., 2005), except for GFAP which was evaluated in total hippocampal membranes. Briefly, after determining the amount of protein, each sample was diluted (final concentration of 2 μg/μl) in SDS-PAGE buffer containing 8 M urea, 100 mM dithiothreitol, 2% (w/v) sodium dodecyl sulfate and 375 mM Tris–HCl pH 6.8 and incubated for 10 min at 98 °C. These samples (15-20 μg of protein per membrane) and the pre-stained molecular weight markers (Amersham) were separated by SDS-PAGE (7.5% with a 4% concentrating gel) under reducing conditions and electro-transferred to polyvinylidene difluoride membranes (0.45 μm, from Amersham). After blocking for 1 h at room temperature with 3% bovine serum albumine (BSA) in Tris-buffered saline, pH 7.6 containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight at 4 °C with commercial antibodies against SNAP-25 (1:5,000 dilution; from Sigma), syntaxin (1:5,000 dilution; from Sigma), synaptophysin (1:2,000 dilution; from Sigma), vesicular glutamate transporters type 1 (vGluT1, 1:5,000 dilution, from Chemicon), vesicular GABA transporter (vGAT, 1:1,000 dilution, from Calbiochem), post-synaptic density 95 kDa protein (PSD-95, 1:20,000 dilution, from Upstate Biotechology), NMDA receptor subunit 1 (NR1, 1:400 dilution, from Chemicon), NMDA receptor subunit 2A (NR2A, 1:800 dilution, from Chemicon), NMDA receptor subunit 2B (NR2B, 1:200 dilution, from BD Biosciences), GluR1 subunit (1:400 dilution, from Upstate Biotechnology), GluR2 subunit (1:400 dilution, from Chemicon) or glial fibrillary acidic protein (GFAP, 1:1,000 dilution; from Cell Signalling). After three washing periods for 15 min with TBS-T, the membranes were incubated

with the alkaline phosphatase-conjugated secondary antibodies (1:10,000 dilution; from Amersham) in TBS-T containing 3% BSA during 90 min at room temperature. After three 15 min-washes in TBS-T, the membranes were incubated with enhanced chemi-fluorescence during 30 seconds and then analyzed with a VersaDoc 3000 (Biorad). The membranes were then re-probed and tested for α-tubulin (1:10,000 dilution, from Sigma) immunoreactivity to confirm that similar amounts of protein were applied to the gels (e.g. Rebola et al., 2005). For each tested protein, we confirmed by loading different amounts of protein in the same gel that we were working under non-saturating conditions enabling to effectively probe changes in the density of these proteins.

Membrane binding assays

Hippocampal nerve terminal membranes were resuspended in an incubation buffer containing 50 mM Tris and 10 mM $MgCl_2$, pH 7.4 and incubated with 2 U/ml adenosine deaminase (Sigma) for 30 min at 37 °C, to remove endogenous adenosine. The mixture was then centrifuged at 14,000 x *g* for 10 min at 4 °C and the pellets resuspended in the incubation buffer. To measure the density of A_1Rs, we incubated a supra-maximal concentration (6 nM) of the selective A_1 receptor antagonist, ^{3}H-1,3-dipropyl-8-cyclopentylxanthine (^{3}H-DPCPX) (specific activity of 109.0 Ci/mmol; from DuPont NEN) for 2 h at 37 °C, with 36-52 µg of protein in a final volume of 200 µl in the incubation solution containing 2 U/mL adenosine deaminase (e.g. Cunha et al., 2006; Rebola et al., 2003. 2005). To evaluate the binding density of A_{2A} receptors, we used 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolol[1,5c]pyrimidine (^{3}H-SCH58261, 6 nM), an A_{2A} receptor antagonist that has been shown to selectively bind cortical A_{2A} receptors and devoid of effects on A_1 receptors and which is supra-maximal at this concentration (Lopes et al., 2004). Binding of ^{3}H-SCH58261 (specific activity of 77 Ci/mmol; prepared by Amersham and generously offered by Dr. Ennio Ongini, Shering-Plough, Italy) was for 1 h at room temperature (23-25 °C) with 94-122 µg of protein in a final volume of 200 µl in the incubation solution containing 4 U/ml adenosine deaminase (see Lopes et al., 2004). Specific binding of either labelled compounds was determined by subtraction of the non-specific binding, which was measured in the presence of 1 µM 8-{4-[(2-aminoethyl)amino]carbonylmethyl-oxyphenyl}xanthine (XAC, Sigma), a mixed A_1/A_{2A} receptor antagonist. Each binding assay data point was performed in triplicate. The binding reactions were stopped by vacuum filtration through glass fiber filters (GF/C filters). The filters were then placed in 4 ml of scintillation liquid (Ready Safe, Pharmacia-Portugal)

and radioactivity was determined after at least 12 h with a counting efficiency of 55–60%. The protein concentration was determined using the bicinchoninic acid method (Pierce).

Immunocytochemistry in purified nerve terminal

The immunocytochemical analysis of hippocampal synaptosomes was performed essentially as previously described (e.g. Rodrigues et al., 2008). Synaptosomes were further purified through a discontinuous Percoll gradient (0.32 M sucrose; 1 mM EDTA; 0.25 mM dithiothreitol, and 3, 10, or 23% Percoll, pH 7.4) to obtain purified nerve terminals largely devoid of post-synaptic contaminants (Rodrigues *et al*, 2005). The gradients were centrifuged at 25,000 x *g* for 11 min at 4°C. Nerve terminals were collected between the 10% and 23% Percoll bands and diluted in 15 ml of HEPES-buffered medium (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4). Nerve terminals were then placed onto coverslips previously coated with poly-L-lysine, fixed with 4% paraformaldehyde for 15 min and washed twice with phosphate-buffered saline (PBS) medium (140 mM NaCl, 3 mM KCl, 20 mM NaH_2PO_4, 15 mM KH_2PO_4, pH 7.4). The nerve terminals were permeabilized in PBS with 0.2% Triton X-100 for 10 min and then blocked for 1 h in PBS with 3% bovine serum albumin (BSA) and 5% normal horse serum. Nerve terminals were then washed twice with PBS and incubated with goat anti-adenosine A_{2A} receptor (1:500; from Santa Cruz Biotechnology) or with rabbit anti-adenosine A_1 receptor antibody (1:400; from Affinity Bioreagents), together with mouse anti-synaptophysin antibody (1:200; from Sigma) for 1 h at room temperature. The nerve terminals were then washed three times with PBS with 3% BSA and incubated for 1 h at room temperature with AlexaFluor-598-(red) labelled donkey anti-goat IgG antibody or AlexaFluor-598-(red) labelled donkey anti-rabbit IgG and AlexaFluor-488-(green) labelled donkey anti-mouse IgG (1:200 dilution, from Molecular Probes). It was confirmed that none of the secondary antibodies produced any signal in preparations to which the addition of the corresponding primary antibody was omitted. After washing and mounting on slides with Prolong Antifade, the preparations were visualized in a Zeiss Axiovert 200 inverted fluorescence microscope equipped with a cooled CCD camera and analyzed with MetaFluor 4.0 software. Each coverslip (3 per animal) was analyzed by counting five different fields and in each field a total amount of at least 100 individualized elements.

Immunohistochemistry analysis

The preparation of brain sections was carried out as previously described (Cunha et al., 2006). Rats were anesthetized with pentobarbital (100 mg/kg), the heart was exposed and, after clamping the descending aorta, a catheter was inserted in the ascending aorta. The animal was then perfused with 200 ml of 0.9% (w/v) NaCl (saline) solution while opening the right atria to allow the outflow of the perfusate. Mice were then perfused with 200 ml of 4% (w/v) paraformaldehyde prepared in saline solution. After its fixation, the brain was removed, maintained for 12 h in the same paraformaldehyde solution and subsequently for 48 h in PBS containing 30% sucrose. The brain was then frozen and sliced in 20 µm coronal sections using a cryostat (Leica CM1850). The sections were stored in PBS containing 0.01% (w/v) sodium azide at 4 °C until mounting in slides coated with 2% gelatine with chromium and potassium sulphate. After drying at room temperature, the mounted sections were stored at -20 °C. Consecutive sections from each animal were used to carry out the histochemical and immunohistochemical procedures described below. The resulting brain slices were then visualized using an Axiovert 200 Zeiss fluorescence microscope (Hitec, Lisbon, Portugal). Microscope photographs were processed and analysed with the software ImageJ 1.37v (NIH, Betshesda, USA).

The general neuronal morphology in hippocampal sections was evaluated by using a Cresyl Violet staining of Nissl bodies, carried out as previously described (Lopes et al., 2003). Briefly, the slices were mounted in slides coated with 2% gelatin with chromium and potassium sulfate. After drying at room temperature, the sections stained for 10 min with Cresyl Violet solution (Sigma; 0.5% in acetate buffer). The sections were then washed twice with acetate buffer, twice in 100% ethanol before being cleared with xylene. Nissl staining was evaluated qualitatively by inspection of the pyramidal cell layer of both CA1 and CA3 regions by three independent researchers.

The population of astrocytes in the hippocampus was evaluated by immunohistochemical detection of glial fibrillary acidic protein (GFAP), a marker of activated astrocytes (Pekny and Nilsson, 2005). The brain sections were immersed in PBS (phosphate buffered saline) for 5 minutes and then in TBS (0.05 M Trizma base buffer containing 150 mM of NaCl, pH 7.2) 3 times for 5 minutes. Slices were blocked with TBS containing 0.2% Triton X-100 and 10% goat serum (Batista Marques) during 45 minutes and then incubated for 72 hours at 4ºC with Cy3-conjugated anti-GFAP mouse monoclonal antibody (1:1,000 dilution in TBS containing 0.2% Triton X-100 and 10% goat serum). Sections were then rinsed twice in TBS for 10 minutes, followed by a 10 minutes wash in distilled water. The sections were dehydrated and cleared in xylol and mounted on slides using Vectaschield

H-1400 mounting medium (Vector Laboratories) for analysis. The numbers of immuno-staining-positive cells in each hippocampal region was averaged from four sections of the hippocampus per rat.

Statistical analysis

Data are mean±SEM of n animals and significant differences were considered at $P<0.05$. Significance was assessed by one-way Student's *t*-test or using a one-way ANOVA followed by Bonferroni's test to compare data of the 4 experimental groups.

RESULTS

A single episode of convulsions in early life causes memory impairment in adult rats

As previously reported (Lynch et al., 2000; Sayin et al., 2004), the administration of kainate (KA) at a dose of 2 mg/kg (i.p.) caused a convulsive-like activity in pups 7 days old (P7). This consisted of hyperactive "bicycling" movements of all extremities with opisthonic arching of the back and tonic limbic extension, as previously described (e.g. Lynch et al., 2000), which occurred with a fast onset (circa 10 min after KA administration), lasted for 80-210 minutes, and disappeared spontaneously.

We then investigated if this single convulsive episode at P7 led to modified behaviour later in adolescence and adulthood. When analysed at P30 (n=6-7), KA-injected rats (at P7) displayed a behaviour in the open field, in the elevated-plus maze and in the modified Y-maze indistinguishable from that of saline-injected rats (Fig.1, first raw of panels). Likewise, when analysed at P60 (n=6-7), KA-injected rats (at P7) displayed a behaviour indistinguishable from that of saline-injected rats (Fig.1, second raw of panels). However, when analysed at P90 (n=6-7), KA-injected rats (at P7) displayed a significantly ($P<0.05$) poorer performance in a spatial memory version of the Y-maze test compared to saline-injected rats (Fig.1, right panel of bottom raw). In fact, in rats tested with all open arms of the Y maze two hours after exploring the maze with one arm closed, both the number of entries in the novel arm as well as the time spent in the novel arm (not shown) of the Y-maze of P90 rats injected with KA at P7 were significantly lower ($P<0.05$) than these observed for saline injected rats. In contrast, the locomotor activity and the anxiogenic-like behaviour of P90 rats injected with KA at P7 was not significantly different ($P>0.05$) from saline injected rats (Fig.1, bottom raw of panels).

This shows that a single KA-induced convulsive period at P7 leads to a delayed memory impairment, which is only observable at P90, but not at P30 or P60.

Caffeine and A_{2A} receptor blockade prevent convulsions-induced memory impairment

Previous studies in different animal models as well as in humans have consistently observed that the chronic consumption of caffeine attenuates memory deficits in a manner mimicked by antagonists of adenosine A_{2A} receptors (reviewed in Cunha, 2008; Takahashi et al., 2008). Thus, we investigated if the consumption of caffeine or of a selective antagonist of adenosine A_{2A} receptor could prevent this delayed deficit in memory impairment caused by a KA-induced convulsive period in early childhood. For this purpose, rats injected with either KA- or saline at P7 were divided into two groups: one group was exposed to caffeine (1 g/l in the drinking water) or to the selective A_{2A} receptor antagonist, KW6002 (3 mg/kg through the drinking water) whereas the non-treated group only drank water from P21 (yawing) until behavioural analysis and subsequent sacrifice.

When analysed at P90, it was again observed that the rats injected with KA at P7 and drinking only water displayed memory impairment in the Y-maze test (Fig.2, right panels). Interestingly, this memory impairment at P90 was abrogated in the group of KA-injected rats that were allowed to drink caffeine (Fig.2A, right panel). In contrast, caffeine was devoid of effect in saline-injected rats since the performance in the Y-maze of saline-injected rats without or with access to caffeine was similar (Fig.2A, right panel). Furthermore, the locomotor activity in the open field as well as the anxiogenic-like behaviour in the elevated-plus maze was similar in all 4 groups (KA- or saline-injected without or with caffeine, n=8-9) (Fig.2A, first two left panel of top raw).

Similar results were obtained when the rats were treated with the selective A_{2A} receptor antagonist, KW002 (Fig.2B). In fact, the memory impairment displayed at P90 by rats injected with KA at P7 was abrogated by the consumption of KW6002 from P21 onwards (Fig.2B, right panel), whereas KW6002 did not modify the Y-maze behaviour of saline-injected rats and the locomotor activity in the open field and the anxiogenic-like behaviour in the elevated-plus maze were similar ($P>0.05$) in all 4 groups of rats (KA- or saline-injected without or with KW6002, n=7-9) (Fig.2B, first two left panel of bottom raw).

Impact of childhood convulsions on the set-up of adenosine receptors in adulthood

We next investigated if the status of adenosine receptors in the hippocampus of P90 rats was modified upon the single convulsive period triggered by KA at P7. Since both A_1 and A_{2A} receptors are concentrated in hippocampal synapses (Rebola et al., 2003, 2005), we compared the binding densities of supra-maximal concentrations of the selective A_1 receptor antagonist ^{3}H-DPCPX (6 nM) and of the selective A_{2A} receptor antagonist ^{3}H-SCH58261 (6 nM) in hippocampal synaptosomal membranes from P90 rats. Binding of the A_{2A} receptor antagonist ^{3}H-SCH58261 showed that A_{2A} receptor density increased by 65.3±4.2% in P90 rats injected with KA at P7 compared to saline-injected rats (n=4, $P<0.05$; Fig.3A). In contrast, binding of the A_1 receptor antagonist ^{3}H-DPCPX showed that A_1 receptor density decreased by 24.0±2.4% in P90 rats injected with KA at P7 compared to saline-injected rats (n=4, $P<0.05$; Fig.3B).

We next confirmed if this modified density of A_1 and A_{2A} receptors corresponded to a modified number of nerve terminals equipped with each receptor. This was evaluated in hippocampal purified nerve terminals of P90 rats by double-labelling immunocytochemistry of A_1 or A_{2A} receptors with a marker of nerve terminals, synaptophysin (Fig.3C). The percentage of synaptophysin-immunopositive terminals endowed with A_1 receptor immunoreactivity was similar in both P90 rats injected with KA at P7 and saline-injected rats (Fig.3D). In contrast, there was an increased number of synaptophysin-immunopositive terminals endowed with A_{2A} receptor immunoreactivity in P90 rats injected with KA at P7 compared to saline-injected rats (Fig.3C,E). This indicates that KA-induced convulsions at P7 led to an increase of nerve terminals endowed with A_{2A} receptors, whereas the number of nerve terminals endowed with A_1 receptors was preserved albeit the density of A_1 receptors per terminal might have decreased, as concluded from the Western blot analysis.

Childhood convulsions lead to features of synaptotoxicity in adulthood

Evidence is accumulating supporting the idea that the deterioration of memory performance is primarily related to synaptic dysfunction and degeneration, i.e. synaptotoxicity (Coleman et al., 2004). Thus, we next tested if P90 rats, subject to a period of KA-induced convulsions at P7, displayed alterations of synaptic proteins integrating the vesicular release machinery, namely SNAP-25, synaptophysin and syntaxin. Western blot analysis (Figs.4A-C) showed a reduction of the immunoreactivity of SNAP-25 (-21.4±3.7%, n=4, $P<0.05$) and syntaxin (-20.2±3.0%, n=4, $P<0.05$) but not of synaptophysin (-6.3±3.0%, n=4, $P>0.05$) in hippocampal membranes of P90 rats that were injected with KA at P7, suggesting the occurrence of synaptic degeneration. To investigate if different

types of nerve terminals were affected in adulthood after KA-induced convulsions in childhood, we compared the density of a glutamatergic marker (vGluT1) and of a GABAergic marker (vGAT) in membranes from P90 rats injected either with KA or saline at P7. Western blot analysis (Figs.4D,E) showed a reduction of the immunoreactivity of vGluT1 (-27.2±3.3%, n=4, $P<0.05$) but not of vGAT (2.3±2.6%, n=4, $P>0.05$) in hippocampal membranes of P90 rats that were injected with KA at P7, suggesting the occurrence of glutamatergic rather than GABAergic synaptic degeneration. Next, we tested if childhood convulsions caused a modification in adulthood of the set-up of a post-synaptic marker (PSD95) as well as of different subunits of ionotropic glutamate receptors. Western blot analysis (Figs.4F,K) showed a lack of modification ($P>0.05$) of the immunoreactivity of PSD95 (n=4, Fig.4F), of the NR1 subunit of NMDA receptors (n=3, Fig.4G), of the NR2A subunit of NMDA receptors (n=4, Fig.4H), of the NR2B subunit of NMDA receptors (n=4, Fig.3I), of the GluR1 subunit of AMPA receptors (n=3, Fig.4J) and of the GluR2 subunit of AMPA receptors (n=2, Fig.4K) in hippocampal membranes of P90 rats that were injected with KA at P7 compared with the control saline-injected rats.

Since it was previously proposed that glial activation links early-life seizures and long-term neurologic dysfunction (Somera-Molina et al., 2007), we next investigated if there were persistent markers of astrocytic activation in the hippocampus of P90 rats that were subject to KA-induced convulsions at P7. Semi-quantitative immunohistochemistry of the astrocytic protein marker GFAP (Pekny and Nilsson, 2005) revealed a similar ($P>0.05$) density of GFAP-positive cells in the hippocampus of P90 rats injected with KA at P7, compared to the P90 rats injected with saline at P7 in the three hippocampal regions (Figs.5B,C). Accordingly, Western blot analysis showed a non-significant ($P>0.05$, n=3) 12.2±3.7% increase of GFAP immunoreactivity in hippocampal membranes of P90 rats that were injected with KA at P7 (Fig.5D), suggesting a lack of evident glial activation.

Caffeine and A_{2A} receptor prevent adulthood synaptotoxicity upon childhood convulsions

Since caffeine and KW6002 prevented the childhood convulsions-related memory impairment in adulthood, which was accompanied by a synaptotoxicity particularly of glutamatergic terminals, we next investigated if caffeine and KW6002 could prevent this synaptotoxicity at P90 resulting from KA administration at P7. As illustrated in Figure 6, the consumption of caffeine or KW6002 from P21 onwards prevented the decreased density of SNAP25 (n=4, $P<0.05$) and of syntaxin (n=4, $P<0.05$) in

hippocampal membranes of P90 rats that were subject to kainate-induced convulsion at P7; likewise, the decrease of vGluT1 was also prevented (n=4, $P<0.05$) by both caffeine and KW6002 (Fig.6), which were devoid of effects on vGAT density that was similar (n=4, $P>0.05$) in all 4 groups (Fig.6).

DISCUSSION

The present results indicate that a single period of convulsions in early childhood (P7) triggers selective memory deficits later in adulthood (P90), which are accompanied by a loss of presynaptic markers, in particular of glutamatergic terminals, without evident modification of postsynaptic markers, astrogliosis or neuronal architecture in the hippocampus. Furthermore, the present study shows that the chronic consumption of caffeine or of a selective A_{2A} receptor antagonist prevents the memory deficits and concurrent synaptotoxicity present in adult rats that were subject to a single period of convulsions early in childhood.

The first conclusion of this study was that a period of convulsions at P7 led to selective memory deficits, which is delayed in time, appearing only in adulthood. Previous studies had already reported that a single administration of kainate at P5-P10 can lead to spatial memory impairment later in life (e.g. Lynch et al., 2000; Sayin et al., 2004; Cornejo et al., 2007; but see Stafstrom et al., 1993). Likewise, induction of convulsive activity with different agents in immature rodents also triggers visuo-spatial memory dysfunction later in life (e.g. de Rogalski-Landrot et al., 2001; Lee et al., 2001; Huang et al., 2002; Cilio et al., 2003; Bo et al., 2004). The present results show that the spatial memory deficits were only observed at P90, but are not evident in late adolescence (P30) or early adulthood (P60). This aspect has not been systematically addressed in previous studies, some confirming that kainate-induced neonatal convulsions-associated memory deficits are present at P90 and later (Lynch et al., 2000; Sayin et al., 2004), whereas others reported memory deficits early in adulthood (P60) albeit they were rather marginal and only observed in some tasks (Cornejo et al., 2007). The relevance of these findings in humans is provocative: although evidence suggest that convulsive episodes early in life might be associated with a poorer neurological outcome (see Holmes and Ben-Ari, 1998; McBride et al., 2000; Shinnar and Hauser, 2002), it is still debatable if early-life convulsions are associated with a poorer memory performance (Dikmen et al., 1975; Chang et al., 2001; Okumura et al., 2006; Vingerhoets, 2006); however, sticking to the results we now report, it is interesting to note that most follow-up studies of children suffering from early life convulsive episodes only

evaluated different aspects of memory performance in late adolescence and early adulthood (Dikmen et al., 1975; Chang et al., 2001; Okumura et al., 2006; Vingerhoets, 2006), leaving room to question whether deficits might become more evidence later in adulthood. Finally, the present study also shows a rather selective affection of memory performance in adult rats that underwent a period of convulsions at P7. In fact, there was no modification of exploratory and locomotor activities (see also Lynch et al., 2000; Sayin et al., 2004), and there was no modification of anxiety-like behaviour in the elevated-plus maze test, in contrast to a previous report (Sayin et al., 2004); this might be related to the use of different rat strains (which were not specified in Sayin et al., 2004).

The second major conclusion of this study is related to the possible neurochemical traits underlying the observed memory deficits in adult rats that experienced a KA-induced convulsive period in early childhood. Previous studies have shown that short convulsive periods early in life do not lead to evident morphological modifications (reviewed in Stafstrom et al., 2002; Holmes, 2005), which can only be observed with repetitive convulsive periods (see Holmes and Ben-Ari, 1998). Likewise, adult animals subject to KA-induced convulsion in early childhood also display minor if any morphological modifications compared to control animals (Nitecka et al., 1984; Lynch et al., 2000; Cornejo et al., 2007). However, neurophysiological modifications such as more exuberant synaptic plasticity (Lynch et al., 2000; Cornejo et al., 2007) were observed in adult animals subject to KA-induced convulsions early in life. Interestingly, the different groups proposed different neurochemical explanations to interpret these neurophysiological changes, namely enhanced efficiency of inhibitory transmission (Lynch et al. 2000), enhanced efficiency of glutamatergic synapses (Cornejo et al., 2007) or persistent astrogliosis (Somera-Molina et al., 2007). In the present study, we did not find evidence for persistent astrogliosis since neither the density of GFAP, nor the number of GFAP-labelled astrocytes, nor the morphology of GFAP-labelled elements in the hippocampus of P90 rats subject to KA-induced convulsions were modified compared to controls. However, we provide the first direct demonstration of presynaptic modifications accompanying memory deficits in adult rats that were subject to a period of convulsions in childhood. In fact, we observed a decrease of two synaptic markers, SNAP-25 and syntaxin. This synaptotoxicity has also been proposed to be a primary and crucial feature responsible for memory impairment occurring in mild cognitive impairment (Scheff et al., 2007) and Alzheimer's disease (reviewed in Selkoe, 2002), since synaptotoxicity is the only morphological parameter that correlates with the degree of memory impairment (e.g. DeKosky and

Scheff, 1990; Terry et al., 1991). Furthermore, features of synaptotoxicity are characteristic of several other brain conditions where memory impairment is also present, such as aging (Canas et al., 2009), Huntington's (Li et al., 2001) or prion's diseases (Ferrer, 2002), HIV infection (Garden et al., 2002) or schizophrenia (Glantz et al., 2006). Most interestingly, we found a reduction of a marker of glutamatergic synapses (vGluT1) whereas a marker of GABAergic synapses (vGAT) was preserved in the hippocampus of adult rats subject to KA-induced convulsions at P7, which is suggestive of selective changes of glutamatergic rather than GABAergic synapses. This is in remarkable agreement with recent results indicating that this memory-related synaptotoxicity might occur particularly in glutamatergic terminals, since the density of vesicular glutamate transporters was found to be decreased in cortical regions of memory-impairment individuals with Alzheimer's disease (Kirvell et al., 2007), as well as in animal models of Alzheimer's disease (Bell et al., 2006; Minkeviciene et al., 2008). Thus, as occurs for Alzheimer's disease, it is likely that the reduced density of hippocampal synaptic proteins, particularly of glutamatergic terminals, may contribute to the memory impairment found in adult rats that suffered from childhood convulsions.

The likely involvement of synaptotoxicity in the mechanism of memory impairment caused in adulthood by early life convulsions is further supported by the main finding of the present work, i.e. that long-term caffeine consumption prevented both memory impairments and loss of nerve terminal markers in the hippocampus of adult rats that suffered from convulsions early in childhood. This ability of caffeine to prevent childhood-induced memory deficits in adulthood is paralleled by the ability of caffeine to prevent memory deficits found in aging, in Alzheimer's and Parkinson's diseases and in attention deficit and hyperactivity disorders (reviewed in Cunha, 2008; Takahashi et al., 2008). This suggests that caffeine is able to interfere with mechanisms playing a key role in demises of memory dysfunction (Cunha, 2008). The only known molecular targets for caffeine at non-toxic concentrations are A_1 and A_{2A} receptors, both antagonised by caffeine (Fredholm et al., 1999). The prevention of memory deficits by caffeine likely result from A_{2A} receptor antagonism since the beneficial effects of caffeine on memory performance deficits upon aging or Alzheimer's disease are mimicked by selective antagonists of A_{2A} receptors (Prediger et al., 2005; Cunha et al., 2008). Accordingly, we now observed that the selective A_{2A} receptor antagonist, KW6002, also prevented both the memory impairment and the loss of nerve terminal markers in the hippocampus of adult rats that suffered a convulsive period in their childhood. Interestingly, A_{2A} receptor antagonism not only

abrogates memory dysfunction but also affords robust neuroprotection against brain damage (Cunha, 2005; Chen et al., 2007). This further supports the involvement of synaptotoxicity in memory dysfunction since A_{2A} receptors have a predominant synaptic localization in the hippocampus (Rebola et al., 2005), where they control synaptotoxicity (Cunha et al., 2006; Silva et al., 2007), which is the most evident morphological change found in the hippocampus of adult rats that suffered a convulsive period early in their childhood. These synaptic A_{2A} receptors undergo a gain of function in noxious brain conditions (reviewed in Cunha, 2005). Accordingly, we now found an increase in the density of synaptic A_{2A} receptors in the hippocampus of adult rats that suffered a convulsive period early in their childhood. However, the mechanism by which A_{2A} receptors control the synaptotoxicity in glutamatergic synapses is still unclear, albeit the control of NMDA receptors (Rebola et al., 2008), of calcium loading (Gonçalves et al., 1997) and of synaptic mitochondria (Silva et al., 2007) are likely candidates.

In conclusion, the present study shows that a convulsive period early in life causes a selective impairment of memory performance later in adulthood, which is accompanied by a loss of synaptic markers, in particular of glutamatergic terminals. Furthermore, the chronic consumption of caffeine or of A_{2A} receptor antagonists starting in adolescence prevented this delayed memory deficit; this widens the prophylactic interest of caffeine and A_{2A} receptor antagonists to manage conditions associated with memory deterioration associated with synaptotoxicity.

ACKNOWLEDGEMENTS

The authors are grateful to Paula M. Canas, Ana Patrícia Simões, Carla G. Silva, Gabriele Ghisleni and Manuella P. Kaster for their help in carrying out some of the experiments. The dedicated and competent help of Alexandre Pires to handle the animals is especially acknowledged. This work was supported by FCT (PTDC/SAU-NEU/74318/2006) and was made possible by a joint Portuguese-Brazilian grant (CAPES-GRICES).

REFERENCES

Abraham WC, Bear MF (1996) Metaplasticity: the plasticity of synaptic plasticity. Trends Neurosci 19: 126-130.

André V, Dubé C, François J, Leroy C, Rigoulot MA, Roch C, Namer IJ, Nehlig A (2007) Pathogenesis and pharmacology of epilepsy in the lithium-pilocarpine model. Epilepsia 48(Suppl. 5): 41-47.

Arendash GW, Schleif W, Rezai-Zadeh K, Jackson EK, Zacharia LC, Cracchiolo JR, Shippy D, Tan J (2006) Caffeine protects Alzheimer's mice against cognitive impairment and reduces brain beta-amyloid production. Neuroscience 142: 941-952.

Bell KF, Ducatenzeiler A, Ribeiro-da-Silva A, Duff K, Bennett DA, Cuello AC (2006) The amyloid pathology progresses in a neurotransmitter-specific manner. Neurobiol. Aging 27:1644-1657.Ben-Ari Y (1985) Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience 14: 375-403.

Bo T, Jiang Y, Cao H, Wang J, Wu X (2004) Long-term effects of seizures in neonatal rats on spatial learning ability and N-methyl-D-aspartate receptor expression in the brain. Dev Brain Res 152: 137-142.

Canas PM, Duarte JM, Rodrigues RJ, Köfalvi A, Cunha RA (2009) Modification upon aging of the density of presynaptic modulation systems in the hippocampus. Neurobiol Aging (in press).

Chang YC, Guo NW, Wang ST, Huang CC, Tsai JJ (2001) Working memory of school-aged children with a history of febrile convulsions: a population study. Neurology 57: 37-42.

Chen JF, Sonsalla PK, Pedata F, Melani A, Domenici MR, Popoli P, Geiger J, Lopes LV, de Mendonça A (2007) Adenosine A_{2A} receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation. Prog Neurobiol 83: 310-331.

Cilio MR, Sogawa Y, Cha BH, Liu X, Huang LT, Holmes GL (2003) Long-term effects of status epilepticus in the immature brain are specific for age and model. Epilepsia 44: 518-528.

Coleman P, Federoff H, Kurlan R (2004) A focus on the synapse for neuroprotection in Alzheimer disease and other dementias. Neurology 63: 1155-1162.

Cornejo BJ, Mesches MH, Coultrap S, Browning MD, Benke TA (2007) A single episode of neonatal seizures permanently alters glutamatergic synapses. Ann Neurol 61: 411-426.

Costa MS, Botton PH, Mioranzza S, Souza DO, Porciúncula LO (2008) Caffeine prevents age-associated recognition memory decline and changes brain-derived neurotrophic factor and tirosine kinase receptor (TrkB) content in mice. Neuroscience 153: 1071-1078.

Cunha RA (2005) Neuroprotection by adenosine in the brain: from A_1 receptor activation to A_{2A} receptor blockade. Purinergic Signal 1: 111-134.

Cunha RA (2008) Caffeine, adenosine receptors, memory and Alzheimer disease. Med Clin 131: 790-795.

Cunha GM, Canas PM, Oliveira CR, Cunha RA (2006) Increased density and synapto-protective effect of adenosine A_{2A} receptors upon sub-chronic restraint stress. Neuroscience 141: 1775-1781.

Cunha GM, Canas PM, Melo CS, Hockemeyer J, Müller CE, Oliveira CR, Cunha RA (2008) Adenosine A_{2A} receptor blockade prevents memory dysfunction caused by beta-amyloid peptides but not by scopolamine or MK-801. Exp Neurol 210: 776-781.

Dall'Igna OP, Porciúncula LO, Souza DO, Cunha RA, Lara DR (2003) Neuroprotection by caffeine and adenosine A_{2A} receptor blockade of beta-amyloid neurotoxicity. Br J Pharmacol 138: 1207-1209.

Dall'Igna OP, Fett P, Gomes MW, Souza DO, Cunha RA, Lara DR (2007) Caffeine and adenosine A_{2a} receptor antagonists prevent beta-amyloid (25-35)-induced cognitive deficits in mice. Exp Neurol 203: 241-245.

Dawson GR, Tricklebank MD (1995) Use of the elevated plus maze in the search for novel anxiolytic agents. Trends Pharmacol Sci 16: 33-36.

DeKosky ST, Scheff SW (1990) Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. Ann. Neurol. 27: 457-464.

Dellu F, Fauchey V, LeMoal M, Simon H (1997) Extension of a new two-trial memory task in the rat: influence of environmental context on recognition processes. Neurobiol Learn Mem 67: 112-120.

de Rogalski-Landrot I, Minokoshi M, Silveira DC, Cha BH, Holmes GL (2001) Recurrent neonatal seizures: relationship of pathology to the electroencephalogram and cognition. Dev Brain Res 129: 27-38.

Dikmen S, Matthews CG, Harley JP (1975) The effect of early versus late onset of major motor epilepsy upon cognitive-intellectual performance. Epilepsia 16: 73-81.

Dubé CM, Brewster AL, Richichi C, Zha Q, Baram TZ (2007) Fever, febrile seizures and epilepsy. Trends Neurosci 30: 490-496.

El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM (2008) Evidence for the involvement of the adenosine A_{2A} receptor in the lowered susceptibility to pentylenetetrazol-induced seizures produced in mice by long-term treatment with caffeine. Neuropharmacology 55: 35-40.

Eskelinen MH, Ngandu T, Tuomilehto J, Soininen H, Kivipelto M (2009) Midlife coffee and tea drinking and the risk of late-life dementia: a population-based CAIDE study. J Alzheimers Dis 16: 85-91.

Ferrer I (2002) Synaptic pathology and cell death in the cerebellum in Creutzfeldt-Jakob disease. Cerebellum 1: 213-222.

Fredholm BB, Bättig K, Holmén J, Nehlig A, Zvartau EE (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 51: 83-133.

Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM (2005) Adenosine and brain function. Int Rev Neurobiol 63: 191-270.

Georgiev V, Johansson B, Fredholm BB (1993) Long-term caffeine treatment leads to a decreased susceptibility to NMDA-induced clonic seizures in mice without changes in adenosine A1 receptor number. Brain Res 612: 271-277.

Glantz LA, Gilmore JH, Lieberman JA, Jarskog LF (2006) Apoptotic mechanisms and the synaptic pathology of schizophrenia. Schizophr Res 81: 47-63.

Gonçalves ML, Cunha RA, Ribeiro JA (1997) Adenosine A_{2A} receptors facilitate $^{45}Ca^{2+}$ uptake through class A calcium channels in rat hippocampal CA3 but not CA1 synaptosomes. Neurosci Lett 238: 73-77.

Haas KZ, Sperber EF, Opanashuk LA, Stanton PK, Moshé SL (2001) Resistance of immature hippocampus to morphologic and physiologic alterations following status epilepticus or kindling. Hippocampus 11: 615-625.

Holmes GL (2005) Effects of seizures on brain development: lessons from the laboratory. Pediatr Neurol 33: 1-11.

Holmes GL, Ben-Ari Y (1998) Seizures in the developing brain: perhaps not so benign after all. Neuron 21: 1231-1234.

Huang LT, Yang SN, Liou CW, Hung PL, Lai MC, Wang CL, Wang TJ (2002) Pentylenetetrazol-induced recurrent seizures in rat pups: time course on spatial learning and long-term effects. Epilepsia 43: 567-573.

Hughes RN (2004) The value of spontaneous alteration behavior (SAB) as a test of retention in pharmacological investigations of memory. Neurosci Behav Rev 28: 497-505.

Johansson B, Georgiev V, Kuosmanen T, Fredholm BB (1996) Long-term treatment with some methylxanthines decreases the susceptibility to bicuculline- and pentylenetetrazol-induced seizures in mice. Relationship to c-fos expression and receptor binding. Eur J Neurosci 8: 2447-2458.

Kirvell SL, Esiri M, Francis PT (2007) Down-regulation of vesicular glutamate transporters precedes cell loss and pathology in Alzheimer's disease. J Neurochem 98: 939-950.

Koh S, Storey TW, Santos TC, Mian AY, Cole AJ (1999) Early-life seizures in rats increase susceptibility to seizure-induced brain injury in adulthood. Neurology 53: 915-921.

Lee CL, Hannay J, Hrachovy R, Rashid S, Antalffy B, Swann JW (2001) Spatial learning deficits without hippocampal neuronal loss in a model of early-onset epilepsy. Neuroscience 107: 71-84.

Li H, Li SH, Yu ZX, Shelbourne P, Li XJ (2001) Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. J Neurosci 21: 8473-8481.

Lopes LV, Rebola N, Pinheiro PC, Richardson PJ, Oliveira CR, Cunha RA (2003) Adenosine A3 receptors are located in neurons of the rat hippocampus. Neuroreport 14: 1645-1648.

Lopes LV, Halldner L, Rebola N, Johansson B, Ledent C, Chen JF, Fredholm BB, Cunha RA (2004) Binding of the prototypical adenosine A_{2A} receptor agonist, CGS 21680, to the cerebral cortex of adenosine A_1 and A_{2A} receptor knockout mice. Br J Pharmacol 141: 1006-1014.

Lynch M, Sayin U, Bownds J, Janumpalli S, Sutula T (2000) Long-term consequences of early postnatal seizures on hippocampal learning and plasticity. Eur J Neurosci 7: 2252-2264.

Maia L, de Mendonça A (2002) Does caffeine intake protect from Alzheimer's disease? Eur J Neurol 9: 377-382.

McBride MC, Laroia N, Guillet R (2000) Electrographic seizures in neonates correlate with poor neurodevelopmental outcome. Neurology 55: 506-513.

Minkeviciene R, Ihalainen J, Malm T, Matilainen O, Keksa-Goldsteine V, Goldsteins G, Iivonen H, Leguit N, Glennon J, Koistinaho J, Banerjee P, Tanila H (2008) Age-related decrease in stimulated glutamate release and vesicular glutamate transporters in APP/PS1 transgenic and wild-type mice. J Neurochem 105: 584-594.

Nitecka L, Tremblay E, Charton G, Bouillot JP, Berger ML, Ben-Ari Y (1984) Maturation of kainic acid seizure-brain damage syndrome in the rat. II. Histopathological sequelae. Neuroscience 13: 1073-1094.

Okumura A, Watanabe K, Negoro T, Hayakawa F, Kato T, Maruyama K, Kubota T, Suzuki M, Kurahashi H, Azuma Y (2006) Long-term follow-up of patients with benign partial epilepsy in infancy. Epilepsia 47: 181-185.

Pekny M, Nilsson M (2005) Astrocyte activation and reactive gliosis. Glia 50: 427-434.

Prediger RD, Batista LC, Takahashi RN (2005) Caffeine reverses age-related deficits in olfactory discrimination and social recognition memory in rats. Involvement of adenosine A_1 and A_{2A} receptors. Neurobiol Aging 26: 957-964.

Rebola N, Pinheiro PC, Oliveira CR, Malva JO, Cunha RA (2003) Subcellular localization of adenosine A_1 receptors in nerve terminals and synapses of the rat hippocampus. Brain Res 987: 49-58.

Rebola N, Canas PM, Oliveira CR, Cunha RA (2005) Different synaptic and subsynaptic localization of adenosine A_{2A} receptors in the hippocampus and striatum of the rat. Neuroscience 132: 893-903.

Rebola N, Lujan R, Cunha RA, Mulle C (2008) Long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses: an essential role for adenosine A_{2A} receptors. Neuron 57: 121-134.

Rigoulot MA, Leroy C, Koning E, Ferrandon A, Nehlig A (2003) Prolonged low-dose caffeine exposure protects against hippocampal damage but not against the occurrence of epilepsy in the lithium-pilocarpine model in the rat. Epilepsia 44: 529-535.

Ritchie K, Carrière I, de Mendonca A, Portet F, Dartigues JF, Rouaud O, Barberger-Gateau P, Ancelin ML (2007) The neuroprotective effects of caffeine: a prospective population study (the Three City Study). Neurology 69: 536-545.

Rodrigues RJ, Almeida T, Richardson PJ, Oliveira CR, Cunha RA (2005) Dual presynaptic control by ATP of glutamate release via facilitatory P2X1, P2X2/3, and P2X3 and inhibitory P2Y1, P2Y2, and/or P2Y4 receptors in the rat hippocampus. J Neurosci 25: 6286-6295.

Rodrigues RJ, Canas PM, Lopes LV, Oliveira CR, Cunha RA (2008) Modification of adenosine modulation of acetylcholine release in the hippocampus of aged rats. Neurobiol Aging 29: 1597-1601.

Rutten A, van Albada M, Silveira DC, Cha BH, Liu X, Hu YN, Cilio MR, Holmes GL (2002) Memory impairment following status epilepticus in immature rats: time-course and environmental effects. Eur J Neurosci 16: 501-513.

Sayin U, Sutula TP, Stafstrom CE (2004) Seizures in the developing brain cause adverse long-term effects on spatial learning and anxiety. Epilepsia 45: 1539-1548.

Scheff SW, Price DA, Schmitt FA, DeKosky ST, Mufson EJ (2007) Synaptic alterations in CA1 in mild Alzheimer disease and mild cognitive impairment. Neurology 68:1501-1508.

Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. Science 298: 789-791.

Shinnar S, Hauser WA (2002) Do occasional brief seizures cause detectable clinical consequences? Prog Brain Res 135: 221-235.

Silva CG, Porciúncula LO, Canas PM, Oliveira CR, Cunha RA (2007) Blockade of adenosine A_{2A} receptors prevents staurosporine-induced apoptosis of rat hippocampal neurons. Neurobiol Dis 27: 182-189.

Somera-Molina KC, Robin B, Somera CA, Anderson C, Stine C, Koh S, Behanna HA, Van Eldik LJ, Watterson DM, Wainwright MS (2007) Glial activation links early-life seizures and long-term neurologic dysfunction: evidence using a small molecule inhibitor of proinflammatory cytokine upregulation. Epilepsia 48: 1785-1800.

Stafstrom CE (2002) Assessing the behavioral and cognitive effects of seizures on the developing brain. Prog Brain Res 135: 377-390.

Stafstrom CE, Thompson JL, Holmes GL (1992) Kainic acid seizures in the developing brain: status epilepticus and spontaneous recurrent seizures. Dev Brain Res 65: 227-236.

Stafstrom CE, Chronopoulos A, Thurber S, Thompson JL, Holmes GL (1993) Age-dependent cognitive and behavioral deficits after kainic acid seizures. Epilepsia 34: 420-432.

Takahashi RN, Pamplona FA, Prediger RD (2008) Adenosine receptor antagonists for cognitive dysfunction: a review of animal studies. Front Biosci 13: 2614-2632.

Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R (1991) Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann. Neurol. 30: 572-580.

Vingerhoets G (2006) Cognitive effects of seizures. Seizure 15: 221-226.

Figure 1 Rats suffering from a kainate-induced convulsive period early in childhood display selective memory impairment in adulthood, but not in late adolescence or early adulthood, without modification of locomotion, exploratory or anxiogenic behaviour. Pups with 7 days of age received an i.p. injection of either saline (open bars) or kainate (2 mg/kg, black bars), which caused a period of convulsions. Rats were then behaviourally analysed when adolescent (P30), young adults (P60), and adults (P90-100). Locomotion was evaluated in an open field arena (first column from the left) by counting the number of crossings and rearings of the rats during 5 min. The degree of anxiety was determined by their preference for the closed and open arms of an elevated-plus maze (second column from the left). The reaction to novelty was tested in a Y-maze by measuring the number of entrances in an arm that had been closed in a previous exploration of the maze 2 min before the test (2 min ITI, third column from the left). Spatial memory was also evaluated using a Y-maze by measuring the number of entrances in an arm that had been closed in a previous exploration of the maze now 2 hours before the test (2 h ITI, first column from the right). Note that a period of convulsions in childhood selectively affected spatial memory and only in adult rats. Data are means±SEM of n=6-7 rats per group; * $P<0.05$ using a Student's *t* test.

Figure 2 Consumption of caffeine or of the selective adenosine A_{2A} receptor antagonist KW6002 beginning in adolescence prevents the memory impairment present in adult rats that suffered from a convulsive period early in their childhood. Pups with 7 days of age received an i.p. injection of either saline (open bars) or kainate (2 mg/kg, filled bars), which caused a period of convulsions. From day 21 onwards each of these two groups of rats were further sub-divided into two groups, one drinking water (black bars) and the other (grey bars) drinking either caffeine (1 g/L, upper raw of panels) or the A_{2A} receptor antagonist KW6002 (3 mg/kg, bottom raw of panels). Rats were then behaviourally analysed when adults, between 90-100 days of age. Locomotion was evaluated in an open field arena (first column from the left) by counting the number of crossings and rearings of the rats during 5 min. The degree of anxiety was determined by their preference for the closed and open arms of an elevated-plus maze (second column from the left). Spatial memory was also evaluated using a Y-maze by measuring the number of entrances in an arm that had been closed in a previous exploration of the maze 2 hours before the test (2 h ITI, first column from the right). Note that a period of convulsions in

childhood selectively affected spatial memory and this deficit was abrogated by consumption of either caffeine or KW6002. Data are means±SEM of n=7-9 rats per group; * P<0.05 using a one-way ANOVA followed by Bonferroni's test.

Figure 3 Adult rats that suffered from a convulsive period in early childhood display an increased density of synaptic adenosine A_{2A} receptors and a decreased density of synaptic adenosine A_1 receptors. Panel A and B compare the binding density (presented as specific binding) of a supra-maximal concentration (6 nM) of a selective antagonist of adenosine A_1 receptors (^{3}H-DPCPX, A) or of a supra-maximal concentration (6 nM) of the selective antagonist of adenosine A_{2A} receptors (^{3}H-SCH58261, B) to membranes of hippocampal synaptosomes of adult (P90) rats that were either injected with saline (open bars) or with 2 mg/kg of kainate at P7 (filled bars) to trigger a period of convulsions. Panel C shows immunocytochemical double labelling of synaptophysin (a synaptic marker, left photographs) and of A_{2A} receptors (right photographs) in hippocampal purified nerve terminals from adult (P100) rats that were either injected with saline (top photographs) or with 2 mg/kg of kainate at P7 (bottom photographs) to trigger a period of convulsions. The average quantification of the number of nerve terminals endowed with A_{2A} receptors is displayed in panel E, whereas panel D shows the number or nerve terminals endowed with A_1 receptors in hippocampal purified nerve terminals of adult (P100) rats that were either injected with saline (open bars) or with 2 mg/kg of kainate at P7 (filled bars) to trigger a period of convulsions. Data are means±SEM of n=4 rats per group. * P<0.05 using a Student's *t* test.

Figure 4 Adult rats that suffered from a convulsive period in early childhood display a decreased density of synaptic markers, in particular of glutamatergic but not GABAergic terminals. Pups with 7 days of age received an i.p. injection of either saline (open bars) or kainate (2 mg/kg, black bars), which caused a period of convulsions. Rats were sacrificed as adults (P100) and synaptosomal membranes prepared from their hippocampi for Western blot analysis of the immunoreactivity (IR) of 3 presynaptic proteins, SNAP-25 (A) syntaxin (B) and synaptophysin (C), markers of glutamatergic (vesicular glutamate transporter type 1, vGluT1, D) and GABAergic nerve terminals (vesicular GABA transporter, vGAT, E), markers of the post-synaptic density (PSD-95, F) and different subunits of different glutamate ionotropic receptors expected to be mainly located post-synaptically, such as NMDA receptor subunit 1 (NR1, G),

NMDA receptor subunit 2A (NR2A, H), NMDA receptor subunit 2B (NR2B, I), AMPA receptor GluR1 subunit (J), AMPA receptor GluR2 subunit (K). Data are means±SEM of n=4 rats per group, except data in panel J (n=3) and the data in panel K (which are individual data from 2 experiments); * P<0.05 using a Student's *t* test.

Figure 5 Adult rats that suffered from a convulsive period in early childhood do not display modified astrocytic reactivity. Pups with 7 days of age received an i.p. injection of either saline (open bars) or kainate (2 mg/kg, black bars), which caused a period of convulsions. Rats were sacrificed as adults (P100) to prepare either brain sections or total hippocampal membranes. Staining of the sections with cresyl violet (A) revealed a pattern of cell body organization in the hippocampal formation similar in the two groups. Immunohistochemical staining of the section for GFAP (an astrocytic marker, B) revealed a similar number of stained elements (quantified in C) with similar morphology in the three main hippocampal regions, CA1 CA3 and dentate gyrus (DG). This lack of modification of astrocytic density was confirmed by Western blot analysis (D) of GFAP immunoreactivity (IR) in total membranes from the 2 groups of rats. Histochemical analysis was performed in 3 rats per group, whereas the Western bots were carried out in membranes derived from 4 rats per group. Data in the bar graphs are means±SEM.

Figure 6 Consumption of caffeine or of the selective A_{2A} receptor antagonist KW6002 prevents the loss of synaptic markers, namely of glutamatergic terminals, in the hippocampus of adult rats that suffered from a convulsive period in early childhood. Pups with 7 days of age received an i.p. injection of either saline (open bars) or kainate (2 mg/kg, filled bars), which caused a period of convulsions. From day 21 onwards each of these 2 groups of rats were further sub-divided into 2 groups, one drinking water (black bars) and the other (grided bars) drinking either caffeine (1 g/L, upper raw of panels) or the A_{2A} receptor antagonist KW6002 (3 mg/kg, bottom raw of panels). Rats were sacrificed as adults (P100) and synaptosomal membranes prepared from their hippocampi for Western blot analysis of the immunoreactivity (IR) of 2 presynaptic proteins (SNAP-25 and syntaxin), a marker of glutamatergic (vesicular glutamate transporter type 1, vGluT1) and a marker of GABAergic nerve terminals (vesicular GABA transporter, vGAT). Data are means±SEM of n=4 rats per group; * P<0.05 using a one-way ANOVA followed by Bonferroni's test.

FIGURE 1

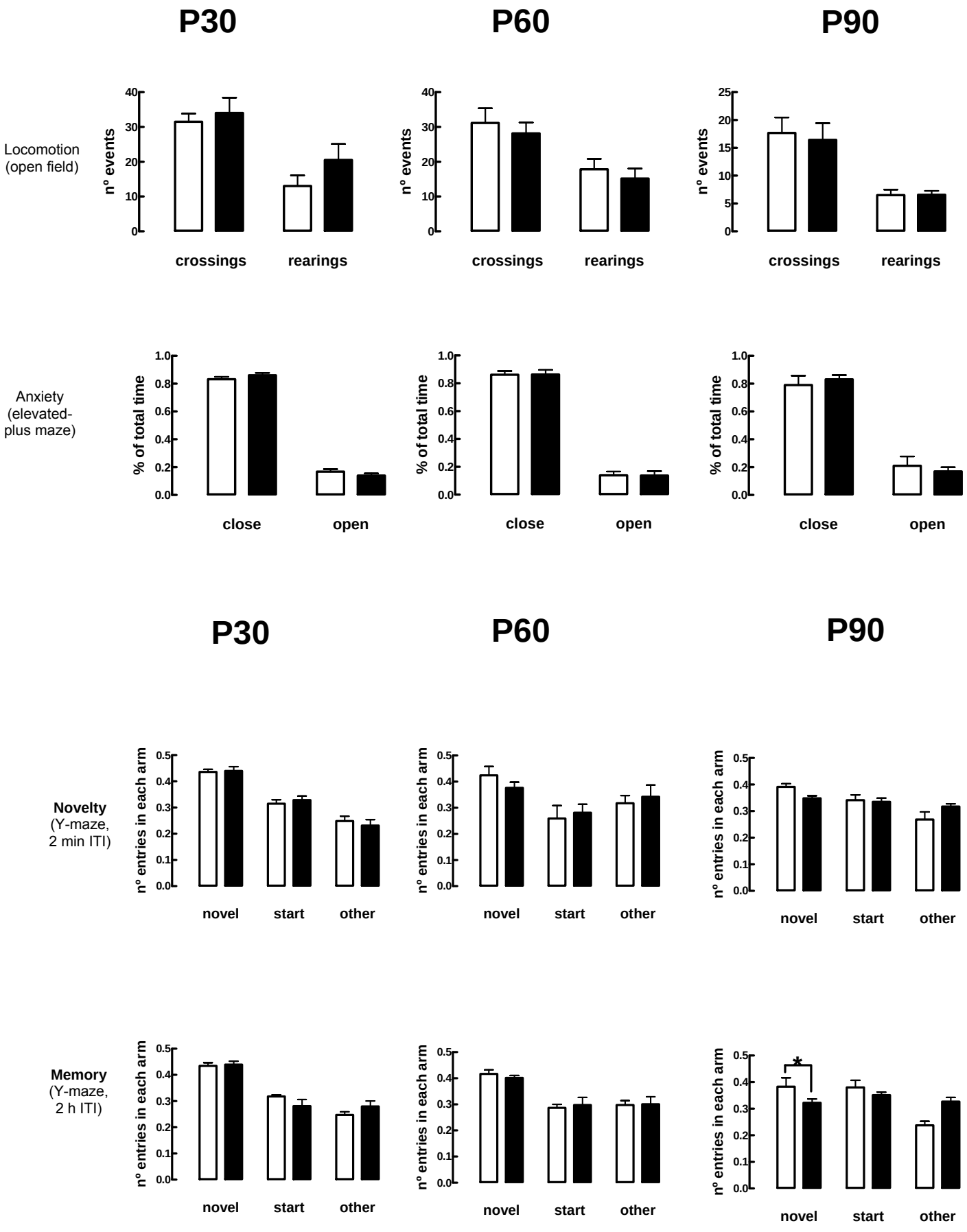

P30
P60
P90
Locomotion (open field)
n° events
crossings
rearings
Anxiety (elevated-plus maze)
% of total time
close
open
Novelty (Y-maze, 2 min ITI)
Memory (Y-maze, 2 h ITI)
n° entries in each arm
novel
start
other
*

FIGURE 2

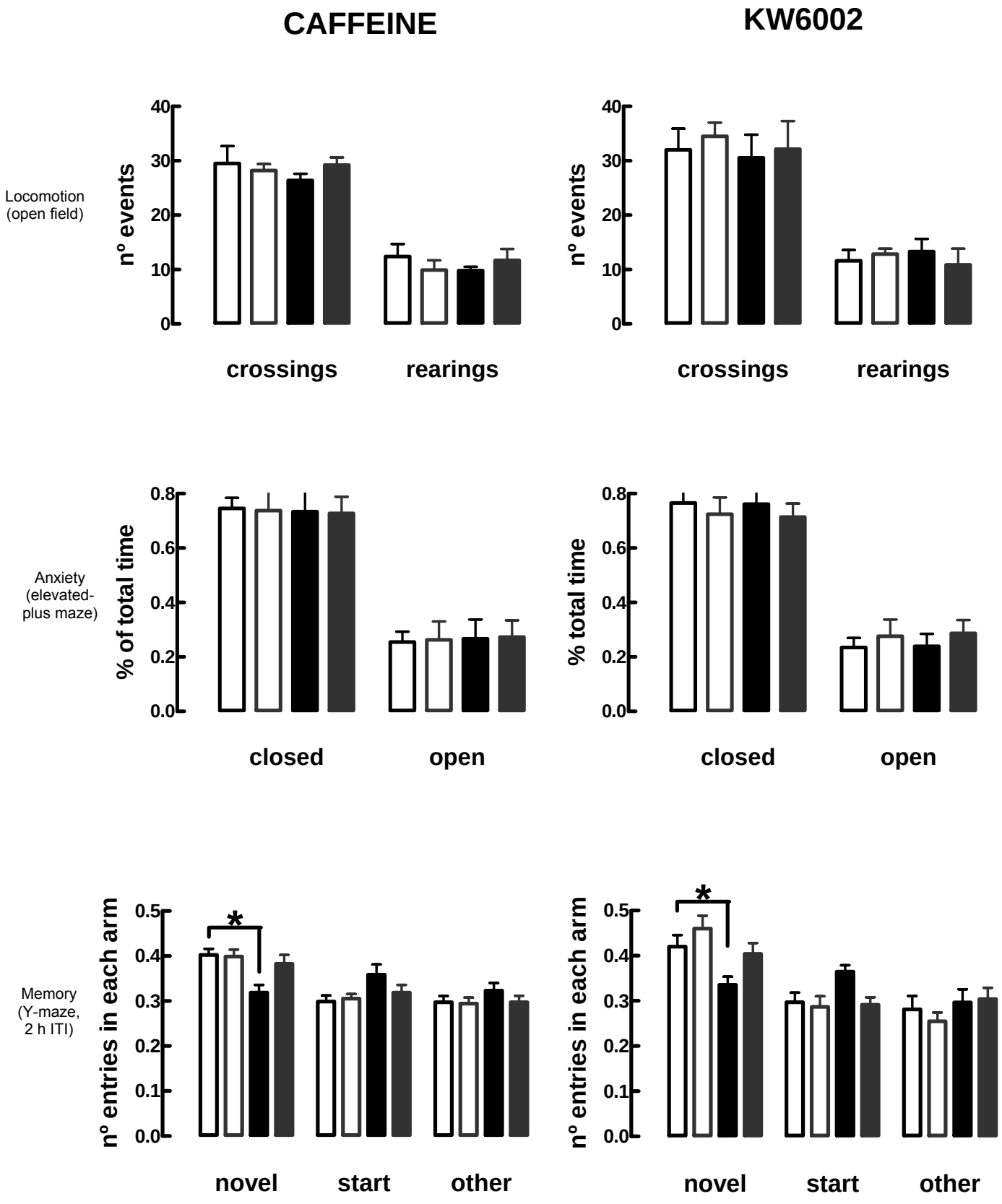

CAFFEINE
KW6002
Locomotion (open field)
nº events
40
30
20
10
0
crossings
rearings
Anxiety (elevated-plus maze)
% of total time
0.8
0.6
0.4
0.2
0.0
closed
open
Memory (Y-maze, 2 h ITI)
nº entries in each arm
0.5
0.4
0.3
0.2
0.1
0.0
*
novel
start
other

FIGURE 3

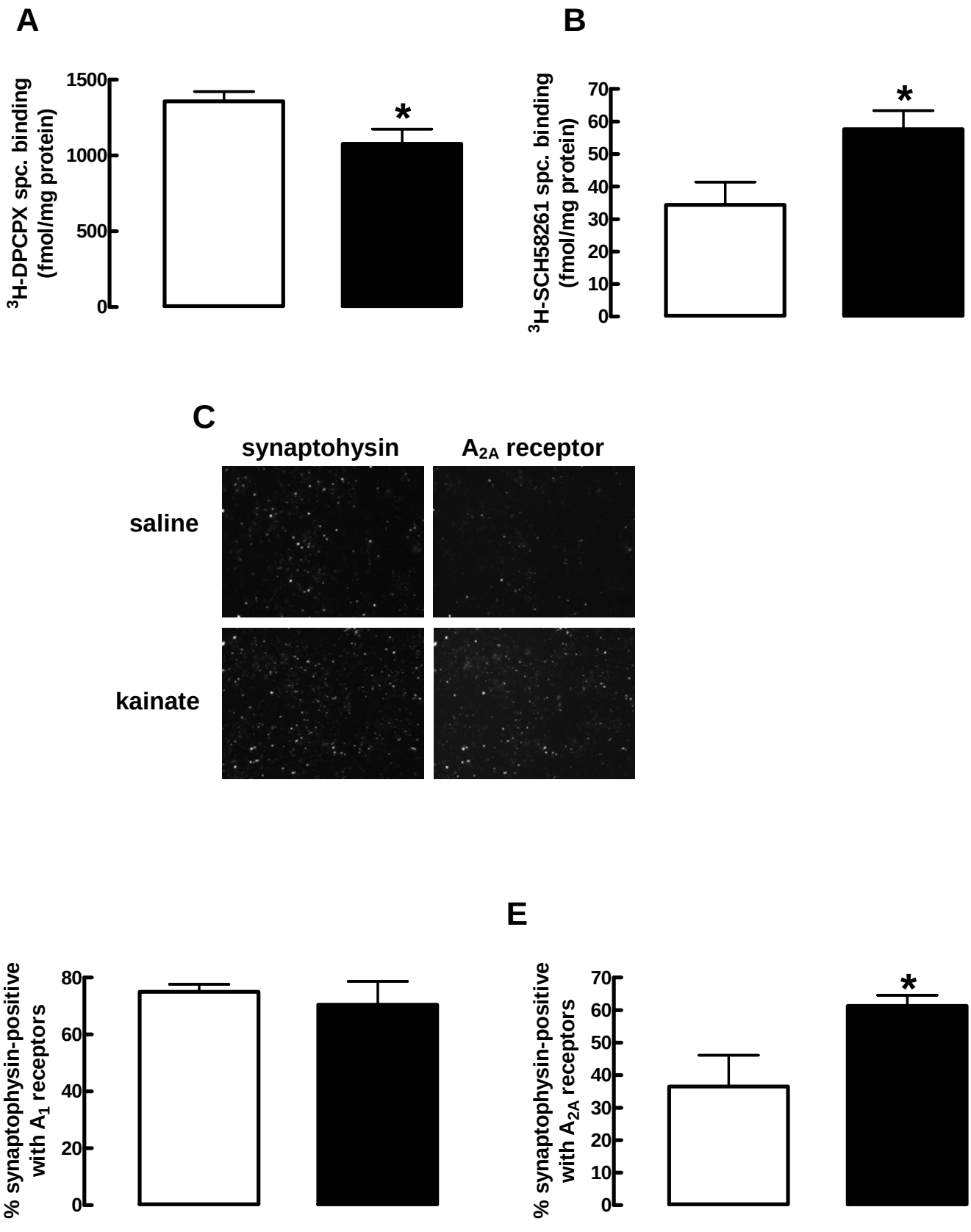

A
B
C
D
E
^{3}H-DPCPX spc. binding (fmol/mg protein)
1500
1000
500
0
*
^{3}H-SCH58261 spc. binding (fmol/mg protein)
70
60
50
40
30
20
10
0
*
synaptohysin
A_{2A} receptor
saline
kainate
% synaptophysin-positive with A_1 receptors
80
60
40
20
0
% synaptophysin-positive with A_{2A} receptors
70
60
50
40
30
20
10
0
*

FIGURE 4

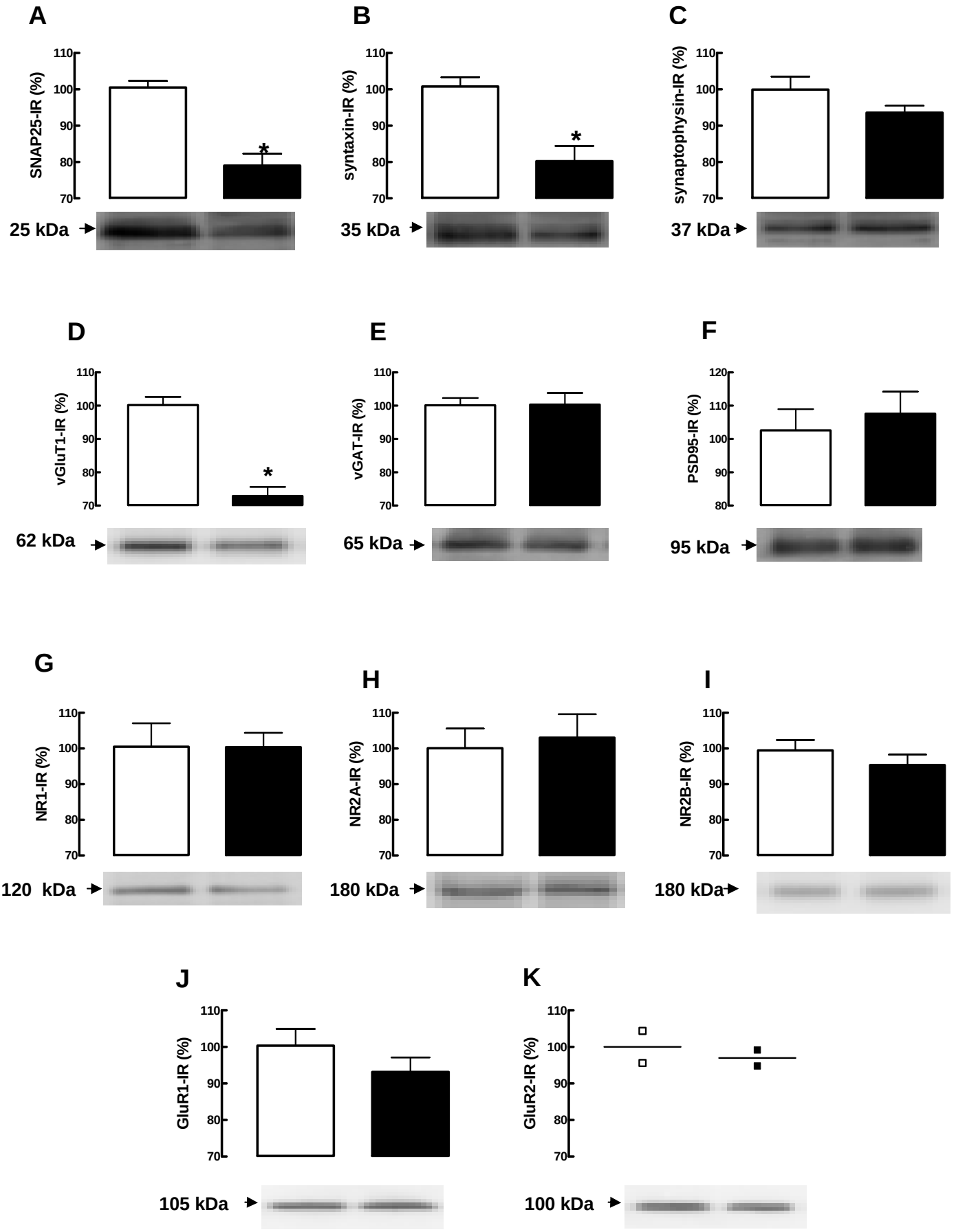

A
SNAP25-IR (%)
110
100
90
80
70
*
25 kDa
B
syntaxin-IR (%)
110
100
90
80
70
*
35 kDa
C
synaptophysin-IR (%)
110
100
90
80
70
37 kDa
D
vGluT1-IR (%)
110
100
90
80
70
*
62 kDa
E
vGAT-IR (%)
110
100
90
80
70
65 kDa
F
PSD95-IR (%)
120
110
100
90
80
95 kDa
G
NR1-IR (%)
110
100
90
80
70
120 kDa
H
NR2A-IR (%)
110
100
90
80
70
180 kDa
I
NR2B-IR (%)
110
100
90
80
70
180 kDa
J
GluR1-IR (%)
110
100
90
80
70
105 kDa
K
GluR2-IR (%)
110
100
90
80
70
100 kDa

FIGURE 5

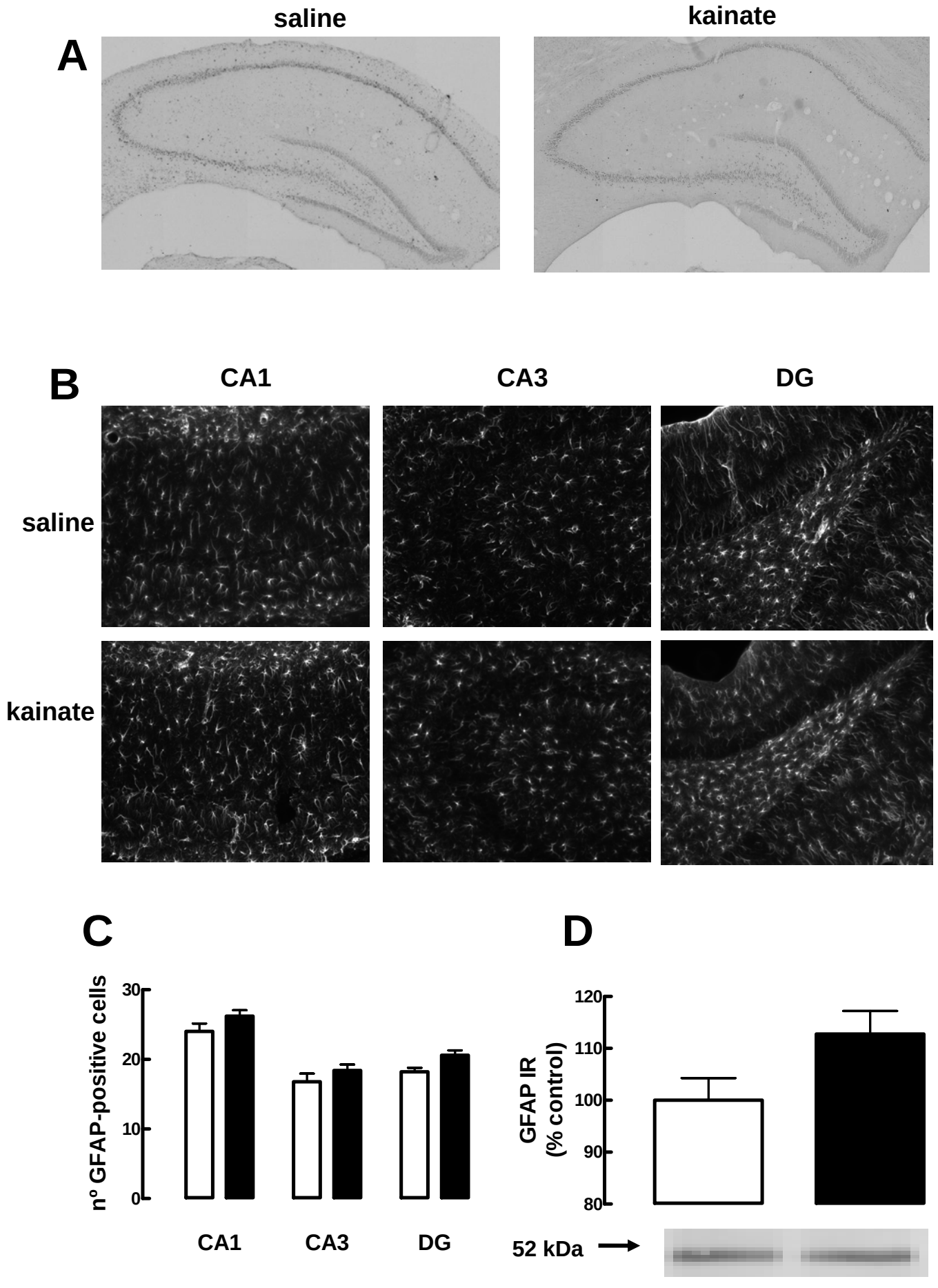
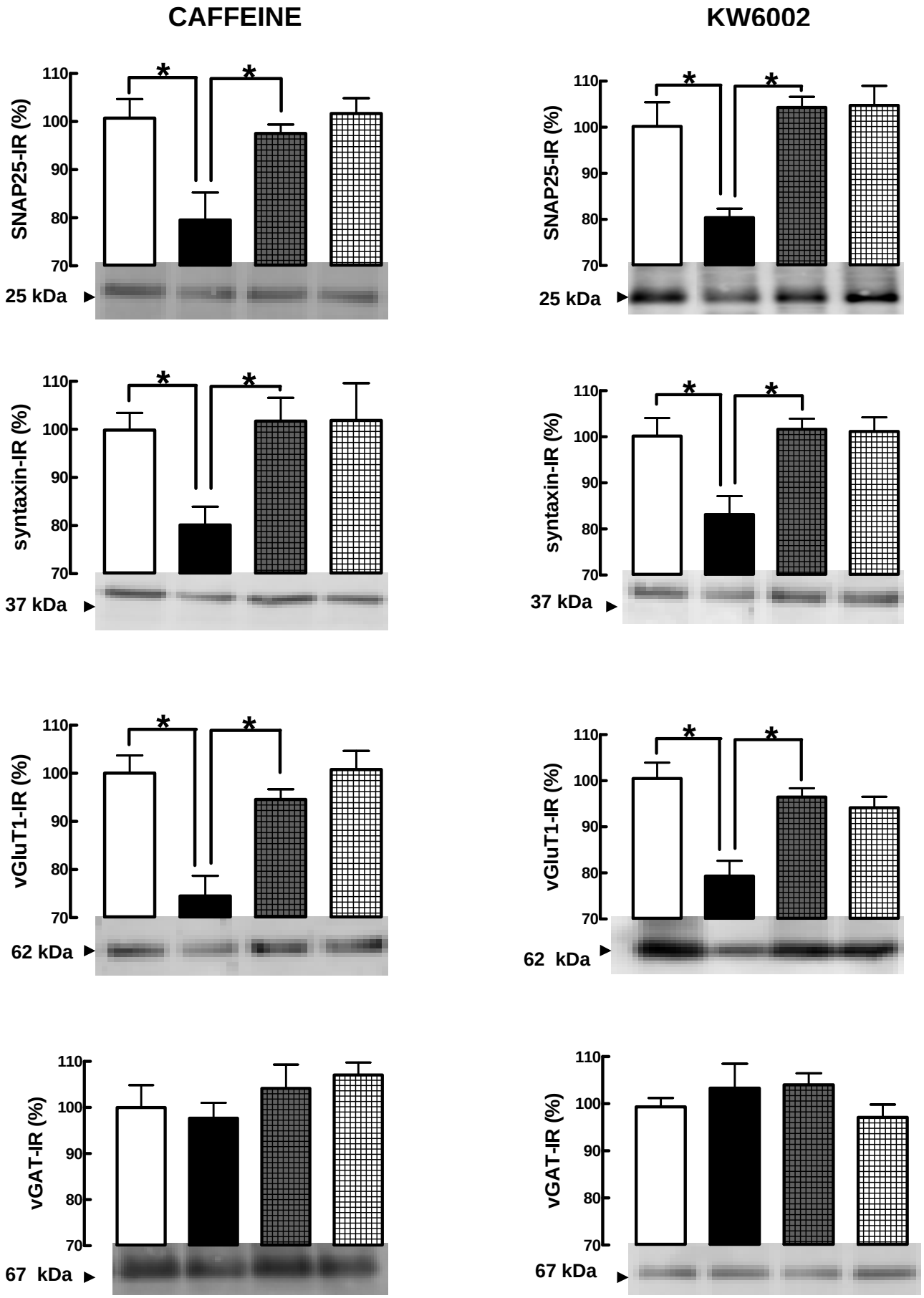

A
saline
kainate
B
CA1
CA3
DG
saline
kainate
C
nº GFAP-positive cells
30
20
10
0
CA1
CA3
DG
D
GFAP IR
(% control)
120
110
100
90
80
52 kDa

FIGURE 6


CAFFEINE
KW6002
SNAP25-IR (%)
110
100
90
80
70
25 kDa
syntaxin-IR (%)
37 kDa
vGluT1-IR (%)
62 kDa
62 kDa
vGAT-IR (%)
67 kDa
67 kDa
*

IV. DISCUSSÃO

A sinalização via nucleotídeos extracelulares é um importante mecanismo de comunicação no SNC e envolve todos os tipos celulares desde glia até neurônios. Este tipo de sinalização se torna muito peculiar uma vez que a hidrólise extracelular dos nucleotídeos é feita por um grande número de enzimas com diferentes propriedades catalíticas. Dependendo do ligante, do subtipo de receptor purinérgico e do tipo de ectonucleotidase presente, a hidrólise extracelular dos nucleotídeos pode resultar na inativação de um ligante, na produção de um ligante adicional ou alternativo ou na produção de um nucleosídeo (YEGUTKIN, 2008). Portanto, a investigação dos tipos de ectonucleotidases que podem potencialmente contribuir com o controle da sinalização purinérgica em regiões cerebrais distintas se torna importante.

Ao longo dos anos, diversos estudos vêm sendo feitos com o intuito de elucidar a localização das ectonucleotidases no SNC (BRAUN et al., 2003; BELCHER et al., 2006; BJELOBABA et al., 2006, 2007; LANGER et al., 2008). Na ontogenia, alguns trabalhos já demonstraram a presença das ectonucleotidases ao longo do desenvolvimento cerebral. Estudos mostraram que as atividades de hidrólise de ATP, ADP e AMP aumentam com a idade (MÜLLER et al., 1990; FUCHS, 1991; BANJAC et al., 2001; de PAULA COGNATO et al., 2005). Além disso, alguns estudos têm relacionado as NPPs com funções importantes no desenvolvimento cerebral (FUSS et al., 1997; BLASS-KAMPMANN et al., 1997). Estes estudos evidenciam que as ectonucleotidases podem exercer um papel importante nos processos de desenvolvimento cerebral e no envelhecimento.

Tendo como referência as informações citadas acima e que poucos estudos foram feitos relacionando a ontogenia com as NPPs, o capítulo I desta tese de doutorado teve como objetivo elucidar o perfil de expressão das NPPs 1-3 em diferentes regiões cerebrais (hipocampo, cerebelo, estriado, córtex e bulbo olfatório) ao longo do desenvolvimento do SNC. Neste estudo foi observada uma expressão bem distribuída da NPP1 nas cinco regiões cerebrais avaliadas ao longo do desenvolvimento, com uma crescente expressão até aos 60 dias e decaimento aos 150 dias em bulbo olfatório, estriado e cerebelo. Sabendo que a NPP1 é expressa em capilares cerebrais (HARAHAP & GODING, 1988), nossos resultados corroboram com estudos que descrevem que a maior vascularização do SNC se dá por angiogênese durante o desenvolvimento (MARIN-PADILLA, 1985) e que este fenômeno é quase inexistente em tecidos maduros (PLATE et al., 1994). A expressão da NPP2 se mostrou constante ao longo do desenvolvimento cerebral das cinco regiões cerebrais analisadas. Essa enzima é responsável pela produção do ácido lisofosfatídico, um lipídeo mediador de várias funções biológicas, incluindo ações importantes no desenvolvimento cerebral, tais como neurogênese e padrões de formação corticais (KINGSBURY et al., 2003). Além disso, o ácido lisofosfatídico também está envolvido com a maturação do sistema vascular no cérebro (van MEETEREN et al., 2006). Portanto, a presença desta enzima no SNC ao longo do desenvolvimento parece ser essencial e onipresente. Nossos resultados também mostraram que a expressão da NPP3 foi diminuindo à medida que o animal envelhecia em todas as regiões cerebrais analisadas. Nossos resultados parecem corroborar com os dados da literatura que sugerem que a NPP3 representa um importante

fator nos processos de proliferação glial (ZIMMERMANN et al., 2006b) e que esta enzima só foi detectada em astrócitos imaturos (BLASS-KAMPMANN et al., 1997).

Considerando a importância de investigar os tipos de ectonucleotidases que podem potencialmente contribuir para o controle da sinalização purinérgica e que vários estudos explorando a distribuição das ectonucleotidases no tecido cerebral têm sido incapazes de identificar quais enzimas estão presentes nas sinapses (por exemplo, BELCHER et al., 2006; BJELOBABA et al., 2007; LANGER et al., 2008), o capítulo II desta tese apresenta a primeira demonstração da presença de diferentes ectonucleotidases identificadas molecularmente nas sinapses hipocampais e estriatais. Este estudo demonstra que as NTPDases expressas no cérebro (NTPDase 1, 2 e 3), bem como a 5'-nucleotidase estão presentes nestas sinapses, embora com diferentes níveis de enriquecimento.

A estratégia usada neste estudo foi o fracionamento dos terminais pré-sinapticos que permitiu avaliar a distribuição das ectonucleotidases e identificar algumas diferenças na localização destas enzimas. Os resultados apresentados neste capítulo contrastam com os achados prévios de estudos envolvendo imunohistoquímica, onde possivelmente os epítopos presentes nas sinapses permitem um baixo acesso à anticorpos. Assim, nós observamos que a NTPDase 3 possui um alto enriquecimento nas sinapses de hipocampo e estriado de ratos, dados que não estão de acordo com achados prévios, mostrando uma baixa densidade da NTPDase 3 fora da parte medial e posterior do cérebro (BELCHER et al., 2006). Além disso, nosso estudo identifica a NTPDase 1 como sendo a principal ectonucleotidase localizada

sinapticamente, enquanto que estudos prévios identificaram a presença desta enzima em apenas fibras tracteis (BJELOBABA et al., 2007). Nosso estudo também confirmou a presença da 5'-nucleotidase em terminais sinápticos e mostra que a sua densidade é tão intensa quanto em células gliais, local anteriormente proposto como sendo o mais abundante em termos de 5'-nucleotidase (KREUTZBERG et al., 1996).

Os resultados mostrados no capítulo II também revelam uma associação particular das diferentes ectonucleotidases em diferentes terminais sinápticos no hipocampo e estriado. De fato, foi observado que a NTPDase 1 está mais associada com os terminais GABAérgicos e que a NTPDase 3 e 5'-nucleotidase estão mais associadas com os terminais sinápticos glutamatérgicos. Este cenário está de acordo com o atual conhecimento da modulação purinérgica destes terminais sinápticos. Foi demonstrado que o ATP afeta as sinapses glutamatérgicas através dos receptores P2, controlando a liberação de neurotransmissores (KHAKH & HENDERSON, 1998; RODRIGUES et al., 2005; LEÓN et al., 2008) e a plasticidade sináptica (PANKRATOV et al., 2002; ALMEIDA et al., 2003; SIM et al., 2006), sendo que este sinal seria terminado pela ação de uma NTPDase localizada sinapticamente. Entretanto, os terminais glutamatérgicos também parecem ser modulados pela adenosina provavelmente produzida por ectonucleotidases localizadas no terminal sináptico (CUNHA et al., 1996; REBOLA et al., 2008). Portanto, a presença da ecto-5'-nucleotidase, bem como de uma NTPDase (no caso a NTPDase3) também é esperada nestes terminais. Em contraste com os terminais glutamatérgicos, as sinapses GABAérgicas são controladas pelo ATP através dos receptores P2 (HUGEL & SCHLICHTER, 2000; GÓMEZ-

VILLAFUERTES et al., 2001; DONATO et al., 2008; XIAO et al., 2008), mas não existem evidencias claras quanto a regulação destas sinapses por adenosina (FREDHOLM et al., 2005). Portanto, a presença de uma NTPDase para controlar a disponibilidade de ATP nos terminais GABAérgicos é esperada.

O enriquecimento das NTPDases e da ecto-5'-nucleotidase observados em sinapses de hipocampo e estriado reforçam a importância da sinalização purinérgica localizada nas sinapses. De fato, existem muitos estudos mostrando o papel do ATP extracelular no controle da plasticidade sináptica pela ativação dos receptores P2 (PANKRATOV et al., 2002; ALMEIDA et al., 2003; SIM et al., 2006) ou pela ação das ecto-proteínas quinases (FUJJI et al., 2002). Em ambas as situações, as ectonucleotidases sinápticas poderiam determinar o tempo de sinalização mediada pelo ATP. Em paralelo, a formação de adenosina a partir do ATP pode ocorrer de maneira confinada em sinapses ativadas para a direta ativação dos A_{2A}, receptor este que está envolvido em algumas formas de plasticidade sináptica (CUNHA, 2008b) em hipocampo (REBOLA et al., 2008) e estriado (D'ALCANTARA et al., 2001). Portanto, a cascata das ectonucleotidases controla a sinalização do ATP e da adenosina na fenda sináptica. A importância destas ectonucleotidases sinápticas é enfatizada por vários trabalhos na literatura mostrando que essas atividades enzimáticas se encontram alteradas em adaptações fisiológicas (CUNHA et al., 2001; PEREIRA et al., 2002; PEDRAZZA et al., 2007) e em condições patológicas (SCHETINGER et al., 1998; BONAN et al., 2000a, 2000b; BRUNO et al., 2005; COGNATO et al., 2007; DUARTE et al., 2007). Assim, a identificação de natureza molecular das ectonucleotidases presentes em

sinapses hipocampais e estriatais abre caminho para um entendimento mais detalhado dos papéis patofisiológicos destas ectonucleotidases no controle da sinalização purinérgica.

Tendo em vista que (i) as NTPDases se encontram em terminais sinápticos (dados do capítulo II), que (ii) o ATP pode ser um modulador nos fenômenos plásticos requeridos para o aprendizado e memória e que (iii) convulsões no período neonatal podem levar a déficits cognitivos na vida adulta, o capítulo III desta tese investigou a hidrólise de nucleotídeos em sinaptossomas de hipocampo de ratos previamente submetidos a um único episódio convulsivo aos 7 dias de vida (PN7) em tempos diferentes após o período de crise epiléptica. Primeiramente, este estudo mostrou que ratos adultos (PN90) desenvolveram um prejuízo de memória no labirinto em Y após serem submetidos a uma convulsão neonatal (PN7) induzida por KA. Estudos prévios já haviam mostrado que uma única administração de KA em ratos com PN5-PN10 pode levar ao déficit cognitivo na vida adulta em diferentes tarefas de memória (LYNCH et al., 2000; SAYIN et al., 2004; CORNEJO et al., 2007). Da mesma forma, a indução de convulsões com diferentes agentes convulsivos em roedores jovens também leva à disfunção da memória espacial no período maduro (De ROGALSKI-LANDROT et al., 2001; LEE et al., 2001; HUANG et al., 2002; CILIO et al., 2003; BO et al., 2004).

Uma vez que os déficits cognitivos gerados pela convulsão neonatal foram confirmados na vida adulta, as hidrólises de nucleotídeos foram testadas em sinaptossomas de hipocampo de ratos previamente administrados com KA aos 7 dias de vida. As hidrólises foram testadas quando os ratos tinham 7 (doze horas após a injeção de KA), 14, 21 e 90 dias de vida. Nossos

resultados não demonstraram mudança significativa na hidrólise de nucleotídeos após um episódio convulsivo em idades de maturação cerebral (PN7, PN14 e PN21). Muitos estudos têm descrito que o cérebro imaturo é menos vulnerável às alterações morfológicas e fisiológicas após o *status epilepticus* quando comparado com cérebro adulto (HAUT et al., 2004; CILIO et al., 2003; HAAS et al., 2001), apesar de mudanças no comportamento e nas conexões cerebrais serem detectadas na vida adulta (HOLMES, 2004). A hidrólise de nucleotídeos em ratos com PN7, PN14 e PN21 que se encontra inalterada pelo episódio convulsivo neonatal está de acordo com estudos prévios que revelaram que as hidrólises de ATP, ADP e AMP não sofreram mudanças em ratos jovens expostos ao modelo de epilepsia da pilocapina (COGNATO et al., 2005). Nossos resultados também demonstraram que as hidrólises de ATP e ADP dos ratos controle aumentaram ao longo do desenvolvimento (do PN7 ao PN90). Estudos anteriores já haviam demonstrado que as hidrólises destes nucleotídeos em preparações sinaptossomais de córtex cerebral de ratos aumentam significativamente do nascimento até a segunda semana de vida pós-natal (MÜLLER et al., 1990). Além disso, a 5'-nucleotidase é expressa em células do sistema nervoso em desenvolvimento e, por essa razão, foi definida como uma possível proteína marcadora do desenvolvimento cerebral (BRAUN & ZIMMERMANN, 1998). Além disso, foi observado que a atividade de hidrólise dessa enzima aumenta com o envelhecimento (FUCHS et al., 1991). Nossos resultados estão de acordo com esse estudo, uma vez que foi observado um aumento na hidrólise de AMP em sinaptossomas hipocampais ao longo do envelhecimento dos animais.

Os resultados do capítulo III também mostram que a hidrólise de ATP em sinaptossomas hipocampais de ratos com PN90 previamente submetidos a um episódio convulsivo neonatal se encontra aumentada significativamente. Estudos anteriores de nosso laboratório já demonstraram que a hidrólise de nucleotídeos em SNC se encontra aumentada após a indução de diferentes modelos de epilepsia ou convulsão em ratos adultos (BONAN et al., 2000a, 2000b; COGNATO et al., 2005; COGNATO et al., 2007; OSES et al., 2007). Entretanto, esse é o primeiro estudo demonstrando um aumento na hidrólise de ATP passado um longo período após um único episódio convulsivo em idade pós-natal. Apesar de curtos períodos convulsivos durante o desenvolvimento cerebral não levarem a modificações morfológicas evidentes (STAFSTROM et al., 2002; HOLMES, 2005), tem sido observado que a atividade neuronal durante o desenvolvimento não somente determina a organização dos circuitos neuronais, mas também pode influenciar na capacidade plástica do circuito na vida adulta (ABRAHAM & BEAR, 1996; FELDMAN & KNUDSEN, 1998). Uma vez que o ATP parece estar relacionado com a indução de LTP e plasticidade sináptica (WIERASZKO, 1996; FUJJI et al., 2004), o aumento na hidrólise desse nucleotídeo observado nos ratos adultos com prejuízo cognitivo pode ser um dos mecanismos cerebrais alterados pela crise convulsiva no período neonatal. De fato, LYNCH e colaboradores (2000) observaram que ratos adultos previamente submetidos a convulsão neonatal apresentaram um prejuízo na indução de LTP em hipocampo. Uma vez que nossos resultados mostram um aumento na hidrólise de ATP e esse nucleotídeo parece ser importante para a formação de LTP, pode-se assumir que o prejuízo cognitivo observado em ratos adultos que

sofreram um episódio convulsivo na vida neonatal pode ser uma conseqüência do aumento na atividade de hidrólise de ATP. Interessantemente, foi descrita uma diminuição da hidrólise de nucleotídeos em sinaptossomas hipocampais de ratos imediatamente sacrificados após a sessão de treino da tarefa de esquiva inibitória (BONAN et al., 1998). Esse achado indica que a presença do ATP na fenda sináptica pode ser necessária nos mecanismos de retenção de memória e evidenciam uma situação oposta com a observada por nosso estudo, onde a hidrólise de ATP se encontra aumentada e existe um prejuízo cognitivo.

O aumento na atividade de hidrólise do ATP observado no capítulo III não foi acompanhado pelo aumento na expressão relativa das possíveis ectonucleotidases responsáveis por essa hidrólise (neste caso as NTPDases 1, 2 e 3), sugerindo que a hidrólise aumentada de ATP não ocorreu devido ao aumento na síntese destas proteínas. Um estudo anterior também descreveu que as atividades de hidrólise de nucleotídeos alteradas por crises convulsivas não foram acompanhadas por mudanças na expressão relativa das ectonucleotidases (OSES et al., 2007).

Ainda buscando entender melhor o papel do sistema purinérgico e de outros sistemas correlacionados nos mecanismos neuroquímicos que participam do prejuízo de memória no cérebro maduro causado por convulsões no período neonatal, o capítulo IV desta tese avaliou alterações morfológicas e fisiológicas em ratos adultos (PN90) previamente submetidos ao tratamento neonatal com KA e o efeito de antagonistas de receptores de adenosina. Nossos resultados mostraram que existe uma perda de marcadores sinápticos, particularmente em terminais glutamatérgicos, sem evidentes modificações nos

marcadores pós-sinápticos, na astrogliose ou na arquitetura neuronal do hipocampo. Além disso, os resultados mostraram que o consumo crônico de cafeína ou de um antagonista seletivo para receptor A_{2A} preveniu o déficit cognitivo e a decorrente sinaptotoxicidade presente em ratos adultos que foram submetidos a uma única convulsão no período neonatal. No último capítulo desta tese, também foi avaliado se ratos com 30 ou 60 dias de vida apresentavam o déficit cognitivo causado pela crise convulsiva neonatal. Nossos resultados demonstraram que esses déficits só estão presentes em ratos de 90 dias.

Alguns trabalhos têm evidenciado que curtos períodos convulsivos no período neonatal não causam modificações morfológicas evidentes (HOLMES, 2005; STAFSTROM et al., 2002), sendo que essas modificações só podem ser observadas após períodos convulsivos repetitivos (HOLMES & BEN-ARI, 1998). Similarmente, animais adultos submetidos a convulsões induzidas por KA no período neonatal apresentam pouca ou nenhuma modificação morfológica quando comparados ao grupo controle (NITECKA et al., 1984; LYNCH et al., 2000; CORNEJO et al., 2007). Entretanto, modificações neurofisiológicas, tais como plasticidade sináptica mais excitada (LYNCH et al., 2000; CORNEJO et al., 2007), foram observadas em animais adultos submetidos a convulsões neonatais. Nos resultados do capítulo IV, não foram encontradas evidências para uma astrogliose persistente, uma vez que a densidade da GFAP, o número de astrócitos marcados com GFAP e a morfologia de hipocampo marcada com GFAP de ratos adultos que sofreram convulsão neonatal não foram alterados quando comparados com os controles. Entretanto, os resultados do capítulo IV providenciam a primeira

demonstração direta de modificações pré-sinápticas acompanhadas de prejuízo da memória em ratos adultos previamente submetidos à crise convulsiva neonatal. De fato, foi observada uma diminuição dos marcadores sinápticos SNAP-25 e sintaxina. Interessantemente, foi encontrada uma redução dos marcadores de sinapses glutamatérgicas (vGluT1), enquanto que o vGAT, marcador de sinapses GABAérgicas, foi preservado em ratos adultos submetidos a convulsão induzida por KA aos PN7. Este dado sugere que mudanças seletivas em sinapses glutamatérgicas, porém não em sinapses GABAérgicas, estão ocorrendo. A sinaptotoxicidade também tem sido proposta como sendo uma característica primária e crucial responsável por prejuízos na memória (SCHEFF et al., 2007), sendo o único parâmetro morfológico conhecido que correlaciona com o prejuízo cognitivo (DEKOSKY & SCHEFF, 1990; TERRY et al., 1991). Além disso, a sinaptotoxicidade é uma característica de muitas outras condições cerebrais onde o déficit de memória está presente, tais como doença de Huntington (LI et al., 2001), doença de Alzheimer (SELKOE, 2002), doenças causadas por prion (FERRER, 2002), infecção por HIV (GARDEN et al., 2002) ou esquizofrenia (GLANTZ et al., 2006). Em particular, estudos recentes indicam que a sinaptotoxicidade relacionada com o déficit de memória pode ocorrer em terminais glutamatérgicos, uma vez que a densidade dos transportadores de glutamato se mostrou diminuída em regiões corticais de indivíduos com doença de Alzheimer (KIRVELL et al., 2007), bem como em modelos animais desta doença (BELL et al., 2006; MINKEVICIENE et al., 2008). Assim como na doença de Alzheimer, parece ser possível que a redução das proteínas sinápticas em hipocampo, em particular dos terminais glutamatérgicos, possa

contribuir para os prejuízos na memória encontrados em ratos adultos previamente submetidos à convulsão neonatal.

O provável envolvimento da sinaptotoxicidade nos mecanismos de prejuízo de memória na vida adulta causados por convulsão neonatal é sustentado pelos resultados principais deste trabalho: o consumo crônico de cafeína foi capaz de prevenir o déficit cognitivo e a perda de marcadores sinápticos hipocampais observados na vida adulta causados por uma única convulsão no período neonatal, bem como foi capaz de prevenir a sinaptotoxicidade. Essa habilidade da cafeína de prevenir déficits de memória na vida adulta causados por convulsão neonatal se correlaciona com sua habilidade em prevenir déficits de memória encontrados no envelhecimento, na doença de Alzheimer e na doença de Parkinson, assim como em desordens de déficit de atenção e hiperatividade (CUNHA, 2008a; TAKAHASHI et al., 2008). Os resultados sugerem que a cafeína é capaz de interferir em mecanismos neuroquímicos e possui um papel chave em diminuir as disfunções de memória (CUNHA, 2008a). Os únicos alvos moleculares conhecidos para a cafeína são os receptores A_1 e A_{2A} de adenosina, ambos antagonizados pela cafeína (FREDHOLM et al., 1999). A prevenção da disfunção de memória pela cafeína provavelmente resulta da sua interação com os receptores A_{2A}, uma vez que os efeitos benéficos da cafeína em disfunção de memória foram mimetizados por antagonistas seletivos destes receptores em doença de Alzheimer (PREDIGER et al., 2005; CUNHA et al., 2008a). De acordo com esses achados, o antagonista seletivo de receptors A_{2A}, KW6002, também foi capaz de prevenir os prejuízos de memória da vida adulta causados por convulsão neonatal, bem como a perda de marcadores de terminais sinápticos no

hipocampo. Foi demonstrado que o antagonismo dos receptores A_{2A} não apenas abole a disfunção de memória, mas também proporciona uma robusta neuroproteção contra o dano cerebral (CUNHA, 2005; CHEN et al., 2007). Este dado suporta o envolvimento da sinaptotoxicidade na disfunção de memória uma vez que os receptores A_{2A} possuem uma localização predominantemente sináptica no hipocampo (REBOLA et al., 2005), onde podem controlar a sinaptotoxicidade (CUNHA et al., 2006; SILVA et al., 2007), o evento morfológico mais marcante achado em hipocampo de ratos adultos previamente submetidos a crise epiléptica induzida por KA aos 7 dias de vida. Os resultados também mostram um aumento na densidade destes receptores no hipocampo de ratos adultos que sofreram convulsão neonatal. Já foi descrito um aumento na função dos receptores em condições cerebrais nocivas (CUNHA, 2005). Entretanto, os mecanismos pelos quais os receptores controlam a sinaptotoxicidade em sinapses glutamatérgicas ainda não estão claros, embora fortes candidatos para explicar este fenômeno sejam o controle dos receptores NMDA (REBOLA et al., 2008), o aporte de cálcio (GONÇALVES et al., 1997) e o controle da mitocôndria sináptica (SILVA et al., 2007).

Nossos resultados evidenciam a presença das NPPs em diferentes regiões cerebrais durante o desenvolvimento, uma localização sináptica das ectonucleotidases com possíveis diferenças na degradação de ATP e formação de adenosina em sinapses excitatórias e inibitórias, uma participação do sistema purinérgico nos mecanismos envolvidos no déficit cognitivo causado por uma única crise epiléptica durante o desenvolvimento e a cafeína

como possível agente profilático para a prevenção dos prejuízos de memória causados por crise convulsiva neonatal.

V. CONCLUSÕES GERAIS

Os resultados obtidos e apresentados no presente estudo nos permitem concluir que:

- As NPPs 1-3 estão presentes durante a maturação cerebral em bulbo olfatório, hipocampo, estriado, cerebrelo e córtex cerebral. O padrão de expressão dessas enzimas é diferente ao longo do desenvolvimento. A expressão relativa da NPP1 parece aumentar com o envelhecimento e esse fato parece estar relacionado com o aumento da vascularização cerebral a medida que o animal envelhece. A expressão relativa das duas isoformas da NPP2 se apresenta constante durante o desenvolvimento, indicando que a presença essa enzima parece ser importante nas idades avaliadas. A expressão relativa da NPP3 diminui ao longo do desenvolvimento, o que corrobora com estudos anteriores que detectaram a NPP3 apenas em astrócitos imaturos.

- As NTPDases 1, 2, 3 e 5'-nucleotidases estão presentes em terminais sinápticos. Além disso, essas enzimas estão diferentemente associadas à sinapses excitatórias e inibitórias. A NTPDase 1 está mais associada à terminais GABAérgicos enquanto que a NTPDase 3 e 5'-nucleotidase estão mais associadas aos terminais glutamatérgicos.

- Uma única convulsão neonatal induzida por KA pode levar a prejuízos cognitivos em ratos adultos testados em labirinto em Y. Outros parâmetros comportamentais, tais como atividade locomotora e ansiedade, não foram alterados pelo episódio convulsivo neonatal.

- Existe um aumento da hidrólise de ATP em sinaptossomas hipocampais de ratos adultos previamente submetidos a uma convulsão neonatal e que apresentaram déficits cognitivos em labirinto em Y. O aumento na hidrólise desse nucleotídeo parece estar envolvido com os déficits cognitivos causados pela convulsão neonatal.

- Os prejuízos cognitivos na vida adulta induzidos por convulsão neonatal podem ser prevenidos pelo consumo crônico de cafeína ou KW6002. A diminuição nas densidades das proteínas sinápticas induzidas pela crise epiléptica no período de desenvolvimento cerebral também podem ser prevenidas pelo consumo crônico de antagonistas dos receptores A_{2A} (cafeína e KW6002).

VI. PERSPECTIVAS

1. Estudar a localização subsináptica das NPPs em hipocampo e estriado de ratos adultos.
2. Estudar o envolvimento das NPPs na hidrólise de nucleotídeos em sinaptossomas de hipocampo de ratos adultos previamente submetidos a crise convulsiva neonatal.
3. Estudar o envolvimento das NTPDases, das NNPs e da 5'-nucleotidase na hidrólise de nucleotídeos em sinaptossomas de córtex pré-frontal (outra região cerebral importante no processamento da memória) de ratos adultos submetidos a convulsão neonatal.
4. Estudar se o consumo crônico de cafeína pode prevenir déficits cognitivos relacionados a convulsão neonatal em outros testes de memória, tais como water maze e radial maze.

VII. REFERENCIAS BIBLIOGRÁFICAS

ABBRACCHIO, M.P.; BURNSTOCK, G.; BOEYNAEMS, J.M.; BARNARD, E.A.; BOYER, J.L.; KENNEDY, C.; KNIGHT, G.E.; FUMAGALLI, M.; GACHET, C.; JACOBSON, K.A.; WEISMAN, G.A. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev. 58(3):281-341, 2006.

ABRAHAM, W.C.; BEAR, M.F. Metaplasticity: the plasticity of synaptic plasticity. Trends Neurosci. 19: 126-130, 1996.

AIRAS, L.; HELLMAN, J.; SALMI, M.; BONO, P.; PUURUNEN, T.; SMITH, D.J.; JALKANEN, S. CD73 is involved in lymphocyte binding to the endothelium: characterization of lymphocyte-vascular adhesion protein 2 identifies it as CD73. J Exp Med. 182(5):1603-1608, 1995.

ALMEIDA, T.; RODRIGUES, R.J.; DE MENDONÇA, A.; RIBEIRO, J.A.; CUNHA, R.A. Purinergic P_2 receptors trigger adenosine release leading to adenosine A_{2A} receptor activation and facilitation of long-term potentiation in rat hippocampal slices. Neuroscience. 122: 111-121, 2003.

ANGELATOU, F.; PAGONOPOULOU, O.; KOSTOPOULOS, G. Changes in seizure latency correlate with alterations in A1 adenosine receptor binding during daily repeated pentylentetrazol-induced convulsions in different mouse brain areas. Neurosci Lett. 132(2):203-206, 1991.

ANGELATOU, F.; PAGONOPOULOU, O.; MARAZIOTIS, T.; OLIVIER, A.; VILLEMEURE, J.G.; AVOLI, M. & KOSTOPOULOS, G. Upregulation of A1

adenosine receptors in human temporal lobe epilepsy: a quantitative autoradiographic study. *Neurosci Lett* 163(1):11-14, 1993.

ANTLE, M.C.; STEEN, N.M.; MISTLBERGER, R.E. Adenosine and caffeine modulate circadian rhythms in the Syrian hamster. Neuroreport. 12(13):2901-2905, 2001.

BANJAC, A.; NEDELJKOVIC, N.; HORVAT, A.; KANAZIR, D. & NIKEZIC, G. Ontogenetic profile of ecto-ATPase activity in rat hippocampal and caudate nucleus synaptic plasma membrane fractions. Physiol Res. 50(4):411-7, 2001.

BARAM, T.Z. Long-term neuroplasticity and functional consequences of single versus recurrent early-life seizures. *Ann Neurol* 54:701–705, 2003.

BARAM, T.Z.; EGHBAL-AHMADI, M.; BENDER, R.A. Is neuronal death required for seizure-induced epileptogenesis in the immature brain? *Prog Brain Res* 135:365–375, 2002.

BATTASTINI, A.M.O.; OLIVEIRA, E.M.; MOREIRA, C.M.; BONAN, C.D.; SARKIS J.J.F.; DIAS, R.D. Solubilization and characterization of an ATP diphosphohydrolase (EC 3.6.1.5.) from rat brain synaptic plasma membranes. Biochem Mol Biol Int, 37: 209-219, 1995.

BATTASTINI, A.M.O.; ROCHA, J.B.T.; BARCELLOS, C.K.; DIAS, R.D.; SARKIS, J.J.F. Characterization of an ATP diphosphohydrolase (EC 3.6.1.5) in synaptosomes from cerebral cortex of adult rats. Neurochem Res. 16: 1303-1310, 1991.

BELCHER, S.M.; ZSARNOVSZKY, A.; CRAWFORD, P.A.; HEMANI, H.; SPURLING, L.; KIRLEY, T.L. Immunolocalization of ecto-nucleoside

triphosphate diphosphohydrolase 3 in rat brain: implications for modulation of multiple homeostatic systems including feeding and sleep-wake behaviors. Neuroscience. 137: 1331-1346, 2006.

BELL, K.F.; DUCATENZEILER, A.; RIBEIRO-DA-SILVA, A.; DUFF, K.; BENNETT, D.A.; CUELLO, A.C. The amyloid pathology progresses in a neurotransmitter-specific manner. Neurobiol. Aging 27:1644-1657, 2006.

BENARROCH, E.E. Adenosine and its receptors: multiple modulatory functions and potential therapeutic targets for neurologic disease. Neurology. 70(3):231-236, 2008.

BETOURNE, A.; BERTHOLET, A.,.; LABROUE, E.; HALLEY, H. SUN, H.S.; LORSIGNOL, A.; FENG, Z.P.; FRENCH, R.J.; PENICAUD, L.; LASALLE, J.M.; FRANCES, B. Involvement of hippocampal CA3 KATP channels in contextual memory. Neuropharmacol, 56: 615-625, 2009.

BIGONNESSE, F.; LÉVESQUE, S.A.; KUKULSKI, F.; LECKA, J.; ROBSON, S.C.; FERNANDES, M.J.; SÉVIGNY, J. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-8. Biochemistry. 43: 5511-5519, 2006.

BJELOBABA, I., NEDELJKOVIC, N., SUBASIC, S., LAVRNJA, I., PEKOVIC, S., STOJKOV, D., RAKIC, L., STOJILJKOVIC, M. Immunolocalization of ecto-nucleotide pyrophosphatase/ phosphodiesterase 1 (NPP1) in the rat forebrain. Brain Res. 1120, 54–63, 2006.

BJELOBABA, I.; STOJILJKOVIC, M.; PEKOVIC, S.; DACIC, S.; LAVRNJA, I.; STOJKOV, D.; RAKIC, L.; NEDELJKOVIC, N. Immunohistological determination of ecto-nucleoside triphosphate diphosphohydrolase1

(NTPDase1) and 5′-nucleotidase in rat hippocampus reveals overlapping distribution. Cell Mol. Neurobiol. 27: 731-743, 2007.

BLASS-KAMPMANN, S., KINDLER-ROHRBORN, A., DEISSLER, H., D'URSO, D., RAJEWSKY, M.F. In vitro differentiation of neural progenitor cells from prenatal rat brain: common cell surface glycoprotein on three glial cell subsets. J. Neurosci. Res.. 48, 95–111., 1997.

BO, T.; JIANG, Y.; CAO, H.; WANG, J.; WU, X. Long-term effects of seizures in neonatal rats on spatial learning ability and N-methyl-D-aspartate receptor expression in the brain. Dev. Brain Res. 152: 137-142, 2004.

BONAN, C.D, DIAS, M.M., BATTASTINI, A.M., DIAS, R.D., SARKIS, J.J. Inhibitory avoidance learning inhibits ectonucleotidases activities in hippocampal synaptosomes of adult rats. Neurochem Res, 23: 977-982, 1998.

BONAN, C.D., AMARAL, O.B., ROCKENBACH, I.C., WALZ, R., BATTASTINI, A.M., IZQUIERDO, I., SARKIS, J.J. Altered ATP hydrolysis induced by pentylenetetrazol kindling in rat brain synaptosomes. Neurochem. Res, 25: 775-779, 2000b.

BONAN, C.D.; WALZ, R.; PEREIRA, G.S.; WORM, P.V.; BATTASTINI, A.M.; CAVALHEIRO, E.A.; IZQUIERDO, I.; SARKIS, J.J. Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy. Epilepsy Res. 39: 229-238, 2000a.

BRAUN, N.; BRENDEL, P.; ZIMMERMANN, H. Distribution of 5'-nucleotidase in the developing mouse retina. Brain Res Dev Brain Res. 88(1): 79-86, 1995.

BRAUN, N.; LENZ, C.; GILLARDON, F.; ZIMMERMANN, M.; ZIMMERMANN, H. Focal cerebral ischemia enhances glial expression of ecto-5'-nucleotidase. Brain Res. 766(1-2): 213-226, 1997.

BRAUN, N.; SÉVIGNY, J.; ROBSON, S.C.; ENJYOJI, K.; GUCKELBERGER, O.; HAMMER, K.; DI VIRGILIO, F.; ZIMMERMANN, H. Assignment of ecto-nucleoside triphosphate diphosphohydrolase-1/cd39 expression to microglia and vasculature of the brain. Eur J Neurosci. 12(12): 4357-4366, 2000.

BRAUN, N.; SÉVIGNY, J.; MISHRA, S.K.; ROBSON, S.C.; BARTH, S.W.; GERSTBERGER, R.; HAMMER, K.; ZIMMERMANN, H. Expression of the ecto-ATPase NTPDase2 in the germinal zones of the developing and adult rat brain. Eur. J. Neurosci. 17:1355-1364, 2003.

BRAUN, N.; ZIMMERMANN, H. Association of ecto-5'-nucleotidase with specific cell types in the adult and developing rat olfactory organ. J Comp Neurol, 393: 528-537, 1998.

BRUNDEGE, J.M.; DUNWIDDIE, T.V. Role of adenosine as a modulator of synaptic activity in the central nervous system. Adv Pharmacol. 39: 353-391, 1997.

BRUNO, A.N.; DINIZ, G.P.; RICACHENEVSKY, F.K.; POCHMANN, D.; BONAN, C.D.; BARRETO-CHAVES, M.L.; SARKIS, J.J. Hypo-and hyperthyroidism affect the ATP, ADP and AMP hydrolysis in rat hippocampal and cortical slices. Neurosci Res, 52: 61-68, 2005.

BRUNO, A.N.; OSES, J.P.; AMARAL, O.; COITINHO. A.; BONAN, C.D.; BATTASTINI, A.M.; SARKIS, J.J. Changes in nucleotide hydrolysis in rat blood serum induced by pentylenetetrazol-kindling. Brain Res Mol Brain Res. 114(2): 140-145, 2003.

BRUNO, A.N.; OSES, J.P.; BONAN, C.D.; WALZ, R.; BATTASTINI, A.M.; SARKIS, J.J. Increase of nucleotidase activities in rat blood serum after a single convulsive injection of pentylenetetrazol. Neurosci Res. 43(3):283-288, 2002.

BURNSTOCK, G. Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev. 87: 659-797., 2007.

BURNSTOCK, G. Purinergic nerves. Pharmacol Rev. 24(3):509-81, 1972.

CASEL, D.; BROCKHAUS, J.; DEITMER, J.W. Enhancement of spontaneous synaptic activity in rat Purkinje neurones by ATP during development. J. Physiol. 568, 111–122, 2005.

CHEN, J.F.; SONSALLA, P.K.; PEDATA, F.; MELANI, A.; DOMENICI, M.R.; POPOLI, P.; GEIGER, J.; LOPES, L.V.; DE MENDONÇA, A. Adenosine A_{2A} receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation. Prog Neurobiol. 83: 310-331, 2007.

CILIO, M.R.; SOGAWA, Y.; CHA, B.H.; LIU, X.; HUANG, L.T.; HOLMES, G.L. Long-term effects of status epilepticus in the immature brain are specific for age and model. Epilepsia 44: 518-528, 2003.

CIMPEAN, A.; STEFAN, C.; GIJSBERS, R.; STALMANS, W.; BOLLEN, M. Substrate-specifying determinants of the nucleotide pyrophosphatases / phosphodiesterases NPP1 and NPP2. Biochem. J. 381, 71–77, 2004.

CLAES, P.; VAN KOLEN, K.; ROYMANS, D.; BLERO, D.; VISSENBERG, K.; ERNEUX, C.; VERBELEN, J.P.; ESMANS, E.L.; SLEGERS, H. P2Y(AC)(--)-receptor agonists enhance the proliferation of rat C6 glioma cells through activation of the p42/44 mitogen-activated protein kinase. Br. J. Pharmacol. 134, 402–408, 2001.

COGNATO, G. de P.; BRUNO, A.N.; DA SILVA, R.S.; BOGO, M.R.; SARKIS, J.J.; BONAN, C.D. Antiepileptic drugs prevent changes induced by pilocarpine model of epilepsy in brain ecto-nucleotidases Neurochem Res. 32: 1046-1055, 2007.

COGNATO, G.P.; BRUNO, A.N.; VUADEN, F.C.; SARKIS, J.J.; BONAN, C.D. Ontogenetic profile of ectonucleotidase activities from brain synaptosomes of pilocarpine-treated rats. Int J Dev Neurosci. 23: 703-709, 2005.

CORNEJO, B.J.; MESCHES, M.H.; COULTRAP, S.; BROWNING, M.D.; BENKE, T.A. A single episode of neonatal seizures permanently alters glutamatergic synapses. Ann Neurol. 61: 411-426, 2007.

COULTER, D.A.; DE LORENZO, R.J. Basic mechanisms of status epilepticus. Adv Neurol. 79: 725–733, 1999.

CUNHA, G.M.; CANAS, P.M.; MELO, C.S.; HOCKEMEYER, J.; MÜLLER, C.E.; OLIVEIRA, C.R.; CUNHA, R.A. Adenosine A_{2A} receptor blockade prevents memory dysfunction caused by beta-amyloid peptides but not by scopolamine or MK-801. Exp Neurol. 210: 776-781, 2008.

CUNHA, G.M.; CANAS, P.M.; OLIVEIRA, C.R.; CUNHA, R.A. Increased density and synapto-protective effect of adenosine A_{2A} receptors upon sub-chronic restraint stress. Neuroscience 141: 1775-1781, 2006.

CUNHA, R.A. Caffeine, adenosine receptors, memory and Alzheimer disease. Med Clin. 131: 790-795, 2008a.

CUNHA, R.A. Different cellular sources and different roles of adenosine: A_1 receptor-mediated inhibition through astrocytic-driven volume transmission and synapse-restricted A_{2A} receptor-mediated facilitation of plasticity. Neurochem Int. 52: 65-72, 2008b.

CUNHA, R.A. Neuroprotection by adenosine in the brain: from A_1 receptor activation to A_{2A} receptor blockade. Purinergic Signal. 1: 111-134, 2005.

CUNHA, R.A. Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals. Neurochem Res. 26: 979-991, 2001.

CUNHA, R.A.; ALMEIDA, T.; RIBEIRO, J.A. Parallel modification of adenosine extracellular metabolism and modulatory action in the hippocampus of aged rats. J Neurochem 76: 372-382, 2001.

CUNHA, R.A.; CORREIA-DE-SÁ, P.; SEBASTIÃO, A.M.; RIBEIRO, J.A. Preferential activation of excitatory adenosine receptors at rat hippocampal and neuromuscular synapses by adenosine formed from released adenine nucleotides. Br J Pharmacol. 119: 253-260, 1996.

D'ALCANTARA, P.; LEDENT, C.; SWILLENS, S.; SCHIFFMANN, S.N. Inactivation of adenosine A_{2A} receptor impairs long term potentiation in the accumbens nucleus without altering basal synaptic transmission. Neuroscience 107: 455-464, 2001.

De MENDONÇA, A.; RIBEIRO, J.A. Adenosine and neuronal plasticity. Life Sci. 60: 245-251, 1997.

De ROGALSKI-LANDROT, I.; MINOKOSHI, M.; SILVEIRA, D.C.; CHA, B.H.; HOLMES, G.L. Recurrent neonatal seizures: relationship of pathology to the electroencephalogram and cognition. Dev Brain Res. 129: 27-38, 2001.

DEISSLER, H.; BLASS-KAMPMANN, S.; BRUYNEEL, E.; MAREEL, M.; RAJEWSKY, M.F. Neural cell surface differentiation antigen gp130 (RB13-6) induces fibroblasts and glioma cells to express astroglial proteins and invasive properties. FASEB J. 13(6):657-666, 1999.

DEKOSKY, S.T.; SCHEFF, S.W. Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. Ann. Neurol. 27: 457-464, 1990.

DONATO, R.; RODRIGUES, R.J.; TAKAHASHI, M.; TSAI, M.C.; SOTO, D.; MIYAGI, K.; VILLAFUERTES, R.G.; CUNHA, R.A.; EDWARDS, F.A. GABA release by basket cells onto Purkinje cells, in rat cerebellar slices, is directly controlled by presynaptic purinergic receptors, modulating Ca^{2+} influx. Cell Calcium 44: 521-532, 2008.

DRAGUNOW, M. Purinergic mechanisms in epilepsy. Prog Neurobiol. 31(2):85-108, 1988.

DRAGUNOW, M. Endogenous anticonvulsant substances. Neurosci Biobehav Rev. 10(3):229-44, 1986.

DUARTE, J.M.; OSES, J.P.; RODRIGUES, R.J.; CUNHA, R.A. Modification of purinergic signaling in the hippocampus of streptozotocin-induced diabetic rats. Neuroscience 149: 382-391, 2007.

DUBÉ, C.M.; BREWSTER, A.L.; RICHICHI, C.; ZHA, Q.; BARAM, T.Z. Fever, febrile seizures and epilepsy. Trends Neurosci 30: 490-496, 2007.

DUBYAK, G.R.; EL-MOATASSIM, C. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. Am J Physiol. 265(3 Pt 1):C577-606, 1993.

DUNWIDDIE, T.V.; MASINO, S.A. The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci. 2001;24:31-55, 2001.

FELDMAN, D.E.; KNUDSEN, E.I. Experience dependent plasticity and the maturation of glutamatergic synapses. Neuron, 20: 1067-71, 1998.

FERRER, I. Synaptic pathology and cell death in the cerebellum in Creutzfeldt-Jakob disease. Cerebellum 1: 213-222, 2002.

FIDLER, E.P.; MARKS, M.J.; COLLINS, A.C. Postnatal development of cholinergic enzymes and receptors in mouse brain. J.Neurochem. 49(3): 983-990, 1987.

FISCHER, R.S.; BOAS, W.V; BLUME, W.; ELGER, C.; GENTON, P.; LEE, P. ENGEL, J.Jr. Epilepstic seizures and epilepsy: definitions proposed by the international league against eilepsy (ILAE) and the international bureau for epilepsy (IBE). Epilepsia, 46: 470-472, 2005.

FRANKE, H.; GRUMMICH, B.; HÄRTIG, W.; GROSCHE, J.; REGENTHAL, R.; EDWARDS, R.H.; ILLES, P.; KRÜGEL, U. Changes in purinergic signaling after cerebral injury -- involvement of glutamatergic mechanisms? Int J Dev Neurosci. 24(2-3):123-32, 2006.

FREDHOLM, B.B.; BÄTTIG, K.; HOLMÉN, J.; NEHLIG, A.; ZVARTAU, E.E. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 51: 83-133, 1999.

FREDHOLM, B.B.; CHEN, J.F.; CUNHA, R.A.; SVENNINGSSON, P.; VAUGEOIS, J.M. Adenosine and brain function. Int Rev Neurobiol 63: 191-270, 2005.

FREDHOLM, B.B. Adenosine receptors as targets for drug development. Drug News Perspect. 16(5): 283-289, 2003.

FREDHOLM, B.B.; IJZERMAN, A.P.; JACOBSON, K.A.; KLOTZ, K.N.; LINDEN, J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev. 53(4): 527-552, 2001.

FRITTITTA, L.; SPAMPINATO, D.; SOLINI, A.; NOSADINI, R.; GOLDFINE, I.D.; VIGNERI, R.; TRISCHITTA, V. Elevated PC-1 content in cultured skin fibroblasts correlates with decreased in vivo and in vitro insulin action in nondiabetic subjects: evidence that PC-1 may be an intrinsic factor in impaired insulin receptor signaling. Diabetes. 47(7): 1095-1100, 1998.

FUCHS, J.L. 5'-nucleotidase activity increases in aging rat brain. Neurobiol Aging, 12: 523-530, 1991.

FÜRSTENAU, C.R.; TRENTIN, D.da S.; GOSSENHEIMER, A.N.; RAMOS, D.B.; CASALI, E.A.; BARRETO-CHAVES, M.L.; SARKIS, J.J. Ectonucleotidase activities are altered in serum and platelets of L-NAME-treated rats. Blood Cells Mol Dis. 41(2): 223-229, 2008.

FUJII, S. ATP- and Adenosine-Mediated Signaling in the Central Nervous System: The Role of Extracellular ATP in Hippocampal Long-Term Potentiation. J Pharmacol Sci. 94: 103 – 106, 2004.

FUJII, S.; KATO, H.; FURUSE, H.; et al. The mechanism of ATP-induced long-term potentiation involves extracellular phosphorylation of membrane proteins in guinea-pig hippocampal CA1 neurons. Neurosci Lett. 187:130–132, 1995.

FUJII, S.; KATO, H.; KURODA, Y. Cooperativity between extracellular adenosine 5'-triphosphate and activation of N-methyl-D-aspartate receptors in long-term potentiation induction in hippocampal CA1 neurons. Neuroscience 113: 617-628, 2002.

FUSS, B.; BABA, H.; PHAN, T.; TUOHY, V.K.; MACKLIN, W.B. Phosphodiesterase I, a novel adhesion molecule and/or cytokine involved in oligodendrocyte function. J. Neurosci. 17: 9095–9103, 1997.

GARDEN, G.A.; BUDD, S.L.; TSAI, E.; HANSON, L.; KAUL, M.; D'EMILIA, D.M.; FRIEDLANDER, R.M.; YUAN, J.; MASLIAH, E.; LIPTON, S.A. Caspase cascades in human immunodeficiency virus-associated neurodegeneration. J Neurosci. 22(10): 4015-4024, 2002.

GIJSBERS, R.; AOKI, J.; ARAI, H.; BOLLEN, M. The hydrolysis of lysophospholipidsand nucleotides by autotaxin (NPP2) involves a single catalytic site. FEBS Lett. 538: 60–64, 2003.

GIJSBERS, R.; CEULEMANS, H.; STALMANS, W.; BOLLEN, M. Structural and catalytic similarities between nucleotide pyrophosphatases / phosphodiesterases and alkaline phosphatases. J Biol Chem. 276(2): 1361-1368, 2001.

GLANTZ, L.A.; GILMORE, J.H.; LIEBERMAN, J.A.; JARSKOG, L.F. Apoptotic mechanisms and the synaptic pathology of schizophrenia. Schizophr Res. 81: 47-63, 2006.

GLASS, M.; FAULL, R.L.; BULLOCK, J.Y.; JANSEN, K.; MEE, E.W.; WALKER, E.B.; SYNEK, B.J.; DRAGUNOW, M. Loss of A_1 adenosine receptors in human temporal lobe epilepsy. Brain Res. 710(1-2): 56-68, 1996.

GODING, J.W.; GROBBEN, B.; SLEGERS, H. Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase / phosphodiesterase family. Biochim. Biophys. Acta 1638: 1–19, 2003.

GÓMEZ-VILLAFUERTES, R.; GUALIX, J.; MIRAS-PORTUGAL, M.T. Single GABAergic synaptic terminals from rat midbrain exhibit functional P2X and dinucleotide receptors, able to induce GABA secretion. J Neurochem. 77: 84-93, 2001.

GONÇALVES, M.L.; CUNHA, R.A.; RIBEIRO, J.A. Adenosine A_{2A} receptors facilitate $^{45}Ca^{2+}$ uptake through class A calcium channels in rat hippocampal CA3 but not CA1 synaptosomes. Neurosci Lett. 238: 73-77, 1997.

GROBBEN, B.; ANCIAUX, K.; ROYMANS, D.; STEFAN, C.; BOLLEN, M.; ESMANS, E.L.; SLEGERS, H. An ecto-nucleotide pyrophosphatase is one of the main enzymes involved in the extracellular metabolism of ATP in rat C6 glioma. J. Neurochem. 72: 826–834, 1999.

GU, J.G.; FOGA, I.O.; PARKINSON, F.E.; GEIGER, J.D. Involvement of bidirectional adenosine transporters in the release of L-[3H]adenosine from rat brain synaptosomal preparations. J Neurochem. 64(5): 2105-2110, 1995.

HAAS, K.Z.; SPERBER, E.F.; OPANASHUK, L.A.; STANTON, P.K.; MOSHÉ, S.L. Resistance of immature hippocampus to morphologic and physiologic alterations following status epilepticus or kindling. Hippocampus 11: 615-625, 2001.

HANDA, M.; GUIDOTTI, G. Purification and cloning of a soluble ATP-diphosphohydrolase (apyrase) frompotato tubers (Solanum tuberosum). Biochem Biophys Res Commun. 218(3): 916-923, 1996.

HARAHAP, A.R., GODING, J.W. Distribution of the murine plasma cell antigen PC-1 in non-lymphoid tissues. J. Immunol. 141: 2317–2320, 1988.

HAUSER, W.; HERSDORFFER, D. Epilepsy: frequency, causes and consequences. New York: Demos, 1990.

HAUSER, W.A.; ANNEGERS, J.F.; ROCCA, W.A. Descriptive epidemiology of epilepsy: contributions of population-based studies from Rochester, Minnesota.Mayo Clin Proc. 71(6): 576-586, 1996.

HAUT, S.R.; VELISKO´VA, J.; MOSHE´, S.L. Susceptibility of immature and adult brains to seizure effects. Lancet Neurobiol. 3: 608–617, 2004.

HEILBRONN, A.; MAIENSCHEIN, V.; CARSTENSEN, K.; GANN, W.; ZIMMERMANN, H. Crucial role of ecto-5'-nucleotidase in differentiation and survival of developing neural cells. Neuroreport. 7(1): 257-261, 1995.

HEILBRONN, A.; ZIMMERMANN, H. 5'-nucleotidase activates and an inhibitory antibody prevents neuritic differentiation of PC12 cells. Eur J Neurosci. 7(6): 1172117-9, 1995.

HOLMES, G.L. Effects of seizures on brain development: lessons from the laboratory. Pediatr Neurol. 33: 1-11, 2005.

HOLMES, G.L.; BEN-ARI, Y. Seizures in the developing brain: perhaps not so benign after all. Neuron 21: 1231-1234, 1998.

HOLMES, G.L. Epilepsy in the developing brain: lesson from the laboratory and clinic. Epilepsia. 38: 12-30, 1997.

HOLMES, G.L. Effects of early seizures on later behavior and epileptogenicity. Ment Retard Dev Disabil Res Rev. 10: 101-5, 2004.

HOLMES, G.L. Effects of seizures on brain development: lessons from the laboratory. Pediatr Neurol. 33: 1-11, 2005.

HUANG, L.T., YANG, S.N., LIOU, C.W., HUNG, P.L., LAI, M.C., WANG, C.L., WANG, T.J. Pentylenetetrazol-induced recurrent seizures in rat pups: time course on spatial learning and long-term effects. Epilepsia 43: 567-573, 2002.

HUGEL, S.; SCHLICHTER, R. Presynaptic P2X receptors facilitate inhibitory GABAergic transmission between cultured rat spinal cord dorsal horn neurons. J Neurosci 20: 2121-2130, 2000.

JAMES, G.; BUTT, A.M. P2Y and P2X purinoceptor mediated Ca2+ signalling in glial cell pathology in the central nervous system. Eur J Pharmacol. 447(2-3):247-60, 2002.

JOHANSSON B, HALLDNER L, DUNWIDDIE TV, MASINO SA, POELCHEN W, GIMÉNEZ-LLORT L, ESCORIHUELA RM, FERNÁNDEZ-TERUEL A, WIESENFELD-HALLIN Z, XU XJ, HÅRDEMARK A, BETSHOLTZ C, HERLENIUS E, FREDHOLM BB. Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor.Proc Natl Acad Sci U S A. 98(16): 9407-1942, 2001.

JOHNSON-KOZLOW, M.; KRITZ-SILVERSTEIN, D.; BARRETT-CONNOR, E.; MORTON, D. Coffee consumption and cognitive function among older adults. Am J Epidemiol. 156(9):842-50, 2002.

KHAKH, B.S.; HENDERSON, G. ATP receptor-mediated enhancement of fast excitatory neurotransmitter release in the brain. Mol Pharmacol 54: 372-378, 1998.

KINGSBURY, M.A.; REHEN, S.K.; CONTOS, J.J.; HIGGINS, C.M.; CHUN, J.. Nonproliferative effects of lysophosphatidic acid enhance cortical growth and folding. Nat. Neurosci. 12: 1292–1299, 2003.

KIRVELL, S.L.; ESIRI, M.; FRANCIS, P.T. Down-regulation of vesicular glutamate transporters precedes cell loss and pathology in Alzheimer's disease. J Neurochem. 98: 939-950, 2007.

KOGA, K.; KUROKAWA, M.; OCHI, M.; NAKAMURA, J.; KUWANA, Y. Adenosine A(2A) receptor antagonists KF17837 and KW-6002 potentiate rotation induced by dopaminergic drugs in hemi-Parkinsonian rats. Eur J Pharmacol. 408(3): 249-255, 2000.

KOH, S.; STOREY, T.W.; SANTOS, T.C.; MIAN, A.Y.; COLE, A.J. Early-life seizures in rats increase susceptibility to seizure-induced brain injury in adulthood. Neurology 53: 915-921, 1999.

KREUTZBERG, G.W.; HEYMANN, D.; REDDINGTON, M. 5’-Nucleotidase in the nervous system. In ‘Cellular Biology of Ectoenzymes’ (Kreutzberg GW, Reddington M, Zimmermann H, Eds), pp.147-164, 1986.

KUKULSKI, F.; LÉVESQUE, S.A.; LAVOIE, E.G.; LECKA, J.; BIGONNESSE, F.; KNOWLES, A.F.; ROBSON, S.C.; KIRLEY, T.L.; SÉVIGNY, J. Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. Purinergic Signal. 1(2):193-204, 2005.

LAGAE, L. What's new in: "genetics in childhood epilepsy". Eur J Pediatr. 167(7):715-22, 2008.

LANGER, D.; HAMMER, K.; KOSZALKA, P.; SCHRADER, J.; ROBSON, S.; ZIMMERMANN, H. Distribution of ectonucleotidases in the rodent brain revisited. Cell Tissue Res 334: 199-217, 2008.

LAVOIE, E.G.; KUKULSKI, F.; LÉVESQUE, S.A.; LECKA, J.; SÉVIGNY, J. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-3. Biochem Pharmacol. 67(10):1917-26, 2004.

LEE, C.L.; HANNAY, J.; HRACHOVY, R.; RASHID, S.; ANTALFFY, B.; SWANN, J.W. Spatial learning deficits without hippocampal neuronal loss in a model of early-onset epilepsy. Neuroscience, 107: 71-84, 2001.

LEÓN, D.; SÁNCHEZ-NOGUEIRO, J.; MARÍN-GARCÍA, P.; MIRAS-PORTUGAL, M.A. Glutamate release and synapsin-I phosphorylation induced by P2X7 receptors activation in cerebellar granule neurons. Neurochem Int 52: 1148-1159, 2008.

LI, H.; LI, S.H.; YU, Z.X.; SHELBOURNE, P.; LI, X.J. Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. J Neurosci 21: 8473-8481, 2001.

LINDEN, J. Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. Annu Rev Pharmacol Toxicol. 41: 775-787, 2001.

LIE, A.A.; BLÜMCKE, I.; BECK, H.; WIESTLER, O.D.; ELGER, C.E.; SCHOEN, S.W. 5'-Nucleotidase activity indicates sites of synaptic plasticity and reactive synaptogenesis in the human brain. J Neuropathol Exp Neurol. 58(5): 451-458, 1999.

LYNCH, M.; SAYIN, U.; BOWNDS, J.; JANUMPALLI, S.; SUTULA, T. Long-term consequences of early postnatal seizures on hippocampal learning and plasticity. Eur J Neurosci, 7: 2252-2264, 2000.

MAIA, L.; de MENDONÇA, A. Does caffeine intake protect from Alzheimer's disease? Eur J Neurol 9: 377-382, 2002.

MARIN-PADILLA, M. Early vascularization of the embryonic cerebral cortex: Golgi and electron microscopic studies. J. Comp. Neurol. 241: 237–249, 1985.

MATHERN, G.W.; ADELSON, P.D.; CAHAN, L.D.; LEITE, J.P. Hippocampal neuron damage in human epilepsy: Meyer's hypothesis revisited. Prog Brain Res. 135: 237–252, 2002.

MINKEVICIENE, R.; IHALAINEN, J.; MALM, T.; MATILAINEN, O.; KEKSA-GOLDSTEINE, V.; GOLDSTEINS, G.; IIVONEN, H.; LEGUIT, N.; GLENNON, J.; KOISTINAHO, J.; BANERJEE, P.; TANILA, H. Age-related decrease in stimulated glutamate release and vesicular glutamate transporters in APP/PS1 transgenic and wild-type mice. J Neurochem. 105: 584-594, 2008.

MÜLLER, J.; ROCHA, J.B.; BATTASTINI, A.M.; SARKIS, J.J.; DIAS, R.D. Ontogeny of ATP and ADP hydrolysis by cerebral cortex synaptosomes from rats. Braz. J. Med. Biol. Res. 23: 935–939, 1990.

NADLER, J.V. The recurrent mossy fiber pathway of the epileptic brain. Neurochem Res 28:1649–1658, 2003.

NAFFAH-MAZZACORATTI, M.G. Alterações neuroquímicas associadas às epilepsias do lobo temporal. IN: Fundamentos neurobiológicos das epilepsias: aspectos clínicos e cirúrgicos, eds. Da Costa, J.C.; Palmini, A.; Yacubian, E.M.T., Cavalheiro, E.A., pp 75 – 100, São Paulo, 1998.

NAM, S.W.; CLAIR, T.; CAMPO, C.K.; LEE, H.Y.; LIOTTA, L.A.; STRACKE, M.L. Autotaxin (ATX), a potent tumor motogen, augments invasive and metastatic potential of ras-transformed cells. Oncogene. 19(2):241-247, 2000.

NAM, S.W.; CLAIR, T.; KIM, Y.S.; MCMARLIN, A.; SCHIFFMANN, E.; LIOTTA, L.A.; STRACKE, M.L. Autotaxin (NPP-2), a metastasis-enhancing motogen, is an angiogenic factor. Cancer Res. 61(18):6938-6944, 2001.

NEWBY, A.C. The interaction of inhibitors with adenosine metabolising enzymes in intact isolated cells. Biochem Pharmacol. 30(18):2611-2615, 1981.

NITECKA, L.; TREMBLAY, E.; CHARTON, G.; BOUILLOT, J.P.; BERGER, M.L.; BEN-ARI, Y. Maturation of kainic acid seizure-brain damage syndrome in the rat. II. Histopathological sequelae. Neuroscience 13: 1073-1094, 1984.

OSES, J.P.; CARDOSO, C.M.; GERMANO, R.A.; KIRST, I.B.; RÜCKER, B.; FÜRSTENAU, C.R.; WINK, M.R.; BONAN, C.D.; BATTASTINI, A.M.; SARKIS, J.J. Soluble NTPDase: An additional system of nucleotide hydrolysis in rat blood serum. Life Sci. 74(26):3275-84, 2004.

OSES, J.P.; VIOLA, G.G.; de PAULA COGNATO, G.; JÚNIOR, V.H.; HANSEL, G.; BÖHMER, A.E.; LEKE, R.; BRUNO, A.N.; BONAN, C.D.; BOGO, M.R.; PORTELA, L.V.; SOUZA, D.O.; SARKIS, J.J. Pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid. Epilepsy Res. 75: 104-111, 2007.

OTTMAN, R.; RISCH, N.; HAUSER, W.A.; PEDLEY, T.A.; LEE, J.H.; BARKER-CUMMINGS, C.; LUSTENBERGER, A.; NAGLE, K.J.; LEE, K.S.; SCHEUER, M.L. Localization of a gene for partial epilepsy to chromosome 10q. Nat Genet. 10(1):56-60, 1995.

PAGONOPOULOU, O.; ANGELATOU, F.; KOSTOPOULOS, G. Effect of pentylentetrazol-induced seizures on A1 adenosine receptor regional density in the mouse brain: a quantitative autoradiographic study. Neuroscience. 56(3): 711-716, 1993.

PAGONOPOULOU, O.; EFTHIMIADOU, A.; ASIMAKOPOULOS, B.; NIKOLETTOS, N.K. Modulatory role of adenosine and its receptors in epilepsy: possible therapeutic approaches. Neurosci Res. 56(1):14-20, 2006.

PANKRATOV, Y.V.; LALO, U.V.; KRISHTAL, O.A. Role for P2X receptors in long-term potentiation. J Neurosci 22: 8363-8369, 2002.

PEDATA, F.; CORSI, C.; MELANI, A.; BORDONI, F.; LATINI, S. Adenosine extracellular brain concentrations and role of A2A receptors in ischemia. Ann N Y Acad Sci. 939:74-84, 2001.

PEDRAZZA, E.L.; RIBOLDI, G.P.; PEREIRA, G.S.; IZQUIERDO, I.; BONAN, C.D. Habituation to an open field alters ecto-nucleotidase activities in rat hippocampal synaptosomes. Neurosci Lett 413: 21-24, 2007.

PEREIRA, G.S.; MELLO, E.; SOUZA, T.; BATTASTINI, A.M.; IZQUIERDO, I.; SARKIS, J.J.; BONAN, C.D. Effects of inhibitory avoidance training and/or isolated foot-shock on ectonucleotidase activities in synaptosomes of the anterior and posterior cingulate cortex and the medial precentral area of adult rats. Behav Brain Res 128: 121-127, 2002.

PHILLIS, J.W.; WU, P.H. The role of adenosine and its nucleotides in central synaptic transmission. Prog Neurobiol. 16(3-4):187-239, 1981.

PITKANEN, A.; SUTULA, T.P. Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. *Lancet Neurol* 1:173–181, 2002.

PLATE, K.H., BREIER, G., RISAU, W. Molecular mechanisms of developmental and tumor angiogenesis. Brain Pathol. 4, 207–218, 1994.

PLESNER, L. Ecto-ATPases: identities and functions. Int Rev Cytol. 158:141-214, 1995.

POTTER, P.; WHITE, T.D. Release of adenosine 5'-triphosphate from synaptosomes from different regions of rat brain. Neuroscience. 5(7):1351-6, 1980.

PREDIGER, R.D.; BATISTA, L.C.; TAKAHASHI, R.N. Caffeine reverses age-related deficits in olfactory discrimination and social recognition memory in rats. Involvement of adenosine A_1 and A_{2A} receptors. Neurobiol. Aging. 26: 957-964, 2005.

RALEVIC, V.; BURNSTOCK, G. Receptors for purines and pyrimidines. Pharmacol. Rev. 50, 413–492, 1998.

RATHBONE, M.P.; MIDDLEMISS, P.J.; GYSBERS, J.W.; ANDREW, C.; HERMAN, M.A.; REED, J.K.; CICCARELLI, R.; DI IORIO, P.; CACIAGLI, F. Trophic effects of purines in neurons and glial cells. Prog Neurobiol. 59(6):663-90, 1999.

REBOLA, N.; CANAS, P.M.; OLIVEIRA, C.R.; CUNHA, R.A. Different synaptic and subsynaptic localization of adenosine A_{2A} receptors in the hippocampus and striatum of the rat. Neuroscience 132: 893-903, 2005.

REBOLA, N.; LUJAN, R.; CUNHA, R.A.; MULLE, C. Adenosine A_{2A} receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses. Neuron 57: 121-134, 2008.

RIBEIRO, J.A.; SEBASTIAO, A.M.; de MENDONCA, A. Participation of adenosine receptors in neuroprotection. Drug News Perspect. 16(2):80-6, 2003.

RICHARDSON, P.J.; BROWN, S.J. ATP release from affinity-purified rat cholinergic nerve terminals. J Neurochem. 48(2):622-30. 1987

ROBSON, S.; SÉVIGNY, J.; ZIMMERMANN, H. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal. 2, 409–430, 2006

RODRIGUES, R.J.; ALMEIDA, T.; RICHARDSON, P.J.; OLIVEIRA, C.R.; CUNHA, R.A. Dual presynaptic control by ATP of glutamate release via facilitatory P2X1, P2X2/3 and P2X3, and inhibitory P2Y1, P2Y2 and/or P2Y4 receptors in the rat hippocampus. J Neurosci 27: 6286-6295, 2005.

RYU, J.K.; MCLARNON, J.G. Block of purinergic P2X(7) receptor is neuroprotective in an animal model of Alzheimer's disease. Neuroreport. 19(17):1715-9, 2008.

SAFIULINA, V.F.; KASYANOV, A.M.; SOKOLOVA, E.; CHERUBINI, E.; GINIATULLIN, R. ATP contributes to the generation of network-driven giant depolarizing potentials in the neonatal rat hippocampus. 565(Pt 3):981-92, 2005.

SAKAGAMI, H.; AOKI, J.; NATORI, Y.; NISHIKAWA, K.; KAKEHI, Y.; NATORI, Y.; ARAI, H. Biochemical and molecular characterization of a novel cholinespecific glycerophosphodiester phosphodiesterase belonging to the

nucleotide pyrophosphatase/phosphodiesterase family. J. Biol. Chem. 280, 23084– 23093, 2005.

SAYIN, U.; SUTULA, T.P.; STAFSTROM, C.E. Seizures in the developing brain cause adverse long-term effects on spatial learning and anxiety. Epilepsia, 45: 1539-1548, 2004.

SCHEFF, S.W.; PRICE, D.A.; SCHMITT, F.A.; DEKOSKY, S.T.; MUFSON, E.J. Synaptic alterations in CA1 in mild Alzheimer disease and mild cognitive impairment. Neurology 68:1501-1508, 2007.

SCHETINGER, M.R.; BONAN, C.D.; SCHIERHOLT, R.C.; WEBBER, A.; ARTENI, N.; EMANUELLI, T.; DIAS, R.D.; SARKIS, J.J.F.; NETTO, C.A. Nucleotide hydrolysis in rats submitted to global cerebral ischemia: A possible link between preconditioning and adenosine production. J Stroke Cerebrovasc Dis. 7: 281-286, 1998.

SCHOEN, S.W.; EBERT, U.; LÖSCHER, W. 5'-Nucleotidase activity of mossy fibers in the dentate gyrus of normal and epileptic rats. Neuroscience. 93(2):519-26, 1999.

SCHOEN, S.W.; KREUTZBERG, G.W. Synaptic 5'-nucleotidase activity reflects lesion-induced sprouting within the adult rat dentate gyrus. Exp Neurol. 127(1):106-18, 1994.

SCHRIER, S.M.; FLOREA, B.I.; MULDER, G.I.; NAGELKERKE, J.F.; IJZERMAN, A.P. Apoptosis induced by extracellular ATP in mouse neuroblastomacell line N1E-115: studies on involvement of P2 receptors andadenosine, Biochem. Pharmacol. 63: 1119– 1126, 2002.

SEBASTIÃO, A.M.; RIBEIRO, J.A. Adenosine A2 receptor-mediated excitatory actions on the nervous system. Prog Neurobiol. 48(3):167-189, 1996.

SELKOE, D.J. Alzheimer's disease is a synaptic failure. Science 298: 789-791, 2002.

SHIOZAKI, S.; ICHIKAWA, S.; NAKAMURA, J.; KITAMURA, S.; YAMADA, K.; KUWANA, Y. Actions of adenosine A2A receptor antagonist KW-6002 on drug-induced catalepsy and hypokinesia caused by reserpine or MPTP. Psychopharmacology (Berl). 147(1):90-5, 1999.

SHUKLA, V.; ZIMMERMANN, H.; WANG, L.; KETTENMANN, H.; RAAB, S.; HAMMER, K.; SÉVIGNY, J.; ROBSON, S.C.; BRAUN, N. Functional expression of the ecto-ATPase NTPDase2 and of nucleotide receptors by neuronal progenitor cells in the adult murine hippocampus.J Neurosci Res. 80(5):600-10, 2005.

SILVA, C.G.; PORCIÚNCULA, L.O.; CANAS, P.M.; OLIVEIRA, C.R.; CUNHA, R.A. Blockade of adenosine A_{2A} receptors prevents staurosporine-induced apoptosis of rat hippocampal neurons. Neurobiol Dis. 27: 182-189, 2007.

SIM, J.A.;CHAUMONTS, J.O.J.; ULMANN, L.; YOUNG, M.T.; CHO, K.; BUELL, G.; NORTH, R.A.; RASSENDREN, F. Altered hippocampal synaptic potentiation in P2X4 knock-out mice. J Neurosci 26: 9006-9009, 2006.

SLOVITER, R.S. The functional organization of the hippocampal dentate gyrus and its relevance to the pathogenesis of temporal lobe epilepsy. Ann Neurol 35:640–654, 1994.

SMITH, T.M.; KIRLEY, T.L. Cloning, sequencing, and expression of a human brain ecto-apyrase related to both the ecto-ATPases and CD39 ecto-apyrases1. Biochim Biophys Acta. 1386(1):65-78, 1998.

STAFSTROM, C.E. Assessing the behavioral and cognitive effects of seizures on the developing brain. Prog Brain Res, 135: 377-390, 2002.

STAFSTROM, C.E.; THOMPSON, J.L.; HOLMES, G.L. Kainic acid seizures in the developing brain: status epilepticus and spontaneous recurrent seizures. Dev Brain Res, 65: 227-236, 1992.

TAKAHASHI, R.N.; PAMPLONA, F.A.; PREDIGER, R.D. Adenosine receptor antagonists for cognitive dysfunction: a review of animal studies. Front Biosci .13: 2614-2632, 2008.

TCHEKALAROVA, J.; SOTIRIOU, E.; GEORGIEV, V.; KOSTOPOULOS, G.; ANGELATOU, F. Up-regulation of adenosine A1 receptor binding in pentylenetetrazol kindling in mice: effects of angiotensin IV. Brain Res. 1032(1-2):94-103, 2005.

TERRY, R.D.; MASLIAH, E.; SALMON, D.P.; BUTTERS, N.; DETERESA, R.; HILL, R.; HANSEN, L.A.; KATZMAN, R. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann. Neurol. 30: 572-580, 1991.

TODOROV, L.D.; MIHAYLOVA-TODOROVA, S.; WESTFALL, T.D.; SNEDDON, P.; KENNEDY, C.; BJUR, R.A.; WESTFALL, D.P. Evaluation of a method for controlling molecular scaffold diversity in de novo ligand design. J. Comput. Aid. Mol. Des. 11, 175–192, 1997.

van MEETEREN, L.A.; RUURS, P.; STORTELERS, C.; BOUWMAN, P.; VAN ROOIJEN, M.A.; PRADÈRE, J.P.; PETTIT, T.R.; WAKELAM, M.J.; SAULNIER-BLACHE, J.S.; MUMMERY, C.L.; MOOLENAAR, W.H.; JONKERS, J. Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. Mol Cell Biol. 26(13):5015-22, 2006.

VANORE, G.; GIRALDEZ, L.; RODRÍGUEZ, de LORES ARNAIZ G.; GIRARDI E. Seizure activity produces differential changes in adenosine A1 receptors within rat hippocampus. Neurochem Res. 26(3):225-30, 2001.

VIANNA, E.P.; FERREIRA, A.T.; DONÁ, F.; CAVALHEIRO, E.A.; da SILVA FERNANDES, M.J. Modulation of seizures and synaptic plasticity by adenosinergic receptors in an experimental model of temporal lobe epilepsy induced by pilocarpine in rats.Epilepsia. 46(5):166-73, 2005.

VORHOFF, T.; ZIMMERMANN, H.; PELLETIER, J.; SÉVIGNY, J.; BRAUN, N. Cloning and characterization of the ecto-nucleotidase NTPDase3 from rat brain: Predicted secondary structure and relation to other members of the E-NTPDasefamily and actin Purinergic Signal. 1(3):259-70, 2005.

WARDAS, J.; KONIECZNY, J.; LORENC-KOCI, E. SCH 58261, an A(2A) adenosine receptor antagonist, counteracts parkinsonian-like muscle rigidity in rats. Synapse. 41(2):160-71, 2001.

WETHERINGTON, J.P.; LAMBERT, N.A. Differential desensitization of responses mediated by presynaptic and postsynaptic A1 adenosine receptors.J Neurosci. 22(4):1248-55, 2002.

WIERASZKO, A. Extracellular ATP as a neurotransmitter: its role in synaptic plasticity in the hippocampus. Acta Neurobiol 56:637-648, 2006.

WINK, M.R.; BRAGANHOL, E.; TAMAJUSUKU, A.S.; LENZ, G.; ZERBINI, L.F.; LIBERMANN, T.A.; SÉVIGNY, J.; BATTASTINI, A.M.; ROBSON, S.C. Nucleoside triphosphate diphosphohydrolase-2 (NTPDase2/CD39L1) is the dominant ectonucleotidase expressed by rat astrocytes. Neuroscience 138:421-432, 2006.

WINK, M.R.; LENZ, G.; RODNIGHT, R.; SARKIS, J.J.; BATTASTINI, A.M. Identification of brain ecto-apyrase as a phosphoprotein. Mol Cell Biochem. 213(1-2):11-6, 2000.

WIRKNER, K.; FRANKE, H.; INOUE, K.; ILLES, P. Differential age-dependent expression of alpha2 adrenoceptor- and P2 purinoceptor-functions in rat locus coeruleus neurons. Naunyn Schmiedebergs Arch Pharmacol. 357(2):186-9, 1998.

WWW.CRRI.CA/IMAGES/FIGURE_SEVIGNY_E.GIF

XIANG, Z.; BURNSTOCK, G. Changes in expression of P2X purinoceptors in rat cerebellum during postnatal development. Brain Res Dev Brain Res. 156(2):147-57, 2005a.

XIANG, Z.; BURNSTOCK, G. Expression of P2X receptors in rat choroid plexus. Neuroreport.. 16(9):903-7, 2005b.

XIAO, C.; ZHOU, C.; LI, K.; DAVIES, D.L.; YE, J.H. Purinergic type 2 receptors at GABAergic synapses on ventral tegmental area dopamine neurons are targets for ethanol action. J. Pharmacol. Exp. Ther. 327: 196-205, 2008.

YAAR, R.; JONES, M.R.; CHEN, J.F.; RAVID, K. Animal models for the study of adenosine receptor function. J Cell Physiol. 202(1):9-20, 2005.

YEGUTKIN, G.G. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochim Biophys Acta. 1783(5):673-94, 2008.

ZIMMERMANN, H.; BRAUN, N.; KEGEL, B.; HEINE, P. New insights into molecular structure and function of ectonucleotidases in the nervous system. Neurochem Int. 32(5-6):421-5, 1998.

ZIMMERMANN, H. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Prog. Neurobiol. 49: 587-618, 1996.

ZIMMERMANN, H. Ectonucleotidases: some developments and a note on nomenclature. Drug Developmental Res. 52: 44-56, 2001.

ZIMMERMANN, H. 5'-Nucleotidase: molecular structure and functional aspects, Biochem J 285: 345–365, 1992.

ZIMMERMANN, H. Ectonucleotidases in the nervous system. Novartis Found Symp. 276, 113–128, 2006a.

ZIMMERMANN, H. Nucleotide signaling in nervous system development. Eur. J. Physiol. 452, 573–588, 2006b.