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RECEPTORES EP1 E EP3 MODULAM AS CRISES EPILÉPTICAS INDUZIDAS POR PENTILENOTETRAZOL E ÁCIDO CAÍNICO EM CAMUNDONGOS

TESE DE DOUTORADO

Cristina Ruedell Reschke Banderó

Santa Maria, RS, Brasil

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RECEPTORES EP1 E EP3 MODULAM AS CRISES EPILÉPTICAS INDUZIDAS POR PENTILENOTETRAZOL E ÁCIDO CAÍNICO EM CAMUNDONGOS

por

Cristina Ruedell Reschke Banderó

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Orientador: Prof. Dr. Carlos Fernando de Mello
Co-orientador: Prof. Dr. Mauro Schneider de Oliveira

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Como requisito parcial para obtenção do grau de
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DEDICATÓRIA

À minha família e
àqueles que futuramente
possam ser beneficiados por esta
pesquisa.

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“Há um tempo em que é preciso abandonar as roupas usadas, que já tem a forma do nosso corpo, e esquecer os nossos caminhos, que nos levam sempre aos mesmos lugares. É o tempo da travessia: e, se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos.”

Fernando Pessoa

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Farmacologia
Universidade Federal de Santa Maria, RS, Brasil

RECEPTORES EP1 E EP3 MODULAM AS CRISES EPILÉPTICAS INDUZIDAS POR PENTILENOTETRAZOL E ÁCIDO CAÍNICO EM CAMUNDONGOS

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Co-orientador: Prof. Dr. Mauro Schneider de Oliveira
Local e Data da Defesa: Santa Maria, 27 de Junho de 2013.

A epilepsia é uma das disfunções neurológicas mais comuns. Tem sido sugerido que as crises epilépticas podem ser facilitadas pela ocorrência de inflamação. A PGE_2 é um dos mediadores inflamatórios mais importantes que, agindo por meio dos receptores EP1 e EP3, facilita as convulsões induzidas por pentilenotetrazol (PTZ). Contudo, até a presente data, nenhum estudo investigou, de maneira sistêmica, se a ativação ou bloqueio de receptores EP1 e EP3 facilitam as convulsões induzidas por outros agentes; tampouco se alterações na atividade da Na^+,K^+-ATPase estão envolvidas nesse efeito. Assim, no presente estudo, investigamos se ligantes (agonistas e antagonistas) de receptores EP1 e EP3 modificam as crises induzidas por PTZ e ácido caínico (KA), e se tais efeitos estão associados a alterações na atividade da enzima Na^+,K^+-ATPase, em camundongos. Os antagonistas EP1 e EP3 (ONO-8713 e ONO-AE3-240, respectivamente, 10 µg/Kg, s.c.) atenuaram as convulsões induzidas por PTZ (60 mg/Kg, i.p.) e KA (20 mg/Kg). Os seus respectivos agonistas (ONO-DI-004 e ONO-AE-248 de 10 µg/Kg, s.c.) facilitaram as convulsões em ambos modelos agudos de crises epilépticas e, em doses não efetivas para gerar crises, preveniram os efeitos dos antagonistas. Os animais submetidos à administração de PTZ apresentaram, ao final do experimento, a atividade Na^+,K^+-ATPásica diminuída no córtex cerebral e hipocampo. Por outro lado, animais tratados com KA apresentaram um aumento na atividade Na^+,K^+-ATPásica nestas mesmas estruturas, que se correlacionou positivamente com a vigência de *status epilepticus* no momento do sacrifício. Os achados divergentes no que diz respeito à alteração da atividade da Na^+,K^+-ATPase nos dois modelos de crises agudas sugere que tais alterações estejam relacionadas ao tipo de agente convulsivante utilizado, e dificultam estabelecer, de forma inequívoca, uma relação entre atividade desta ATPase e sensibilidade à crises agudas. Ademais, a administração de antagonistas EP1 e EP3 aboliu as alterações da atividade da Na^+,K^+-ATPase induzidas tanto por PTZ como por KA, de tal forma que estas parecem estar mais associadas com o fenômeno ictal em si, do que com os mecanismos de indução da crise. Contudo, os resultados mostram de forma clara que os receptores EP1 e EP3 podem se constituir possíveis novos alvos para o desenvolvimento de drogas antiepilépticas, pois antagonistas EP1 e EP3 diminuíram as crises, independente do agente convulsivante utilizado.

Palavras-chave: epilepsia, prostaglandina E_2, receptores EP, PTZ, ácido caínico

ABSTRACT

Ph.D. Thesis
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EP1 AND EP3 RECEPTORS MODULATE PENTYLENETETRAZOL- AND KAINIC ACID-INDUCED SEIZURES IN MICE

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Date and place of the defense: Santa Maria, June 27th, 2013.

Epilepsy is one of the most common neurologic disorders. It has been suggested that seizures may be facilitaded by inflammation. PGE_2 is one of the most important inflammatory mediators, and facilitates pentylenetetrazol (PTZ)-induced seizures by stimulating EP1 and EP3 receptors. However, up to the present moment, no study has investigated whether EP1 and EP3 receptors blocking attenuate seizures induced by convulsants other than PTZ. It is also unknown whether Na^+,K^+-ATPase activity alterations are involved in such an effect. Therefore, in the current study we investigated whether EP1 and EP3 ligands (agonists and antagonists) modulate PTZ- and kainic acid (KA)-induced seizures, and whether alterations in Na^+,K^+-ATPase activity mediate such a protective effect, in mice. EP1 and EP3 antagonists (ONO-8713 and ONO-AE3-240, respectively, 10 µg/kg, s.c.) attenuated PTZ (60 mg/kg, i.p.)- and KA (20 mg/kg, i.p.)-induced seizures. The respective agonists (ONO-DI-004 and ONO-AE-248, 10 µg/kg, s.c.) facilitated seizures in both acute models, and at non-effective doses, prevented the protective effects of the antagonists. Animals injected with PTZ presented decreased Na^+,K^+-ATPase activity in the cerebral cortex and hippocampus. On the other hand, animals injected with KA presented increased Na^+,K^+-ATPase activity in the same cerebral structures at the end of the experiment. These divergent findings suggest that alterations in Na^+,K^+-ATPase activity in both acute models depends on the convulsant agent used and make difficult to establish a relationship between Na^+,K^+-ATPase activity and seizure development. Moreover, EP1 and EP3 antagonists administration abolished Na^+,K^+-ATPase activity alterations induced by PTZ and KA, in such a way that these alterations seem to be related more to the presence of ictal phenomenon itself than to the seizure induction mechanisms. Notwithstanding, the currrent results clearly show that EP1 and EP3 receptors might constitute novel targets for anticonvulsants development, since EP1 and EP3 decreased seizures, regardless of the convulsant agent used.

Keywords: epilepsy, prostaglandin E_2, EP receptors, PTZ, kainic acid

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

AINEs – anti-inflamatórios não esteroidais

AMPA – ácido α-amino-3-hidróxi-5-metil-4-isoxazol propiônico

AMPc – adenosina monofosfato cíclico

ANOVA – análise de variância

ATP – trifosfato de adenosina

C – carbono

Ca^{2+} – íon cálcio

CA1 e CA3 – subregiões hipocampais CA1 e CA3

Cl^- – íon cloreto

COX – ciclooxigenase

DAMPs – padrões moleculares de reconhecimento de dano celular

DP – receptores para prostaglandina D_2

EEG – eletroencefalograma

EP – receptores para prostaglandina E_2

ERG-1 – proteína 1 de resposta de crescimento precoce

ERK – proteína quinase regulada por sinal extracelular

GABA – ácido γ-aminobutírico

GFAP – proteína glial fibrilar ácida

HMGB1 – *high mobility group Box* 1

i.c.v. – intracerebroventricular

IFN – interferon

IL – interleucina

i.p. – intraperitoneal

IP – receptors para prostaciclina

K^+ – íon potássio

KA – ácido caínico ou cainato

Kg – kilograma

LCR – líquido cefalorraquidiano

LOX – lipooxigenase

LPS – lipopolissacarídio

LT – leucotrieno

mg – miligrama

MMA – ácido metilmalônico ou metilmalonato

Na^+ – íon sódio

NFkB – fator de transcrição nuclear kappa B

NMDA – N-metil-D-aspartato

PAMPs – padrões moleculares de reconhecimento de patógenos

PEPS – potencial excitatório pós-sináptico

PG – prostaglandina

PGD_2 – prostaglandina D_2

PGDS – prostaglandina D_2 sintase

PGE_2 – prostaglandina E_2

PGES – prostaglandina E_2 sintase

$PGF_{2\alpha}$ – prostaglandina $F_{2\alpha}$

PGFS – prostaglandina $F_{2\alpha}$ sintase

PGG_2 – prostaglandina G_2

PGH_2 – prostaglandina H_2

PGHS – prostaglandina H_2 sintase

PGI_2 – prostaciclina

PGIS – prostaciclina sintase

PIPS – potencial inibitório pós-sináptico

PI3K – fosfatidil inositol 3-quinase

PKA – proteína quinase A

PKC – proteína quinase C

PLC – fosfolipase C

Poly I:C – ácido poliinosínico:policitidílico

PTZ – pentilenotetrazol

RNA – ácido ribonucléico

RNAm – RNA mensageiro

s.c. – subcutâneo

SE – *Status epilepticus*

SNC – sistema nervoso central

TLE – epilepsia do lobo temporal

TLR – receptores tipo *Toll*

TNF-α – fator de necrose tumoral α

TX - tromboxano

TXA_2 – tromboxano A_2

TXAS – tromboxano A_2 sintase

μL – microlitro

μM – micromolar

$[\ ]_i$ e $[\ ]_o$ – concentração intracelular e concentração extracelular

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1 INTRODUÇÃO

1 INTRODUÇÃO

1.1 Epilepsia

A epilepsia é um distúrbio cerebral crônico caracterizado pela ocorrência de crises epilépticas recorrentes (FISHER et al., 2005; DUNCAN et al., 2006). Por definição, crise epiléptica é a ocorrência de sinais e sintomas decorridos de uma hipersincronia neuronal cerebral. Por sua vez, convulsão é a manifestação motora da crise epiléptica (FISHER et al., 2005). A epilepsia é um dos transtornos neurológicos graves mais comuns, afetando mundialmente aproximadamente 50 milhões de pessoas. Aproximadamente 30% dos indivíduos epilépticos são refratários ao tratamento com os anticonvulsivantes atuais, apesar da ampla variedade de drogas antiepilépticas disponíveis (LÖSCHER & SCHMIDT, 2004; PERUCCA et al., 2007; CREMER et al., 2009).

As causas da epilepsia são diversas, e incluem mutações genéticas, defeitos em canais iônicos e/ou distúrbios metabólicos, entre outras (CREMER et al., 2009). As crises epilépticas podem ser (1) focais, com preservação da consciência do paciente; (2) parciais, com perda da consciência alterada; (3) generalizadas, com elevado ou total comprometimento dos hemisférios cerebrais de forma simultânea, simétrica e sincrônica; (4) não classificadas ou desconhecidas (ENGEL & PEDLEY, 2008; BERG et al., 2010; SHORVON, 2011). O *status epilepticus* (SE) é caracterizado como uma crise duradoura, e pode ser desencadeado por diversos fatores como trauma, infecção, hipóxia e febre (ENGEL & PEDLEY, 2008).

1.1.1 Crises epilépticas

Os neurônios são células únicas, pois sua função é modificar e transmitir mensagens de uma célula para outra e/ou entre as diferentes partes de uma mesma célula através do potencial de ação ou impulso nervoso (LEVITAN & KACZMAREK,

1997). Quando neurônios disparam de forma excessiva e sincronizada, dá-se a crise epiléptica. Desta forma, o controle do potencial de repouso dos neurônios é fundamental para a prevenção de convulsões (MACHADO, 2006). O glutamato e o ácido γ-aminobutírico (GABA) são os principais neurotransmissores, respectivamente, excitatório e inibitório do sistema nervoso central. Como tais, ambos neurotransmissores estão envolvidos na gênese e propagação de crises epilépticas (ENGEL & PEDLEY, 2008). A transmissão GABAérgica é mediada por dois subtipos de receptores: o receptor $GABA_A$, que consiste em um canal iônico pós-sináptico ativado por ligante, permeável a íons Cl^- e responsável por induzir um estado de hiperpolarização; e o $GABA_B$, receptor metabotrópico acoplado à proteína G, presente tanto na pré quanto na pós-sinapse, capaz de inativar os canais de Ca^{++} dependentes de voltagem, e abrir os canais de K^+ (MacNAMARA, 2006). Os receptores glutamatérgicos são classificados em receptores metabotrópicos (acoplados à proteína G) e receptores ionotrópicos (acoplados a canais iônicos). Estes últimos são capazes de conduzir tanto correntes de Na^+ quanto de Ca^{2+}. O receptor glutamatérgico ionotrópico do subtipo NMDA (que tem alta afinidade por N-metil-D-aspartato, o que lhe confere esta acronímia) conduz correntes de Ca^{2+}. O receptor AMPA (que tem alta afinidade por ácido α-amino-3-hidroxi-5-metil-4-isoxazol) conduz preferentemente Na^+, e a sua ativação (ENGEL Jr. & PEDLEY, 2008) produz uma corrente deste íon que se constitui no estímulo despolarizante inicial da membrana plasmática. Quando o potencial de membrana atinge o limiar para abertura dos canais de sódio dependentes de voltagem (em torno de -40 mV), ocorre influxo massivo de Na^+, que culmina com a despolarização da membrana (ENGEL Jr. & PEDLEY, 2008; KLEEN & HOLMES, 2008). Tanto a diminuição nos potenciais inibitórios pós-sinápticos (PIPS) quanto um aumento nos potenciais excitatórios pós-sinápticos (PEPS), seriam os prováveis mecanismos responsáveis pelo início de uma crise epiléptica (ENGEL Jr. & PEDLEY, 2008).

Células gliais também têm sido implicadas nos mecanismos de gênese e propagação das crises epilépticas, dado o papel fundamental destas células no controle dos níveis extracelulares de íons e neurotransmissores, particularmente de glutamato e GABA (DEVINSKY et al., 2013). Na homeostase, o glutamato é captado para o interior dos astrócitos ativados por meio de transportadores, e então convertido a glutamina pela enzima glutamina sintase. Um defeito na captação de glutamato pelos transportadores e/ou distúrbio da homeostase celular de potássio

podem contribuir para um estado de hiperexcitabilidade e, consequentemente, desencadear crises epilépticas (FRIEDMAN, 2011; DEVINSKY et al., 2013). Além disso, a ativação de astrócitos e micróglia, decorrente de processo inflamatório, pode resultar na produção de mediadores que provocam rompimento da barreira hematoencefálica, e consequente extravasamento de componentes plasmáticos que facilitam a despolarização, como o potássio e albumina (WETHERINGTON et al., 2008; FRIEDMAN, 2011).

1.1.2 Modelos experimentais de crises epilépticas

Apesar do aumento nas possibilidades de pesquisa não-invasiva no cérebro humano, através da neuroimagem, a utilização de modelos experimentais ainda é essencial para a pesquisa em epilepsia. Além da compreensão dos processos fisiopatológicos envolvidos nos diferentes tipos de manifestações epilépticas, os modelos experimentais são importantes para a identificação de novos fármacos com potencial antiepiléptico (VELISEK, 2006).

Para uma melhor compreensão dos modelos experimentais, estes são classificados em *in vitro* e *in vivo.* Principalmente através de técnicas de eletrofisiologia, os experimentos *in vitro* utilizam as técnicas de exposição a altas concentrações de K^+ (DUDEK et al., 1994), aplicação de antagonistas GABAérgicos, como Bicuculina (TASKER & DUDEK, 1991) e redução nas concentrações de Mg^{2+} (MODY et al., 1987). Já os modelos *in vivo* utilizados para o estudo da epilepsia e de crises epilépticas se classificam em agudos ou crônicos. Através do estudo das crises agudas pode-se elucidar os processos celulares e moleculares potencialmente importantes nas crises. Já os modelos crônicos, permitem investigar alterações persistentes relacionadas ao fenômeno epileptogênico que se apresentam durante o período interictal (ENGEL & SCHWARTZKROIN, 2006).

Dentre os vários modelos experimentais, destacam-se os modelos genéticos determinados por deleção ou alteração disfuncional de genes selecionados; modelos de estimulação elétrica, como o abrasamento e o eletrochoque máximo; e modelos farmacológicos, como PTZ, KA (que pode ser utilizado como crônico ou agudo), pilocarpina, penicilina, 4-aminopiridina, toxina colérica, bicuculina, picrotoxina, entre

outros (CREMER et al., 2009). Neste estudo optou-se pela utilização de dois modelos farmacológicos agudos de crises epilépticas: PTZ e KA.

1.1.2.1 Pentilenotetrazol (PTZ)

Considerado de alto valor preditivo na detecção de anticonvulsivantes eficazes na clínica (VELISEK, 2006), o modelo de indução de crises epilépticas pela administração de PTZ é utilizado tanto para o desenvolvimento de novos agentes anticonvulsivantes como para o entendimento dos mecanismos moleculares envolvidos no SE (ENGEL, 2001; VELISEK, 2006).

O PTZ, originalmente um cardioestimulante, é um antagonista do receptor $GABA_A$ que, ligando-se aos sítios de reconhecimento da picrotoxina e dos benzodiazepínicos, inibe as correntes de cloreto associadas a este canal. Nestas condições, há uma redução dos efeitos endógenos do GABA, o que resulta em um estado de hiperexcitabilidade do SNC (VELISEK, 2006; CREMER et al., 2009). A injeção de PTZ induz quatro fenômenos comportamentais: *reação de congelamento*, espasmos mioclônicos, convulsões clônicas e, convulsões tônico-clônicas generalizadas (VELISEK, 2006), que variam de acordo com o modelo animal e a dose administrada (VELISEK, 2006; CREMER et al., 2009). As doses usualmente administradas em modelos animais de experimentação variam entre 10 a 110 mg/Kg, dependendo do objetivo do estudo (CREMER et al., 2009). As principais vantagens do uso do PTZ são o curto período de latência para a primeira convulsão tônico-clônica generalizada, e a degeneração neuronal mínima (ENGEL & PEDLEY, 2008; CREMER et al., 2009).

1.1.2.2 Ácido caínico (KA)

O ácido caínico é uma neurotoxina (McGEER et al., 1978) análoga ao glutamato (Figura 2) que se liga preferencialmente aos receptores glutamatérgicos ionotrópicos do subtipo cainato (COYLE, 1987). Esta neurotoxina tem sido

comumente utilizada para produzir SE e alterações neuropatológicas associadas (VELISEK, 2006). A injeção de KA induz a uma crise inicial (COYLE, 1983) que dura horas, seguida por um período de 3 a 4 semanas sem episódios convulsivos (período silencioso). Após este período, ocorre o desenvolvimento de crises espontâneas focais e recorrentes e, concomitantemente, neurodegeneração, principalmente em áreas específicas do hipocampo, como as regiões CA1 e CA3, e estriado (SPERK, 1994; McCORD et al., 2008), que são similares àquelas observadas na epilepsia do lobo temporal em humanos (ENGEL, 2001; BRANDT et al., 2003). Por esta razão, este modelo tem sido considerado um modelo experimental importante para o estudo de crises agudas e como de um modelo de epileptogênese (crônico) (ENGEL, 2001).

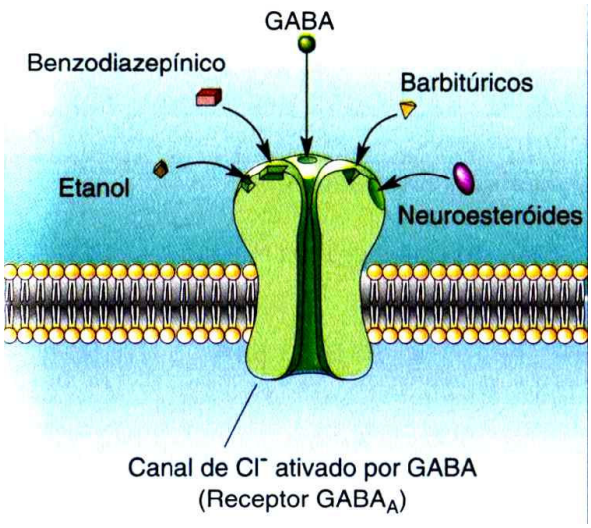

Figura 1 – Receptor GABA$_A$. Alvo de ligação do PTZ na pós-sinapse. Modificado de VELISEK, 2006.

As crises epilépticas induzidas por KA normalmente ocorrem em até 60 minutos após sua administração, e devem ser visualizadas no eletroencefalograma (EEG). A necessidade de acompanhamento por EEG se justifica pelo fato de que há uma correlação fraca entre o início da atividade ictal e o aparecimento de manifestações motoras induzidas por KA. Além disso, a atividade ictal pode permanecer por horas após o término das manifestações motoras associadas à crise

(GIORGI et al., 2005). Doses de KA são específicas para cada espécie e dependentes da idade do animal experimental. Normalmente, crises epilépticas são induzidas em ratos adultos com doses entre 10 a 14 mg/Kg. Porém, para ratos imaturos, doses entre 1 a 4 mg/Kg podem ser suficientes. Camundongos requerem doses consideravelmente mais elevadas, aproximadamente 20 a 60 mg/Kg (VELISEK, 2006).

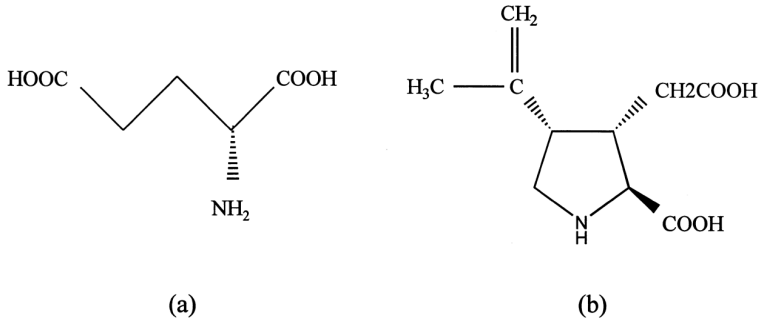

Figura 2 – Analogia estrutural entre glutamato e ácido caínico. Ácido caínico (b) promove um potencial excitatório 30 a 100 vezes maior que o glutamato (a) (FAROOQUI et al., 2001).

1.1.3 Epilepsia e neuroinflamação

Dado o elevado número de pacientes com epilepsia que apresentam convulsões refratárias às terapias disponíveis, torna-se importante a busca por novos fármacos anticonvulsivantes. Para isto, é fundamental o entendimento dos mecanismos de indução e manutenção de convulsões (PERUCCA et al. 2007). Principalmente nos últimos 10 anos, evidências clínicas e experimentais sugerem que o processo inflamatório no cérebro poderia constituir um mecanismo importante na fisiopatologia de crises epilépticas e epilepsia, podendo ser tanto causa quanto consequência das mesmas (VEZZANI et al., 2011). Neste contexto, esforços tem sido dedicados para elucidar as relações entre a ocorrência de crises epilépticas e o processo inflamatório no sistema nervoso central (SNC).

A presença de mediadores inflamatórios em tecido cerebral (*post mortem*) de pacientes com epilepsias refratárias, como a epilepsia do lobo temporal (TLE) e a

displasia cortical focal é um achado recorrente (CHOI et al., 2009; RIAZI et al., 2010). Experimentalmente, tem sido mostrado que crises epilépticas *per se* podem induzir neuroinflamação, e que crises recorrentes perpetuam a inflamação (VEZZANI et al., 2012). Além disso, através da utilização de modelos experimentais de infecção, sistêmica ou do SNC, tem sido sugerido que a neuroinflamação pré-existente aumenta a predisposição a crises epilépticas (revisar em VEZZANI et al., 2012). Tais achados têm sido associados a alterações na excitabilidade neuronal e ao aumento das alterações neuropatológicas induzida pelas crises, como morte neuronal, perda da plasticidade sináptica e redução da neurogênese (VEZZANI et al., 2011; VEZZANI & FRIEDMAN, 2011).

Estudos epidemiológicos mostraram que até 80% dos pacientes com malária apresentam convulsões durante a fase aguda da inflamação (SINGH & PRABHAKAR, 2008). Além disso, pacientes com encefalite apresentam convulsões que podem persistir mesmo após eliminação do agente infeccioso, e são atenuadas por anti-inflamatórios (SINGH & PRABHAKAR, 2008).

A reação inflamatória no SNC se inicia com a ativação dos sistemas imunológicos inato e adaptativo. Mediadores inflamatórios são produzidos durante a atividade epiléptica pela microglia, astrócitos no cérebro de roedores, como parte do mecanismo imunológico inato (ROTHWELL & LUHESHI, 2000). Moléculas pró-inflamatórias, astrócitos reativos, microglia ativada e outros indicadores de inflamação têm sido encontrados em hipocampo de pacientes com TLE, *post mortem* (CRESPEL et al., 2002; ARONICA et al., 2007; VAN GASSEN et al., 2008). Em particular, citocinas como a interleucina (IL)-1β, o fator de necrose tumoral (TNF)-α e a IL-6 são rapidamente expressos após convulsões, bem como após isquemia e trauma (VEZZANI, 2005). Em contrapartida, estes mediadores inflamatórios não foram encontrados em tecidos obtidos de pacientes saudáveis (revisar em VEZZANI et al., 2011; 2012). Assim, citocinas e mediadores inflamatórios também atuam como neuromoduladores no SNC, afetando o limiar de excitabilidade neuronal, além de participarem na ativação da imunidade inata e adaptiva periférica (VEZZANI et al., 2012). Clinicamente foi evidenciado que doenças autoimunes como lúpus eritematoso sistêmico, vasculite, esclerose múltipla e síndromes paraneoplásticas podem causar convulsões recorrentes e, consequentemente, epilepsia (NAJJAR et al., 2008).

Estudos bioquímicos têm mostrado que a IL-1β pode facilitar a neurotransmissão glutamatérgica, por diminuir a recaptação de glutamato pelos astrócitos, aumentando os níveis extracelulares de glutamato, que promove a ativação dos receptores NMDA e consequentemente o influxo de Ca^{2+} (VEZZANI, 2008). Além disso, a IL-1β também diminui as correntes mediadas por GABA em culturas de neurônios hipocampais (WANG et al., 2000) aumentando assim a excitabilidade no SNC. Já, o efeito do TNF-α sobre as convulsões depende dos níveis desta citocina no cérebro e dos subtipos de receptores ativados. A administração i.c.v. de TNF-α, em baixas doses, reduz as convulsões por interagir com o receptor TNF-α p75 (BALOSSO et al., 2005). Uma direta interação entre TNF-α e receptor AMPA foi demonstrada em neurônios hipocampais. O TNF-α, por meio dos receptores p55, aumenta a disponibilidade de receptores AMPA na membrana celular, mas reduz a densidade de receptores $GABA_A$ (STELLWAGEN, 2005), amplificando as respostas glutamatérgicas (VEZZANI, 2008). A superexpressão de TNF-α e IL-6 no cérebro de camundongos foi associada com a ocorrência de mudanças neurodegenerativas dependentes da idade e presença de convulsões espontâneas esporádicas (CAMPBELL et al., 1993; AKASSOGLOU et al., 1997). Ainda, camundongos transgênicos que superexpressam IL-6 nos astrócitos apresentam um aumento na sensibilidade às crises induzidas por agonistas glutamatérgicos (SAMLAND et al. 2003) e crises espontâneas (CAMPBELL et al., 1993).

A injeção sistêmica de lipopolissacarídio (LPS) induz inflamação periférica e central em ratos e camundongos e, consequentemente, reduz o limiar da indução de convulsão (SAYYAH et al., 2005). Este efeito pode ser bloqueado pela administração de anti-inflamatórios não esteroidais (AINEs) (SAYYAH et al., 2005), e se dá através da ativação, pelo LPS, dos receptores *Toll-like* 4 (TLR4). O receptor TLR4 é um membro da família de receptores TLR, conhecida pelas suas ações na resposta imune inata e a capacidade de reconhecer padrões moleculares de patógenos (PAMPs) e de dano celular (DAMPs) (revisar em MOYNAGH, 2005; O'NEILL, 2008; LEHNARDT, 2010). Assim como o LPS, uma proteína chamada HMGB1 (*high mobility group box* 1), se liga aos receptores TLR4 e induz inflamação. Porém, a HMGB1 é uma proteína liberada pelos neurônios quando danificados e/ou estressados. Tem sido sugerido que a HMGB1 liberada pelos neurônios interage com os TLR4, diminuindo o limiar para crises epilépticas, que, por sua vez,

promovem uma onda adicional de liberação de HMGB1 dos astrócitos e micróglia ativados, levando a um ciclo de *feedback* positivo destas crises e do processo inflamatório (MAROSO et al. 2010). Sendo assim, esta nova via tem sido proposta como um mecanismo subjacente crucial para a ocorrência de crises epilépticas recorrentes (MAROSO et al. 2010).

Os efeitos da HMGB1 e da IL-1β são aparentemente semelhantes, principalmente por ambos serem bloqueados por ifenprodil (VIVIANI et al., 2003; RAVIZZA et al., 2008; MAROSO et al., 2010), um antagonista seletivo dos receptores NMDA que contém a subunidade NR2B (YU et al., 1997). Por sua vez, este receptor também tem sido relacionado com a atividade epiléptica induzida por inflamação (VIVIANI et al., 2003; BALOSSO et al., 2008).

Outro membro da família de receptores TLR que tem sido implicado na indução/facilitação de atividade ictal é o TLR3 (GALIC et al., 2009; COSTELLO & LYNCH, 2013). O TLR3 reconhece RNA de cadeia dupla (um produto da replicação viral) e regula as respostas a diversos vírus através da produção de interferons (IFN) do tipo I (IFNα e IFNβ) (revisar em COSTELLO & LYNCH, 2013). Enquanto a ativação do TLR4 mimetiza uma infecção bacteriana (SAYYAH et al., 2005), o poly I:C, um agonista sintético TLR3, ao se ligar no respectivo receptor, mimetiza os efeitos de uma infecção viral sistêmica (CUNNIGHAM et al., 2007) elevando os níveis de IFNα e IFNβ. Estudos *in vitro* sugerem que estes IFNs aumentam a excitabilidade de neurônios hipocampais (MULLER et al., 1993) e corticais (BEYER et al., 2009). Além disso, foi verificado que a ativação dos TLR3, pela administração i.c.v. de poly I:C, aumenta a susceptibilidade a crises epilépticas (GALIC et al., 2009). Embora a injeção sistêmica de poly I:C não altere o limiar de indução do potencial de ação em fatias de hipocampo de camundongos, ele induz atividade interictal espontânea e sustentada nestas preparações, mediada por IFNβ (COSTELLO & LYNCH, 2013).

Além da produção de IFNs, a ativação do TLR3 leva à produção de TNFα e IL-6, dentre outras citocinas pró-inflamatórias, pela micróglia (LI et al., 2011). Esta produção de TNFα and IL-6 pode ser suprimida pelo bloqueio não seletivo da COX-2, e ainda, pelo bloqueio ou ausência do receptor para prostaglandina (PG) E_2 subtipo 1 (EP1) (LI et al., 2011), sugerindo a importância da manutenção da via das PGs para a produção de citocinas a partir da micróglia após a ativação da imunidade inata.

Em contrapartida, associada à estimulação dos receptores TLR, há a ativação e o aumento da expressão da ciclooxigenase (COX)-2 por S-nitrosilação (TIAN et al., 2008), devido ao aumento na produção de óxido nítrico pela estimulação dos receptores NMDA (BARAÑANO et al., 2001), como pode ser visualizado na Figura 3.

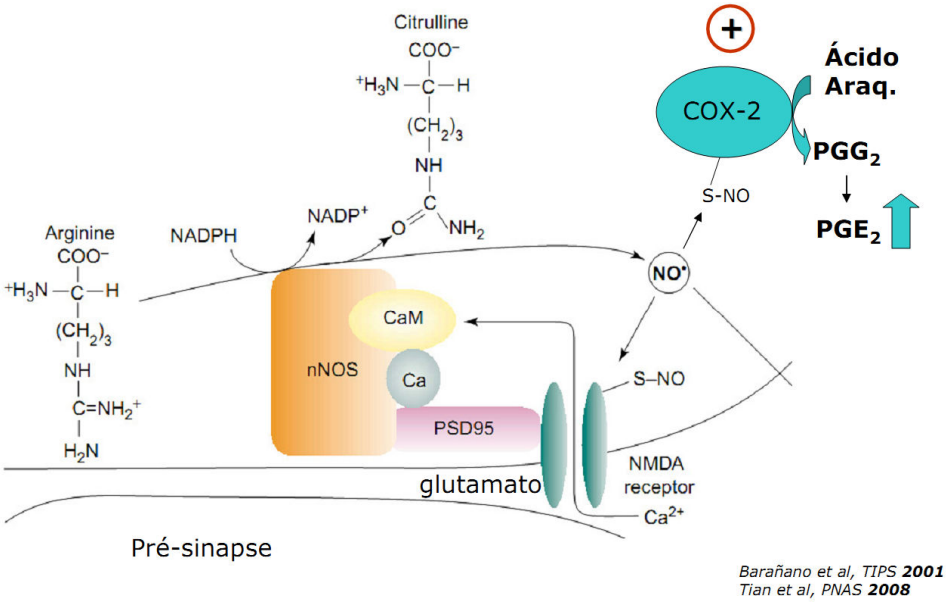

Figura 3 – Ativação e aumento da expressão da COX-2, por S-nitrosilação, devido ao aumento na produção de óxido nítrico pela estimulação dos receptores NMDA (adaptado de: BARAÑANO et al., 2001; TIAN et al., 2008).

Com a indução da COX-2 ocorre a formação de prostaglandinas e, a super-regulação dos componentes do sistema do complemento na microglia, astrócitos e neurônios. Embora a relação de muitos mediadores inflamatórios permaneça inconclusiva, é clara a evidência da influência direta da IL-1β, do TNF-α, da IL-6, dos INFs tipo I, e da PGE_2 na geração e exacerbação das convulsões (OLIVEIRA et al. 2008a; OLIVEIRA et al. 2008b; VEZZANI et al., 2012; COSTELLO & LYNCH, 2013).

A COX-2 é constitutivamente expressa, em nível baixo a moderado, nos neurônios hipocampais, sendo regulada (KAUFMANN et al., 1996) e regulando (COLE-EDWARDS & BAZAN, 2005) a atividade sináptica via PGE_2. Esta enzima é marcadamente induzida nos neurônios em aproximadamente uma hora após uma convulsão (MARCHESELLI & BAZAN, 1996), através dos receptores NMDA

(YAMAGATA et al., 1993). Foi demonstrado que uma redução quantitativa de PGE_2 endógena, pela inibição seletiva da COX-2, diminui significativamente a excitabilidade neuronal, verificada em fatias da região hipocampal CA1 (CHEN & BAZAN, 2005). Neste contexto, foi verificado que a administração de um análogo da PGE_2 reverte a neuroproteção exercida pela inibição da COX-2 na excitotoxicidade induzida por NMDA *in vitro* e *in vivo* (CARLSON et al., 2003; MANABE et al., 2004). Além disso, a aplicação de PGE_2, mas não de PGD_2 ou $PGF_{2\alpha}$, aumenta a frequência de disparos e a amplitude dos potenciais excitatórios pós-sinápticos (PEPS) em fatias de hipocampo de ratos incubadas com um inibidor seletivo da COX-2, possivelmente pela redução das correntes de potássio (CHEN & BAZAN, 2005). Mais evidências de que a PGE_2 pode ser importante para o controle da excitabilidade no SNC vêm dos estudos que mostraram que a administração i.c.v. de anticorpos monoclonais anti-PGE_2 atenua as convulsões induzidas por PTZ (OLIVEIRA et al., 2008a) e, ainda, que a injeção i.c.v. de PGE_2 facilita o aparecimento das convulsões induzidas por este agente convulsivante (OLIVEIRA et al., 2008a), bem como por ácido metilmalônico (SALVADORI et al., 2012).

Interessantemente, foi demonstrado que os níveis hipocampais de PGE_2 estão aumentados durante as convulsões induzidas por PTZ e KA (BERCHTOLD-KANZ et al., 1981; BARAN et al., 1987), e que inibidores da COX-2 facilitam as convulsões induzidas por esse agente convulsivante (BAIK et al., 1999; KUNZ & OLIW, 2001; GOBBO e O'MARA, 2004), sugerindo um papel inibitório para a PGE_2.

Estes resultados aparentemente contraditórios sugerem que o papel da via COX-2/PGE_2 em diferentes tipos de convulsões é complexo, e pode estar relacionado à contribuição diferencial de cada subtipo de receptor EP para as convulsões induzidas por PTZ ou KA ou, alternativamente, que a inibição da COX-2 pode induzir a produção de agentes pró-convulsivantes por outras vias que utilizam o ácido araquidônico como substrato, como a via das lipoxigenases (LOX). Considerando que a PGE_2 é uma das principais prostaglandinas produzidas no cérebro, via COX-2 (VIDENSKY et al., 2003; SANG et al., 2005), e sua importância na modulação da neuroexcitabilidade (CHEN et al., 2002; CHEN & BAZAN, 2005; COLE-EDWARDS & BAZAN, 2005; VEZZANI et al., 2013), torna-se importante estudar o envolvimento de cada subtipo de receptor EP nas convulsões induzidas por diversos agentes, visto que diferentes ligantes de receptores EP podem ser úteis para tipos específicos de convulsões.

1.2 Prostaglandina E_2

Eicosanoides são substâncias, com 20 átomos de C (do grego, "*eicosi*" significa "vinte") formadas a partir de ácidos graxos poli-insaturados de cadeia longa (BERGSTRÖEM et al., 1964), como por exemplo o ácido araquidônico. Os eicosanoides são considerados autacoides, ou seja, substâncias que são geradas e agem localmente, com meia-vida curta (segundos). Através de estímulos mecânicos, físicos e/ou produzidos por diversos mediadores, o ácido araquidônico é liberado dos fosfolipídios de membrana por ação de acil-hidrolases, particularmente da fosfolipase A_2 citosólica (PLA_{2c}) (NEEDLEMAN et al., 1986; AKIBA & SATO, 2004). O ácido araquidônico livre, liberado pela ação da PLA_2, pode ser utilizado pelas enzimas COX e/ou LOX para produzir prostanoides (prostaglandinas – PG e tromboxanos – TX) e leucotrienos (LT), respectivamente (YEDGAR et al., 2000). O ácido araquidônico pode dar origem a cinco prostanoides bioativos pela via da COX-2 *in vivo*: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 e TXA_2 (Figura 4) (SMITH, 1992; BREYER et al., 2001).

A COX, também chamada de prostaglandina H_2 sintase (PGHS), foi purificada em 1976 a partir de vesículas seminais de ovelha (HEMLER, 1976; MIYAMOTO, 1976), e é considerada limitante da velocidade na rota de biossíntese de PG e TX. Duas isoformas de COX foram identificadas em mamíferos: COX-1 e COX-2 (SIMMONS et al., 2004). As PG produzidas via COX-1 são usualmente relacionadas à homeostase fisiológica incluindo a manutenção do tônus da musculatura lisa, agregação plaquetária, e proteção da mucosa gástrica, devido a esta isoforma ser constitutiva em muitos tecidos (SMITH et al., 2000; SIMMONS et al., 2004). Já a COX-2 é induzida na maioria dos tecidos por citocinas, fatores tumorais e fatores de crescimento (CAO et al., 1996), contribuindo para o reparo da lesão e cronificação (SMITH et al., 2000; SIMMONS et al., 2004). Porém, esta isoforma pode ser encontrada também na forma constitutiva, sendo expressa no fígado, estômago e cérebro (HOFFMANN, 2000). Uma variante de *splicing* da COX-1, que retém o íntron 1 do seu RNAm, e é expressa principalmente no SNC (MITCHELL & WARNER, 2006) também foi identificada. Contudo, ainda há controvérsias a respeito desta isoforma ser cataliticamente ativa e sobre suas funções no organismo (DAVIES et al., 2004; SIMMONS et al., 2004; KIS et al., 2005).

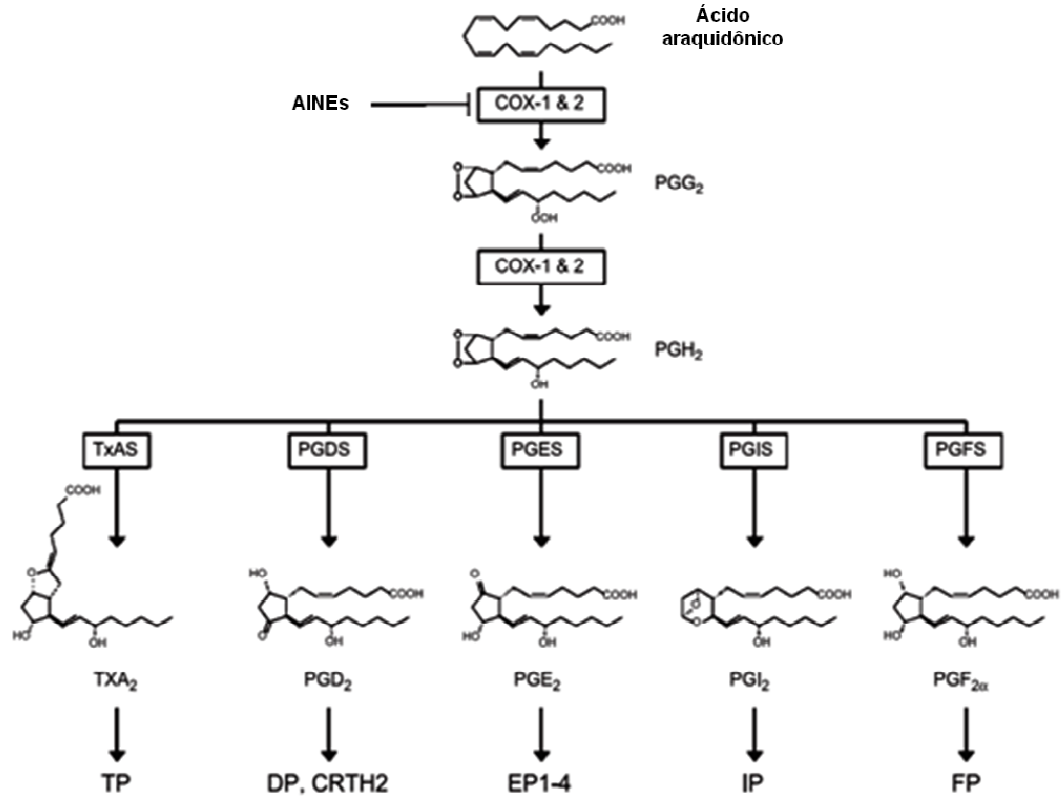

Figura 4 – Biossíntese das prostaglandinas. Ácido araquidônico é metabolizado, pelas COX-1 ou 2, em PGH_2, um endoperóxido instável. Este é o precurssor comum dos cinco principais prostanoides: PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 e TXA_2, que são produzidos a partir de enzimas prostaglandina sintase específicas (PGES, PGFS, PGDS, PGIS e TXAS, respectivamente). Estas PG e TX formados agem pela ativação de receptores metabotrópicos de membrana (HATA & BREYER, 2004).

A COX-2, isoforma induzível, também é expressa de maneira constitutiva em alguns tecidos, como o encéfalo (SIMMONS et al., 2004). Há evidências do envolvimento da COX-2 na regulação da temperatura corporal (IVANOV & ROMANOVSKY, 2004), controle do ciclo sono/vigília (HAYAISHI & MATSUMURA, 1995), aprendizado e memória (TEATHER et al., 2002), isquemia (COLLACO-MORAES et al., 1996), trauma crânio-encefálico (DASH et al., 2000) e epilepsia (VEZZANI & GRANATA, 2005). No SNC, esta enzima é encontrada principalmente no córtex, hipocampo, amígdala (YAMAGATA et al., 1993), hipotálamo, neurônios, mas também em células não-neuronais, como astrócitos, células da micróglia, células da meninge e do plexo coroide (VANE et al., 1998; TOMIMOTO et al., 2000). Quantitativamente, a COX-2 é mais abundante em neurônios glutamatérgicos do

hipocampo e córtex cerebral, e nestas células esta enzima está localizada nos espinhos dendríticos, onde ocorre a transmissão sináptica (KAUFMANN et al., 1996). A COX-2 também pode usar endocanabinoides como anandamida e 2-araquidonil-glicerol como substratos. Tem sido proposto a COX-2 determina a inativação destes lipídios neuroativos, limitando suas ações fisiológicas (KIM & ALGER, 2004; SLANINA & SCHWEITZER, 2005a; SLANINA et al., 2005b).

Uma das principais prostaglandinas produzidas no cérebro por esta via é a PGE_2 (VIDENSKY et al., 2003; SANG et al., 2005), isto porque a COX-2 está metabolicamente acoplada à PGE_2 sintase (BOSETTI et al., 2004; UENO et al., 2005). Esta PG exerce importantes funções no processo inflamatório, como a regulação da expressão de citocinas pró-inflamatórias em vários tipos celulares (HINSON et al., 1996; FIEBICH et al., 1997; WILLIAMS & SHACTER, 1997). A PGE_2 exerce suas ações por interagir com receptores prostanoides (EP) metabotrópicos específicos (COLEMAN et al., 1994; USHIKUBI et al., 1998; NARUMIYA et al., 1999; SUGIMOTO & NARUMIYA, 2007). Além disso, sabe-se que essa PG modula, via receptores EP, a atividade da enzima Na^+,K^+-ATPase no hipocampo de ratos (OLIVEIRA et al., 2009).

1.2.1 Receptores EP para PGE_2

A diversidade de ações biológicas da PGE_2 em diferentes tecidos decorre da sua ligação a receptores específicos acoplados à proteína G. Estes receptores, responsáveis por diferentes vias de sinalização e são divididos em quatro subtipos: EP1, EP2, EP3 e EP4 (TSUBOI et al., 2002; SUGIMOTO & NARUMIYA, 2007).

1.2.1.1 Estrutura e localização no SNC

Através da clonagem dos receptores EP foi predita a presença de 405, 357, 366 e 488 resíduos de aminoácidos, em ratos (BOIE et al.,1997) e, 405, 362, 266 e 513 resíduos em camundongos, para EP1, EP2, EP3 e EP4, respectivamente. O

receptor EP1 de ratos apresenta aproximadamente 96% de homologia com o receptor de camundongos e 83% de homologia com o receptor humano (FUNK et al., 1993). Porém, a semelhança entre as sequências primárias de aminoácidos dos receptores EP é limitada (NARUMIYA et al., 1999; SUGIMOTO & NARUMIYA, 2007). O receptor EP4 apresenta sequência mais longa na cadeia carboxiterminal quando comparado com o receptor EP2, e contém 38 resíduos de serina e de treonina, que atuam como múltiplos sítios de fosforilação enquanto o receptor EP2 apresenta uma pequena região carboxiterminal. Isto sugere que o receptor EP4 pode ser um alvo para fosforilação dependente de agonistas bem como dessensibilização, enquanto que o receptor EP2 é insensível a esses efeitos. De fato, Nishigaki e colaboradores (1996) demonstraram que o receptor EP4, mas não o receptor EP2, sofre rápida dessensibilização após estimulação por agonista (BASTEPE & ASHBY, 1999).

Estruturalmente, o receptor EP4 possui a terceira alça intracelular relativamente longa, bem como o domínio carboxiterminal mais longo em relação aos demais receptores EP. O receptor EP1 também possui uma terceira alça intracelular longa, enquanto que os receptores EP2 e EP3 possuem uma estrutura mais compacta. O receptor EP3 possui múltiplas variantes, porém com semelhante afinidade pela PGE_2, geradas por *splicing* alternativo do RNA da porção carboxiterminal. Estas variantes podem apresentar diferenças na fosforilação e dessensibilização do receptor e, variações nas vias de transdução de sinais. Com isto, cada isoforma do receptor EP3 ativa vias de sinalização diferentes, embora todas sejam ativadas pela PGE_2. Em humanos, ao menos oito variantes de *splicing* já foram identificadas e múltiplas variantes de *splicing* existem em outras espécies como camundongos, coelho e vacas (BREYER et al., 2001). Em camundongos, as três isoformas (α, β e γ) apresentam afinidades aos agonistas idênticas, mas são funcionalmente diferentes na eficiência de ativação da proteína G (SUGIMOTO et al.,1994) e, na susceptibilidade à dessensibilização induzida por agonistas (NEGISHI et al., 1993). Por exemplo, as isoformas EP3α e EP3β se acoplam à proteína G_i, mas a EP3β necessita de concentrações mais elevadas de agonista para a ativação da proteína G do que a EP3α. Além disso, a isoforma EP3α, mas não a EP3β, sofre dessensibilização induzida por agonista (ICHIKAWA et al., 1996).

A semelhança encontrada entre o receptor EP1 e os receptores EP2, EP3 e EP4 é apenas de 30, 33 e 28 %, respectivamente. Mesmo os receptores EP2 e EP4,

que estão acoplados a um mesmo tipo de proteína G, possuem somente 31% de identidade na sequência primária. De fato, o receptor EP2 é mais parecido com os receptores para prostaciclina (IP) e PGD_2 do subtipo 1 (DP1) do que com os outros receptores EP, considerando-se a sequência primária de aminoácidos. A baixa identidade encontrada entre os receptores EP é provavelmente devida à relação filogenética destes receptores (KATOH et al., 1996).

Os receptores EP1-4 são encontrados em diversos tecidos de mamíferos, incluindo o cérebro, podendo ser expressos em neurônios sensoriais (SOUTHALL & VASKO, 2001; NARUMIYA, 2009). Principalmente o receptor EP3 é amplamente distribuído em neurônios do córtex, hipocampo, tálamo e hipotálamo (SUGIMOTO et al., 1994; NAKAMURA et al., 2000). Adicionalmente, os receptores EP1-4 são expressos no hipocampo, uma área cerebral importante para vários processos fisiológicos e patológicos. Zhu et al. (2005) analisaram o padrão de expressão dos receptores EP no hipocampo e sua modulação por alterações na atividade neuronal através de técnicas como RT-PCR, western blotting e immunohistoquímica. Foi mostrado que todos os subtipos de receptores EP são expressos no hipocampo, tanto em neurônios quanto em astrócitos, visto que a imunoreatividade para os diferentes receptores EP está co-localizada com a imunoreatividade do marcador neuronal NeuN e do marcador astrocitário GFAP (ZHU et al., 2005). Já na micróglia, foi verificada a presença somente dos subtipos EP1 e EP2 (CAGGIANO & KRAIG, 1999). Quantitativamente, os receptores EP2 e EP3 são os subtipos mais abundantes no hipocampo, seguido pelos receptores EP1 e EP4. Apesar destas diferenças, tem sido sugerido que todos os receptores EP estão presentes em quantidades suficientes para modular a transmissão sináptica (ZHU et al., 2005).

Dentre os quatro receptores EP, a PGE_2 possui maior afinidade com os receptores EP3 e EP4 (constante de dissociação (K_d) < 1 nM), em relação ao EP1 e ao EP2 (K_d > 10 nM) (ABRAMOVITZ et al., 2000). Porém, há uma diferença significativa de afinidade dos receptores EP e seus ligantes entre as espécies. Por exemplo, o receptor EP1 humano liga o SC-19220 e o AH-6809, antagonistas clássicos EP1, com um K_i de 4,5 μM e 333 μM, respectivamente. Enquanto os mesmos antagonistas não apresentam afinidade pelo receptor EP1 de camundongo (revisado por NARUMIYA et al., 1999; TSUBOI et al. 2002).

1.2.1.2 Vias de sinalização

Como pode ser visualizado na Figura 5, o receptor EP1 está acoplado à proteína heterodimérica $G_{q/11}$ e, medeia uma elevação da concentração intracelular de Ca^{2+}. Esta ação deve-se à ativação da fosfolipase C (PLC), a qual, por sua vez leva à produção de IP_3 e diacilglicerol, e consequente mobilização de Ca^{2+} no citosol com a ativação a proteína quinase C (PKC) dependente de Ca^{2+} (HEBERT et al., 1990; NARUMYIA, 2009; KAWABATA, 2011).

O receptor EP2 é farmacologicamente definido por ser sensível ao butaprost. Este receptor está acoplado a uma proteína G_s, levando a ativação da adenilil ciclase e a consequente produção do segundo mensageiro AMPc, seguido pela ativação da proteína quinase A (PKA) (SUGIMOTO & NARUMIYA, 2007; KAWABATA, 2011).

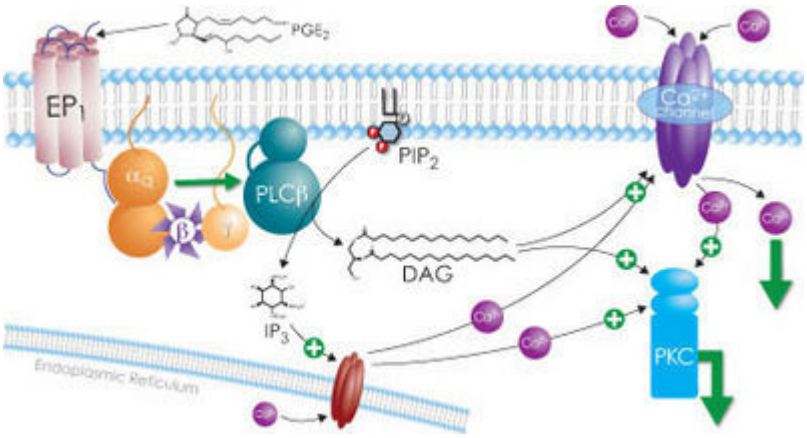

Figura 5 – Vias de sinalização do receptor EP1. A ativação do receptor EP1 leva a um aumento na concentração de Ca^{++} intracelular e ativação da PKC. (fonte: www.caymanchem.com/app/ template/ Article/2144/ figure/1).

Já os receptores EP3, que apresentam diferentes isoformas (α, β e γ, no caso de camundongos), divergem quanto à fosforilação e dessensibilização do receptor, e as vias de transdução de sinal. As isoformas EP3α e EP3β, por exemplo, são acoplados a uma proteína G_i, e ativação destes receptores inibe a adenilil ciclase,

inibindo a geração de AMPc (NARUMIYA et al., 1999; SUGIMOTO & NARUMIYA, 2007). Em contrapartida, EP3γ tem ambos efeitos, estimulatório e inibitório, sobre a adenilil ciclase via ativação de proteínas G_s e G_i, respectivamente (SUGIMOTO & NARUMIYA, 2007; TOBER et al., 2007). Apesar das isoformas geralmente inibirem a geração de AMPc via ativação da proteína G_i, existem evidências de que outras vias de sinalização como G_s, G_{13} e G_q possam ser ativadas pelo receptor EP3 (HATA & BREYER, 2004). As diferenças na transdução de sinal das isoformas do receptor EP3 podem também resultar de diferenças na dessensibilização dos receptores induzidas pelo agonista. Embora a importância funcional das diferentes isoformas pode ser claramente observada, sua significância fisiológica individual permanece incerta (KAWABATA, 2011).

Assim como no caso do receptor EP2, a via de sinalização do receptor EP4 está relacionada com o aumento dos níveis de AMPc (REGAN et al., 1994). Apesar de ambos os receptores EP2 e EP4 serem acoplados à proteína G_s resultando em estimulação da adenilil ciclase e aumento dos níveis de AMPc (HONDA et al., 1993; REGAN et al., 1994), o receptor EP4 se acopla mais fracamente do que o receptor EP2 (FUJINO et al., 2002). A estimulação dos receptores EP4, mas não dos receptores EP2, pela PGE_2 leva à fosforilação da ERK 1/2 (proteína quinase regulada por sinal extracelular), através de mecanismo dependente de PI3K (FUJINO et al., 2003). Além disso, esta ativação da sinalização PI3K/ERK pelos receptores EP4 induz a expressão funcional da proteína 1 de resposta de crescimento precoce (ERG-1). Sob as mesmas condições, a indução da ERG-1 não foi observada após a ligação da PGE_2 nos receptores EP2. Estas diferenças de sinalização entre os receptores EP2 e EP4 são significantes em relação ao potencial envolvimento dos receptores EP4 em inflamação e câncer (FUJINO et al., 2003).

Estas diferenças funcionais sugerem a importância única de cada receptor EP. Assim, estes podem ser importantes alvos para o tratamento de diversas situações patológicas, potencialmente com menos efeitos adversos que a inibição da COX (ZEILHOFER & BRUNE, 2006). Devido a isso, agentes farmacológicos seletivos para os diferentes receptores EP tem sido amplamente utilizados para investigar os efeitos da PGE_2. Uma série de novos ligantes EP tem sido desenvolvida incluindo os agonistas ONO-DI-004, ONO-AE1-259, ONO-AE-248, e ONO-AE1-329 (EP1, EP2, EP3, e EP4, respectivamente) e antagonistas ONO-8713, ONO-AE3-237, ONO-AE3-240, e ONO-AE-208 (EP1, EP2, EP3 e EP4,

respectivamente) (SUZAWA et al., 2000; WATANABE et al., 2000; KABASHIMA et al., 2002).

Neste contexto, tem sido mostrado que a manipulação farmacológica ou genética da função dos receptores EP oferece neuroproteção em modelos animais de doenças neurodegenerativas. De fato, a administração sistêmica de antagonistas de receptores EP1 e EP3 diminui a extensão da lesão cerebral induzida por isquemia (AHMAD et al., 2007; KAWANO et al., 2006). Camundongos transgênicos sem o receptor EP2 apresentam menor índice de morte neuronal após injeção i.c.v. de LPS (SHIE et al., 2005), reforçando a ideia que os receptores EP podem ser um alvo importante para o tratamento de doenças neurodegenerativas. Além disso, foi verificado que a administração i.c.v. de antagonistas de receptores EP1(SC-19220), EP3 (L-826266) e EP4 (L-161982) e um agonista EP2 (butaprost) atenuam as convulsões induzidas por PTZ (OLIVEIRA et al., 2008b).

1.3 Envolvimento da Na^+,K^+-ATPase nas crises epilépticas

Estruturalmente, a Na^+,K^+-ATPase (E.C. 3.6.3.9) é um complexo proteico heterotrimérico (α, β, γ) de membrana plasmática (KAPLAN, 2002), presente nas células eucarióticas, e fundamental na manutenção da homeostase iônica celular (SKOU & ESMANN, 1992), sendo que α é a subunidade catalítica de transporte iônico (KAPLAN, 2002). Neurônios expressam duas isoformas α: α1, constitutiva, e α3, que é restritamente expressa (HIEBER et al, 1991; McGRAIL et al. 1991). Ambas são importantes para a função neuronal basal, porém α3 controla, em maior proporção, o reestabelecimento das concentrações intracelulares de Na^+ após disparos sustentados (AZARIAS et al, 2013).

A reação básica catalisada pela Na^+,K^+-ATPase (conforme esquema na Figura 6) é o transporte de 3 íons Na^+ para o meio extracelular e de 2 íons K^+ para o meio intracelular, usando energia proveniente da hidrólise de 1 ATP (SKOU & ESMANN, 1992). Ao regular o gradiente de Na^+ e K^+ através da membrana plasmática a Na^+,K^+-ATPase regula também, de maneira indireta a concentração

intracelular de outros íons, como Ca^{2+}, Cl^- e H^+, e substâncias como água e glicose (SKOU & ESMANN, 1992).

A atividade da Na^+,K^+-ATPase no cérebro é fundamental para a manutenção do gradiente eletroquímico responsável pelos potenciais de repouso e ação e captação e liberação de neurotransmissores (STAHL & HARRIS, 1986). Consequentemente, alterações na atividade da Na^+,K^+-ATPase afetam a sinalização celular e a atividade neuronal (MOSELEY et al., 2007). Neste contexto, a supressão genética da Na^+,K^+-ATPase causa prejuízo ao aprendizado espacial e aumento no comportamento típico de ansiedade em camundongos (MOSELEY et al., 2007). Além disso, uma redução no funcionamento da Na^+,K^+-ATPase ocasiona aumento ou diminuição da excitabilidade neuronal, dependendo do grau de inibição induzido e do tipo neuronal afetado (GRISAR et al., 1992).

A ouabaína, um inibidor da Na^+,K^+-ATPase, aumenta o influxo de Ca^{2+} em fatias de cérebro de ratos (FUJISAWA et al., 1965) e causa convulsões em camundongos (JAMME et al., 1995), liberação de glutamato por reversão do transportador dependente de Na^+ (LI & STYS, 2001) e morte celular no hipocampo de ratos (LEES et al., 1990). O PTZ é capaz de, diretamente, inibir a enzima Na^+,K^+-ATPase, ligando-se preferencialmente a forma E1 (DUBBERKE et al., 1998). Também é importante mencionar que a atividade da Na^+,K^+-ATPase está diminuída no cérebro *post mortem* de pacientes com epilepsia (GRISAR et al., 1992). O grau de inibição da atividade da Na^+,K^+-ATPase induzido pela administração intraestriatal de MMA, de ácido glutárico ou injeção intraperitoneal de PTZ se correlaciona positivamente com a duração das convulsões induzidas por estes agentes (FIGHERA et al., 2006; FURIAN et al., 2007; SOUZA et al., 2009), reforçando o papel importante da Na^+,K^+-ATPase nas convulsões. Ainda, a injeção i.c.v. de PGE_2 diminui a atividade da Na^+,K^+-ATPase no hipocampo de ratos (OLIVEIRA et al., 2009). Sendo assim, hipotetizamos que a PGE_2, através dos receptores EP, pode facilitar crises epilépticas por inibir a atividade da enzima Na^+,K^+-ATPase.

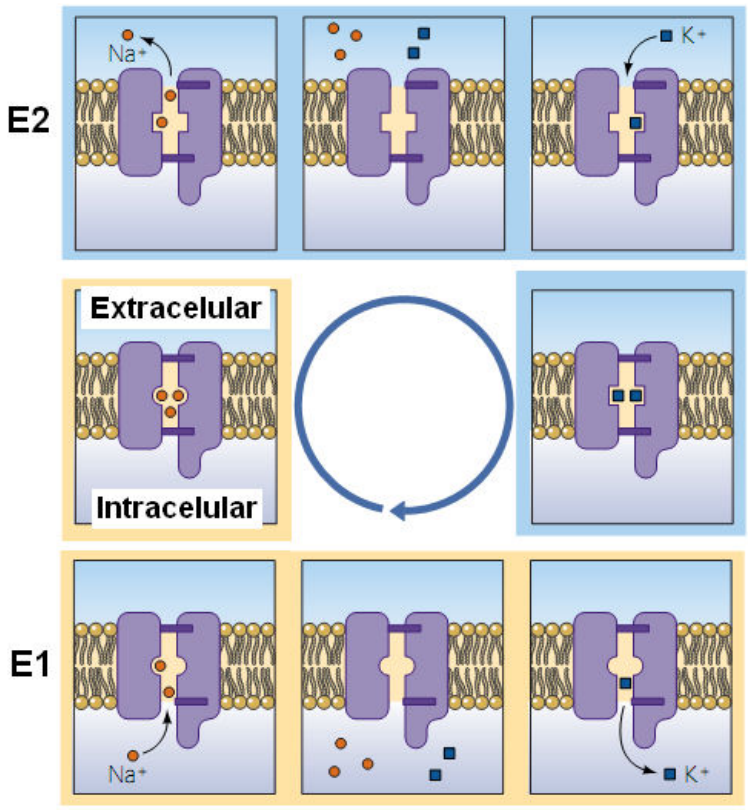

Figura 6 – Esquema de funcionamento da Na^+,K^+-ATPase. Os sítios de ligação para os cátions são acessíveis alternadamente do lado citoplasmático (conformação E1) e do lado extracelular (conformação E2) da membrana. O processo inicia-se com a ligação de 3 Na^+ aos sítios de alta afinidade, na superfície interna da membrana no interior da célula (E1). A fosforilação da enzima altera sua conformação e diminui sua afinidade por Na^+, levando a liberação deste no meio extracelular e a ligação de 2 K^+ em sítios de alta afinidade presentes na porção extracelular (E2). Após a ligação do K^+ a enzima é desfosforilada e retorna a sua configuração anterior. Desta forma ocorre a liberação do K^+ no meio intracelular (E1) e a proteína está pronta para um novo ciclo de bombeamento. Fonte: adaptado de HORISBERGER, 2004.

JUSTIFICATIVA

Dado o elevado número de pacientes com epilepsia, que apresentam convulsões refratárias aos fármacos disponíveis, torna-se importante a busca por novas terapias anticonvulsivantes. Para isto, é necessário o entendimento dos mecanismos de indução e manutenção de convulsões.

Neste sentido, considerando a associação entre o processo inflamatório e o aumento da excitabilidade, que culmina com o aparecimento de crises epilépticas, o presente estudo tem como objetivo investigar o envolvimento dos receptores EP1 e EP3 para PGE_2 nas convulsões induzidas por PTZ e KA, dois modelos com alto valor preditivo na clínica para o desenvolvimento de novos agentes com potencial anticonvulsivantes.

Considerando os mecanismos moleculares envolvidos na modulação de convulsões por ligantes de receptores EP, e o relevante papel dos receptores EP1 e EP3 exacerbando isquemia e excitotoxicidade cerebral (AHMAD, A.S. et al., 2006, AHMAD, M. et al., 2007) nossa hipótese é que a PGE_2 e os receptores EP1 e EP3 podem facilitar convulsões por inibir a atividade da enzima Na^+,K^+-ATPase no córtex e hipocampo, áreas cerebrais envolvidas na geração e manutenção das convulsões induzidas por PTZ e KA (BEN-ARI & COSSART, 2000, BREVARD et al., 2006). Além disso, a hipótese que a PGE_2 e seus receptores modulem a atividade da Na^+,K^+-ATPase também é relevante não só para o estudo da epilepsia, mas também para outras patologias nas quais são encontrados níveis elevados de PGE_2 e diminuição da atividade da Na^+,K^+-ATPase, como doença de Alzheimer (TOWNSEND & PRATICO, 2005).

OBJETIVO GERAL

Avaliar o envolvimento dos subtipos de receptores prostanoides para PGE_2 EP1 e EP3 nas convulsões induzidas por PTZ e KA, e na modulação da atividade da enzima Na^+,K^+-ATPase.

Objetivos específicos – Parte I

1. Verificar os efeitos dos agonistas para receptores EP1 e EP3, ONO-DI-004 e ONO-AE-248, respectivamente, sobre as convulsões induzidas por PTZ em camundongos;
2. Verificar os efeitos dos antagonistas para receptores EP1 e EP3, ONO-8713 e ONO-AE3-240, respectivamente, sobre as convulsões induzidas por PTZ em camundongos;
3. Verificar se a atividade da enzima Na^+,K^+-ATPase é modulada pela ação dos agonistas e antagonistas dos receptores EP1 e EP3, nas convulsões induzidas por PTZ em córtex e hipocampo de camundongos;
4. Verificar se os agonistas para os receptores EP1 e EP3 são capazes de prevenir as ações dos respectivos antagonistas nas convulsões induzidas por PTZ, *in vivo*, e na modulação da atividade da enzima Na^+,K^+-ATPase, *ex vivo*.

Objetivos específicos – Parte II

1. Verificar os efeitos dos agonistas para receptores EP1 e EP3 sobre as convulsões induzidas por KA, em camundongos;
2. Verificar os efeitos dos antagonistas para receptores EP1 e EP3 sobre as convulsões induzidas por KA, em camundongos;
3. Verificar se a atividade da enzima Na^+,K^+-ATPase é modulada pela ação dos agonistas e antagonistas dos receptores EP1 e EP3, nas convulsões induzidas por KA em córtex e hipocampo de camundongos;
4. Verificar se os agonistas para os receptores EP1 e EP3 são capazes de prevenir as ações dos respectivos antagonistas nas convulsões induzidas por KA, *in vivo*, e na modulação da atividade da enzima Na^+,K^+-ATPase, *ex vivo*.

APRESENTAÇÃO

O presente trabalho consiste na apresentação dos resultados obtidos sob a forma de ARTIGOS CIENTÍFICOS, que contém os MANUSCRITOS I e II, correspondentes às Partes I e II da Tese de Doutorado. Ambos manuscritos estão sob avaliação pela indústria farmacêutica japonesa *ONO Pharmaceutical* para a submissão de patente. No caso de posterior publicação, serão submetidos às revistas: Experimental Neurology (ISSN: 0014-4886, fator de impacto: 4,7) e, Neurobiology of Disease (ISSN: 0969-9961, fator de impacto: 5,4), respectivamente. Em cada manuscrito encontram-se as respectivas seções: INTRODUÇÃO, MATERIAIS e MÉTODOS, RESULTADOS, DISCUSSÃO e REFERÊNCIAS BIBLIOGRÁFICAS.

Com a finalidade de integrar e discutir os resultados dos MANUSCRITOS I E II uma nova seção DISCUSSÃO é apresentada. As REFERÊNCIAS BIBLIOGRÁFICAS correspondem somente às citações realizadas nas seções, exceto ARTIGOS CIENTÍFICOS.

Na seção pós-textual APÊNDICE são apresentados RESULTADOS PRELIMINARES, complementares ao assunto desta tese, sob a forma de Materiais e Métodos e, Resultados. Adicionalmente, são apresentados os resultados referentes ao estágio no exterior, sob forma de manuscrito (MANUSCRITO III). Este manuscrito foi aceito para publicação na revista Neurobiology of Disease, ISSN: 0969-9961, fator de impacto: 5,4.

2 ARTIGOS CIENTÍFICOS

2. ARTIGOS CIENTÍFICOS

2.1 Manuscrito I

“Selective blockade of EP1 and EP3 receptors attenuate pentylenetetrazole-induced seizures in mice”

2.1.1 Título em português

“O bloqueio seletivo dos receptores EP1 e EP3 atenua as convulsões induzidas por pentilenotetrazol em camundongos”

Selective blockade of EP1 and EP3 receptors attenuate pentylenetetrazole-induced seizures in mice

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Abstract

Epileptic process is mediated by changes in both excitatory and inhibitory circuits leading to the formation of hyperexcitable seizure networks. Neuroinflammation is a major issue in epilepsy, but the impact of specific inflammatory mediators on neuronal excitability is incompletely understood. The role of PGE_2 receptors (EP) in seizure development remains unclear. In the current study we investigated whether EP1 and EP3 agonists and antagonists modulate PTZ-induced seizures, through Na^+,K^+-ATPase activity, in mice. Systemic administration of EP1 and EP3 antagonists (ONO-8713 and ONO-AE3-240, respectively, 10 µg/kg, s.c.) attenuate PTZ-induced seizures. Nevertheless, their respective agonists (ONO-DI-004 and ONO-AE-248, 10 µg/kg, s.c.) facilitate seizures elicited by PTZ, and at a non-effective dose for generate ictal activity, prevented the antagonist effects on this acute model. Thus, whilst EP1 and EP3 agonists exacerbate Na^+,K^+-ATPase activity decreasing, their respective antagonists prevent it. Accordingly, antagonists effects were prevented by a non-effective agonist dose previously administrated. Moreover, our findings suggest a relation between Na^+,K^+-ATPase activity, ictogenic activity, and PGE_2 pathway, even if it cannot be definitely established at the moment. In order to fully evaluate the anticonvulsant role of these compounds, and their use in epilepsy therapy, more studies are necessary, also extending to others experimental seizure models. EP1 and EP3 ligands may also represent novel targets for anti-epileptogenic drugs with an impact on the disease progression.

Key words: epilepsy, prostaglandin E_2, EP1 receptor, EP3 receptor, pentylenetetrazol

Introduction

Epilepsy affects about 1% of the general population and is characterized by recurring and unprovoked seizures. This disabling disorder is the second most common neurological disorder after stroke (Engel & Pedley, 2008; McNamara, 1999; Porter, 1993). One third of these patients do not respond to currently available treatments. Thus unraveling novel putative anticonvulsant targets becomes essential, as well as a better understanding of the causes of underlying seizure episodes in epilepsy (Perucca et al., 2007; Galanopoulou et al., 2012).

Current evidence strongly supports the involvement of inflammatory process in the etiopathogenesis of seizures and epilepsy development (Vezzani & Granata, 2005). Proinflammatory cytokines, complement factors (Ravizza et al. 2008) and prostaglandins (Oliveira et al, 2008a) contribute to cell loss and seizures in experimental models. Consequently, inhibiting their production or blocking their receptors decreases seizure activity (Oliveira et al., 2008a; Vezzani et al., 2011).

Prostaglandins are produced from arachidonic acid via cyclooxygenases (COXs) pathway (Simmons et al., 2004), and are directly related to inflammatory responses (Phillis et al., 2006). Prostaglandin E_2 (PGE_2) is a crucial mediator of responses to illness synthesized by COX-2 pathway in the brain (Chen & Bazan, 2005; Milatovic et al., 2011). PGE_2 plays an important role in dynamically maintaining membrane excitability, synaptic transmission, integration, and plasticity in the hippocampus. In fact, significant reduction of the membrane input resistance and frequency of firing has been found during endogenous PGE_2 depletion in hippocampal CA1 pyramidal neurons. Such a decrease of membrane excitability is reversed by the exogenous application of PGE_2 (Chen & Bazan, 2005). In addition, PGE_2 facilitates PTZ- and methylmalonate-induced seizures (Oliveira et al., 2008b; Salvadori et al., 2012). PGE_2 actions are mediated by G-protein-coupled E-prostanoid (EP) receptors, which are

divided into EP1, EP2, EP3, and EP4 (Furuyashiki & Narumiya, 2009). The existence of EP receptors subtypes may account for the multiplicity of biological responses exerted by PGE_2, since each EP subtype has a different structure and is coupled to a different signaling pathway (Tsuboi et al., 2002). It has been shown that EP1 receptor mediates a G-protein coupled increase of free Ca^{++} concentration by Ca^{++} channel gate regulation (Tsuboi et al., 2002). This receptor has a splicing isoform that presents a defective signal transduction, suppressing the EP signaling pathway, even though it shows a ligand-binding specificity similar to EP1 receptor (Okuda-Ashitaka et al., 1996). EP2 and EP4 receptors are coupled to G_s-protein, and their activation increase cAMP production (Katsuyama et al., 1995; Sugimoto & Narumiya, 2007). However, these receptors seem to have different sensitivity to phosphorylation and agonist-dependent desensitization, due structural differences (Nishigaki et al., 1996). The EP3 receptor has three splicing isoforms (α, β and γ) in mouse, with similar ligand-binding specificities (Sugimoto & Narumiya, 2007). These isoforms are functionally different, as they bind distinct G-proteins (G_i, G_q and G_s) (Sugimoto & Narumiya, 2007). However, the major EP3 receptor signaling pathway involves G_i activation with consequent adenylate cyclase inhibition (Namba et al., 1993; Tsuboi et al., 2002).

Na^+,K^+-ATPase (EC 3.6.3.9) is a heterodimeric integral membrane protein responsible for maintaining the homeostatic ionic equilibrium in almost all tissues, contributing to preserve the resting membrane potential in cell membranes (Kaplan, 2002). Na^+,K^+-ATPase activity is reduced in the cerebral tissue of epileptic patients (Rapport et al., 1975; Grisar et al. 1992), and a mutation in a subunit gene of this enzyme has been linked with epilepsy in humans (Jurkat-Rott et al., 2004). It has been reported a diminished activity of the glial Na^+,K^+-ATPase in seizure focus compared to non epileptogenic area, in experimental models of focal epilepsy (Grisar et al., 1992). Moreover, the same has been observed in patients with intractable temporal lobe epilepsy (Grisar et al., 1992). Na^+,K^+-ATPase activity is also

decreased after PTZ- (Fighera et al., 2006; Silva et al., 2011), glutaric acid- (Fighera et al., 2006), and methylmalonic acid-induced seizures (Malfatti et al., 2003; Royes et al., 2007; Ribeiro et al., 2009). In line with this view, ouabain, an irreversible Na^+,K^+-ATPase inhibitor, may activate various intracellular pathways and induce seizures (Donaldson et al., 1971; Haas et al., 2000).

Considering that the role of EP receptors in epilepsy is still not well established, and the challenging search for new pharmacological targets in epilepsy, in the current study was investigated whether systemic administration of EP1 or EP3 receptors ligands modulate PTZ-induced seizures in mice.

Material and Methods

Animals and Reagents

Adult male Swiss mice (25-35 g) maintained under controlled light and environment (12:12 h light–dark cycle, 24±1 °C, 55% relative humidity) with free access to food (Supra™, Santa Maria, Brazil) and water were used. All experimental protocols were designed aiming to keep the number of animals used to a minimum, as well as their suffering. These were conducted in accordance with national and international legislation (guidelines of Brazilian Council of Animal Experimentation – CONCEA – and of U.S. National Institute of Health Guide for the Care and Use of Laboratory Animals – NIH Publications Nº 80-23, revised 1996), and with the approval of the Ethics Committee for Animal Research of the Federal University of Santa Maria (process number 078/2010).

ONO-8713 (an EP1 antagonist), ONO-DI-004 (an EP1 agonist), ONO-AE3-240 (an EP3 antagonist), and ONO-AE- 248 (an EP3 agonist), were generously donated by Ono Pharmaceutical Co. (Osaka, Japan). ONO-8713, ONO-DI-004, ONO-AE3-240, and ONO-

AE-248 were dissolved in dimethylsulfoxide (DMSO) and then diluted with sterile saline, in such a way that DMSO concentration did not exceed 1%. PTZ and all other reagents were purchased from Sigma (St. Louis, MO, USA).

Surgical procedures

All animals were anesthetized with ketamine (5 mg/kg) and xylazine (50 mg/kg) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, two screw electrodes were placed over the right and left parietal cortices (coordinates in mm: AP -4.5 and L 2.5), along with a ground lead positioned over the nasal sinus (Franklin & Paxinos, 2008). The electrodes were connected to a multipin socket for electroencephalogram (EEG) recordings, and were fixed to the skull with dental acrylic cement. Chloramphenicol (200 mg/kg, i.p.) was administered immediately before the surgical procedure. After surgery, all mice received a single subcutaneous (s.c.) injection of 0.01 mg/kg buprenorphine hydrochloride for amelioration of pain.

Drug administration protocol and EEG recordings

The effect of EP ligands on PTZ-induced seizures was assessed 5-7 days after surgery. Mice were habituated for at least 10 minutes and connected to the lead socket of a swivel, which was connected to a digital encephalographic equipment (Neuromap EQSA260, Neurotec, Brazil) inside a Faraday's cage. Routinely, a 10 min baseline recording was obtained to establish an adequate control period. After this period, ONO-8713 (10 µg/kg), ONO-DI-004 (10 µg/kg), ONO-AE3-240 (10 µg/kg), ONO-AE-248 (10 µg/kg), or their respective vehicle (1% DMSO in saline) were administrated subcutaneously. The animals were injected with PTZ (60 mg/kg, i.p.) 30 minutes after antagonist / agonist administration and followed up for 30 min after PTZ administration for the appearance of seizures, by electrographic and

behavioral methods. Latency to myoclonic jerks and tonic-clonic seizures were recorded, in seconds. We have attributed a cut-off time of 1800 seconds for those animals that did not present EEG seizures during the observation period, for statistical purposes. Doses and time elapsed between drug injection and PTZ injections were selected based on pilot experiments. EEG signals were amplified, filtered (0.1 to 70.0 Hz, bandpass), digitalized (sampling rate 256 Hz) and stored in a PC for off-line analysis, as described below. Seizures were defined by the occurrence of ictal episodes characterized by the following alterations in the recording leads: spikes (≥ 2 X baseline) plus slow waves, multispikes (≥ 2X baseline, ≥ 3 spikes/complex) plus slow waves, multiple sharp waves (≥ 2X baseline) in long spindle episodes (≥ 5 s) or major seizure (repetitive spikes plus slow waves, ≥ 5 sec) (Oliveira et al., 2008a). Rhythmic scratching of the electrode headset rarely caused artifacts, which were easily identified and discarded.

A separated set of animals was used to assess if the EP1 and EP3 agonists (ONO-DI-004 and ONO-AE-248) might pharmacologically prevent the anticonvulsant actions of ONO-8713 and ONO-AE3-240 (EP1 and EP3 antagonists, respectively). This possibility was investigated by injecting a non-effective dose of EP1 (3 µg/kg, s.c.), EP3 (3 µg/kg, s.c.) agonists or their respective vehicles, followed by EP1 (10 µg/kg, s.c.), EP3 (10 µg/kg, s.c.) antagonists or their respective vehicles, 30 and 15 min before PTZ (60 mg/kg, i.p.) seizure induction, respectively.

Na^+, K^+ - ATPase activity measurements

Na^+,K^+-ATPase has been identified as a target for PGE_2-mediated signaling in adult rat hippocampal slices (Oliveira et al., 2009). The effect of EP1 and EP3 ligands on Na^+,K^+-ATPase activity was measured. Immediately after the EEG recordings, the animals were sacrificed. Cerebral cortices and hippocampi were dissected, weighted and immediately

frozen at -80ºC. On the experimental day, each area was taken in separate and gently homogenized (7-10 strokes) in ice-cold 10 mM Tris-HCl (pH 7.4) for Na^+,K^+-ATPase activity determination (Wyse et al., 2000). Briefly, the assay medium consisted of 30 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$ and 50 µg of protein in the presence or absence of ouabain (1 mM), in a final volume of 350 µL. The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 5 mM. After 30 min, the reaction was stopped by the addition of 70 µL of 50 % (w/v) trichloroacetic acid. Appropriate controls were included in the assay for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified by the colorimetric method described by Fiske & Subbarow (1925), using KH_2PO_4 as reference standard. Specific Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nmol Pi/mg protein/min. Protein content was measured colorimetrically by the Bradford (1976) method using bovine serum albumin (1 mg/ml) as standard.

Statistical analyses

Latencies to myoclonic jerks and to tonic-clonic seizures were analyzed by Kruskall-Wallis, followed by nonparametric Dunn's multiple comparison test, when indicated. Data are presented as median and interquartile ranges. Total time spent in seizures, mean amplitude of EEG recordings and Na^+,K^+-ATPase activity were analyzed by one- or two-way ANOVA followed by Bonferroni's test, depending on the experimental design. Data are expressed as mean + S.E.M. A probability of $P < 0.05$ was considered significant, and H and F values are shown only if $P < 0.05$.

Results

Figure 1 shows the effect of ONO-DI-004 (10 µg/kg, s.c.) and ONO-8713 (10 µg/kg, s.c.) on PTZ-induced seizures (60 mg/kg, i.p.), measured as the latency to the first isolated myoclonic jerk, with concomitant spike activity on EEG recordings (A), latency to generalized tonic-clonic seizures (B), and total time spent in generalized seizures (C). Statistical analysis revealed that ONO-8713 increased the latency to myoclonic jerks [$H(2) = 14.49$, $P < 0.05$, Fig. 1A], as well as to generalized seizures [$H(2) = 11.77$, $P < 0.05$, Fig. 1B]. ONO-DI-004 did not significantly alter these parameters (Fig. 1A and 1B). Figure 1C shows that the EP1 antagonist decreased total time spent in generalized seizures, when compared with vehicle-injected group [$F(2,15) = 22.17$, $P < 0.05$]. However, mice that received EP1 agonist spent more time in seizures than the respective control group [$F(2,15) = 22.17$, $P < 0.05$]. Quantitative analyses of EEG trace amplitudes before and after PTZ injection are shown in Figures 2A and 2B, for ONO-DI-004 and ONO-8713, respectively. Although ONO-DI-004 did not alter seizures onset, it significantly increased the mean amplitude (in µV) of EEG ictal traces, when compared with the vehicle group [$F(2,18) = 11.86$, $P < 0.05$]. Statistical analysis also revealed a significant decrease in mean amplitude (in µV) of EEG ictal traces of ONO-8713-treated animals, when compared with the vehicle group [$F(2,24) = 20.93$, $P < 0.05$]. Representative EEG patterns are presented in figures 2C, 2D and 2E (for vehicle-, ONO-DI-004-, and ONO-8713-treated groups, respectively), showing the ictal activity induced by PTZ injection. PTZ injection caused the appearance of multispike plus slow waves and major seizure activity, which coincided with myoclonic jerks. Generalized seizures appeared in the EEG recordings as the major seizure activity, and were characterized by 2–3 Hz high-amplitude activity (Fig. 2C, 2D and 2E). After the ictal discharge, postictal EEG suppression and slow waves were observed, correlating with behavioral catalepsy.

ONO-DI-004 (3 µg/kg, s.c.) prevented the protective effect of ONO-8713 (10 µg/kg, s.c.) against PTZ-induced seizures, measured as the latency to the first generalized tonic-clonic seizure [H(3) = 11.50, $P < 0.05$, Fig. 3], but not as the latency to myoclonic jerks ($P > 0.05$, data not shown).

Figure 4 shows the effect of ONO-DI-004 (10 µg/kg, s.c., Fig 4A) and ONO-8713 (10 µg/kg, s.c., Fig 4B) on Na^+,K^+-ATPase activity of cerebral cortex and hippocampus homogenates. The subcutaneous administration of ONO-DI-004 or ONO-8713 did not alter the Na^+,K^+-ATPase activity *per SE*. ONO-DI-004 accentuated PTZ-induced Na^+,K^+-ATPase activity decrease in homogenates of cerebral cortex and hippocampus [F(1,16) = 5.645 $P < 0.05$, for cerebral cortex, and F(1,16) = 11.88, $P < 0.05$, for hippocampus, Fig 4A]. The EP1 antagonist ONO-8713 prevented PTZ-induced decrease of Na^+,K^+-ATPase activity [F(1,16) = 13.03, $P < 0.05$, for cerebral cortex, and F(1,16) = 19.93, $P < 0.05$, for hippocampus, Fig 4B]. Accordingly, ONO-DI-004 (3 µg/kg, s.c.) did not alter Na^+,K^+-ATPase activity *per SE* ($P > 0.05$, Fig 5A), but also accentuated the PTZ-induced decrease of Na^+,K^+-ATPase activity. Moreover, the EP1 agonist ONO-DI-004 prevented the protective effect of the EP1 antagonist ONO-8713 on Na^+,K^+-ATPase activity in cortical and hippocampal homogenates [F(1,12) = 10.39, $P < 0.05$, for cerebral cortex, and F(1,12) = 7.678, $P < 0.05$, for hippocampus, Fig 5B].

The effect of the EP3 agonist ONO-AE-248 (10 µg/kg, s.c.) and of the EP3 antagonist ONO-AE3-240 (10 µg/kg, s.c.) on PTZ-induced seizures is shown in Figure 6. Statistical analysis revealed that the EP3 receptor agonist decreased the latency to clonic [H(2) = 14.34, $P < 0.05$, Fig. 6A] and to generalized seizures [H(2) = 18.78, $P < 0.05$, Fig. 6B]. The EP3 receptor antagonist significantly increased both onset parameters [H(2) = 14.34, $P < 0.05$, Fig. 6A, for myoclonic jerks; H(2) = 18.78, $P < 0.05$, Fig. 6B, for generalized seizures]. ONO-AE-248- and ONO-AE3-240-injected animals respectively spent less and more [F(2,21) = 13.6, $P <$

0.05] time in generalized seizures compared with control group (Fig. 6C). Two-way ANOVA followed by Bonferroni's test revealed that ONO-AE-248 and ONO-AE3-240 respectively increased and decreased the mean amplitude of EEG recordings of PTZ-induced seizures [$F(2,24) = 41.9$, $P < 0.05$, Fig. 7A; and $F(2,24) = 184.8$, $P < 0.05$, Fig. 7B, respectively]. Representative EEG recordings of animals treated with PTZ and vehicle, ONO-AE-248 or ONO-AE3-240 are shown in Figures 7C, 7D and 7E, respectively.

ONO-AE-248 (3 µg/kg, s.c.) prevented ONO-AE3-240-induced protection against PTZ-induced seizures, measured as the latency to myoclonic jerks [$H(3) = 5.745$, $P < 0.05$, Fig. 8A], and to generalized seizure [$H(3) = 11.90$, $P < 0.05$, Fig. 8B].

Similarly to the findings obtained with EP1 ligands, the EP3 receptor agonist decreased Na^+,K^+-ATPase activity only in those mice which had PTZ-induced seizures [$F(1,16) = 7.082$, $P < 0.05$, for cerebral cortex and $F(1,16) = 6.238$, $P < 0.05$, for hippocampus, Fig 9A]. Accordingly, the EP3 receptor antagonist prevented the expected decrease of Na^+,K^+-ATPase activity elicited by PTZ [$F(1,16) = 7.024$, $P < 0.05$, for cerebral cortex, and $F(1,16) = 4.615$, $P < 0.05$, for hippocampus, Fig 9B]. Additionally, ONO-AE-248 (3 µg/kg, s.c.) accentuated PTZ-induced decrease of Na^+,K^+-ATPase activity, and prevented the protective effect of the EP3 antagonist (ONO-AE3-240, 10 µg/kg, s.c.) on Na^+,K^+-ATPase activity in homogenates of cerebral cortex and hippocampus [$F(1,14) = 9.637$, and $F(1,14) = 9.244$, respectively, $P < 0.05$, Fig 10B].

Discussion

The current study shows that systemic administration of EP1 and EP3 antagonists attenuate PTZ-induced seizures. Accordingly, the systemic administration of EP1 and EP3 agonists

facilitated PTZ-induced seizures and, at doses that had no effect on seizures *per se*, prevented the protective effects of EP1 and EP3 antagonists.

Systemic administration of EP1 and EP3 antagonists also prevented PTZ-induced decrease of Na^+,K^+-ATPase activity in the cerebral cortex and hippocampus *ex vivo*. Accordingly, EP1 and EP3 agonists potentiated PTZ-induced decrease of Na^+,K^+-ATPase activity in both cerebral structures.

The currently reported inhibition of PTZ-induced seizures by EP1 and EP3 antagonists (ONO-DI-004 and ONO-AE3-240, respectively) not only fully agrees with the previous finding that the i.c.v. administration of an EP1 antagonist (SC-19220) or an EP3 antagonist (L-826266) decreases PTZ-induced seizures in rats (Oliveira et al, 2008), but importantly extends these findings to mice and reveals that the systemic administration of these antagonists decrease PTZ-induced seizures, a crucial information from the pre-clinical point of view.

From the pharmacological point of view, the current study provides strong evidence for a role of EP1 and EP3 receptors in seizure initiation and/or propagation, because: (1) the anticonvulsant effect initially observed for EP1 and EP3 antagonists (Oliveira et al, 2008b) was confirmed with chemically diverse compounds, of the same pharmacological class; (2) EP1 and EP3 agonists facilitated PTZ-induced seizures, the expected effect if these receptors were involved in seizure initiation and/or propagation; (3) The protective effect of the EP1 and EP3 antagonists were prevented by the respective agonists at doses that had no effect *per se* on PTZ-induced seizures (noneffective doses), indicating its specificity and further indicating a role for both EP1 and EP3 receptors in this seizure model. In this respect, early evidence that EP1 and EP3 receptors are involved in PTZ-induced seizures came from an experiment that has shown that the protective effect of EP1 and EP3 antagonists against PTZ-induced seizures was prevented by the nonspecific agonist PGE_2. It is well known that

introducing a nonspecific agonist in a biological system in which a specific antagonist was previously administered, may potentiate the action of co-occurring counter regulatory receptor subtypes, i.e., administering epinephrine to a propranolol-treated patient causes severe hypertension by stimulating alpha$_1$-adrenergic mechanisms in the absence of the beta$_2$-adrenergic counter regulatory actions (Goodman & Gilman, 2012). Therefore, one might reasonably propose that previously reported antagonism of EP1 and EP3 blockade by PGE_2 could be due to an action of this agonist at EP2 receptors, exacerbated by the lack of counter regulatory EP1- or EP3-mediated mechanisms. The current results practically rule out this possibility, since the agonists used to prevent the effect of the EP1 and EP3 antagonists have a very low affinity for EP2 receptors (EP1 antagonist ONO-8713: *Ki* = 0.3 nM for EP1 receptor, and *Ki* = 3000 nM for EP2 receptor; EP3 antagonist ONO-AE-240: *Ki* = 0.23 nM for EP3 receptor, and *Ki* >10^4 nM for EP2 receptor) (Sugimoto & Narumiya, 2007).

The currently reported PTZ-induced decrease of Na^+,K^+-ATPase activity in cerebral cortex of mice reproduces previous data by Fighera et al. (2006), and Souza and colleagues, who have shown a dose-dependent deleterious effect of PTZ on the sodium pump (Souza et al., 2009). Moreover, the protective effect of EP1 and EP3 antagonists on PTZ-induced decrease of Na^+,K^+-ATPase activity in cerebral cortex and hippocampus *ex vivo* is also, to some degree, in agreement with a previous study that has shown that EP1 and EP3 antagonists prevent PGE_2-induced decrease of Na^+,K^+-ATPase activity in slices of rat hippocampus (Oliveira et al, 2009). While one cannot define whether the currently observed decrease of Na^+,K^+-ATPase activity is a cause or consequence of the convulsive activity, it is intriguing that manipulation of PGE_2 signaling through EP1 and EP3 receptors alter convulsive activity and sodium pump activity with notable congruence. While EP1 and EP3 agonists potentiate PTZ-induced decrease of sodium pump activity and facilitate seizures, the respective antagonists have the opposite effect. In fact, current experimental and clinical evidence suggests that

increased neuronal activity increases cyclooxygenase activity by NMDA receptor-mediated activation of nitric oxide synthase and S-nitrosylation of the enzyme (Tian et al, 2008), with consequent increased production of PGE_2. Therefore, it is possible that the currently observed inhibitory effect of PTZ on Na^+,K^+-ATPase activity may involve primary excitatory-induced production of PGE_2, with consequent activation of EP1 and EP3 receptors.

In summary, we showed that EP1 and EP3 receptors modulate PTZ-induced seizures and Na^+,K^+-ATPase activity in mice. Interestingly, Rumià and colleagues (2012) have shown an important increase in PGE_2 and TXA_2 levels in epileptic patients, supporting a role for inflammation in epilepsy, and suggesting that selective blockade of prostanoid receptors may constitute a novel antiepileptic strategy for human epilepsy.

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FIGURE 1

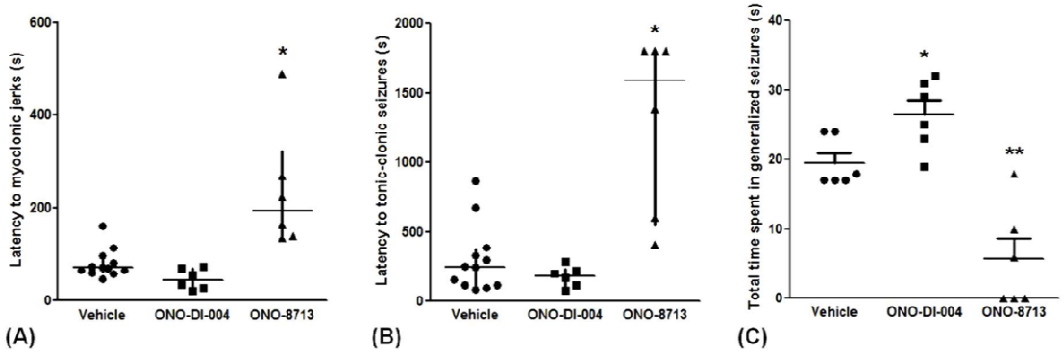

Figure 1 - Effect of EP1 receptor agonist and antagonist (ONO-DI-004 and ONO-8713, respectively; 10 µg/kg, s.c.) on PTZ-induced seizures (60 mg/kg, i.p.). (A) Latency to the first myoclonic episode. (B) Latency to the first tonic-clonic generalized seizure. (C) Total time spent in generalized seizures. Data expressed as median and interquartile range (A and B), and mean + SEM (C), for n = 6-12 in each experimental group. A probability of $P < 0.05$ was considered significant. * $P < 0.05$ and ** $P < 0.01$, when compared with vehicle group.

FIGURE 2

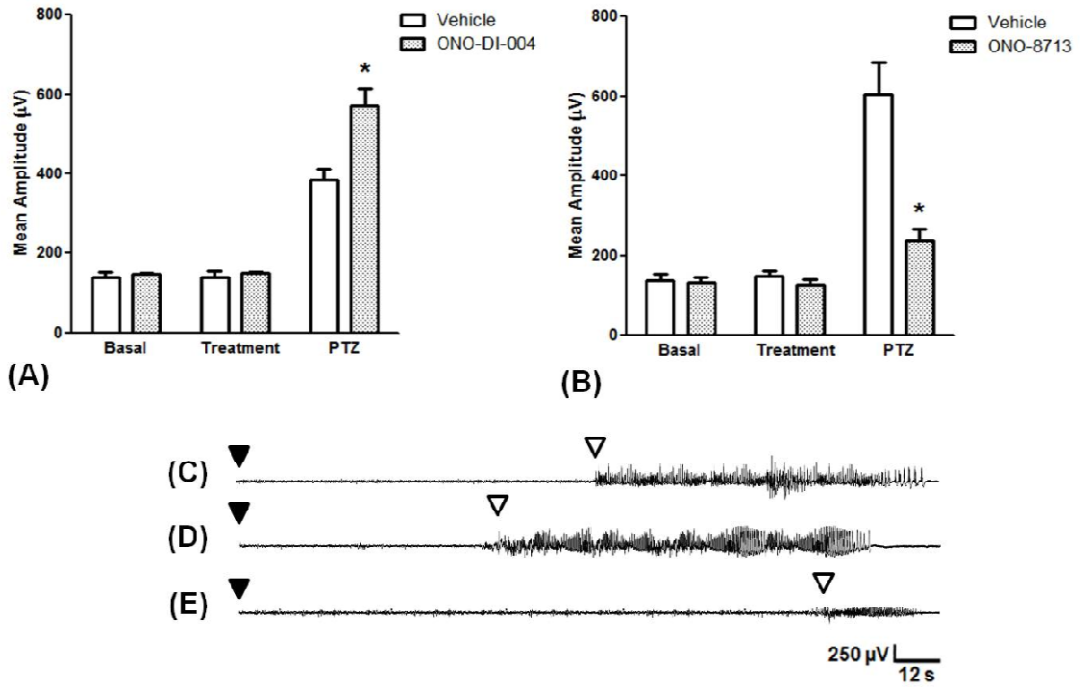

Figure 2 – Effect of EP1 receptor agonist (A) and antagonist (B) (ONO-DI-004 and ONO-8713, respectively; 10 µg/kg, s.c.) on the mean amplitude of EEG recordings in the parietal cortex of animal injected with PTZ (60 mg/kg, i.p.). Mean amplitude of EEG recordings was analyzed by two-way ANOVA followed by the Bonferroni's test and expressed as mean + S.E.M., for n = 6-12 in each group. * $P < 0.05$, when compared with respective vehicle. Representative electrocorticographic recordings of animals after PTZ injection are represented as follows: (C) vehicle, (D) ONO-DI-004, and (E) ONO-8713. Black and white arrowheads indicate PTZ injection and seizures latency, respectively, and the y-axis (amplitude) and x-axis (time) calibration bar is the same for all traces.

FIGURE 3

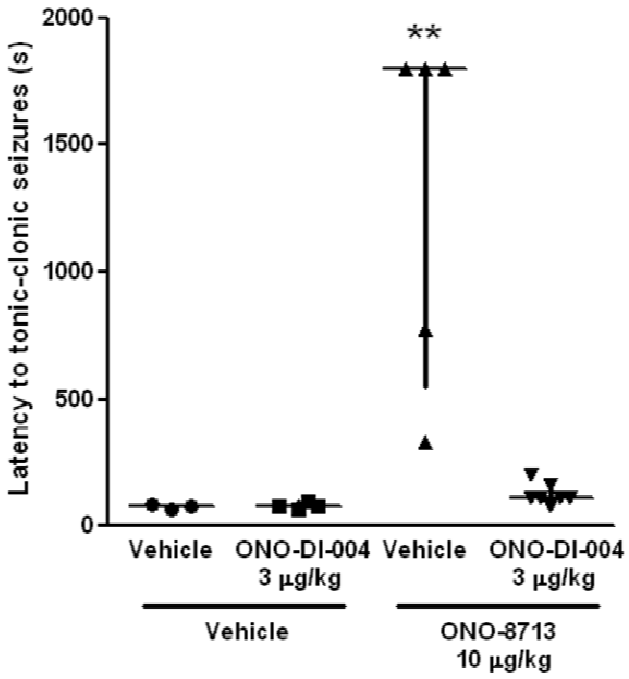

Figure 3 – Effect of ONO-DI-004 (3 µg/kg, s.c.) administration, followed or not by ONO-8713 (10 µg/kg, s.c.), on PTZ-induced seizures, measured as latency to the first tonic-clonic seizure. ONO-DI-004 (3 µg/kg, s.c.) prevented the protective effect of ONO-8713 (10 µg/kg, s.c.) against PTZ-induced seizures. Data expressed as median and interquartile range for n = 3-7 in each group. A probability of $P < 0.05$ was considered significant. ** $P < 0.01$, when compared with vehicle + vehicle group.

FIGURE 4

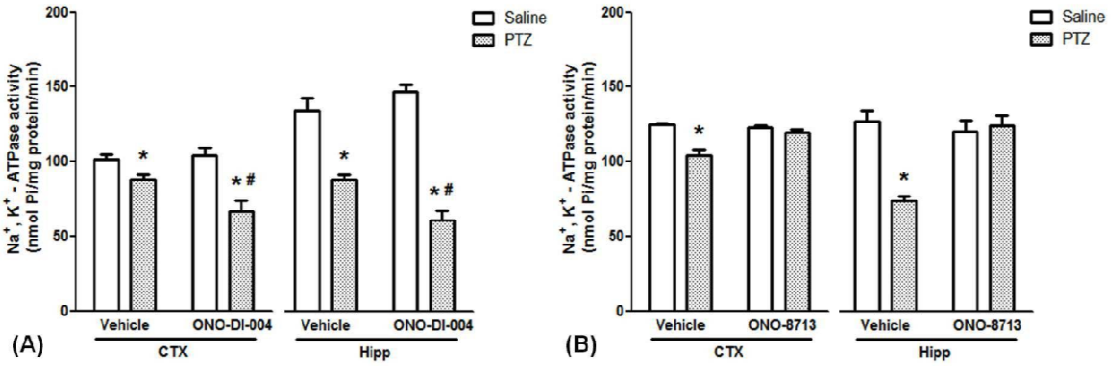

Figure 4 – ONO-DI-004 (10 µg/kg, s.c.) intensify (A) and ONO-8713 (10 µg/kg, s.c.) prevents (B) PTZ-induced decrease of Na^+,K^+-ATPase activity in cerebral cortex and hippocampus homogenates in mice. Data are presented in nmol Pi/mg protein/min, as mean + S.E.M., for $n = 5$ in each group. The Na^+,K^+-ATPase activity was analyzed by two-way ANOVA followed by the Bonferroni's test. * Indicates a significant difference within group compared with the respective control; # Indicates a significant difference between groups compared with the respective control ($P < 0.05$).

FIGURE 5

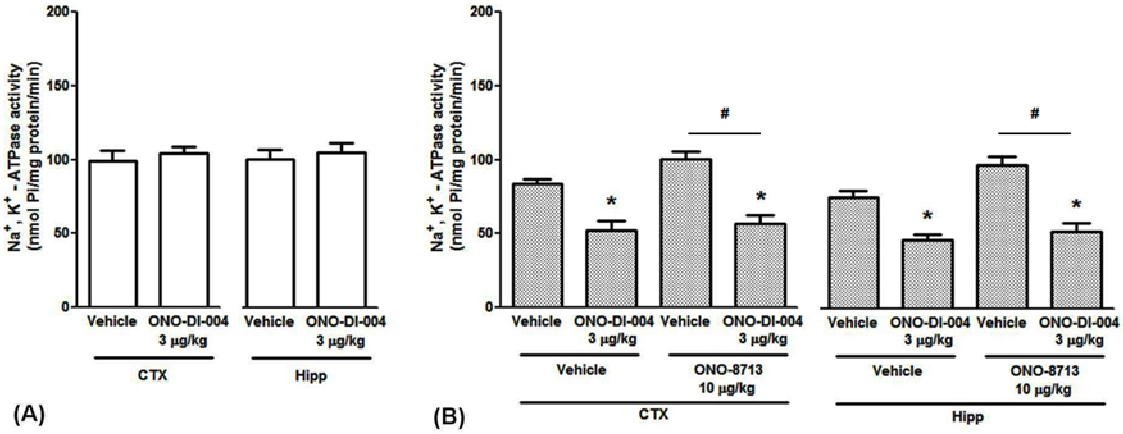

Figure 5 – (A) ONO-DI-004 (3 µg/kg, s.c.) does not present *per SE* effect on Na^+,K^+-ATPase ($P > 0.05$ by Student's t-test, for $n = 5$). (B) Two-way ANOVA followed by the Bonferroni's test revealed that ONO-DI-004 (3 µg/kg, s.c.) accentuated PTZ-induced decrease of Na^+,K^+-ATPase activity and prevented ONO-8713 (10 µg/kg, s.c.) effect on this enzyme activity in cerebral cortex and hippocampus homogenates in mice. Data are presented in nmol Pi/mg protein/min, as mean + S.E.M., for $n = 5$ in each experimental group. * $P < 0.05$, when compared with the respective control group; # $P < 0.05$, when compared with vehicle group.

FIGURE 6

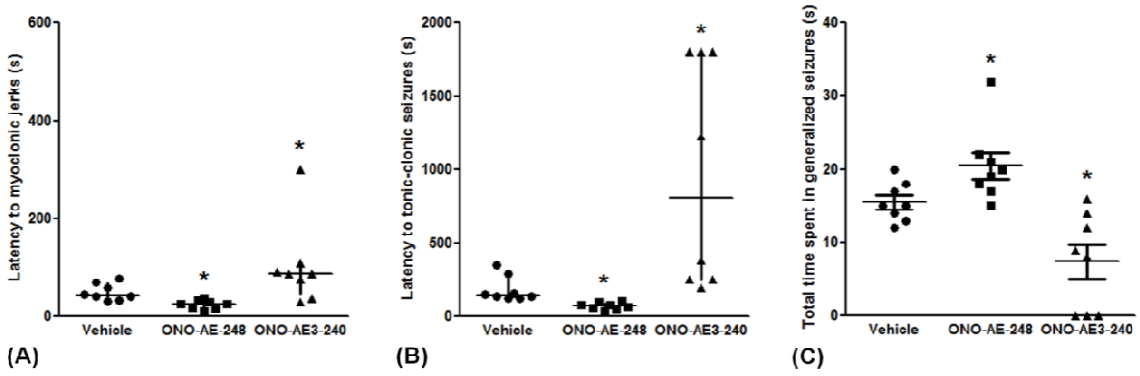

Figure 6 - Effect of EP3 receptor agonist and antagonist (ONO-AE-248 and ONO-AE3-240, respectively; 10 µg/kg, s.c.) on PTZ-induced seizures (60 mg/kg, i.p.). (A) Latency to the first myoclonic episode. (B) Latency to the first tonic-clonic generalized seizure. (C) Total time spent in generalized seizures. Data expressed as median and interquartile range (A and B), and mean + SEM (C), for $n = 8$ in each experimental group. A probability of $P < 0.05$ was considered significant. * $P < 0.05$, when compared with vehicle group.

FIGURE 7

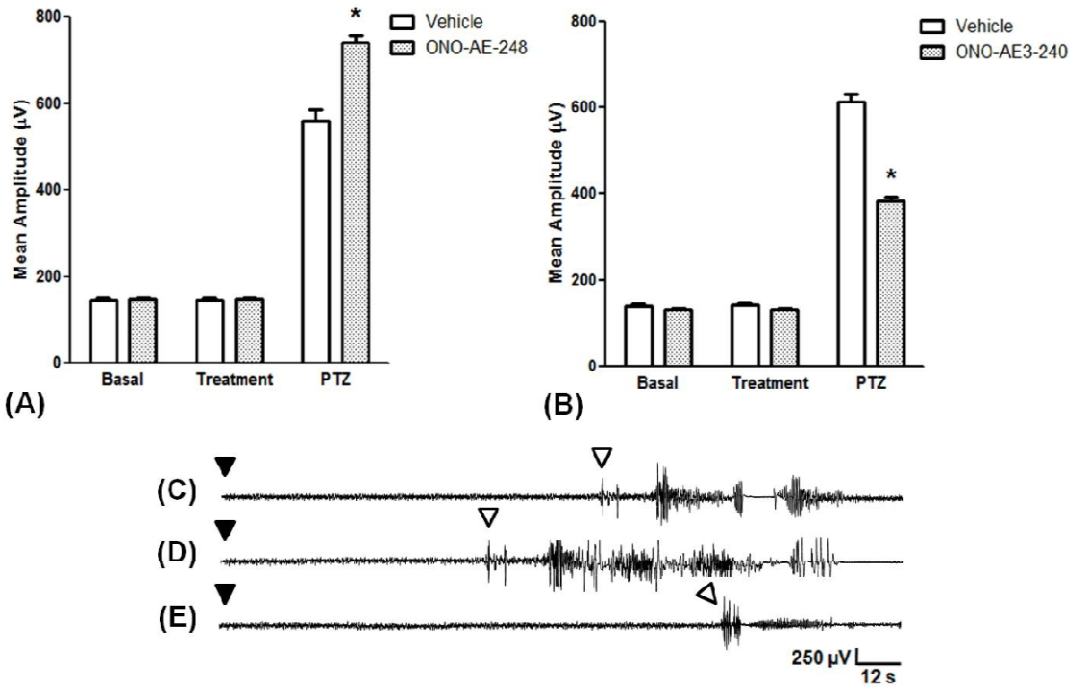

Figure 7 – Effect of EP3 receptor agonist (A) and antagonist (B) (ONO-AE-248 and ONO-AE3-240, respectively; 10 µg/kg, s.c.) on the mean amplitude of EEG recordings in the parietal cortex of animal injected with PTZ (60 mg/kg, i.p.). Mean amplitude of EEG recordings was analyzed by two-way ANOVA followed by the Bonferroni's test and expressed as mean + S.E.M., for $n = 8$ in each group. * $P < 0.05$, when compared with respective vehicle. Representative electrocorticographic recordings of animals after PTZ injection are represented as follows: (C) vehicle, (D) ONO-AE-248, and (E) ONO-AE3-240. Black and white arrowheads indicate PTZ injection and seizures latency, respectively, and the y-axis (amplitude) and x-axis (time) calibration bar is the same for all traces.

FIGURE 8

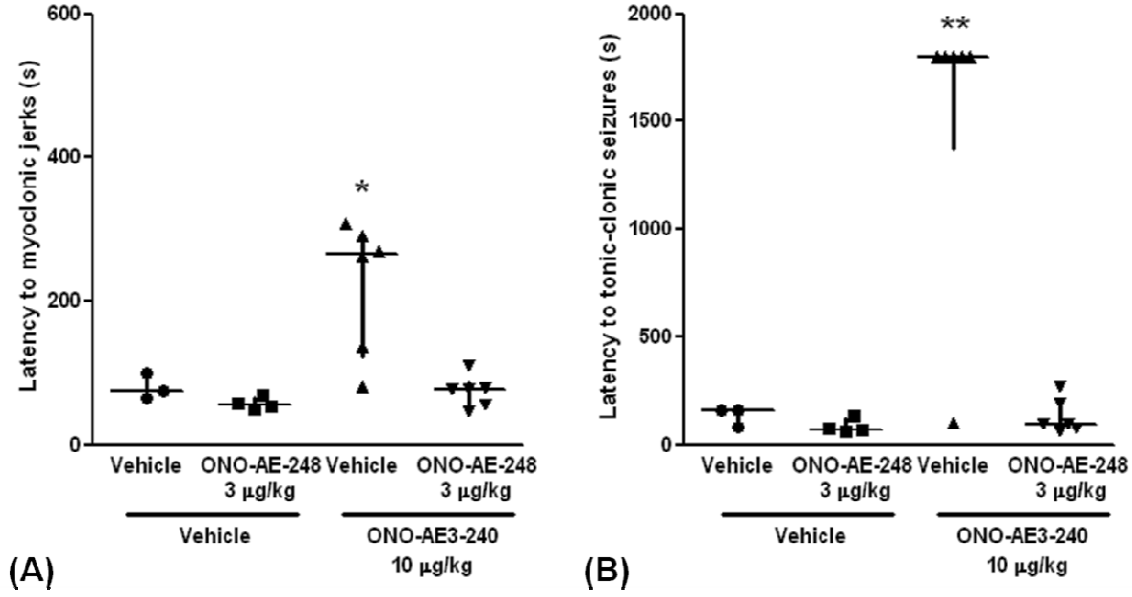

Figure 8 – Effect of ONO-AE-248 (3 µg/kg, s.c.) administration, followed or not by ONO-AE3-240 (10 µg/kg, s.c.), on PTZ-induced seizures, measured as latency to the first myoclonic jerks (A), and to the first tonic-clonic seizure (B). ONO-AE-248 (3 µg/kg, s.c.) prevented the protective effect of ONO-AE3-240 (10 µg/kg, s.c.) against PTZ-induced seizures. Data expressed as median and interquartile range for n = 3-6 in each group. A probability of $P < 0.05$ was considered significant. * $P < 0.05$, and ** $P < 0.01$, when compared with vehicle + vehicle group.

FIGURE 9

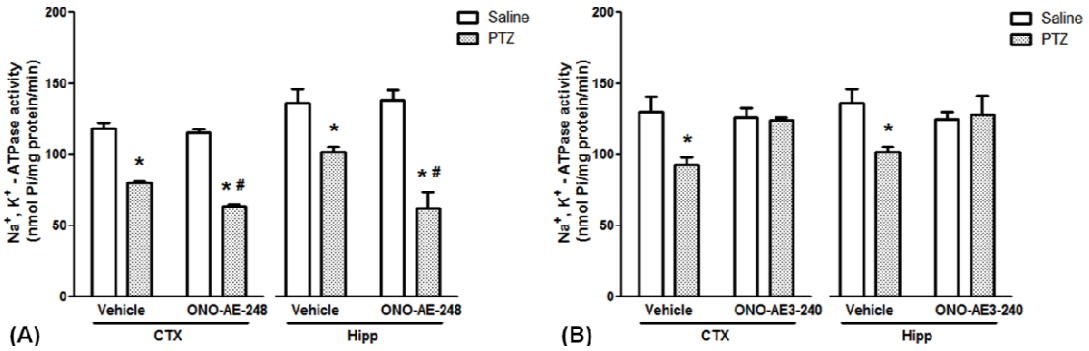

Figure 9 – ONO-AE-248 (10 µg/kg, s.c.) intensify (A) and ONO-AE3-240 (10 µg/kg, s.c.) prevents (B) PTZ-induced decrease of Na^+,K^+-ATPase activity in cerebral cortex and hippocampus homogenates in mice. Data are presented in nmol Pi/mg protein/min, as mean + S.E.M., for n = 5 in each group. The Na^+,K^+-ATPase activity was analyzed by two-way ANOVA followed by the Bonferroni's test. * Indicates a significant difference within group compared with the respective control; # Indicates a significant difference between groups compared with the respective control ($P < 0.05$).

FIGURE 10

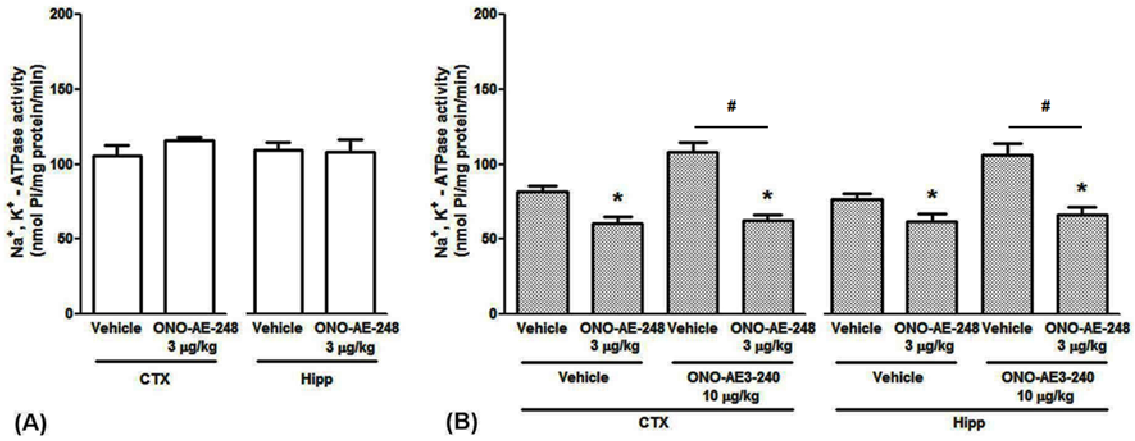

Figure 10 – (A) ONO-AE-248 (3 µg/kg, s.c.) does not present *per SE* effect on Na^+,K^+-ATPase ($P > 0.05$ by Student's t-test, for $n = 5$). (B) Two-way ANOVA followed by the Bonferroni's test revealed that ONO-AE-248 (3 µg/kg, s.c.) accentuated PTZ-induced decrease of Na^+,K^+-ATPase activity and prevented ONO-8713 (10 µg/kg, s.c.) effect on this enzyme activity in cerebral cortex and hippocampus homogenates in mice. Data are presented in nmol Pi/mg protein/min, as mean + S.E.M., for $n = 5$ in each experimental group. * $P < 0.05$, when compared with the respective control group; # $P < 0.05$, when compared with vehicle group.

2.2 Manuscrito II

“Selective EP1 and EP3 antagonists attenuate kainic acid-induced seizures in mice”

2.1.1 Título em português

“Antagonistas seletivos dos receptores EP1 e EP3 atenuam as convulsões induzidas por ácido caínico em camundongos”

Selective EP1 and EP3 antagonists attenuate kainic acid-induced seizures in mice

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Abstract

Epilepsy is a disabling disorder characterized by recurring and unprovoked seizures. One third of these patients are refractory to the currently available therapy. In line with this view, it is important to elucidate the mechanisms involved in seizures, aiming to develop novel pharmacological treatments and/or approaches for the existing ones. It has been established that inflammatory mediators, markedly prostaglandin E_2 ($PGE_{2)}$, play a relevant facilitatory role in the etiopathogenesis of seizures. Considering that EP1 and EP3 receptors activation exacerbates PTZ-induced seizures, in this study we evaluate wether another acute model of seizures, with a different mechanism, such as kainic acid (KA), shares the same facilitatory effects. The effect of EP1 and EP3 ligands on KA-induced seizures was assessed by subcutaneous administration of their agonists (ONO-DI-004 and ONO-AE-248, respectively; 10 µg/kg) and antagonists (ONO-8713 and ONO-AE3-240, respectively; 10 µg/kg). Ratifying PTZ model findings, EP1 and EP3 receptor blocking attenuates KA-induced seizures, whilst their activation facilitate seizures elicited by KA. Moreover, EP1 and EP3 agonist administration, at a non-effective dose for generate ictal activity, prevented the respective antagonists effects. Notwithstanding, the correlation previously observed between a diminished activity of Na^+,K^+-ATPase, a crucial enzyme for ionic gradients maintenance, and presence of PTZ-induced seizures was not reproduced with KA model. Interestingly, Na^+,K^+-ATPase activity was increased in mice cerebral cortex and hippocampus after KA injection, which positively correlated with *status epilepticus* presence at sacrifice. Thus, whilst EP1 and EP3 agonists exacerbate Na^+,K^+-ATPase activity increasing, their respective antagonists not only prevent, but also decrease this ATPase activity. In accordance, antagonists effects were prevented by a non-effective agonist dose previously administrated. Therefore, in this studywe presented a clear role for EP1 and EP3 receptors in seizures, suggesting that these receptors are relevant novel targets for anticonvulsants development.

Key words: epilepsy, PGE_2, EP receptors, kainic acid, Na^+,K^+-ATPase

Introduction

Epilepsy is the second most common neurological disorder after stroke (Engel & Pedley, 2008; McNamara, 1999). About 30% of affected patients are refractory to pharmacological treatments currently available (Perucca et al., 2007), and the search for novel targets is a challenge in this disorder therapy. Current evidences support the involvement of inflammatory and immune processes in the etiopathogenesis of seizures (Vezzani & Granata, 2005). Prostaglandin E_2 (PGE_2) is a crucial mediator of responses to illness synthesized by COX-2 pathway in the brain (Chen & Bazan, 2005; Milatovic et al., 2011), and is directly related to inflammatory responses (Phillis et al., 2006). COX-2 is constitutively expressed and also markedly induced in neurons within an hour after seizures (Marcheselli & Bazan, 1996).

In the CNS, PGE_2 has been implicated in the regulation of body temperature and sleep–wake activity, and is involved in hyperalgesic responses as part of sickness behavior (Yamagata et al., 1993; Gerozissis et al., 1995; Boulant et al., 1997; Ivanov & Romanovsky, 2004). PGE_2 also plays an important role in dynamically maintaining membrane excitability, synaptic transmission, integration, and plasticity in the hippocampus. In fact, significant reduction of the membrane input resistance and frequency of firing has been found during endogenous PGE_2 depletion in hippocampal CA1 pyramidal neurons. Such a decrease of membrane excitability is reversed by the exogenous application of PGE_2 (Chen & Bazan, 2005). cAMP-PKA and PKC pathways mediate synaptic modulation PGE_2-induced in rat hippocampal neurons, once the excitatory postsynaptic potential was eliminated by blocking both PKA and PKC activities (Chen & Bazan, 2005). Accordingly, PGE_2 facilitates PTZ- (Oliveira et al., 2008b) and methylmalonic-induced seizures (Salvadori et al., 2012), meanwhile anti-PGE_2 antibodies attenuate seizures elicited by PTZ (Oliveira et al., 2008b). The multiple biological responses exerted by PGE_2 are mediated by G-protein-coupled E-prostanoid receptors (EP1,

EP2, EP3, and EP4) (Furuyashiki & Narumiya, 2009), which present different structures and are coupled to different signaling pathways (Tsuboi et al., 2002). Through EP1, EP3 and EP4 receptor-mediated activation of PKA and PKC, PGE_2 facilitates phosphorylation of Na^+,K^+-ATPase (EC 3.6.3.9) of the alpha subunit, decreasing its activity (Oliveira et al., 2009).

Na^+,K^+-ATPase is a heterodimeric integral membrane protein responsible for maintaining the homeostatic ionic equilibrium in almost all tissues, including CNS (Kaplan, 2002). The impairment of its activity results in neurotoxicity (Benarroch, 2011). Accordingly,various neurologic disorders are associated with impaired Na^+,K^+-ATPase activity, including epilepsy, reflecting that the pump plays a fundamental role in maintenance of neuronal excitability, synaptic homeostasis, and cell volume regulation (Benarroch, 2011).

It has been shown the relevant role of EP1 and EP3 receptors in PTZ-induced seizures and in Na^+,K^+-ATPase activity modulation, in rats and mice (Oliveira et al., 2008; Oliveira et al., 2009; Banderó et al., *manuscript I*). Additionally, it seems plausible that PGE_2 (or COX-2 inhibitors) may have pro- or anticonvulsant action, depending on the seizure model used (Claycomb et al., 2012, Salvadori et al., 2012). In line with this view, we investigated the involvement of EP1 and EP3 receptors in kainic acid (KA)-induced seizures, and whether these receptors modulate Na^+,K^+-ATPase activity in mice cerebral cortex and hippocampus after KA exposure.

Materials and Methods

Animals and Reagents

Adult male Swiss mice (25-35 g) maintained under controlled light and environment (12:12 h light–dark cycle, 24±1 °C, 55% relative humidity) with free access to food (Supra™, Santa Maria, Brazil) and water were used. All experimental protocols were designed aiming to keep

the number of animals used to a minimum, as well as their suffering. These were conducted in accordance with national and international legislation (guidelines of Brazilian Council of Animal Experimentation – CONCEA – and of U.S. National Institute of Health Guide for the Care and Use of Laboratory Animals – NIH Publications Nº 80-23, revised 1996), and with the approval of the Ethics Committee for Animal Research of the Federal University of Santa Maria (process number 078/2010).

ONO-8713 (an EP1 antagonist), ONO-DI-004 (an EP1 agonist), ONO-AE3-240 (an EP3 antagonist), and ONO-AE- 248 (an EP3 agonist), were generously donated by Ono Pharmaceutical Co. (Osaka, Japan). ONO-8713, ONO-DI-004, ONO-AE3-240, and ONO-AE-248 were dissolved in dimethylsulfoxide (DMSO) and then diluted with sterile saline, in such a way that DMSO concentration did not exceed 1%. KA and all other reagents were purchased from Sigma (St. Louis, MO, USA).

Surgical protocol and EEG recordings

All animals were anesthetized with ketamine (5 mg/kg) and xylazine (50 mg/kg) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, two screw electrodes were placed over the right and left parietal cortices (coordinates in mm: AP -4.5 and L 2.5), along with a ground lead positioned over the nasal sinus (Franklin & Paxinos, 2008). The electrodes were connected to a multipin socket for electroencephalogram (EEG) recordings, and were fixed to the skull with dental acrylic cement. Chloramphenicol (200 mg/kg, i.p.) was administered immediately before the surgical procedure. After surgery, all mice received a single subcutaneous (s.c.) injection of 0.01 mg/kg buprenorphine hydrochloride for amelioration of pain.

After 5-7 days after surgery, the effect of EP ligands on KA-induced seizures was assessed. Mice were habituated for at least 10 minutes and connected to the lead socket of a swivel,

which was connected to a digital encephalographic equipment (Neuromap EQSA260, Neurotec, Brazil) inside a Faraday's cage. Routinely, a 10 min baseline recording was obtained to establish an adequate control period. After this period, ONO-8713 (10 µg/kg), ONO-DI-004 (10 µg/kg), ONO-AE3-240 (10 µg/kg), ONO-AE-248 (10 µg/kg), or their respective vehicle (1% DMSO in saline) were administrated subcutaneously. Animals were injected with KA (20 mg/kg, i.p.) 30 minutes after antagonist / agonist administration and followed up for 120 min after KA administration for the appearance of seizures, by electrographic and behavioral methods. Latency to myoclonic jerks and tonic-clonic seizures were recorded in seconds. We have attributed a cut-off time of 7200 seconds for those KA-injected animals that did not present EEG seizures during the observation period, for statistical purposes. Doses and time elapsed between drug injection and KA injections were selected based on pilot experiments. EEG signals were amplified, filtered (0.1 to 70.0 Hz, bandpass), digitalized (sampling rate 256 Hz) and stored in a PC for off-line analysis, as described below. Seizures were defined by the occurrence of ictal episodes characterized by the following alterations in the recording leads: spikes (≥ 2 X baseline) plus slow waves, multispikes (≥ 2X baseline, ≥ 3 spikes/complex) plus slow waves, multiple sharp waves (≥ 2X baseline) in long spindle episodes (≥ 5 s) or major seizure (repetitive spikes plus slow waves, ≥ 5 sec) (Oliveira et al., 2008a). No significant behavioral changes were observed during EEG seizures except for freezing and stereotypies. Rhythmic scratching of the electrode headset rarely caused artifacts, which were easily identified and discarded.

Na^+, K^+ - ATPase activity measurements

Immediately after the EEG recordings, the animals were sacrificed. The effect of EP1 and EP3 ligands and of KA on Na^+,K^+-ATPase activity was measured. Cerebral cortices and hippocampi were dissected, weighted and immediately frozen at -80ºC. On the experimental

day, each area was taken in separate and gently homogenized (7-10 strokes) in ice-cold 10 mM Tris-HCl (pH 7.4) for Na^+,K^+-ATPase activity determination (Wyse, et al. 2000). Briefly, the assay medium consisted of 30 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$ and 50 μg of protein in the presence or absence of ouabain (1 mM), in a final volume of 350 μL. The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 5 mM. After 30 min, the reaction was stopped by the addition of 70 μL of 50 % (w/v) trichloroacetic acid. Appropriate controls were included in the assay for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified by the colorimetric method described by Fiske & Subbarow (1925), using KH_2PO_4 as reference standard. Specific Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nmol Pi/mg protein/min. Protein content was measured colorimetrically by the Bradford (1976) method using bovine serum albumin (1 mg/ml) as standard.

Statistical analyses

Latencies to myoclonic jerks and to tonic-clonic seizures were analysed by Kruskall-Wallis tests. Post hoc analysis was carried out by nonparametric Dunn's multiple comparison test, when indicated. Data are presented as median and interquartile ranges. Total time spent in seizures, mean amplitude of EEG recordings and Na^+,K^+-ATPase activity were analysed by one- or two-way ANOVA followed by Bonferroni's test, depending on the experimental design. Correlation between *status epilepticus* at sacrifice and Na^+,K^+-ATPase activity was verified by Spearman's rank correlation coefficient. Data are expressed as mean + S.E.M. A probability of $P < 0.05$ was considered significant, and H and F values are shown only if $P < 0.05$.

Results

In order to evaluate the effect of EP1 ligands (ONO-DI-004 or ONO-8713, 10 µg/kg) on KA (20 mg/kg, i.p.)-induced seizures we measured the latency to generalized seizures (Fig 1A), total time spent in epileptic activity (Fig 1B), and number of ictal events elicited by this excitotoxin, with concomitant EEG recording (Fig 1C). Statistical analysis revealed that the EP1 antagonist ONO-8713 increased [$H(2) = 15.61$, $P < 0.05$, Fig. 1A], and that the EP1 agonist ONO-DI-004 decreased [$H(2) = 12.83$, $P < 0.05$, Fig. 1A] the latency to generalized seizures compared with the control group. Additionally, ONO-DI-004-treated mice spent more time in ictal activity [$F(2,16) = 8.196$, $P < 0.05$, Fig 1B], and presented more ictal events [$F(2,16) = 7.587$, $P < 0.05$, Fig 1C] than control animals. Accordingly, ONO-8713-treated animals spent less time seizing [$F(2,16) = 9.632$, $P < 0.05$, Fig 1B] and presented a less ictal events [$F(2,16) = 4.889$, $P < 0.05$, Fig 1C] than vehicle-treated animals. The mean amplitude (in µV) of EEG recordings before and after KA injection are shown in Figure 2. While the EP1 agonist ONO-DI-004 increased [$F(2,27) = 50.68$, $P < 0.05$, Fig 2A], the EP1 antagonist ONO-8713 [$F(2,27) = 20.36$, $P < 0.05$, Fig 2B], decreased mean amplitude of KA-induced ictal episodes, compared with vehicle group. Representative EEG recordings (from one hour after KA administration) of vehicle- (Fig 2C), ONO-DI-004- (Fig 2D) and ONO-8713-treated (Fig 2E) animals are shown. The protective effect of ONO-8713 (10 µg/kg, s.c.) against KA-induced seizures was prevented by a non-effective dose administration of its agonist (ONO-DI-004, 3 µg/kg, s.c.), measured as latency to generalized seizures [$H(3) = 8.680$, $P < 0.05$, Fig 3A], total time spent in ictal activity [$F(1,8) = 49.21$, $P < 0.05$, Fig 3B], but not as number of seizures ($P > 0.05$, Fig 3C).

Na^+,K^+-ATPase activity was measured in cortical and hippocampal homogenates of mice injected with ONO-DI-004 or ONO-8713, and subjected (or not) to KA injection, as described

above. Kainic acid injection increased Na^+,K^+-ATPase activity in cortical and hippocampal homogenates of vehicle-treated animals (Fig 4). ONO-DI-004 slightly, but significantly, potentiated KA-induced increase of Na^+,K^+-ATPase activity in the hippocampus [$F(1,17) = 15.01$, $P < 0.05$, Fig 4A]. The EP1 antagonist ONO-8713 decreased KA-induced increase of Na^+,K^+-ATPase activity in cerebral cortex and hippocampus [$F(1,26) = 4.771$, $P < 0.05$, for cerebral cortex and $F(1,26) = 9.742$, $P < 0.05$, for hippocampus, Fig 4B]. ONO-DI-004 (3 µg/kg, s.c.) *per SE* did not modify Na^+,K^+-ATPase activity in absence ($P > 0.05$, Fig 5A), or presence of KA ($P > 0.05$, Fig 5B) in cerebral cortex and hippocampus. Moreover, the non-effective dose of EP1 agonist ONO-DI-004 prevented the expected decrease on Na^+,K^+-ATPase activity after EP1 antagonist ONO-8713 (10 µg/kg, s.c.) administration in cortical and hippocampal homogenates [$F(1,27) = 4.218$, $P < 0.05$, for cerebral cortex and $F(1,27) = 13.25$, $P < 0.05$, for hippocampus, Fig 5B].

Figure 6 shows the effect of the EP3 agonist ONO-AE-248 (10 µg/kg, s.c.) and of the EP3 antagonist ONO-AE3-240 (10 µg/kg, s.c.) on KA-induced seizures (20 mg/kg, i.p.). Statistical analysis (Kruskall-Wallis test, followed by nonparametric Dunn's multiple comparison test) revealed that while the EP3 antagonist increased, the EP3 agonist decreased latency to generalized seizures [$H(2) = 12.5$, $P < 0.05$, Fig. 6A], compared with control group. While EP3 agonist-treated animals spent more [$F(2,12) = 9.00$, $P < 0.05$, Fig 6B], EP3 antagonist-treated animals spent less [$F(2,12) = 20.02$, $P < 0.05$, Fig 6B] time in ictal activity. In addition, EP3 agonist- and EP3 antagonist-treated animals presented respectively more [$F(2,12) = 5.83$, $P < 0.05$, Fig 6B] and less [$F(2,12) = 4.50$, $P < 0.05$, Fig 6B] ictal episodes than vehicle-treated ones. The EP3 agonist ONO-AE-248 increased [$F(2,21) = 34.34$, $P < 0.05$, Fig 7A] mean amplitude (in µV) of KA-induced ictal traces. Accordingly, the EP3

antagonist ONO-AE-240 decreased [F(2,21) = 109.0, $P < 0.05$, Fig 7B] mean amplitude of ictal recordings compared with vehicle group. Figure 7 shows representative EEG traces obtained one hour after KA administration, for vehicle (C), ONO-AE-248 (D) and ONO-AE3-240 (E).

ONO-AE-248 (3 µg/kg, s.c.) prevented ONO-AE3-240 (10 µg/kg, s.c.)-induced protection against KA-induced seizures, measured as the latency to generalized seizures [H(3) = 49.28, $P < 0.05$, Fig 8A], total time spent in ictal activity [F(1,9) = 57.21, $P < 0.05$, Fig 8B], and number of seizures [F(1,9) = 7.715, $P < 0.05$, Fig 8C].

Figure 9 shows the effect of the EP3 agonist ONO-AE-248 (A) and of the EP3 antagonist ONO-AE3-240 (B), on Na^+,K^+-ATPase activity of vehicle- or KA-injected mice. The EP3 agonist potentiated KA-induced increase of Na^+,K^+-ATPase activity in hippocampal [F(1,14) = 22.23, $P < 0.05$, Fig 9A], but not in cortical homogenates ($P > 0.05$). Notwithstanding, the EP3 antagonist not only prevented the KA-induced increase of Na^+,K^+-ATPase activity, but also significantly decreased this enzyme activity in both cerebral structures [F(1,13) = 13.12, $P < 0.05$, for cerebral cortex and F(1,13) = 17.66, $P < 0.05$, for hippocampus, Fig 9B]. Additionally, the non-effective dose of EP3 agonist (ONO-AE-248, 3 µg/kg, s.c.), which did not present *per SE* effect on Na^+,K^+-ATPase activity in absence or presence of KA (Fig 10A and B, respectively), prevented the EP3 antagonist-induced decrease of Na^+,K^+-ATPase activity in cerebral cortex and hippocampal homogenates [F(1,26) = 5.658, $P < 0.05$, for cerebral cortex and F(1,26) = 6.633, $P < 0.05$, for hippocampus, Fig 10B]. Figure 11 shows a highly significant positive correlation between *status epilepticus* at sacrifice and Na^+,K^+-ATPase activity (r_s= 0.762, $P < 0.0001$).

Discussion

The main findings of the current study are that while EP1 and EP3 receptor agonists (ONO-DI-004 and ONO-AE-248, respectively) facilitate, antagonists of these receptors (ONO-8713 and ONO-AE3-240, respectively) decrease KA-induced seizures. These results strongly support a role for these receptors in KA-seizures, as far as the inhibitory effects of the antagonists are prevented by non-effective doses of the respective agonists. Accordingly, the same antagonists and agonists, respectively, attenuate and facilitate PTZ-induced seizures (Banderó et al., *manuscript I*). Furthermore, it has been shown that other EP1 and EP3 antagonists (SC-19220 and L-826266, respectively) also prevent seizures elicited by PTZ (Oliveira et al., 2008b). It is important to point out that structurally different antagonists shared the same pharmacological properties, suggesting that their common mechanism of action (EP1 and EP3 receptor blockade) underlie the currently reported anticonvulsant effect. Although the mechanism by which EP1 and EP3 antagonists decrease seizures is unknown, previous studies have suggested that EP1 and EP3 antagonists maintain Na^+,K^+-ATPase activity in the brain of PTZ-injected animals, a finding that is associated with seizure protection (Oliveira et al., 2008a; Oliveira et al., 2009).

In the current study kainic acid administration caused seizures and increased Na^+,K^+-ATPase activity in cerebral cortex and hippocampus. While the systemic administration of EP1 and EP3 receptor antagonists prevented, EP1 and EP3 agonists potentiated KA-induced increase of Na^+,K^+-ATPase activity in both cerebral structures.

Na^+,K^+-ATPase has been considered the main regulator of intracellular ion homeostasis, being responsible for generating and maintaining transmembrane ionic gradients that support regulation of cell volume, pH maintenance, generation of action potentials and secondary active transport (Kaplan, 2002). Reduced Na^+,K^+-ATPase activity has been reported in the

cerebral tissue of epileptic patients (Rapport et al., 1975; Grisar et al. 1992). Additionally, decreased glial Na^+,K^+-ATPase activity has been reported in epileptogenic zones in experimental models of focal epilepsy (Grisar et al., 1992).

It has been known that PTZ injection decreases Na^+,K^+-ATPase activity in the striatum, cerebral cortex and hippocampus (Fighera et al. 2006; Banderó et al. *manuscript I*), and suggested that such a decreased enzyme activity could contribute to both seizure generation and termination (Krishnan et al., 2012). We have found that Na^+,K^+-ATPase activity is increased two hours after KA injection, a finding that agrees with those obtained by Bortolatto and colleagues (2011), who have observed an increased Na^+,K^+-ATPase activity in rat hippocampal and cortical homogenates three hours after KA injection. This is also in agreement with the study of Sztriha and colleagues (1987), who have found increased Na^+,K^+-ATPase activity in the parietal cortex, hippocampus, and thalamus 3 hours following systemic KA injection. However, a decreased enzyme activity has been observed 6 and 24 hours after KA administration, a period that coincides with edema formation and neuropathological damage in hippocampus and thalamus (Sztriha et al., 1987; Fariello et al.,1989). It has also been shown that Na^+,K^+-ATPase activity is diminished on the third and fifth days after KA injection in the hippocampus and cerebral cortex, respectively (Vitezić et al., 2008). This is in agreement with the finding that chronic KA injection decreases Na^+,K^+-ATPase mRNA levels in rat hippocampus (Anderson et al., 1994). Therefore, current evidence supports a biphasic effect of KA on Na^+,K^+-ATPase activity along time. In this regard, it is particularly interesting that, computing all data obtained in the current study, a highly significant positive correlation between *status epilepticus* at sacrifice and Na^+,K^+-ATPase activity was found (Fig. 11, r_s= 0.762, $P < 0.0001$). Moreover, computation of correlation values in single groups (where n>1) were also significant. Therefore, one might argue that increased Na^+,K^+-ATPase activity may be associated with *status epilepticus* and full activation of kainate receptors.

Kainate receptor activation increases intracellular sodium concentrations ($[Na^+]i$) due to repeated discharges and excessive prolonged depolarization in response to KA (Sztriha et al., 1987). In fact, it has been long known that increased $[Na^+]i$ activates Na^+,K^+-ATPase (Vaillend et al., 2002; Anderson et al., 2010). In neurons, the α-3 Na^+,K^+-ATPase isoform rapidly restores $[Na^+]i$, controlling membrane potential (Azarias et al., 2013). In line with this view, it has been reported that Na^+,K^+-ATPase activity increases after electrical stimulation (Bignami et al., 1966; Harmony et al., 1968), and during the chronic period of the pilocarpine epilepsy model (Fernandes et al., 1996; Kinjo et al., 2007), two conditions associated with increased electrical activity, and $[Na^+]i$ and $[K^+]o$ accumulation. In what concerns the effect of $[Na^+]i$ accumulation on cell membrane depolarization, there are *in silico* and *in vitro* findings supporting that $[Na^+]i$ increases above 23 mM lead neurons to a silent state, corresponding to depolarization block (Krishnan & Bazhenov, 2011, Azarias et al., 2013). Therefore, maintenance of Na^+,K^+-ATPase activity (and sodium concentrations below 23 mM) is necessary for maintaining membrane depolarization capacity, *a sine qua non* condition to elicit and maintain seizures. This is in agreement with the fact that kainic acid elicits long-lasting seizures. Additionally, α-3 Na^+,K^+-ATPase isoform inhibition nearly abolishes the capacity of dendrites to restore 20-40 mM $[Na^+]i$ increases (Azarias et al., 2013). Furthermore, it has been shown that increased $[Ca^{++}]i$ also regulates Na^+,K^+-ATPase activity (Ishii & Takeyasu, 1995; Blanco & Mercer, 1998; Kim et al., 2007), and the α-3 isoform reduces to half of its maximum activity if $[Ca^{++}]i$ exceeds 5–10 μM in the Na^+,K^+-ATPase surrounding medium (Blanco & Mercer, 1998).

In accordance with our findings, SC-19220 or L-826266 did not alter Na^+,K^+-ATPase activity *in vitro per se* (Oliveira et al., 2009). The fact that neither agonists nor antagonists of EP1 and EP3 receptors alter Na^+,K^+-ATPase activity might suggest that there is no direct molecular/metabolic link between these receptors and Na^+,K^+-ATPase *in vivo*. However, it

has been shown that incubation of hippocampal slices with SC-19220 or L-826266 prevents PGE_2-induced decrease of Na^+,K^+-ATPase activity (Oliveira et al., 2009). Therefore, Na^+,K^+-ATPase activity alterations in seizure states seems to be determined by the form of seizure induction (including mechanism of action of the convulsant) and/or occurrence of *status epilepticus* (probably due the balance of $[Ca^{++}]i$ and $[Na^+]i$ and triggering of post-translational regulatory mechanisms, which persist after tissue homogenization) at sacrifice, and its synergism with PGE_2 pathway activation. In summary, our data strongly support a role for EP1 and EP3 receptors in *status epilepticus*.

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FIGURE 1

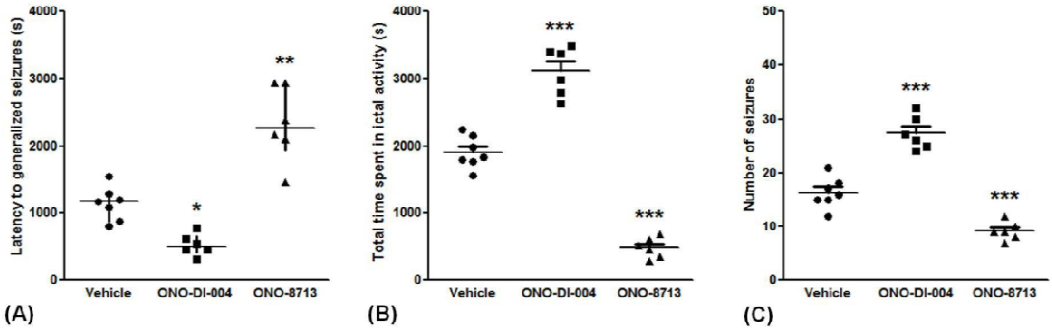

Figure 1 – Effect of EP1 receptor agonist and antagonist (ONO-DI-004 and ONO-8713, respectively; 10 µg/kg, s.c.) on KA-induced seizures (20 mg/kg, i.p.). (A) Latency to the first generalized seizure. (B) Total time spent in ictal activity. (C) Number of ictal events. Data expressed as median and interquartile range (A), and mean + SEM (B and C), for n = 6-7 in each experimental group. A probability of $P < 0.05$ was considered significant. * $P < 0.05$, and *** $P < 0.001$, when compared with vehicle group.

FIGURE 2

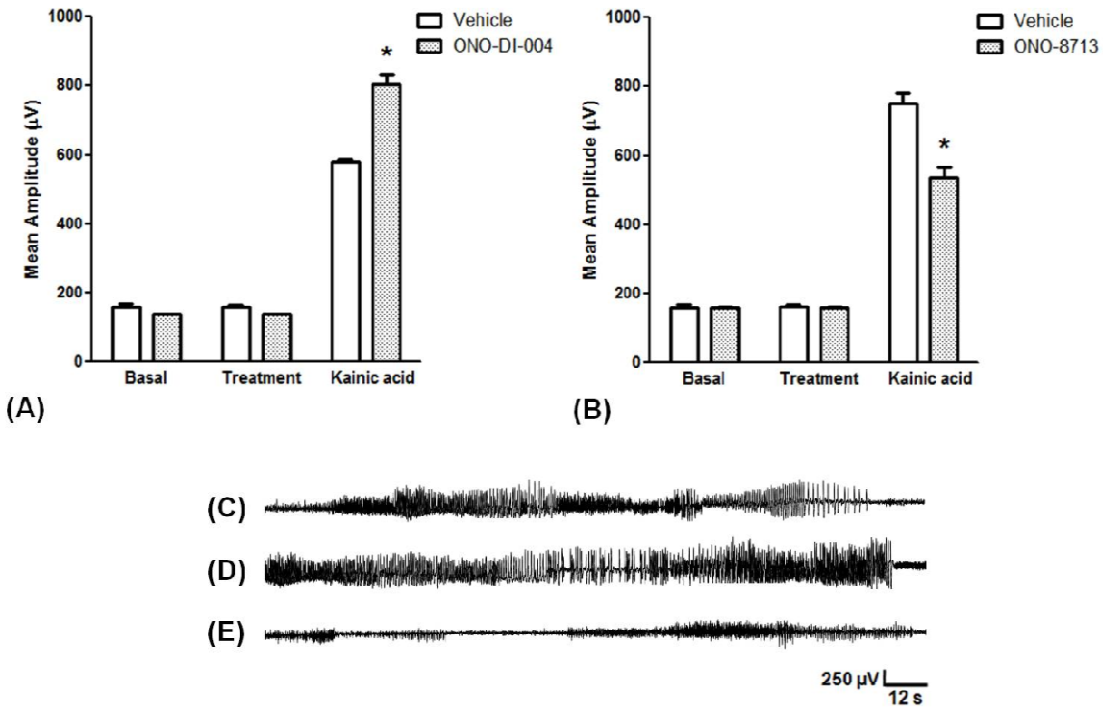

Figure 2 – Effect of EP1 receptor agonist (A) and antagonist (B) (ONO-DI-004 and ONO-8713, respectively; 10 µg/kg, s.c.) on the mean amplitude of EEG recordings in the parietal cortex of animals injected with KA (20 mg/kg, i.p.). Mean amplitude of EEG recordings was analyzed by two-way ANOVA followed by the Bonferroni's test and expressed as mean + S.E.M., for n = 6-7 in each group. * $P < 0.05$, when compared with respective vehicle. Representative electrocorticographic recordings of animals after KA injection are represented as follows: (C) vehicle, (D) ONO-DI-004, and (E) ONO-8713. EEG traces represent one hour after KA injection, and the y-axis (amplitude) and x-axis (time) calibration bar is the same for all traces.

FIGURE 3

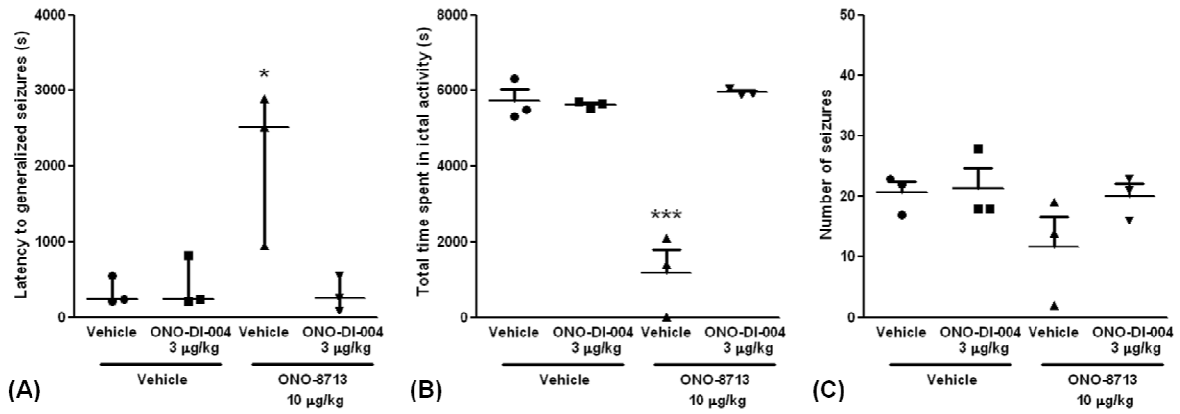

Figure 3 – Effect of ONO-DI-004 (3 µg/kg, s.c.) administration, followed or not by ONO-8713 (10 µg/kg, s.c.), on KA-induced seizures, measured as latency to generalized seizures (A), total time spent in ictal activity (B), and number of seizures (C). ONO-DI-004 (3 µg/kg, s.c.) prevented the protective effect of ONO-8713 (10 µg/kg, s.c.) against KA-induced seizures. Data are expressed as median and interquartile range (A), and mean + S.E.M. (B, C) for $n = 3$ in each group. A probability of $P < 0.05$ was considered significant. * $P < 0.05$, and *** $P < 0.001$, when compared with vehicle + vehicle group.

FIGURE 4

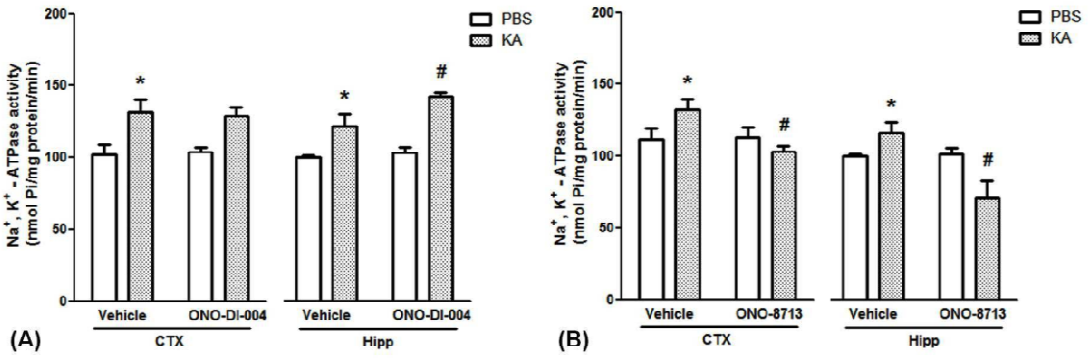

Figure 4 – ONO-DI-004 (10 µg/kg, s.c.) intensify (A) and ONO-8713 (10 µg/kg, s.c.) reverts (B) KA-induced increase of Na^+,K^+-ATPase activity in cerebral cortex and hippocampus homogenates in mice. Data are presented in nmol Pi/mg protein/min, as mean + S.E.M., for n = 5 in each group. The Na^+,K^+-ATPase activity was analyzed by two-way ANOVA followed by the Bonferroni's test. * Indicates a significant difference within group compared with the respective control; # Indicates a significant difference between groups compared with the respective control ($P < 0.05$).

FIGURE 5

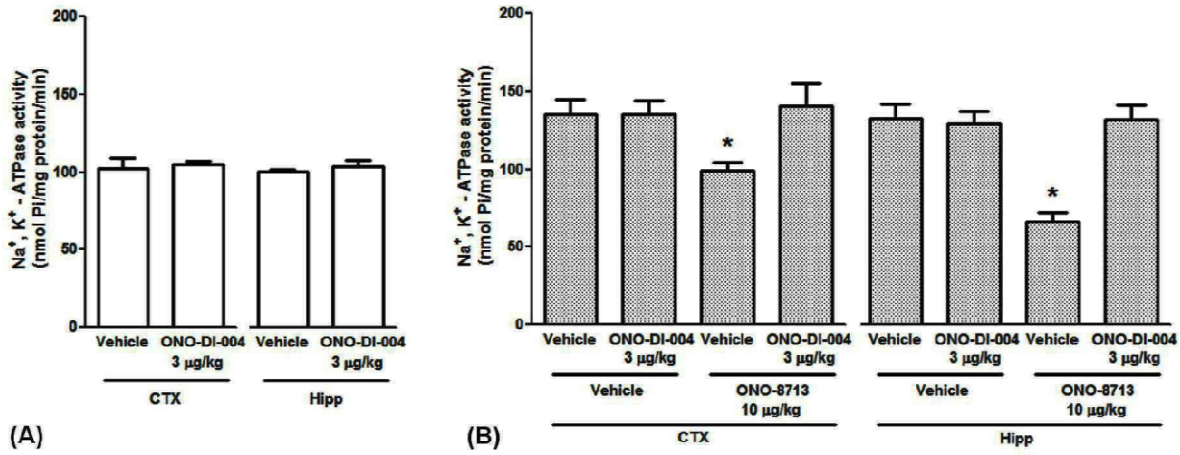

Figure 5 - (A) ONO-DI-004 (3 µg/kg, s.c.) does not present *per SE* effect on Na^+,K^+-ATPase ($P > 0.05$ by Student's t-test, for $n = 5 - 6$). (B) Two-way ANOVA followed by the Bonferroni's test revealed that ONO-DI-004 (3 µg/kg, s.c.) prevented ONO-8713 (10 µg/kg, s.c.) effect on Na^+,K^+-ATPase activity induced by KA in cerebral cortex and hippocampus homogenates in mice. Data are presented in nmol Pi/mg protein/min, as mean + S.E.M., for $n = 6 - 9$ in each experimental group.

FIGURE 6

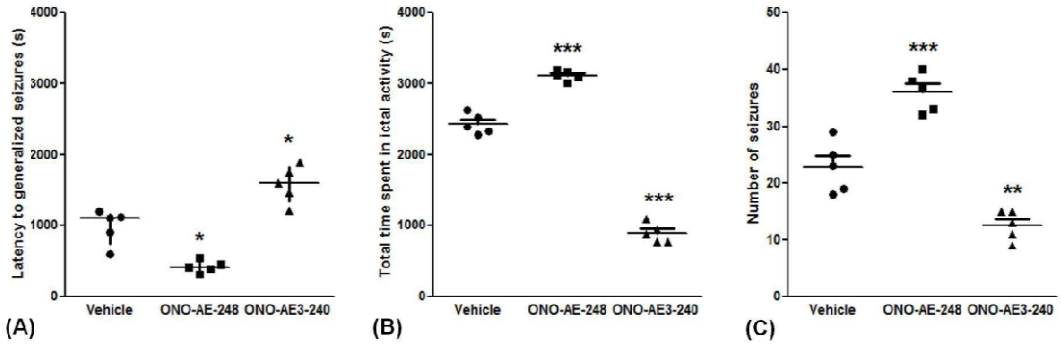

Figure 6 - Effect of EP3 receptor agonist and antagonist (ONO-AE-248 and ONO-AE3-240, respectively; 10 µg/kg, s.c.) on KA-induced seizures (20 mg/kg, i.p.). (A) Latency to generalized seizures. (B) Total time spent in ictal activity. (C) Number of seizures. Data are expressed as median and interquartile range (A), and mean + SEM (B and C), for $n = 5$ in each experimental group. A probability of $P < 0.05$ was considered significant. * $P < 0.05$, when compared with vehicle group.

FIGURE 7

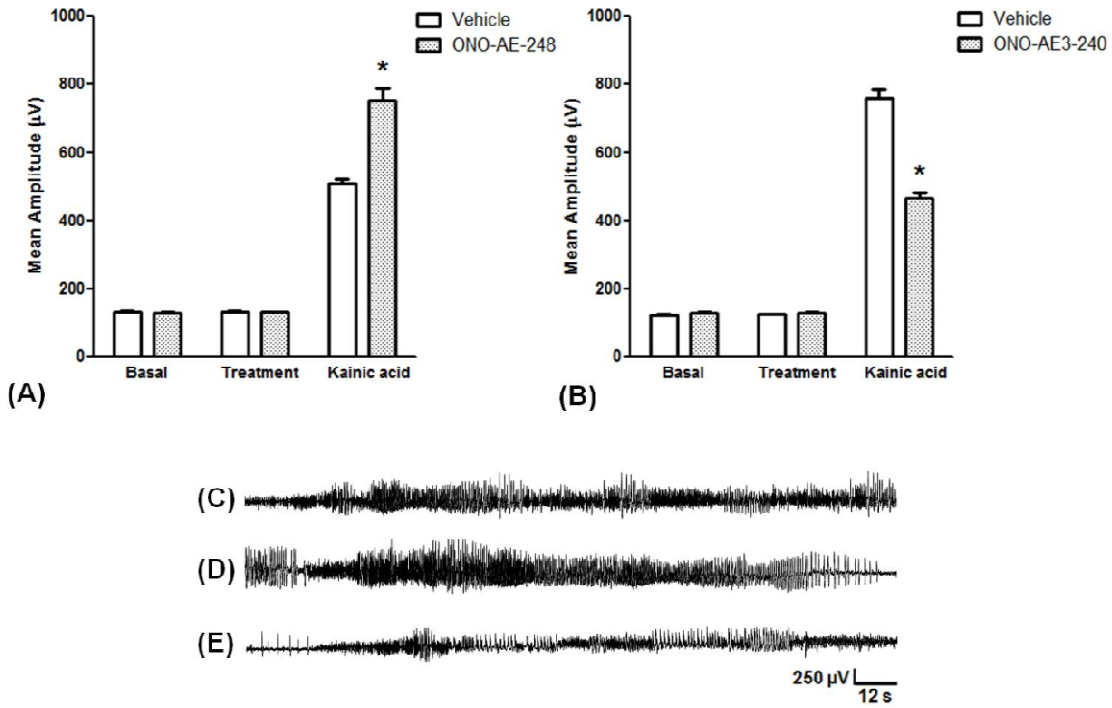

Figure 7 – Effect of EP3 receptor agonist (A) and antagonist (B) (ONO-AE-248 and ONO-AE3-240, respectively; 10 µg/kg, s.c.) on the mean amplitude of EEG recordings in the parietal cortex of animal injected with KA (20 mg/kg, i.p.). Mean amplitude of EEG recordings was analyzed by two-way ANOVA followed by the Bonferroni's test and expressed as mean + S.E.M., for $n = 5$ in each group. * $P < 0.05$, when compared with respective vehicle. Representative electrocorticographic recordings of animals after KA injection are represented as follows: (C) vehicle, (D) ONO-AE-248, and (E) ONO-AE3-240. EEG traces represent one hour after KA injection, and the y-axis (amplitude) and x-axis (time) calibration bar is the same for all traces.

FIGURE 8

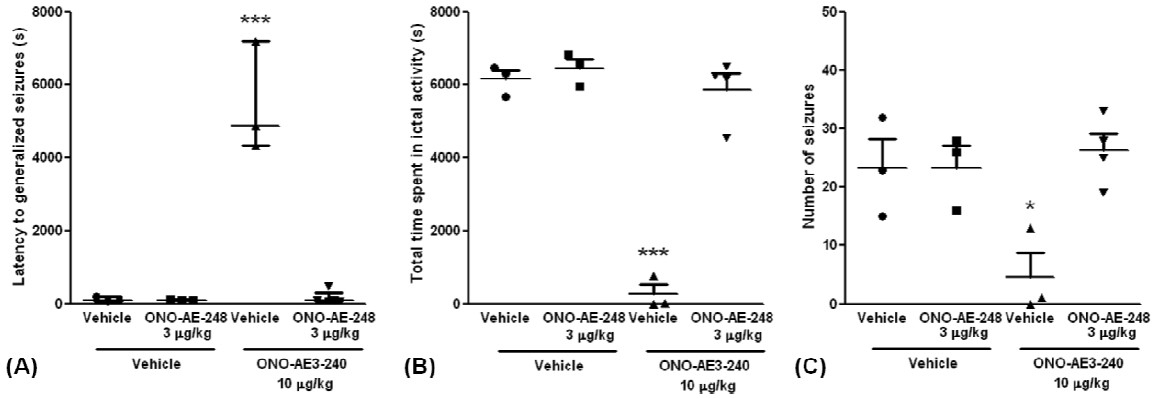

Figure 8 – Effect of ONO-AE-248 (3 µg/kg, s.c.) administration, followed or not by ONO-AE3-240 (10 µg/kg, s.c.), on KA-induced seizures, measured as latency to generalized seizures (A), total time spent in ictal activity (B), and number of seizures (C). ONO-AE-248 (3 µg/kg, s.c.) prevented the protective effect of ONO-AE3-240 (10 µg/kg, s.c.) against KA-induced seizures. Data are expressed as median and interquartile range (A), and mean + S.E.M. (B and C) for n = 3-5 in each group. A probability of $P < 0.05$ was considered significant. * $P < 0.05$, and *** $P < 0.001$, when compared with vehicle + vehicle group.

FIGURE 9

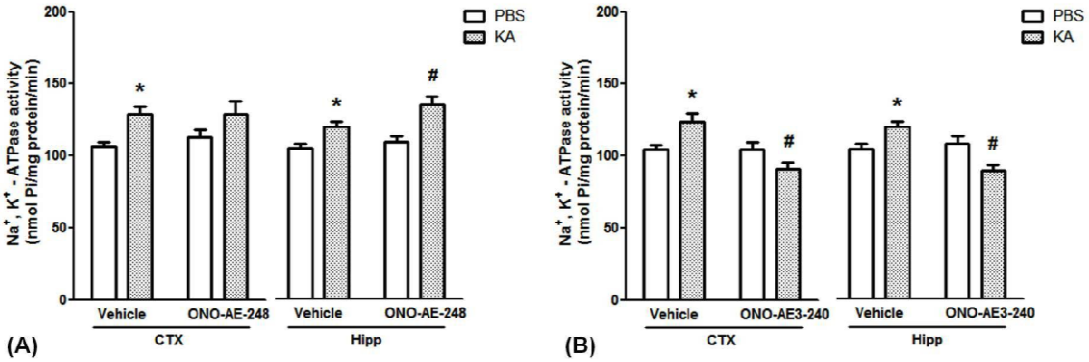

Figure 9 – ONO-AE-248 (10 µg/kg, s.c.) intensify (A) and ONO-AE3-240 (10 µg/kg, s.c.) reverts (B) KA-induced increase of Na^+,K^+-ATPase activity in cerebral cortex and hippocampus homogenates in mice. Data are presented in nmol Pi/mg protein/min, as mean + S.E.M., for n = 5 in each group. The Na^+,K^+-ATPase activity was analyzed by two-way ANOVA followed by the Bonferroni's test. * Indicates a significant difference within group compared with the respective control; # Indicates a significant difference between groups compared with the respective control ($P < 0.05$).

FIGURE 10

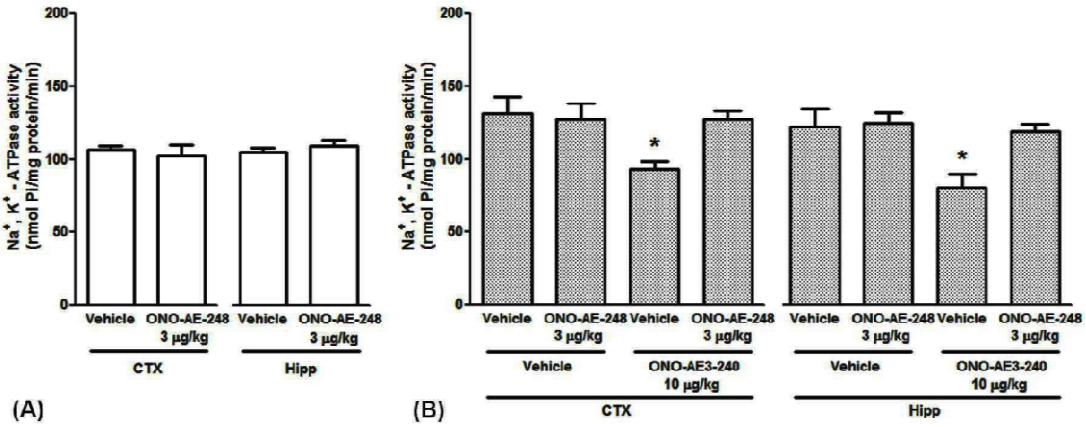

Figure 10 – (A) ONO-AE-248 (3 µg/kg, s.c.) does not present *per SE* effect on Na^+,K^+-ATPase ($P > 0.05$ by Student's t-test, for $n = 5$). (B) Two-way ANOVA followed by the Bonferroni's test revealed that ONO-AE-248 (3 µg/kg, s.c.) prevented ONO-AE3-240 (10 µg/kg, s.c.) effect on Na^+,K^+-ATPase activity induced by KA in cerebral cortex and hippocampus homogenates in mice. Data are presented in nmol Pi/mg protein/min, as mean + S.E.M., for $n = 6$ - 10 in each experimental group.

FIGURE 11

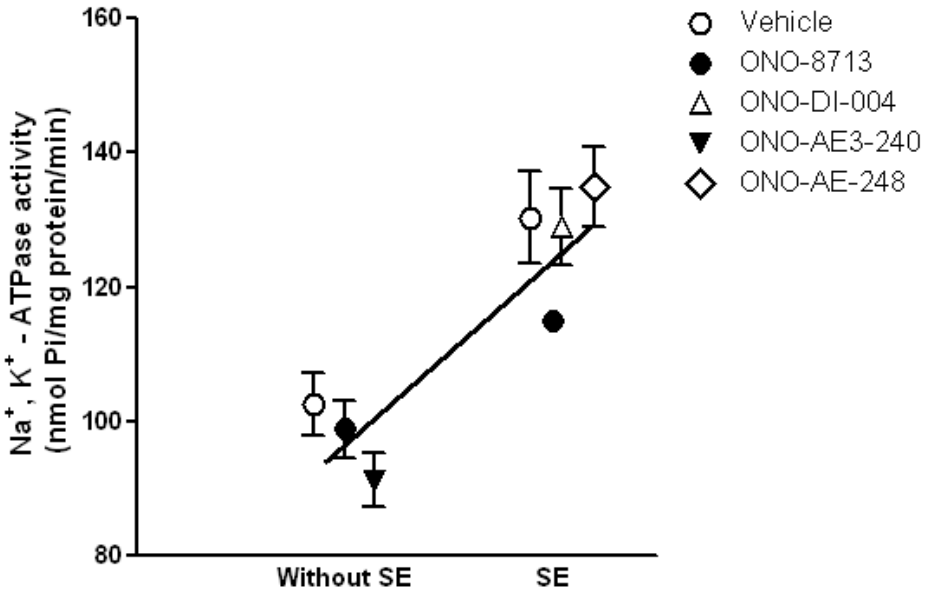

Figure 11 – Correlation plot of ranked data of KA-induced *status epilepticus* (SE) at sacrifice and Na^+,K^+-ATPase activity. Spearman's test revealed a highly significant positive correlation between SE at sacrifice and Na^+,K^+-ATPase activity (r_s= + 0.762, $P < 0.0001$), for ONO-8713 (EP1 antagonist, 10 µg/kg, s.c., n = 6), ONO-DI-004 (EP1 agonist, 10 µg/kg, s.c., n = 6), ONO-AE3-240 (EP3 antagonist, 10 µg/kg, s.c., n = 4), ONO-AE-248 (EP3 agonist, 10 µg/kg, s.c., n = 4). None animals from EP3 antagonist group presented SE at the sacrifice time. Data are presented in nmol Pi/mg protein/min, as mean ± S.E.M..

3 DISCUSSÃO

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O presente estudo mostra que o bloqueio dos receptores EP1 e EP3, induzido pela administração sistêmica dos antagonistas para estes receptores ONO-8713 e ONO-AE3-240, respectivamente, atenua as crises epilépticas induzidas por PTZ e KA. Conforme esperado do ponto de vista farmacológico, a administração sistêmica de ONO-DI-004 ou ONO-AE-248, respectivos agonistas dos receptores EP1 e EP3, facilita estas crises e, em doses não efetivas sobre a atividade ictal, previne os efeitos dos antagonistas nos modelos do PTZ e KA.

Além disso, este estudo mostrou que a administração de antagonistas EP1 ou EP3, *in vivo,* previnem a esperada diminuição da atividade da enzima Na^+,K^+-ATPase induzida por PTZ, *ex vivo*; e, que a administração dos respectivos agonistas intensifica este decréscimo.

Nossos experimentos também mostraram que a injeção de KA promove um aumento na atividade da Na^+,K^+-ATPase *per se*. A administração dos antagonistas para os receptores EP1 ou EP3 preveniu este efeito, enquanto que a administração dos agonistas o intensificou.

Os mecanismos pelos quais os antagonistas EP1 e EP3 reduzem as crises epilépticas ainda são desconhecidos. A hipótese que a ativação da Na^+,K^+-ATPase poderia ser um mecanismo convergente pelo qual os antagonistas EP1 e EP3 atenuam as convulsões induzidas por PTZ e KA não foi confirmada. Na verdade, a atividade da Na^+,K^+-ATPase variou em direções opostas, dependendo do agente convulsivante utilizado. Baseado nestes resultados, poderíamos assumir que a atividade da Na^+,K^+-ATPase não está envolvida na geração e/ou término das crises epilépticas, já que a atividade ictal ocorreu em ambos estados da enzima: ativado (quando KA foi usado como agente convulsivante) e, inibido (quando PTZ foi usado para gerar convulsões). Entretanto, a premissa de que a inibição da atividade da Na^+,K^+-ATPase seria linearmente correlacionada com a atividade ictal revelou-se incongruente (VAILLEND et al., 2002). Estudos *in vitro* and *in silico* demonstraram que o impacto das alterações da atividade da Na^+,K^+-ATPase depende do grau de inibição e das alterações de $[Na^+]_i$ e $[K^+]_o$ (KRISHNAN & BAZHENOV, 2011; AZARIAS et al., 2013). Enquanto uma fraca inibição da atividade facilitaria crises

epilépticas por reduzir o limiar de despolarização, um excessivo aumento na $[Na^+]_i$ desencadearia mecanismos de inativação do influxo de sódio e refratariedade neuronal. Além disso, uma forte inibição enzimática poderia, subsequentemente, desfazer os gradientes iônicos comprometendo a função neuronal. Neste sentido, o segundo pressuposto de que diferentes agentes convulsivantes, com diferentes mecanismos de ação, poderiam induzir crises epilépticas por um mecanismo convergente também é igualmente vulnerável. Em outras palavras, é possível que a redução da atividade da Na^+,K^+-ATPase induzida pelo PTZ contribua para as crises epilépticas induzidas por este agente quimioconvulsivante, e que isso não tenha nenhuma relação com o achado experimental de que as convulsões induzidas por KA foram relacionadas com um aumento na atividade da Na^+,K^+-ATPase. De fato, o PTZ desencadeia eventos intracelulares que não são compartilhados pelo KA, como por exemplo um decréscimo na $[Ca^{++}]_o$ que precede as crises induzidas por PTZ (LEWEKE et al., 1990). Considerando que uma redução na $[Ca^{++}]_o$ indica um considerável aumento na $[Ca^{++}]_i$, poderíamos supor que os efeitos mediados pelo cálcio intracelular poderiam contribuir para a atividade ictal induzida por PTZ. Neste contexto, é interessante salientar que aumentos na concentração intracelular de cálcio estão associados com uma diminuição da atividade da Na^+,K^+-ATPase (BLANCO & MERCER, 1998) e fosforilação desta enzima pela PKC (CHENG et al., 1999). Em síntese, considerando todos os argumentos listados acima, nossos resultados não nos permitem confirmar ou excluir a hipótese que a atividade da Na^+,K^+-ATPase reduzida desempenhe um papel nas convulsões induzidas por PTZ e/ou que antagonistas EP1 e EP3 atenuem as convulsões induzidas por PTZ, por prevenir a inibição da Na^+,K^+-ATPase.

As crises epilépticas induzidas por KA, particularmente o SE, foram associadas com um aumento na atividade da Na^+,K^+-ATPase. Porém, o mecanismo pelo qual a estimulação dos receptores de KA aumenta a atividade desta enzima ainda não está esclarecido. Não obstante, a ativação dos receptores de KA aumenta as $[Na^+]_i$ em resposta às repetidas despolarizações (SZTRIHA et al., 1987). Embora a atividade da Na^+,K^+-ATPase tenha sido medida em um meio de incubação contendo uma concentração padrão de sódio (50 mM NaCl), é possível que um aumento de $[Na^+]_i$, *in vivo,* desencadeie mecanismos pós-traducionais regulatórios, os quais persistem após a homogeneização dos tecidos. Entretanto, também é possível que o KA tenha aumentado a atividade da Na^+,K^+-ATPase por outros

mecanismos. Por esta razão, estudos mais específicos devem ser realizados para definir se alterações pós-traducionais na enzima são, ou não, induzidas por KA.

Independentemente do mecanismo pelo qual o KA aumenta a atividade da Na^+,K^+-ATPase, está bem estabelecido que a Na^+,K^+-ATPase determina os gradientes iônicos através da membrana. Portanto, seria razoável supor que uma atividade enzimática aumentada seria necessária para que ocorressem despolarizações recorrentes em presença de estímulos adequados (por exemplo, um foco ictal). Se a ocorrência de crises epilépticas depende das despolarizações neuronais recorrentes e sincronização dos disparos, é bem possível que crises epilépticas induzidas por KA, particularmente o SE, dependam de uma atividade aumentada da Na^+,K^+-ATPase. O fato que o KA provoca crises de longa duração está de acordo com esta suposição. Concisamente, nossos dados sugerem um papel para os receptores EP1 e EP3 nas atividades ictais induzidas por PTZ e KA. Porém, ainda permanece a ser determinado se a atividade da Na^+,K^+-ATPase modula os mecanismos pelos quais os antagonistas EP1 e EP3 atenuam estas crises.

Em resumo, os receptores EP1 e EP3, administrados sistemicamente, modulam as convulsões induzidas por PTZ e KA, em camundongos. Com estes achados estendemos os prévios estudos do grupo em relação a atividade da Na^+,K^+-ATPase, PGE_2 e modulação das convulsões induzidas por PTZ, bem como obtivemos interessantes resultados em outro modelo agudo de crise epiléptica, o do KA, ratificando o envolvimento dos receptores EP1 e EP3 na epilepsia. Além disso, é importante ressaltar os achados em relação à modulação da atividade da Na^+,K^+-ATPase pelo KA *per* se, assim como pelos receptores EP1 e EP3, nas crises induzidas por este quimioconvulsivante.

4 CONCLUSÕES

4. CONCLUSÕES

De acordo com os resultados obtidos nesta tese, pode-se concluir que a ativação dos receptores EP1 e EP3 facilita e, o bloqueio, atenua as crises epilépticas induzidas por PTZ e KA, dois modelos experimentais considerados de alto valor preditivo na detecção de anticonvulsivantes eficazes na clínica. Entretanto, os mecanismos responsáveis por estes efeitos ainda são desconhecidos, bem como o papel da enzima Na^+,K^+-ATPase. Pois, especificamente:

1. A administração sistêmica dos agonistas para receptores EP1 e EP3, ONO-DI-004 e ONO-AE-248, respectivamente, facilitam as crises epilépticas induzidas por PTZ e KA;
2. A administração sistêmica dos antagonistas para receptores EP1 e EP3, ONO-8713 e ONO-AE3-240, respectivamente, atenuam as crises epilépticas induzidas por PTZ e KA;
3. A administração sistêmica dos agonistas EP1 e EP3, em doses não efetivas para gerar atividade ictal, previnem as ações dos respectivos antagonistas nas crises epilépticas induzidas por PTZ e KA, *in vivo*;
4. A atividade da enzima Na^+,K^+-ATPase está diminuída após a indução das crises epilépticas por PTZ e aumentada após SE induzido por KA;
5. Embora os efeitos do PTZ e KA sobre a atividade da enzima Na^+,K^+-ATPase são opostos, ambos são prevenidos pela administração sistêmica dos antagonistas EP1 e EP3. Estes resultados sugerem que os efeitos sobres a atividade da Na^+,K^+-ATPase dependem do agente quimioconvulsivante e, e podem influenciar de uma maneira indireta a gênese e/ou manutenção da atividade ictal.

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APÊNDICE

Resultados preliminares

“Effect of EP2 and EP4 agonists and antagonists on PTZ-induced seizures in mice”

Título em português

“Efeito dos agonistas e antagonistas EP2 e EP4 sobre as convulsões induzidas por PTZ em camundongos”

Effect of EP2 and EP4 agonists and antagonists on PTZ-induced seizures in mice

Material and Methods

Animals and Reagents

Adult male Swiss mice (25-35 g) maintained under controlled light and environment (12:12 h light–dark cycle, 24±1 °C, 55% relative humidity) with free access to food (Supra^{TM}, Santa Maria, Brazil) and water were used. All experimental protocols were designed aiming to keep the number of animals used to a minimum, as well as their suffering. These were conducted in accordance with national and international legislation (guidelines of Brazilian Council of Animal Experimentation – CONCEA – and of U.S. National Institute of Health Guide for the Care and Use of Laboratory Animals – NIH Publications Nº 80-23, revised 1996), and with the approval of the Ethics Committee for Animal Research of the Federal University of Santa Maria (process number 078/2010).

ONO-AE3-237 (an EP2 antagonist), ONO-AE1-259-01 (an EP2 agonist), ONO-AE3-208 (an EP4 antagonist), and ONO-AE1-329 (an EP4 agonist), were generously donated by Ono Pharmaceutical Co. (Osaka, Japan). ONO-AE3-237, ONO-AE1-259-01, ONO-AE3-208, and ONO-AE1-329 were dissolved in dimethylsulfoxide (DMSO) and then diluted with sterile saline, in such a way that DMSO concentration did not exceed 1%. PTZ and all other reagents were purchased from Sigma (St. Louis, MO, USA).

Surgical procedures

All animals were anesthetized with ketamine (5 mg/kg) and xylazine (50 mg/kg) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, two screw electrodes were placed over the right (ipsilateral) and left (contralateral) parietal cortices (coordinates in mm: AP -4.5 and L 2.5), along with a ground lead positioned over the nasal sinus (Franklin & Paxinos, 2008). The electrodes were connected to a multipin socket for electroencephalogram (EEG) recordings, and were fixed to the skull with dental acrylic cement. Chloramphenicol (200 mg/kg, i.p.) was administered immediately before the surgical procedure. After surgery, all mice received a single subcutaneous (s.c.) injection of 0.01 mg/kg buprenorphine hydrochloride for amelioration of pain.

Drug administration protocol and EEG recordings

The effect of EP ligands on PTZ-induced seizures was assessed 5-7 days after surgery. Mice were habituated for at least 10 minutes and connected to the lead socket of a swivel, which was connected to a digital encephalographic equipment (Neuromap EQSA260, Neurotec, Brazil) inside a Faraday's cage. Routinely, a 10 min baseline recording was obtained to establish an adequate control period. After this period, ONO-AE3-237 (10 µg/kg), ONO-AE1-259-01 (10 µg/kg), or their respective vehicle (1% DMSO in saline) were administrated subcutaneously. The animals were injected with PTZ (60 mg/kg, i.p.) 30 minutes after antagonist / agonist administration and followed up for 30 min after PTZ administration for the appearance of seizures, by electrographic and behavioral methods. The same pharmacological protocol, without EEG recording, was performed with EP4 ligands (ONO-AE3-208 (10 µg/kg), and ONO-AE1-329 (10 µg/kg), agonist and antagonist, respectively). These animals were followed up for the appearance of seizures by behavioral analysis.

Latency to myoclonic jerks and tonic-clonic seizures were measured, in seconds. We have attributed a cut-off time of 1800 seconds for those animals that did not present seizures during the observation period, for statistical purposes. Doses and time elapsed between drug injection and PTZ injections were selected based on pilot experiments. EEG signals were amplified, filtered (0.1 to 70.0 Hz, bandpass), digitalized (sampling rate 256 Hz) and stored in a PC for off-line analysis, as described below. Seizures were defined by the occurrence of ictal episodes characterized by the following alterations in the recording leads: spikes (≥ 2 X baseline) plus slow waves, multispikes (≥ 2X baseline, ≥ 3 spikes/complex) plus slow waves, multiple sharp waves (≥ 2X baseline) in long spindle episodes (≥ 5 s) or major seizure (repetitive spikes plus slow waves, ≥ 5 sec) (Oliveira et al., 2008a). Rhythmic scratching of the electrode headset rarely caused artifacts, which were easily identified and discarded.

Statistical analyses

Latencies to myoclonic jerks and to tonic-clonic seizures were analyzed by Kruskall-Wallis, followed by nonparametric Dunn's multiple comparison test, when indicated. Data are presented as median and interquartile ranges. Total time spent in seizures, mean amplitude of EEG recordings were analyzed by one- or two-way ANOVA followed by Bonferroni's test, depending on the experimental design. Data are expressed as mean + S.E.M. A probability of $P < 0.05$ was considered significant, and H and F values are shown only if $P < 0.05$.

Results

Figure 1 shows the effect of ONO-AE1-259-01 (EP2 agonist, 10 μg/kg, s.c.) and ONO-AE3-237 (EP2 antagonist, 10 μg/kg, s.c.) on PTZ-induced seizures (60 mg/kg, i.p.), measured as the latency to the first isolated myoclonic jerk, with concomitant spike activity on EEG recordings (A), latency to generalized tonic-clonic seizures (B), and total time spent in generalized seizures (C). Statistical analysis revealed that EP2 agonist (ONO-AE1-259-01) increased the latency to myoclonic jerks [$H(2) = 10.77$, $P < 0.05$, Fig. 1A], as well as to generalized seizures [$H(2) = 10.27$, $P < 0.05$, Fig. 1B]. EP2 antagonist (ONO-AE3-237) did not significantly alter these parameters ($P > 0.05$, Fig. 1A and 1B). Figure 1C shows that the EP2 agonist decreased total time spent in generalized seizures, when compared with vehicle-injected group [$F(2,15) = 4.009$, $P < 0.05$]. However, mice that received EP2 antagonist did not present any change ($P >0.05$, Fig. 1C). Quantitative analyses of EEG trace amplitudes before and after PTZ injection are shown in Figures 2A and 2B, for ONO-AE1-259-01 and ONO-AE3-237, respectively. Statistical analysis revealed a significant decrease in mean amplitude (in μV) of EEG ictal traces of ONO-AE1-259-01-treated animals, when compared with the vehicle group [$F(2,12) = 30.67$, $P < 0.05$]. Although ONO-AE3-237did not alter seizures onset, it significantly increased the mean amplitude (in μV) of EEG ictal traces, when compared with the vehicle group [$F(2,12) = 12.98$, $P < 0.05$]. Representative EEG patterns are presented in figures 2C, 2D and 2E (for vehicle-, ONO-AE1-259-01-, and ONO-AE3-237-treated groups, respectively), showing the ictal activity induced by PTZ injection. Generalized seizures appeared in the EEG recordings as the major seizure activity, and were characterized by 2–3 Hz high-amplitude activity (Fig. 2C, 2D and 2E). After the ictal discharge, postictal EEG suppression and slow waves were observed, correlating with behavioral catalepsy.

The effect of the EP4 agonist ONO-AE1-329 (10 μg/kg, s.c.) and of the EP4 antagonist ONO-AE3-208 (10 μg/kg, s.c.) on PTZ-induced seizures is shown in Figure 3. Statistical analysis revealed that the EP4 receptor antagonist increased the latency to myoclonic jerks [$H(2) = 9.625$, $P < 0.05$, Fig. 3A] and to generalized seizures [$H(2) = 9.953$, $P < 0.05$, Fig. 3B]. ONO-AE1-329- and ONO-AE3-208-injected animals spent similar time in generalized seizures compared with control group (Fig. 3C).

FIGURE 1

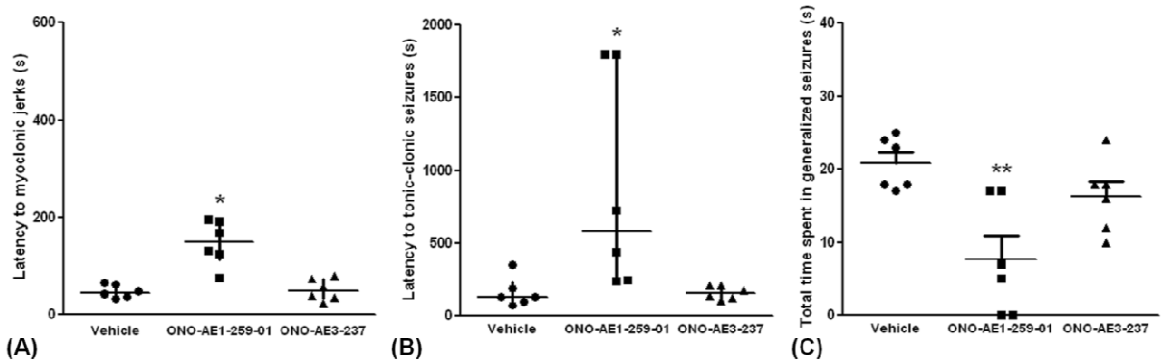

Figure 1 - Effect of EP2 receptor agonist and antagonist (ONO-AE1-259-01 and ONO-AE3-237, respectively; 10 µg/kg, s.c.) on PTZ-induced seizures (60 mg/kg, i.p.). (A) Latency to the first myoclonic episode. (B) Latency to the first tonic-clonic generalized seizure. (C) Total time spent in generalized seizures. Data expressed as median and interquartile range (A and B), and mean + SEM (C), for $n = 6$ in each experimental group. A probability of $P < 0.05$ was considered significant. * $P < 0.05$ and ** $P < 0.01$, when compared with vehicle group.

FIGURE 2

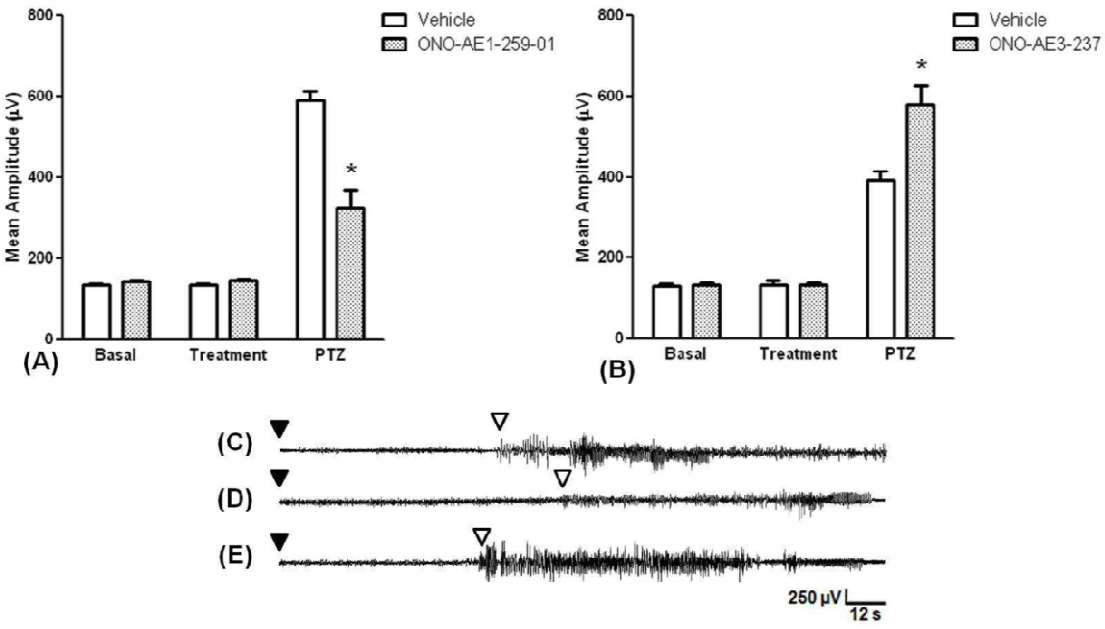

Figure 2 – Effect of EP2 receptor agonist (A) and antagonist (B) (ONO-AE1-259-01 and ONO-AE3-237, respectively; 10 µg/kg, s.c.) on the mean amplitude of EEG recordings in the parietal cortex of animal injected with PTZ (60 mg/kg, i.p.). Mean amplitude of EEG recordings was analyzed by two-way ANOVA followed by the Bonferroni's test and expressed as mean + S.E.M., for $n = 6$ in each group. * $P < 0.05$, when compared with respective vehicle. Representative electrocorticographic recordings of animals after PTZ injection are represented as follows: (C) vehicle, (D) ONO-AE1-259-01, and (E) ONO-AE3-237. Black and white arrowheads indicate PTZ injection and seizures latency, respectively, and the y-axis (amplitude) and x-axis (time) calibration bar is the same for all traces.

FIGURE 3

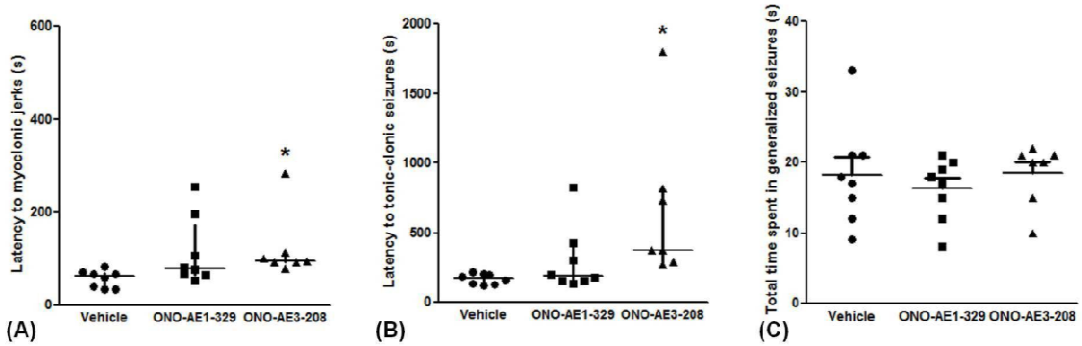

Figure 3 - Effect of EP4 receptor agonist and antagonist (ONO-AE1-329 and ONO-AE3-208, respectively; 10 µg/kg, s.c.) on PTZ-induced seizures (60 mg/kg, i.p.). (A) Latency to the first myoclonic episode. (B) Latency to the first tonic-clonic generalized seizure. (C) Total time spent in generalized seizures. Data expressed as median and interquartile range (A and B), and mean + SEM (C), for n = 7-8 in each experimental group. A probability of $P < 0.05$ was considered significant. * $P < 0.05$ and ** $P < 0.01$, when compared with vehicle group.

Manuscrito III

“Blockade of IL-1β/IL-1 receptor type 1 axis during epileptogenesis provides neuroprotection in two rat models of temporal lobe epilepsy”

Título em português

“O bloqueio do eixo IL-1β/ receptor IL-1 tipo I confere neuroproteção durante epileptogênese em dois modelos de epilepsia do lobo temporal em ratos”

Blockade of IL-1β/IL-1 receptor type 1 axis during epileptogenesis provides neuroprotection in two rat models of temporal lobe epilepsy

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Summary

We studied whether pharmacological blockade of the IL-1 β-mediated signaling, rapidly activated in forebrain by epileptogenic injuries, affords neuroprotection in two different rat models of status epilepticus (SE). As secondary outcome, we measured treatment's effect on SE-induced epileptogenesis. IL-1 β signaling was blocked by systemic administration of two anti-inflammatory drugs, namely human recombinant IL-1 receptor antagonist (anakinra), the naturally occurring and clinically used competitive IL-1 receptor type 1 antagonist, and VX-765 a specific non-peptide inhibitor of IL-1 β cleavage and release. Antiinflammatory drugs were given 60 min after antiepileptic drug-controlled SE induced by pilocarpine, or 180 min after unrestrained electrical SE, for 7 days, using a protocol yielding therapeutic drug levels in brain. This drug combination significantly decreased both IL-1 β expression and cell loss in rat forebrain. Neuroprotection and the antiinflammatory effect were more pronounced in the electrical SE model. Onset of epilepsy, and frequency and duration of seizures 3 months after electrical SE were not significantly modified. Transcriptomic analysis in the hippocampus showed that the combined treatment did not affect the broad inflammatory response induced by SE during epileptogenesis. In particular, the treatment did not prevent the induction of the complement system and Toll-like receptors, both contributing to cell loss and seizure generation. We conclude that the IL-1 β signaling represents an important target for reducing cell loss after SE. The data highlight a new class of clinically tested agents affording neuroprotection after a delayed post-injury intervention. Early blockade of this rapid onset inflammatory pathway during SE, or concomitant treatment with antiinflammatory drugs targeting additional components of the broad inflammatory response to SE, or co-treatment with AEDs, is likely to be required for optimizing beneficial outcomes.

Introduction

Increased levels of inflammatory molecules and upregulation of their cognate receptors in glia, neurons and microvessels have been demonstrated in human brain specimens of drug-resistant forms of epilepsy, thus suggesting that proinflammatory pathways are activated in seizure foci (Aronica et al., 2012; Choi et al., 2009; Vezzani et al., 2011a). Induction of the same inflammatory molecules occurs after epileptogenic injuries and during recurrent seizures in epilepsy models (Aronica et al., 2012; Vezzani et al., 2012b). Inhibition of experimental seizures and neuroprotection have been attained by pharmacological blockade of specific proinflammatory signalings (Aronica et al., 2012; Friedman and Dingledine, 2011; Kwon et al., 2013; Maroso et al., 2011a; Vezzani et al., 2012a). In particular, the activation of the IL-1 receptor 1 (IL-1R1)/Toll-like receptor (TLR) innate immune signaling in glia and neurons by the endogenous ligands IL-1 β and High Mobility Group Box 1 (HMGB1) plays a key role in ictogenesis. In fact, treatment with the IL-1 receptor antagonist (Ra), TLR4 blockers, or specific inhibitors of Interleukin Converting Enzyme (ICE), the biosynthetic enzyme producing the releasable form of IL-1 β, results in drastic reduction of acute and chronic seizures in various epilepsy models (Akin et al., 2011; Auvin et al., 2010a; Marchi et al., 2009; Maroso et al., 2011a; Maroso et al., 2010; Ravizza et al., 2006; Ravizza et al., 2008b; Vezzani et al., 1999; Vezzani et al., 2000; Vezzani et al., 2002). Supportive evidence is provided by studies in transgenic mice with perturbed IL-1R1/TLR4 signals (Dubé et al., 2005; Maroso et al., 2010; Maroso et al., 2011b; Ravizza et al., 2006; Spulber et al., 2009; Vezzani et al., 2011b; Vezzani et al., 2000). There is compelling evidence that the activation of the IL-1R1/TLR4 signaling is also involved in excitotoxicity (Allan et al., 2005; Vezzani et al., 2013; Vezzani et al., 2011b). In particular, IL-1 β and HMGB1 enhance NMDA-induced hippocampal cell loss by increasing receptor-gated Ca^{2+} influx into neurons (Bernardino et al., 2008; Iori et al., 2013; Maroso et al., 2010; Viviani et al., 2003), a mechanism also involved in their proictogenic effects (Balosso et al., 2008; Maroso et al., 2010). Neuroprotective effects were observed using antagonists of the IL-1 β /IL-1R1 axis in organotypic cell cultures exposed to AMPA (Bernardino et al., 2008).

The activation of the IL-1R/TLR signaling is pivotal for initiating the complex brain inflammatory response to various CNS injuries, including status epilepticus (SE) (Bartfai et al., 2007; Clausen et al., 2009; Dinarello, 2011; Gao et al., 2012b; Hua et al., 2011; Lei et al., 2013; Ravizza et al., 2008a). This occurs by induction of NF-kB- and AP-1-dependent

transcription of a large array of inflammatory genes in target cells (O'Neill and Bowie, 2007; Vezzani et al., 2011b). IL-1R1/TLR4 signaling induction after chemical or electrical SE in rodents is rapid (<30 min) and persists until the onset of spontaneous seizures (De Simoni et al., 2000; Dhote et al., 2007; Kuteykin-Teplyakov et al., 2009; Librizzi et al., 2012; Marcon et al., 2009; Maroso et al., 2010; Ravizza et al., 2008b; Vezzani et al., 2011b; Voutsinos-Porche et al., 2004).

Beneficial outcomes, such as decreased cell loss and reduced spontaneous seizure frequency/severity, are induced by non-steroidal antiinflammatory drugs blocking pathways downstream IL-1R/TLR, when administered to rodents after SE (*reviewed by* Löscher and Brandt, 2010; Pitkanen, 2010; Ravizza et al., 2011; Gao et al., 2012b). This evidence suggests that the various inflammatory pathways activated during epileptogenesis contribute in concert to the adverse outcomes. Preventing the activation of this inflammatory cascade by blockade of the upstream IL-1R/TLR pathway, therefore, represents a promising strategy to attain improved therapeutic effects.

In this study, we used a novel treatment combination of clinically tested and safe anti-inflammatory drugs (Dinarello et al., 2012; Vezzani et al., 2010), namely human recombinant (hr)IL-1Ra (anakinra) and the ICE inhibitor VX-765, in order to block the IL-1 β /IL-1R1 axis in two SE rat models. Each drug, individually, mediates neuroprotection in acute injury models (Allan et al., 2005; Brough et al., 2011; Ross et al., 2007), displays anti-ictogenic properties (Akin et al., 2011; Maroso et al., 2011a; Maroso et al., 2010; Ravizza et al., 2006) and inhibits kindling epileptogenesis (Auvin et al., 2010b; Ravizza et al., 2008b).

Our primary endpoint was to assess whether the combined treatment affords neuroprotection by preventing IL-1 β actions during epileptogenesis. As a secondary outcome, we evaluated the effect of treatment on epilepsy development.

Materials and Methods

Animals

The experiments were carried out in two distinct laboratories: rats exposed to electrically-induced SE were prepared in Milano (male Sprague-Dawley rats, 275-300 g; Charles-River, Calco, VA, Italy) while rats exposed to lithium/pilocarpine were prepared in Hannover (female Sprague-Dawley rats, 250-275 g; Harlan, Horst, The Netherlands). Rats were housed

at constant temperature (23 ± 1°C) and relative humidity (60 ± 5%) with free access to food and water and a fixed 12 h light/dark cycle. Procedures involving animals and their care were conducted in conformity with the institutions guidelines that are in compliance with national (D.L.n.116, G.U., Suppl. 40, February 18, 1992; German Tierschutzgesetz, Dec. 9, 2010) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Experimental groups

We compared drug combination effects in two experimental models of SE induced by either hippocampal electrical stimulation or systemic administration of the chemoconvulsant pilocarpine. The models were established in either gender by the two experimental groups. The intent was to exclude model specific treatment effects.

Electrically-induced SE

Animals were randomly divided into 3 experimental groups: 1. Sham-operated rats implanted with electrodes but not electrically stimulated (*Sham*); 2. Rats experiencing SE and treated with vehicle (*Vehicle*); 3. Rats experiencing SE, and treated with a combination of anakinra and VX-765 (*Treatment*). Drugs were administered for 5 or 7 consecutive days starting 3 h after the end of electrical stimulation (Supplementary Figure 1, see later for details).

Experiment 1 measured the human recombinant (hr)IL-1Ra (anakinra) concentration in rat blood and cerebrospinal fluid (CSF) after 7 consecutive days of antiinflammatory treatment (n=5 rats in each vehicle and treatment group).

Experiment 2 assessed the effect of antiinflammatory treatment on IL-1 β induction in the hippocampus and frontoparietal and entorhinal cortices 7 days post-SE, by immunohistochemistry (n=5 sham rats; n=5 rats in each vehicle and treated group). This time point was chosen since it represents the minimum number of days preceding the onset of spontaneous seizures as indicated by our previous experience with this model (Noe' et al, 2008).

Experiment 3 studied the differential expression of inflammatory genes during anti-inflammatory treatment by microarray analysis of gene transcription, 5 days post-SE (n=5 sham rats; n=5 rats in each vehicle and treated group). This time point is within the temporal window of the plateau increase of IL-1β after SE in this model, then decling afterwards as

assessed by immunohistochemistry (*data not shown*). Moreover, at this time VX-765 has already reached its maximal antiinflammatory effect (Maroso et al, 2011a).

Experiment 4 studied the effect of antiinflammatory treatment (i) on forebrain cell loss, and (ii) on epileptogenesis using as outcome measures the onset of spontaneous seizures (n=11 rats in each vehicle and treated group) and their frequency and duration, as assessed 3 months post-SE by EEG analysis. Six out of 11 chronic epileptic rats in vehicle or treatment group were EEG recorded at 3 months, because of the loss of the EEG implant before analysis in some rats. Neuropathology and glia activation were assessed by quantitative immunohistochemistry in brain sections of epileptic rats at the end of the EEG evaluation. Sham rats (n=5) were used as controls for immunohistochemical analysis.

Pilocarpine-induced SE

In the *Experiment 5 r*ats were divided into 3 groups: 1. Rats implanted with electrodes receiving vehicle (*Sham*, n=4); 2. Rats implanted with electrodes and exposed to SE receiving vehicle (*Vehicle*, n=12); 3. Rats implanted with electrodes and exposed to SE receiving a combination of anakinra and VX-765 (*Treatment,* n=7). Drugs or vehicle were administered for 5 or 7 days starting after 1 h of SE (see later for details). For histological analysis, we included naive rats (n=6) without any treatment. In this model, we studied specifically the effect of treatment on forebrain cell loss and IL-1 β induction by quantitative immunohistochemistry. Although chronic epilepsy was not assessed in this model, the same rats were evaluated for early seizures occurrence during the first week post-SE.

Induction of SE and spontaneous seizure recordings

Electrical stimulation

Fifty two rats (*Experiments 1-4*) were implanted under 1.5% isofluorane anaesthesia with 2 bipolar Teflon-insulated stainless-steel depth electrodes placed bilaterally into the temporal pole of the hippocampus (from bregma, mm: AP –4.7; L ± 5.0; –5.0 below dura, Paxinos and Watson, 2005).

Two screw electrodes were positioned over the nasal sinus and the cerebellum, and used as ground and reference electrodes, respectively. Electrodes were connected to a multipin socket and secured to the skull by acrylic dental cement. After surgical procedures, rats were treated

locally with Cicatrene powder (Neomicyn; Bacitracin; Glicyne; L-Cistein; DL-Treonin) and injected with Ampicillin (100 mg/kg, s.c.) for 4 days to prevent infections. Rats were allowed to recover from surgery in their home cage for 7-10 days. Before electrical stimulation, EEG baseline hippocampal activity was recorded in freely-moving rats for 24 h. Then, rats were unilaterally stimulated (50 Hz, 400 µA peak-to-peak, 1ms biphasic square waves in 10 s trains delivered every 11 s) in the CA3 region of the ventral hippocampus for 90 min to induce SE according to a well established protocol (De Simoni et al., 2000; Noé et al., 2008). EEG was recorded in each rat every 10 min epoch for 1 min in the absence of electrical stimulation, i.e. the "stimulus-off" period. All rats used for subsequent analysis showed an EEG pattern of uninterrupted bilateral spikes in the hippocampi during the "stimulus-off" period, starting between the 1st and the 4th epoch of stimulation onwards.

These criteria selected rats developing SE, that remitted spontaneously within 24 h from the initial stimulation, leading to subsequent epilepsy development (De Simoni et al., 2000; Noé et al., 2008). SE was defined as the presence of continuous spike activity with a frequency higher than 0.5 Hz intermixed with high amplitude and frequency discharges lasting for at least 5 sec, with a frequency of >8 Hz and an amplitude 2 fold-higher than the baseline. Spikes were defined as sharp waves with amplitude of at least 2.5-fold higher than the baseline and duration lower than 100 ms, or as a spikeand-wave with a duration lower than 200 ms (Pitkanen et al., 2005). End of SE was defined when inter-spike interval was longer than 2 sec. No pharmacological intervention was done to stop SE since no mortality is observed in this model. SE was evaluated by measuring its total duration and the number of spikes during the first 24 h after induction using Clampfit 9.0 program (Axon Instruments, Union City, CA, U.S.A.).

Spontaneous seizures following electrical stimulation

In *Experiment 4*, rats exposed to electrical SE were analysed for spontaneous seizures occurrence. Rats were continuously EEG recorded (24 h/day) from SE induction until 2 spontaneous EEG seizures occurred. No video-recording was done since all EEG seizures are associated with Racine's scale (Racine, 1972) stage 3-4 motor seizures (Gorter et al., 2001; van Vliet et al., 2004). After epilepsy onset, EEG was discontinued and resumed 3 months later to determine spontaneous seizures frequency and duration by continuous EEG monitoring for 2 weeks (24/7). Spontaneous seizures were discrete EEG ictal episodes lasting

on average ≥60 sec, characterized by high frequency and high-voltage synchronous spike activity and/or multi-spike complexes (Noé et al., 2008). EEG was recorded using the TWin EEG Recording System connected with a Comet AS-40 32/8 Amplifier (sampling rate 400 Hz, high-pass filter 0.3 Hz, low-pass filter 70 Hz, sensitivity 2000 mV/cm; Grass-Telefactor, West Warwick, R.I., U.S.A.) Digitized EEG data were processed using the TWin record and review software (Noé et al., 2010). EEG were visually inspected and quantified by two independent operators blinded to the treatments. Deviation from concordance was considered acceptable since it was ≤5%.

SE induced by lithium/pilocarpine

In *Experiment 5,* stainless steel screws for EEG recording were stereotaxically implanted into the left and right primary somatosensory cortex under chloral hydrate anesthesia (360 mg/kg, intraperitoneally, i.p.) at the following coordinates (from bregma): AP, -2.2 mm; L, ±3.2 mm. An additional ground screw electrode was implanted 5 mm posterior and 2.6 mm lateral to bregma. Two weeks after electrode implantation, rats received lithium chloride (127 mg/kg p.o.) 12-17 h before pilocarpine. Furthermore, methyl-scopolamine (1 mg/kg, i.p.) was administered 30 min prior to the first pilocarpine injection to prevent peripheral effects of pilocarpine. Then, each rat received pilocarpine using a modified version of a ramping-up dosing protocol previously described to allow individual dosing with lower mortality compared to single dose administration (Glien et al., 2001).

Treatment was started by injecting 30 mg/kg pilocarpine i.p. followed by repeated injection of 10 mg/kg at 30 min intervals. SE consisting of ongoing limbic or generalized convulsive seizures, typically started after the 3rd injection of pilocarpine. Only rats developing a self-sustained SE with generalized convulsive seizures were used for further experiments. After 60 min, SE was terminated by diazepam (10 mg/kg into the tail vein, i.v.) and phenobarbital (25 mg/kg, i.v.) administration. The application of these drugs was repeated i.p. after 4 and 8 h to prevent recurrence of SE and to reduce mortality.

Spontaneous seizures after lithium/pilocarpine

Following SE, the rats were continuously video/EEG recorded (24 h/day) by a combined video-EEG detection system as described in detail (Rattka et al., 2012). Continuous monitoring was done for 5 or 7 days over the period of treatment. For detection of early spontaneous seizures, the EEG recordings were visually analysed for abnormal (most likely

paroxysmal) events. High-frequency synchronized firing with an amplitude of at least twice the EEG baseline and a duration of at least 8 sec was considered an abnormal event. To verify that this abnormal EEG event was a seizure, the corresponding video-recording was viewed. For rating of spontaneous seizure severity, a modified Racine's scale (Racine, 1972) was used. Videos were used to decide whether seizures were focal (head nodding, stage 2, or unilateral forelimb clonus, stage 3) or generalized convulsive (stage 4-5).

If the EEG showed typical seizure-like paroxysmal activity, but the camera did not point towards the front of the animal, only generalized convulsive seizures could be definitely seen; otherwise, the seizure was rated as unclassified. In addition to severity rating, duration of spontaneous seizures was analysed in the EEG.

Study design and drug administration

Electrically induced SE

The study protocol of *Experiments 1-4* is summarized in Suppl. Figure 1. Rats developing selfsustained uninterrupted seizure activity (SE) for at least 3 h after the end of electrical stimulation were continuously EEG monitored for 7 days (*Experiments 1 and 2*) or for 5 days (*Experiment 3*). In *Experiment 4*, rats were monitored continuously after SE induction until 2 spontaneous seizures occurred, then EEG analysis was discontinued and resumed for 2 additional weeks at 3 months after SE (Noe' et al., 2008). Drugs or their vehicle were administered either for 5 days (*Experiment 3*) or 7 days (*Experiments 1,2 and 4*), starting 3 h after the end of electrical stimulation. Rats were killed in *Experiments 1,2 and 3* at the end of treatment and in *Experiment 4* at the end of EEG recordings.

For drug administration, SE rats were unplugged from the EEG recording system and randomly divided in *treatment* or *vehicle* groups. Rats were anaesthetized with 1.5% isofluorane and implanted subcutaneously (s.c.) with osmotic minipumps (Alzet 2ML1, Cupertino, CA, USA) preloaded with anakinra (200 mg /2 ml saline; Biovitrum AB, Stockholm, Sweden) or saline. Minipumps had an infusion rate of 10 µl/h resulting in a release of ~24 mg anakinra per day (80 mg/kg/day). After surgical procedures, rats were treated locally with antibiotics as reported above.

Anakinra treatment was based on previous evidence of neuroprotection in rat and human stroke (Clark et al., 2008), and in a Parkinson's disease animal model (Koprich et al., 2008). At the time of minipump implantation, rats were concomitantly injected i.v. with a bolus of

anakinra (33 mg/kg, 10 mg/200 μL) and i.p. with VX-765 (200 mg/kg in 0.1% Tween 80 + 0.5% HEC in water, Vertex Inc., Cambridge, Boston, MA, USA). A second VX-765 injection was done 5 h later, then twice a day for the subsequent 5 (*Experiment 3*) or 7 days (*Experiments 1, 2 and 4*). This treatment regimen was previously shown to reduce both brain IL-1 β and recurrent seizures in animals (Maroso et al., 2011a; Maroso et al., 2010; Ravizza et al., 2006; Ravizza et al., 2008b). EEG recording was resumed at the end of minipump implantation which lasted about 30 min.

Pilocarpine-induced SE

Immediately following pharmacological termination of SE (i.e. 60 min after SE onset), rats were anaesthetized with 1.5% isofluorane and implanted s.c. with osmotic minipumps as described above for *Experiments* 1-4. One group of rats exposed to SE (n=10) received minipumps filled with saline.

In SE rats receiving the combined treatment (n=7), the experimental procedure was similar as for *Experiments* 1-4 except that VX-765 was given twice a day for the first 2 days post-SE, then once a day until day 5 or 7. Since no significant differences in our main outcome measure, i.e. cell loss, were observed in these rats, the two groups were merged and treated as an unique experimental group. In the first 24 h following SE, 4 SE rats died (one rat in the treatment group, 3 rats in the vehicle group). In all experiments the minipump was removed in each rat at the end of treatment and the inside solution was measured for assessing proper drug delivery (according to the manufacturer's instructions).

Analysis of anakinra in plasma and CSF

In *Experiment 1*, anakinra was measured in rat plasma and CSF after 7 days treatment, using a commercially available ELISA kit (Quantikine Human IL-1Ra/IL-1F3 Immunoassay; R&D Systems, Minneapolis, USA). This assay determines recombinant hrIL-1Ra, but it does not crossreact significantly with rat IL-1Ra or IL-1 β as reported in the specificity profile of the product data sheet.

Immunohistochemistry

In *Experiments 2, 4, and 5*, rats were deeply anaesthetized using Equithesin and perfused via the ascending aorta with 50 mM cold phosphate buffered saline (PBS, pH 7.4) followed by chilled 4% paraformaldehyde (PFA) in PBS. The brains were post-fixed in PFA for 90 min at 4°C, then transferred to 20% sucrose in PBS for 24 h at 4°C. Brains were then frozen in -50°C isopentane for 3 min and stored at -80°C until assay.

Serial coronal sections (40 μm) were cut on a cryostat throughout the dorso-ventral extension of the hippocampus (-2.3 to -5.3 mm from bregma; Paxinos and Watson, 2005) and collected in 100 mM PBS. For each marker, we used 1 series of 9 sections in each brain (IL-1 β in *Experiments 2 and 5*; GFAP in *Experiment 4;* NeuN in *Experiments 4 and 5*). Immunohistochemistry was done in freelyfloating slices.

IL-1β staining was done in slices incubated at 4°C for 10 min in 70% methanol and 2% H_2O_2 in Tris-HCl-buffered saline (TBS), followed by 30 min incubation in 10% FCS in 1% Triton X-100 in TBS. The slices were then incubated overnight at 4°C in the same medium with the primary antibody against rat IL-1β (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), then with biotinylated secondary anti-goat antibody (1:200, Vector Labs, Burlingame, CA, U.S.A.). GFAP and NeuN staining were done in slices incubated at 4°C for 30 min in 0.4% Triton X-100 in PBS followed by a 15 min incubation in 3% fetal bovine serum (FBS) in 0.1% Triton X-100 in PBS. The slices were then incubated overnight at 4°C in 3% FBS in 0.1% Triton X-100 in PBS, with mouse anti-GFAP primary antibody (1:6500, Chemicon Int. Inc., Billerica, MA, U.S.A.) or with mouse anti-NeuN primary antibody (1:1000, Chemicon). Immunoreactivity was tested and evaluated as previously described (Ravizza et al., 2008a). Specificity of the immunohistochemical signals was previously demonstrated (Ravizza et al., 2008a).

Assessment of cell loss and cell quantification

Neuronal cell loss was assessed as previously described in detail (Langer et al., 2011) using 2 NeuN-stained coronal sections for each rat brain. In both SE models, neurons were counted in CA1, CA3, dentate hilus, basolateral amygdala, frontoparietal cortex, and in the entorhinal cortex (only in the electrical SE). Cell loss was not detected in basolateral amygdala in electrical SE rats and only in 3 out of 6 rats treated with pilocarpine (not shown). Entorhinal cortex after pilocarpine-induced SE was not damaged due to the neuroprotective effect of

antiepileptic drug treatment given to rats after the first hour of SE (Du et al., 1995). Briefly, in each hemisphere/slice, one high-power 10X magnification field was acquired in the hippocampal CA1 subfield, in the hilus and in the frontoparietal and entorhinal corteces. One high-power 20X magnification field was acquired in the hippocampal CA3 subfield and in the basolateral amygdala (Olympus BX61, Germany). NeuN positive cells were marked by one investigator blinded to the identity of the samples, and an automated cell count was generated using Fiji software. The number of cells was divided by the corresponding area (mm^2) of cell counting, thus providing a value of cell density (neurons/mm^2). No significant changes in the area of the regions analysed were measured in SE rats in both models, except for the hilar area that was significantly increase in electrical SE rats (see Results). Data obtained from the 2 sections and in the two hemispheres were averaged in each brain and used for statistical analysis.

IL-1β-positive astrocytes were quantified using 2 representative sections for each rat brain, as previously described (Ravizza and Vezzani, 2006). Briefly, in each hemisphere/slice, one highpower 10X magnification field was acquired in the hippocampal CA1, CA3 subfields, in the hilus and in the frontoparietal cortex while two high-power 10X magnification fields were acquired in the entorhinal cortex. One high-power 20X magnification field was acquired in the central part of the basolateral amygdala (Olympus BX61, Germany). IL-1β immunopositive astrocytes were marked by one investigator blinded to the identity of the samples, and an automated cell count was generated. Data obtained in the 2 fields of the entorhinal cortex in each hemisphere were added together providing one value *per* slice/hemisphere. Values obtained in the 2 slices of each rat brain and in the two hemispheres were averaged providing a single value for each rat. In each experimental group, this value was used for statistical analysis of data.

GFAP staining was quantified in the hilus using 3 coronal sections in each rat brain, as previously described (Filibian et al., 2012). High-power 20X magnification non-overlapping fields were positioned in each hemisphere/slice to encompass the total hilar region (Olympus BX61). These images were acquired and digitized by an operator unaware of the identity of the samples. GFAP immunostained area was measured using Fiji software (Schindelin et al., 2012). Data were expressed as percentage of area occupied by GFAP-positive pixels in each field, then these values were averaged providing a single value per hilus/hemisphere. Values obtained in the 3 sections/brain/hemisphere were averaged providing a single value for each

at, and this value was used for statistical analysis. For all these evaluations, data obtained in the two hemispheres/slice were averaged since they did not differ.

Microarray analysis

Rats were deeply anaesthetized using Equithesin, then perfused via the ascending aorta with 50 mM ice-cold PBS (pH 7.4) for 3 min to remove blood from brain vessels. Rats were decapitated and the electrically stimulated hippocampi were dissected out at 4°C in a RNAse free environment, immediately frozen on dry ice and stored at -80°C. Frozen tissues (30 mg) were homogenized in a TissueLyser LT (Qiagen, Milan, Italy) and the total RNA purified using RNeasy Mini Kit isolation system (Qiagen), following manufacturer's protocols. Total RNA concentration and proteins contamination were determined by Nanodrop spectrophotometer (Nanodrop Technologies, Ambion). Only samples with a RNA Integrity Number (RIN) larger than 6 and a Nanodrop A260:280 ratio between 1.8 and 2.1 were further processed and aliquots stored at -80°C until use.

Array experiments were performed using standardised procedures, as previously described (Marchini et al., 2011; Marchini et al., 2012). Briefly, 100 ng of total RNA was reverse transcribed into Cy3-labeled cRNA using LowInput QuickAmp labelling kit (Agilent Technologies, Palo Alto, CA, U.S.A.) and hybridized with a RNA labelling and hybridization kit, according to the manufacturer's instructions (Agilent Technologies). We used the commercially available G4858A rat whole GE Microarray kit (Agilent Technologies) which consists of 60K features printed in an 8-plex format (8x60 array). The arrays were washed and scanned with a laser confocal scanner (G2565B, Agilent Technologies) according to the manufacturer's instructions. mRNA microarrays underwent standard post hybridization processing and the intensities of fluorescence were calculated by Feature Extraction software v.11 (Agilent Technologies). Scanner generated data were pre-processed, filtering out probes that were flagged as unreliable by the scanning software, and then normalized with quantile normalization. The procedure employed the "Agi4x44PreProcess" package, part of the Bioconductor software suite (Gentleman et al., 2004).

Differentially expressed genes were calculated with the Rank Product algorithm (Breitling et al., 2004), considering genes as significantly differentially expressed if their percentage of false positives ("pfp") was less than 5%. Gene Ontology (GO) enrichment analysis was performed with the topGO Bioconductor package (Alexa et al., 2006) over the Biological

process (BP) GO hierarchy: calculated p-values were then corrected for multiple testing following the procedure described by Benjamini and Hochberg (Benjamini and Hochberg, 1995), and terms were included if their False Discovery Rate (FDR) was less or equal than 0.05. Microarray raw data have been submitted to ArrayExpress (experiment number E-MTAB-1567), in accordance with MIAME (minimum information about microarray experiment) guidelines.

Statistical analysis

Data are presented as mean ± SE (n= number of animals). Student's t-test, Mann-Whitney U test or Fisher's exact test for two independent groups or one-way ANOVA followed by Bonferroni test for more than two independent groups were used for statistical analysis of data as appropriate. In Fig. 6 A,B, data were analysed by Fisher's exact test and by Rank Product, respectively. A probability of $p<0.05$ was considered significant. Statistical tests were performed using GraphPad Prism 5 (GraphPad Software, USA) for Windows.

Results

Plasma and CSF concentrations of anakinra after combined treatment

Figure 1 depicts anakinra levels attained in plasma (A) and CSF (B) withdrawn from electrical SE rats undergoing combined treatment with VX-765 and anakinra for 7 days (*Experiment 1*). Anakinra was not detectable in SE rats treated with vehicle because the ELISA antibody recognizes only the human recombinant form, therefore it does not cross-react significantly with rat IL-1Ra. This treatment protocol induced a significant increase both in plasma and CSF anakinra levels. The attained concentrations were similar to those previously reported to be neuroprotective in a rat model of stroke and in patients with subarachnoid haemorrhage (Clark et al., 2008). Notably, anakinra level in CSF was higher than in plasma suggesting drug accumulation in the brain possibly due to increased BBB permeability during SE (Frigerio et al., 2012; van Vliet et al., 2007).

Antiinflammatory effect of combined treatment in the rat brain

We assessed first whether the combined treatment protocol blocked the IL-1 β upregulation induced by SE. Immunohistochemical analysis of the hippocampus and cortex of rats exposed to electrical SE (*Experiment 2*) showed the typical induction of IL-1 β in astrocytes as previously reported (De Simoni et al., 2000; Ravizza et al., 2008b). We identified astrocytes on morphological basis, however, we previously demonstrated that 3 to 7 days after electrical or pilocarpine SE, IL-1 β and GFAP staining fully co-localized (Ravizza et al., 2008b). At the end of treatment, rats injected with drug combination (Figure 2B,E,H,K,N) displayed a reduced number of IL-1 β -positive cells as compared to SE rats treated with vehicle (Figure 2A,D,G,J,M). Cell quantification showed a significant reduction of IL-1 β -positive cells in treated- vs vehicle-injected rats in all areas, except in the frontoparietal cortex where there was a trend decrease in IL-1 β -positive cells which was not statistically significant ($p=0.068$ vs vehicle, by Mann Whitney U test).

The combined treatment given for 7 days after pilocarpine SE (*Experiment 5)* significantly reduced the number of IL-1 β -positive cells in CA1, dentate hilus and frontoparietal cortex, but not in the CA3 region (Figure 3B) or in the amygdala (Suppl. Table 1). Notably, the extent of SE induced inflammation in vehicle-injected rats and the residual number of IL-1 β -positive cells after treatment were significantly higher after pilocarpine than after electrical stimulation (Supplementary Table 1).

Effects of combined treatment on pathophysiological outcomes

Status epilepticus

In *Experiments 1-4*, the combined treatment begun after 3 h of self-sustained SE since this time is required for developing epilepsy in this model. The treatment did not modify the total duration of SE (vehicle, 817 ± 197 min; treatment, 785 ± 111 min, n=11 each group, *Experiment 4*).

Retrospective analysis of the total number of spikes in the first 3 h before rat randomization, showed similar spike numbers in vehicle and treatment groups (Figure 4A). The total number of spikes in EEG recording from the beginning until the end of SE did not differ between vehicle and treatment groups (Figure 4A). We found, however, a significant decrease in the number of spikes in the first 60 min following the i.v. bolus of anakinra (vehicle, number of spikes/h, mean ± SE, 4.190 ± 588; anakinra, 1.736 ± 628, $p<0.05$). It is unlikely that this effect is due to the concomitant first injection of VX-765 since its antiictogenic effects in

acute and chronic seizure models develop following multiple drug administrations (Maroso et al., 2011a; Ravizza et al., 2006; Ravizza et al., 2008b).

Cell loss

In vehicle-injected rats killed 3 months after electrical SE (*Experiment 4*), neurodegeneration was observed in CA1, CA3, hilus, frontoparietal and entorhinal cortices (Figure 5). The combined treatment reduced cell loss significantly vs vehicle-treated rats in CA1 ($p<0.01$, panel A) and frontoparietal cortex ($p<0.05$, panel C). In CA3 and entorhinal cortex, the treatment group was not significantly different from either the sham- or the vehicle-group (B,D). In the hilus, significant neuroprotection was observed in 4 out of 6 rats (Fig. 5F, neurons/hilus: sham, 167.4 ±10.8 (6); vehicle, 96.5 ± 3.5 (6); treated 134.4 ± 6.5 (4), $p<0.01$ treated vs vehicle by one-way ANOVA).

Two out of 6 treated rats (circled symbols in panel E,F,G) did not differ from vehicle rats. If all treated rats were included in the experimental group (n=6), no significant difference was observed in the number of neurons/hilus as compared to vehicle group (Figure 5F). Both the hilar area (μm^2) and corresponding GFAP-positive area were significantly increased in SE-vehicle vs sham rats while in SE-treated rats both parameters did not differ from sham rats (Figure 5G,H).

Vehicle-injected rats killed 5 or 7 days after pilocarpine SE (*Experiment 5*) showed significant cell loss in CA1, CA3, hilus, and frontoparietal cortex (Figure 3C). Treatment with anakinra and VX-765 prevented neuronal loss in cortex, but not in the hippocampal formation (Figure 3C). Only 50% of rats developed significant cell damage in the amygdala which was similar both in vehicle and treatment group (not shown).

Spontaneous seizures

In *Experiment 4,* we assessed the effect of combined treatment on epilepsy development after electrical SE by evaluating the onset of spontaneous seizures, and their frequency and duration 3 months after SE (2 weeks of 24/7 EEG recording). The onset of the first spontaneous seizure typically occurs in this model 14.3 ± 3.1 days after induction of SE, and this parameter was not significantly modified by the combined treatment (Figure 4B). Similarly, the frequency (0.76 ± 0.2 seizures/day) and average duration (121.1 ± 31.8 sec) of spontaneous seizures in vehicle-injected rats were not modified by the treatment (Figure 4C,D).

In *Experiment 5*, 7 vehicle and 6 treated rats were video/EEG monitored to detect spontaneous recurrent seizures. Six out of 7 rats injected with vehicle exhibited seizures during the first week, and their total number was similar to that measured in drug-injected rats (Figure 3A). In all vehicle injected rats, early seizures occurred between day 2 and day 4 but in 50% of rats seizures were detected also between day 4 and 7 (Supplementary Figure 2). In drug-treated rats, 5 out of 6 rats developed early seizures (Figure 3A) only during the first 4 days after SE; none of these rats had seizures between day 4 and 7 (Supplementary Figure 2). Onset time of seizures, severity or duration of seizures were not significantly different between groups (not shown). Most spontaneous seizures in both groups were generalized convulsive (stage 4) seizures. However, stage 5 seizures, which were observed in 4/7 vehicle-treated rats were not observed in drug-treated rats (0/6; $p = 0.069$ by Fisher's exact test).

Differentially expressed genes (DEGs) after combined treatment

In order to determine if the drug treatment inhibited the broad inflammatory response during SE, we carried out transcriptomic analysis in hippocampal samples from sham rats not exposed to SE, and in rats exposed to SE and treated with the combined drugs or their vehicle (*Experiment 3*). Rats were killed after 5 days of treatment since at this time VX-765 attains its maximal anti-inflammatory effects, as shown in previous experiments (Maroso et al., 2011a). Comparison of vehicle and treatment groups yielded to a relatively small number of DEGs (183 genes, Supplementary Table 2) with no significant functional enrichment, thus indicating a substantial similarity between these two groups. Individual comparison of vehicle or treatment groups with sham rats (not exposed to SE) showed 1.611 and 1.721 significant DEGs, respectively. Functional enrichment with GO terms showed a large similarity in the two experimental SE groups either receiving vehicle or the combined treatments. In particular, the most common affected functions related to the immunemediated inflammatory response were unaltered by the treatment (Figure 6A). We screened the DEGs in each experimental group for key inflammatory molecules with established proictogenic properties (Figure 6B), thus confirming that the combined treatment did not affect the broad inflammatory response induced by SE, although it was effective in reducing IL-1 β expression in astrocytes (Figure 2). The IL-1 system genes were not modified by the combined treatment (Figure 6B) which acts downstream of gene transcription.

Discussion

This study shows that combined treatment with two clinically tested antiinflammatory drugs specifically blocking the IL-1 β /IL-1R1 signaling during epileptogenesis, provides neuroprotection in forebrain areas although not preventing epilepsy development.

We postulated that the novel strategy of blocking the upstream IL-1 β /IL-1R1 proinflammatory signaling induced by SE would prevent the activation and amplification of downstream inflammatory events involved in excitotoxicity and seizure generation (Vezzani et al., 2011b). The rationale for choosing such pharmacological intervention was manifold: (1) the IL-1R1 signaling, together with TLRs, is a key mechanism of fast activation of the innate immune response and the associated generation of inflammatory molecules (Vezzani et al., 2011b). This signaling, first characterized in macrophages, is rapidly activated in neurons (within a few min) and in glia (>30 min) (De Simoni et al., 2000; Ravizza and Vezzani, 2006) by IL-1 β released in the brain following SE (Aronica et al., 2012; Vezzani et al., 2011b). It represents, therefore, a promising target for preventing the generation of the brain inflammatory cascade. (2) Both anakinra and VX-765 have neuroprotective properties (Allan et al., 2005 ; Dinarello et al., 2012; Ross et al., 2007 ; Vezzani et al., 2010). (3) The activation of the IL-1R1 signaling significantly contributes to ictogenesis in acute seizure models (Maroso et al., 2011a; Vezzani et al., 2011b), and to kindling development (Auvin et al., 2010b; Ravizza et al., 2008b).

We used two modalities of SE induction in rats for limiting the possibility that drug-associated effects could be model-specific. First, we used the electrical SE model to evaluate the antinflammatory effect of the combined treatment protocol, and its impact on neuronal cell loss. We replicated these findings in the chemoconvulsant pilocarpine model which has the following peculiar characteristics as compared to electrical SE: spontaneous seizures occur more rapidly, thus denoting a much shorter latent phase; inflammation in forebrain develops to a larger extent likely due to the more severe SE, and to the early spontaneous seizures occurring during the first week after SE. In fact, it is well established that recurrent seizures induce IL-1 β in rodent brain (Librizzi et al., 2012; Vezzani et al., 2011b). A further difference between the two SE models, as highlighted by our findings, is the prominent astrocytosis induced in the hilus following electrical SE, likely contributing to the increased hilar area, a phenomenon which was not observed in the pilocarpine model.

The combined treatment was initiated 60 and 180 min after the onset of SE in the pilocarpine and electrical model, respectively. This protocol was adopted to allow sufficient time in SE for promoting significant cell loss and priming epilepsy development in all rats. In spite of this delayed post-injury treatment, both the induction of IL-1 β and neurodegeneration in forebrain areas were significantly reduced. The neuroprotective effect of the anti-IL-1 β treatment is compatible with the well established role of this cytokine in excitotoxicity (Allan et al., 2005; Bernardino et al., 2008; Viviani et al., 2003). Notable differences, however, were observed between the two models. Drug combination reduced IL-1 β expression to a larger extent in the electrical vs the pilocarpine model.

This might be due to the less extensive IL-1 β induction by electrical SE, which is more easily controlled, therefore, by the antiinflammatory intervention. In electrical SE rats, neuroprotection was observed in CA1 area, and in 70% of rats in the hilus. Cell loss was also significantly reduced in the frontoparietal cortex as in the pilocarpine model. Differently, neuroprotection was not significant in CA3 area or in the entorhinal cortex, in spite of a significant reduction of IL-1 β levels. This evidence suggests that IL-1 β plays a more prominent role in cell damage in brain areas where hyperexcitability and ictal activity spread (CA1, hilus) as compared to areas where seizures are generated (CA3 and entorhinal cortex; Du et al., 1995). In contrast, no neuroprotection was observed in the hippocampus of pilocarpine rats where IL-1 β was massively induced by both SE and the subsequent early seizures. Although the treatment significantly reduced IL-1 β levels in the hippocampus of pilocarpine treated rats, the residual number of cytokine-positive astrocytes exceeded by 3.5 fold on average the levels measured in electrical SE rats receiving the vehicle (Supplementary Table 1). No reduction of IL-1 β was attained in the amygdala where the treatment did not afford neuroprotection. These findings underscore that IL-1 β level should be reduced below a critical threshold specific for each brain region, to provide significant neuroprotecion.

The comparison of the outcomes in the two SE models raises important considerations: (1) a more prolonged antiinflammatory drug administration might be required after severe seizures (such as in pilocarpine-treated rats) to reduce the level of inflammation below the neurotoxicity threshold; (2) the areas of seizure generation appear to be less sensitive to the neuroprotective effects of the antiinflammatory treatment than those secondarily recruited by epileptic activity (as inferred by the electrical SE model). This peculiar beneficial effect of IL-1 β /IL-1R1 signaling blockade in areas where hyperexcitability spreads from a focal site of onset was previously reported. Thus, transgenic mice overexpressing (hr)IL-1Ra in astrocytes

showed massive reduction of *c-fos* activation in the frontoparietal cortex after intrahippocampal bicuculline injection (Vezzani et al., 2000), and generalizized convulsive seizures were decreased in rats injected with anakinra during the first hour of electrical SE (De Simoni et al., 2000). Moreover, VX-765 administered during rapid electrical kindling suppressed seizure generalization without interfering with hippocampal afterdischarge (Ravizza et al., 2008b). At variance with the antiictogenic effects of either anakinra or VX-765 given alone in various acute and chronic seizure models (Akin et al., 2011; Auvin et al., 2010b; De Simoni et al., 2000; Marchi et al., 2009; Maroso et al., 2011a; Ravizza et al., 2006; Vezzani et al., 2000), the combination of these drugs following a therapeutic dose regimen for 7 days, did not prevent the development of spontaneous seizures in these SE models. The apparent lack of antiepileptogenic effects of the combined drugs does not exclude, however, that a more prolonged antiinflammatory treatment is required to affect spontaneous seizure generation in these severe brain injury models. Indeed, both drugs were shown to arrest or delay kindling epileptogenesis in adult and immature rats (Auvin et al., 2010b; Ravizza et al., 2008b), representing milder epileptogenesis conditions. One additional consideration is that the combined treatment protocol, although decreasing SE-induced IL-1 β in forebrain, did not prevent the activation of the broad inflammatory cascade, as demonstrated by DEGs analysis in the treatment vs vehicle groups. In particular, complement system components and TLRs genes were still induced after the combined treatment similarly to SE vehicle-injected rats. Considering that these pathways play a significant role in experimental seizure generation (Kharatishvili et al., 2013; Maroso et al., 2010; Sakatani et al., 2008; Shetty, 2011; Xiong et al., 2003), it is likely that their persisting activation has compensated for the IL-1 β signaling blockade, therefore precluding any genuine antiepileptogenic effect. Indeed, TLRs activation can *per se* sustain NF-kB-mediated transcription of inflammatory genes, including the IL-1 β / IL-1R1 genes (O'Neill and Bowie, 2007; Vezzani et al., 2011b), possibly explaining why the concomitant blockade of IL-1 β / IL-1R1 activation did not affect the broad inflammatory cascade induced by SE.

Complement upregulation may also account for cell loss in CA3 area where high level of neurotoxic C1q are induced by SE (Aronica et al., 2007; Rozovsky et al., 1994; Xiong et al., 2003).

The lack of a broad antiinflammatory effect of our combined treatment is likely to depend on the delayed pharmacological intervention. In fact, the IL-1 β / IL-1R1 signaling is rapidly activated within 30 min from SE onset (De Simoni et al., 2000; Dhote et al., 2007), therefore,

this time could be sufficient to trigger an irreversible cascade of downstream inflammatory events. This evidence suggests that earlier intervention may lead to improved therapeutic effects, including increased neuroprotection. In favour of a better outcome after a rapid anti-IL-1 β drug administration in SE, there is evidence that if anakinra is given before, or shortly after SE, both the incidence and/or the severity of SE in rats are reduced (De Simoni et al., 2000; Marchi et al., 2009). Differently, we found a transient reduction of spiking activity when anakinra was given 3 h after the onset of SE.

Therapeutic effects of other antiinflammatory drugs were studied after SE. COX-2 inhibitors (celocoxib, parecoxib, rofecoxib; Jung et al., 2006; Kunz and Oliw, 2001; Ma et al., 2013; Polascheck et al., 2010), prostanoid EP2 receptor antagonist (Jiang et al., 2012) or inhibitors of glia activation (minocycline, fingolimod; Abraham et al., 2010; Gao et al., 2012a) decreased SEinduced cell loss. Some of these treatments reduced spontaneous seizures frequency or severity, but none of them prevented epilepsy development (*reviewed by* Löscher and Brandt, 2010; Pitkanen, 2010; Ravizza et al., 2011). A recent study showed that a combination of anakinra and COX-2 inhibitor was required to produce both neuroprotective and antiepileptogenic effects in young rats exposed to pilocarpine SE (Kwon et al., 2013).

In conclusion, our findings raise two important considerations for optimizing the therapeutic effects of antinflammatory interventions after epileptogenic injuries: 1. early intervention is likely to be required when rapid onset inflammatory pathways are targeted such as the IL-1 β /IL-1R1; 2. Timely administration of combined drugs antagonizing parallel or sequential proinflammatory signals (e.g. TLR4, complement system) should be considered for maximizing the neuroprotection attained by IL-1 β system blockade, and possibly impact effectively on spontaneous seizures generation. Although our findings relate to SE models, similar considerations could be applied to other epileptogenic injuries associated with time- and region-specific induction of similar sets of inflammatory molecules (e.g. stroke, neurotrauma, infection). Finally, our study emphasizes the important finding that significant neuroprotection can be attained, even with a delayed post-injury intervention, using anti-IL-1 β drugs. This finding opens up a novel therapeutic opportunity for rescuing neurons and the associated cognitive deficits (Kotloski et al., 2002) in clinical cases of unremitting SE, by administration of clinically tested and safe drugs (Dinarello et al., 2012; Vezzani et al., 2010) that could importantly complement the action of the available antiepileptic drugs.

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FIGURE 1

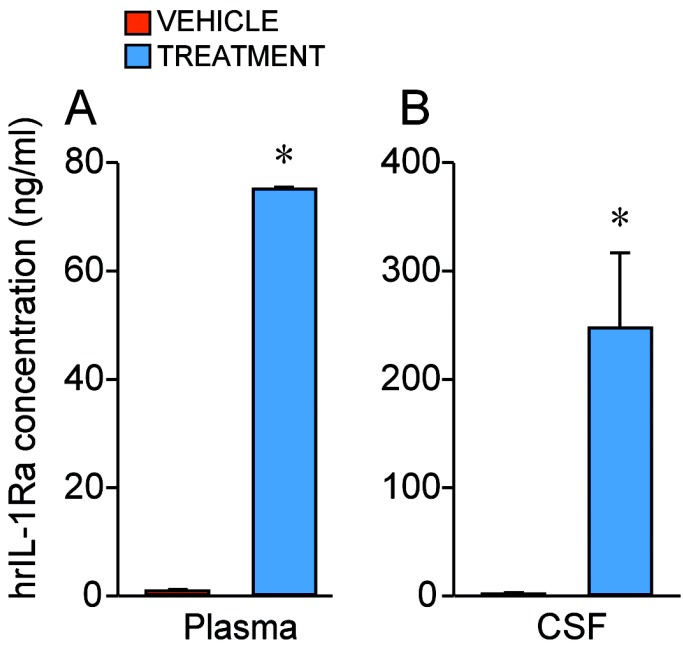

Figure 1
Human recombinant (hr)IL-1Ra (anakinra) concentration in plasma and CSF of rats exposed to electrical status epilepticus, 7 days after the combined anakinra and VX-765 treatment Data are the mean ± SE (n=5 rats in each group). Experimental (TREATMENT) and control (VEHICLE) rats were subcutaneously implanted with osmotic minipumps preloaded with (hr)IL-1Ra (Anakinra, 200 mg/2 ml; 10 µl/h, ~24 mg/day) or saline, respectively, 3 h after ongoing status epilepticus (SE). Minipumps were left in place for 7 days. At the same time of minipumpimplantation, rats were i.v. injected with a bolus of 10 mg Anakinra or saline. VX-765 (200 mg/kg, i.p) or its vehicle (0.1% Tween 80 + 0.5% HEC) was administered at the time of bolus injection, then twice a day for the subsequent 7 days. At the end of the 7th day, treatment was discontinued and the minipump removed. CSF was withdrawn from cisterna magna, and blood collected from heart puncture. (hr)IL-1Ra was measured by ELISA in plasma (A) and CSF (B). *$p<0.05$ vs vehicle by Mann Whitney U test for two independent groups.

FIGURE 2

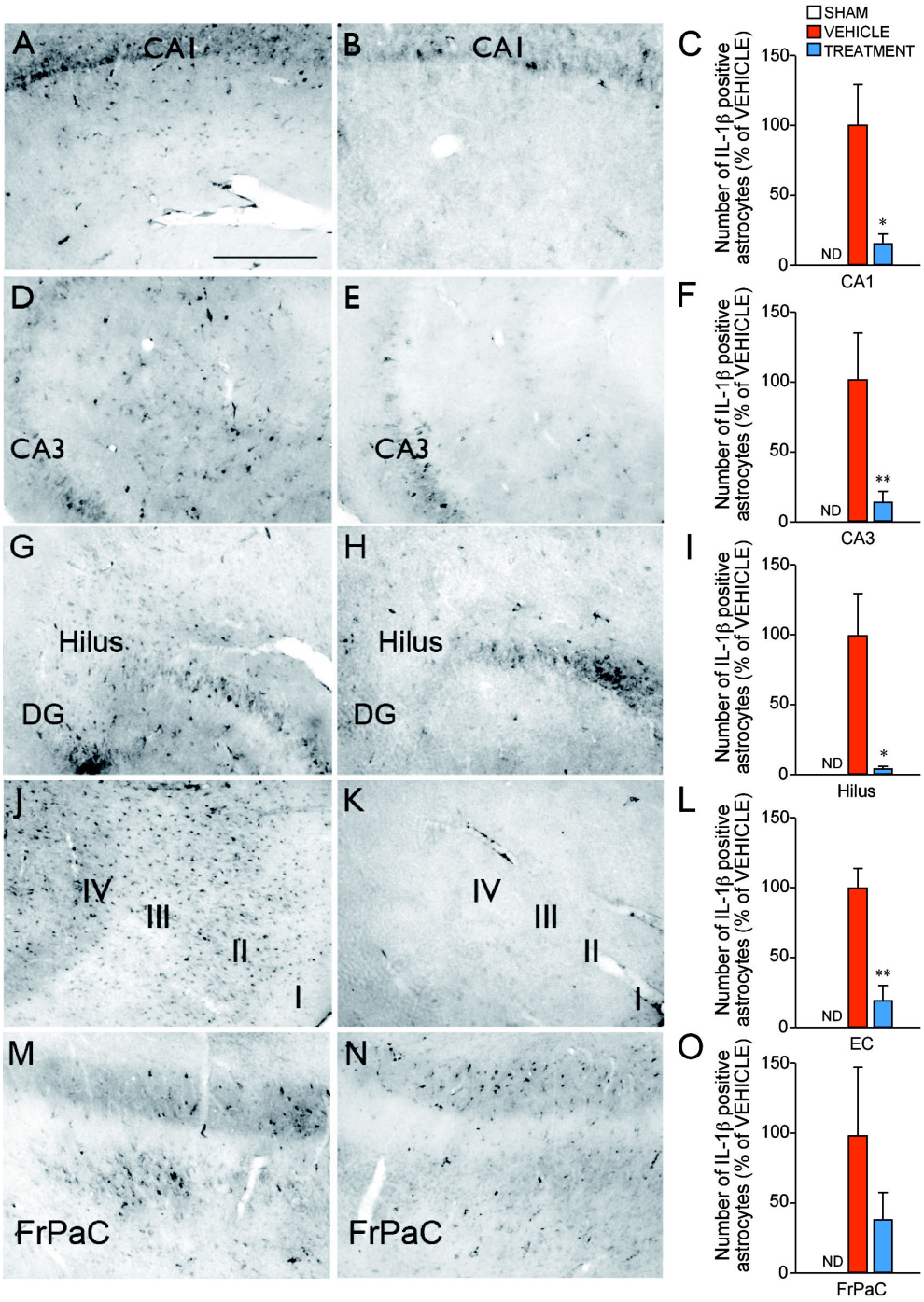

Figure 2
IL-1β immunostaining in forebrain areas of rats exposed to electrical status epilepticus and treated with the combined anakinra and VX-765 treatment during epileptogenesis Representative micrographs depicting immunohistochemical evidence of IL-1β induction in astroglial cells in the hippocampus (A,D,G), entorhinal cortex (J) and frontoparietal cortex (M), 7 days after SE induction in vehicle-treated rats (VEHICLE). Notably, rats undergoing combined anti-inflammatory treatment during epileptogenesis (B,E,H,K,N) showed a strong IL-1β signal reduction. Bargrams (C,F,I,L,O; mean ± SE) report the number of IL-1β-positive cells in each area analysed in control (VEHICLE; n=5) and combined treatment (TREATMENT; n=5) groups. Shamimplanted rats (n=5) were killed at the same time as experimental rats and showed no IL-1β signal (ND). *p<0.05, **p<0.01 vs vehicle by Mann Whitney U test for two independent groups. Scale bar 100 µm.

FIGURE 3

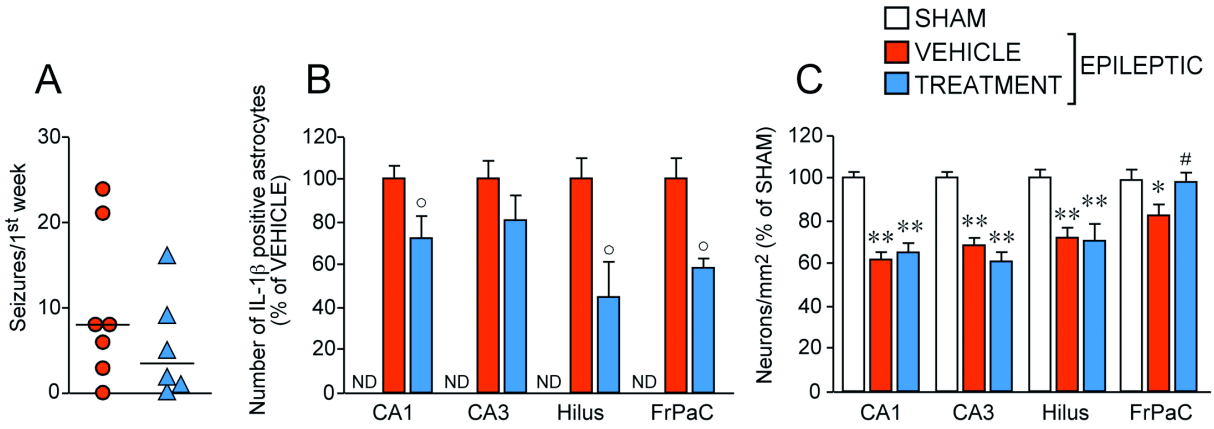

Figure 3
Effects of anakinra and VX-765 treatment in the lithium/pilocarpine model of SE in rats Panel A shows the seizure frequency in SE rats treated with vehicle (n=7) or receiving the combined treatment (n=6), as assessed by continuous video/EEG recording (24 h/day) for 5 or 7 days after SE (see also Suppl. Figure 2). Panel B illustrates the number of IL-1β-positive cells in hippocampus and frontoparietal cortex in SE rats treated with vehicle (n=7) or anakinra and VX-765 (n=6). Rats were killed after the end of EEG recording (see above). All bars were significantly different from Sham (ND) ($p<0.01$). Panel C illustrates the effect of treatment on SE-induced neurodegeneration. Data from both hemispheres were averaged in each rat since they did not differ (Mann-Whitney). *$p<0.05$; **$p<0.01$ vs SHAM; °$p<0.05$ vs VEHICLE; #$p=0.05$ vs VEHICLE by one-way ANOVA followed by Bonferroni test.

FIGURE 4

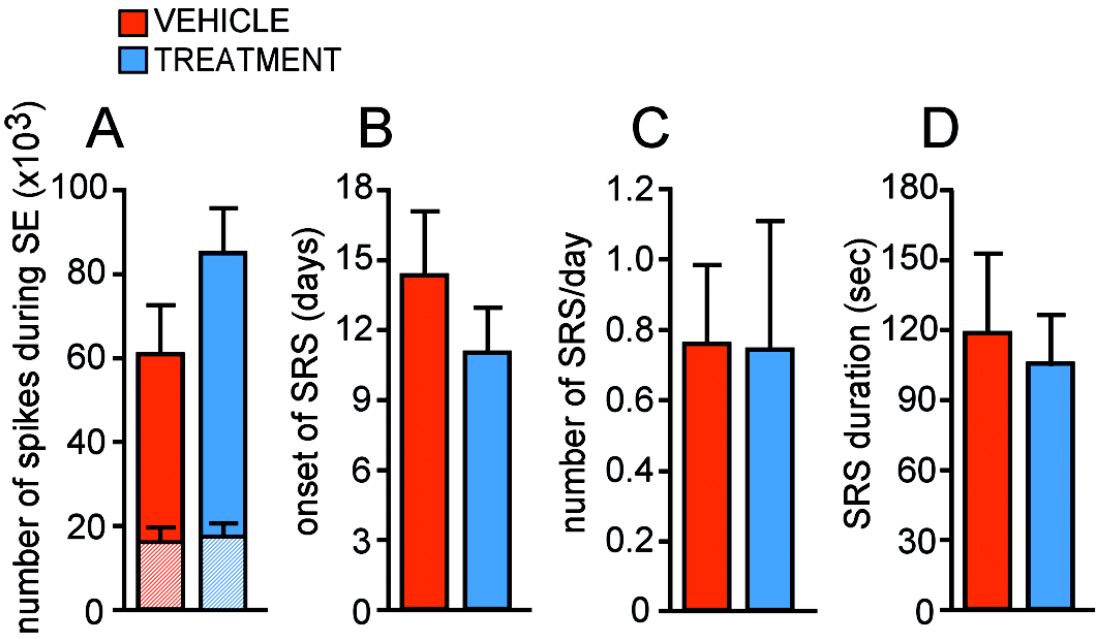

Figure 4
EEG evaluation of electrical status epilepticus and spontaneous recurrent seizures in rats receiving the combined anakinra and VX-765 treatment during epileptogenesis. Data are the mean ± SE. Panel (A) depicts the total number of epileptiform spikes during SE measured by EEG for 24 h from SE onset in vehicle (n=11) and treated (n=11) rats. Dashed inner bars in panel A represent the number of spikes in each group during 3 h preceding treatment. Panels B, C and D depict the onset time of the first spontaneous seizure ensuing after SE (n=11 each experimental group) and the average frequency and duration of spontaneous recurrent seizures (SRS) in epileptic animals (n=6 each experimental group), as assessed by 2 weeks EEG recording (24/7), 3 months after SE.

FIGURE 5

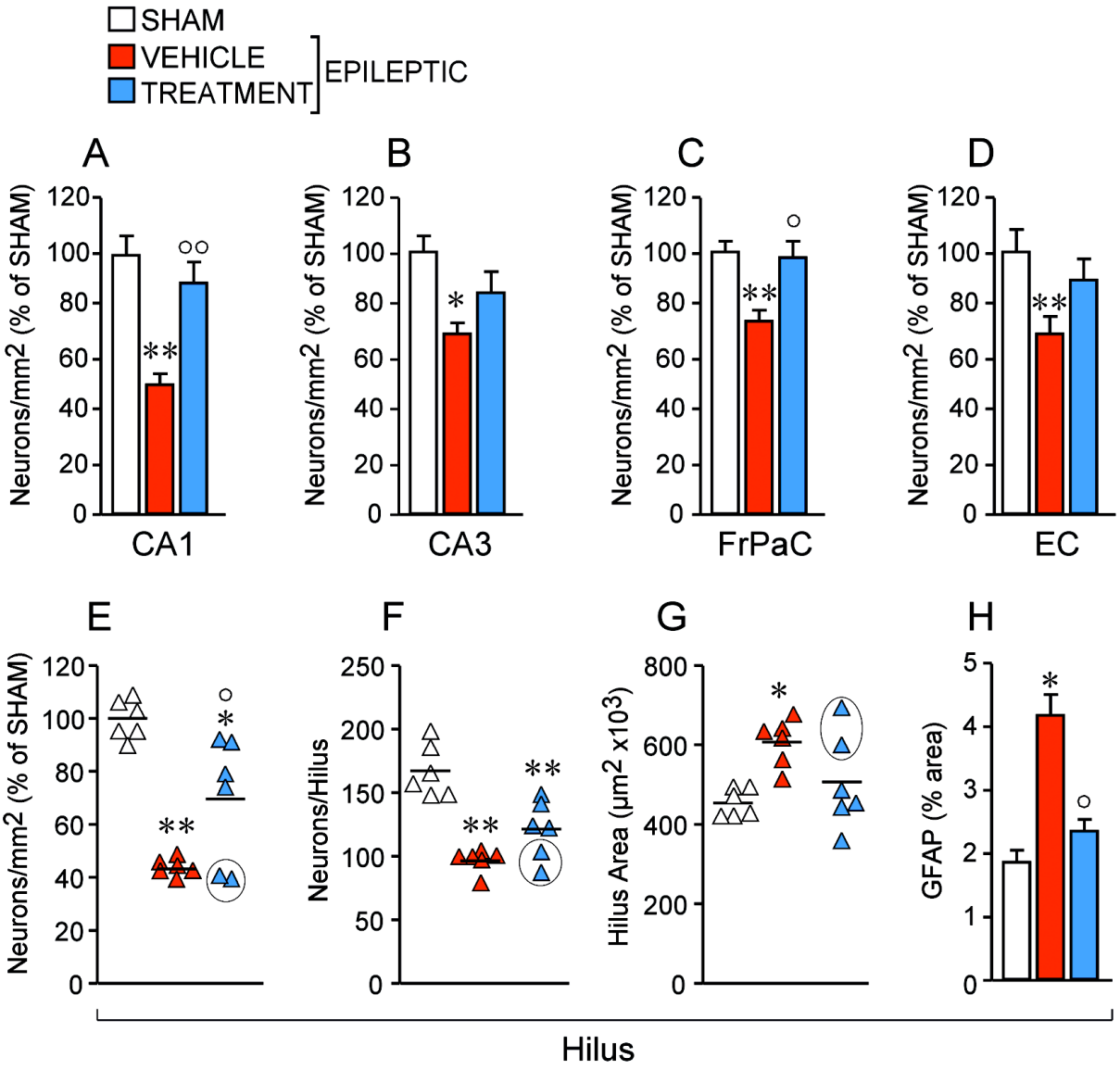

Figure 5

Treatment effects on neuronal density in forebrain areas and morphological changes in the hilus of epileptic rats after electrical status epilepticus. Bargrams represent quantification of neuronal density (A-E), total number of hilar interneurons (F), hilar area (G) and related astrogliosis (H) in epileptic rats receiving either vehicle or treatment for 7 days after SE, and killed 3 months later (after EEG evaluation of SRS, same rats as in Fig. 4). Data in panel H represent the area covered by the GFAP staining expressed as percentage of the total area analysed (see Methods). Data are mean ± SE (n=6). Statistical analysis refers to data including all rats (n=6). *p<0.05, **p<0.01 vs sham rats (implanted with electrodes but not stimulated); °p<0.05, °°p<0.01, vs vehicle by one-way ANOVA followed by Bonferroni test. Circled symbols in panels E,F,G denote the 2 rats in the treatment group (n=6) which did not differ from vehicle rats. In panels F,G, when these rats were excluded from the treatment group (n=4), a significant difference was observed with vehicle rats (p<0.01).

FIGURE 6

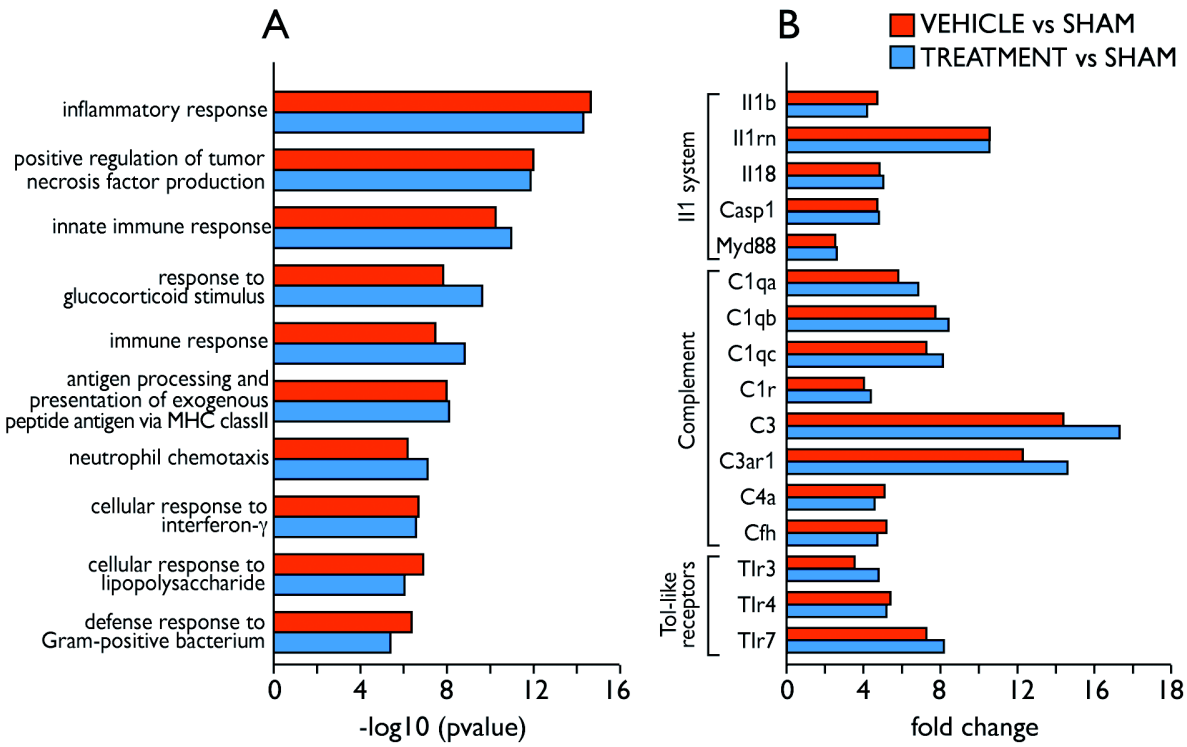

Figure 6

Bar plots of Gene Ontology (GO) terms and differentially expressed genes (DEGs) involved in the inflammation process Transcriptomic analysis in hippocampal samples from sham rats (implanted with electrodes but not stimulated, n=5) and rats exposed to SE and treated with the combined drugs (n=5) or their vehicle (n=5). Treatment or vehicle was given for 5 days after 3 h of SE, then rats were killed for biochemical analysis. Panel A shows bar plot of top 10 common significant ($p<0.05$ by Fisher's exact test) GO terms found after enrichment on DEGs between vehicle-injected and sham rats, and between combined treatment and sham rats; p values are expressed as -log10(p). Panel B shows the bar plot of significant ($p<0.05$ by Rank Product) DEGs involved in the inflammatory processes grouped by signaling pathways in the two experimental groups vs sham rats.