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Effects of SC58236, a selective COX-2 inhibitor, on epileptogenesis and spontaneous seizures in a rat model for temporal lobe epilepsy

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Summary Inflammation is an important biological process that is activated after status epilepticus and could be implicated in the development of epilepsy. Here we tested whether an anti-inflammatory treatment with a selective cox-2 inhibitor (SC58236) could prevent the development of epilepsy or modify seizure activity during the chronic epileptic phase. SC58236 was orally administered (10 mg/kg) during the latent period for 7 days, starting 4 h after electrically induced SE. Seizures were monitored using EEG/video monitoring until 35 days after SE. Cell death and inflammation were investigated using immunocytochemistry (NeuN and Ox-42). Sprouting was studied using Timm's staining after 1 week and after 4—5 months when rats were chronic epileptic. SC58236 was also administered during 5 days in chronic epileptic rats. Hippocampal EEG seizures were continuously monitored before, during and after treatment. SC58236 effectively reduced PGE_2 production but did not modify seizure development or the extent of cell death or microglia activation in the hippocampus. SC58236 treatment in chronic epileptic rats did not show any significant change in seizure duration or frequency of daily seizures. The fact that cox-2 inhibition, which effectively reduced prostaglandin levels, did not modify epileptogenesis or chronic seizure activity suggests that this type of treatment (starting after SE) will not provide an effective anti-epileptogenic or anti-epileptic therapy.

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Introduction

In most patients who suffer from human mesial temporal lobe epilepsy (MTLE), the development of epilepsy is an ongoing process. The disease process follows an initial insult (e.g. trauma, complex febrile seizures or status epilepticus) and after a latency period — that may last many years — leads to subsequent spontaneous seizures that might worsen over time, even to a stage that seizures become intractable (Engel, 1996; Sutula and Hermann, 1999). Control of epilepsy has primarily focused on suppressing seizure activity after epilepsy has developed. However it is even more challenging to avoid the development of epilepsy (acquired by an initial event) by preventing or stopping epileptogenesis, the process by which the brain becomes epileptic. Only a few experimental studies have focused on the effect of drugs and the risk of epilepsy after status epilepticus (SE). Chronic treatment with an alpha2 adrenergic agonist did not prevent epileptogenesis although the epilepsy became milder (Pitkanen et al., 2004). Furthermore, treatment during status epilepticus with high doses of diazepam has shown some anti-epileptogenic effects (Pitkanen et al., 2005). However, clinical trials using conventional anti-epileptic drugs (AEDs) have been disappointing and did not reveal an anti-epileptogenic effect (Temkin, 2001). Therefore it is important to test potential new targets which could be used to control the epileptogenic process that occurs after an initial insult.

Effects of drugs on epileptogenesis can be tested in animal models for MTLE in which epilepsy develops after a chemically or electrically induced SE. In our laboratory we use the rat post-SE model in which epilepsy gradually develops after an electrically evoked SE. In a large-scale microarray study we have investigated gene expression in hippocampus (CA3) and entorhinal cortex at different epileptogenic stages. We found many genes that dynamically change their expression at specific time points after SE (Gorter et al., 2006, 2007). Biological processes such as proteolysis, inflammation, immune and defense response were most conspicuously upregulated in the acute and latent phase and some in the chronic epileptic phase.

Recently, using microarrays we found prominent changes in prostaglandin synthesis and regulation shortly after SE (Fig. 1 and Gorter et al., 2006). An activity-dependent increase of cyclooxygenase-2 (Cox-2) expression has been observed within one day after SE and during the chronic epileptic phase (Gorter et al., 2006). Activation of Cox-2 has also been observed during kindling epileptogenesis (Chen et al., 1995; Tu and Bazan, 2003),. This enzyme is responsible for the activation of prostanoids including PGE_2. Increased activity of the prostanoid pathway produces neuroinflammation involving release of cytokines and oxidative stress. Moreover, activation of prostanoids (via Cox-2) can induce glutamate release (Bezzi et al., 1998) and can increase excitability via modulation of potassium channels (Chen and Bazan, 2005). All these conditions could destabilize neuronal network activity. Effects on development of epilepsy have also been studied in genetically modified mice: Cox-2 deficiency decreases the incidence of afterdischarges in a rapid kindling model (Takemiya et al., 2003), while Cox-2 overexpression leads to increased lethality after kainate induced seizures (Kelley et al., 1999). Together these studies suggest that cox-2 inhibitors are possible candidates that merit being investigated since the modification of these targets may counteract the development of the epileptic condition or modify seizure frequency during the chronic epileptic phase.

Material and methods

Experimental animals

Adult male Sprague—Dawley rats (Harlan Netherlands, Horst, The Netherlands) weighing 400—600 g were used in this study which was approved by the University Animal Welfare committee. In total 90 rats were used. The rats were housed individually in a controlled environment ($21 \pm 1\,^{\circ}C$; humidity 60%; lights on 12 h/day; food and water available *ad libitum*).

Electrode implantation and status epilepticus induction

Rats were anesthetized with an i.p. injection of ketamine (57 mg/kg; Alfasan, Woerden, The Netherlands) and xylazine (9 mg/kg; Bayer AG, Leverkusen, Germany), and placed in a stereotaxic frame. A pair of stimulation electrodes was implanted in the angular bundle; recording electrodes were aimed at the hippocampus as described previously (Gorter et al., 2001). Two weeks after recovery from the operation, each rat was transferred to a recording cage (40 cm × 40 cm × 80 cm) and connected to a recording and stimulation system (NeuroData Digital Stimulator, Cygnus Technology Inc., USA) with a shielded multi-strand cable and electrical swivel (Air Precision, Le Plessis Robinson, France). Hippocampal EEG signals were amplified (10×) via a FET transistor that connected the headset to a differential amplifier (20×; CyberAmp, Axon Instruments, Burlingame, CA, USA), filtered (1—60 Hz) and digitized by a computer. EEG software (Harmonie, Stellate Systems, Montreal, Canada) sampled the incoming signal at a frequency of 200 Hz per channel. A week after habituation to the new condition, rats underwent tetanic stimulation (50 Hz) of the hippocampus in the form of a succession of trains of pulses every 13 s. Each train had a duration of 10 s and consisted of biphasic pulses (pulse duration 0.5 ms, maximal intensity 500 μA). Stimulation was stopped when the rats displayed sustained forelimb clonus and salivation for a few minutes, which usually occurred within 1 h. Behavior was observed during electrical stimulation and several hours thereafter. Immediately after termination of the stimulation, *periodic epileptiform discharges* (PEDs) occurred at a frequency of 1—2 Hz and were accompanied by behavioral and EEG seizures (status epilepticus) which lasted for several hours. In order to administer the drug (or vehicle), SE was interrupted at 4 h after the start using isoflurane anesthesia (4 vol%). Untreated control rats were implanted with electrodes and handled like experimental rats but they were not electrically stimulated.

Treatment with Cox-2 inhibitor SC58236

Latent period

We previously described that in untreated post-SE rats epilepsy starts to develop after a latency of ~8—13 days, after which spontaneous seizures start to occur with increasing frequency that stabilizes after 3—4 months in the majority of rats (Gorter et al., 2001; van Vliet et al., 2004). Therefore we decided to treat the animals with the Cox-2 inhibitor during the first week of epileptogenesis. In this study we used SC58236 (Pfizer-Kalamazoo, Michigan, USA, 10 mg/kg, p.o.), a highly selective Cox-2 inhibitor, that was orally administered at a dosage of 10 mg/kg as previously described (Govoni et al., 2001). These authors found that a single treatment at a dosage of 10 mg/kg (and higher) is neuroprotective in

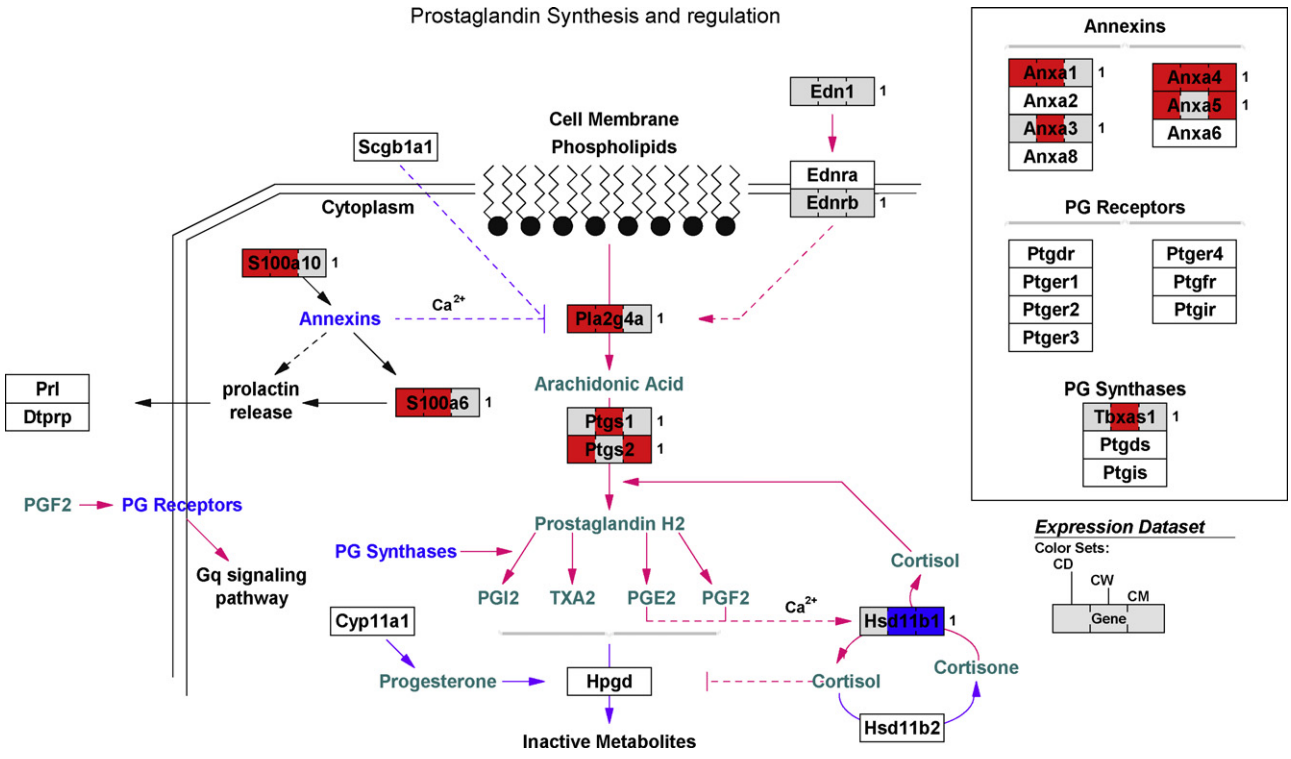

Figure 1 Schematic representation of the genes involved in the prostaglandin synthesis pathway in the entorhinal cortex at three time points after status epilepticus in the rat (obtained with the web-accessible program Genmapp, Doniger et al., 2003). Gene expression was detected using Affymetrics microarrays (RAE230A). Each box is divided in 3 parts which represent 3 different time points at which gene expression is studied (=time points, D, 1 day post-SE; W, 1 week post-SE; M, 3—4 months post-SE) which are color coded to indicate significance level when compared with control (C) expression ($p < 0.05$): Grey: not different from control; red: significantly upregulated in entorhinal cortex compared to control expression; blue: significantly downregulated compared to control expression; white: the gene is not present on the array. Note the biphasic upregulation of Ptgs2 (=Cox-2). Part of the data was presented in Gorter et al. (2006) in which the analysis and statistics are explained in more detail. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

rats after a focally induced ischemic insult. SC58236 was suspended in 0.5% methylcellulose and daily applied (in the early afternoon) for 7 days after SE starting 4 h after SE ($n = 7$). A gastric tube was used to administer the drug solution (under isoflurane anesthesia). A post-SE control group ($n = 7$) was treated similarly except that only methylcellulose was administered.

Chronic epileptic phase

SC58236 was applied for 5 days during the chronic epileptic phase using a gastric tube. Hereto we selected chronic epileptic rats ($n = 15$) that had a relatively stable number of daily seizures (4—5 months after SE; at average ~15 seizures per day). Each rat was first treated with vehicle for 5 days after which it was treated with SC58236 for 5 days (1× early afternoon); in this way each rat served as its own control. Recovery of the treatment was measured in 8 rats. The other 7 rats were sacrificed to obtain brain material that was stored for other experiments. In the recovery phase they were injected with vehicle for another 4 days after which they were recorded for 3 days without any treatment.

Prostaglandin analysis

In order to confirm that SC58236 effectively inhibited Cox-2, a prostaglandin E_2 (PGE_2) assay was performed in brain tissue of 22 additional rats (control rats (C; $n = 6$), 1 day after SE ($n = 4$), 1 day after SE treated with SC58236 4 h after SE and 5 h before decapitation (1 day SC; $n = 4$), chronic epileptic rats ($n = 4$) and chronic epileptic rats treated for 5 days during the chronic phase (chronic SC; $n = 4$)). The hippocampus, and cerebellum were dissected and homogenated by pottering in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 10 μM indomethacin. Hereafter PGE_2 was purified using C-18 Solid Phase Extraction Cartridges (Cayman Chemical, Ann Arbor, USA) and the amount of PGE_2 in these brain regions was determined in duplo by a competitive enzyme immunoassay, the PGE_2 Express EIA kit (Cayman Chemical, Ann Arbor, USA), according to the manufacturer's instructions.

Histology and immunocytochemistry

Histology and immunostainings were performed on brain sections from a separate group of rats (i.e. not included in the EEG measurements), and that had been sacrificed at different time points after SE (in total, $n = 39$).

Rats were deeply anesthetized with pentobarbital (Nembutal, intraperitoneally, 60 mg/kg). The animals were perfused through the ascending aorta with 300 ml 0.37% Na_2S solution followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were post-fixed *in situ* overnight at 4 °C. For cox-2 staining ($n = 12$) brains were embedded in paraffin (see below). For all other immunostainings brains were dissected and cryoprotected in 30% phosphate-buffered sucrose solution, pH 7.4. After overnight incubation at 4 °C, the brains were frozen in isopentane (−30 °C) and stored at −80 °C until sectioning. The brains were cut in sections (40 μm) on a sliding microtome.

Table 1 Summary of clinical details of cases studied according to pathology.

Pathology type	Number of cases	Male/female	Mean age at surgery or death (range/years)	Mean duration of epilepsy (range/years)	Engel class
HS (S)	6	3/3	27.0 (17—34)	21.3 (6—29)	I A
Non-HS (S)	4	2/2	30.3 (17—42)	17.4 (8—24)	I A
Controls (PM)	5	3/2	36.4 (18—51)	NA	NA

S = Surgical tissue; PM = post mortem tissue; HS = hippocampal sclerosis; NA = not applicable.

NeuN and OX-42 immunocytochemistry in rats treated during the latent period

SC58236 treated and vehicle (methylcellulose; MC) treated post-SE rats were sacrificed for immunocytochemistry at 1 week (SC, $n = 8$; MC, $n = 6$), and 4—5 months after the initial SE ($n = 4$ in each group); a control group that was not stimulated was also included ($n = 5$). Horizontal sections of treated and vehicle treated post-SE and control rats were stained with different immunocytochemical markers. Sections were washed in 0.05 M phosphate buffered saline (PBS), pH 7.4 and incubated for 30 min in 0.3% hydrogen peroxide in PBS to inactivate endogenous peroxidase. Sections were then washed (2× 10 min) in 0.05 M PBS, followed by washing (1× 60 min) in PBS + 0.4% Bovine Serum Albumin (BSA). Sections were incubated with OX-42 as marker for activated microglia (mouse anti-rat CD11b/c (OX-42), 1:100, PharMingen, CA, USA) and a neuronal marker (NeuN; mouse clone MAB377; Chemicon, Temecula, CA, USA; 1:2000), in PBS + 0.4% BSA. Twenty-four hours after the incubation with the primary antibody, the sections were washed in PBS (3× 10 min) and then incubated for 1.5 h in biotinylated sheep anti-mouse Ig (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), diluted 1:200 in PBS. Sections were washed in PBS (3× 10 min) and incubated for 60 min with AB-mix (Vectastain ABC kit, Peroxidase Standard pk-4000, Vector Laboratories, Burlingame, CA, USA). After washing (3× 10 min) in 0.05 M Tris—HCl, pH 7.9, the sections were stained with 3,3′-diaminobenzidin tetrahydrochloride (30 mg DAB, Sigma—Aldrich, Zwijndrecht, The Netherlands) and 2.5 μl 30% hydrogen peroxide in a 10 ml solution of Tris—HCl. The staining reaction was followed under the microscope and stopped by washing the sections in Tris—HCl. After mounting on superfrost plus slides, the sections were air dried, dehydrated in alcohol and xylene and coverslipped with Entellan (Merck, Darmstadt, Germany).

Cox-2 immunostaining in rat brain

Cox-2 immunostaining in rat brain was performed on paraffin sections of post-SE rats and control rats (3 sections per rat). In total 12 rats were used using the polyclonal anti-Cox2 antibody (Cayman Chemical, Ann Arbor, USA; #160106; 1:200). The tissue was sectioned at 6 μm (sagittal orientation), mounted on organosilane-coated slides (SIGMA, St. Louis, MO) and used for immunocytochemical reactions. Sections were deparaffinated in xylene, rinsed in ethanol (100%, 95%, 70%) and incubated in 0.3% hydrogen peroxide diluted in methanol for 20 min. After that, slides were placed into sodium citrate buffer (0.01 M, pH 6.0). Autoclaving was used for antigen retrieval (10 min, 120 °C). Slides were then washed with phosphate-buffered saline (PBS; 10 mM, pH 7.4). After incubation in primary antibody for 24 h, sections were washed in PBS and stained with PowerVision Peroxidase system (ImmunoVision, Brisbane, CA, USA). After washing, sections were stained with 3,3′-diaminobenzidin tetrahydrochloride (50 mg DAB, Sigma—Aldrich, Zwijndrecht, The Netherlands) and 5 μl 30% hydrogen peroxide in a 10 ml solution of Tris—HCl.

Cox-2 immunoreactivity in human brain

In order to test the possible relevance of increased Cox-2 expression we also tested whether Cox-2 expression was changed in tissue of patients with hippocampal sclerosis. We used brain material that was obtained from the files of the department of neuropathology of the Academical Medical Center (University of Amsterdam). Patients underwent resection of the hippocampus ($n = 6$) for medically intractable epilepsy. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. This material was compared to normal-appearing hippocampi of 5 autopsy specimens from patients without history of seizures or other neurological diseases. We also included hippocampal surgical specimens of patients without HS (non-HS; with a focal lesion not involving the hippocampus proper). The clinical characteristics derived from the patient's medical records are summarized in Table 1. Brain tissue was fixed in 10% buffered formalin, paraffin embedded, sectioned at 6 μm and mounted on organosilane-coated slides (Sigma, St. Louis, MO, USA). Representative sections of all specimens were processed for immunocytochemistry. Sections were deparaffinated in xylene, rinsed in ethanol (100%, 96%) and incubated in 0.3% hydrogen peroxide diluted in methanol for 20 min. Slides were then washed with phosphate-buffered saline (PBS; 10 mM, pH 7.4) and overnight incubated at 4 °C in anti-cox-2 (mouse, clone CX229, Cayman, 160112; 1:100). Hereafter, sections were washed in PBS and stained with PowerVision Peroxidase system (ImmunoVision, Brisbane, CA, USA). After washing, sections were stained with 3,3′-diaminobenzidin tetrahydrochloride (50 mg DAB, Sigma—Aldrich, Zwijndrecht, The Netherlands) and 5 μl 30% hydrogen peroxide in a 10 ml solution of Tris—HCl. Sections were counterstained with hematoxylin, dehydrated in alcohol and xylene and coverslipped. Sections incubated without anti-cox-2 or with preimmune serum were essentially blank. Characterization of the antibody used in human brain tissue has been documented previously (Hoozemans et al., 2001). The specificity of the antibodies used, was further tested performing western blot analysis of total homogenates of human and rat control hippocampus (data not shown).

Timm's staining

A part of the sections (40 μm) were either processed for immunohistochemistry (see below for details) or mounted on superfrost plus slides and processed by a modified Timm method (for details see (Sloviter, 1982). We processed sections of 1 week ($n = 4$ in each group) SC- and vehicle treated rats, chronic epileptic SC-treated and vehicle treated epileptic rats ($n = 4$ in each group) and control rats; $n = 5$). Sections were processed at the same time with the same development time (55—58 min) to enable comparison between groups. The extent of synaptic reorganization of the mossy fibers was evaluated by two observers according to a standardized 0—5 scale according to the extent and density of zinc stained granules (Cavazos et al., 1992). 0: No zinc staining between the tips and crest of the dentate gyrus (DG). 1: Sparse zinc staining in the supragranular region in a patchy distribution between the tips and crest of the DG. 2: More abundant staining in the supragranular region with patchy distribution between tips and crest of the DG and a continuous pattern near the tips. 3: Prominent staining in the supragranular region in a continuous pattern between the tips and crest of the DG. 4: Prominent staining in the supra-

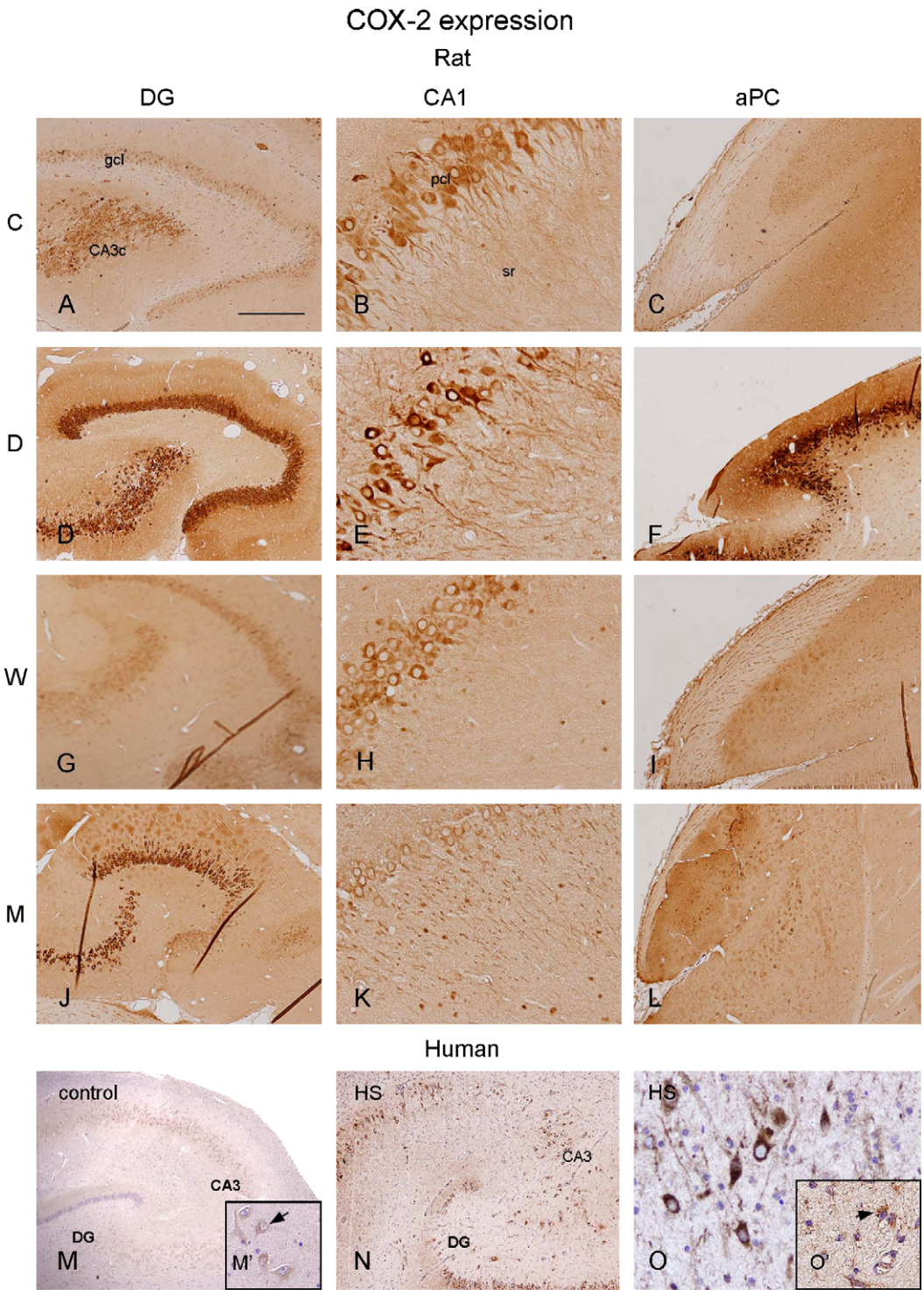

Figure 2 Cox-2 immunostaining on sagittal paraffin sections in the *rat* in DG (A, D, G, J), CA1 (B, E, H, K) and anterior piriform cortex (C, F, I, L) show clear neuronal upregulation at 1 day (D) and 4 months (M) after SE. M-O: Cox-2 immunostaining on paraffin sections in *human* shows low Cox-2 expression in control (M-M′) and increased cox-2 expression in neurones (N-O) and astrocytes (O′).

granular region that forms a confluent dense laminar band in the inner molecular layer (IML) but does not completely fill it. 5: Confluent dense laminar band of staining in the supragranular region that completely occupies the IML. Intermediary values were scored in case the suprapyramidal blade had a different score than the infrapyramidal blade.

Sections were photographed using bright-field illumination on an Olympus microscope, equipped with a digital camera (Olympus, DP11, Paes Nederland, Zoeterwoude, The Netherlands), and imported into Adobe Photoshop (version 7.0). This program was used to optimize contrast and brightness, but not to enhance or change the image content in any way.

Statistical comparisons

Analysis of brain sections used for immunohistochemistry (with NeuN) was performed on alternate sections at the midlevel of the hippocampus. The investigator performing the quantification was unaware of the treatment of the animals. The number of neurons in the hilus was counted from the NeuN stained sections (three sections per rat) at 200× magnification. The hilus was defined as the region enclosed by the granule cell layer and by two imaginary straight lines connecting the two tips of the granule cell layer with the proximal end of the CA3 pyramidal cell layer. Thus the neuron counts as performed in this study are relative estimates of the amount of neurons counted at the specific level and not absolute calculations of the number of hilar neurons in each rat. Cell counts were normalized to those of control sections (100%). Values are given as mean value ± SEM. These data are expressed as mean ± SEM. Statistical comparisons were performed on the absolute number of NeuN positive cells using Student's t test. $P < 0.05$ was assumed to indicate a significant difference. The intensity and the number of OX-42 immunoreactive cells were estimated semiquantitatively. The intensity of immunoreactivity was classified as: (+) = weak, + = moderate, ++ = strong. The frequency of immunoreactive cells was classified as: 1 = sparse, 2 = moderate, 3 = high, 4 = very high in different hippocampal and entorhinal cortex subfields (layers).

Results

Severity of status epilepticus

Four hours after electrically induced SE, SC58236 (or vehicle) was administered to the rats under isoflurane anesthesia. Before the anesthesia, all rats exhibited moderate behavioral SE with often continuous head bobbing, regularly interrupted by stage V seizures. Although the behavioral manifestation had become milder after recovery from anesthesia in both groups, the electrographic SE continued for several hours more. SE duration did not significantly differ between the SC-treated and vehicle treated groups (SC ($n = 7$): 9.5 ± 0.4 h; vehicle ($n = 7$) 8.9 ± 0.7).

Cox-2 expression

Rat

In panels A—L of Fig. 2, details are shown of cox-2 immunostaining performed on brain sections in the rat. Immunostaining on sections from 1 day (D) post-SE rats confirmed the upregulation of Cox-2 in the somata and dendrites of hippocampal CA and granule neurons and in the molecular dendritic layer of the dentate gyrus. As reported previously after SE (Joseph et al., 2006), upregulation of Cox-2 was found in neurons of other limbic regions and the superficial cortical layers as well; the (anterior) piriform region (aPC) was the most intensely stained region (Fig. 2C, F, I, L). During the latent period (W) Cox-2 expression was reduced and in the hippocampus resembled control expression; in the chronic epileptic phase (M), Cox-2 upregulation was found in CA3 and granule cells but to lesser extent than shortly (1 day) after SE.

Human

Cox-2 immunostaining on hippocampal sections from patients with hippocampal sclerosis (HS) showed that strong Cox-2 expression was present in remaining CA3 hippocampal neurons (detail in Fig. 2O). Astrocytic Cox-2 labeling was observed in the hilar region (Fig. 2O′). Histologically, non-sclerotic hippocampus (non-HS) did not show changes in Cox-2 protein expression compared with autopsy specimens from cases with no history of epilepsy.

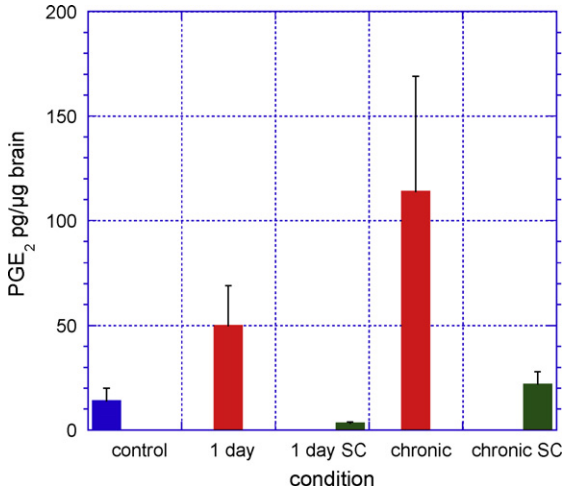

Figure 3 PGE_2 production in ventral rat hippocampus at different time points after SE with or without the Cox-2 inhibitor SC58236.

PGE_2 production after orally administered SC58236

In order to test whether the Cox-2 inhibition protocol with SC58236 (10 mg/kg p.o., 1× per day) was effective in suppressing prostaglandin production, we measured PGE_2 production using a specific Enzyme Immuno Assay kit. We used samples of the hippocampus and cerebellum of post-SE rats (that were treated with SC58236 or methylcellulose only). We measured PGE_2 at two different time points after SE: 1 day and 4—5 months after SE (treated during the chronic phase). Hippocampal tissue showed an increased PGE_2 production at both time points after SE, which was significant at 1 day with a more than 7 fold increase ($p < 0.05$; Fig. 3) and did not reach significance ($p < 0.11$) in the chronic phase due to a large variability in PGE_2 production. Since Cox-2 expression is seizure-driven, the variability of the PGE_2 production among this chronic group of rats could be due to the different times that have elapsed between last spontaneous seizures and sacrifice. SC58236 treatment reduced PGE_2 production at both time points measured. PGE_2 production in cerebellum was lower (~6 pg/µg brain) than in hippocampus and did not increase after SE or change after SC58236.

Development of epilepsy

After the induction of SE we measured the latency to the first seizure and the number of seizures during the last week before sacrifice (day 28—35) in SC-treated and untreated rats using EEG/video recordings. In total 14 rats were monitored (SC58236, $n = 7$; vehicle treated, $n = 7$). One rat in each group died within a few weeks after the treatment. All rats developed seizures. In the SC58236 post-SE

Figure 4 Evaluation of cell death (NeuN), mossy fiber sprouting (Timm) and microglial activation (OX-42) in control (A—C), 1 week (D—F), 1 week SC-treated (G—I), chronic epileptic (J—L), and chronic epileptic SC-treated during latent period (M—O). Calibration in A, D, G, J, M = 700 μm. All others: 300 μm.

rats the first seizure appeared after 8.8 ± 0.8 days. This was not significantly different from the untreated post-SE rats (8.0 ± 1.1 days). We monitored 7.0 ± 2.1 seizures in SC-treated rats on average during the last week before sacrifice; this number was larger but not significantly different from the vehicle post-SE rats (3.8 ± 2.4 seizures).

Cell death and inflammation after status epilepticus in SC58236 treated rats

In order to assess the extent of inflammation and cell death we used other groups of SC-treated and vehicle rats that had been treated during the latent period and that were sacrificed at 1 week (W) and at 4—5 months after SE (M; chronic). Inflammation was assessed semi-quantitatively, using immunocytochemistry with a microglia (inflammation) marker (OX-42). In both groups of rats, we found considerable activation of OX-42 (Fig. 4). The intensity scores did not differ between groups in any region (Table 2). Hilar neuronal loss was also estimated in treated and untreated rats at 1 week after SE. An unstimulated control group was also included ($n = 5$). We did not detect a significant difference in hilar cell count between treated and untreated post-SE rats (Table 3: SC-treated 37 ± 8% of control values; untreated 42 ± 5% of control values; Student-t performed on absolute hilar cell numbers, n.s.).

Table 2 Immunoreactivity score of OX-42 at 1 week (W) after SE in vehicle treated (−) and SC58236 treated rats (+), ($n = 6$ in each group). Immunoreactivity (IR) was classified as: (+) = weak, + = moderate, ++ = strong. Frequency (fr) was classified as: 1 = sparse, 2 = moderate, 3 = high, 4 = very high. DG = dentate gyrus, Sub = subiculum, PrS = presubiculum, PaS = parasubiculum, EC = entorhinal cortex.

	Control		W (−)		W (+)	
	IR	fr	IR	fr	IR	fr
DG	(+)	2	++	3—4	++	3—4
CA3	(+)	2	++	3—4	++	3—4
CA1	(+)	2	++	3—4	++	3—4
Sub	(+)	2	+	2—3	+	2—3
PrS	(+)	2	(+)	2	(+)	2
PaS	(+)	2	++	3—4	++	3
EC II/III	(+)	2	++	3—4	++	3—4
EC V/VI	(+)	2	+	2	+	2

Table 3 Effects of SC58236 treatment on neuronal hilar cell loss (NeuN counts; $n = 6$ in SC-group and $n = 8$ in vehicle group; $n = 5$ in control group) and mossy fiber sprouting at 1 week (W) and 4—5 months (M) after SE ($n = 4$ in each group).

	Control	Vehicle	SC-treated
Hilar cell loss	100% ± 7	42% ± 5	37% ± 8
Timm score (W)	0.25 ± 0.2	0.75 ± 0.2	1.0 ± 0.1
Timm score (M)	0.25 ± 0.2	4.5 ± 0.3	4.75 ± 0.3

Timm's staining in SC58236 treated rats

The effects of SC treatment during the latent period on mossy fiber sprouting were also studied. Timm's staining performed on sections obtained at 1 week after SE did not reveal a difference in the extent of sprouting (both groups: sparse zinc staining in the supragranular region in a patchy distribution between the tips and crest of the DG; Fig. 4E and H; Table 3). At 4—5 months after SE ($n = 4$ in each group), sprouted fibers completely filled the inner molecular layer of the DG in treated as well as untreated rats (Fig. 4K and N; Table 3).

SC58236 treatment during the chronic epileptic phase

We previously showed that during the chronic epileptic phase a number of inflammatory related genes, including Cox-2, present increased expression compared to the expression in controls (Gorter et al., 2006). PGE_2 production was also still increased in the chronic phase (Fig. 3). This suggests that prostaglandin activation could play a role in seizure progression during the chronic epileptic phase. To find out whether chronic seizure activity could be modulated by Cox-2 inhibition we administered SC58236 for 5 days during the chronic epileptic phase (see Fig. 3). During the week before the treatment started, rats experienced 14.7 ± 2.7 seizures per day. Vehicle injection did not modify seizure frequency or duration (not shown). During the treatment, seizure frequency became more variable but the effects were not significant (Fig. 5A). Duration of seizures during the week before the treatment started was at average 64.7 ± 3.7 s. Individual seizures tended to last longer during the first day of the five day treatment but this was not significant (Fig. 5B).

Discussion

Cox-2, a key enzyme that plays an important role in inflammation, was extensively upregulated after SE in rat and during the chronic epileptic phase both in rat and human. Treatment with the selective cox-2 inhibitor SC58236 during the latent period did not have a significant effect on seizure development in the rat. Although it effectively reduced PGE_2 production, it did not reduce the extent of microglial cell activation, cell death or development of mossy fiber sprouting. Cox-2 inhibition in chronic epileptic rats did not lead to a significant effect on the number or duration of daily seizures.

Cox-2 expression is both upregulated in epileptic rats and human

In the present study we demonstrate that Cox-2 induction was exclusively present in neurons at 1 day after SE as well as in the chronic epileptic phase. There are several studies that have demonstrated an induction of Cox-2

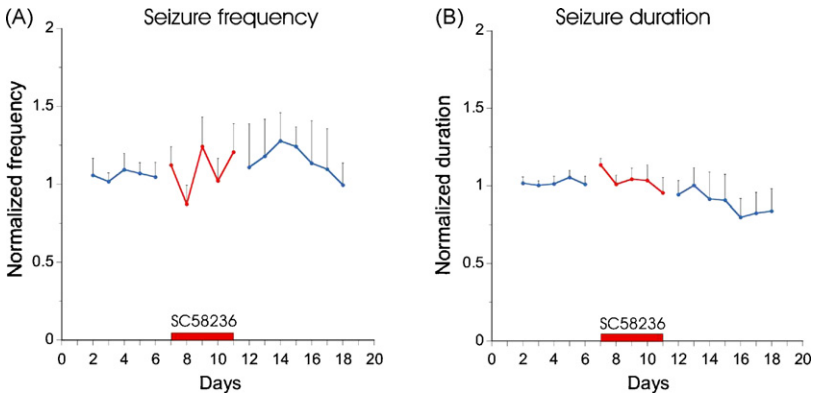

Figure 5 Effects of 5 day Cox-2 inhibition on seizure frequency (seizures/day, mean ± SEM) and seizure duration (seconds; mean ± SEM) during baseline ($n = 15$), SC58236 treatment ($n = 15$) and during recovery phase ($n = 8$). Data are normalized to average baseline levels.

after status epilepticus or kindling stimulations (Chen et al., 1995; Sandhya et al., 1998; Tu et al., 2003; Voutsinos-Porche et al., 2004; Kawaguchi et al., 2005). Upregulation is also observed in our post-SE model, both at the level of gene expression in hippocampal CA3 and entorhinal cortex subregions as at the protein level (Gorter et al., 2006). Cox-2 was regulated in a ''neuronal-activity'' dependent way, since Cox-2 gene expression was highest 1 day after SE, decreased in the latent period, but increased expression again in the chronic epileptic phase when rats had frequent daily seizures. We did not detect Cox-2 induction in astrocytes at any time point after SE, which corresponds with the findings reported after electrical kindling (Tu et al., 2003) and chemically induced SE (Voutsinos-Porche et al., 2004; Joseph et al., 2006), although several studies also report an increased expression in astrocytes after pilocarpine or kainate induced SE (Sandhya et al., 1998; Jung et al., 2006; Lee et al., 2007). In the latter study an early (4 h) wave of CRE-mediated Cox-2 expression was observed in neurons and a later (48 h) long-lasting wave in reactive astrocytes.

The possible relevance of Cox-2 expression was supported by the increased expression in human hippocampal tissue of patients with hippocampal sclerosis. Also here Cox-2 expression was mainly observed in neurons. Astrocytic Cox-2 labeling was observed in the hilar region but was less extensive as has been reported previously (Desjardins et al., 2003).

Cox2 inhibition did not affect the duration of SE or the development of epilepsy

SE and epileptogenesis

We did not detect an effect on SE duration or seizure development after SC58236 administration. Latency to the first seizure did not differ between groups and the number of seizures at 1 month after SE in the SC-treated rats was also not different from vehicle treated rats. In a recent study, a mild antiepileptogenic effect of celecoxib, another highly selective Cox-2 inhibitor, was reported when the inhibitor was administered during the latent period that followed the pilocarpine-induced SE in rats starting 1 day after SE. These authors also reported a mild neuroprotection and reduction of microglial activation (Jung et al., 2006). No other studies on the effects of Cox-2 inhibition and epileptogenesis have been published so far.

Epilepsy

Based on the observed reduction of PGE_2 in epileptic rats after SC58236 treatment, we expected an inhibitory effect by the drug on chronic seizure activity. However, although the variability in seizure frequency and duration increased the effects on seizure duration or frequency were not significant. Since inhibitors of the cyclooxygenase pathways are frequently recommended for the prevention and treatment of several inflammatory diseases, including neurodegenerative disorders, clinical and epidemiological studies in epileptic patients could provide insight in whether this drug affects seizure activity.

Cox-2 inhibition did not lead to reduction of cell death and/or inflammation

Cox-2 inhibition with SC58236 significantly reduced PGE_2 production but, contrary to what we expected, did not appear to have any suppressing effect on microglia activation or hilar cell death. Various studies have shown that Cox-2 inhibition can reduce ischemia- or SE-induced neuronal degeneration when the treatment is started after the insult (Chen et al., 1995; Govoni et al., 2001; Kunz and Oliw, 2001; Kawaguchi et al., 2005; Jung et al., 2006) although aggravation of neurodegeneration has also been reported (Kim et al., 2008). When a Cox-2 inhibitor is applied before the SE, aggravation of seizure activity has been reported (Baik et al., 1999; Gobbo and O'Mara, 2004; Kim et al., 2008). However, there are some conflicting results regarding the effects on seizure activity which can be related to the dose and selectivity of the Cox-2 inhibitor (and PGE_2 levels) and seizure type. For instance, the aggravating effect of Cox-2 inhibition on seizure activity is not observed during pentylenetetrazol (PTZ) induced seizures (Dhir et al., 2007; Oliveira et al., 2008). PGE_2 seems to play a crucial role as shown recently in the PTZ seizure model, since an anticonvulsant action of celecoxib was reversed by the intracerebroventricular administration of PGE_2 (Oliveira et al., 2008). The fact that SC58236 effectively reduced PGE_2 production supports the fact that the Cox-2 inhibition was effective so that it is not likely that the observed lack of neuroprotection or lack of reduction on microglia activation was due to insufficient action of the drug. There might be several possible explanations why we could not detect any pronounced effect of the Cox-2 inhibitor while other studies did report effects on Cox-2 inhibition. First: the SE is a too severe insult which might overwhelm the Cox-2 inhibition effect. Since the electrical stimulation model generally produces a less severe behavioral SE than the pilocarpine or kainate models in which neuroprotection by Cox-2 inhibition was reported we believe that this is not a likely explanation. Second: since a transient increase in PGE_2 levels may have a neuroprotective role, the time of the treatment might be crucial as suggested by (Gobbo et al., 2004). For instance, celecoxib treatment 2 h before kainate-induced SE did not lead to a significant effect on histological parameters or learning performance while a 2 h post-kainate treatment led to a significant improved learning performance. Since positive effects of Cox-2 inhibition had been reported at 2 h post-SE (Gobbo et al., 2004), we expected a positive effect using our post-SE cox-2 inhibition protocol. Nevertheless, we cannot exclude that the long-duration SE (~9—10 h) negatively interfered with the outcome of Cox-2 inhibition that started within this period. The third possibility that could explain why neuroprotective effects of Cox-2 inhibition are observed in those other studies, is that these studies are performed in models (kainate- or pilocarpine-induced SE or after ischemia) where astrocytic Cox-2 induction is reported (Sandhya et al., 1998; Jung et al., 2006; Lee et al., 2007). Since Cox-2 induction in astrocytes contributes to astrocytic PGE_2 production which stimulates astrocytic glutamate release (Bezzi et al., 1998) this would exacerbate neurodegeneration. Consequently, Cox-2 inhibition would have a more pronounced protective effect as observed in those studies than when Cox-2 is only induced in neurons (as in the electrical stimulation post-SE model) where effects of

Cox-2 inhibition might be much smaller or not detectable. Nimesulide, another Cox-2 inhibitor, was also not able to significantly inhibit the development of electrical kindling, a model in which only neuronal Cox-2 activation is observed (Tu et al., 2003).

Conclusion

In summary, we showed that treatment with the Cox-2 inhibitor SC58236, starting shortly after electrically induced SE, did not have pronounced effects on the extent of neurodegeneration, sprouting or microglial activation. More importantly, Cox-2 inhibition did not lead to a reduced rate of epileptogenesis. Treatment during the chronic epileptic phase also did not lead to a significant change in seizure frequency or duration. The lack of any substantial effects of this selective compound during the latent period or during chronic epilepsy, suggests that Cox-2 inhibition might not provide an effective anti-epileptogenic or anti-epileptic therapy. Whether other anti-inflammatory treatments are more effective will be investigated in the near future.

Conflict of interest

The authors have no conflicts of interest.

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References

Baik, E.J., Kim, E.J., Lee, S.H., Moon, C., 1999. Cyclooxygenase-2 selective inhibitors aggravate kainic acid induced seizure and neuronal cell death in the hippocampus. Brain Res. 843, 118—129.

Bezzi, P., Carmignoto, G., Pasti, L., Vesce, S., Rossi, D., Rizzini, B.L., Pozzan, T., Volterra, A., 1998. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 391, 281—285.

Cavazos, J.E., Golarai, G., Sutula, T.P., 1992. Septotemporal variation of the supragranular projection of the mossy fiber pathway in the dentate gyrus of normal and kindled rats. Hippocampus 2, 363—372.

Chen, C., Bazan, N.G., 2005. Endogenous PGE2 regulates membrane excitability and synaptic transmission in hippocampal CA1 pyramidal neurons. J. Neurophysiol. 93, 929—941.

Chen, J., Marsh, T., Zhang, J.S., Graham, S.H., 1995. Expression of cyclo-oxygenase 2 in rat brain following kainate treatment. Neuroreport 6, 245—248.

Desjardins, P., Sauvageau, A., Bouthillier, A., Navarro, D., Hazell, A.S., Rose, C., Butterworth, R.F., 2003. Induction of astrocytic cyclooxygenase-2 in epileptic patients with hippocampal sclerosis. Neurochem. Int. 42, 299—303.

Dhir, A., Naidu, P.S., Kulkarni, S.K., 2007. Neuroprotective effect of nimesulide, a preferential COX-2 inhibitor, against pentylenetetrazol (PTZ)-induced chemical kindling and associated biochemical parameters in mice. Seizure 16, 691—697.

Doniger, S.W., Salomonis, N., Dahlquist, K.D., Vranizan, K., Lawlor, S.C., Conklin, B.R., 2003. MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. Genome Biol. 4, R7.

Engel Jr., J., 1996. Clinical evidence for the progressive nature of epilepsy. Epilepsy Res. Suppl. 12, 9—20.

Gobbo, O.L., O'Mara, S.M., 2004. Post-treatment, but not pre-treatment, with the selective cyclooxygenase-2 inhibitor celecoxib markedly enhances functional recovery from kainic acid-induced neurodegeneration. Neuroscience 125, 317—327.

Gorter, J.A., van Vliet, E.A., Aronica, E., Breit, T., Rauwerda, H., Lopes da Silva, F.H., Wadman, W.J., 2006. Potential new antiepileptogenic targets indicated by microarray analysis in a rat model for temporal lobe epilepsy. J. Neurosci. 26, 11083—11110.

Gorter, J.A., Van Vliet, E.A., Aronica, E., Lopes da Silva, F.H., 2001. Progression of spontaneous seizures after status epilepticus is associated with mossy fibre sprouting and extensive bilateral loss of hilar parvalbumin and somatostatin-immunoreactive neurons. Eur. J. Neurosci. 13, 657—669.

Gorter, J.A., Van Vliet, E.A., Rauwerda, H., Breit, T., Stad, R., van Schaik, L., Vreugdenhil, E., Redeker, S., Hendriksen, E., Aronica, E., Lopes da Silva, F.H., Wadman, W.J., 2007. Dynamic changes of proteases and protease inhibitors revealed by microarray analysis in CA3 and entorhinal cortex during epileptogenesis in the rat. Epilepsia 48, 53—64.

Govoni, S., Masoero, E., Favalli, L., Rozza, A., Scelsi, R., Viappiani, S., Buccellati, C., Sala, A., Folco, G., 2001. The cycloxygenase-2 inhibitor SC58236 is neuroprotective in an in vivo model of focal ischemia in the rat. Neurosci. Lett. 303, 91—94.

Hoozemans, J.J., Rozemuller, A.J., Janssen, I., De Groot, C.J., Veerhuis, R., Eikelenboom, P., 2001. Cyclooxygenase expression in microglia and neurons in Alzheimer's disease and control brain. Acta Neuropathol. 101, 2—8.

Joseph, S.A., Lynd-Balta, E., O'Banion, M.K., Rappold, P.M., Daschner, J., Allen, A., Padowski, J., 2006. Enhanced cyclooxygenase-2 expression in olfactory-limbic forebrain following kainate-induced seizures. Neuroscience 140, 1051—1065.

Jung, K.H., Chu, K., Lee, S.T., Kim, J., Sinn, D.I., Kim, J.M., Park, D.K., Lee, J.J., Kim, S.U., Kim, M., Lee, S.K., Roh, J.K., 2006. Cyclooxygenase-2 inhibitor, celecoxib, inhibits the altered hippocampal neurogenesis with attenuation of spontaneous recurrent seizures following pilocarpine-induced status epilepticus. Neurobiol. Dis. 23, 237—246.

Kawaguchi, K., Hickey, R.W., Rose, M.E., Zhu, L., Chen, J., Graham, S.H., 2005. Cyclooxygenase-2 expression is induced in rat brain after kainate-induced seizures and promotes neuronal death in CA3 hippocampus. Brain Res. 1050, 130—137.

Kelley, K.A., Ho, L., Winger, D., Freire-Moar, J., Borelli, C.B., Aisen, P.S., Pasinetti, G.M., 1999. Potentiation of excitotoxicity in transgenic mice overexpressing neuronal cyclooxygenase-2. Am. J. Pathol. 155, 995—1004.

Kim, H.J., Chung, J.I., Lee, S.H., Jung, Y.S., Moon, C.H., Baik, E.J., 2008. Involvement of endogenous prostaglandin F2alpha on kainic acid-induced seizure activity through FP receptor: the mechanism of proconvulsant effects of COX-2 inhibitors. Brain Res. 1193, 153—161.

Kunz, T., Oliw, E.H., 2001. The selective cyclooxygenase-2 inhibitor rofecoxib reduces kainate-induced cell death in the rat hippocampus. Eur. J. Neurosci. 13, 569—575.

Lee, B., Dziema, H., Lee, K.H., Choi, Y.S., Obrietan, K., 2007. CRE-mediated transcription and COX-2 expression in the pilocarpine model of status epilepticus. Neurobiol. Dis. 25, 80—91.

Oliveira, M.S., Furian, A.F., Royes, L.F., Fighera, M.R., Fiorenza, N.G., Castelli, M., Machado, P., Bohrer, D., Veiga, M., Ferreira, J., Cavalheiro, E.A., Mello, C.F., 2008. Cyclooxygenase-

2/PGE2 pathway facilitates pentylenetetrazol-induced seizures. Epilepsy Res. 79, 14—21.

Pitkanen, A., Kharatishvili, I., Narkilahti, S., Lukasiuk, K., Nissinen, J., 2005. Administration of diazepam during status epilepticus reduces development and severity of epilepsy in rat. Epilepsy Res. 63, 27—42.

Pitkanen, A., Narkilahti, S., Bezvenyuk, Z., Haapalinna, A., Nissinen, J., 2004. Atipamezole, an alpha(2)-adrenoceptor antagonist, has disease modifying effects on epileptogenesis in rats. Epilepsy Res. 61, 119—140.

Sandhya, T.L., Ong, W.Y., Horrocks, L.A., Farooqui, A.A., 1998. A light and electron microscopic study of cytoplasmic phospholipase A2 and cyclooxygenase-2 in the hippocampus after kainate lesions. Brain Res. 788, 223—231.

Sloviter, R.S., 1982. A simplified Timm stain procedure compatible with formaldehyde fixation and routine paraffin embedding of rat brain. Brain Res. Bull. 8, 771—774.

Sutula, T.P., Hermann, B., 1999. Progression in mesial temporal lobe epilepsy. Ann. Neurol. 45, 553—556.

Takemiya, T., Suzuki, K., Sugiura, H., Yasuda, S., Yamagata, K., Kawakami, Y., Maru, E., 2003. Inducible brain COX-2 facilitates the recurrence of hippocampal seizures in mouse rapid kindling. Prostaglandins Other Lipid Mediat. 71, 205—216.

Temkin, N.R., 2001. Antiepileptogenesis and seizure prevention trials with antiepileptic drugs: meta-analysis of controlled trials. Epilepsia 42, 515—524.

Tu, B., Bazan, N.G., 2003. Hippocampal kindling epileptogenesis upregulates neuronal cyclooxygenase-2 expression in neocortex. Exp. Neurol. 179, 167—175.

van Vliet, E.A., Aronica, E., Tolner, E.A., Lopes da Silva, F.H., Gorter, J.A., 2004. Progression of temporal lobe epilepsy in the rat is associated with immunocytochemical changes in inhibitory interneurons in specific regions of the hippocampal formation. Exp. Neurol. 187, 367—379.

Voutsinos-Porche, B., Koning, E., Kaplan, H., Ferrandon, A., Guenounou, M., Nehlig, A., Motte, J., 2004. Temporal patterns of the cerebral inflammatory response in the rat lithium-pilocarpine model of temporal lobe epilepsy. Neurobiol. Dis. 17, 385—402.