doi:10.1093/brain/awn271 *Brain* (2008), **131**, 3256–3265

A novel non-transcriptional pathway mediates the proconvulsive effects of interleukin-1β

Silvia Balosso,[1,*] Mattia Maroso,[1,*] Manuel Sanchez-Alavez,[2] Teresa Ravizza,[1] Angelisa Frasca,[1] Tamas Bartfai[2] and Annamaria Vezzani[1]

[1]Laboratory of Experimental Neurology, Department of Neuroscience, Mario Negri Institute for Pharmacological Research, Milan, Italy and [2]Molecular and Integrative Neurosciences Department (MIND), The Scripps Research Institute, La Jolla, CA, USA

*These authors contributed equally to this work.

Correspondence to: A. Vezzani, PhD, Laboratory of Experimental Neurology, Department of Neuroscience, Mario Negri Institute for Pharmacological Research, Via G. La Masa 19, Milan, Italy
E-mail: vezzani@marionegri.it

Interleukin-1β (IL-1β) is overproduced in human and rodent epileptogenic tissue and it exacerbates seizures upon brain application in rodents. Moreover, pharmacological prevention of IL-1β endogenous synthesis, or IL-1 receptor blockade, mediates powerful anticonvulsive actions indicating a significant role of this cytokine in ictogenesis. The molecular mechanisms of the proconvulsive actions of IL-1β are not known. We show here that EEG seizures induced by intrahippocampal injection of kainic acid in C57BL6 adult mice were increased by 2-fold on average by pre-exposure to IL-1β and this effect was blocked by 3-*O*-methylsphingomyelin (3-O-MS), a selective inhibitor of the ceramide-producing enzyme sphingomyelinase. C2-ceramide, a cell permeable analog of ceramide, mimicked IL-1β action suggesting that ceramide may be the second messenger of the proconvulsive effect of IL-1β. The seizure exacerbating effects of either IL-1β or C2-ceramide were dependent on activation of the Src family of tyrosine kinases since they were prevented by CGP76030, an inhibitor of this enzyme family. The proconvulsive IL-1β effect was associated with increased Tyr^{418} phosphorylation of Src-family of kinases indicative of its activation, and Tyr^{1472} phosphorylation of one of its substrate, the NR2B subunit of the *N*-methyl-D-aspartate receptor, which were prevented by 3-O-MS and CGP76030. Finally, the proconvulsive effect of IL-1β was blocked by ifenprodil, a selective NR2B receptor antagonist. These results indicate that the proconvulsive actions of IL-1β depend on the activation of a sphingomyelinase- and Src-family of kinases-dependent pathway in the hippocampus which leads to the phosphorylation of the NR2B subunit, thus highlighting a novel, non-transcriptional mechanism underlying seizure exacerbation in inflammatory conditions.

Keywords: experimental epilepsy; glia activation; cytokines; NMDA receptor; inflammation

Abbreviations: 3-O-MS = 3-*O*-methylsphingomyelin; BSA = bovine serum albumin; NMDA = *N*-methyl-D-aspartate; DMSO = dimethylsulfoxide; ICE = interleukin-1 converting enzyme; icv = intracerebroventricular; IL-1RI = IL-1 receptor type I; IL-1β = Interleukin-1β; PBS = phosphate-buffered saline

Received June 13, 2008. Revised August 5, 2008. Accepted September 21, 2008. Advance Access publication October 24, 2008

Introduction

Prominent inflammatory processes have been described in epileptogenic brain tissue surgically resected from patients with chronic drug-resistant epilepsies (Vezzani and Granata, 2005), including clinical cases which do not feature a typical inflammatory pathophysiology, such as temporal lobe epilepsy and epilepsy-associated with malformations of cortical development (Crespel *et al.*, 2002; Maldonado *et al.*, 2003; Ravizza *et al.*, 2006*a*; Ravizza *et al.*, 2008). Moreover, brain injuries associated with CNS inflammation lead to the early occurrence of seizures and can result in epilepsy (Pitkanen and Sutula, 2002; Vezzani and Granata, 2005). These observations, together with the clinical evidence that anti-inflammatory treatments provide seizure control in some cases of drug-resistant epilepsies (Vezzani and Granata, 2005), support the possibility that inflammation in the brain is implicated in the development of seizures. Accordingly, the induction of brain inflammation by

lipopolysaccharide in experimental models has been shown to increase seizure susceptibility (Sayyah *et al*., 2003; Heida and Pittman, 2005; Galic *et al*., 2008). Experimental studies also indicate that seizures *per se* are among the most potent inducers of cytokines and other downstream mediators of inflammation in the brain (Vezzani *et al*., 1999; De Simoni *et al*., 2000; Turrin and Rivest, 2004; Vezzani and Granata, 2005; Gorter *et al*., 2006; Aronica *et al*., 2007), suggesting that seizures, upon their occurrence, contribute to perpetuate brain inflammation. Immunohistochemical analysis of epileptogenic areas after induction of seizures in rodents showed a strong and rapid upregulation of interleukin-1β (IL-1β) in astrocytes and microglia. Fast onset neuronal expression of IL-1 receptor type 1 (IL-1R1) was also observed, followed by upregulation of IL-1R1 in astrocytes (Vezzani *et al*., 1999, 2000; Ravizza and Vezzani, 2006; Ravizza *et al*., 2008), suggesting that IL-1β mediates functional glio-neuronal communication during seizures. A similar pattern of IL-1β and IL-1R1 expression was demonstrated in human chronic epileptic tissue (Ravizza *et al*., 2006*a*, 2008).

Pharmacological studies support a crucial role of the IL-1β–IL-1R1 signaling in neuronal network hyperexcitability underlying seizures. Thus, intracerebral application of IL-1β decreases the threshold of seizure induction (Dubé *et al*., 2005; Heida and Pittman, 2005) and prolongs the frequency and duration of seizures (Vezzani *et al*., 1999, 2000). In contrast, IL-1Ra, an endogenous IL-1R1 competitive antagonist (Dinarello, 1998), mediates powerful anticonvulsant effects (De Simoni *et al*., 2000; Vezzani *et al*., 2002), and mice overexpressing the secreted human form of IL-1Ra in astrocytes exhibit decreased seizure susceptibility (Vezzani *et al*., 2000). Importantly, seizures are dramatically reduced when blocking endogenous IL-1β synthesis by interleukin-1 converting enzyme (ICE) inhibition, or in mice with a null mutation of ICE (Ravizza *et al*., 2006*b*). These data indicate that elevated levels of IL-1β in the brain due to a preexisting inflammatory state or to the occurrence of seizures, or both, result in proconvulsive effects.

The effects of IL-1β on seizures have a rapid onset, which is consistent with activation of fast, non-transcription dependent pathways that leads to ion channels modification. We had previously demonstrated that IL-1β activation of IL-1R1 in cultured hippocampal neurons induces Src family of kinases-mediated tyrosine phosphorylation of N-methyl-D-aspartate (NMDA) receptor NR2B subunit which is co-expressed with IL-1R1 on pyramidal hippocampal neurons (Viviani *et al*., 2003). This effect facilitates NMDA receptor-mediated increase in intracellular Ca^{2+} in neurons (Viviani *et al*., 2003), suggesting that this molecular event may be involved in IL-1β proconvulsive actions. Ceramide, the intracellular signaling product of sphingomyelinases (Kolesnick and Golde, 1994) also alters Ca^{2+} currents in neurons through ceramide-activated protein phosphatases and kinases like Src, which in turn changes the phosphorylation state of various receptors and ion channels, including NMDA receptors (Gulbins *et al*., 1997; Bock *et al*., 2003; Salter and Kalia, 2004; Davis *et al*., 2006b). In particular, the ceramide-producing enzyme neutral sphingomyelinase (N-Smase) is activated in mouse forebrain upon application of IL-1β via the IL-1R1–MyD88 complex (Nalivaeva *et al*., 2000).

We tested here whether this pathway, which starts with IL-1β-mediated activation of N-Smase, the production of ceramide and the subsequent phosphorylation of Src-family of tyrosine kinases and the target receptor protein NR2B subunit, mediates the proconvulsive effects of IL-1β. The detailed understanding of the IL-1β-mediated signaling in conditions of elevated cytokine levels is instrumental for understanding the mechanisms underlying seizure exacerbation in inflammatory conditions.

Materials and Methods

Experimental animals

Animals were housed at a constant temperature (23°C) and relative humidity (60%) 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 institutional guidelines that are in compliance with national and international laws and policies.

Cannula and electrodes implantation

Male C57BL6 mice (60-day-old, 25 g, Charles River, Calco, Italy) were surgically implanted with an injection guide cannula and recording electrodes under deep Equithesin anestesia and stereotaxic guidance (Vezzani *et al*., 2000; Balosso *et al*., 2005). Two nichrome-insulated bipolar depth electrodes (60 µm OD) were implanted bilaterally into the dorsal hippocampus [from bregma (mm): nose bar 0; anteroposterior –1.9, lateral 1.5 and 1.8 below dura mater]. A 23-gauge cannula was unilaterally positioned on top of the dura mater and glued to one of the depth electrodes for the intrahippocampal infusion of drugs (see below). In some mice, an additional guide cannula was positioned on dura mater ipsilateral to the hippocampal cannula for intracerebroventricular (icv) injection of drugs [from bregma (mm): nose bar 0; anteroposterior 0.0, lateral 1.0 and 3.0 below dura mater]. The electrodes were connected to a multipin socket and, together with the cannula of injection, secured to the skull by acrylic dental cement. The correct position of the electrodes and injection needle was evaluated in the sections used for immunohistochemical analysis of brain tissue (see below).

Pharmacological treatments

Intracerebral injection of drugs in freely moving mice have been previously described (Vezzani *et al*., 2000; Balosso *et al*., 2005).

Kainic acid (7 ng/0.5 µl; Sigma, Saint Louis, MI, USA) was dissolved in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and injected unilaterally in the dorsal hippocampus by using a needle protruding 1.8 mm from the bottom of the guide cannula. This dose of kainic acid was proven to induce EEG ictal episodes and spiking activity in the hippocampus in 100% of mice without mortality (Balosso *et al*., 2005).

Human recombinant (hr)IL-1β (R&D System, Minneapolis, USA) was dissolved in 0.1 M PBS supplemented with 0.1%

bovine serum albumin (BSA) and injected intrahippocampally (1 ng/0.5 µl) 10 min before kainic acid. This is the same dose causing proconvulsive effects in kainate-injected rats (Vezzani *et al.*, 1999, 2002).

C2-ceramide, a membrane-permeable ceramide analogue, and dihydroceramide, the biologically inactive form of C2-ceramide (Sigma) (Obeid *et al.*, 1993) were dissolved in 10% dimethylsulfoxide (DMSO) in PBS and injected intrahippocampally (0.25–1.0–2.0 µg in 0.5 µl), 10 min before kainic acid.

3-O-Methylsphingomyelin (3-O-MS, N-Smase inhibitor; BioMol Research Laboratories Inc., PA, USA) (Zeng *et al.*, 2005; Tsakiri *et al.*, 2008) was dissolved in 10% DMSO in PBS and injected intrahippocampally (3 µg/0.5 µl) or ICV (15 µg/1 µl), 20 min before kainic acid. These doses were shown to block the rapid phase of febrile response to IL-1β after injection into the mouse preoptic area/anterior hypothalamus (pilot study; see Sanchez-Alavez *et al.*, 2006).

CGP76030, a selective Src-family of tyrosine kinase inhibitor which binds to the catalytic SH1 domain, thus preventing substrate phosphorylation (Susa *et al.*, 2000; Susa *et al.*, 2005; Rucci *et al.*, 2006) was provided as a research tool by Novartis AG, Basel, Switzerland. It was dissolved in 2% DMSO in PBS and injected intrahippocampally (65 ng/0.5 µl) or icv (130 ng/1 µl), 20 min before kainic acid. The dosage of CGP76030 corresponds to the one achieved in mouse brain after oral administration of 30 µmol/kg, as assessed measuring its brain/plasma ratio at 1 and 3 h, reaching a highest ratio at 3 h. This dose blocked the electrophysiological effects of IL-1β on the activity of warm-sensitive hypothalamic neurons *in vivo* (pilot study; see also Sanchez-Alavez *et al.*, 2006).

Ifenprodil [NR2B-selective NMDA antagonist (Chenard and Menniti, 1999), Sigma] was dissolved in 5% DMSO with 9% Tween 80 in PBS and injected intraperitoneally (1 mg/kg), 15 min before kainic acid. We choose this dose since it was shown to block NR2B receptors *in vivo* during tPA facilitation of ethanol withdrawal seizures, although not affecting seizures *per se* (Pawlak *et al.*, 2005).

Control mice were injected with the corresponding volume of vehicles before kainic acid. Pharmacological experiments were carried between 9.00 am and 2.00 pm.

Seizures assessment and quantification

EEG seizures induced by intrahippocampal injection of kainic acid in mice have been extensively described (Balosso *et al.*, 2005). Briefly, a 30 min recording was done before kainic acid injection to assess the basal EEG pattern and for 180 min thereafter. At least a 30 min recording similar to baseline was required before ending the experiment. Ictal episodes are characterized by high-frequency and/or multispike complexes and/or high-voltage synchronized spikes simultaneously occurring in the injected and contralateral hippocampi. Spiking activity is typically observed between seizures and after seizures subside. The EEG recording of each animal was analysed visually by two independent investigators unaware of the treatments to detect any activity different from baseline. Seizure activity was quantified by reckoning the time elapsed from kainic acid injection to the occurrence of the first EEG seizure (onset) and the total number and total duration of seizures (reckoned by summing up the duration of every ictal episode during the EEG recording period). Seizures occurred with an average latency of about 10 min from kainic acid injection, then recurred for about 90 min from their onset. Interictal activity was reckoned by summing up the time spent in EEG spiking.

Immunocytochemistry

At the end of the EEG analysis, mice injected with kainic acid ($n = 5$) and their controls ($n = 5$), were deeply anaesthetized using Equithesin and perfused via ascending aorta with 50 mM cold PBS, pH 7.4 followed by chilled 4% paraformaldehyde in 0.1 M PBS. The brains were post-fixed for 90 min at 4°C, and then transferred to 20% sucrose in PBS for 24 h at 4°C. The brains were rapidly frozen in –50°C isopentane for 3 min and stored at –80°C until assayed. Serial cryostat coronal sections (40 µm) were cut from all brains throughout the septo-temporal extension of the hippocampus (Franklin and Paxinos, 1997) and collected in 0.1 M PBS. Primary and secondary antibodies and experimental procedures were chosen to determine a specific immunohistochemical signal of the protein of interest in rodent brain slices (Ravizza and Vezzani, 2006; Ravizza *et al.*, 2008).

IL-1β. Slices were incubated at 4°C for 10 min in 70% methanol and 2% H_2O_2 in Tris–HCl-buffered saline (TBS, pH 7.4) followed by 30 min incubation in 10% foetal calf serum (FCS) in 1% Triton X-100 in TBS. Then, slices were incubated overnight with the primary antibody against IL-1β (1:200, Santa Cruz Bio., CA, USA) at 4°C in 10% FCS in 1% Triton X-100 in TBS. Immunoreactivity was tested by the avidin-biotin-peroxidase technique (Vector Labs, USA), the sections were reacted using diaminobenzydine (DAB) and the signal was amplified by nickel ammonium.

IL-1R1. IL-1R1 immunostaining was carried out as previously described (Ravizza and Vezzani, 2006). Briefly, sections were incubated at 4°C for 10 min in 0.3% H_2O_2 in 0.3% Triton X-100 in PBS. After three 5 min washes in 0.3% Triton X-100 in PBS, slices were incubated at 4°C for 60 min in 10% FCS in 0.3% Triton X-100 in PBS. Then, slices were incubated with the primary antibody against IL-1R1 (6 µg/ml, R&D System) for 72 h at 4°C in 4% FCS in 0.3% Triton X-100 in PBS. Immunorectivity was tested as described for IL-1β.

Double-immunostaining

Two brain slices in each mouse brain for each cell type marker were randomly chosen to identify the cells expressing IL-1β or IL-1R1. After incubation with the primary IL-1β and IL-1R1 antibodies, slices were incubated in biotinylated secondary anti-goat and anti-hamster antibodies respectively (1:200, Vector Labs), then in streptavidin–HRP and the signal was revealed with tyramide conjugated to Fluorescein using TSA amplification kit (NEN Life Science Products, Boston, MA, USA). Sections were subsequently incubated with the following primary antibodies: mouse anti-glial fibrillary acidic protein (1:2500; GFAP, Chemicon, Temecula, CA, USA), a selective marker of astrocytes, or rat anti-mouse CD11b (1:1000; MAC-1, Serotec, Oxford, UK), a marker of microglia-like cells, or mouse anti-neuronal specific nuclear protein (1:1000; NeuN, Chemicon), a selective neuronal marker. Fluorescence was detected using anti-mouse or anti-rat secondary antibody conjugated with Alexa546 (Molecular Probes, Leiden, The Netherlands). Slide-mounted sections were examined with an Olympus Fluorview laser scanning confocal microscope (microscope BX61 and confocal system FV500; Hamburg, Germany) using dual excitation of 488 nm (Laser Ar) and 546 nm (Laser He–Ne green) for Fluorescein and Alexa546, respectively. The emission of fluorescent probes was collected on separate detectors.

To eliminate the possibility of bleed-through between channels, the sections were scanned in a sequential mode.

Evaluation of neuronal damage

Neuronal loss was evaluated using Nissl-stained slices as previously described (Hoffman *et al*., 2003). A subset of mice treated with kainic acid, or ceramide + kainate, or IL-1β + kainate and their respective vehicle- injected controls, were killed one week after treatment by transcardial perfusion as described above ($n = 5$ mice in each experimental group). Serial cryostat sections (40 μm) were cut coronally from plate 41 to 50 according to Franklin and Paxinos (1997), collected on slides and Nissl stained. Two sections in each brain were analysed, namely at –1.46 mm (corresponding to plate 43) and –2.06 mm (corresponding to plate 48) from bregma.

In each section, neuronal cell loss was quantified by measuring the area occupied by Nissl-stained neurons in CA3 pyramidal cell layers (Hoffman *et al*., 2003). An image of the whole hippocampus was captured with a 4× objective using an Olympus light microscope (BX61; Hamburg, Germany) connected to a ColorView digital camera (Olympus). Using an AnalySIS Image Processing software (Soft Imaging System, Olympus), we divided the areas of interest in non-overlapping fields (each of 700 μm width × 400 μm high) using a 20× magnification, and in these fields we measured the area (in μm^2) occupied by Nissl-stained neurons. By summing up these values, we determined the total area in CA3 occupied by Nissl-stained neurons which reflects neuronal density in each region. The quantification of neuronal loss was performed by two investigators blind to the identification code of the samples.

Western blot

Different groups of mice ($n = 10$–14 in each group) were implanted with electrodes and cannula as described before and injected with hrIL-1β, kainic acid, or their combination ± 3-O-MS or CGP76030 (see before for injection protocol). Control mice ($n = 14$) were implanted with electrodes and cannula and injected with the corresponding vehicles of the various treatments at the appropriate time interval. One hour after the onset of EEG seizures, experimental mice and their controls were decapitated. The injected dorsal hippocampus was dissected out at 4°C and two hippocampi obtained from different mice within the same experimental group were pooled and homogenized in 20 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 5 mM EGTA, 1 mM Na-vanadate, 2 μg/μl aprotinin, 1 μg/μl pepstatin, 2 μg/μl leupeptin (30 mg tissue/150 μl homogenization buffer), thus obtaining five to seven individual samples in the various treatment groups. Total proteins (70 μg per lane; Bio-Rad Protein Assay) were separated using SDS-PAGE, 10% acrylamide and each sample was run in duplicate. Proteins were transferred to Hybond nitrocellulose membranes by electroblotting. For immunoblotting, we used anti-$pTyr^{418}$-Src which is located in the catalytic domain therefore indicative of Src-family of tyrosine kinases activation (1:750; Sigma) or anti-$pTyr^{1472}$-NR2B (1:1000; Affinity Bioreagents Golden, CO, USA) rabbit polyclonal antibodies. Total NR2B levels were assessed using goat polyclonal anti-NR2B antibody (1:1000; Santa Cruz). Immunoreactivity was visualized with enhanced chemiluminescence (ECL, Amersham, UK) using peroxydase-conjugated goat anti-rabbit (1:2000; Sigma) or rabbit anti-goat (1:10 000; Sigma) IgGs as secondary antibodies. Densitometric analysis of immunoblots was done to quantify the changes in protein levels (AIS image analyzer, Imaging Research Inc., Ontario, Canada) using film exposures with maximal signals below the photographic saturation point. Optical density values in each sample were normalized using the corresponding amount of β-actin.

Statistical analysis of data

Data are the mean ± SEM (n = number of individual samples). The effects of treatments were analysed by two-way ANOVA followed by Tukey or Kruskal–Wallis test, or by Student t-test.

Data reporting quantification of cell loss or depicted in Figs 2 and 3 are expressed as % of vehicle-injected mice; however, statistical analysis was carried out using absolute values.

Results

Seizure-mediated induction of IL-1β and IL-1RI in the mouse hippocampus

Immunohistochemical analysis of hippocampal sections 3 h after intrahippocampal injection of kainic acid, showed increased IL-1β immunoreactivity in astrocytes (Fig. 1B) while IL-1R1 was enhanced both in neurons and astrocytes (Fig. 1D); this activation involved the dorsal and temporal poles of the hippocampus bilaterally. This is the first evidence of increased IL-1β and IL-1R1 in mice after kainate-induced seizures which is in accordance with our previous findings showing induction of IL-1β and IL-1R1 by chemically- or electrically-induced seizures in rats (Vezzani *et al*., 1999, Ravizza and Vezzani, 2006; Ravizza *et al*., 2008) or bicuculline-induced seizures in mice (Vezzani *et al*., 2000). IL-1β and

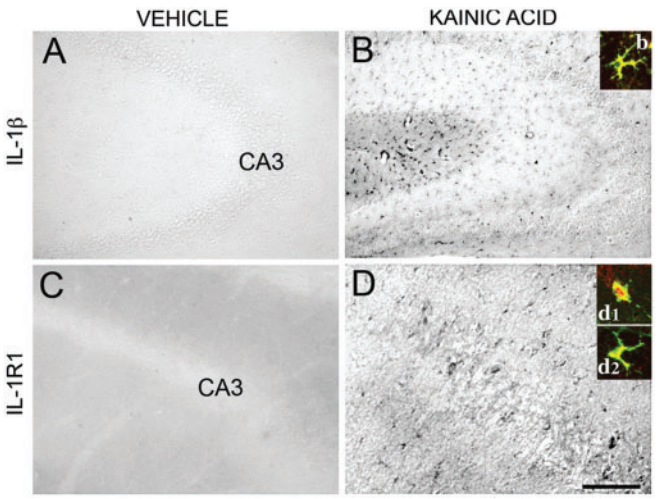

Fig. 1 IL-1β and IL-1RI expression in the mouse hippocampus after kainic acid-induced seizures. Representative photomicrographs of IL-1β (**A–B**) and IL-1RI (**C–D**) immunoreactivity in the CA3 area of the hippocampus, 3 h after seizures induced by intrahippocampal injection of kainic acid (7 ng/0.5 μl; **B** and **D**) and in vehicle-injected C57BL6 mice (**A, C**). IL-1β (**A**) and IL-1RI (**C**) immunostaining was not detectable in control hippocampus. After seizures, IL-1β immunoreactivity is strongly enhanced in GFAP-positive astrocytes (**B**, yellow signal in inset); IL-1RI staining was enhanced both in neurons (**D**, yellow signal in d_1) and astrocytes (**D**, yellow signal in d_2). Scale bar: **A–D** 100 μm; insets, 25 μm.

IL-1R1 (Fig. 1A and C) were not detectable in control hippocampus, as previously shown (Vezzani *et al.*, 1999, 2000; Ravizza and Vezzani, 2006; Ravizza *et al.*, 2008).

N-Smase-Src kinase-NR2B pathway mediates the proconvulsive activity of IL-1β

3-O-MS

We established the role of N-Smase in the proconvulsive effect of IL-1β using the selective N-Smase inhibitor 3-O-MS (Zeng *et al.*, 2005; Tsakiri *et al.*, 2008) (Table 1). The intrahippocampal injection of hrIL-1β, 10 min before kainate, increased by ~1.8-fold the number of seizures and by ~2-fold their duration as compared with vehicle-treated mice ($P<0.01$; Table 1). Three micrograms of 3-O-MS blocked the proconvulsive effect of hrIL-1β when injected into the hippocampus, 10 min before the cytokine (i.e. 20 min before kainate) since it abolished the increase in the number of seizures [$F(1,62)=2.2$; $P<0.05$] and time spent in ictal activity [$F(1,62)=5.9$; $P<0.01$ by two-way ANOVA) induced by hrIL-1β. The latency to the first seizure or the time spent in interictal activity were not affected by these treatments. 3-O-MS injected intrahippocampally (3 μg/0.5 μl) (Table 1) or icv (15 μg/1 μl) (data not shown) 20 min before kainic acid, did not affect seizure parameters; higher doses could not be used because of the limit of solubility of this compound in the volume of injection.

C2-ceramide

Ceramide is produced by N-Smase acting on membrane sphingomyelin and this pathway is rapidly activated in mouse forebrain by IL-1β via IL-1R1 (Nalivaeva *et al.*, 2000). Intrahippocampal injection of C2-ceramide, the cell-penetrating analog of ceramide, 10 min before kainic acid, dose-dependently enhanced the number of seizures and their duration (Fig. 2) without changing the time to onset of seizures and the spiking activity (Table 2). No effect on seizures was observed using 0.25 μg C2-ceramide, 1 μg significantly increased seizures duration by ~2-fold ($P<0.05$) while 2 μg significantly increased the number of seizures by 2-fold and their duration by 2.5-fold on average ($P<0.01$) versus mice injected with dihydroceramide, a membrane-impermeable ceramide analog (Fig. 2A; see Table 2). Thus, C2-ceramide mimics IL-1β proconvulsive effects inducing also a similar EEG pattern of seizures (Fig. 2B). IL-1β or C2-ceramide alone did not induce seizures.

Either C2-ceramide or IL-1β did not affect the pattern or extent of neuronal cell loss induced by kainate in mice, which consists of degeneration of CA3 pyramidal neurons in the injected hippocampus as assessed by Nissl staining in a subset of mice ($n=5$ each group) killed 7 days after seizure induction [–1.46 mm from bregma; data are expressed as percentage of vehicle ($n=5$); 'vehicle', 100 ± 3.7, 'kainate', 86.2 ± 5.4; 'ceramide+kainate', 81.1 ± 8.1; 'IL-1β+kainate', 81.5 ± 7.0 ($P<0.05$ for each treatment groups versus vehicle); –2.06 mm from bregma; 'vehicle', 100 ± 2.0, 'kainate', 67.1 ± 9.7; 'ceramide+kainate', 69.5 ± 9.0; 'IL-1β+kainate', 63.3 ± 5.4 ($P<0.05$ for each treatment groups versus vehicle)].

CGP76030

Tables 1 and 2 show the effect of intrahippocampal injection of the selective inhibitor of Src-family of tyrosine kinase activity, CGP76030 (Susa *et al.*, 2000, 2005; Rucci *et al.*, 2006), on the proconvulsant effect of hrIL-1β (Table 1) and C2-ceramide (Table 2). CGP76030 (65 ng/0.5 μl) injected 20 min before kainate, significantly prevented the 2-fold increase in the number of seizures induced by IL-1β [$F(1,63)=5.9$; $P<0.05$] or C2-ceramide [$F(1,28)=23.9$; $P<0.01$] and in the time spent in ictal activity induced by IL-1β [$F(1,63)=13.3$; $P<0.01$] or C2-ceramide [$F(1,28)=30.9$; $P<0.01$ by two-way ANOVA]. Intrahippocampal CGP76030 did not affect seizures *per se*; however, when 130 ng/1 μl CGP76030 was injected alone icv 20 min before kainate, the number and the duration of

Table 1 Effect of inhibition of the N-Smase-Src-NR2B pathway on the proconvulsive effects of IL-1β

	Dose	Onset (min)	Number of seizures	Time in ictal activity (min)	Time in spikes (min)
Vehicle	–	8.7 ± 0.5	9.0 ± 1.0	5.6 ± 0.5	48.0 ± 3.8
hrIL-1β	1 ng, i.h.	7.6 ± 0.4	16.0 ± 2.0**	11.8 ± 1.1**	50.0 ± 3.7
3-O-MS	3 μg, i.h.	8.6 ± 1.0	7.0 ± 1.0	4.9 ± 0.6	43.9 ± 6.6
3-O-MS + hrIL-1β		9.9 ± 2.1	8.0 ± 1.0†	5.2 ± 0.6††	48.5 ± 4.6
CGP076030	65 ng, i.h.	8.1 ± 1.0	10.0 ± 2.0	6.3 ± 1.1	53.3 ± 7.5
CGP76030 + hrIL-1β		8.8 ± 0.9	7.0 ± 1.0†	3.8 ± 0.6††	47.4 ± 4.7
Ifenprodil	1 mg, i.p.	9.2 ± 2.1	8.0 ± 1.0	6.4 ± 1.3	42.9 ± 6.6
Ifenprodil + hrIL-1β		8.9 ± 1.7	9.0 ± 1.0†	5.0 ± 0.5††	59.4 ± 4.2

Data are the mean ± SE (n = mice in each experimental group). Vehicle ($n=23$ mice) represents C57BL6 mice treated with the corresponding vehicles of the various treatments at the appropriate times before kainic acid application; since these mice did not differ in seizure parameters they were pooled in a single control group. hrIL-1β ($n=28$) are mice injected in the hippocampus (i.h.) with this cytokine (1 ng/0.5 μl) 10 min after receiving the corresponding vehicles of the various treatments, then they were treated 10 min later with kainic acid (7 ng/0.5 μl). Since these mice did not differ in seizure parameters they were pooled in a single group. 3-O-MS, CGP76030 or ifenprodil ($n=7$–11) were injected alone or with hrIL-1β before kanic acid injection (see Methods for details). $^{**}P<0.01$ versus vehicle; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ versus hrIL-1β by two-way ANOVA followed by Tukey's test [F(DF) for each treatment group are reported in the Result section].

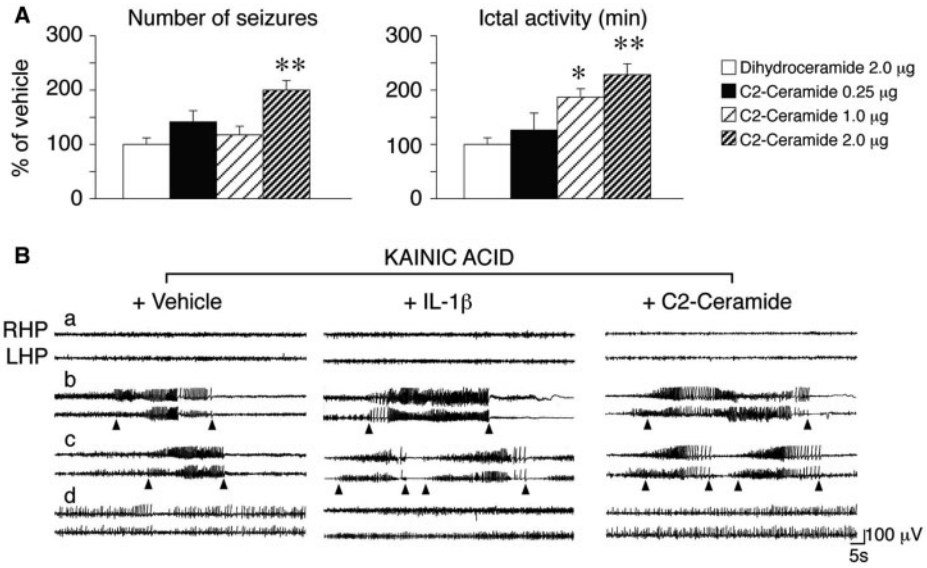

Fig. 2 Effect of C2-ceramide on seizures. (**A**) Bargrams represent the mean ± SE (n = 12). Dihydroceramide, the inactive analogue of ceramide, (2 μg/0.5 μl) or C2-ceramide (the cell-permeable analogue of ceramide; 0.25–1.0–2.0 μg/0.5 μl) were injected intrahippocampally, 10 min before kainic acid. Significant increases in seizure parameters were observed at 1 and 2 μg C2-ceramide. $^{*}P < 0.05$; $^{**}P < 0.01$ versus dihydroceramide by one way ANOVA followed by Tukey test. (**B**) Representative EEG tracings of freely moving C57BL6 mice injected unilaterally in the hippocampus with kainic acid ± IL-1β (1 ng/0.5 μl) or C2-ceramide (2 μg/0.5 μl). Treatments or vehicles were given 10 min before kainic acid. (a) Baseline recording before kainic acid injection; arrowheads in (b) and (c) include representative ictal episodes recorded in the EEG during 90 min after kainic acid injection ± IL-1β or C2-ceramide; tracings in (d) depict spiking activity in the EEG after termination of seizures. Either IL-1β or C2-ceramide alone did not induce seizures. RHP and LHP are right and left (injected) hippocampus, respectively.

Table 2 Effect of inhibition of Src-family of tyrosine kinases on the proconvulsive effects of C2-ceramide

	Dose	Onset (min)	Number of seizures	Time in ictal activity (min)	Time in spikes (min)
Dihydroceramide	2 μg	8.6 ± 0.7	8.0 ± 1.0	5.4 ± 0.5	44.0 ± 3.4
C2-Ceramide	2 μg	8.9 ± 1.4	18.0 ± 2.0**	13.4 ± 1.0**	51.1 ± 3.7
CGP076030	65 ng	8.1 ± 1.0	10.0 ± 2.0	6.3 ± 1.1	53.3 ± 7.5
CGP076030 + C2-Ceramide		8.4 ± 1.6	8.0 ± 1.0††	5.5 ± 0.4††	45.0 ± 3.3

Data are the mean ± SE (n = 7–9 mice in each experimental group). C57BL6 mice were injected intrahippocampally with C2-ceramide (2 μg/0.5 μl) or CGP076030 (65 ng/0.5 μl), or their combination before intrahippocampal kainic acid injection (7 ng/0.5 μl). $^{**}P < 0.01$ versus the inactive analog dihydroceramide; $^{††}P < 0.01$ versus C2-ceramide by two-way ANOVA followed by Tukey's test [F(DF) for each treatment group are reported in the Results section].

seizures were decreased by ~1.8- and 2-fold, respectively, as compared with vehicle-treated mice [number of seizures: vehicle, $n = 8$, 16.0 ± 2.0; CGP, $n = 8$, 9.0 ± 1; $P < 0.01$; time in ictal activity (min): vehicle, 9.9 ± 0.5; CGP, 4.8 ± 0.4; $P < 0.01$ by Student's t-test].

Ifenprodil

Ifenprodil (1 mg/kg), a NR2B-specific NMDA antagonist (Chenard and Menniti, 1999), injected 5 min before hrIL-1β blocked the cytokine-mediated increase in the number [$F(1,61) = 2.2$; $P < 0.05$] and total time in seizures [$F(1,61) = 9.0$; $P < 0.01$ versus hrIL-1β by two-way ANOVA] (Table 1). This dose of ifenprodil did not affect seizures *per se* (Pawlak *et al.*, 2005), although higher doses are known to provide anticonvulsive effects (Kohl and Dannhardt, 2001; Yen *et al.*, 2004).

Effect of pharmacological treatments on Src-family of tyrosine kinases activation and NR2B phosphorylation

We assessed by western blot analysis of hippocampal homogenates, the level of tyrosine phosphorylated (p) forms of Src-family of kinases and the NR2B subunit of NMDA receptor, 60 min after seizures onset in kainate ± IL-1β injected mice, as well as in mice receiving IL-1β alone (Fig. 3). Densitometric analysis of the specific protein bands showed that either hrIL-1β alone (no seizures) or seizures *per se* increased phosphorylation (p) of Src-family of kinases on Tyr^{418} in the catalytic domain denoting their activation (Papp *et al.*, 2008), by 40% on average as compared to vehicle-treated mice ($P < 0.05$ and $P < 0.01$, respectevely) while in the same hippocampi the Tyr^{1472} phosphorylation

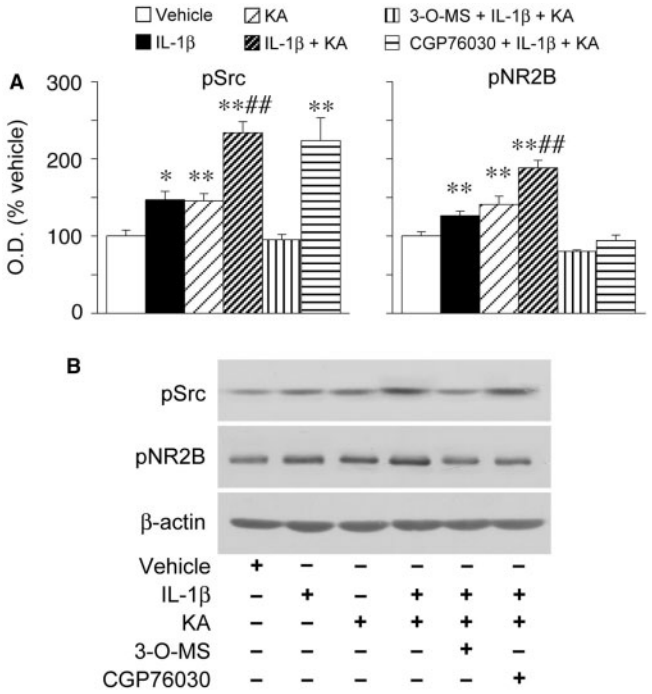

Fig. 3 IL-1β and seizure induced tyrosine phosphorylation of Src-family of kinases and the NR2B subunit of the NMDA receptor: effect of pharmacological treatments. Bargrams show densitometry analysis of the Src kinases and NR2B bands corresponding to their phosphorylated (p) forms (Src-Tyr^{418}; NR2B-Tyr^{1472}) in the various experimental groups, 60 min after the onset of kainate-induced seizures (~70 min after kainate injection), as assessed by western blot analysis of hippocampal homogenates. Vehicle- or IL-1β alone- treated mice were killed 70 min after the injection. Data (means ± SE, n = 5–7) are optical density (O.D.) values of the relevant bands (as depicted in the representative western blot), divided by the corresponding β-actin value (internal standard). Data are expressed as percentage of values measured in corresponding vehicle-treated mice. IL-1β (1 ng/0.5 μl) was injected alone or 10 min before kainic acid; 3-O-MS (3 μg/0.5 μl) and CGP76030 (65 ng/0.5 μl) were injected 20 min before kainic acid (i.e. 10 min before IL-1β). 3-O-MS or CGP76030 alone did not change protein phosphorylation levels as compared to vehicle-injected mice; total NR2B levels were not changed by the various treatments (not shown). $^{*}P < 0.05$; $^{**}P < 0.01$ versus vehicle; $^{\#\#}P < 0.01$ versus 3-O-MS+IL-1+KA and versus CGP76030+IL-1+KA (for pNR2B only) by two-way ANOVA followed by Kruskal–Wallis test. Representative western blot bands corresponding to the specific proteins are depicted in **B**.

of the NR2B form, causing upregulation of channel gating properties (Salter and Kaila, 2004), was increased by 26% and 47% on average, respectively ($P < 0.01$). When hrIL-1β was coinjected with kainic acid, thus producing proconvulsive effects, (p)Src and (p)NR2B levels were increased by 127% and 82% respectively as compared to vehicle-treated mice ($P < 0.01$), denoting additive effects of the single treatments. We also evaluated whether inhibition of N-Smase or Src-family of kinases activity affects the increased (p)Src and (p)NR2B levels observed in proconvulsive conditions. 3-O-MS or CGP76030 at the doses which blocked the proconvulsive effects of IL-1β, reversed NR2B phosphorylation by reducing (p)NR2B to the levels of vehicle-injected mice [3-O-MS: $F(1,17) = 21.9$; $P < 0.01$; CGP76030: $F(1,17) = 10.7$; $P < 0.01$]. In IL-1β+kainate-treated mice, inhibition of N-Smase by 3-O-MS similarly reversed Src-family of kinases phosphorylation [$F(1,17) = 30.5$; $P < 0.01$], which was not affected by CGP76030 treatment. 3-O-MS or CGP76030 alone did not change the basal level of (p)Src and (p)NR2B. The total levels of NR2B were not changed by the various treatments (not shown).

Discussion

This study shows a novel IL-1β-IL-1R1 activated cell signaling that mediates the proconvulsive actions of IL-1β in the mouse hippocampus. IL-1β is a pluripotent proinflammatory cytokine which binds to IL-1R1, a Toll receptor family member, and induces via an NFkB-dependent mechanism, the transcription of various genes encoding several downstream mediators of inflammation, including IL-6 or TNF-α, inducible NO and COX-2 (Dinarello, 2004); the time scale of these effects is included between 30 and 90 min. The activation of this Toll-like pathway requires transcription and translation of the encoded proteins with their subsequent release, therefore it is unlikely to account for the recently described *rapid* effects of IL-1β on NMDA-dependent neuronal Ca^{2+} influx and seizure activity (Vezzani *et al.*, 1999, 2000; Viviani *et al.*, 2003; Ravizza *et al.*, 2006*b*). This consideration raises the hypothesis that the proconvulsant action of IL-1β involves an additional, rapid, non-transcriptional intracellular neuronal pathway leading to fast changes in ion channels. Previous reports have shown neuronal effects of IL-1β in hippocampal and hypothalamic neurons involving synaptic plasticity and thermoregulation which also appear to be independent of transcriptional events (Davis *et al.*, 2006*b*; Sanchez-Alavez *et al.*, 2006; Pickering and O'Connor, 2007; Viviani *et al.*, 2007). In particular, the activation of the 'IL-1β-N-Smase-Src kinase pathway' has been shown to mediate the 'fast actions of IL-1β' on preoptical/anterior warm sensitive hypothalamic neurons underlying the rapid phase of the febrile response to IL-1β (Sanchez-Alavez *et al.*, 2006). When studying these IL-1β effects in hypothalamic neurons *in vivo* and *in vitro*, we found that the effects of IL-1β can be mimicked by C2-ceramide, and that inhibitors of N-Smase in the brain lower or prevent the response to IL-1β (Sanchez-Alavez *et al.*, 2006). Furthermore, the formed ceramide activates the Src-family of tyrosine kinases in hypothalamic neurons, but not in glia (Davis *et al.*, 2006*a*, *b*).

We show here, using a detailed pharmacological approach, that inhibition of N-Smase using 3-O-MS blocks the increase in the frequency and duration of seizures induced by IL-1β and that C2-ceramide faithfully mimics the IL-1β effect on seizures, thus suggesting that the activation of the N-Smase-ceramide pathway mediates the proconvulsive activity of this cytokine. To elucidate the downstream events which follow N-Smase activation, we addressed the possible involvement

of the Src-family of kinases. Thus, this family of enzymes is known to be activated by ceramide (Kolesnick and Golde, 1994; Saklatvala, 1995), and a similar cascade of events was previosly shown to mediate the electrophysiological effects of IL-1β on the activity of warm-sensitive hypothalamic neurons (Davis *et al.*, 2006*b*; Sanchez-Alavez *et al.*, 2006). Our data show that the inhibition of the Src-family of kinases activity prevents the IL-1β and C2-ceramide proconvulsive effects similarly, thus suggesting that Src-family of tyrosine kinases activation is a downstream event subsequent to IL-1β activation of N-Smase. This possibility is also supported by the reversal of IL-1β-induced tyrosine phosphorylation of Src-family of kinases during seizures using the N-Smase inhibitor.

Src-family of tyrosine kinases is abundantly expressed in neurons, and one main function of the activated forms of two members of this family, namely c-Src and Fyn, is to upregulate the activity of NMDA receptors via Tyr^{1472}-phosphorylation of the NR2B subunit (Ali and Salter, 2001). We found that ifenprodil, a selective antagonist of NMDA receptors containing the NR2B subunit, prevented the proconvulsive effects of IL-1β, thus indicating that this subunit is critically involved in IL-1β action on seizures. Our previous findings in primary cultures of hippocampal neurons showed that IL-1β induces the Tyr^{1472}-phosphorylation of the NR2B subunit via Src-family of kinases activation (Viviani *et al.*, 2003), therefore we assessed whether these events occurred *in vivo* when seizures were potentiated by IL-1β. The levels of the phosphorylated forms of Src-family of kinases and the NR2B subunit in the hippocampus were indeed enhanced by IL-1β or seizures, and these effects were additive when seizures were increased by IL-1β. Moreover, NR2B Tyr^{1472}-phosphorylation induced in proconvulsive conditions was reversed to baseline level in mice treated with N-Smase and Src-family of kinases inhibitors, thus supporting the involvement of these enzymes in the activation of this receptor subunit (Yu *et al.*, 1997). Tyr^{1472}-phosphorylation of the NR2B subunit promotes Ca^{2+} influx into neurons (Yu *et al.*, 1997; Viviani *et al.*, 2003), thus resulting in potentiation of NMDA function which is pivotal for evoking neuronal hyperexcitability (Meador, 2007). This molecular event may be the ultimate step of this novel 'IL-1β-N-Smase-Src kinase' pathway responsible for the increased neuronal excitability and subsequent proconvulsive effects of this cytokine. Although CGP76030 selectively inhibits Src-family of tyrosine kinases activation, and it was shown to have higher affinity for c-Src versus other Src-family of tyrosine kinase members (Susa *et al.*, 2000, 2005), no data about the inhibitor IC50 on Fyn activity are available, therefore both c-Src and Fyn may be involved in the IL-1β signaling underlying its proconvulsive effects. The possible specific involvement of c-Src versus Fyn should await the possibility to unequivocally distinguish the involvement of these two kinase activities using pharmacological approaches which are not yet available.

Interestingly, Src-family of tyrosine kinases appears to contribute to seizures also in the absence of proinflammatory conditions since Src-family of kinases inhibition *per se* after icv, but not intrahippocampal injection of CGP76030, reduced seizures number and duration in mice. We could not affect seizures by inhibiting the activity of N-Smase using 3-O-MS either intrahippocampally or icv injected. The lack of effect of intrahippocampal application of these drugs on kainate-induced seizures may be due to the local spread of the inhibitors around the injection site as opposed to seizures-induced IL-1β expression throughout the whole hippocampus. Moreover, we should also consider the possibility that the dose of 3-O-MS allowed to be injected by the limited solubility of this compound is not enough to protect from kainate seizures.

Although hippocampal EEG seizure activity was increased by IL-1β or C2-ceramide, cell death in the hippocampus was not affected. This observation indicates that the increase in seizures was insufficient to induced additional cell loss which was restricted in this model to CA3 pyramidal cells in the injected hippocampus. Moreover, CA3 degeneration has been reported to depend on a direct neurotoxic action of kainate rather than on seizures *per se* (Fariello et al, 1989; Jarrard, 2002), therefore suggesting that kainate-induced cell loss in this model is insensitive to the proneurotoxic actions of IL-1β.

The relevance of our findings for the etiopathogenesis of seizures is based on clinical and experimental evidence showing that the IL-1β system is activated in chronic human epilepsy (Vezzani and Granata, 2005; Ravizza *et al.*, 2006*a*, 2008) and that anti-IL-1β pharmacological treatments result in powerful anticonvulsant effects (De Simoni *et al.*, 2000; Vezzani *et al.*, 2000, 2002; Ravizza *et al.*, 2006*b*). Moreover, several brain injuries in humans are associated with brain inflammation, result in early occurrence of seizures and present a high risk of developing epilepsy (Pitkanen and Sutula, 2002; Vezzani and Granata, 2005). Therefore, the elucidation of this novel IL-1β-activated pathway in the hippocampus adds important insights into the mechanisms of ictogenesis in inflammatory conditions and may allow the development of innovative strategies to block the activation of IL-1β signaling in disease conditions, thus highlighting potential new targets of therapeutic intervention.

Funding

EPICURE (LSH-CT-2006-037315 to AV), Negri Weizmann Programme (to A.V.) and Fondazione Monzino (to A.V.) and the NIH grant NS43501-04 (to T.B.)

References

Ali DW, Salter MW. NMDA receptor regulation by Src kinase signalling in excitatory synaptic transmission and plasticity. Curr Opin Neurobiol 2001; 11: 336–42.

Aronica E, Boer K, van Vliet EA, Redeker S, Baayen JC, Spliet WG, et al. Complement activation in experimental and human temporal lobe epilepsy. Neurobiol Dis 2007; 26: 497–511.

Balosso S, Ravizza T, Perego C, Peschon J, Campbell IL, De Simoni MG, et al. Tumor necrosis factor-alpha inhibits seizures in mice via p75 receptors. Ann Neurol 2005; 57: 804–12.

Bock J, Szabo I, Gamper N, Adams C, Gulbins E. Ceramide inhibits the potassium channel Kv1.3 by the formation of membrane platforms. Biochem Biophys Res Commun 2003; 305: 890–7.

Chenard BL, Menniti FS. Antagonists selective for NMDA receptors containing the NR2B subunit. Curr Pharm Des 1999; 5: 381–404.

Crespel A, Coubes P, Rousset MC, Brana C, Rougier A, Rondouin G, et al. Inflammatory reactions in human medial temporal lobe epilepsy with hippocampal sclerosis. Brain Res 2002; 952: 159–69.

Davis CN, Mann E, Behrens MM, Gaidarova S, Rebek M, Rebek J Jr, et al. MyD88-dependent and -independent signaling by IL-1 in neurons probed by bifunctional Toll/IL-1 receptor domain/BB-loop mimetics. Proc Natl Acad Sci USA 2006a; 103: 2953–8.

Davis CN, Tabarean I, Gaidarova S, Behrens MM, Bartfai T. IL-1beta induces a MyD88-dependent and ceramide-mediated activation of Src in anterior hypothalamic neurons. J Neurochem 2006b; 98: 1379–89.

De Simoni MG, Perego C, Ravizza T, Moneta D, Conti M, Marchesi F, et al. Inflammatory cytokines and related genes are induced in the rat hippocampus by limbic status epilepticus. Eur J Neurosci 2000; 12: 2623–33.

Dinarello CA. Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. Int Rev Immunol 1998; 16: 457–99.

Dinarello CA. Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. J Endotoxin Res 2004; 10: 201–22.

Dubé C, Vezzani A, Behrens M, Bartfai T, Baram TZ. Interleukin-1beta contributes to the generation of experimental febrile seizures. Ann Neurol 2005; 57: 152–5.

Fariello RG, Golden GT, Smith GG, Reyes PF. Potentiation of kainic acid epileptogenicity and sparing from neuronal damage by an NMDA receptor antagonist. Epilepsy Res 1989; 3: 206–13.

Franklin KBJ, Paxinos G. The mouse brain in stereotaxic coordinates. San Diego: Academic Press, 1997.

Galic MA, Riazi K, Heida JG, Mouihate A, Fournier NM, Spencer SJ, et al. Postnatal inflammation increases seizure susceptibility in adult rats. J Neurosci 2008; 28: 6904–13.

Gorter JA, van Vliet EA, Aronica E, Breit T, Rauwerda H, Lopes da Silva FH, et al. Potential new antiepileptogenic targets indicated by microarray analysis in a rat model for temporal lobe epilepsy. J Neurosci 2006; 26: 11083–110.

Gulbins E, Szabo I, Baltzer K, Lang F. Ceramide-induced inhibition of T lymphocyte voltage-gated potassium channel is mediated by tyrosine kinases. Proc Natl Acad Sci USA 1997; 94: 7661–6.

Heida JG, Pittman QJ. Causal links between brain cytokines and experimental febrile convulsions in the rat. Epilepsia 2005; 46: 1906–13.

Hoffman GE, Moore N, Fiskum G, Murphy AZ. Ovarian steroid modulation of seizure severity and hippocampal cell death after kainic acid treatment. Exp Neurol 2003; 182: 124–34.

Jarrard LE. Use of excitotoxins to lesion the hippocampus: update. Hippocampus 2002; 12: 405–14.

Kohl BK, Dannhardt G. The NMDA receptor complex: a promising target for novel antiepilptic strategies. Curr Med Chem 2001; 8: 1275–89.

Kolesnick R, Golde DW. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell 1994; 77: 325–8.

Maldonado M, Baybis M, Newman D, Kolson DL, Chen W, McKhann G, II, et al. Expression of ICAM-1, TNF-alpha, NF kappa B, and MAP kinase in tubers of the tuberous sclerosis complex. Neurobiol Dis 2003; 14: 279–90.

Meador KJ. The basic science of memory as it applies to epilepsy. Epilepsia 2007; 48 (Suppl. 9): 23–5.

Nalivaeva NN, Rybakina EG, Pivanovich I, Kozinets IA, Shanin SN, Bartfai T. Activation of neutral sphingomyelinase by IL-1beta requires the type 1 interleukin 1 receptor. Cytokine 2000; 12: 229–32.

Obeid LM, Linardic CM, Karolak LA, Hannun YA. Programmed cell death induced by ceramide. Science 1993; 259: 1769–71.

Papp S, Szabo E, Kim H, McCullock CA, Opas M. Kinase-dependent adhesion to fibronectin: regulation by calreticulin. Exp Cell Res 2008; 314: 1313–26.

Pawlak R, Melchor JP, Matys T, Skrzypiec AE, Strickland S. Ethanol-withdrawal seizures are controlled by tissue plasminogen activator via modulation of NR2B-containing NMDA receptors. Proc Natl Acad Sci USA 2005; 102: 443–8.

Pickering M, O'Connor JJ. Pro-inflammatory cytokines and their effects in the dentate gyrus. Prog Brain Res 2007; 163: 339–54.

Pitkanen A, Sutula TP. Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. Lancet Neurol 2002; 1: 173–81.

Ravizza T, Boer K, Redeker S, Spliet WG, van Rijen PC, Troost D, et al. The IL-1beta system in epilepsy-associated malformations of cortical development. Neurobiol Dis 2006a; 24: 128–43.

Ravizza T, Gagliardi B, Noe F, Boer K, Aronica E, Vezzani A. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis 2008; 29: 142–60.

Ravizza T, Lucas SM, Balosso S, Bernardino L, Ku G, Noe F, et al. Inactivation of caspase-1 in rodent brain: a novel anticonvulsive strategy. Epilepsia 2006b; 47: 1160–8.

Ravizza T, Vezzani A. Status epilepticus induces time-dependent neuronal and astrocytic expression of interleukin-1 receptor type I in the rat limbic system. Neuroscience 2006; 137: 301–8.

Rucci N, Recchia I, Angelucci A, Alamanou M, Del Fattore A, Fortunati D, et al. Inhibition of protein kinase c-Src reduces the incidence of breast cancer metastases and increases survival in mice: implications for therapy. J Pharmacol Exp Ther 2006; 318: 161–72.

Saklatvala J. Intracellular signalling mechanisms of interleukin 1 and tumour necrosis factor: possible targets for therapy. Br Med Bull 1995; 51: 402–18.

Salter MW, Kalia LV. Src kinases: a hub for NMDA receptor regulation. Nat Rev Neurosci 2004; 5: 317–28.

Sanchez-Alavez M, Tabarean IV, Behrens MM, Bartfai T. Ceramide mediates the rapid phase of febrile response to IL-1beta. Proc Natl Acad Sci USA 2006; 103: 2904–8.

Sayyah M, Javad-Pour M, Ghazi-Khansari M. The bacterial endotoxin lipopolysaccharide enhances seizure susceptibility in mice: involvement of proinflammatory factors: nitric oxide and prostaglandins. Neuroscience 2003; 122: 1073–80.

Susa M, Missbach M, Green J. Src inhibitors: drugs for the treatment of osteoporosis, cancer or both? TiPS 2000; 21: 489–95.

Susa M, Missbach M, Gamse R, Kneissel M, Buhl T, Gasser JA et al. Src as a target for pharmaceutical intervention. In: Fabbro D, McCormick F, editors. Cancer drug discovery and development. Totowa: Humana Press Inc.; 2005. p. 71–92.

Tsakiri N, Kimber I, Rothwell NJ, Pinteaux E. Interleukin-1-induced interleukin-6 synthesis is mediated by the neutral sphingomyelinase/Src kinase pathway in neurones. Br J Pharmacol 2008; 153: 775–83.

Turrin NP, Rivest S. Innate immune reaction in response to seizures: implications for the neuropathology associated with epilepsy. Neurobiol Dis 2004; 16: 321–34.

Vezzani A, Conti M, De Luigi A, Ravizza T, Moneta D, Marchesi F, et al. Interleukin-1beta immunoreactivity and microglia are enhanced in the rat hippocampus by focal kainate application: functional evidence for enhancement of electrographic seizures. J Neurosci 1999; 19: 5054–65.

Vezzani A, Granata T. Brain inflammation in epilepsy: experimental and clinical evidence. Epilepsia 2005; 46: 1724–43.

Vezzani A, Moneta D, Conti M, Richichi C, Ravizza T, De Luigi A, et al. Powerful anticonvulsant action of IL-1 receptor antagonist on intracerebral injection and astrocytic overexpression in mice. Proc Natl Acad Sci USA 2000; 97: 11534–9.

Vezzani A, Moneta D, Richichi C, Aliprandi M, Burrows SJ, Ravizza T, et al. Functional role of inflammatory cytokines and antiinflammatory molecules in seizures and epileptogenesis. Epilepsia 2002; 43 (Suppl 5): 30–5.

Viviani B, Bartesaghi S, Gardoni F, Vezzani A, Behrens MM, Bartfai T, et al. Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. J Neurosci 2003; 23: 8692–700.

Viviani B, Gardoni F, Marinovich M. Cytokines and neuronal ion channels in health and disease. Int Rev Neurobiol 2007; 82: 247–63.

Yen W, Williamson J, Bertram EH, Kapur J. A comparison of three NMDA receptor antagonists in the treatment of prolonged status epilepticus. Epilepsy Res 2004; 59: 43–50.

Yu XM, Askalan R, Keil GJ II, Salter MW. NMDA channel regulation by channel-associated protein tyrosine kinase Src. Science 1997; 275: 674–8.

Zeng C, Lee JT, Chen H, Chen S, Hsu CY, Xu J. Amyloid-beta peptide enhances tumor necrosis factor-alpha-induced iNOS through neutral sphingomyelinase/ceramide pathway in oligodendrocytes. J Neurochem 2005; 94: 703–12.