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The cortical innate immune response increases local neuronal excitability leading to seizures

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Brain glial cells, five times more prevalent than neurons, have recently received attention for their potential involvement in epileptic seizures. Microglia and astrocytes, associated with inflammatory innate immune responses, are responsible for surveillance of brain damage that frequently results in seizures. Thus, an intriguing suggestion has been put forward that seizures may be facilitated and perhaps triggered by brain immune responses. Indeed, recent evidence strongly implicates innate immune responses in lowering seizure threshold in experimental models of epilepsy, yet, there is no proof that they can play an independent role in initiating seizures *in vivo*. Here, we show that cortical innate immune responses alone produce profound increases of brain excitability resulting in focal seizures. We found that cortical application of lipopolysaccharide, binding to toll-like receptor 4 (TLR4), triples evoked field potential amplitudes and produces focal epileptiform discharges. These effects are prevented by pre-application of interleukin-1 receptor antagonist. Our results demonstrate how the innate immune response may participate in acute seizures, increasing neuronal excitability through interleukin-1 release in response to TLR4 detection of the danger signals associated with infections of the central nervous system and with brain injury. These results suggest an important role of innate immunity in epileptogenesis and focus on glial inhibition, through pharmacological blockade of TLR4 and the pro-inflammatory mediators released by activated glia, in the study and treatment of seizure disorders in humans.

Keywords: epilepsy; glial cells; LPS; TLR4; IL1

Abbreviations: AEP = auditory evoked potential; BMI = bicuculline methiodide; $GABA_A$ = gamma-aminobutyric acid ionotrophic receptor; IL-1 = interleukin-1; IL-1ra = interleukin-1 receptor antagonist; LPS = lipopolysaccharide; LPS-RS = LPS derived from the photosynthetic bacterium Rhodobacter sphaeroides; PTZ = pentylenetetrazol; RMS = root mean squared; SEP = somatosensory evoked potential; TBI = traumatic brain injury; TLR4 = toll-like receptor 4; TNFα = tumour necrosis factor-α; X–A = xylazine–acepromazine; K-X-A = ketamine-xylazine-acepromazine

Introduction

Epileptic seizures affect 0.5%–1.5% of the global population and remain the subject of concentrated neuroscientific investigation. Because seizures are characterized by pathologically synchronized interactions between neurons, epilepsy research remains justifiably 'neurocentric' (Robert, 2008). Yet, glial cells far outnumber neurons in the forebrain and are perfectly situated to modulate

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neuronal function by encapsulating neuronal synapses and maintaining neurotransmitter balance (Araque *et al*., 1999; Halassa *et al*., 2007). With recent reports of dynamic neuro-glial interactions (Fields and Stevens-Graham, 2002), the possible modulating role of glial cells in epileptogenicity has received intense interest (Zimmer *et al*., 1997; Wang and White, 1999; Plata-Salaman *et al*., 2000; Vezzani *et al*., 2000; Oprica *et al*., 2003; Rizzi *et al*., 2003; Sayyah *et al*., 2003; Vezzani, 2005; Halassa *et al*., 2007; Choi and Koh, 2008; Ravizza *et al*., 2008; Robert, 2008; Vezzani *et al*., 2008).

Since glial cells also comprise the brain's immune system, it has been proposed that some seizures may result from brain immune responses (Vezzani, 2005; Ravizza *et al*., 2008; Riazi *et al*., 2008). If so, this would suggest the involvement of astrocytes but, additionally, it would predict the involvement of microglia (Aloisi, 2001), a second major class of glial cells which has received less attention by epileptologists. This would be suggested because microglia are distinguished from astrocytes in their singular responsibility for immune surveillance of invading pathogens and danger signals produced by brain damage (Aloisi, 2001), damage that frequently results in seizures. Relatively, quiescent in their basal state (Davalos *et al*., 2005; Nimmerjahn *et al*., 2005), microglia are activated by central infection, trauma and ischaemia, releasing pro- and anti-inflammatory cytokines, chemokines, prostaglandins and nitric oxide that comprise an early inflammatory response (innate immunity) (Nadeau and Rivest, 2000).

Recent evidence indicates that chemically induced visceral inflammation is correlated with widespread activation of cortical glial cells, and decreased thresholds for generalized pentylenetetrazol (PTZ)-induced seizures that can be reversed by cortical application of the anti-inflammatory glial inhibitor minocycline (Riazi *et al*., 2008). These results strongly implicate glial cells and the accompanying innate immune response in the facilitation of generalized seizures when the cortex is challenged by convulsant compounds. While comorbity between peripheral inflammation and epilepsy has been decribed (Tellez-Zenteno *et al*., 2005), more importantly, these results raise the possibility that direct activation of brain innate immunity, characteristic of more commonly observed cortical insult and focal epilepsy, could participate in epileptogenesis. It is possible that innate immune responses alone, in the absence of convulsive agents and prior seizures, could independently increase cortical excitbility and serve as a source as well as potentiator of epileptogenesis. To explore this possibility, we used field potential mapping of sensory evoked potentials and spontaneous activity in rat somatosensory cortex to quantify changes in brain excitability associated with innate immune responses *in vivo*, induced by direct cortical application of lipopolysaccharide (LPS), a gram negative bacteria that mimics the CNS response to insult and infection.

Materials and Methods

Evoked potential studies

Surgery

All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory animals in biological research. Seven adult male Sprague-Dawley rats (300–400 g) were anaesthetized to surgical levels using an intraperitoneal injection of ketamine–xylazine–acepromazine (K-X-A; 45–9–1.5 mg/kg body weight), placed on a regulated heating pad, and maintained with subsequent injections throughout the experiment so that the eye blink reflex could be barely elicited. While ketamine has been shown to attenuate LPS-induced inflammatory responses of glial cultures (Shibakawa *et al*., 2005), it was required here to provide adequate anaesthesia for the large bilateral craniotomy required for hemispheric mapping. The effects reported here may have been larger without ketamine. A bilateral cranotomy was performed over both hemispheres extending 2 mm rostral to bregma to 1 mm caudal of lambda and from the mid-sagittal suture to 2 mm below the zygomatic arch, exposing a maximal area of the surgically accessible cortex. The dura was reflected and the exposed cortex regularly doused with Ringer Solution containing: NaCl 135 mM; KCl 3 mM; MgCl 2 mM and CaCl 2 mM—pH 7.4 at 37°C.

Experimental procedures

Somatosensory responses were evoked by electrical stimulation of the forepaw that was shaved and coated with conductive jelly. A bipolar electrode (500 μm tips; 2 mm separation) attached to a constant current source delivered biphasic current pulses (1.0 ms; 0.5–1.0 mA) to the exposed skin. Auditory click stimuli (0.1 ms monophasic pulses) were delivered using a high frequency piezoelectric speaker placed ~15 cm lateral to the contralateral ear.

Epipial maps of auditory and somatosensory evoked potentials (AEP and SEP, respectively) were recorded using a flat multi-electrode array consisting of 256 stainless steel wires in a 16×16 grid (tip diameter: ~100 μm; inter-electrode spacing: 400 μm) covering a 6.0×6.0 mm area of the left hemisphere in a single placement. The array was pressed against the cortex with sufficient force to establish contact of all electrodes. The required pressure had no effect on evoked potential amplitude, post-stimulus latency, or morphology when compared to potentials recorded previously with more lightly placed small arrays. While both the surgical procedures (i.e. craniotomy and dura removal) have the potential for producing inflammatory effects, we have not noted any progressive changes in SEP amplitudes, morphology or spatial distribution for hours after this procedure in other studies, unlike the acute SEP changes induced by LPS application in this experiment. Recordings were referred to a stainless steel electrode secured over the frontal bone, and were simultaneously amplified (×2000; NerveAmp, Center for Neural Recording, Washington State University, Pullman, WA, USA), analogue filtered (bandpass cut-off = –6 db at 0.1 to 3000 Hz, roll-off = 5 db/octave) and digitized at 10 kHz. Evoked potentials were averaged over 32 stimulus presentations.

Regions of auditory and somatosensory cortex were estimated from interpolated (bicubic spline) maps of the root mean squared (RMS) power of the evoked potential (an example of the mapping procedure is shown in Fig. 1). Evoked potentials were recorded 1 min prior to, and 5, 10, 15, 20, 25 and 30 min subsequent to epicortical suffusion (0.02 μg in 10 μl saline) of the toll-like receptor 4 (TLR4) agonist, LPS (Sigma, St Louis, MO, USA), alone (left hemisphere), or LPS after pre-treatment with TLR4 antagonist, LPS-RS (LPS-derived from the photosynthetic bacterium *Rhodobacter sphaeroides*; InvivoGen, SanDiego, CA, USA; right hemisphere). Manufacturer recommended concentrations of LPS for adequate stimulation of TLR4, and of LPS-RS to block these effects, are 0.0001–0.01 μg/μl. Other reports where LPS was injected into cortex have used concentrations ranging from

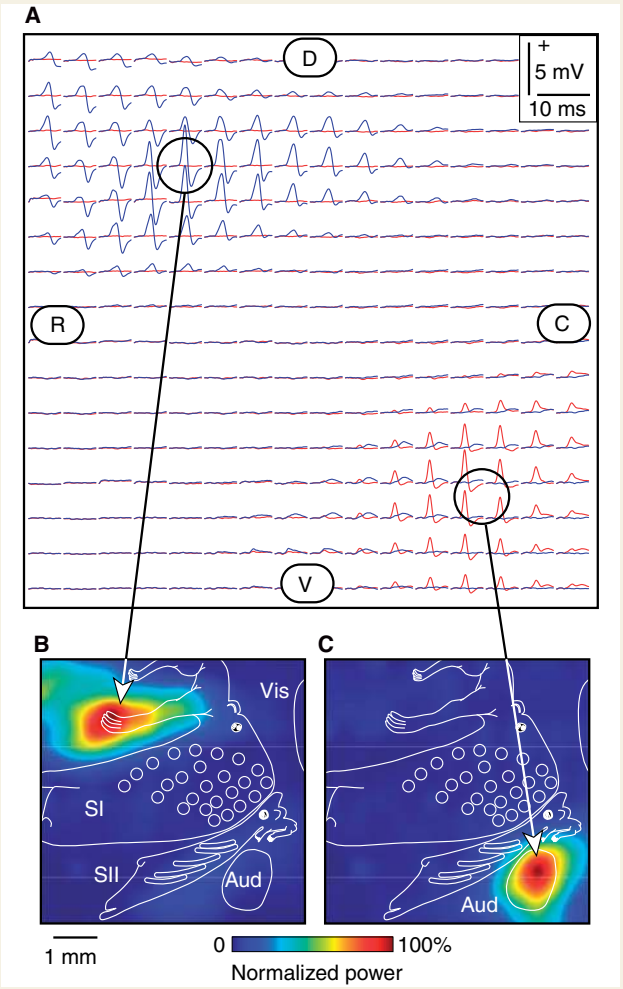

Figure 1 Hemispheric epipial mapping of averaged SEP and AEP recorded from the left hemisphere of seven animals. (**A**) The SEP produced by forepaw stimulation (blue traces) and AEP (red traces) produced by click stimulation. Rostral, caudal, dorsal and ventral are labelled R, C, D and V, respectively. (**B**, **C**) RMS power of the waveform at each electrode site displayed as an interpolated map for the SEP and AEP, respectively. Superimposed is a ratunculus, reflecting the complete body representation.

0.002–1.25 μg/μl and doses of 0.002–5 μg to effect inflammation (Andersson *et al*., 1992; de Pablos *et al*., 2006; Park *et al*., 2005, 2007). Our low LPS concentration of 0.002 μg/μl and high concentration of 0.02 μg/μl (for epileptiform spikes and seizures; see below) were at the low end of previous reports. If all of the LPS penetrated the cortex, our low and high doses of LPS were 0.02 and 0.2 μg, which is also at the low end of the dose range from previous reports. Because the precise spread and penetration of epipially suffused LPS used here were not known, these doses should be considered approximate, but were chosen because they produced consistent functional results across animals.

LPS-RS antagonizes toxic LPS in both human and murine cells and also prevents LPS-induced shock in mice (Qureshi *et al*., 1999). The main mechanism it uses to block LPS-dependent activation of TLR4 consists of direct competition between under-acylated LPS and hexa-acylated LPS for the same binding site on myeloid differentiation factor 2 (MD-2), while the secondary mechanism involves the ability of under-acylated LPS:MD-2 complexes to inhibit hexa-acylated endotoxin:MD-2 complexes function at TLR4 (Saitoh *et al*., 2004; Coats *et al*., 2005; Teghanemt *et al*., 2005; Visintin *et al*., 2005). Suffusion of 10 μl, for both LPS and LPS-RS solutions, was chosen because this quantity completely saturated a 4 × 4 mm pledget of filter paper used to confine applications in our pilot studies. However, direct suffusion was used in the present experiment to permit immediate recording, presumably covering a wider cortical area but sufficiently confined to the approximate area of somatosensory cortex as to produce no effects in auditory cortex, only 4 mm lateral to the application site (see Results section). Here, and in all subsequent cortical applications, 10 μl of dimethyl sulphoxide (DMSO; Sigma; 2.5% in saline) was first applied.

Epilepsy studies

Surgery

An additional 12 adult male Sprague-Dawley rats (300–400 g) were anaesthetized to surgical levels using an intraperitoneal injection of X–A (9–1.5 g/kg body weight) then supplemented with isoflurane (2%) for the duration of surgery (~30 min). At the end of surgery, all surgical wounds were injected with lidocaine (2%) and isoflurane was discontinued. Frequent supplements of xylazine-acepromazine (X-A) were administered for the duration of the experiment (2–3 h), resulting in a highly sedated but unanaesthetized preparation. Both eye blink and toe pinch withdraw reflexes could be easily elicited, but no voluntary movements were observed, and animals were left unrestrained during recording. Recording commenced 1 h following termination of isoflurane. The purpose of this aneasthesia regime was to eliminate ketamine, which can influence epileptogenicity (Velisek *et al*., 1993; Borowicz and Czuczwar, 2003; Midzyanovskaya *et al*., 2004) and also attenuate LPS-induced inflammatory responses of glial cultures (Shibakawa *et al*., 2005). Isoflurane was also cleared because of its possible effects on epileptogenicity (Veronesi *et al*., 2008).

Surgery differed from the prior evoked potential preparation only in that the craniectomy was smaller (extending from bregma to lambda and from the mid-sagittal suture to 2 mm past the temporal ridge) to accommodate a smaller recoding array (8 × 8 electrodes; 500 μm spacing; 3.5 × 3.5 mm recording area).

Experimental procedures

In six animals, a unilateral craniectomy over the left hemisphere was performed and only spontaneous activity recorded (i.e. no evoked potentials). One minute of spontaneous activity was recorded prior to, and at 10 min intervals for 30 min, following epipial application of LPS (0.2 μg in 10 μl saline). In an additional six animals, bilateral craniectomies were performed. One min of spontaneous activity was recorded prior to and immediately following epipial application of interleukin-1 (IL-1) receptor antagonist (IL-1ra) (Amgen, Thousand Oaks, CA, USA; 100 μg in 10 μl saline). In two subsequent 15 min intervals, IL-1ra was reapplied along with LPS (0.2 μg in 10 μl saline) and 1 min of spontaneous activity recorded immediately before and after each application. In the right hemisphere, 1 min of spontaneous activity was recorded before, and at 15 min intervals for 30 min following LPS application (no IL-1ra treatment). If no epileptiform spikes were observed by 15 min following the first LPS application, the same amount of LPS was applied a second time. This was required in two of the animals. Following recording, spontaneous activity in the right hemisphere was silenced by epipial application of lidocaine hydrochloride (2%). Gamma-aminobutyric acid ionotrophic receptor

($GABA_A$) receptor antagonist bicuculline methiodide (BMI; Sigma; 10 μM in 0.9% NaCl) was then applied (10 μl) to the left hemisphere to produce a simple model of cortical epilepsy-independent of neuro-glial effects. One-minute epochs of spontaneous activity were recorded in 10-min intervals for ~20 min following application of BMI.

In four of the bi-hemisphere surgical preparations, SEPs were obtained for comparison to the previous study. Whisker stimulation was provided by 0.1 ms pulses delivered to a solenoid with attached 3 cm armature constructed from hypodermic tubing. Single whisker displacements were ~0.5 mm on the rostro-caudal axis. Whisker stimulation (contralateral to the recorded hemisphere) was used instead of electrical forepaw stimulation because it did not disturb the animals or elicit reflexive responses. Whisker evoked SEPs (32 trials) were obtained immediately following each recording of spontaneous activity.

Analysis

Changes in the SEP were quantified in three ways. First, the RMS power of SEP was computed at single electrode sites in the left and right hemisphere. Electrode sites were chosen based on those revealing the largest amplitude response in the pre-treatment baseline. Second, to examine changes in the spatial distribution or area of the SEP-independent of overall power, the responses across the array were normalized to the maximum power for a given condition (latency pre- or post-treatment) and then the number of electrode sites reaching $\geqslant$50% of this maximum were counted to determine active area in sq mm. Finally, total SEP power in each condition was summed across all electrodes of the array. This measure combined changes in power at each electrode with changes in the area of active cortex. Significant differences in SEP amplitude and spatial distribution are reported as the mean ± standard error and evaluated using two-way repeated measures ANOVA followed by pair-wise post-hoc comparisons.

Results

Innate immune responses increase cortical excitability

The SEP evoked by forepaw stimulation in untreated cortex (Fig. 1A; blue traces; averaged across seven animals) consisted of a typical biphasic (positive/negative) slow wave of maximum amplitude at electrode sites in the dorsal and rostral region of a 16 × 16 epipial electrode array used to map the entire hemisphere, centred on the forepaw representation of primary somatosensory cortex. Click stimulation produced an AEP (Fig. 1A; red traces) of maximum amplitude at electrode sites covering primary and secondary auditory cortex in the ventral and caudal area of the array. To better visualize the amplitude distribution of evoked responses, the RMS power of the waveform at each electrode site was computed, normalized to the electrode with the highest power, and displayed as an interpolated map for the SEP and AEP (Fig. 1B and C, respectively). In these and subsequent maps, a ratunculus has been superimposed as an anatomical reference, reflecting the complete body representation of primary and secondary somatosensory cortex (SI and SII, respectively). The ratunculus was adapted from a previous study (Benison *et al*., 2007) since comprehensive mapping was not performed in the present experiment. The SEP was of largest power over ~7 sq mm of the forepaw representation of SI, and the AEP potential covered a smaller region in auditory cortex (Fig. 1C; Aud).

The same SEP map as shown in Fig. 1B is depicted again in Fig. 2A (left column, top), representing the baseline response prior to cortical application of LPS. Here, the amplitude of the response appears lower than in Fig. 1 because the values were not normalized to the largest response within the map, but rather, to the largest response across experimental conditions and hemispheres. Local suffusion of LPS (0.02 μg in 10 μl saline) at the site of the forepaw representation in the left hemisphere markedly increased cortical excitability, reflected by increases of both the amplitude and spatial distribution of the SEP. By 20 min these parameters had nearly tripled (Fig. 2A; left column).

Excitability changes are specific to TLR4

Pre-application of LPS-RS prevented LPS-induced excitability changes in the contralateral hemisphere (Fig. 2A; right column). However, both the spatial distribution and amplitude of the SEP remained unchanged from baseline. Thus, LPS-RS had only a blocking effect of LPS activation through antagonism of TLR4 and no direct effect on cortical excitability, demonstrating no detectable basal TLR4 activity or that which could potentially have been induced by the surgery. AEP in both hemispheres remained stable (Fig. 2B), suggesting that LPS spread remained largely confined to somatosensory cortex, although this was not histologically verified.

Quantitative analysis of SEP changes at single electrode sites in the left hemisphere (treated with LPS alone) and right hemisphere (treated with LPS + LPS-RS), indicated significant effects of hemisphere group ($P<0.005$), time after LPS application ($P<0.001$), and an interaction between the two factors ($P<0.002$). Pair-wise comparisons revealed no significant increases in SEP power from pre-treatment baseline when TLR4 activation was prevented with LPS-RS. There were also no significant decreases observed from the pre-treatment baseline (Fig. 3A; light grey bars). In contrast, SEP amplitude increased significantly and steadily with LPS alone (Fig 3A; dark grey bars) compared with LPS+LPS-RS, reaching significance at 10 min. While there was a slight decrease in excitability at 20 min, this was not significant, and measurements conducted as late as 30 min revealed sustained power increase. To examine increases in the spatial distribution or area of the SEP, independent of overall power, the responses across the array were normalized to the maximum power for a given condition (latency pre- or post-treatment) and then the number of electrode sites reaching at least 50% of this maximum was counted to quantify the active area in square millimetres. With measurements of spatial distribution, there were significant effects of hemisphere group ($P<0.009$), time after LPS application ($P<0.01$) and an interaction between the two factors ($P<0.002$). Pair-wise comparisons revealed that increases in the area of active cortex with LPS compared with the LPS-RS control mirrored those of power increases with the exception that

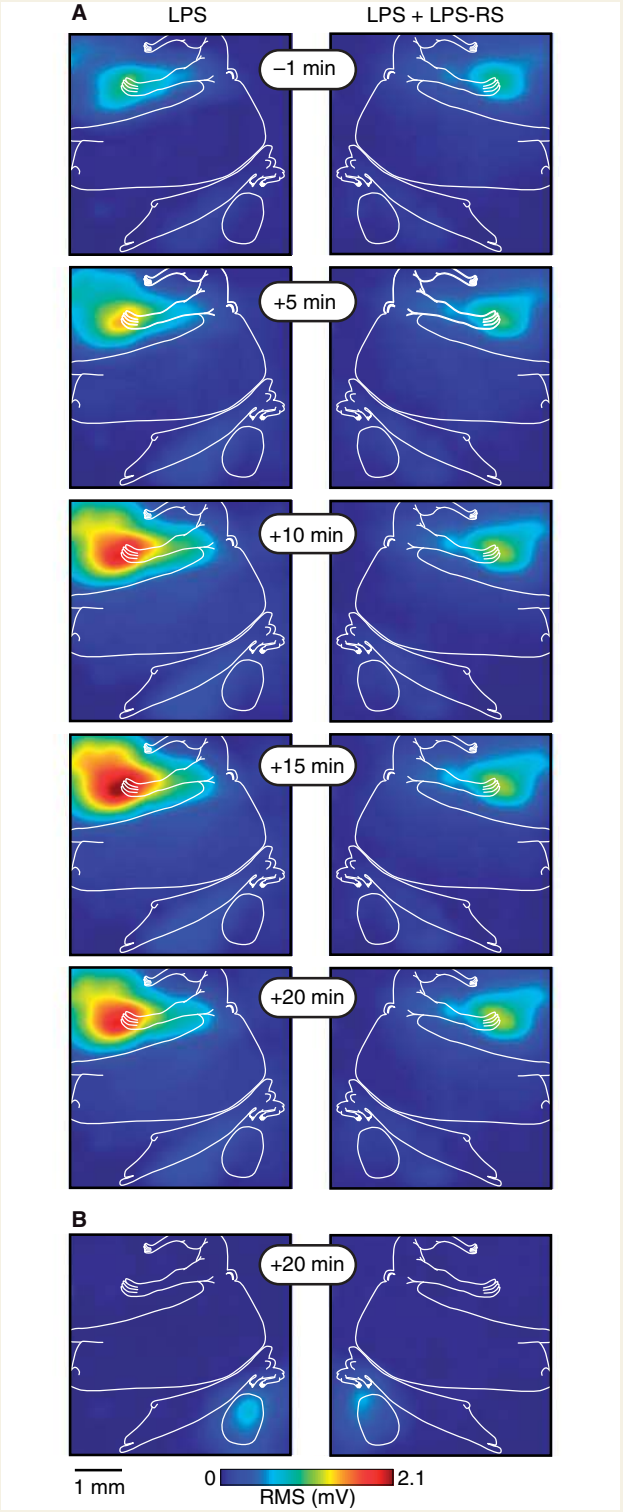

Figure 2 Increases in SEP power and area produced by LPS. (**A**) The left column of maps displays the grand average SEP evoked by forepaw stimulation at time points 1 min prior to, and 5, 10, 15 and 20 min subsequent to application of LPS. Right hemisphere was treated with an LPS-RS before application of LPS (right column). LPS-RS prevented the increased neuronal excitability produced by LPS without affecting the amplitude or area of normal SEPs. (**B**) Mapping of auditory AEPs in both hemispheres following LPS and LPS-RS application revealed no change from baseline.

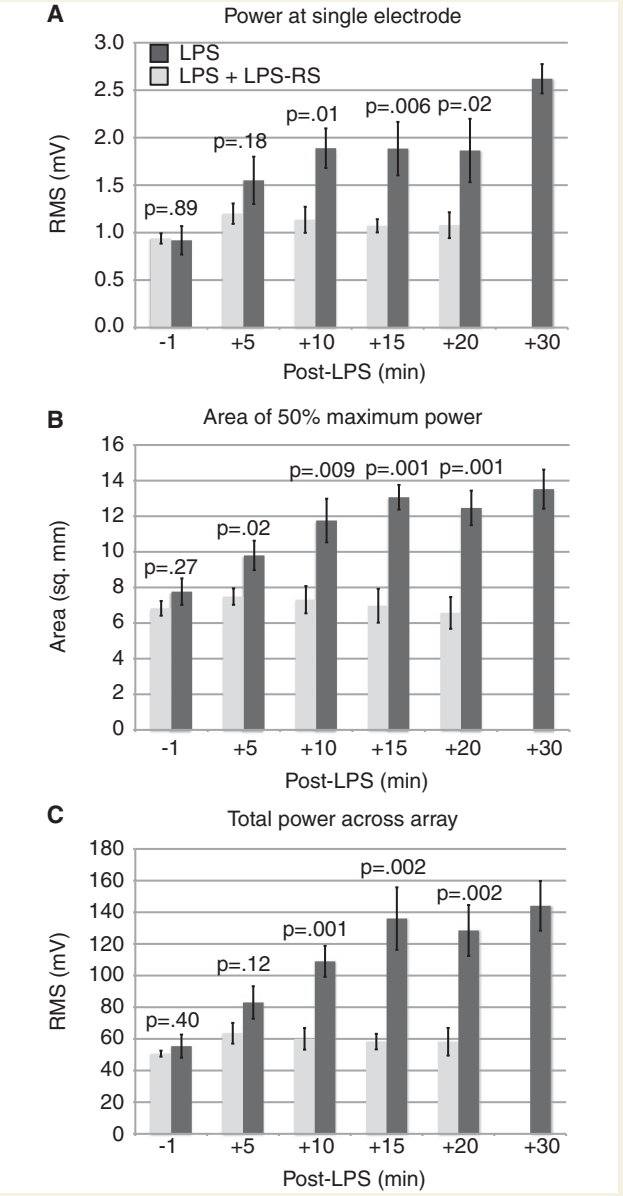

Figure 3 Quantitative analysis of SEP changes induced by LPS. (**A**) The RMS power of SEP at a single electrode site in the left hemisphere and right hemisphere. While no significant increases in SEP power were seen when TLR4 were blocked with LPS-RS (light grey bars), neither were there any significant decreases from the pre-treatment baseline. In contrast, SEP amplitude increased significantly and steadily with LPS alone (dark grey bars). (**B**) To examine increases in the spatial distribution or area of the SEP, independent of overall power, the responses across the array were normalized to the maximum power for a given condition (latency pre- or post-treatment) and then the number of electrode sites reaching $\geqslant$50% of this maximum were counted to determine active area in sq mm. Increases in the area of active cortex with LPS compared with the LPS-RS control mirrored those of power increases (**A**) with the exception that significant changes were detected as early as 5 min post-treatment. (**C**) Similar to (**A**), but reflecting SEP power summed across all 256 electrodes of the array. This measure combines changes in power at each electrode (**A**) with changes in the area of active cortex (**B**).

significant changes were detected as early as 5 min post-treatment (Fig. 3B). Finally, SEP power summed across all 256 electrodes of the array, which combined changes in power at each electrode with changes in the area of active cortex (Fig. 3C), produced significant effects of hemisphere group ($P < 0.007$), time after LPS application ($P < 0.0001$) and an interaction between the two factors ($P < 0.0002$). Pair-wise comparisons mirrored increases of separate analyses of SEP amplitude and spatial distribution with LPS alone or LPS+LPS-RS.

Innate immune responses produce spontaneous epileptiform discharge and seizures

Epicortical application of more concentrated LPS (0.2 μg in 10 μl saline) in an additional 12 animals produced spontaneous epileptiform discharge (Fig. 4A a–l) within 10–20 min. Three animals exhibited motor characteristics of focal seizures, with extension and superimposed twitching of the tail, contralateral hindpaw and/or forepaw. Ipsilateral motor involvement was not observed. Electrographic records were characterized by periodic episodes of high frequency spikes (Fig. 4A a–c; 164 ± 3.2 spikes per min; 3.5 ± 0.25 mV peak to peak) followed by suppression of all spontaneous activity for 10–30 s, but no distinct tonic/clonic phases typical of more generalized seizures. Seizures recurred at ~1–2 min intervals for the 30 min recording period. The remaining nine animals (Fig. 4A d–l) only exhibited continuous lower frequency epileptiform spikes (38.8 ± 0.25 spikes per min; 3.3 ± 0.41 mV) during this period with no accompanying motor signs.

IL-1 is involved in innate immune control of cortical excitability

Epicortical application of IL-1ra prior to LPS prevented both seizures and epileptiform spikes in all animals in which it was tested (Fig. 4B) for the entire recording period, indicating IL-1 as an important mediator of LPS-induced epileptiform activity in these animals. In four bilateral preparations (surgically exposed right and left hemispheres; Fig. 4B; lower four traces) prevention of epileptiform spiking was achieved with IL-1ra in the left hemisphere while subsequent LPS application to the untreated contralateral hemisphere resulted in substantial epileptiform discharges (corresponding to Fig. 4A a, d, e and h, respectively). Yet, effects of IL-1ra were selective to LPS-induced hyper-excitability. Reduced inhibition effected by application of $GABA_A$ receptor antagonist BMI to the IL-1ra treated cortex resulted in epileptiform spikes (42.1 ± 2.75 spikes per min; 4.5 ± 0.16 mV peak to peak), demonstrating that, despite previous findings showing that IL-1ra over-expressing mice are less susceptible to BMI-induced seizures (Vezzani *et al*., 2000), neural circuits remained capable of paroxysmal synchronization when sufficiently challenged (Fig. 4B; right traces). Indeed, SEP amplitudes in the bilateral animals did not differ from baseline after application of either IL-1ra or subsequent application of IL-1ra + LPS in the left hemisphere (Fig. 4C; light grey bars), suggesting normal cortical

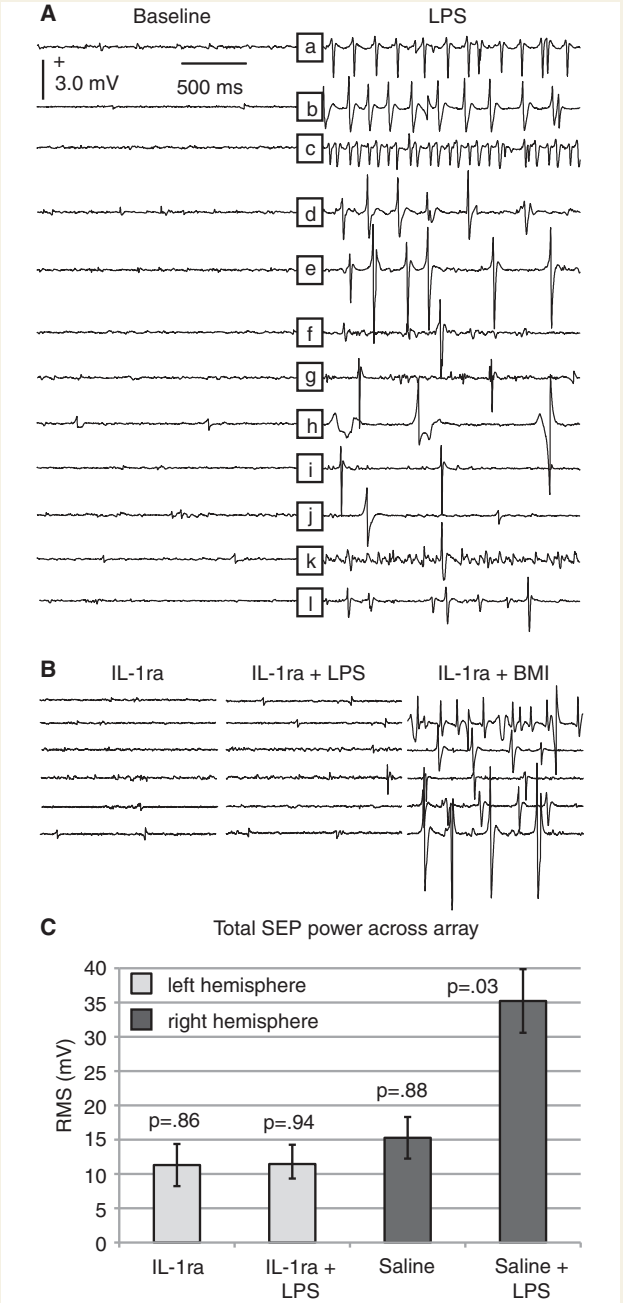

Figure 4 Increased excitability and epileptiform spikes produced by higher LPS concentrations are due to glial release of IL-1. (**A**) Spontaneous activity recorded from an electrode in the centre somatosensory cortex before (left) and 30–40 min after application of higher concentrations of LPS. The top three animals (a–c) exhibited seizures, the others (d–l) produced only lower frequency epileptiform discharges. (**B**) Application IL-1ra prevented LPS-induced hyper-excitability. Spontaneous activity, recorded up to an hour following LPS administration (middle traces), was indistinguishable from baseline (left traces). However, BMI remained effective in evoking epileptiform discharges (right traces). (**C**) In four animals with bi-hemispheric surgeries, LPS approximately doubled the SEP amplitude in the untreated right hemisphere (dark grey bars), whereas pre-application of IL-1ra to the left hemisphere maintained normally responsive SEPs in the presence of LPS (light grey bars).

excitability was maintained despite the IL-1ra treatment. In contrast, LPS application to the untreated contralateral hemisphere resulted in significantly increased SEP amplitude (Fig. 4C; dark grey bars).

Discussion

Glial cells have long been thought to provide only metabolic/structural support in the cerebral cortex and, until recently, have been largely ignored with respect to their potential influence on normal or abnormal electrical activity of neural circuits. With insults to the brain, such as percussive or penetrating wounds, tumour, infarct and infection, glial cells accumulate in the regions of damage (reactive gliosis) for the purpose of repair and reconstruction (Sofroniew, 2005). However, as early as 1970 (Pollen and Trachtenberg, 1970), it was suspected that astrocytes in regions of cortical damage may also function abnormally and alter the excitability of neurons sufficiently to trigger acute or chronically occurring epileptic seizures. This glial hypothesis of epilepsy was based on the theory that damaged astrocytes in an epileptic focus may fail to buffer excess extracellular K^+ accumulated near active neurons, resulting in hyper-excitability and epileptic seizures.

While the glial/K^+ buffering theory of epilepsy fell out of favour for several decades for lack of strong supporting evidence (however, see Frohlich *et al*., 2008) the potential role of glia in epileptogenesis has received a resurgence of interest with discovery of neuro-glial interactions. The continued focus of this work has been on astrocytes since these are known to surround the synaptic junctions between nerve cells, forming a tripartite synapse (Araque *et al*., 1999; Halassa *et al*., 2007) that not only can buffer K^+, but can respond to, release and remove chemical transmitters in these critical zones. It is possible that dysfunction of astrocytes may disrupt regulatory control of neuronal circuits, resulting in runaway excitation (i.e. seizures Parpura *et al*., 1994). In the same light, a repertoire of recently discovered glio-transmitters and pro-inflammatory cytokines [IL-1 and tumour necrosis factor-α (TNFα)], that can be released by microglia, astrocytes and endothelial cells influencing neuronal excitability, are under intense investigation for their possible contributions to epileptogenesis (Merrill, 1992; Vitkovic *et al*., 2000).

The present data indicate that the innate immune response to TLR4 ligands, most likely involving activated microglia, results in increased neuronal excitability sufficient to trigger seizures *in vivo*. While microglia are regarded to be the major cell type in the CNS capable of transducing LPS signals (Lehnardt *et al*., 2002), TLR4 have also been described in astrocyte cultures (Bowman *et al*., 2003; Carpentier *et al*., 2005; Borysiewicz *et al*., 2009; Li *et al*., 2008) and LPS stimulation of cultured astrocytes results in production of IL-1 and TNFα (Lieberman *et al*., 1989; Chung and Benveniste, 1990; Bowman *et al*., 2003; Borysiewicz *et al*., 2009; Konat *et al*., 2008). While TLR4 on astrocytes have not yet been demonstrated *in vivo* (Farina *et al*., 2007), their contribution to the LPS-induced seizures reported here is a distinct possibility. It has also been shown that, although fluorescently tagged LPS binds to microglia but not to neurons (Lehnardt *et al*., 2003), neurons can express and up-regulate TLR4 in response to ischaemia (Tang *et al*., 2007). Yet, unlike microglia and astrocytes, neuronal responses to LPS have not been demonstrated (Tang *et al*., 2007). This does not rule out the potential contribution of neuronal TLR4 to pathological excitability in situations of brain trauma where ischaemia is a distinct possibility. Similarly, while it is not clear that TLR4 expression on endothelial cells in their basal state would be sufficient to contribute to LPS effects observed in the present study (however, see Zeuke *et al*., 2002), TLR4 is markedly up-regulated in endothelial cells of brain capillaries following subarachnoid haemorrhage (Zhou *et al*., 2007). Given the capacity of micro-vascular endothelial cells to secrete pro-inflammatory cytokines IL-1 and TNFα (Behling-Kelly *et al*., 2007), they may also contribute to neuronal hyper-excitability following traumatic injury.

The remarkably rapid-onset of both LPS-induced SEP increases and epileptiform spikes demonstrated here (<10.0 min) may, at least in part, have been facilitated by the requisite tissue trauma and elapsed time of surgical preparation, priming the cells for up-regulation of cytokines. However, in this study, significant changes in excitability were not detectable until administration of LPS and associated cytokine release. In astrocytic cultures, LPS exposure times as short as 15 min result in a sharp up-regulation of IL-1β; however, the time-course of the response is slow, requiring 24 h to reach maximum (Krasowska-Zoladek *et al*., 2007). Yet, studies concerned with the time-course of cytokine expression evoked by LPS focus on changes with a temporal resolution of hours and not minutes. Our data suggest functional responses to LPS reach significance at latencies as short as 5 min, and are sustained for at least an hour (the longest time point evaluated here) without abatement. This rapid LPS response mirrors *in vitro* results from LPS application to slices of adult rat parietal cortex, resulting in increased glutamate and noradrenaline within 10 min, potentially resulting in rapid increases of neuronal excitability (Wang and White, 1999).

Glia are clearly activated following seizures in experimentally induced epileptic foci, producing elevated IL-1 and TNFα (Minami *et al*., 1991; Zimmer *et al*., 1997; Plata-Salaman *et al*., 2000; Oprica *et al*., 2003; Rizzi *et al*., 2003). Our results support the possibility that IL-1 is an important glia-mediated pro-inflammatory cytokine participating in these excitability changes since IL-1ra prevented the effects of LPS, a finding in close agreement with reports of anti-convulsant actions of IL-1ra in experimental models of epilepsy (Vezzani *et al*., 2000). However, IL-1 is only one of several pro-inflammatory cytokines capable of increasing neuronal excitability and lowering seizure threshold. For example, CNS inflammation triggered by peripheral chemically induced visceral inflammation is correlated with widespread activation of cortical glial cells, decreased thresholds for generalized PTZ-induced seizures, increased levels of TNFα and a marked increase in seizure threshold with central antagonism of TNFα (Riazi *et al*., 2008). IL-1 released by microglia may increase neuronal excitability through its activating effect on astrocytes, imbalancing their control of glutamate homeostasis at pyramidal synaptic junctions (Choi and Koh, 2008). However, IL-1 released by microglia and/or astrocytes may also have direct effects

on neuronal channels and excitability. It is notable that studies of seizure-induced IL-1 expression reveal rapid dynamics in agreement with the LPS-induced excitability effects shown here. Expression of IL-1 has been detected within 30 min of PTZ injection and rapid-onset (5–10 min post-injection) seizures (Minami *et al*., 1990). While the molecular mechanisms of the convulsive actions of IL-1 are not known, such a rapid time-course would suggest a non-transcriptional glia to neuron pathway such as recently reported by Balosso *et al*. (2008).

Ravizza *et al*. (2008) have hypothesized that persistent inflammation may be a fundamental mechanism of chronic epilepsy. Our data indicate that early inflammation associated with innate immunity produces large but short-term increases in neuronal excitability. Early inflammatory responses to traumatic brain injury (D'Ambrosio *et al*., 2004) and to infection (Vezzani and Granata, 2005), modelled in this study by LPS-activated innate immunity via TLR4, result in the glial release of a variety of pro-inflammatory cytokines. The rapid modulation of brain excitability demonstrated here would be expected to result in inflammation-induced decreases in seizure threshold, as observed by others in a variety of experimental epilepsy models (Vezzani *et al*., 2008). However, this rapid modulation may also serve as an acute source of epileptiform neuro-excitability with sufficient activation of the innate immune response. In this light, the innate immune response may be viewed, not just as a consequence and possible facilitator of seizures, but as a potential precursor to seizures. The results suggest an important role of innate immunity and focus on glial inhibition, through pharmacological blockade of TLR4 and the pro-inflammatory mediators released by activated glia, in the study and possible prevention of epilepsy in humans.

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References

Aloisi F. Immune function of microglia. Glia 2001; 36: 165–79.

Andersson PB, Perry VH, Gordon S. The acute inflammatory response to lipopolysaccharide in CNS parenchyma differs from that in other body tissues. Neuroscience 1992; 48: 169–86.

Araque A, Parpura V, Sanzgiri RP, Haydon PG. Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 1999; 22: 208–15.

Balosso S, Maroso M, Sanchez-Alavez M, Ravizza T, Frasca A, Bartfai T, et al. A novel non-transcriptional pathway mediates the proconvulsive effects of interleukin-1beta. Brain 2008; 131: 3256–65.

Behling-Kelly E, Kim KS, Czuprynski CJ. Haemophilus somnus activation of brain endothelial cells: potential role for local cytokine production and thrombosis in central nervous system (CNS) infection. Thromb Haemost 2007; 98: 823–30.

Benison AM, Rector DM, Barth DS. Hemispheric mapping of secondary somatosensory cortex in the rat. J Neurophysiol 2007; 97: 200–7.

Borowicz KK, Czuczwar SJ. Effects of etomidate, ketamine or propofol, and their combinations with conventional antiepileptic drugs on amygdala-kindled convulsions in rats. Neuropharmacology 2003; 45: 315–24.

Borysiewicz E, Fil D, Konat GW. Rho proteins are negative regulators of TLR2, TLR3, and TLR4 signaling in astrocytes. J Neurosci Res 2009; 87: 1565–72.

Bowman CC, Rasley A, Tranguch SL, Marriott I. Cultured astrocytes express toll-like receptors for bacterial products. Glia 2003; 43: 281–91.

Carpentier PA, Begolka WS, Olson JK, Elhofy A, Karpus WJ, Miller SD. Differential activation of astrocytes by innate and adaptive immune stimuli. Glia 2005; 49: 360–74.

Choi J, Koh S. Role of brain inflammation in epileptogenesis. Yonsei Med J 2008; 49: 1–18.

Chung IY, Benveniste EN. Tumor necrosis factor-alpha production by astrocytes. Induction by lipopolysaccharide, IFN-gamma, and IL-1 beta. J Immunol 1990; 144: 2999–3007.

Coats SR, Pham TT, Bainbridge BW, Reife RA, Darveau RP. MD-2 mediates the ability of tetra-acylated and penta-acylated lipopolysaccharides to antagonize Escherichia coli lipopolysaccharide at the TLR4 signaling complex. J Immunol 2005; 175: 4490–8.

D'Ambrosio R, Fairbanks JP, Fender JS, Born DE, Doyle DL, Miller JW. Post-traumatic epilepsy following fluid percussion injury in the rat. Brain 2004; 127 (Pt 2): 304–14.

Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, et al. ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 2005; 8: 752–8.

de Pablos RM, Villaran RF, Arguelles S, Herrera AJ, Venero JL, Ayala A, et al. Stress increases vulnerability to inflammation in the rat prefrontal cortex. J Neurosci 2006; 26: 5709–19.

Farina C, Aloisi F, Meinl E. Astrocytes are active players in cerebral innate immunity. Trends Immunol 2007; 28: 138–45.

Fields RD, Stevens-Graham B. New insights into neuron-glia communication. Science 2002; 298: 556–62.

Frohlich F, Bazhenov M, Iragui-Madoz V, Sejnowski TJ. Potassium dynamics in the epileptic cortex: new insights on an old topic. Neuroscientist 2008; 14: 422–33.

Halassa MM, Fellin T, Haydon PG. The tripartite synapse: roles for gliotransmission in health and disease. Trends Mol Med 2007; 13: 54–63.

Konat GW, Krasowska-Zoladek A, Kraszpulski M. Statins enhance toll-like receptor 4-mediated cytokine gene expression in astrocytes: implication of Rho proteins in negative feedback regulation. J Neurosci Res 2008; 86: 603–9.

Krasowska-Zoladek A, Banaszewska M, Kraszpulski M, Konat GW. Kinetics of inflammatory response of astrocytes induced by TLR 3 and TLR4 ligation. J Neurosci Res 2007; 85: 205–12.

Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, et al. The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. J Neurosci 2002; 22: 2478–86.

Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, et al. Activation of innate immunity in the CNS triggers neurodegeneration through a toll-like receptor 4-dependent pathway. Proc Natl Acad Sci USA 2003; 100: 8514–9.

Li Q, Michaud M, Stewart W, Schwartz M, Madri JA. Modeling the neurovascular niche: murine strain differences mimic the range of responses to chronic hypoxia in the premature newborn. J Neurosci Res 2008; 86: 1227–42.

Lieberman AP, Pitha PM, Shin HS, Shin ML. Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. Proc Natl Acad Sci USA 1989; 86: 6348–52.

Merrill JE. Tumor necrosis factor alpha, interleukin 1 and related cytokines in brain development: normal and pathological. Dev Neurosci 1992; 14: 1–10.

Midzyanovskaya IS, Salonin DV, Bosnyakova DY, Kuznetsova GD, van Luijtelaar EL. The multiple effects of ketamine on electroencephalographic activity and behavior in WAG/Rij rats. Pharmacol Biochem Behav 2004; 79: 83–91.

Minami M, Kuraishi Y, Yamaguchi T, Nakai S, Hirai Y, Satoh M. Convulsants induce interleukin-1 beta messenger RNA in rat brain. Biochem Biophys Res Commun 1990; 171: 832–7.

Minami M, Kuraishi Y, Satoh M. Effects of kainic acid on messenger RNA levels of IL-1 beta, IL-6, TNF alpha and LIF in the rat brain. Biochem Biophys Res Commun 1991; 176: 593–8.

Nadeau S, Rivest S. Role of microglial-derived tumor necrosis factor in mediating CD14 transcription and nuclear factor kappa B activity in the brain during endotoxemia. J Neurosci 2000; 20: 3456–68.

Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 2005; 308: 1314–8.

Oprica M, Eriksson C, Schultzberg M. Inflammatory mechanisms associated with brain damage induced by kainic acid with special reference to the interleukin-1 system. J Cell Mol Med 2003; 7: 127–40.

Park KW, Lee DY, Joe EH, Kim SU, Jin BK. Neuroprotective role of microglia expressing interleukin-4. J Neurosci Res 2005; 81: 397–402.

Park KW, Lee HG, Jin BK, Lee YB. Interleukin-10 endogenously expressed in microglia prevents lipopolysaccharide-induced neurodegeneration in the rat cerebral cortex in vivo. Exp Mol Med 2007; 39: 812–9.

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG. Glutamate-mediated astrocyte-neuron signalling. Nature 1994; 369: 744–7.

Plata-Salaman CR, Ilyin SE, Turrin NP, Gayle D, Flynn MC, Romanovitch AE, et al. Kindling modulates the IL-1beta system, TNF-alpha, TGF-beta1, and neuropeptide mRNAs in specific brain regions. Brain Res Mol Brain Res 2000; 75: 248–58.

Pollen DA, Trachtenberg MC. Neuroglia: gliosis and focal epilepsy. Science 1970; 167: 1252–3.

Qureshi N, Jarvis BW, Takayama K. Nontoxic RsDPLA as a potent antagonist of toxic lipopolysaccharide. In: Brade H, Opal SM, Vogel SN, Morrison DC, editors. Endotoxin in health and disease. New York: Marcel Dekker; 1999. p. 687–98.

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.

Riazi K, Galic MA, Kuzmiski JB, Ho W, Sharkey KA, Pittman QJ. Microglial activation and TNFalpha production mediate altered CNS excitability following peripheral inflammation. Proc Natl Acad Sci USA 2008; 105: 17151–6.

Rizzi M, Perego C, Aliprandi M, Richichi C, Ravizza T, Colella D, et al. Glia activation and cytokine increase in rat hippocampus by kainic acid-induced status epilepticus during postnatal development. Neurobiol Dis 2003; 14: 494–503.

Robert S. Early glial dysfunction in epilepsy. Epilepsia 2008; 49: 1–2.

Saitoh S, Akashi S, Yamada T, Tanimura N, Kobayashi M, Konno K, et al. Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. Int Immunol 2004; 16: 961–9.

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.

Shibakawa YS, Sasaki Y, Goshima Y, Echigo N, Kamiya Y, Kurahashi K, et al. Effects of ketamine and propofol on inflammatory responses of primary glial cell cultures stimulated with lipopolysaccharide. Br J Anaesth 2005; 95: 803–10.

Sofroniew MV. Reactive astrocytes in neural repair and protection. Neuroscientist 2005; 11: 400–7.

Tang SC, Arumugam TV, Xu X, Cheng A, Mughal MR, Jo DG, et al. Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits. Proc Natl Acad Sci USA 2007; 104: 13798–803.

Teghanemt A, Zhang D, Levis EN, Weiss JP, Gioannini TL. Molecular basis of reduced potency of underacylated endotoxins. J Immunol 2005; 175: 4669–76.

Tellez-Zenteno JF, Matijevic S, Wiebe S. Somatic comorbidity of epilepsy in the general population in Canada. Epilepsia 2005; 46: 1955–62.

Velisek L, Vondrickova R, Mares P. Models of simple partial and absence seizures in freely moving rats: action of ketamine. Pharmacol Biochem Behav 1993; 45: 889–96.

Veronesi MC, Kubek DJ, Kubek MJ. Isoflurane exacerbates electrically evoked seizures in amygdala-kindled rats during recovery. Epilepsy Res 2008; 82: 15–20.

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. Inflammation and epilepsy. Epilepsy Curr 2005; 5: 1–6.

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

Vezzani A, Balosso S, Ravizza T. The role of cytokines in the pathophysiology of epilepsy. Brain Behav Immun 2008; 22: 797–803.

Visintin A, Halmen KA, Latz E, Monks BG, Golenbock DT. Pharmacological inhibition of endotoxin responses is achieved by targeting the TLR4 coreceptor, MD-2. J Immunol 2005; 175: 6465–72.

Vitkovic L, Bockaert J, Jacque C. ''Inflammatory'' cytokines: neuromodulators in normal brain? J Neurochem 2000; 74: 457–71.

Wang YS, White TD. The bacterial endotoxin lipopolysaccharide causes rapid inappropriate excitation in rat cortex. J Neurochem 1999; 72: 652–60.

Zeuke S, Ulmer AJ, Kusumoto S, Katus HA, Heine H. TLR4-mediated inflammatory activation of human coronary artery endothelial cells by LPS. Cardiovasc Res 2002; 56: 126–34.

Zhou ML, Shi JX, Hang CH, Zhang FF, Gao J, Yin HX. Expression of toll-like receptor 4 in the brain in a rabbit experimental subarachnoid haemorrhage model. Inflamm Res 2007; 56: 93–7.

Zimmer LA, Ennis M, Shipley MT. Soman-induced seizures rapidly activate astrocytes and microglia in discrete brain regions. J Comp Neurol 1997; 378: 482–92.