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Postnatal Inflammation Increases Seizure Susceptibility in Adult Rats

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There are critical postnatal periods during which even subtle interventions can have long-lasting effects on adult physiology. We asked whether an immune challenge during early postnatal development can alter neuronal excitability and seizure susceptibility in adults. Postnatal day 14 (P14) male Sprague Dawley rats were injected with the bacterial endotoxin lipopolysaccharide (LPS), and control animals received sterile saline. Three weeks later, extracellular recordings from hippocampal slices revealed enhanced field EPSP slopes after Schaffer collateral stimulation and increased epileptiform burst-firing activity in CA1 after 4-aminopyridine application. Six to 8 weeks after postnatal LPS injection, seizure susceptibility was assessed in response to lithium–pilocarpine, kainic acid, and pentylenetetrazol. Rats treated with LPS showed significantly greater adult seizure susceptibility to all convulsants, as well as increased cytokine release and enhanced neuronal degeneration within the hippocampus after limbic seizures. These persistent increases in seizure susceptibility occurred only when LPS was given during a critical postnatal period (P7 and P14) and not before (P1) or after (P20). This early effect of LPS on adult seizures was blocked by concurrent intracerebroventricular administration of a tumor necrosis factor α (TNFα) antibody and mimicked by intracerebroventricular injection of rat recombinant TNFα. Postnatal LPS injection did not result in permanent changes in microglial (Iba1) activity or hippocampal cytokine [IL-1β (interleukin-1β) and TNFα] levels, but caused a slight increase in astrocyte (GFAP) numbers. These novel results indicate that a single LPS injection during a critical postnatal period causes a long-lasting increase in seizure susceptibility that is strongly dependent on TNFα.

Key words: **development; lipopolysaccharide; seizure; cytokine; tumor necrosis factor α; interleukin-1β**

Introduction

Seizures in adults can be caused by any number of genetic factors or acquired clinical pathologies. However, in the face of such factors, there is substantial variation in both the likelihood that an individual will develop a seizure and in the severity of the condition. The relative contributions of genetic, environmental, and traumatic conditions to this diversity remain unknown. It has been postulated that early life events, for example a postnatal seizure brought about by whole-body hyperthermia, may predispose the brain to epilepsy later in life (Dubé et al., 2006).

Mounting evidence indicates that brief systemic inflammation during critical periods of development, although not associated with obvious CNS injury, may result in long-lasting cerebral and peripheral vulnerability (programming or sensitization) well into adulthood (Eklind et al., 2005; Hagberg and Mallard, 2005; Godbout and Johnson, 2006). For example, adult rats that had been treated at postnatal day 14 (P14) with the immune activator lipopolysaccharide (LPS) showed increased brain NMDA receptor mRNA (Harré et al., 2008) and greater neuronal loss after global cerebral ischemia (Spencer et al., 2006a). Moreover, a similar postnatal treatment was also sufficient to evoke differences in pain sensitivity (Boissé et al., 2005), memory performance (Bilbo et al., 2005), and neuroimmune responses (Boissé et al., 2004) in adulthood. Consequently, it appears that the immature brain can be permanently modified after a single inflammatory episode in a manner that may contribute to a number of behavioral and physiological abnormalities.

The inflammatory response brought about by LPS is characterized by the generation of cytokines in the periphery and concomitant synthesis in the CNS (Layé et al., 1994; Nguyen et al., 1998; Verma et al., 2006). Among the proinflammatory cytokines produced in the brain are tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) (Turrin et al., 2001), which are now accepted as bona fide modulators of both normal and abnormal

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neuronal transmission within the brain (Merrill, 1992; Mehler and Kessler, 1998; Vitkovic et al., 2000).

In the adult, clinical and experimental evidence suggests that infection or inflammation may be a potent contributor to seizure predisposition and occurrence, as well as seizure-related brain injury (Vezzani and Granata, 2005; Ravizza et al., 2006; Bartfai et al., 2007; Vezzani and Baram, 2007). Several reports have revealed that proinflammatory cytokines are involved in the pathophysiology of seizures and may be new targets for therapies against epilepsy (De Simoni et al., 2000; Virta et al., 2002; Turrin and Rivest, 2004; Dubé et al., 2005; Heida and Pittman, 2005; Bartfai et al., 2007; Somera-Molina et al., 2007). Because the early postnatal brain also generates cytokines in response to peripheral inflammation and seizures (Heida and Pittman, 2005; Ravizza et al., 2005), it is likely that the interaction of these cytokines with neuronal elements during development may alter the brain in a manner that makes it more susceptible to seizures as an adult. We therefore tested the hypothesis that postnatal inflammation may make adult animals more vulnerable to a challenge by convulsant drugs via an interaction with cytokines.

Materials and Methods

Animals and drugs. Timed pregnant Sprague Dawley rats were obtained from Charles River Laboratories and maintained in the Medical Sciences vivarium at the University of Calgary under standard specific pathogen-free (SPF) environmental conditions. They were housed at a constant temperature (20–21°C) with food and water available *ad libitum*. The light/dark cycle was 12/12 h with photophase onset at 7:00 A.M. local time. Pregnant females were monitored for the parturition date that was taken as P0 at which time all litters were culled to 12. After the postnatal treatments as described below, animals were returned to their dams, weaned at P21, and housed two per cage, where they were subjected to regular SPF husbandry until additional testing. All experiments involved male rats only to exclude any sex-specific interactions between treatments, and all rats were derived from multiple litters to preclude possible differences in individual maternal behaviors as a mitigating factor in any subsequent long-lasting changes induced in the offspring (Meaney and Szyf, 2005). All procedures were approved by the local Animal Care Committee and were compliant with the guidelines of the Canadian Council on Animal Care.

Kainic acid (KA) was obtained from A.G. Scientific, lithium and pilocarpine, pentylenetetrazol (PTZ), 4-aminopyridine (4-AP), and LPS were purchased from Sigma-Aldrich. The TNFα antibody Infliximab was obtained from Centocor, and the IL-1 receptor antagonist (IL-1ra) Anakinra was purchased from Amgen. Rat recombinant TNFα (rrTNFα) was bought from PeproTech. All compounds were administered at 1 ml/kg unless otherwise stated.

Lipopolysaccharide injections. To determine whether a single inflammatory event during development can influence seizure susceptibility in later life, male rats were injected intraperitoneally on P14 with LPS (*Escherichia coli*, serotype O26:B6; 25, 100, or 250 μg/kg) or pyrogen-free saline. We previously established that 100 μg/kg LPS generates a mild inflammatory response in the host that lasts for ~6–8 h (Heida et al., 2004; Ellis et al., 2006). Additional groups of rats were also injected on P14 with saline or LPS (100 μg/kg) to evaluate both the acute and chronic effects of LPS on cytokine and glial cell activity in the hippocampus (a seizure-vulnerable region). P14 is considered by some to be developmentally equivalent to a human infant of ~1–2 years of age (Gottlieb et al., 1977; Avishai-Eliner et al., 2002). To determine whether there was a critical age at which LPS could cause long-term changes to seizure susceptibility, rats were also treated with saline or LPS (100 μg/kg) at P1, P7, or P20.

In vitro *hippocampal electrophysiology.* Hippocampal slices ($n = 43$) were prepared from ~5-week-old male rats that had been treated with either saline or LPS (100 μg/kg) on P14. Under halothane anesthesia, brains were removed and placed in cold (0–4°C) slicing solution (in mM): 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 7 $MgCl_2$, 1.25 NaH_2PO_4, 25 D-glucose, 20 sucrose, and 0.48 $CaCl_2$ that was bubbled with 5% CO_2/95% O_2. Horizontal hippocampal slices (400 μm) were cut using a vibratome and maintained for 45 min in a warm (32°C) recovery solution composed of artificial CSF (aCSF) (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$, 11 D-glucose, and 1.5 kynurenic acid, and continuously bubbled with 5% CO_2/95% O_2 to maintain a pH of 7.4. After 45 min, slices were transferred into a second chamber that contained aCSF (but without kynurenic acid) for 3–4 h at room temperature. Slices were then transferred to a recording chamber that was continuously perfused with aCSF at 32°C. Extracellular field potentials were recorded with glass micropipettes filled with aCSF (2–3 MΩ) and signals were acquired using an Axopatch 200B amplifier (Molecular Devices; low-pass filter, 5 kHz; high-pass filter, 1 Hz; acquisition frequency, 10 kHz; gain, 500×). Evoked field EPSPs (fEPSPs) were elicited in the CA1 stratum radiatum region of the hippocampus by electrically stimulating the Schaffer collaterals (CA3) with a concentric bipolar electrode at 0, 25, 50, 75, and 100% maximal stimulation. The point at which the fEPSP slope did not increase further with increasing stimulation was taken as 100% maximal stimulation. To record spontaneous field activity, recording electrodes were placed in the CA1 stratum pyramidale. Spontaneous epileptiform activity (burst-firing) was induced by bath perfusion with aCSF containing 75 μM 4-AP, a potassium channel blocker (Perreault and Avoli, 1991; Yonekawa et al., 1995; Motamedi et al., 2006), for 30 min. The epileptiform activity was quantified by determining the frequency of bursts during the last 10 min of 4-AP application, when burst-firing is stable and most robust (Salazar et al., 2003).

Lithium–pilocarpine seizure susceptibility. Six to 8 weeks after P14 injections of either 25, 100, or 250 μg/kg of LPS or saline, 37 rats received subcutaneous injections of lithium chloride (3 mEq/kg) followed 4 h later by pilocarpine (30 mg/kg) (LI-PILO). The latency to the first behavioral seizure after pilocarpine administration, referred to as the seizure onset time (SOT), was defined by the occurrence of forelimb clonus, rearing, and loss of balance, and was recorded to the nearest second for each animal by an individual blind to the postnatal treatment of the animal. Seizure onset time is a commonly used measure to describe seizure susceptibility to convulsant compounds in rats (Galic and Persinger, 2005; Lian et al., 2007). Electroencephalographic recordings at the time of overt convulsive behaviors have previously been shown to reveal clear, paroxysmal epileptiform discharges (Cavalheiro et al., 1987; Turski et al., 1989).

Pentylenetetrazol seizure susceptibility. To explore the developmental window in which LPS could produce changes in seizure onset, and to extend our findings to another chemoconvulsant with a different mechanism of action, we examined the seizure susceptibility of adult rats treated at either P1, P7, P14, or P20 with LPS (100 μg/kg) or saline ($n =$ 5–6/group) and given intravenous PTZ 6–8 weeks later. Jugular vein catheters were surgically implanted under ketamine/xylazine (85:15) anesthesia 3–4 d before seizure threshold testing with PTZ. This intravenous PTZ seizure protocol is considered an accurate way to assess seizure susceptibility (Ramzan and Levy, 1985; Mandhane et al., 2007). The clonic seizure threshold was determined by intravenous infusion of a 1% PTZ solution to the unrestrained rat through the catheter at a rate of 0.58 ml/min using an infusion pump. The infusion was terminated when generalized (full-body) clonus was observed. The amount of PTZ (in milligrams per kilogram) required to induce a generalized clonic seizure was calculated and used as an index of seizure susceptibility.

Kainic acid seizure susceptibility and hippocampal cell degeneration. We further asked whether postnatal LPS treatment could generate a comparable susceptibility to a different chemoconvulsant that also causes a well defined pattern of neuronal damage in the hippocampus, yet through an entirely different mechanism of action as LI-PILO and PTZ. Male rats received either saline or LPS (100 μg/kg) on P14 as described above ($n =$ 6–7/group), and then 2 months later received 15 mg/kg KA intraperitoneally (Haas et al., 1996). Seizure onset time was recorded as defined above for LI-PILO, and pentobarbital (20 mg/kg, i.p.) was given 1 h later to suppress convulsions, promote survival, and importantly to equalize seizure duration. Rats were killed 24 h after SOT to determine the extent of neuronal degeneration using Fluoro-Jade histochemistry (Schmued et al., 1997). This time point is optimal for assessing the acute neuronal

consequences (degeneration) of KA-induced seizures (Fujikawa et al., 2000).

Under pentobarbital anesthesia (60 mg/kg), rats were perfused with PBS followed by 4% neutral-buffered paraformaldehyde. The brains were placed into 10% neutral-buffered formalin for 48 h before processing. Brains were embedded in paraffin, and 10 μm sections were cut through the hippocampus in the coronal plane and mounted on potassium dichromate gelatin-coated slides. Fluoro-Jade histochemistry was conducted on alternating sections through the hippocampus from −3.30 to −3.80 mm to bregma (Paxinos and Watson, 1998), which included the dentate gyrus (DG), CA3, and CA1 regions. Sections stained with Fluoro-Jade were then examined using an epifluorescent microscope with a fluorescein isothiocyanate filter at 100× magnification. Degenerating neurons fluoresced bright green, and the total number of neurons (pyramidal cells from the CA1 and CA3 regions and granule cells from the DG) from three sections was tabulated for the brain regions above and for each subject (n = 3–4/group) by an observer blind to the experimental treatments.

Cytokine assay and glial cell immunohistochemistry. To assess the acute effects of LPS on P14 rat microglia and cytokine (IL-1β and TNFα) levels, we collected blood plasma at 2 h after injection, as well as hippocampal tissue 3 and 6 h after LPS (100 μg/kg) or saline injection (n = 4–6/group). Previous reports show that cytokine expression is increased within the adult hippocampus ~6 h after peripheral LPS (Nguyen et al., 1998; Turrin et al., 2001; Oprica et al., 2006).

We also collected hippocampal tissue from adult rats treated at P14 with saline or LPS (100 μg/kg) to examine whether any chronic or persistent differences exist in microglia or cytokine levels after postnatal injection. Moreover, we asked whether there were any differences in cytokine or microglia expression in the hippocampus 6 h after seizure induction in adult rats with a history of LPS exposure. Rats were seized with KA and treated with pentobarbital to abate seizure activity 1 h after seizure onset as above. We chose 6 h to assay for cytokines and microglia because this appears to be the peak time to inflammation in the hippocampus after seizures (Vezzani et al., 2002). Briefly, rats were deeply anesthetized with pentobarbital and perfused with PBS, brains were quickly removed, and the hippocampal tissue was rapidly dissected out. One hippocampus was snap frozen in liquid nitrogen and stored at −80°C to be used for the cytokine assays, whereas the other was immersed in 10% neutral-buffered formalin and immunostained for quantification of microglia.

Measurements of the cytokines TNFα and IL-1β from the hippocampus of rats treated with saline or LPS was assessed using ELISA kits (BioSource). Interassay and intraassay variability (represented as coefficient of variation) for IL-1β were 8.7–9.7 and 6.7–8.2%, respectively, with a lower limit of detection at <3 pg/ml. Interassay and intraassay variability for TNFα were 3.5–4.3 and 2.6–8.2%, respectively, with a lower limit of detection at <4 pg/ml. All hippocampal samples were adjusted according to the protein content and presented as picograms per milligram.

For microglial measurements, the hippocampus was paraffin embedded and cut (10 μm) in the coronal plane [−3.30 to −3.80 mm to bregma (Paxinos and Watson, 1998)]. Sections were incubated with a primary Iba1 antibody (overnight; 1:500; rabbit; Wako Chemicals) followed by a secondary antibody [2 h; 1:1000; donkey anti-rabbit IgG (CY3); Jackson ImmunoResearch Laboratories]. The total number of microglia, as well as the percentage of activated cells for each subject, was counted (200× magnification) in the CA1 hippocampus as previously described (Spencer et al., 2007) by an examiner blind to the treatment history of the animals. We quantified microglial activation in CA1 because both the electrophysiology experiments and Fluoro-Jade histochemistry indicated robust effects within the CA1 region after LPS treatment. Activated microglia were defined as those with shorter less ramified processes, perikaryal hypertrophy, and ameboid appearance.

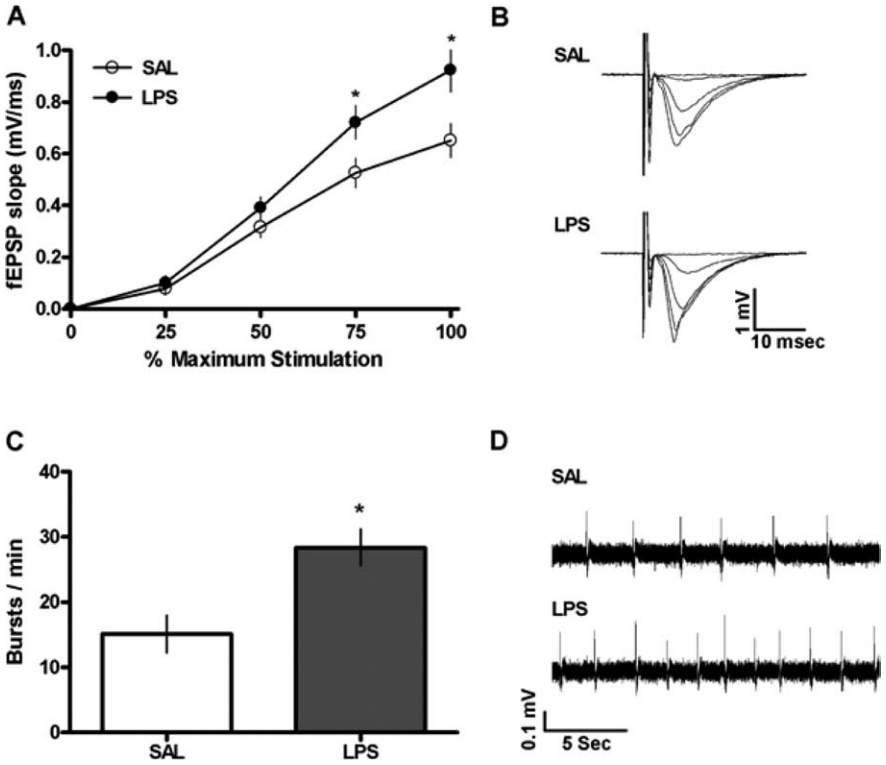

Figure 1. *In vitro* hippocampal slice electrophysiological recordings from 5-week-old rats treated postnatally with either SAL or LPS (100 μg/kg). ***A***, CA1 recordings of fEPSP slopes to stimulation of afferent fibers in the Schaffer collateral pathway from rats treated with either SAL or LPS at P14 showed significantly ($p < 0.05$) greater slopes (in microvolts per millisecond) at 75 and 100% maximum stimulation in the LPS-treated group compared with controls (n = 9–12/group). ***B***, Representative fEPSP traces from the two treatment conditions. ***C***, The number of epileptiform bursts per minute from the pyramidal cell layer of CA1 during bath application of 4-AP in aCSF from SAL- or LPS-treated rats (n = 8–14/group). There were significantly ($p < 0.05$) more burst-firing episodes in the LPS-treated group compared with controls. ***D***, Representative traces of 4-AP-induced epileptiform activity from the two treatment conditions. An asterisk denotes LPS-treated groups that differ significantly ($p < 0.05$) from controls. Error bars indicate SEM.

We also asked whether astrocyte numbers [identified by glial fibrillary acidic protein (GFAP) expression] were altered in adulthood after a P14 injection of LPS. Adult rats that received saline or LPS (n = 4/group) on P14 were given an overdose of pentobarbital and transcardially perfused with cold physiological saline followed by 10% neutral-buffered formalin. Brains were removed and postfixed for 72 h and sectioned in the coronal plane at 50 μm on a vibrating microtome. Sections were stored in a cryoprotectant solution [30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, 30% (v/v) ethylene glycol] at −25°C until processing.

Every sixth section through the entire hippocampal formation was processed using the free-floating method. Sections were incubated overnight at 4°C with a mouse monoclonal antibody raised against GFAP (1:3000; Sigma-Aldrich) followed by biotinylated horse anti-mouse IgG antibody (1:500; Vector Laboratories). Sections were placed in avidin–biotin–peroxidase complex (1:500; Vectastain Elite; Vector Laboratories) for 1 h, and peroxidase activity was visualized using 3,3′-diaminobenzidine and H_2O_2 in PBS. The specificity of the antibodies was determined by running the staining protocol in the absence of the primary antibodies. No staining was observed for any of these sections.

A single examiner who was blind to each animal's treatment performed the data collection. The total number of GFAP-positive cells in

the left DG, CA3, and CA1 subfields of the hippocampal formation was estimated using the unbiased optical fractionator method (West et al., 1991), with assistance from a computerized stereology system (StereoInvestigator; MicroBrightField). All regions were traced at low power (40×), demarcated using methodology previously used (Long et al., 1998; Grady et al., 2003), and counted at 400× magnification.

Intracerebroventricular injections. Cytokines are synthesized and released in the brain after a peripheral injection of LPS (Nguyen et al., 1998; Turrin et al., 2001). To evaluate the contribution of the central cytokine responses to peripheral LPS on the programming of the long-term effect on seizure susceptibility after peripheral LPS administration, 65 P14 rats were given intracerebroventricular injections of the natural antagonist for IL-1, IL-1ra (Anakinra; 10 or 50 μg/5 μl), or the TNFα neutralizing antibody (Infliximab; 50 μg/5 μl), concurrently with LPS (100 μg/kg, i.p.) or saline. We chose these dosages of IL-1ra because previous reports indicate that 4 μg intracerebroventricularly effectively antagonizes effects of peripheral pyrogens on rat behavior (Kent et al., 1992) and that 10 μg intracerebroventricularly significantly reduces the incidence of febrile seizures in rat pups (Heida and Pittman, 2005). In other P14 rats, rrTNFα was administered intracerebroventricularly (bilaterally) at 1 μg/2.5 μl per side to determine the role of this cytokine in promoting long-term seizure susceptibility ($n = 5$–7/group). Dosages of TNFα similar to this have been shown to influence neuronal transmission in the absence of negative side effects (Ignatowski et al., 1999; Reynolds et al., 2004). The surgical procedure, described in detail previously (Heida and Pittman, 2005), was performed under halothane anesthesia and required <10 min to conduct. Animals were returned to their dams, and seizure thresholds were examined 6–8 weeks later using the PTZ paradigm described above.

Statistical analysis. All analyses were completed using Statistical Package for the Social Sciences software (SPSS, version 13). Between group differences in seizure susceptibility, cell counts, cytokine concentrations, and burst-firing frequency were analyzed using independent *t* tests or a one-way ANOVA with Student–Newman–Keuls *post hoc* tests. The fEPSP slopes were examined using a two-way ANOVA followed by independent *t* tests. Results are expressed as the mean (M) ± SEM. The criterion for statistical significance was set at $p < 0.05$.

Results

Enhanced hippocampal excitability and epileptiform activity after LPS

Electrophysiological recordings from hippocampal slices of ~5-week-old rats treated at P14 with saline or LPS revealed that the fEPSPs slopes recorded in the stratum radiatum of CA1 were significantly ($F_{(4,95)} = 2.60$; $p < 0.05$) different between the postnatal treatments depending on stimulation intensity. Subsequent analyses determined that the major effects occurred at 75% ($t = 2.16$; $p < 0.05$) and 100% ($t = 2.38$; $p < 0.05$) maximum stimulation that displayed significant enhancement in the LPS-treated animals (Fig. 1*A*,*B*). We also quantified the number of spontaneous epileptiform discharges from the stratum pyramidale layer of CA1 in response to 30 min bath application of 4-AP in rats treated with saline or LPS at P14. There was a significant difference in epileptiform discharge frequency between the two treatments ($t = 3.05$; $p < 0.05$), such that rats given LPS postnatally showed consistently more burst-firing discharges than controls (Fig. 1*C*,*D*).

Postnatal LPS increases adult seizure susceptibility to LI-PILO

To determine whether postnatal LPS treatment altered adult seizure threshold, rats at 6–8 weeks of age were administered LI-PILO. All rats developed the expected behavioral seizures that were similar between the different postnatal treatments. Rats given LPS (100 μg/kg) on P14, however, showed ~30% faster mean SOT (in minutes) compared with controls [saline (SAL) (M, 29.24; SEM, 2.58); LPS (M, 20.63; SEM, 1.20); $t = 3.13$; $p < 0.01$]. A similar increase in seizure susceptibility was seen after postnatal LPS at either 25, 100 (replicate), or 250 μg/kg. A one-way ANOVA determined a significant difference between the postnatal treatments (three dosages of LPS and saline) and SOT ($F_{(3,19)} = 5.97$; $p < 0.01$). *Post hoc* analysis showed that the mean SOTs of all P14 LPS-treated groups differed significantly from controls, but did not differ significantly from each other (Fig. 2*A*).

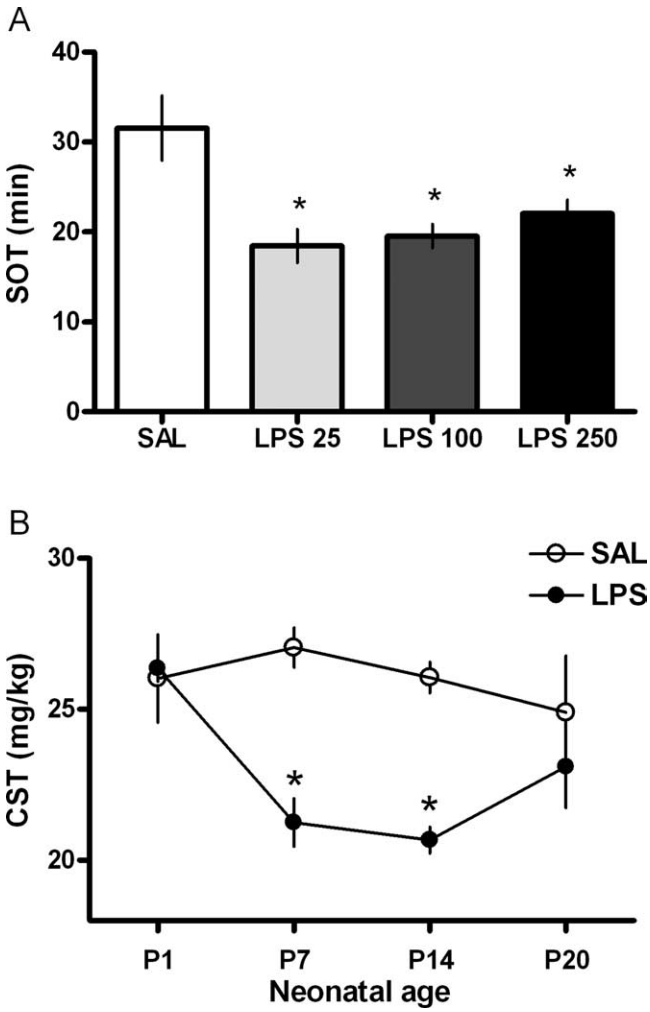

Figure 2. Adult seizure susceptibility to LI-PILO and PTZ after postnatal treatments of either SAL or LPS. ***A***, Latency in minutes to SOT in adult male rats that received LI-PILO after a P14 injection of either SAL ($n = 6$), or LPS at 25, 100, or 250 μg/kg ($n = 4$–5/group). All rats that received LPS regardless of the dosage showed significantly ($p < 0.05$) faster SOTs compared with controls. Error bars indicate SEM. ***B***, Adult seizure susceptibility to PTZ after postnatal treatments of either SAL or LPS (100 μg/kg) on either P1, P7, P14, or P20 ($n = 5$–6/group). Data are presented as means ± SEM for PTZ-induced clonic seizure threshold (CST) in mg/kg. Rats that received LPS on P7 and P14, but not on P1 or P20, showed significantly ($p < 0.05$) lower CSTs compared with controls. An asterisk denotes LPS-treated groups that differ significantly ($p < 0.05$) from controls.

LPS increases adult seizure susceptibility to PTZ during a critical postnatal period

To examine the critical developmental period during which LPS could alter seizure susceptibility, we determined the clonic seizure threshold to PTZ for rats treated at P1, P7, P14, and P20, with either saline or LPS (Fig. 2*B*). Both P7 and P14 LPS-treated rats showed seizure thresholds that were significantly lower than their respective controls ($t = 5.65$, $p < 0.01$; $t = 7.79$, $p < 0.01$).

In other words, they required less PTZ to induce generalized clonus. However, P1 and P20 LPS-treated rats showed no differences in seizure susceptibility compared with saline-treated controls.

Increased seizure susceptibility, cytokine expression, and neuronal degeneration after KA-induced seizures in LPS-treated rats

To determine whether postnatal LPS altered sensitivity to a different convulsant, we examined SOT in response to KA. Postnatal treatment (P14) with LPS resulted in ~30% faster mean SOT ($t = 2.28$; $p < 0.05$) compared with controls (Fig. 3*A*). We also observed an interaction between postnatal treatment and cytokine levels in the hippocampus after KA treatment. Adult rats that received LPS at P14 showed significantly more IL-1β ($t = 2.21$; $p < 0.05$) and a trend toward increased TNFα ($t = 1.72$; $p = 0.1$) levels 6 h after seizure (Fig. 3*B*). There was no interaction between postnatal treatment and microglia number or percentage of activated microglia 6 h after seizure (data not shown). In a subset of animals chosen for Fluoro-Jade histochemistry (to evaluate the effects on neuronal injury), there was a statistically significant difference between the saline and LPS-treated rats with respect to the number of degenerating neurons within the CA3 ($t = 7.61$; $p < 0.01$) and the CA1 region ($t = 3.28$; $p < 0.05$) of the hippocampus, but not in the DG. The postnatal LPS-treated rats showed greater numbers of degenerating neurons, despite similar seizure durations than controls 24 h after KA seizure onset (Fig. 3*C*,*D*).

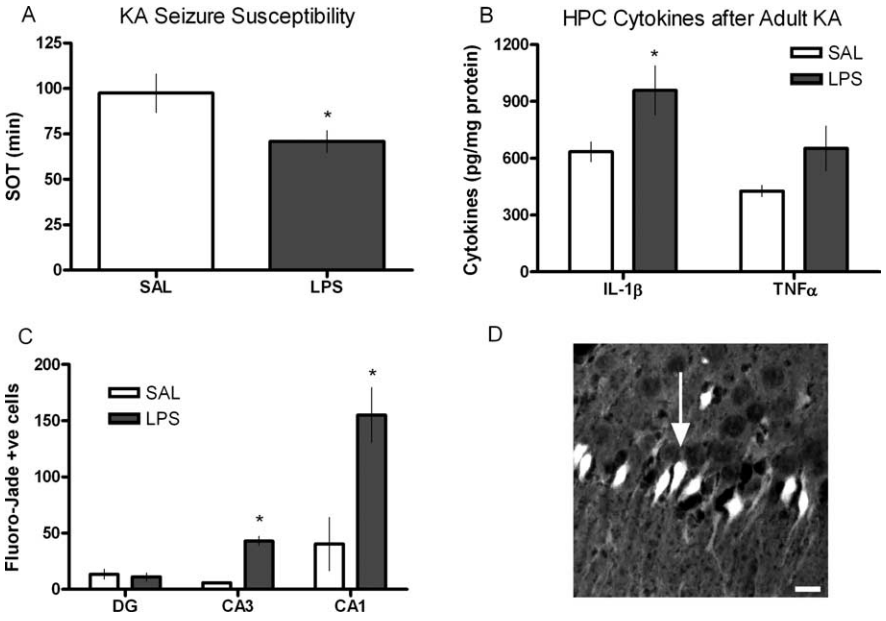

Figure 3. Adult seizure susceptibility to KA after postnatal treatments of either SAL or LPS (100 μg/kg). ***A***, Latency in minutes to SOT in adult rats that received KA after a P14 injection of SAL or LPS ($n = 6–7$/group). Rats that received LPS at P14 showed significantly ($p < 0.05$) faster SOTs compared with controls. ***B***, Levels of IL-1β and TNFα (in picograms per milligram of protein) within the hippocampus (HPC) 6 h after seizure onset in adult animals that received KA after a postnatal injection of either SAL or LPS ($n = 5–7$/group). ***C***, Total number of Fluoro-Jade-staining neurons within the DG, CA3, and CA1 hippocampus of adult rats that received KA after a postnatal injection of either SAL or LPS ($n = 3–4$/group). Significantly ($p < 0.05$) more degenerating neurons were found in the CA3 and CA1 region of the hippocampus 24 h after KA in LPS-treated rats compared with controls. ***D***, Photomicrograph of degenerating neurons (arrow) stained with Fluoro-Jade from the CA1 hippocampus of a postnatally LPS-treated rat after adult KA. Scale bar, 25 μm. An asterisk denotes LPS-treated groups that differ significantly ($p < 0.05$) from controls. Error bars indicate SEM.

TNFα mediates the long-term effect of LPS on adult seizure susceptibility

To determine whether the neural proinflammatory cytokines TNFα or IL-1β were involved in the LPS-induced reduction in seizure threshold, P14 rats were given a single intracerebroventricular dose of IL-1ra or TNFα antibody at the same time as peripheral saline or LPS in an attempt to prevent the LPS-induced reduction in seizure threshold. We found that postnatal treatment with IL-1ra (10 μg/5 μl) plus LPS did not block the significant reduction in adult seizure thresholds to PTZ (in milligrams per kilogram) compared with those displayed by postnatal saline-treated rats given the IL-1ra [SAL plus IL-1ra (M, 31.54; SEM, 0.70); LPS plus IL-1ra (M, 21.36; SEM, 1.54); $t = 4.78$; $p < 0.01$]. In an additional series of experiments, the dose of IL-1ra was increased to 50 μg/5 μl and this also did not reverse the reduced seizure threshold [LPS plus SAL (M, 25.18; SEM, 2.36); LPS plus IL-1ra (M, 25.94; SEM, 1.54); $t < 1$; $p > 0.05$]. In contrast, the TNFα antibody blocked the LPS-induced change in seizure threshold (Fig. 4), because this group did not differ from controls ($t < 1$; $p > 0.05$) given saline plus TNFα antibody at P14. Thus, TNFα antibody, but not IL-1ra, in the P14 rat blocked the reduction in adult seizure threshold brought about by LPS. When we injected the rrTNFα (intracerebroventricularly) alone into P14 rats and induced seizures in adulthood, we found a significant ($t = 2.52$; $p < 0.05$) reduction in seizure threshold to PTZ when compared with P14 intracerebroventricular saline controls (Fig. 4). The intracerebroventricular treatment with rrTNFα at P14 did not influence the gross development of the rats as inferred by their body weights that were not different from controls (data not shown).

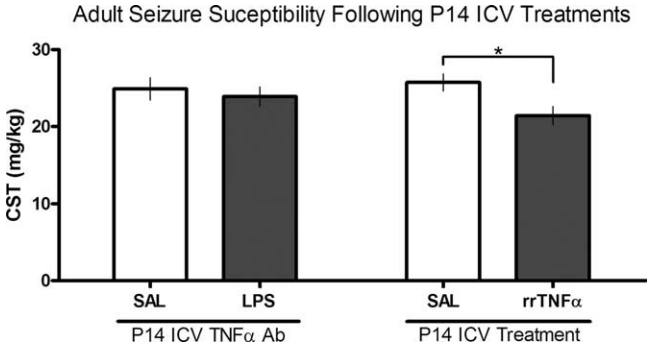

Figure 4. Adult seizure susceptibility to PTZ after P14 treatments of either SAL or LPS (100 μg/kg) and intracerebroventricular (ICV) injections of TNFα antibody (Ab) (50 μg) ($n = 9–11$/group) or intracerebroventricular injection of SAL or rrTNFα (2 μg) ($n = 5–7$/group). Data are presented as means ± SEMs for the clonic seizure threshold (CST) in milligrams per kilogram for adult rats that received PTZ. Rats that received LPS and the TNFα Ab showed no difference in CST compared with SAL controls suggesting that this drug was capable of blocking the LPS-induced facilitation of seizure susceptibility. Moreover, P14 intracerebroventricular rrTNFα alone can reduce adult seizure threshold similar to LPS. The asterisk denotes that the rrTNFα-treated group differed significantly ($p < 0.05$) from controls.

Hippocampal cytokines and microglia are not chronically upregulated in LPS-treated rats

We examined cytokine levels (IL-1β and TNFα) in the plasma (2 h after injection) and hippocampus (3 and 6 h after injection) of

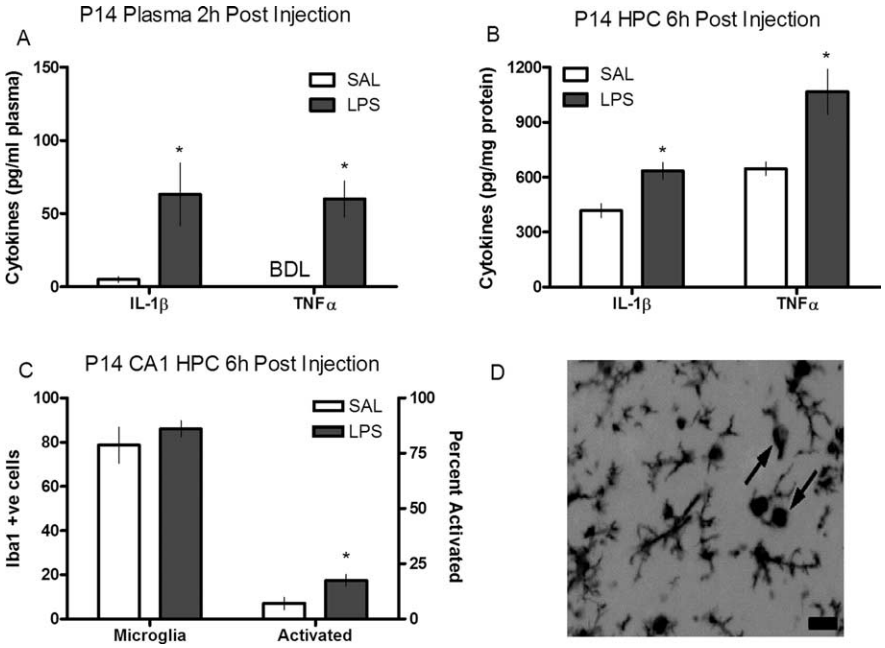

Figure 5. Acute cytokine (IL-1β and TNFα) and microglia (Iba1 immunohistochemistry) data from P14 SAL- or LPS (100 μg/kg)-treated rats ($n = 6$/group). ***A***, IL-1β and TNFα concentrations in the plasma (in picograms per milliliter) of P14-treated rats 2 h after injection. Both cytokines are significantly ($p < 0.05$) increased in LPS-treated rats compared with controls. Values lower than the detection limit of the ELISA kit are labeled as below detectable levels (BDL). ***B***, IL-1β and TNFα concentrations (in picograms per milligram of protein) in the hippocampus (HPC) of P14-treated rats 6 h after injection. Both cytokines are significantly ($p < 0.05$) increased in LPS-treated rats compared with controls. ***C***, Total number of microglia (Iba1-positive) cells (left axis) and the percentage activated microglia count (right axis) of P14 SAL- or LPS-treated rats 6 h after injection. There is no difference in the number of Iba1-positive cells between LPS and control tissues; however, there are significantly ($p < 0.05$) greater numbers of activated microglia within the CA1 HPC of LPS-treated rats 6 h after injection. ***D***, Photomicrograph of activated microglia cells (arrows) from the CA1 HPC of P14 LPS-treated rats. Scale bar, 20 μm. An asterisk denotes LPS-treated groups that differ significantly ($p < 0.05$) from controls. Error bars indicate SEM.

P14-treated rats to determine the amount of acute inflammation after LPS. We found a significant increase in both IL-1β ($t = 2.69$; $p < 0.05$) and TNFα ($t = 4.44$; $p < 0.01$) levels in the plasma 2 h after LPS administration (Fig. 5*A*). In the hippocampus, we found no significant difference in either cytokine at 3 h after LPS (data not shown); but at 6 h later, both IL-1β ($t = 3.42$; $p < 0.05$) and TNFα ($t = 2.82$; $p < 0.05$) were significantly increased in the hippocampus of LPS-treated rats compared with controls (Fig. 5*B*). In adult rats that received saline or LPS on P14, and no subsequent interventions, we observed no significant differences in either cytokine levels in the hippocampus (both values of $t < 1$; $p > 0.05$) (Fig. 6*A*).

Because microglia synthesize and release cytokines, we measured the total number of microglial cells (Iba1 immunopositive) and the percentage of activated microglia within the CA1 hippocampus acutely (6 h) after LPS injection at P14, and in adult rats after P14 saline or LPS. Six hours after peripheral LPS administration to P14 rats, there were no significant ($t < 1$; $p > 0.05$) differences in the total number of Iba1-positive cells within the CA1 hippocampus; however, the percentage of activated microglia was significantly ($t = 2.64$; $p < 0.05$) increased in LPS-treated rats (Fig. 5*C*,*D*). In adults that received saline or LPS on P14, and no subsequent interventions, we observed no significant differences in either the number of microglia or the percentage of activated microglia (both values of $t < 1$; $p > 0.05$) between treatments (Fig. 6*B*).

To examine baseline astrocyte numbers, we immunostained adult hippocampal sections from rats treated at P14 with either saline or LPS. We found no difference between saline and LPS-treated rats in the number of GFAP-positive cells in the DG and CA3 regions of the hippocampus (Fig. 6*C*). However, there were significantly ($t = 2.56$; $p < 0.05$) greater numbers of GFAP-positive cells counted in the CA1 region of the hippocampus of LPS-treated rats compared with controls (Fig. 6*C*,*D*). These cells appeared to be more hypertrophied with thickened processes in the LPS-treated group compared with controls.

Discussion

The most exciting finding of the present study is that a mild inflammatory response evoked by LPS during a critical period of development causes a long-lasting increase in hippocampal excitability *in vitro*, and enhanced seizure susceptibility to the convulsants LI-PILO, KA, and PTZ *in vivo*. The latter effect was observed over a range of mildly inflammatory doses of LPS and was only evident if administered during the second postnatal week (P7 and P14), and not before (P1) or after (P20) this time. Importantly, inactivation of the proinflammatory cytokine TNFα with an intracerebroventricular TNFα antibody blocked the long-term changes to seizure susceptibility induced by LPS, whereas intracerebroventricular administration of rrTNFα alone mimicked the effect of LPS on seizure susceptibility. These novel results indicate that a single transient inflammatory episode during development can modify the brain through a TNFα-dependant mechanism, making it more susceptible to generate seizures in adulthood.

There was some concern that the increased susceptibility to convulsant drugs might be attributable to changes in the access of these convulsants into the brain. For example, LPS can acutely alter blood–brain barrier permeability (Mayhan, 1998), and it is possible that such a change in the developing rat may be permanent. Other possibilities include LPS-induced alterations in hepatic enzymes (Agrawal and Shapiro, 2005) that result in reduced peripheral metabolism and therefore higher effective concentrations of the convulsants. There is also evidence that postnatal LPS increases hypothalamic–pituitary–adrenal axis and corticosterone reactivity (Ellis et al., 2005), which in turn may promote seizure activity (Roberts and Keith, 1995; Baram and Hatalski, 1998). However, the fact that the hippocampus of LPS-treated rats displayed increased excitability *in vitro* argues against these possibilities and suggests that enduring changes in the structural or functional properties of the brain may occur after selective postnatal treatment with LPS.

Possible mechanisms leading to increased neuronal excitability are so numerous that their elucidation is beyond the scope of this study. However, the fact that the fEPSP is increased makes the Schaffer collateral–CA1 pyramidal cell synapse one possible target. This is an AMPA-mediated potential that can be modified through alterations in presynaptic function, leading to increased transmitter release or alterations in the transduction mechanisms of postsynaptic receptors (Bredt and Nicoll, 2003). For example, hyperthermia-induced seizures in postnatal rats cause a decrease

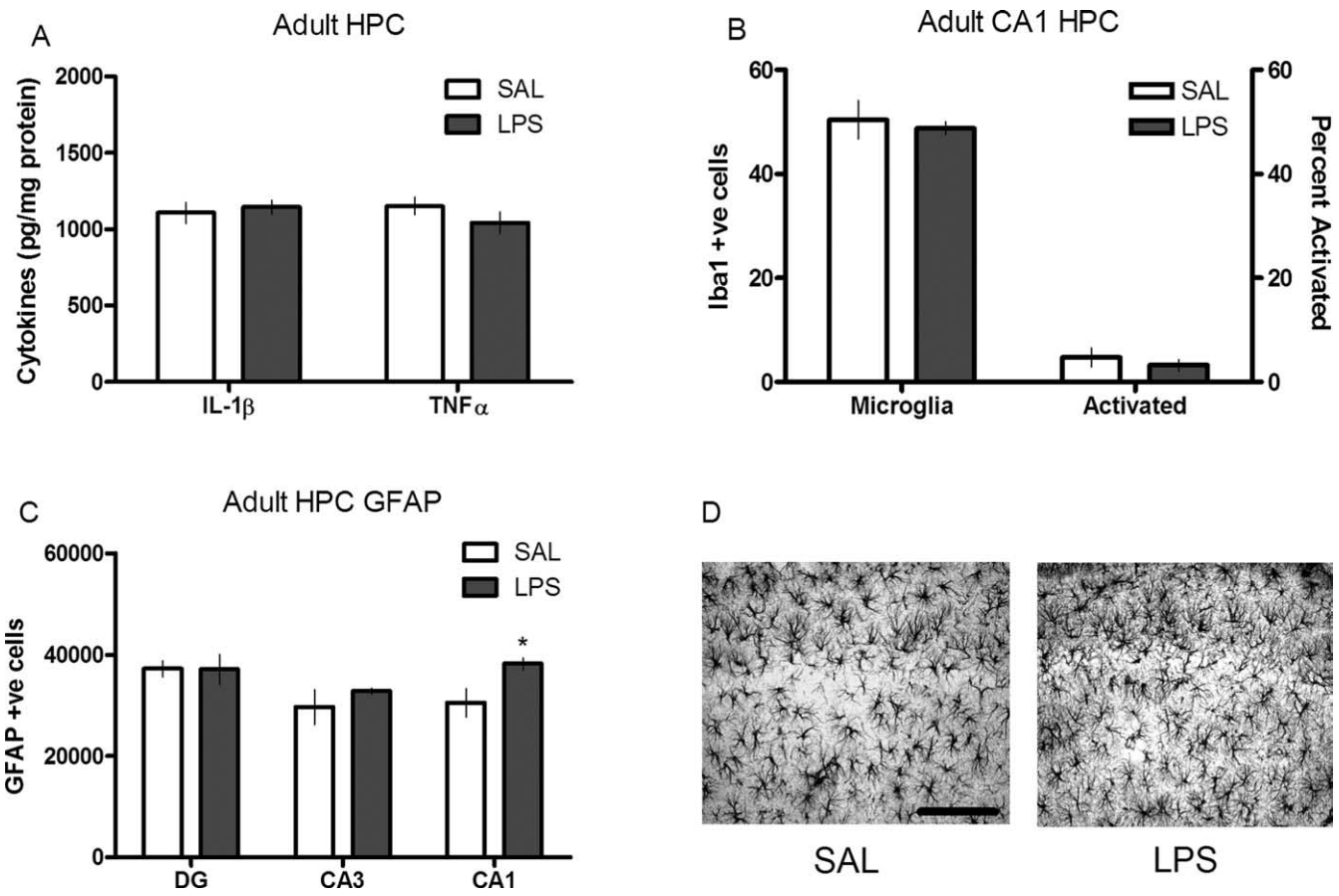

Figure 6. Adult cytokine (IL-1β and TNFα), microglia (Iba1), and GFAP immunohistochemistry data from rats treated with SAL or LPS (100 μg/kg) on P14 ($n = 4–6$/group). ***A***, IL-1β and TNFα concentrations in adult hippocampus (HPC) of rats treated at P14 with either SAL or LPS. Both cytokines are not significantly different between SAL- and LPS-treated groups. ***B***, Total number of microglia (Iba1-positive) cells (left axis) and the percentage activated microglia count (right axis) of adult rats treated at P14 with SAL or LPS. There is no difference in the number of Iba1-positive cells or percentage activated between LPS and control tissues. ***C***, Total number of GFAP-immunopositive cells within the DG, CA3, and CA1 regions of the HPC of adult animals treated at P14 with either SAL or LPS. There was a significant ($p < 0.05$) increase in the number of GFAP-positive cells in the CA1 HPC of rats treated at P14 with LPS compared with controls. ***D***, Photomicrographs of GFAP-positive cells in the CA1 HPC of adult SAL- and LPS-treated rats. Scale bar, 150 μm. An asterisk denotes LPS-treated groups that differ significantly ($p < 0.05$) from controls. Error bars indicate SEM.

in $GABA_B$-mediated inhibition at the Schaffer collateral synapse (Leung and Wu, 2006). Alternatively, changes in intrinsic membrane excitability, such as through modification of hyperpolarization activated current channels, could be equally altered as suggested by other postnatal interventions (Brewster et al., 2002).

We were surprised that our data resulting from intracerebroventricular injection of the IL-1ra did not reveal a role for IL-1β in the long-term effect of LPS on seizure thresholds. IL-1β is released in the hippocampus in a similar manner to TNFα after peripheral LPS treatment (Fig. 5*B*) and is known to have acute effects on neuronal excitability and excitotoxicity leading to seizure development (Heida et al., 2005; Vezzani and Baram, 2007). However, our data implicating TNFα in the programming response of LPS point to other potential causative mechanisms. TNFα can independently enhance excitatory synaptic strength by increasing membrane insertion of AMPA receptors in cultured hippocampal pyramidal neurons (Beattie et al., 2002; Stellwagen et al., 2005), and reduce the overall inhibitory currents mediated through $GABA_A$ receptors *in vitro* (Wang et al., 2000; Stellwagen et al., 2005). TNFα is significantly increased in the blood and brain (hippocampus) after a peripheral LPS injection at P14. Because our data with the TNFα antibody and recombinant agonist preparations imply that TNFα is necessary and sufficient to program the long-term changes in neuronal excitability, we suggest that TNFα-induced changes, such as those described above, may be permanent when established during a vulnerable developmental period.

We showed that the LPS-precipitated changes to adult seizure susceptibility are linked to a critical developmental period (P7 and P14). This time window is similar to the period we previously observed for other physiological changes caused by postnatal LPS (Spencer et al., 2006b). Interestingly, this is also the time when long-term potentiation-induced plasticity first becomes apparent (e.g., P7) and shows maximum responsivity (e.g., P15) (Harris and Teyler, 1984). Thus, we may suspect that some permanent change has taken place during this relatively sensitive period of development in which the effects of LPS (and TNFα) can modify synapses. Alternatively, TNFα is known to be essential in the normal development of the hippocampus through regulation of neurotrophic factors such as nerve growth factor and brain-derived neurotrophic factor (Golan et al., 2004). The possibility exists that "optimal" levels of TNFα may be perturbed after LPS-induced inflammation, even at potentially low doses (Fig. 2*A*). Future studies will be needed to address the potential mechanisms through which postnatal TNFα permanently alters neuronal function to enhance excitability and seizure propensity.

Although cytokines and microglia were not chronically perturbed in the LPS-treated rats, immunostaining for GFAP indi-

cated greater numbers of astrocytes within the CA1 hippocampus of rats given LPS. In other words, LPS administration at P14 resulted in a chronic, although mild form of astrogliosis, a feature commonly found in patients with mesial temporal lobe epilepsy (Eid et al., 2008). Other reports have also implicated increased astrocyte numbers and distributions to a heightened susceptibility to seizures in a number of seizure models (Somera-Molina et al., 2007; Oberheim et al., 2008). Like microglia, astrocytes can also synthesize and release proinflammatory cytokines (Dong and Benveniste, 2001; Kipp et al., 2008; Vezzani et al., 2008; Wetherington et al., 2008). It is possible that they may contribute to the reduced seizure thresholds and other pathological sequelae in the adult brain, but it remains to be determined how LPS (and possibly TNFα) might induce long-lasting changes in astrocytic number, morphology, or function.

Our results show that when postnatal LPS-treated rats were subjected to KA as adults, they displayed greater cytokine production (IL-1β in particular) and greater neuronal injury in the CA3 and CA1 hippocampus, regions that are typically affected by KA seizures (Ben Ari, 1985; Silveira et al., 2002). In keeping with the argument that cytokines (primarily IL-1β) are central to enhanced seizure susceptibility (Vezzani and Baram, 2007), other work has identified proinflammatory cytokines as mediators of CNS injury (Allan and Rothwell, 2001; Allan et al., 2005; Sherwin and Fern, 2005), possibly via facilitation of apoptotic processes in the brain (Thornton et al., 2006). Because our observed increases in IL-1β appear at 6 h after seizure onset, this could easily account for the greater neuronal injury (as inferred by Fluoro-Jade histochemistry) in the KA-treated rats seen the next day, because proinflammatory cytokines can exacerbate excitotoxic insults (Bernardino et al., 2008; Vezzani et al., 2008). In addition, as both our current data and previous experiments suggest (Bilbo et al., 2005), IL-1β can be specifically primed to generate a more robust response in adulthood by exposure to inflammation during development. Such priming may explain why we did not observe an abrupt increase in microglial activation, but recorded pronounced cytokine levels after KA seizures (Fig. 3*B*). One possibility is that the increased proliferation of hippocampal CA1 astrocytes induced by LPS [an effect potentially mediated by TNFα (Barna et al., 1990)] may be important in providing the IL-1β responsible in facilitating seizure onset and the magnitude of neuronal injury after seizure.

Clinically, CNS infections occur with some frequency in young children and may be associated with an increased risk for late unprovoked seizures (Rantakallio et al., 1986; Herman, 2002). In those patients surviving CNS infections, the risk for epilepsy was highest during the first 5 years after infection and remained elevated for the next 15 years (Annegers et al., 1988). Currently, there is limited clinical evidence to suggest that peripheral childhood infection contributes to the etiology of adult epilepsies. However, based on the novel data presented here, relatively mild early-life inflammation may be associated with permanent modifications in seizure susceptibility that persist well into adulthood. This may warrant additional patient history review to ascertain whether such a relationship exists. Our data raise the possibility that the known differences in adult seizure susceptibility to very similar insults may have their etiology in a common postnatal infection.

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