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Immunomodulatory Effect of Toll-Like Receptor-3 Ligand Poly I:C on Cortical Spreading Depression

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Abstract The release of inflammatory mediators following cortical spreading depression (CSD) is suggested to play a role in pathophysiology of CSD-related neurological disorders. Toll-like receptors (TLR) are master regulators of innate immune function and involved in the activation of inflammatory responses in the brain. TLR3 agonist poly I:C exerts anti-inflammatory effect and prevents cell injury in the brain. The aim of the present study was to examine the effect of systemic administration of poly I:C on the release of cytokines (TNF-α, IFN-γ, IL-4, TGF-β1, and GM-CSF) in the brain and spleen, splenic lymphocyte proliferation, expression of GAD65, $GABA_A\alpha$, $GABA_A\beta$ as well as Hsp70, and production of dark neurons after induction of repetitive CSD in juvenile rats. Poly I:C significantly attenuated CSD-induced production of TNF-α and IFN-γ in the brain as well as TNF-α and IL-4 in the spleen. Poly I:C did not affect enhancement of splenic lymphocyte proliferation after CSD. Administration of poly I:C increased expression of $GABA_A\alpha$, $GABA_A\beta$ as well as Hsp70 and decreased expression of GAD65 in the entorhinal cortex compared to CSD-treated tissues. In addition, poly I:C significantly prevented production of CSD-induced dark neurons. The data indicate neuroprotective and anti-inflammatory effects of TLR3 activation on CSD-induced neuroinflammation. Targeting TLR3 may provide a novel strategy for developing new treatments for CSD-related neurological disorders.

Keywords Spreading depolarization · Migraine · Stroke · Cell death · Immunotherapy · Neuroprotection

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Introduction

Cortical spreading depression (CSD) is a slowly propagated wave of profound neuronal and glial depolarization. CSD is characterized by a high amplitude negative DC shift associated with a transient reduction of bioelectrical activity, dramatic failure of brain ion homeostasis, efflux of excitatory amino acids from nerve cells, increased energy metabolism, and changes in cerebral blood flow [1, 2]. Occurrence of CSD is reported in different cerebrovascular diseases (such as intracranial hemorrhage, stroke, and subarachnoid hemorrhage) and migraine aura is the human equivalent of CSD. In addition, clinical and experimental studies indicated that CSD may have a role in epilepsy, spinal cord disorders, and transient global amnesia [3, 4].

A series of evidence suggests that the pain of migraine may be a form of sterile neurogenic inflammation [5]. An increase in calcitonin gene-related peptide in the jugular vein [6] and

vasogenic leakage next to small vessels within the brain during aura phase of migraine attacks [7, 8] as well as the role of blocking neurogenic inflammation in prevention of migraine attacks [9] indicate the potential involvement of neuroinflammation in the pathogenesis of migraine headache. The neuroinflammatory response in humans, which is characterized in part by activation of innate immune cells, has been implicated as a fundamental component for the progression of damage to the brain following ischemic brain attacks [10].

Propagation of CSD has been suggested to produce local neurogenic inflammation via the activation of macrophages and mast cells and subsequent release of different types of inflammatory mediators [11]. Repetitive CSD triggered a significant rise in different cytokines (after 6 hours to 3 days), mostly localize in activated microglial cells and in some instances the surrounding media; suggesting a crucial role of CSD in ischemia-mediated neuronal injury in the brain [12]. Changes of cytokines by CSD could contribute to the early exacerbating and later abating of neuronal injury during ischemia [13]. Increased cytokine production and microglial activation by CSD in association with release of other factors, such as brain-derived neurotrophic factor, may affect brain bioelectrical activity and play a role in the transformation of episodic to chronic migraine [12]. Modulation of cerebral inflammation by manipulation of cytokine production in the brain is suggested to improve outcome after an ischemic insult [14]. Cytokines may influence migraine pain by changing the sensitivity of intracranial meningeal nociceptors [11, 15].

The immunopotentiator polyinosinic:polycytidylic acid (poly I:C), a mimic of double-stranded viral RNA, is known to stimulate toll-like receptor 3 (TLR3) and leads to the activation of antigen presenting cells, such as B cells, dendritic cells, and macrophages [16, 17]. Poly I:C has been considered as an excellent adjuvant in different vaccine and immunotherapy models for improving humoral and cellular immunity [18–20]. TLR3 is constitutively expressed in astrocytes, oligodendrocytes, and neurons and can be upregulated upon inflammatory stimulation [21]. Activation of TLR3 plays a key role in the growth of axons and dendrites, dendrite branching, and proliferation of embryonic neural progenitor cells [21, 22]. In addition, TLR3-mediated signaling triggers production of several immunoregulatory mediators and factors that enhance neuronal survival, promote angiogenesis as well as remyelination, and prevent gliosis [21, 23, 24]. Poly I:C pretreatment exerts neuroprotective and anti-inflammatory effects in simulated cerebral ischemia models through reduction of pro-inflammatory cytokine production and enhancement of anti-inflammatory cytokine production from the ischemic astrocytes [25]. TLR-4 polymorphism has been reported as a genetic risk factor for migraine [26]. The aim of the present study was to investigate whether systematic administration of poly I:C may modulate CSD-induced inflammation and neuronal injury in juvenile rats.

Materials and Methods

Animals

Young adult male Wistar rats (25–35 days old; 60–90 g) were housed under constant temperature (20–22 °C) and a 12:12 h light–dark cycle and during this time food pellets and water were available ad libitum. All experiments were carried out according to the protocol approved by the Ethics Committee of Shefa Neuroscience Center, Tehran, Iran.

Surgical Procedure and Histological Assessment

Animals were anesthetized with intraperitoneal (i.p.) injection of chloral hydrate 3.5 % (dissolved in normal saline) and the head of each rat was placed in the stereotaxic frame (Stoelting Instruments, USA). The scalp was incised and retracted, a guide cannula was placed in the skull at the following coordinates: anterior–posterior, +1.8 mm anterior to the bregma; medial–lateral, −3.1 mm lateral to sagittal suture; and dorsal–ventral, 0.7 mm down from the skull surface [27]. A stylet was placed into guide cannula to maintain patency. Two silver electrodes were implanted over the somatosensory cortex and reference electrodes were placed on the nasal bulb (Fig. 1). Electrodes and cannula were fixed with dental acrylic cement. The scalp was sutured and animals were returned to their cages. Before the beginning of the experimental procedures, rats were kept for 1 week in well-ventilated boxes in an air-conditioned room to recover from surgery. Then animals were anesthetized with chloral hydrate and stylet was withdrawn from the guide cannula and a microneedle was inserted into the cannula. The injection microneedle was connected to a 10 μl Hamilton syringe by a polyethylene tube (Harvard Apparatus, USA).

KCl solution (3 mol/l) was injected in a total volume of 10 μl within 60 s (CSD group; $n=8$). The injection needle was retained in the guide cannula for an additional 60 s after injection to facilitate diffusion of the solution. In sham injected controls, 10 μl of Ringer solution was injected according to the same injection procedure (sham group; $n=8$). Four KCl or Ringer- injections were carried out (with an interval of 1 week). Anesthesia was continued for 60 min after each injection of KCl or Ringer solution. Recording of electrocorticographic (ECoG) signals was performed in anesthetized rat for 60 min after each injection.

Five weeks after the first application of KCl or Ringer, rats were decapitated and the brains were removed. Before decapitation, the animals were given a deep anesthesia with chloral hydrate 3.5 % and perfused transcardially with 200 ml of saline followed by 600 ml of 1 % paraformaldehyde (PFA) solution. The brains were extracted and kept in 1 % PFA for 10 days and were then processed for histological assessment. Coronal uniform random sections were cut through the

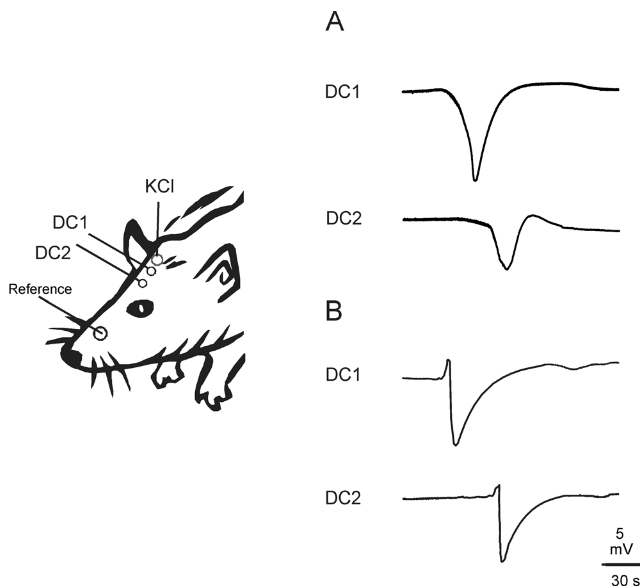

Fig. 1 Recordings of negative direct currents (DC) after injection of KCl (3 mol/l) in juvenile rat brain. A stainless steel, 23-gauge guide cannula and two silver recording electrodes (2–3 mm apart) were implanted above the somatosensory neocortex and fixed with dental acrylic cement. Cortical spreading depression (CSD) was recorded from the somatosensory cortex of juvenile rats without (*A*) and with (*B*) administration of poly I:C

ipsilateral site to the hemisphere in which KCl was injected. Ten pairs of successive sections were selected by random systemic sampling from each animal and stained by toluidine blue. Slides were studied under a light microscope (BX51, Olympus, Japan) linked to a digital camera. Digital photographs were taken using a ×100 oil immersion objective lens (Olympus, Japan). The magnification was calculated using an objective micrometer. For quantitative analysis of dark neurons, the physical dissector method was carried out [28, 29]. The first section of each pair was designated as the reference and the second one was used for comparison. On each pair of sections, at least ten microscopic fields were selected by uniform systematic random sampling in the entorhinal cortex (EC). Using unbiased frame and physical dissector counting rule, the counting of dark neurons in each field was carried out [28, 29].

Poly I:C Administration

Poly I:C was obtained from InvivoGen (San Diego, CA, USA). Poly I:C was stored at −20 °C until use. It was prepared for injection by resuspending in sterile saline, heating to 50 °C at a concentration of 2 mg/ml to ensure complete solubility and then allowing cooling naturally at room temperature. Six hours after the third and fourth KCl injections, rats were treated with poly I:C (PIC/CSD group; $n=8$; 0.3 mg/kg per i.p. injection) to examine systemic and brain inflammatory responses to its systemic administration.

Splenic Cytokine Assay

One week after the last KCl or Ringer injection, the spleens were removed and their splenocytes were isolated. The resultant cell suspension was washed by centrifuging at 600×*g* for 10 min at 4 °C and red blood cells lysed with red blood cell lysis buffer (0.16 M NH_4Cl, 10 mM $KHCO_3$, and 0.13 mM ethylenediaminetetraacetic acid; EDTA). Debris was removed from the suspension via a 70 μm nylon mesh filter and after washing, the cells were then suspended in RPMI 1,640 (containing 0.075 % sodium bicarbonate, 10 mM HEPES buffer, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 1.5 mM L-glutamine, and 2-mercaptoethanol) +10 % heat-inactivated FBS. Cell numbers were counted using trypan blue exclusion method and the percentage of cell viability was calculated. Cell suspensions were added in triplicate to flat-bottom 96-well plates at a volume of 200 μl/well, and plates were incubated at 37 °C and 5 % CO_2. To stimulate the cells, 1 μg/ml phytohemagglutinin (PHA; Sigma Aldrich, St. Louis, MO) was added. Supernatants were collected after 48 h in culture and stored frozen at −80 °C for cytokine analysis by ELISA. Tumor necrosis factor alpha (TNF-α; Santa Cruz, Germany), interferon gamma (IFN-γ), interleukin-4 (IL-4), transforming Growth Factor beta 1 (TGF-β1), and granulocyte-macrophage colony-stimulating factor (GM-CSF; Abcam, Cambridge, UK) were measured following the manufacturer's instructions. Cytokine concentrations were quantified by using standard curves generated using serial dilutions of recombinant proteins. The values were measured with a microplate reader at 490 nm.

CNS Cytokine Assay

One week after the last KCl or Ringer injection, rats were decapitated and the brains were removed. Before decapitation, the animals were given a deep anesthesia with chloral hydrate (350 mg/kg; Sigma Aldrich) and perfused transcardially with 200 ml of saline followed by 600 ml of 1 % PFA solution. Rat brain was homogenized in ice-cold buffer (8 ml of buffer per g of brain) consisting of 20 mM Tris HCl (pH 7.4), 5 mM EDTA, 5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, chymostatin (1 μg/ml), pepstatin (1 μg /ml), and trypsin inhibitor (1 μg/ml; all from Sigma) in phosphate-buffered saline solution (pH 7.2) containing 0.5 % Triton X-100. Brain homogenate was spun at 4 °C for 10 min at 1000×*g*. The levels of IFN-γ, IL-4, TGF-β1, GM-CSF (all from Abcam, Cambridge, UK) and TNF-α (Santa Cruz, Germany) were determined in brain supernatant using sandwich-base ELISA kit following the manufacturer's instruction. All tests were performed in triplicate for each rat.

Lymphocyte Proliferation Assay

One week after the fourth KCl or Ringer application, the splenocytes at a concentration of 2×10^5 cells/well (after red blood cells clearance) were cultured with supplemented RPMI-1,640 with 10 % FBS in 96-well flat-bottom culture plates (Nunc, Denmark) in the presence of T cell mitogen PHA (Sigma, USA) at a concentration of 1 μg/ml per well at 37 °C in 5 % CO_2. After 48 h of incubation, 10 μg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Invitrogen, USA) was added to each well and incubated for 4 h at 37 °C in 5 % CO_2. Following incubation, the supernatant from each well was aspirated carefully and formazan crystals were solubilized by adding 100 μl dimethyl sulfoxide into each well. The absorbance of each well was then determined at a wavelength of 540 nm, and the results expressed as a stimulation index (SI). All tests were performed in triplicate for each rat.

Immunohistochemistry

Animals were deeply anesthetized with 3.5 % chloral hydrate and perfused transcardially with 200 ml of saline followed by 200 ml of 1 % PFA solution. The brains were extracted and kept in 1 % PFA for at least 10 days at 4 °C and were then processed for immunohistochemical studies. The serial (8 μm) coronal sections were prepared. Three paraffin embedded sections were selected by random systematic sampling from each animal and rehydrated through a series of xylol and alcohol and washed with phosphate-buffered saline (PBS) three times and incubated in blockage solution (3 % H_2O_2/methanol for 5 min). Then slides were heated in sodium citrate buffer at 95 °C for 10 min. After cooling at room temperature for 20 min, slides were washed with PBS three times and incubated overnight at 4 °C with commercial rabbit polyclonal anti-rat antibody (Abcam, Cambridge, UK) against $GABA_A\alpha$, $GABA_A\beta$, and glutamic acid decarboxylase 65 (GAD65) overnight separately. Its concentration for HSP-70 was 1:300, $GABA_A\alpha$ was 1:100, for $GABA_A\beta$ was 1: 50, and for GAD65 was 1:200 in a solution containing 1–5 % NGS in 0.3 % Triton X-100 and 0.1 M PBS at pH of 7.4. The sections were then rinsed three times in PBS (10 min each) and incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, United Kingdom) diluted at 1:400 in PBS with 0.3 % Triton X-100 and 5 % NGS at 22 °C for 1 h. After several washings with PBS, the slides were incubated and developed in 3-3′-diaminobenzidine (DAB, Roche; 0.5 μl DAB and 1.5 μl peroxide buffer) for 5–10 min. Sections were counterstained with hematoxylin. Control for the specificity of immunostaining was performed by the omission of the primary antibody. Images for analysis were acquired with a digital camera attached to a microscope (BX51, Olympus, Japan).

Statistical Analysis

Data were expressed as mean±S.E.M. Statistical analysis was carried out by using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The criterion for statistical significance was $P<0.05$.

Results

Application of KCl into the brain of juvenile rats caused negative DC fluctuations, which were followed by positive waves. The mean number of amplitude and duration of the first neocortical CSD-like waves were 10.8±2.1 mV and 112±9 s and the velocity of propagation of these negative DC shifts were 3±0.2 mm/min (n=8; Fig. 1(A)). The amplitude and duration as well as the speed of propagation of CSD events that were repeatedly induced in the following 4 weeks of experiments were not significantly different from the characteristic features of the first CSD waves. However, the mean number of CSD induced by KCl injection was statistically increased during 4 weeks of experiments. The number of CSD recorded during 60 min after KCl injection was 1.4±0.2 in the first week, 1.8±0.3 in the second week, 2.1±0.2 in the third week, and 2.5±0.3 in the fourth week (P=0.036). Application of *i.p.* poly I:C did not affect the amplitude, duration, and repetition rate of CSD-like waves. The same pattern of negative DC deflections was observed in PIC/CSD rats compared to CSD group (n=8; Fig. 1(B)). KCl injection did not induce epileptiform burst discharges in any of tested animals.

CNS Cytokine Assay

We investigated whether poly I:C treatment could influence changes of cytokine patterns after induction of CSD in the brain. The cytokine production was evaluated in brain supernatant using sandwich-base ELISA assay. In the brain homogenates, no significant differences were observed in the level of IL-4 in CSD (n=6; 58±6.3) and PIC/CSD (n=6; 60±5.2) groups compared to sham rats (n=6; 52.4±9.9). However, induction of repetitive CSD significantly increased IFN-γ (188.8±18.3; $P\leq0.001$), TNF-α (211.7±40.6; $P\leq0.001$), TGF-β1 (154.1±22; $P\leq0.001$), and GM-CSF (67.6±19; $P\leq0.05$) production compared to sham group (IFN-γ, 86.5±18.5; TNF-α, 70.7±16; TGF-β1, 61.7±16.07; and GM-CSF, 48.2±6; Fig. 2a). Administration of poly I:C significantly reduced the levels of TNF-α (109±13.4; $P\leq0.001$) and IFN-γ (143±21.1; $P\leq0.05$) in the brain homogenates compared to CSD group (Fig. 2a).

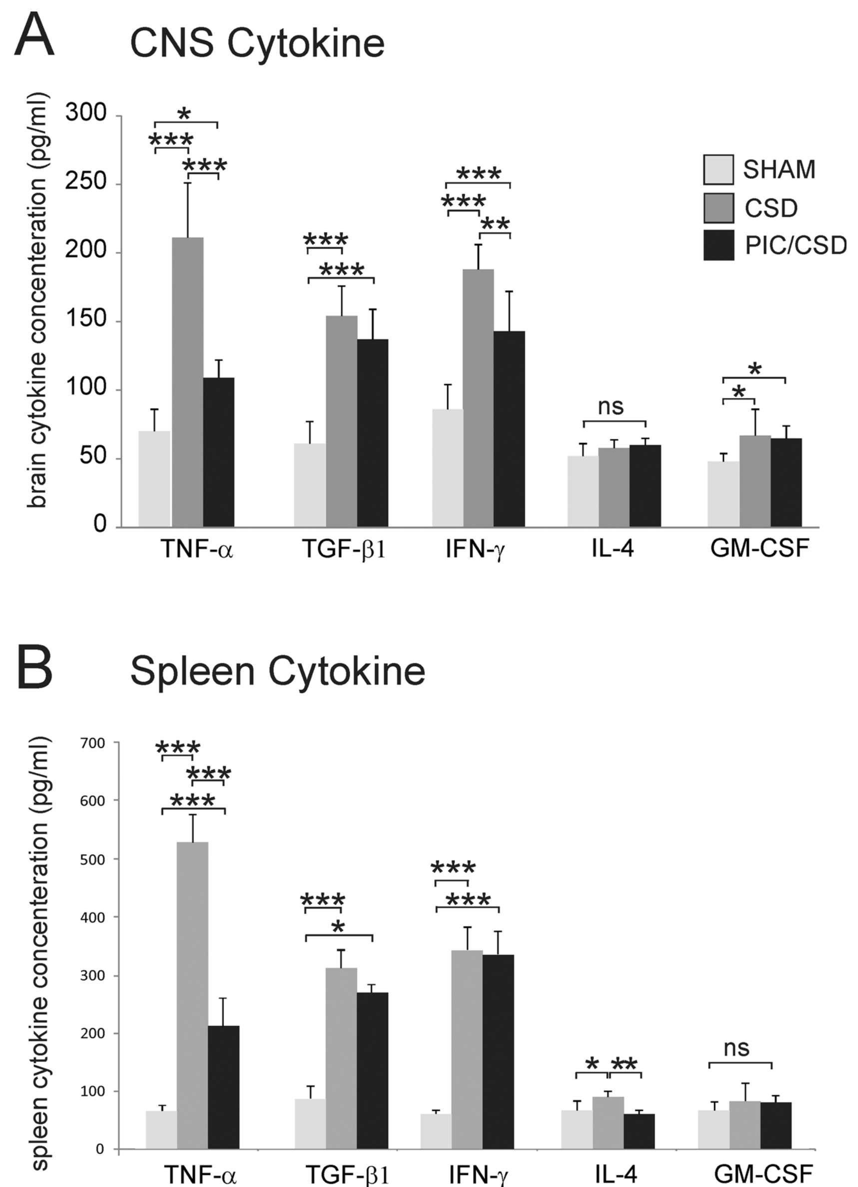

Fig. 2 Enzyme-linked immunosorbent assay (ELISA) of IFN-γ, IL-4, TNF-α, TGF-β1, and GM-CSF in the brain (**a**) and spleen (**b**) of sham animals, rats affected by repetitive cortical spreading depression (CSD) as well as rats affected by CSD and treated with poly I:C (PIC/CSD). CSD was induced by application of KCl on dura mater of juvenile rats and ECoG was recorded by implanted silver electrodes. **a** Induction of CSD significantly increased the production of IFN-γ, TNF-α, TGF-β1, and GM-CSF in the brain but did not affect IL-4. Administration of Poly I:C after CSD significantly reduced the production of IFN-γ and TNF-α compared to CSD group. **b** Induction of CSD significantly enhanced the production of IFN-γ, TNF-α, IL-4, and TGF-β1 in the spleen but did not affect GM-CSF. Administration of poly I:C after CSD significantly reduced the production of IL-4 and TNF-α compared to CSD group. Data are from four independent experiments (mean±S.E.M.). *, **, and ** indicate significant at $P \leq 0.05$, 0.01, and $P \leq 0.001$, respectively (one-way ANOVA test)

Spleen Cytokine Assay

Cellular immune responses were evaluated by cytokine secretion assay of the in vitro stimulated splenocytes in CSD, PIC/CSD and sham groups. IFN-γ secretion is a marker of Th1 type cellular immune responses, while IL-4 is recognized as Th2-biased response. In addition to these cytokines, we also evaluated pro-inflammatory (TNF-α and GM-CSF) and anti-inflammatory (TGF-β1) cytokine profiles. As shown in Fig. 2b, production of IFN-γ (338.2±39.7), TNF-α (638.6±43), and TGF-β1 (305.2±31.8) in splenocyte of CSD group (n=6) was significantly higher than the levels of IFN-γ (58.4±8.6), TNF-α (65.5±9.9), and TGF-β1 (83.6±22.4) in sham rats (n=6; $P<0.001$; Fig. 2b). Furthermore, production of splenic IL-4 (87.6±8.9) after CSD induction was significantly higher than IL-4 values in sham groups (65±21.5, $P \leq 0.05$; Fig. 2b). Administration of poly I:C (PIC/CSD group, n=6) in the third and fourth weeks of the experiments significantly reduced production of TNF-α (215±53, $P \leq 0.001$) and IL-4 (59±6.1, $P \leq 0.05$) compared to CSD group. PIC/CSD group also showed a significant increase of IFN-γ (331.1±40, $P<0.001$), TNF-α (215±53, $P \leq 0.001$), and TGF-β1 (264±13, $P<0.05$) compared to sham rats. There were no significant differences in splenic GM-CFS production among different groups (CSD, 81.5±29.3; PIC/CSD, 80±11; sham, 64.5±16.7; Fig. 2b).

Lymphocyte Proliferation Assay

To determine whether splenocyte proliferation response could be affected by CSD and systemic administration of poly I:C, lymphocytes from CSD and PIC/CSD rats were evaluated and

compared with sham-treated animals. As shown in Fig. 3, induction of CSD significantly increased lymphocyte proliferation compared to sham animals ($P \leq 0.001$). However, administration of poly I:C did not change lymphocyte stimulation index compared to CSD group ($n=6$ for each group; Fig. 3).

Immunohistochemical Distribution of Hsp70, GAD65, and $GABA_A$

Hsp70 plays a crucial role in protecting neurons from cellular stresses [30]. Our results showed a significant higher expression of Hsp70 immunoreactivity in rats treated with poly I:C compared to CSD and sham groups ($P \leq 0.001$; Fig. 4). The mean number of the reacted cells per square millimeter of Hsp70 in EC was 0.008±0.002 in CSD, 0.012±0.002 in sham, and 0.025±0.002 in PIC/CSD rats. There was no significant difference in expression of Hsp70 between CSD and sham groups ($n=6$ for each group; Fig. 4a, b).

It is suggested that the production of some cytokines, such as TNF-α, during CSD may affect neuronal inhibitory tone in the brain [12]. GAD, an enzyme that catalyzes the decarboxylation of glutamate to GABA, exists in two isoforms encoded by two different isoforms; GAD67 and GAD65. In CSD animals, GAD65 displayed a significant higher immunoreactivity in EC compared to sham rats ($P \leq 0.001$). Administration of poly I:C markedly decreased the expression of the mean number of GAD65 reacted neurons compared to CSD group ($P \leq 0.05$; Fig. 4a, b). To clarify the changes in $GABA_A$ receptors after CSD with and without treatment with poly I:C, we analyzed the distribution of $GABA_A\alpha$ and $GABA_A\beta$ in EC. The expression of both $GABA_A\alpha$ and $GABA_A\beta$ did not change after induction of CSD compared to sham group.

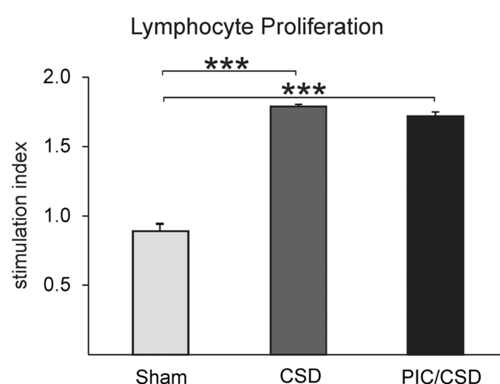

Fig. 3 Lymphocyte proliferation levels in the spleen of sham animals, rats affected by repetitive cortical spreading depression (CSD) as well as rats affected by CSD and treated with poly I:C (CSD/PIC). One week after final CSD induction, spleens of individual rats were removed and lymphocyte proliferation was evaluated. Induction of CSD significantly increased the stimulation index compared to sham-treated rats. Administration of poly I:C after induction of CSD did not change this index. Data are from four independent experiments (mean±S.E.M.). *** indicates significant at $P \leq 0.001$ (one-way ANOVA test)

However, treatment with poly I:C significantly enhanced the expression of $GABA_A\alpha$ and $GABA_A\beta$ in EC compared to CSD and sham animals ($n=6$ for each group; $P \leq 0.001$; Fig. 4a, b).

Number of Dark Neurons

Dark neurons were identified by neuronal shrinkage, nuclear pyknosis, and surrounding spongiosis [28, 29]. The mean number of dark neurons in EC was significantly increased after induction of CSD compared to sham rats ($P \leq 0.001$). Treatment with poly I:C significantly decreased the number of dark cells compared to CSD group ($P \leq 0.001$). The mean number of dark neurons in PIC/CSD rats, however, was higher than in sham group ($n=8$ for each group; $P \leq 0.05$; Fig. 5a, b).

Discussion

The present study indicates the TLR3-mediated modulatory effects on CSD-induced production of pro- and anti-inflammatory cytokines in the brain and spleen as well as the expression of Hsp70, GAD, $GABA_A\alpha$, and $GABA_A\beta$ in EC of juvenile rats. In addition, our data demonstrate the protective role of TLR3 activation on neuronal injury induced by repetitive CSD in EC. Our previous studies demonstrated that changes of synaptic plasticity of EC is important for CSD penetration to deeper brain structures and therefore EC may be a target of therapy to reduce the neurological deficits occurred in CSD-related disorders [31, 32].

In agreement with our results, it has been shown that CSD alters expression of different inflammatory pathways in the brain. CSD induced persistent upregulation of TNF-α and IL-1 mRNA whereas IL-2, IL-10, IL-12, and C4 complement protein mRNA were downregulated in rat brain after CSD [33, 34]. The upregulation of IL-1α, -1β, -2, -4, -6, and -10 as well as IFN-γ and TNF-α at the protein level following spreading depression in hippocampal organotypic cultures has been reported [13]. CSD causes activation of caspase-1 which in turn initiates inflammation by releasing high-mobility group box 1 (HMGB1) and IL-1β from neurons and nuclear factor κB (NF-κB) from astrocytes. HMGB1 behaves like a cytokine and promotes inflammation. Activation of NF-κB after CSD can also provide the sustained inflammatory mediator release to the subarachnoid space [35]. Increases in extracellular K^+ concentrations during CSD augments lipopolysaccharide-elicited neurotoxicity in neuroglia mixed cultures via enhanced production of inflammatory factors, such as TNF-α [36]. Free arachidonic acid also significantly increased during initiation and propagation of CSD [37].

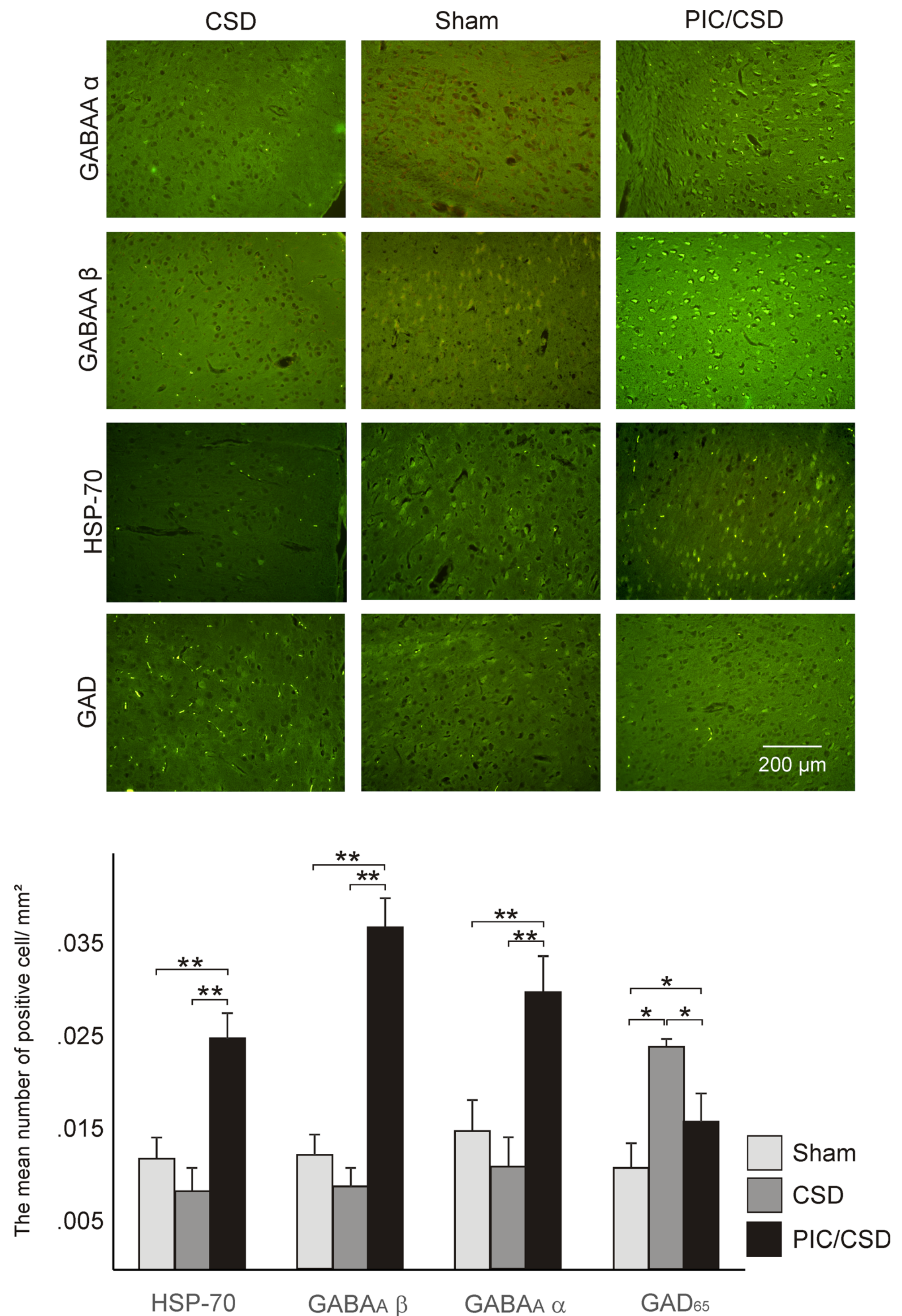

Fig. 4 Immunohistochemistry assay of Hsp70, GAD, $GABA_A\alpha$, and $GABA_A\beta$ in the entorhinal cortex of sham animals, rats affected by repetitive cortical spreading depression (CSD) as well as rats affected by CSD and treated with poly I:C (CSD/PIC). **a** Photomicrographs of immunohistochemistry of Hsp70, GAD, $GABA_A\alpha$, and $GABA_A\beta$ expression in the entorhinal cortex in sham, CSD, and CSD/PIC rats. **b** The *bar graph* shows the quantitative results (mean± SEM) of Hsp70, GAD65, $GABA_A\alpha$, and $GABA_A\beta$ expression. Data are expressed as number of Hsp70, GAD, $GABA_A\alpha$ and $GABA_A\beta$ expression per square millimeter. Compared with sham animals, there was a significant increase in Hsp70, $GABA_A\alpha$ and $GABA_A\beta$ expression in the entorhinal cortex of CSD/PIC rats. The expression of GAD65, however, was significantly higher compared to sham rats. Administration of poly I:C significantly reduced the expression of GAD65 compared to CSD group. Values represent mean±SEM. * and ** indicate significant at $P \leq 0.05$ and $P \leq 0.001$, respectively (one-way ANOVA test)

Our study for the first time revealed that, in addition to the brain, CSD influences the systemic immune system. CSD enhanced production of IFN-γ, TNF-α, TGF-β1, and IL-4 in the spleen and increased proliferation response of splenic lymphocytes. To date, there has been little interest in exploring the possibility of systemic immune responses to CSD. There are, however, several studies demonstrating occurrence of systemic immune responses in CSD-related neurological disorders, i.e., stroke and migraine [38]. Alterations in function of polymorphonuclear cells, monocytes, and natural killer cells (phagocytosis/chemotaxis) were reported in migrainures [39]. Lymphocytes of patients with migraine exhibited increased dopamine D_5-receptor expression [40], decreased β-adrenergic receptor sensitivity [41], and decreased β-

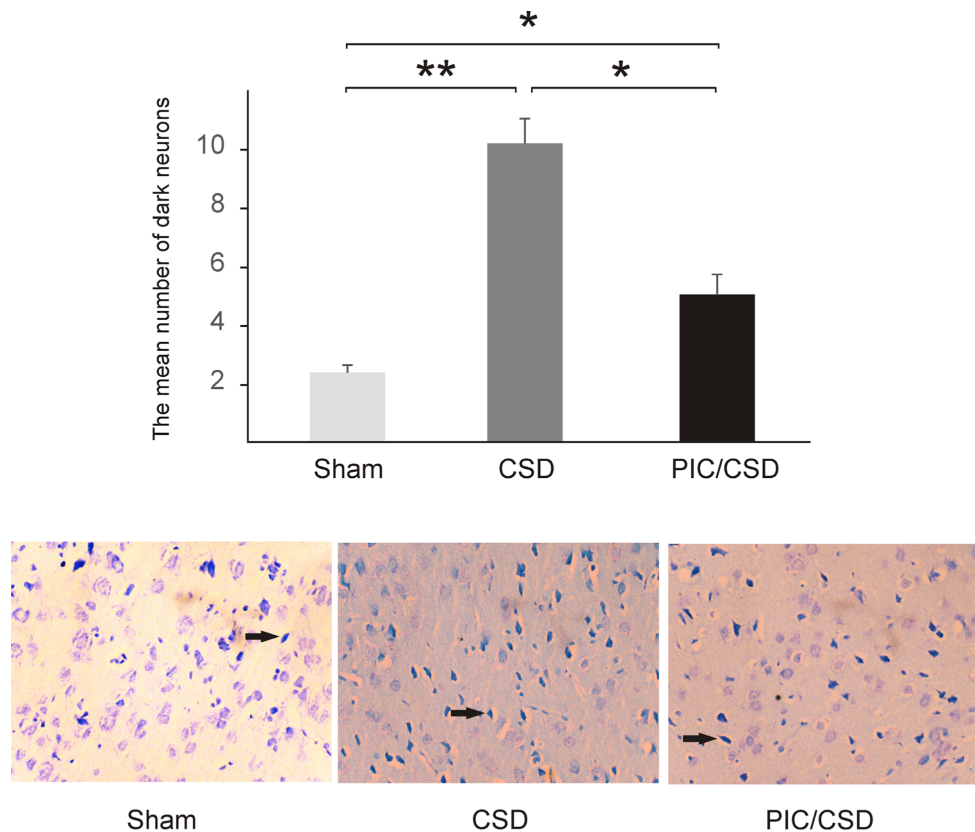

Fig. 5 The effects of repetitive cortical spreading depression (CSD) with and without administration of poly I:C (CSD/PIC) on the mean number of dark neurons in the entorhinal cortex in juvenile rats after 4 weeks of repetitive CSD induction. CSD was induced by application of KCl on dura mater and ECoG was recorded by implanted silver electrodes. **a** Light-microscopic appearance (×40 magnification) of transverse sections of the rat brain in CSD, CSD/PIC, and sham groups stained with toluidine blue, revealing an increase of dark neurons in the entorhinal cortex after CSD induction and decrease after administration of poly I:C. **b** The mean number of dark neurons in the entorhinal cortex of sham, CSD and CSD/PIC rats. Number of dark neurons was compared between experimental groups and analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. Values represent mean±SEM. * and ** indicate significant at $P \leq 0.05$ and $P \leq 0.001$, respectively

endorphin levels [42]. Migraine patients showed higher serum levels of TNF-α and IL-1β interictally [43]. A decrease in plasma IL-4 and IL-6 levels and an increase in plasma GM-CSF and IFN-γ values has been reported after a challenge test in patients with migraine [44]. Lymphocytes from stroke survivors showed more activity against antigens (such as myelin basic protein) compared to the lymphocytes from patients with multiple sclerosis [45]. Myelin-reactive T cells were found in higher numbers among patients with cerebrovascular disease [46]. Further studies are needed to elucidate whether CSD play a role on systemic immune responses of these neurological disorders.

The up- or downregulation of the systemic immune response has the ability to modulate brain dysfunctions [47]. Modulation of microglia/macrophages and lymphocytes infiltration in the brain has been suggested to play a role in treatment of neurological disorders (such as multiple sclerosis, Alzheimer's disease, and amyotrophic lateral sclerosis) by preventing neuronal damage, improving repair, and eliminating toxic proteins [48–50]. Systemic administration of poly I:C, in our study, suppressed production of TNF-α and IFN-γ in the brain as well as splenic TNF-α and IL-4 after marked enhancement of these cytokines by CSD. Neuronal activities trigger low-level pro-inflammatory signaling involving TNF-α, resulting in an enhanced resilience of the brain to withstand injury [12]. The activation of TNF receptor two on inhibitory interneurons may reduce normal brain activity in neurological diseases that are associated with blocking effect on CSD [51]. Conversely, high-level TNF-α possesses the brain's intrinsic capacity to enhance neuronal injury and death [12, 52, 53]. An increased IFN-γ from microglia after induction of CSD has been suggested as the first signal heralding a cytokine-cascade-based modulation of brain ischemic injury [13]. IL-4 plays a major role on activation and proliferation of B-cell, modulation of macrophage functions, and differentiation of T cell [54]. Anti-inflammatory effects of IL-4 on activated microglia have been noted, including inhibition of the expression of TNF-α and IFN-γ [55, 56].

Previous studies have shown that a limited number of CSD failed to influence the expression of Hsp72 [57–59].

Multifocal regions of increased Hsp70 expression were observed in both ipsilateral and contralateral hemispheres after induction of CSD by cerebral embolic events [60]; failed to prove whether increased expression of Hsp70 was due to CSD or ischemic insults. Indeed, preconditioning with brief ischemia can lower the threshold signal for the induction of heat shock proteins [61]. Our results showed a significant reduction of Hsp70 after 4 weeks of repetitive CSD. Expression of this neuroprotective protein, however, significantly increased after TLR3 activation by administration of poly I:C. Due to Hsp70 chaperone role and/or to its ability to protect brain against neurotoxic factors, inflammation, and apoptosis, it has been suggested that Hsp70 may play a neuroprotective role in some neurological disorders, such as multiple sclerosis, epilepsy, ischemic brain injury, etc.[30]. It has been shown that TNF-α is involved in modulating the failure of Hsp70-positive astrocytes to provide functional support to neuritic outgrowth in neuron-astrocyte cultures [62]. Our previous studies demonstrated occurrence of neuronal injury after repetitive CSD in juvenile rats [28, 29]. The present data indicate that administration of poly I:C significantly decreased the mean number of dark neurons; an index of neuronal injury [63]. This may be due to decreases in production of TNF-α [64] and IFN-γ [65] and/or enhancement of Hsp70 expression [66] after application of poly I:C.

Partial $GABA_A$-mediated inhibition plays a role in maintaining the late excitatory period following the depression phase of CSD [67, 68] and contributes to CSD-linked epileptiform activity [4, 69]. It has been shown that the expression of GAD65 was increased in response to an increased neuronal activity induced by partial inhibition of $GABA_A$ receptors [70] or neuronal injury induced by pilocarpine [71]. In addition to increases of GAD65, our data revealed a significant enhancement of $GABA_A\alpha$ and $GABA_A\beta$ expression in EC after administration of poly I:C. Administration of TNF-α produced a rapid and persistent decrease of inhibitory synaptic strength and a downregulation of $GABA_A$ receptors [72]. IFN-γ stimulation of human macrophage lineage cells increased gamma aminobutyric acid transporter-2 expression and reduced extracellular GABA levels [73]; conversely, activation of GABA receptors inhibited IFN-γ production [74]. Triggering of CSD requires activation of NMDA receptors [3] and the brain releases glutamate during CSD propagation [75]. Neuronal injury induced by exposure to NMDA, AMPA, or kainate was attenuated by activation of $GABA_A$ receptors in mouse cortical cell cultures [76]. Administration of poly I:C enhanced the expression of $GABA_A\alpha$ and $GABA_A\beta$ and reduced the expression of GAD. It has been shown that release of both $GABA_A\alpha$ and $GABA_A\beta$ downregulates GAD activity in neuronal tissues [77], possibly via influence of GABA on the translation of GAD mRNA [78].

Activation of different TLR subtypes can initiate either immunostimulatory or immunomodulatory responses in the brain [79]. Although TLR stimulation increases the production of the inflammatory molecules, such as TNF-α, and exacerbates tissue damage, activation of these receptors prior to neuronal injury protects the brain from damage [79]. Activation of TLR generates tumor-specific immune responses to brain tumors, and modulation of these receptors suppresses the damaging inflammatory responses to brain ischemia and enhances the endogenous protection of neuronal injury [80, 81]. Modulation of TLR9 significantly reduced ischemic damage in a nonhuman primate model of stroke [82] and TLR2 [83] as well as TLR4 [84] plays a crucial role in cerebral ischemic/reperfusion injury in different animal models and that their activation leads to exacerbation of infarct volume and mortality. Activation of TLR3, in our study, reduced the mean number of CSD-induced dark neurons. Our previous studies indicate that repetitive CSD in juvenile rats causes neuronal damage and death in both cortical and subcortical structures of the brain [28, 29]. The neuroprotective effects of TLR3 by application of poly I:C has been shown in brain in vivo and in vitro ischemia models. Reduction of pro-inflammatory cytokine production and enhancement of anti-inflammatory cytokine production from the injured astrocytes have been suggested as the underlying neuroprotective mechanism of poly I:C in brain ischemia [25, 81, 85]. Poly I:C improved neuronal survival in organotypic human brain slice cultures [24] and reduced oxygen-glucose deprivation-mediated cell death in mouse mixed cortical cultures [79]. It is suggested that poly I:C decreases in cerebral ischemia/reperfusion injury via TLR3 by preventing the interaction of apoptosis antigen 1 and its associated protein with death domain and reduction of microglial cell caspase-3 and -8 activities [86]. Activation of TLR3 increases synaptic transmission in the spinal cord and plays a critical role in regulating sensory neuronal excitability as well as central sensitization [87].

Conclusion

In addition to the previous data indicating cytokine signaling after CSD in the brain, our results demonstrate that CSD modulates the adaptive immunity and systemic inflammatory response. Furthermore, we have identified TLR3 as a critical signaling molecule that can modulate CSD-induced neuroinflammation and neuronal injury. Targeting TLR3 may provide a novel strategy for developing new treatments for CSD-related neurological disorders.

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