Recent studies indicate that Toll-like receptors (TLRs), originally identified as infectious agent receptors, also mediate sterile inflammatory responses during tissue damage. In this study, we investigated the role of TLR2 in excitotoxic hippocampal cell death using TLR2 knockout (KO) mice. TLR2 expression was up-regulated in microglia in the ipsilateral hippocampus of kainic acid (KA)-injected mice. KA-mediated hippocampal cell death was significantly reduced in TLR2 KO mice compared with wild-type (WT) mice. Similarly, KA-induced glial activation and proinflammatory gene expression in the hippocampus were compromised in TLR2 KO mice. In addition, neurons in organotypic hippocampal slice cultures (OHSCs) from TLR2 KO mouse brains were less susceptible to KA excitotoxicity than WT OHSCs. This protection is partly attributed to decreased expression of proinflammatory genes, such as TNF-α and IL-1β in TLR2 KO mice OHSCs. These data demonstrate conclusively that TLR2 signaling in microglia contributes to KA-mediated innate immune responses and hippocampal excitotoxicity.

**Toll-like receptors (TLRs)** are a group of transmembrane proteins that play a central role in innate immune responses. To date, more than 10 different TLR members have been identified that each recognizes a specific set of pathogen-associated molecular patterns (PAMPs) expressed by microorganisms (1, 2). Interestingly, emerging data indicate that TLRs function as receptors not only for PAMPs, but also for endogenous molecules released from damaged tissue or cells. For example, TLR2 and -4 recognize various endogenous molecules including heat shock proteins, hyaluronan, and high mobility group box-1 (HMGB-1) (3–6). In addition, TLR3 binds miRNA released from necrotic cells (7). It is possible that TLR recognition of these endogenous molecules is involved in the inflammatory response during “sterile” tissue damage.

In the central nervous system (CNS), TLRs including TLR2, -3, and -4 are expressed in microglia and astrocytes, suggesting a role as innate immune cells in the CNS (8). Based on their function as receptors for “danger signals” (9), TLR expression in glial cells is implicated in various “sterile” neurological disorders including mouse cerebral ischemia/reperfusion injury (10, 11), spinal cord injury (12), and axonal transection (13). We have also reported that TLR2 plays a critical role in nerve injury-induced spinal cord glial activation and subsequent pain hypersensitivity (14), and traumatic brain injury (15). In mouse epileptic seizure model, TLR2 transcripts are up-regulated in hippocampal microglia/macrophages upon pilocarpine injection (16) implicating TLR2 in hippocampal excitotoxicity, although this possibility has not yet been explored.

Excitotoxicity is an underlying mechanism of various neurological disorders including traumatic brain injury and stroke, and is also implicated in chronic neurodegenerative diseases such as amyotrophic lateral sclerosis and epileptic seizure (17). NMDA and AMPA/kainate glutamate receptor overstimulation is known to be responsible for excitotoxic neuronal cell death. Moreover, administration of kainic acid (KA) produces characteristic patterns of epileptic seizure be-

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Behavior in mice (18, 19), and triggers acute and delayed neuronal death in the hippocampal CA1 and CA3 regions (20, 21). Studies suggest that microglial activation in the hippocampi and the subsequent production of neurotoxic mediators such as reactive oxygen species and iNOS are responsible for KA-induced delayed cell death (20, 22, 23); however, it is not known how hippocampal microglia are activated during excitotoxic brain damage. Previous reports of TLR2 functioning as a receptor for dying neurons (14) suggest that TLR2 mediates excitotoxin-induced microglial activation. In this study, we used TLR2 KO mice to test this hypothesis and demonstrated that microglial TLR2 expression contributes to KA-induced glial cell activation and hippocampal neuron excitotoxicity.

EXPERIMENTAL PROCEDURES

Animals—TLR2 KO mice (24) on a C57BL/6 background and WT (C57BL/6) mice purchased from Koatech (Pyeongtaek, Korea) were housed at 23 ± 2 °C with a 12 h light-dark cycle and fed food and water ad libitum. All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University.

Stereotaxic Injection and Tissue Processing—For intracerebroventricular (i.c.v.) KA injection, WT and TLR2 KO mice (8–12 week-old male, 22–25 g) were anesthetized by sodium pentobarbital (30 mg/kg, body weight, intraperitoneal), and placed on a stereotaxic apparatus (myNeuroLab, St. Louis, MO). Animals were injected with PBS or KA (0.2 μg in 4.0 μl of PBS) at the speed of 0.5 μl/min into the right ventricle (stereotaxic coordinates in mm with reference to bregma: AP, −2.0; ML, −2.9; DV, −3.8) using a 26-gauge needle. After 5 min, the needle was removed in three intermediate steps for 3 min to minimize backflow. The incision was cleaned with saline and sutured, and animals were kept on a warm pad until recovery. Animals deeply anesthetized with sodium pentobarbital were perfused intracardially with saline followed by 4% PFA in 0.1M phosphate buffer (PB) at the indicated time after surgery. The brains were removed, post-fixed in the same fixative at 4 °C, rinsed twice with PBS, and placed in 10, 20, and 30% sucrose in PBS, serially, for 48 h at 4 °C. The brains were then frozen quickly and cut into serial coronal sections (30-μm thickness) on a cryostat (CM3050S, Leica, Germany). Sections were collected as fresh-floating sections in PBS and stored at −20 °C until use for histochemical studies.

Cell Cultures and RT-PCR—Primary hippocampal neurons and cortical neurons were prepared from E18 mouse embryos as described previously (25). Primary glial cultures were prepared and maintained according to previously established procedures (26). Total RNA was isolated with TRIzol reagents (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA synthesis and RT-PCR were performed as described previously (27). The amplified DNA products were separated by electrophoresis on a 1.5% agarose gel. The sequences of the primers used are as follows: TLR2 forward 5’-GAC TCA CAG CAG TGA AA-3’; TLR2 reverse 5’-AGA GAA GTC AGC CCA GCA AA-3’; GAPDH forward: 5’-CAG CCT GTT GCT GTA GCC GTA T-3’; GAPDH reverse: 5’-AGG TCA TCC CAG AGC TGA ACG-3’.

Stereological Cell Counts—Total number of neurons in the CA1 and CA3 subregions of hippocampal formation was obtained using the optical fractionator as described previously (28) with some modifications. This sampling technique is not affected by tissue volume changes and does not require reference volume determinations (29). Every eighth section (8–9 sections) was selected from the sections (50 μm-thick, coronal plane) of entire hippocampus (AP, −0.94 to −3.80 mm from bregma) in a systematic-random manner, stained with cresyl violet, and used for counting. Sampling was done using the Computer-Assisted Stereological Toolbox system, version 2.1.4 (Olympus Denmark A/S, Ballerup, Denmark), using an Olympus BX51 microscope, a motorized microscope stage (Prior Scientific, Rockland, MA) run by an IBM-compatible computer, and a microcator (Heidenhain ND 281B) connected to the stage and feeding the computer with the distance information in the z axis. According to the mouse brain atlas (30), the CA1 and CA3 subregions were delineated at a 1.25× objective and generated counting areas of 150 × 150 μm. A counting frame (1755 μm²) was placed randomly on the first counting area and systemically moved through all counting areas until the entire delineated area was sampled. Actual counting was performed using a 100× oil objective. Guard volumes (2 μm from the top and the bottom of the section) were excluded from both surfaces to avoid the problem of lost caps, and only the profiles that came into focus within the counting volume (with a depth of 26 μm) were counted. The estimate of the total number of neurons in the CA1 and CA3 of hippocampus of ipsilateral hemisphere was calculated according to the optical fractionator formula (29).

Immunofluorescence Staining—Immunostaining was carried out according to previously established procedures (26). Briefly, sections were incubated in blocking solution (5% normal donkey serum, 2% BSA, and 0.1% Triton X-100) for 1 h at room temperature. The sections were then incubated overnight at 4 °C with the following antibodies: mouse anti-NeuN (1:2000; Millipore, Billerica, MA), rabbit anti-IBA-1 (1:2,000; Wako, Japan), rat anti-CD11b (1:50; Serotec Inc., Oxford, UK), mouse anti-GFAP (1:500; DAKO, Denmark), mouse anti-iNOS (1:200; BD bioscienes, NJ), and rabbit anti-HO-1 antibodies (1:1000; Stressgen, BC, Canada). The sections were incubated for 1 h at room temperature with Cy3- and/or FITC-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA), and then mounted on gelatin-coated slides and coverslipped with VectaShield medium (Vector Labs, Burlingame, CA). Images were captured using confocal laser scanning microscopy (LSM5 PASCAL; Carl Zeiss, Germany).

Hippocampal Slice Preparation and Long-term Potentiation Induction—At 3 days after KA or PBS injection, hippocampal slices were prepared from WT and TLR2 KO mice (8–12-week-old male, 22–25 g). After decapitation, brains were removed rapidly and placed in cold oxygenated (95% O₂ and 5% CO₂) low-Ca²⁺/high-Mg²⁺ dissection buffer composed of 5 mM KCl, 1.23 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM dextrose, and 212.7 mM sucrose. Slices were cut at a thickness of 200 μm and transferred to a slicing chamber containing the dissection buffer with 2 mM Ca²⁺. Briefly, sections were incubated in blocking solution (5% normal donkey serum, 2% BSA, and 0.1% Triton X-100) for 1 h at room temperature. The sections were then incubated overnight at 4 °C with the following antibodies: mouse anti-NeuN (1:2000; Millipore, Billerica, MA), rabbit anti-IBA-1 (1:2,000; Wako, Japan), rat anti-CD11b (1:50; Serotec Inc., Oxford, UK), mouse anti-GFAP (1:500; Millipore), rabbit anti-GFAP (1:10,000; DAKO, Denmark), mouse anti-iNOS (1:200; BD biosciences, NJ), and rabbit anti-HO-1 antibodies (1:1000; Stressgen, BC, Canada). The sections were incubated for 1 h at room temperature with Cy3- and/or FITC-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA), and then mounted on gelatin-coated slides and coverslipped with VectaShield medium (Vector Labs, Burlingame, CA). Images were captured using confocal laser scanning microscopy (LSM5 PASCAL; Carl Zeiss, Germany).
400 μm and transferred to a holding chamber in an incubator containing oxygenated (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF) composed of 124 mM NaCl, 5 mM KCl, 1.23 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgSO₄, 26 mM NaHCO₃, and 10 mM dextrose at 28–30 °C for more than 1 h before recording. After recovery, slices were transferred to a recording chamber where they were perfused continuously with oxygenated ACSF (26–28 °C) at a flow rate of 2 ml/min. Field excitatory postsynaptic potential (fEPSP) in CA1 region evoked by stimulation of CA3 region with bipolar electrode were recorded with ACSF-filled microelectrodes (1 to 3 MΩ). Responses were quantified as the slope of field potentials. Baseline responses were collected at 0.07 Hz with a stimulation intensity that yielded a 40% maximal response. Long-term potentiation (LTP) was induced by theta burst stimulation (TBS), which consisted of four trains containing ten bursts (each with four pulses at 100 Hz) of stimuli delivered every 200 ms. Average responses (mean ± S.E.) are expressed as percent of baseline response.

**Quantitative Analysis of Immunofluorescence**—To measure Iba-1- and GFAP-immunoreactive area, images (460 μm x 460 μm; 3 sections per animal, 6–7 animals per group) were acquired from the hippocampal CA1 and CA3 subfields with confocal laser scanning microscopy. Iba-1- and GFAP-immunofluorescent areas were measured using LSM5 PASCAL software (Carl Zeiss) and expressed as the area (μm²/10,000 μm²).

**Flow Cytometry**—For flow cytometry analysis of microglia and macrophages, the ipsilateral hippocampi of WT and TLR2 KO mice were carefully dissected and dissociated as previously described (31). Briefly, a single-cell suspension was prepared and fixed with 2% paraformaldehyde. Cells were then washed with 2% fetal bovine serum (FBS) in PBS, incubated with CD16/32 antibody (2.4G2, BD Bioscience) for 10 min to block the Fc receptor and washed twice with 2% FBS in PBS. Macrophages and microglia can be identified based on their relative CD45 expression levels (32). Accordingly, cells were incubated with PE-conjugated CD45 (30-F11, BD Bioscience) and FITC-conjugated CD11b (M1/70, BD Bioscience) antibody for 30 min at 4 °C. The cells were then washed twice with 2% FBS in PBS and used for flow cytometry. Data were collected on a FACSCalibur flow cytometer (BD biosciences) and analyzed using CellQuest Pro software (BD Biosciences).

**Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Analysis**—WT mice were i.c.v. injected with either PBS or KA. After 3 days, brains were removed and cryosections were incubated with blocking solution (3% H₂O₂ in methanol) for 10 min and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The sections were then incubated with a TUNEL reaction mixture for 1 h at 37 °C and followed by converter-POD (peroxidase-conjugated anti-fluorescein antibody) for 30 min. After washing in PBS, sections were incubated in DAB substrate (0.5 mg/ml 3,3′-diaminobenzidine and 0.003% H₂O₂ in 50 mM Tris, pH 7.4). Thereafter, sections were mounted on gelatin-coated slides and examined under a bright-field microscope (Carl Zeiss).

**Organotypic Hippocampal Slice Cultures**—Organotypic hippocampal slice cultures (OHSs) were prepared and maintained as described previously (26). After 14 days in culture, slices were transferred to dishes containing fresh culture media supplemented with 50 μM KA following the previously reported protocol (33). After a 3-h incubation in KA-containing media, slices were either immediately fixed in 4% PFA (Re 0 h), or recovered in fresh culture media for 24 h (Re 24 h) and then fixed. One hour prior to fixation, propidium iodide (PI; 0.2 μg/ml, Sigma) was added to the culture medium. Neuronal degeneration was quantified by the uptake of PI into the damaged cells (34). Fluorescent images were taken immediately before KA treatment and at 0 and 24 h after media change using fluorescence microscopy (Carl Zeiss Axiovert200). Separately, cultures were incubated at 4 °C for 24 h in the presence of PI to determine the fluorescence level of 100% cell death. Images were stored in a computer and the regions of interest (ROI) in the CA1 and CA3 pyramidal cell layers were analyzed. A numerical value for PI fluorescence intensity was calculated and analyzed using the Image J program (NIH). PI intensity, indicating cell death, is expressed as a percentage of the final fluorescence of 100% cell death (F₀). Cell death (%) = (F₀ - Fₙ)/(F₀ - Fₐ) × 100, where F₀ is the PI fluorescence of hippocampal slices measured at 0 and 24 h after the change of medium, and Fₐ is the background fluorescence prior to KA treatment. To evaluate the effects of cytokines on KA-induced neuronal cell death, cultures were co-treated with KA and TNF-α/IL-1β or anti-TNF-α/IL-1β antibodies (R&D Systems, Minneapolis, MN) for 3 h, and then, incubated in recovery medium for 24 h with or without cytokines or blocking antibodies.

**Real-time RT-PCR**—Real-time RT-PCR was performed using SYBR Green PCR Master Mix as previously described (26). Reactions were performed in duplicate in a total volume of 10 μl containing 10 pm primer, 4 μl cDNA, and 5 μl SYBR Green PCR Master Mix. The mRNA levels of each target gene were normalized to that of GAPDH mRNA. Fold-induction was calculated using the 2⁻ΔΔC_t method as previously described (35). All real-time RT-PCR experiments were performed at least three times, and presented as mean ± S.E. unless otherwise noted. The following sequences of primers were used for real-time RT-PCR. TLR2 forward: 5′-CCT AGA AGT GGA GGA CAT GTC GGT CA-3′; TLR2 reverse: 5′-GAA GAA AAC GGA ATT CTC TTT TTC ACG A-3′; TNF-α forward: 5′-AGC AAA CCA CCA AGT GGA GGA-3′; TNF-α reverse: 5′-GCT GGC ACC ACT AGT TGG TTG T-3′; IL-1β forward: 5′-TTG TGG CTG TGG AGA AGC TGT-3′; IL-1β reverse: 5′-AAC GTC ACA CAC CAG CAG GGT-3′; IL-6 forward: 5′-TCC ATC CAG TGT CCT TCT GGG-3′; IL-6 reverse: 5′-CCA CGA TTT CCC AGA GAA CAT G-3′; iNOS forward: 5′-GCC AAA CCC AAG GTC TAC GTT-3′; iNOS reverse: 5′-TGG CTC ACG TTG AGC TTG GT-3′; HO-1 forward: 5′-TCA CAG ATG GCG TCA CCT CGT-3′; HO-1 reverse: 5′-TGT TGC CAA CAG GAA GCT GAT-3′; CD11b forward: 5′-TAA TGA CTC TGC GTT...
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TLR2 Expression Is Up-regulated in Activated Microglia of KA-injected Mouse Brain—To test the involvement of TLR2 in KA-induced hippocampal excitotoxicity, we first measured TLR2 expression in the ipsilateral hippocampus of KA-treated mice. At 6 h after i.c.v. KA injection, the level of TLR2 mRNA in hippocampal tissue had increased 32-fold (Fig. 1A). TLR2 protein expression was confirmed by immunohistochemistry, with a strong TLR2-immunoreactive (IR) signal evident in the hippocampal area at 1 day after KA administration (Fig. 1E). Immunostaining with cell type-specific markers indicated that most TLR2-IR cells were Iba-1-IR microglia (Fig. 1, E–G). TLR2 expression was rarely detected and the Iba-1 expression was significantly lower in PBS-treated hippocampi (Fig. 1, B–D). Co-staining of TLR2 and astrocyte-specific GFAP or neuron-specific NeuN was not detected (data not shown). These data indicate that KA stimulation up-regulates TLR2 expression in microglia, but not in astrocytes or neurons. Recent studies showed that TLR2 is not only expressed in microglia but also on cortical neurons in mouse brains (36). To test whether TLR2 is expressed on hippocampal neurons at a very low level that is not detected by immunostaining in vivo, we examined TLR2 mRNA expression in primary cultured hippocampal neurons by RT-PCR. Although TLR2 transcripts were detected in cortical neurons and glial cells of WT mice, they were not detected in primary hippocampal neurons of WT mice (Fig. 1H), or in glial cells of TLR2 KO mice (Fig. 1H). We also failed to detect TLR2 expression in KA-stimulated hippocampal neurons (data not shown). These data suggest that TLR2 is expressed in microglia in the hippocampus and that this expression is up-regulated by KA stimulation.

KA-induced Hippocampal Neuronal Cell Death Is Reduced in TLR2 KO Mice—To investigate the role of TLR2 in hippocampal excitotoxicity, we counted live hippocampal neurons after KA administration using unbiased stereological methods. Three days after KA stimulation, the number of live neurons in the CA1 and CA3 areas of WT hippocampi (supplemental Fig.S1A–D) was reduced to 69 and 54%, respectively, compared with the number in the same areas of PBS-injected mice. However, in TLR2 KO mice, the number of live neurons in the CA1 and CA3 areas of KA-injected WT mice was only 12 and 20% lower than that in PBS-injected mice (Fig. 2G). We performed TUNEL staining to characterize the nature of neuronal death. TUNEL+ cells were detected in the ipsilateral CA1 and CA3 areas of the KA-injected WT mice (Fig. 2H). These TUNEL+ signals colocalized with cresyl violet-stained condensed hippocampal neurons (Fig. 2, K and L). TUNEL+ cells were also found in the TLR2 KO hippocampi (supplemental Fig. S1F), but the number of TUNEL+ cells was ~30% lower than that of WT hippocampi (supplemental Fig. S1E), which is in line with the cresyl violet staining data.

KA-induced hippocampal cell death usually results in impaired hippocampal synaptic function (37), which is experimentally assessed by measuring long-term potentiation.
After a typical LTP-inducing protocol, the field excitatory postsynaptic potential (fEPSP) in the Schaffer collateral pathway (CA3 to CA1) of WT hippocampi increased by 37% above the baseline (Fig. 2, M and N). This increase was attenuated by KA in WT hippocampi, showing impairment of hippocampal synaptic function, whereas KA stimulation did not inhibit LTP induction in TLR2 KO hippocampi (Fig. 2, M and N). The baseline fEPSP in WT and TLR2 KO hippocampi were comparable (1.10 ± 0.07 mV in WT slices versus 1.03 ± 0.08 mV in TLR2 KO slices) (Fig. 2O). This shows that the KA-induced change in hippocampal synaptic function is abrogated in the TLR2 KO mice. Taken together, these data indicate that TLR2 expression contributes to KA-induced neuronal degeneration and is required for the subsequent degeneration.

**FIGURE 2. KA-induced hippocampal neuronal cell death is decreased in TLR2 KO mice.** A–G, WT (A, D, E) and TLR2 KO (C, F) mice were i.c.v. injected with either PBS (A, D) or KA (B, C, E, F). Cryosections (50 μm thick) were stained with cresyl violet. Scale bars: 100 μm. Total numbers of neurons in the CA1 and CA3 subfields of the ipsilateral hippocampus were estimated using optical fractionator method (G). Data are presented as mean ± S.E. (ANOVA test with a Fisher’s post hoc test; ***, p < 0.001; **, p < 0.01; *, p < 0.05 versus PBS- or KA-injected mice). H–L, sections from KA-treated WT mice were stained with TUNEL (brown) and cresyl violet. Arrowheads, cresyl violet-positive live cells; arrows, cresyl violet/TUNEL-double positive dead cells. Scale bars: 500 μm (H), 20 μm (I–L). M–O, hippocampal slices were prepared from WT and TLR2 KO mice at 3 days after KA (KA-WT and KA-TLR2 KO) or PBS injection (PBS-WT and PBS-TLR2 KO), and then LTP was monitored in Schaffer collateral pathway with theta burst stimulation (TBS). M, time courses of field potential mean slopes. An overlay of representative field potential traces taken at baseline (red) and during the last few minutes of recording (blue) is shown for each group. Scale bars: 0.5 mV, 10 ms. The number of mice and slices used for each condition is denoted in parentheses. N, averaged fEPSP slopes of each group are calculated as percent of baseline at the last 10 min of recording. Data are expressed as mean ± S.E. (Student’s t test; *, p < 0.05; **, p < 0.01, versus KA-WT mice; ns, not significant). O, baseline responses were collected at 0.07 Hz with a stimulation intensity that yielded a 40% maximal response. Data are expressed as mean ± S.E. Student’s t test; ns, not significant.
impairment of synaptic function. We also tested KA-induced seizure activity by measuring hippocampal electroencephalography (EEG), but did not observe any discernible differences between WT and TLR2 KO mice (supplemental Fig. S2).

The lack of TLR2 expression in hippocampal neurons suggests that TLR2 expressed on other cell types affects KA excitotoxicity. However, we cannot exclude the possibility that transient TLR2 expression on neuronal stem cells during development (38) makes differentiated hippocampal neurons more susceptible to KA-induced cell death. To test this possibility, we cultured primary hippocampal neurons from WT and TLR2 KO mice and compared KA-induced cell death rates. Incubating hippocampal neurons in 50 μM KA for 24 h resulted in cell death in both WT and TLR2 KO mice (Fig. 2, A–H). When we counted the surviving neurons, there was no difference in the death rate between WT and TLR2 KO neurons (supplemental Fig. S3A). Similarly, there was no significant difference in cell death rate between WT and TLR2 KO neurons treated with other KA concentrations (10 and 100 μM) (data not shown). These data suggest that the reduced KA excitotoxicity observed in TLR2 KO mice is not likely due to an intrinsic difference between WT and TLR2 KO hippocampal neurons, but involves TLR2 expression in other cell types.

**KA-induced Microglia Activation Is Reduced in TLR2 KO Mice**—To address the effects of TLR2 deficiency in other cell types, we first analyzed KA-induced microglia and astrocyte activation in hippocampus sections using anti-Iba-1 and anti-GFAP antibodies, respectively (Fig. 3). In KA-injected WT mice, the number of Iba-1-IR cells increased remarkably in both CA1 and CA3 regions of the ipsilateral hippocampus (Fig. 3, C and G). These Iba-1-IR cells showed typical morphology of activated microglia, with an enlarged cell body and short thick processes (22). In contrast, in PBS-treated mice, Iba-1-IR microglia showed a resting morphology, with small ramified processes (Fig. 3, B and F). Upon quantification, the Iba-1 fluorescence intensities in the CA1 and CA3 subfields of the ipsilateral hippocampus of WT mice were 47 and 57% that of WT, respectively (Fig. 3, J). We tested to determine whether the increase in Iba-1 expression is due to microglia proliferation by BrdU-incorporation assay. Three days after KA stimulation, BrdU-incorporated Iba-1+ cells were detected in the hippocampi of WT mice (supplemental Fig. S4, A, C, and E). The number of BrdU+/Iba-1+ cells in TLR2 KO mice was much lower (supplemental Fig. S4, B, D, and E). These data indicate that TLR2 is involved in microglia proliferation after KA injection, which may account for the reduced number of Iba-1+ cells in TLR2 KO mice. Astrocytes were also activated in the ipsilateral hippocampus of KA-administered TLR2 KO and
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WT mice (Fig. 3, K–R). Although astrocyte activation was slightly attenuated in CA1 of TLR2 KO mice compared with WT mice, it was not statistically significant (Fig. 3S). These results show that TLR2 deletion suppresses in vivo KA-induced activation of microglia but minimally affect astrocyte activation, in the hippocampus.

It is likely that both activation of resident hippocampal microglia and infiltration of blood-derived macrophages account for the increased Iba-1 immunoreactivity. We differentiated these two cell populations by flow cytometry. Upon KA activation, the number of CD11b+/CD45low cells representing the microglia population (31) in WT mice increased to 25%, whereas it increased only to 17% in TLR2 KO mice (Fig. 4, A–E). The CD11b+/CD45high population representing macrophages was also slightly increased indicating that macrophages had also infiltrated the KA-injected hippocampi (0.03% to 1.4%). However, the increase in macrophage population was not attenuated in TLR2 KO mice (Fig. 4, B, D, and E). Taken together, these data show that the reduction in Iba-1-IR observed in TLR2 KO mice compared with WT mice is mainly due to differences in microglia proliferation and activation, but not differences in macrophage infiltration.

Hippocampal Neurons in Organotypic Slice Culture from TLR2 KO Mice Are Less Susceptible to KA Excitotoxicity—Because TLR2 is expressed in other immune cells, it is still possible that the reduced excitotoxicity is due to TLR2 ablation in other immune cells, rather than microglia. To exclude this possibility and define the microglia-specific role of TLR2 in hippocampal excitotoxicity, we used an organotypic hippocampal slice culture (OHSC) system. Hippocampal slices from WT and TLR2 KO mice were maintained in culture medium for 2 weeks before KA stimulation, so that the effects of blood-derived immune cells are excluded (Fig. 5). Excitotoxic KA profiles were evaluated using PI uptake as a measure of neuronal death in OHSCs, in which 100% uptake represents 100% cell death. After a 3-h KA treatment, PI uptake in the CA1 and CA3 areas in OHSCs from WT mice increased to 16 and 13%, respectively (Fig. 5A, D, E, H, and I). After additional 24 h in fresh recovery media, PI uptake further increased to 30 and 23%, respectively (Fig. 5A, F, G, H, and I). In OHSCs from TLR2 KO mice, PI uptake was significantly attenuated compared with WT slices; PI uptake in the CA1 and CA3 areas increased to 8 and 5% after a 3-h KA treatment, and 18 and 10% after 24-h recovery, respectively. Similar to KA administration in vivo, KA stimulation in OHSCs induced microglial activation in WT slices, which was attenuated in TLR2 KO slices (Fig. 5, compare L and M, and N). Taken together, these data indicate that TLR2 expression in CNS-resident microglia contributes to KA-induced microglial activation and hippocampal neuronal cell death ex vivo.

KA-induced Proinflammatory Gene Expression in Microglia Is Reduced in TLR2 KO Mice—To elucidate the mechanisms underlying the differences in excitotoxic susceptibility in WT and TLR2 KO mice, we measured mRNA expression of proinflammatory genes TNF-α, HO-1, IL-1β, IL-6, and iNOS in the ipsilateral hippocampus of KA-injected mice. These proinflammatory genes are implicated in excitotoxicity (23, 39, 40). Thirty-six hours after injection with KA, expression of TNF-α, HO-1, IL-1β, IL-6, and iNOS mRNA in WT hippocampi increased 69-, 59-, 8-, 6-, and 7-fold, respectively (Fig. 6A). In TLR2 KO mice, KA-induced expression of these proinflammatory genes was attenuated by 43–64%, depending on the target gene (Fig. 6A). To identify the cells expressing these genes, the expression of iNOS and HO-1, cytoplasmic proteins among above genes, in the KA-injected ipsilateral hippocampus was assayed by immunohistochemistry. iNOS-IR cells were rarely detected in PBS-injected control mice (data not shown), but were identified adjacent to the demised pyramidal cell layer after KA stimulation (Fig. 6B). Double immunostaining with cell type-specific markers showed that most of the cells showing iNOS-IR were Iba-1-IR microglia. Likewise, HO-1 expression was detected in Iba-1-IR microglia adjacent to the pyramidal cell layer in KA-injected WT mice (Fig. 6C). In TLR2 KO mice, the number of HO-1-IR cells was significantly reduced compared with WT mice (Fig. 6, compare C and D). These data demonstrate that TLR2 is required for proinflammatory gene expression in microglia upon KA stimulation.
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**FIGURE 5. Neurons in OHSCs from TLR2 KO mice are less susceptible to KA-induced excitotoxicity.** A, schematic representation of the protocols used to study KA excitotoxicity in OHSCs. OHSCs were maintained in slice culture medium for 2 weeks, then exposed to KA (50 μM) for 3 h. After medium change, OHSCs were fixed in paraformaldehyde (D, E) or allowed to recover in fresh medium for 24 h (F, G) before fixation. One hour prior to fixation, PI (0.2 μg/ml) was added to the medium. Neuronal cell death in OHSCs was determined by PI uptake in the CA1 and CA3 pyramidal cell layers. The extent of neuronal degeneration was quantified by PI fluorescence intensity (H, h) and representative images are shown (B–G). Scale bars: 200 μm. Data are presented as mean ± S.E. (ANOVA test with a Fisher’s post hoc test, **, p < 0.01 versus control WT OHSCs; +, p < 0.05, 2+, p < 0.01, versus control TLR2 KO OHSCs; #, p < 0.05; ##, p < 0.01, versus KA-treated WT OHSCs). J–N, OHSCs of WT (J, L) and TLR2 KO (K, M) mice before (control) and 24 h after 3-h KA treatment (Re 24 h) were immunostained with anti-Iba-1 antibody. Scale bars: 50 μm. The Iba-1-IR fluorescence intensities were quantified and presented in a graph (N). (Student’s t test, **, p < 0.01, versus KA-treated WT OHSCs.)

**DISCUSSION**

This study provides the first evidence that TLR2 contributes to KA-induced hippocampal cell death. It has been reported that TLR2 is constitutively expressed in microglia (41, 42); however, we rarely detected TLR2 expression in unstimulated brain tissue by immunohistochemistry, probably due to the detection limits of this method. We did, however, observe TLR2 expression in microglia following treatment with KA. TLR2 was recently shown to be constitutively expressed in cortical neurons and to be required for oxygen/glucose deprivation-induced neuronal death (36). This report suggested that TLR2 expression on hippocampal neurons is directly involved in KA-induced neuronal death; however, in our study we failed to detect TLR2 expression in hippocampal neurons. Furthermore, we did not observe any difference in KA excitotoxicity between WT hippocampal neurons and TLR2-deficient neurons in vitro. Although these in vitro data do not completely negate the possibility of neuronal TLR2 effects in vivo, these data strongly suggest that TLR2 expression in other cell types may be involved in the KA excitotoxicity. In-
deed, we observed a significant decrease in KA-induced hippocampal microglia activation in TLR2 KO mice compared with WT mice.

Microglial activation during excitotoxic neuronal death is a well-known phenomenon (43). In the model for KA-induced hippocampal neuronal death, activation of microglia was proposed to promote delayed neuronal cell death in the hippocampus (43, 44). However, it is not completely understood how microglial cells are activated during KA-induced hippocampal neuronal death. In this regard, it should be noted that necrotic neurons activate microglia through TLR2 activation (14). Based on this, one can conjecture that endogenous TLR2 agonists are released from excitotoxic hippocampal neurons and activate nearby microglia via TLR2. Thus far, several endogenous molecules have been proposed to activate TLR2; for example, heat shock proteins (4, 5) and HMGB-1 (6) have been shown to bind to TLR2. Notably, HMGB-1 has recently been shown to play a critical role in microglia activation and subsequent delayed cortical neuronal death in a mouse stroke model, and the authors suggested that HMGB-1 released from damaged neurons activates microglia (45). Based on previous reports, it is tempting to speculate that following KA treatment, damaged hippocampal neurons release HMGB-1, which activates nearby microglia via TLR2 and thereby further promotes delayed neuronal cell death. This possibility can be addressed in future studies using HMGB-1 KO mice.

In addition to microglia activation, we observed macrophage infiltration in KA-injected hippocampi. Because TLR2 is expressed on blood-borne macrophages or other immune cells, the effects of TLR2 deletion in these cells cannot be completely ruled out. To differentiate between the role of blood-borne immune cells and brain-resident glial cells, we adopted an OHSC system in which hippocampal slices were cultured ex vivo for 2 weeks before KA stimulation to ensure that no blood-derived cells were present in the slices during the process.
KA stimulation. Data from this ex vivo system demonstrate that TLR2 potentiates KA-mediated excitotoxicity in the absence of blood-derived immune cells, and strongly suggest that TLR2 in microglia plays the major role in KA excitotoxicity. Still, these data do not completely rule out the putative contribution of blood-derived macrophages in vivo. A future study testing KA-excitotoxicity in TLR2 KO bone marrow-reconstituted WT mice or in microglia-specific TLR2 conditional KO mice would better define the relative contributions of TLR2 in glia and blood-borne immune cells.

In this study, we also assessed the functional damage to the hippocampus after KA stimulation by measuring LTP. In accordance with a previous report (37), KA administration inhibited LTP induction in hippocampus of the WT slices. The difference between LTP in the KA-treated WT hippocampus compared with that of LTP in the PBS-treated WT hippocampus is in line with the decreased cell rate in the CA3 area after KA administration in WT mice. Interestingly, although KA injection reduced the neuronal number in CA3 of TLR2 KO mice by 20%, LTP induced in those slices was not affected at all by KA administration. Although there is no clear explanation for this discrepancy, it can be speculated that a certain level is required for hippocampal cell death to be detected in LTP assessment. In our study, KA-induced seizure activity measured by EEG was not significantly different between WT and TLR2 KO mice. Thus it seems that hippocampal excitotoxicity due to i.c.v. KA injection is not directly related to seizure activity.

In an attempt to elucidate the mechanism underlying the TLR2 effects, we found that KA-induced expression of proinflammatory genes such as TNF-α and IL-1β is reduced in TLR2 KO mouse hippocampi. We tested the role of these proinflammatory cytokines in KA excitotoxicity using the OHSC system. Blocking cytokine effects in WT slices using neutralizing antibodies demonstrated that the cytokines potentiate KA-mediated hippocampal neuronal cell death ex vivo. These data are in line with previous reports showing the neurotoxic effects of these cytokines in vitro (46, 47). Of interest, neither TNF-α nor IL-1β alone induced neurotoxicity in the TLR2 KO slice at the concentrations used, but enhanced KA-induced cell death rate. This suggests that these cytokines make neurons more susceptible to KA excitotoxicity, although the mechanisms have not been elucidated in this study. In our work, we used an ex vivo slice culture system because in vivo experiments requiring repeated i.c.v. administration of neutralizing antibody together with KA injection were not technically feasible. Nevertheless, our ex vivo data strongly suggest that attenuation of these cytokines in TLR2 KO mice may be partly responsible for suppression of KA-induced hippocampal cell death in vivo. Besides proinflammatory cytokines, we observed that KA-induced iNOS and HO-1 expression is attenuated in TLR2 KO mice. The neurotoxic effect of NO in excitotoxicity is well-known (39). Also, recent studies indicate that overexpression of HO-1, an antioxidant enzyme in the brain, may have neurotoxic effects (48, 49). In this regard, it is conceivable that reduced NO production and HO-1 expression in microglia may also contribute to the attenuation of KA neurotoxicity in TLR2 KO mice.

Emerging evidence from several studies implies that innate immune receptors, such as TLR, play a pivotal role in the development of various neurodegenerative diseases. As previously described, TLR2 is required for cortical neuronal death in a mouse cerebral ischemia model (36) and is involved in neuronal cell death in a mouse Alzheimer disease model and in spinal cord injury (50, 51). In this study, we demonstrated that TLR2 contributes to KA-induced hippocampal neuronal cell death and, furthermore, that TLR2-mediated microglia activation and subsequent proinflammatory cytokine expression potentiates KA-induced hippocampal cell death. These data suggest that TLR2 might be a novel therapeutic target for treatment of delayed neuronal cell death caused by excitotoxic stimuli.

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