Toll-like receptor 3 deficiency decreases epileptogenesis in a pilocarpine model of SE-induced epilepsy in mice

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SUMMARY

Objective: Epilepsy affects 60 million people worldwide. Despite the development of antiepileptic drugs, up to 35% of patients are drug refractory with uncontrollable seizures. Toll-like receptors (TLRs) are central components of the nonspecific innate inflammatory response. Because TLR3 was recently implicated in neuronal plasticity, we hypothesized that it may contribute to the development of epilepsy after status epilepticus (SE).

Methods: To test the involvement of TLR3 in epileptogenesis, we used the pilocarpine model for SE in TLR3-deficient mice and their respective wild-type controls. In this model, a single SE event leads to spontaneous recurrent seizures (SRS). Two weeks after SE, mice were implanted with wireless electroencephalography (EEG) transmitters for up to 1 month. The impact of TLR3 deficiency on SE was assessed using separate cohorts of mice regarding EEG activity, seizure progression, hippocampal microglial distribution, and expression of the proinflammatory cytokines tumor necrosis factor (TNF)α and interferon (IFN)β.

Results: Our data indicate that TLR3 deficiency reduced SRS, microglial activation, and the levels of the proinflammatory cytokines TNFα and IFNβ, and increased survival following SE.

Significance: This study reveals novel insights into the pathophysiology of epilepsy and the contribution of TLR3 to disease progression. Our results identify the TLR3 pathway as a potential future therapeutic target in SE.

KEY WORDS: TLR3, Neuroinflammation, Microglia, SE, Epileptogenesis, Toll-like receptors.

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Key Points

- TLR3 deficiency reduces acute mortality resulting from pilocarpine-induced SE
- TLR3 deficiency reduces the development of chronic SRS following SE and attenuates the expression of proinflammatory cytokines in the hippocampus
- TLR3 deficiency reduces the activation of hippocampal microglial cells

Epilepsy is the third most common chronic brain disorder, affecting approximately 60 million people worldwide.[1] Despite the development of antiepileptic drugs, up to 35% of patients are drug refractory with uncontrollable seizures.[2] In some cases, surgical intervention may be beneficial; however, it involves the risk of perisurgical morbidity and possible cognitive and neurologic deficits.[3] Temporal lobe epilepsy (TLE), the most prevalent partial epilepsy, is unresponsive to pharmacologic treatment in a significant number of patients.[4,5] TLE is associated with structural and cellular changes in hippocampal and parahippocampal regions, resulting from neuronal hyperexcitability,[6] astrocyte activation, and proliferative and migratory microglia.[7]

Trauma, infections, stroke, status epilepticus (SE), and febrile convulsions are associated with inflammation and seizures during the acute phase of brain damage and hold an increased risk of developing chronic epilepsy.[8,9] Microglia are crucial for an efficient immune response[10] in which they phagocytose fragments of dying cells, release cytokines and neurotoxic proteins,[10] and affect neuronal survival.[11] In neuroinflammatory conditions such as epilepsy, microglia undergo significant alterations, including changes in molecular composition and proliferation.[12] This transformation from resting to activated microglia in response to insult is fundamental to maintaining homeostasis and limiting injury.[13] Both microglia and other glial cells are activated following local neuronal injuries, leading to the release of proinflammatory effector proteins.[12] Pharmacologic and genetic studies in animal models indicate that specific proinflammatory cytokines, such as tumor-necrosis factor (TNF)α and interferon (IFN)β, contribute significantly to seizure pathology. Furthermore, interfering with these molecules or their receptors can reduce seizure frequency and severity.[14,15]

Toll-like receptors (TLRs) are a central component of the innate inflammatory response. TLRs are activated in response to exogenous ligands such as microbial-associated molecular patterns (MAMPs) and endogenous damage-associated molecular patterns (DAMPs).[16] TLRs are potent inducers of proinflammatory molecules[17] and are implicated in the neuroinflammatory process that occurs in epilepsy. Activation of TLR4 using the MAMP lipopolysaccharide (LPS) or the DAMP high-mobility group B1 protein (HMGB1) is proconvulsive and increases seizure frequency in kainic-acid-induced seizure models.[18] Although HMGB1 activates both TLR4 and receptor for advanced glycation end-products (RAGE), the contribution of RAGE to seizures is less prominent than that of TLR4.[19] Unlike TLR4, TLR3 acts as a sensor for double-stranded RNA (dsRNA), primarily as a product of replicating viruses, but also from endogenous RNA.[20] TLR3 is expressed in the brain, where it regulates immunity, neurogenesis, neuronal plasticity, and the proliferation of embryonic neural progenitor cells.[17] Moreover, neurite outgrowth is prevented by TLR3, causing the collapse of growth cones.[21] TLR3 activation in astrocytes, neurons, oligodendrocytes,[22] and microglia[23] leads to the production of type I IFNs,[22,24] which are essentially neuromodulatory and enhance neuronal excitability of CA3 pyramidal cells,[25] the hypothalamus, amygdala, hippocampus, somatosensory cortex,[26] and neocortical neurons.[27] Of interest, TLR3 activation by synthetic polyinosinic-polycytidylic acid (poly(I:C)) causes a long-lasting increase in seizure susceptibility in rats.[28] Poly(I:C) mimics the effects of systemic viral infections, in part by leading to the production of the type I interferons IFNα and IFNβ as well as TNFα. In addition, TLR3 activation leads to elevated glial production of IFNβ, stimulation of type I IFN receptors, impairment of glutamate homeostasis, and activation extrasynaptic *N*-methyl-D-aspartate (NMDA) receptors.[29]

Here we investigated the contribution of TLR3 to the development of pilocarpine-induced chronic epilepsy. To accomplish this, we induced SE in TLR3−/− mice, recorded electroencephalography (EEG), and analyzed proinflammatory cytokine production and microgliosis in the hippocampus. Our results indicate that activation of TLR3, presumably by DAMPs, adversely affects the pathology and progression of epilepsy by affecting neuronal excitability, microglial activation, and neuroinflammation.

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Thus TLR3 may be a potential therapeutic target for intervention in chronic epilepsy.

Methods

Mice

TLR3-deficient mice (B6N.129s1-Tlr3tm1Flv/J, on a C57bl/6J background, TLR3−/−) and their respective wild-type (C57BL/6J, TLR3+/+) were obtained from the Jackson Laboratories (Maine, U.S.A.). All mice were maintained on a reversed 12-h light/12-h dark cycle with food and water provided ad libitum. All experiments were performed on 8- to 9-week-old male mice. All experimental protocols, methods, and procedures described in this manuscript including the pilocarpine injection protocol, EEG transmitter implantation, and subsequent stereologic analyses were approved by and are in accordance with the guidelines of the Bar-Ilan University's Institutional Animal Care and Use Committee, which requires minimal pain to the animals and minimal use of animals.

Induction of SE with pilocarpine

SE was induced in mice as previously reported.[30–33] Briefly, a single convulsant dose of pilocarpine (340 mg/kg, Sigma, Israel) was injected subcutaneously. SE was defined as a sustained series of generalized tonic–clonic convulsions (stage V; see below for description of stages). Diazepam (4 mg/kg, Teva, Israel) was injected intraperitoneally 40 min after the onset of SE to terminate seizures. To minimize peripheral muscarinic stimulation, methylscopolamine (1 mg/kg, Sigma, Israel) was administered subcutaneously prior to pilocarpine injection. In this model, after a latent period of 1–2 weeks, the initial SE then triggers the process of epileptogenesis, leading to chronic epilepsy and SRS. Only mice developing clinical SE after pilocarpine injection, including whole body tonic–clonic seizures with loss of posture or jumping,[34] were subsequently included in additional phenotypic and correlative analyses. Naive mice were used as control mice for the described experiments.

Telemetric EEG recordings and seizure classification

Implantable telemetric EEG transmitters (Data Science International (DSI), MN, U.S.A.) coupled with a video recording system were used to monitor initial SE development of spontaneous recurrent seizures after SE. This EEG/video telemetry system allows simultaneous data acquisition from multiple animals in parallel for long durations, including EEG and cage activity. For EEG transmitter implantation, the mice were deeply anesthetized with ketamine, (10 mg/kg; Vetoquinol, France) and xylazine (100 mg/kg; Eurovet, The Netherlands). Transmitters were implanted into the right side of the abdominal cavity. Electrodes were then positioned at the stereotaxic coordinates −1.5 posterior, −1.5 lateral relative to Bregma in contact with the cerebral cortex and fixed using dental cement (methylmethacrylate resin, Unifast-Trad, GC America, IL, U.S.A.).

Long-term video-EEG recordings were performed in two animal groups. The first group was used to examine the electrographic and clinical features of SE by pilocarpine. Mice were implanted with EEG electrodes 3 days prior to SE induction (TLR3+/+: n = 5; TLR3−/−: n = 4). SE was monitored using EEG and video, and mice were explanted 2 days later. The second group of mice was implanted with EEG electrodes and transmitters to monitor the development of spontaneous recurrent seizures following pilocarpine-induced SE (TLR3+/+: n = 9; TLR3−/−: n = 8). Mice that experienced and survived SE as described earlier were implanted with EEG electrodes and transmitters 12 days after SE. Following a recovery period of 48 h, video and EEG recordings were made with a sampling rate of 1 kHz (on day 14 after SE). Once stable EEG signals were obtained, monitoring was performed continuously for 14 days (on day 28 after SE). EEG recordings were analyzed using Fast Fourier transformation analysis (FFT). To perform a time-dependent analysis, we used a moving-window approach. EEG signals were divided into nonoverlapping segments of 8,192 sampling points each, corresponding to a window length of 8.2s at the given sampling rate. Prior to FFT calculation, data preprocessing was performed for each window. The duration of increased EEG activity was determined as the time during which band power was increased to 10% of maximal values. All spontaneous seizures were classified according to five stages: stage I (immobility, rigid posture), stage II (repetitive movements, head bobbing), stage III (severe seizures with rearing without falling), stage IV (severe seizures with rearing and falling/loss of righting ability), and stage V (continuous tonic–clonic seizures during SE).

Microglial immunofluorescence staining

To assess the number, distribution, and activation of microglial cells in the hippocampal formation, we have utilized a stereologic approach. To accomplish this, wild-type (TLR3+/+) and TLR3−/− mice (n = 4 in each experimental group) were randomly chosen from cohorts of mice induced with SE using pilocarpine. These mice were monitored for seizure activity during the SE induction phase only. Six weeks after SE induction, mice were anesthetized using ketamine/xylazine (10 mg/kg and 100 mg/kg, respectively) and perfused transcardially with cold 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). Brains were removed and postfixed in 4% PFA overnight and then sequentially cryoprotected in 20% and 30% sucrose in 0.1 M PBS until brains sank in the solution. Brains were then sectioned into 40-μm-thick coronal slices on a freezing microtome. Slices were kept in tissue-collecting solution (TCS, 25% glycerol, 30% ethylene glycol, and NaP04 0.1 M, pH = 7.2). Immunohistochemistry was

completed on free-floating sections and mounted on slides for analysis. Slices were then washed (all washes were done five times in 0.1% Triton X-100 in PBS for a total of 25 min). Nonspecific binding was blocked with 20% normal horse serum and 0.1% Triton X-100 in PBS for 60 min. The microglia marker, ionized calcium-binding adapter molecule 1 (IBA-1; 1:1,000; Cat # 019-19741, WAKO, Japan) was diluted in PBS supplemented with 0.1% Triton-X-100 with 2% horse serum. Following a 24 h incubation with the primary antibody, slices were washed and subsequently incubated with a fluorescent-tagged secondary antibody (Alexa-488, 1:500, Invitrogen, OR, U.S.A.), diluted in PBS supplemented with 0.1% Triton X-100 for 1 h at room temperature. Slices were then stained with Hoechst 33342 (Invitrogen) diluted 1:1,000 in 0.1% Triton X-100 in PBS for 10 min and washed once in 0.1% Triton X-100 in PBS for 10 min. Slices were then placed on glass slides and dried for 15 min before immersed in a mounting solution (Aqua Poly/Mount, Polyscience Inc., PA, U.S.A.) and covered with a cover slip.

Microgliosis assessment

The hippocampus was outlined according to the Paxinos atlas of the mouse brain. Quantification of stained cells was evaluated by stereologic counts using the optical dissector method as described previously.[35] Optical fractionator sampling was carried out using a Leica DM6000 microscope (Leica Microsystems, Germany) coupled to a controller module and a high-sensitivity 3CCD video camera system (MBF Biosciences, VT, U.S.A.), and an Intel Xeon workstation (Intel, CA, U.S.A.). Sampling was implemented using the Stereo Investigator software (MBF Biosciences). Analyzed brain sections spanned from −0.94 mm to −4.04 mm from Bregma. Every 9th to 10th section (360–400 μm apart) was used for quantification from each animal. After outlining a contour of the hippocampus at low magnification (10× objective), the contour was imaged with 18–25 μm thick Z-stack images using a 40× oil immersion objective (numerical aperture = 1.4). Acquired images depicting IBA-1 staining were first iterated 10 times using the Huygens deconvolution software (SVI, The Netherlands) in order to improve resolution and signal to-noise ratio for later offline processing using the optical dissector method.[26] Section thickness was measured by focusing on the top of the section, setting the Z-axis to 0, and then refocusing to the bottom of the section and recording the actual thickness at every counting location. Only fully identified microglial cells with round body shape and clearly visible processes were counted. In addition, cells were counted only if they did not intersect with the lines of exclusion on the counting grid. The total number of positive cell population was estimated based on the section volume and extrapolated for the total volume of the hippocampus. The following parameters were set for cell counts: the counting frame was 100 × 100 × 20 μm (height × width × dissector height), the sampling grid size was defined as a mean of 25 sites, and a guard zone height was 2 μm. These parameters were determined in a preliminary pilot study aimed at determining suitable counting frame and sampling grid parameters prior to counting. An experimenter blind to all treatment groups performed the stereologic counts. The coefficient of error Gunderson (m = 1) values was between 0.02 and 0.03 for all animals. Microglial activation state was measured by quantifying microglial branching. Number of branches per cell and branch complexity were assessed using the WIS-NeuroMath algorithm.[36]

Quantitative real-time polymerase chain reaction (qRT-PCR) to assess messenger RNA (mRNA) expression levels of proinflammatory cytokines and TLRs

RNA was extracted from either the hippocampus or spleen using TRIzol Reagent (Ambion, Life Technologies, CA, U.S.A.) of randomly chosen naive mice, as well as from mice at 6 weeks post pilocarpine-induced SE. Mice were monitored during SE induction period only. Complementary DNA (cDNA) was generated using RevertAid-H minus first strand cDNA synthesis kit (Thermo Scientific, Lithuania). RT-PCR reactions were performed using Fast-SYBR Green (Applied Biosystems, CA, U.S.A.) in a StepOnePlus instrument (Applied Biosystems, CA, U.S.A.). Primers were calibrated, and a negative control was performed for each primer pair. Samples were measured in triplicate and values were normalized according to mRNA levels of β-actin. Denaturing was performed at 94°C for 30 s, annealing for 10 s (temperature for each primer pair is indicated in Table 1), and elongation was performed at 72°C for 10 s. Quantification was assessed at the logarithmic phase of the PCR reaction using the 2-ΔΔCT method.

Data analysis and statistical analysis

Results were analyzed using analysis of variance (ANOVA) and Bonferroni post hoc correction to determine pairwise comparisons among multiple data sets. Significance was set at $p < 0.05$. Statistical analysis was carried out using GraphPad Prism 5 software and IBM SPSS statistics using either the Student's *t*-test to compare two groups or Mann-Whitney *U* and χ^2-test where applicable and indicated.

RESULTS

We have assessed the involvement of TLR3 in epileptogenesis following SE using an established model of pilocarpine-induced SE in both TLR3+/+ and TLR3−/− mouse strains. The acute and chronic impact of TLR3 deficiency on the induction and severity of SE was evaluated using brain EEG measurement, quantification of proinflammatory cytokine mRNA in the hippocampus, and hippocampal microgliosis in TLR3+/+ and TLR3−/− mice up to 1 month after SE induction.

Table 1. Primers used for quantitative real-time-PCR to assess mRNA expression of the proinflammatory cytokines tumor necrosis factor (TNF)α, interferon (IFN)β, β-actin, and TLR2–5, 7, 9

Gene	Accession no.	Annealing temperature	Primer sequences
TNFα	NM 013693	54°C	Forward: 5′ TGCCTATGTCTCAGCCTCTTC 3′ Reverse: 5′ GAGGCCATTTGGGAACTTCT 3′
IFNβ	NM 010510.1	56°C	Forward: 5′ TGACGGAGAAGATGCAGAAGAG 3′ Reverse: 5′ TGGAGTTCATCCAGGAGACGTA 3′
β-Actin	NM 007393.3	56°C	Forward: 5′ TTCTTTGCAGCTCCTTCGTT 3′ Reverse: 5′ ATGGAGGGGAATACAGCCC 3′
TLR2	NM_011905.3	56°C	Forward: 5′ GGTGCGGACTGTTTCCTTCT 3′ Reverse: 5′ AGATTTGACGCTTTGTCTGAGG 3′
TLR3	NM_126166.4	53°C	Forward: 5′ GAGCCAGAACTGTGCCAAAT 3′ Reverse: 5′ CCCGTTCCCAACTTTGTAGA 3′
TLR4	NM_021297.3	57°C	Forward: 5′ ATCCCTGCATAGAGGTAGTTCC 3′ Reverse: 5′ GGTGGTGTAAGCCATGCCA 3′
TLR5	NM_016928.3	57°C	Forward: 5′ CTCTCCAGACGCCTCATCTC 3′ Reverse: 5′ TGATCCTGCTGAAGTGTGGT 3′
TLR7	NM_001290755.1	54°C	Forward: 5′ TCATGGTGTTTTCGATGTGG 3′ Reverse: 5′ GGTGGTGTTAGTGGGAATGC 3′
TLR9	NM_031178.2	57°C	Forward: 5′ TCCATCTCCCAACATGGTTCTC 3′ Reverse: 5′ GCAGAGAAACGGGGTACAGA 3′

TLR3 deficiency does not alter the severity of electrographic SE

To assess differences in SE induction between TLR3+/+ and TLR3−/− mice, TLR3+/+ (n = 4) and TLR3−/− (n = 5) mice were implanted with EEG electrodes 3 days prior to SE induction by pilocarpine. Initial EEG recordings following pilocarpine injection show a period of baseline activity without epileptogenic potentials (Fig. 1A$_1$), followed by spikes and sharp waves with increasing frequency (Fig. 1A$_2$). Clinical convulsions and SE follow with a clear correlate of high-amplitude, high-frequency discharges (Fig. 1A$_3$). Forty minutes following SE induction, diazepam was administered to interrupt the electrical seizure activity. This induced a postictal period of clinical comatose state, and was followed by spikes and sharp waves at a lower frequency (Fig. 1A$_4$). No significant difference between TLR3+/+ and TLR3−/− mice was observed in the frequency distribution of events, suggesting that SE was induced at a similar clinical and electrographic severity in both groups (p > 0.05, Fig. 1B). Clinical seizures were classified following pilocarpine injections by video analysis using the Racine score for seizure severity as described earlier (a I–V scale). Scores were averaged in 5 min intervals, and slope and timing of clinical seizure scores did not differ between TLR3+/+ and TLR3−/− mice (p > 0.05, Fig. 1C). As mentioned, following 40 min of SE, diazepam was applied, explaining the rapid drop in seizure activity at 80 min following pilocarpine administration (Fig. 1C).

SE-related mortality is reduced in TLR3−/− mice

Pilocarpine injections are known to lead to SE in most, but not all, animals and are associated with high mortality rates depending on the pilocarpine dose, strain, and age of the mice. Following pilocarpine injection to induce SE, TLR3−/− mice survived more frequently (TLR3−/−: 51.5%, n = 58/103 vs. TLR3+/+: 35.2%, n = 69/202; p = 0.0057, χ²-analysis; Fig. S1A). The survival rates of the TLR3+/+ mice are consistent with other reports on SE induction in young mice.[30,37,38] No correlation was found between the time of onset of SE, and survival (39.5 ± 3.6 min in mice that survived vs. 41.9 ± 4.2 min in mice that subsequently died; p = 0.54; Fig. S1B). Focusing only on those animals that had developed SE after pilocarpine injection, TLR3−/− mice presented higher survival rates compared with TLR3+/+ mice (70.7%, n = 58/82 vs. 52.3%, n = 69/132; p = 0.0057, χ²-analysis; TLR3−/− and TLR3+/+ respectively, Fig. 1D). Onset of SE was slightly increased in TLR3+/+ mice compared to TLR3−/− mice (44.3 ± 1.9 min and 36.6 ± 2.4 min, p = 0.0082 Mann-Whitney, SE; Fig. 1E). Thus, these data suggest that TLR3 deficiency reduces mortality following pilocarpine-induced SE.

TLR3 deficiency reduces the development of spontaneous recurrent seizures

Following SE induction, mice developed chronic SRS. Long-term telemetric EEG and video monitoring were conducted to determine the frequency and severity of seizure activity in TLR3+/+ (n = 9) and TLR3−/− (n = 8) mice during days 14–28 after SE induction. TLR3 deficiency significantly reduced the development of SRS 2 weeks after SE. During continuous video-EEG monitoring (days 14–28 following SE induction), SRS were observed in all TLR3+/+ and TLR3−/− mice (n = 9 and n = 8 respectively); however, the occurrence of any clinical seizure type (stage I–IV) was reduced by ~75% in TLR3−/− mice (16.7 ± 5.4 per

Figure 1.
TLR3 deficiency reduces SE-related mortality without affecting SE severity. (**A**) Examples of EEG recordings of SE (1) 1 min after pilocarpine injection, (2) 8 m after injection (before any clinical detectable seizures), (3) during SE, and (4) following diazepam injection. (**B**) Frequency histogram of SE in pilocarpine-treated TLR3+/+ (n = 4, gray bars) and TLR3−/− (n = 5, white bars; p > 0.05) mice. (**C**) Clinical seizure scores displayed over time after pilocarpine injection. No difference was detected between TLR3−/− (gray; n = 5) and TLR3+/+ (black; n = 4) mice. (**D**) TLR3−/− mice exhibit higher survival rates following pilocarpine-induced SE. Black segment: Mice not surviving SE. White segment: mice developing SE and surviving. (**E**) Duration to SE following pilocarpine injection was slightly longer (44.3 ± 1.9 min) in TLR3+/+ mice compared to TLR3−/− mice (36.6 ± 2.4 m; p = 0.0082). Error bars indicate standard error of mean SE. Asterisk indicating significance level p < 0.05.
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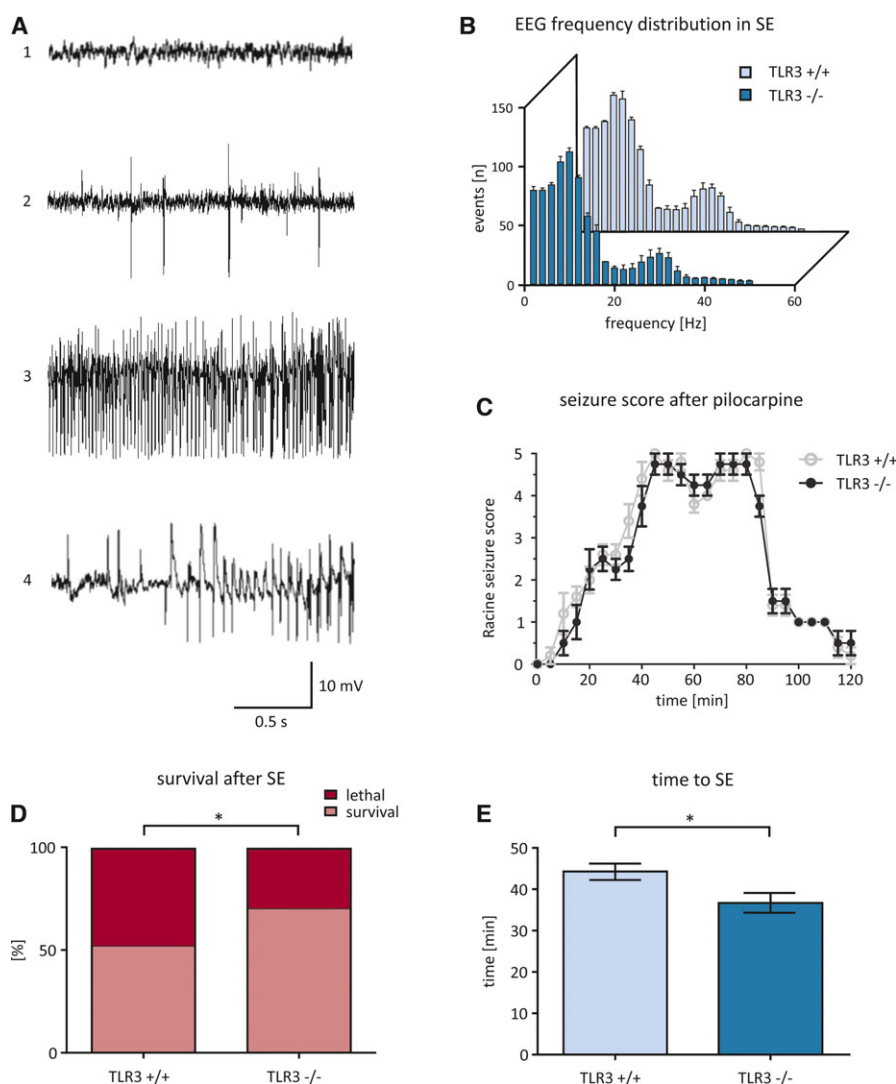
day vs. 4.1 ± 2.7 per day in TLR+/+ mice, p < 0.0001, Fig. 2A). Furthermore, TLR3−/− mice exhibited significantly fewer stage III and stage IV seizures (TLR3+/+ vs. TLR3−/−: stage III: 4.0 ± 1.9 per day vs. 0.6 ± 0.5 per day; p = 0.002; stage IV: 3.0 ± 2.6 per day vs. 0.5 ± 0.6 per day, p = 0.019; Fig. 2B). This suggests that TLR3 deficiency reduces the development of SRS. In addition to the strong reduction in the development of clinical seizures, the frequency of spontaneous seizure EEG activity was also significantly reduced in TLR3−/− mice (21.43 ± 5.6 per day and 6.0 ± 4.3 per day respectively, p < 0.0001, Fig. 2C). Electrographic seizures lasted significantly longer in TLR3+/+ mice: 36.1s ± 1.2s versus 26.5s ± 1.9s in TLR3+/+ and TLR3−/− mice respectively, p < 0.0001; Fig. 2D). The majority of electrographic seizure events in TLR3−/− mice were short and lasted <20 s (<20 s: 61.1% vs. 47.4%; TLR3−/− and TLR+/+ respectively, p < 0.001, Fig. 1E), with few lasting longer than 30 s (>30 s: 16.0% vs. 30.3%; TLR3−/− and TLR+/+ respectively, p < 0.001, Fig. 2E).

TLR3 deficiency attenuates SE-induced expression of IFNβ and TNFα

Seizure activity induces neuroinflammation, and the expression of proinflammatory cytokines has been reported in various experimental models of epilepsy.[15] To assess the inflammatory state of the hippocampus, two central proinflammatory cytokines, TNFα and IFNβ, were quantified. Expression of proinflammatory cytokines following SE induction was quantified using qRT-PCR on hippocampal tissue from TLR3+/+ and TLR3−/− mice at 6, 48 h, and 2 months post-SE (n = 3 for each group and time point). Hippocampal IFNβ expression was significantly increased in TLR3+/+ mice at every time point examined (Fig. 3A, p < 0.05, two-way ANOVA). This result was not unexpected, as TLR3 is a known inducer of IFNβ.[29] Similarly, TNFα expression was significantly augmented in TLR3+/+ hippocampus compared to TLR3−/− mice (Fig. 3B, p < 0.01, two-way ANOVA). In addition, this expression increased at each time point during SE induction, suggesting that seizure activity increases TNFα levels in TLR3+/+
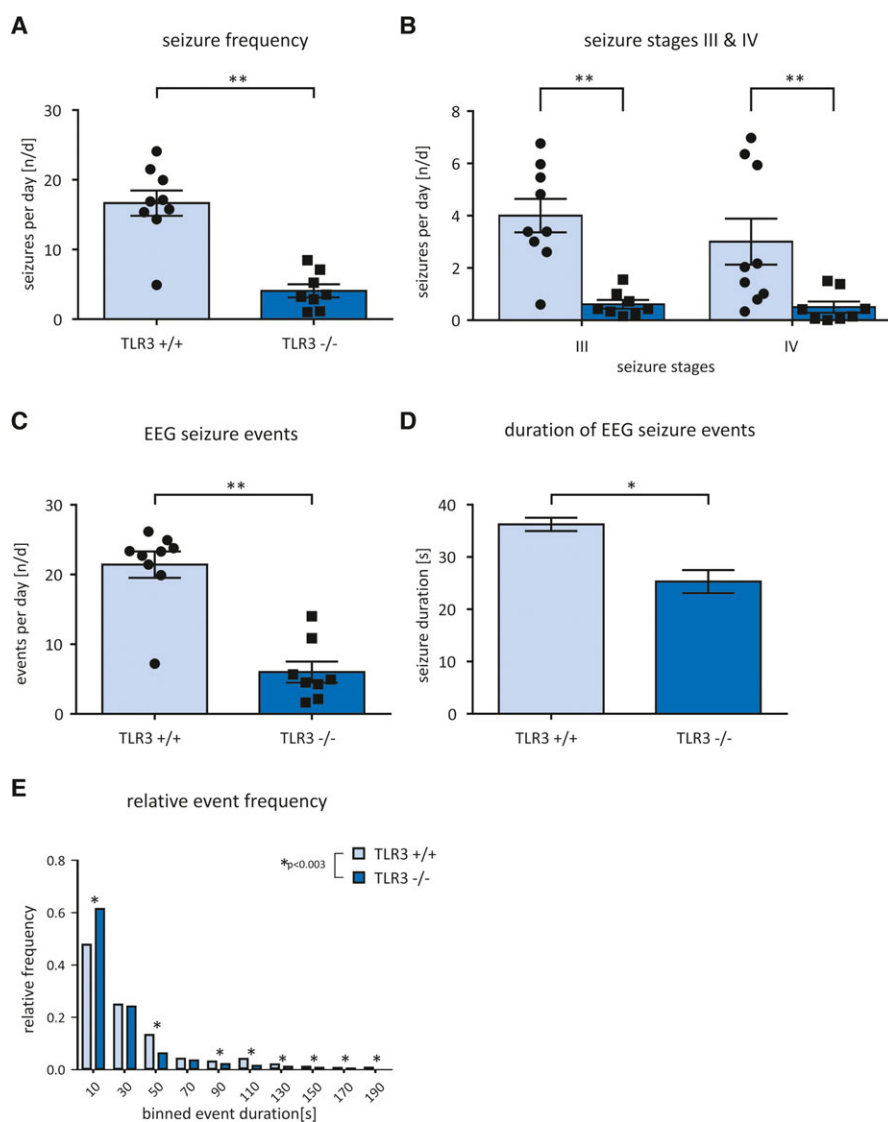
Figure 2.
TLR3 deficiency reduces spontaneous recurrent seizures. (**A**) Spontaneous recurrent seizures are significantly reduced in TLR3−/− mice (2–4 weeks after SE; TLR3+/+, n = 9, gray bars; TLR3−/−, n = 8, white bars; p < 0.0001). (**B**) TLR3−/− mice exhibited a significantly lower number of stage III and stage IV SRS per day compared with TLR3+/+ mice. (**C**) EEG recorded seizure events including clinical and subclinical seizure events are significantly reduced in TLR3−/− mice. (**D**) Duration of electrographic seizures recorded are significantly longer in TLR3+/+ mice. (**E**) Relative frequency histogram of epileptic events duration in TLR3+/+ and TLR3−/− mice. The event duration is binned to present data more clearly. The asterisk indicates significance between both groups p < 0.05. Gray bars indicate TLR3+/+; white bars indicate TLR3−/− mice; * and ** indicate significance level p < 0.03 and p < 0.01, respectively.
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hippocampus in a time-dependent manner. Of note, pilocarpine-treated TLR3+/+ mice exhibited decreased levels of TLR2 and TLR7, but not TLR4 or TLR3 (Fig. S2A–D, p < 0.05). In TLR3−/− mice, on the other hand, no significant difference was observed following pilocarpine treatment (Fig. S2A–D). TLR5 and TLR9 could not be detected in hippocampal tissues. These data suggest that no compensatory response to TLR3 deficiency was involved.

TLR3 deficiency reduces microgliosis following SE

Microglial activation has been observed in both animal models of epilepsy and in human epilepsy patients, suggesting microglial involvement in the pathologic process of epilepsy.[7] Pilocarpine-induced SE is associated with significant hippocampal pathology including astrogliosis, neuronal cell loss, and microglial morphology (thickened and shortened processes).[38] We used IBA-1 immunostaining to analyze whether TLR3 deficiency alters microglial

numbers and morphology in TLR3+/+ (n = 4) and TLR3−/− (n = 4) mice following SE induction. We have assessed whether hippocampal microglial distribution and activation is chronically altered following SE induction. Our analysis revealed a significant baseline difference between hippocampi of naive TLR3+/+ and naive TLR3−/− mice. TLR3−/− mice exhibited a larger total number of microglial cells than TLR3+/+ mice (165,254 ± 7,259 and 147,132 ± 4,150 microglia respectively; p = 0.028, two-way ANOVA; Fig. 4A,B). Pilocarpine treatment did not significantly alter the total number of microglia in TLR3+/+ or TLR3 −/− hippocampi (p > 0.05, Fig. 4A). Unbiased quantification of the number of branches on microglial cells was conducted to assess their activation state. Although control microglial cells exhibited a normal distribution of branch number, which was similar between TLR3+/+ and TLR3−/− mice (Fig 4C,D, dark line), microglial cells from pilocarpine-treated mice exhibited a biphasic distribution of
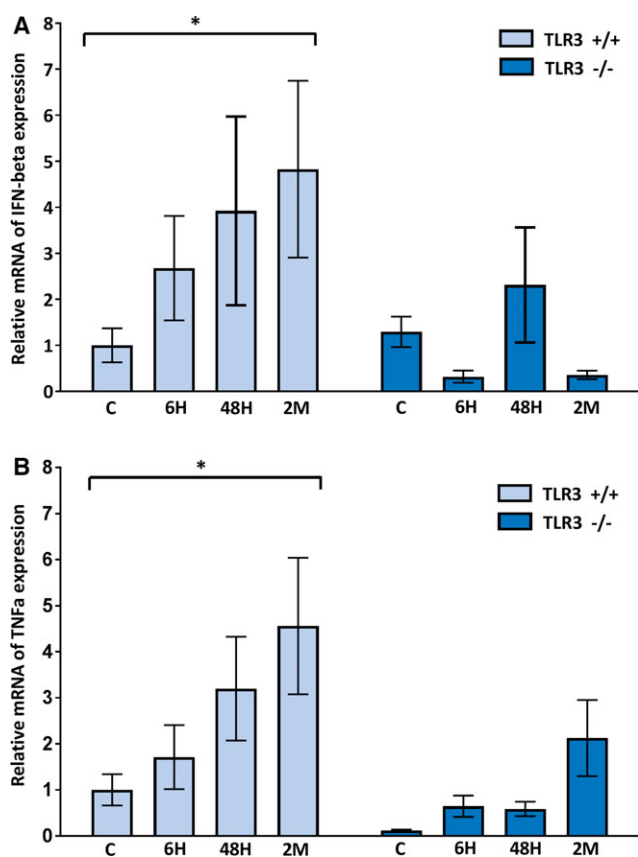
Figure 3.
TLR3−/− mice exhibit depressed production of TNFα and IFNβ following pilocarpine-induced SE. (**A**) TLR3+/+ exhibit significant expression of IFNβ compared to TLR3−/− mice (p < 0.05, two-way ANOVA; n = 3). (**B**) TNFα is highly expressed in hippocampi from TLR3+/+ mice (p < 0.01, two-way ANOVA; n = 3).
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branches number (modus A: low branches number and modus B: high branches number, Fig 4C,D, gray line). Of interest, pilocarpine treatment increased the number of branches on the majority of microglial cells in TLR3−/− mice (modus B, Fig. 4D) compared with pilocarpine-treated TLR3+/+mice (modus B, Fig. 4C, p < 0.05).

These results suggest that TLR3 deficiency plays a protective role by reducing microglial activation during neuroinflammation. Thus TLR3 deficiency increases baseline microglial cell numbers while decreasing their activation state.

Discussion

Although TLRs are reported to contribute to the development of seizures,[18,19,28] the role of TLR3 in the development and progression of epilepsy is largely unknown. We investigated the role of TLR3 in the pilocarpine model of epilepsy and its influence on SE and SRS. Our study has three main results. First, TLR3 deficiency reduced acute mortality resulting from pilocarpine-induced SE. Second, TLR3 deficiency reduced the development of chronic SRS following SE and attenuated the expression of proinflammatory cytokines in the hippocampus. Finally, TLR3 deficiency reduced the activation of hippocampal microglial cells. Taken together, these results suggest that a lack of TLR3 depresses the development of chronic seizures after SE, lessens neuroinflammation following SE, and implicate TLR3 in the development of SE and chronic SRS. Thus the absence of TLR3 reduced epileptogenesis in a pilocarpine-induced SE model. The effects of TLR3 on the development of SE and in light of previous findings that TLR3 deficiency exerts pleotropic effects on hippocampal-dependent cognitive behavior,[17,39] emphasize the TLR3 pathway as central to central nervous system (CNS) plasticity in physiology and disease. Although the magnitude of SE was clinically and physiologically similar between TLR3+/+ and TLR3−/− mice, TLR3 deficiency significantly reduced seizure frequency and severity during the chronic phase (SRS). TLR3−/− mice experienced 75% less SRS than their wild-type counterparts. It is important to note that in addition to the reduction of chronic seizures, acute mortality rates after SE were reduced in TLR3−/− mice. Onset of SE after pilocarpine injection was reduced slightly in the TLR3−/− mice, possibly indicating a difference in susceptibility to pilocarpine in those animals; however, EEG and clinical seizure stages during SE did not differ from the wild-type mice. Taken together, these observations suggest that TLR3 facilitates the development and progression of epilepsy.

Other TLRs have been previously associated with the development of epilepsy. TLR4 activation by LPS acts rapidly as a proconvulsive and increases seizure frequency in kainic-acid-induced seizure models.[18] Studies that utilized genetic interference or pharmacologic inhibition of the TLR4 pathway have provided evidence for the relevance of the TLR4 pathway to epilepsy. TLR4 deficient mice show a delayed seizure onset in kainate and bicuculline-treated mice that exhibit acute seizures.[18,40,41] Moreover, microglia, astrocytes, and neurons secrete HMGB1, which promotes seizures and secretion of proinflammatory factors (e.g. IL-1β) in a TLR4-mediated manner as shown in a kainate-model of focal epilepsy. Furthermore, in chronically epileptic mice, antagonists for either HMGB1 or TLR4 reduce spontaneous seizures.[18,42] Thus TLR4 is significantly implicated in the pathologic onset and progression of epilepsy.

SE and chronic SRS are associated with inflammatory markers such as astrocyte and microglial activation and the secretion of inflammatory molecules.[12,40] TLR3 deficiency attenuated hippocampal expression of the proinflammatory cytokines TNFα and IFNβ following SE. Indeed, TLR3 activation by Poly(I:C) leads to elevated glial production of IFNβ, activation of type-I IFN receptors, and impairment of glutamate homeostasis, and enables activation of
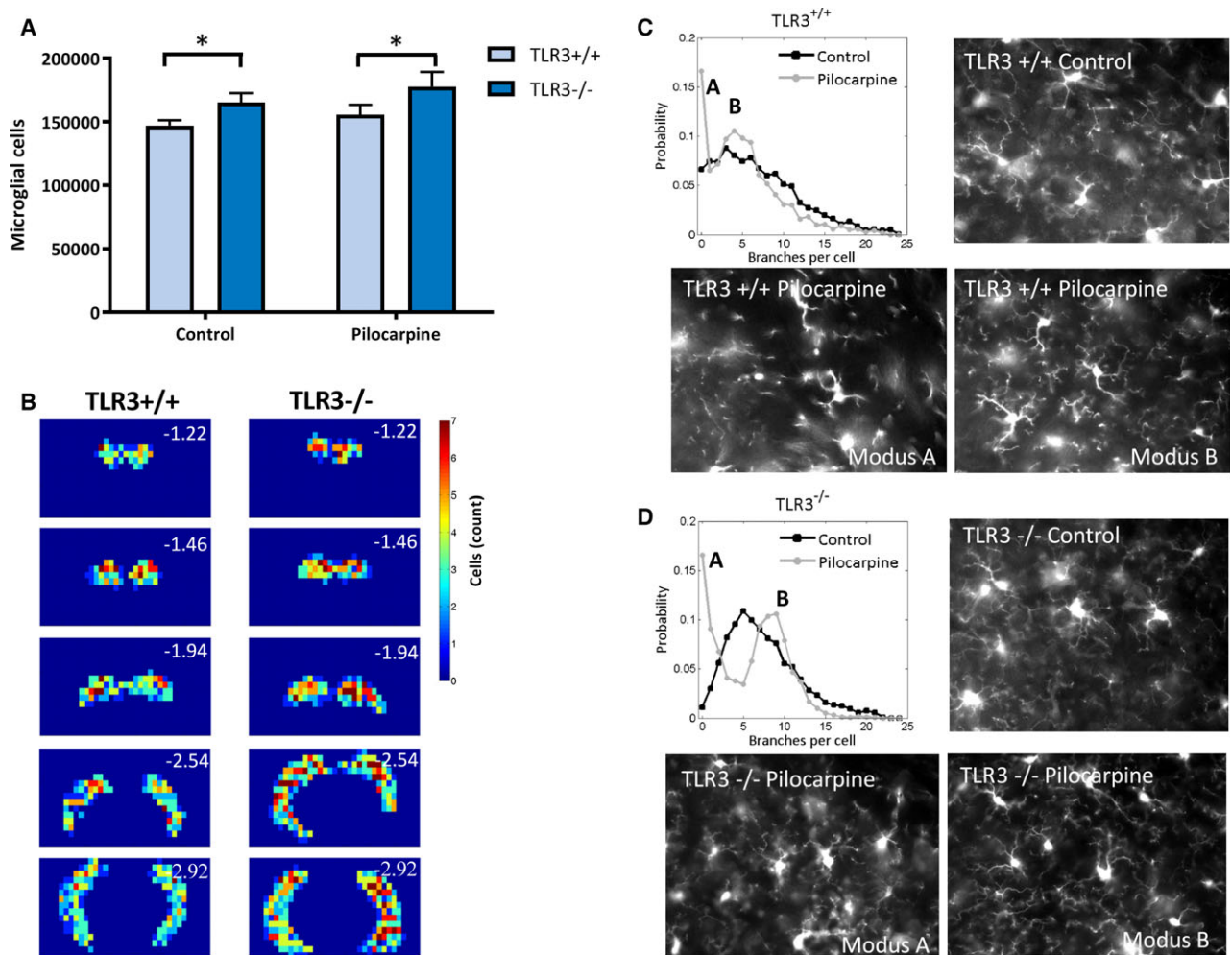
Figure 4.
Microglial cells are less activated in TLR3−/− mice. (**A**) Microglial cells were counted in the hippocampus of naive (n = 4) and pilocarpine-treated (n = 4) mice at 6 weeks following SE induction using optical fractionator probe (stereo investigator software, MBF Biosciences). (**B**) Heat maps representing the probability density function of microglial cell-scatter in the hippocampus of TLR3$^{+/+}$ and TLR3−/− mice in different distances from bregma (from rostral to caudal): −1.22, −1.46, −1.94, −2.54. Bin-size = 300 microns, n = 4 per group. (**C**) Distribution of branches per cell in naive and pilocarpine-treated TLR3$^{+/+}$ mice. Representative images of microglial cells indicate microglial morphology in control TLR3+/+ mice and pilocarpine-treated mice. Two microglial cell populations that exhibit low branches number (modus A) and high branches number (modus B). (**D**) Distribution of branches per cell in naive and pilocarpine-treated TLR3−/− mice. Representative images of microglial cells indicate microglial morphology in control TLR3+/+ mice and pilocarpine-treated mice. Two microglial cell populations that exhibit low branch number (modus A) and high branch number (modus B). Branches per microglial cell were counted using the WisNeuroMath algorithm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, two-way ANOVA, Kolmogorov-Smirnov test.
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extrasynaptic NMDA receptors. The blunted inflammatory response correlated with reduced microglial activation we observed in TLR3−/− mice suggests that TLR3 deficiency reduced chronic neuronal damage resulting from spontaneous seizures. The blunted inflammatory response occurred in TLR3−/− mice without apparent compensatory effect by other TLRs such as TLRs 2, 4, 5, 7, or 9. This may provide an explanation for the protective effect of TLR3 deficiency during the development of chronic recurrent seizures.

The etiology of epilepsy is unknown, and treatments, although highly effective in a number of cases, are mostly symptomatic. This study aimed to understand the role of TLR3 in the onset and progression of seizures. Although the exacerbation of chronic spontaneous recurrent seizures by TLR3 may be linked to increased hippocampal inflammation, the cause of accelerated onset of SE in TLR3−/− mice remains unknown. Nevertheless, these findings suggest that the "vicious cycle" between neuroinflammation and seizure development in epilepsy is indeed initiated by increased

excitability followed by aggravating neuroinflammatory events.

Acknowledgments

Disclosure

The authors declare no competing interests. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Additional Contributors

AG carried out the pilocarpine-induction experiments, microglial assessment, and cytokine analysis experiments; participated in its design and coordination; and helped to draft the manuscript. FB carried out the pilocarpine-induction experiments, EEG transmitter implantation, EEG monitoring, video tracking; participated in study design; and helped to draft the manuscript. RM carried out pilocarpine injection experiments, participated in its design and coordination, and helped to draft the manuscript. TI conducted computational analysis of hippocampal microglial distribution and activation. DO conceived of the study and participated in its design and coordination and helped to draft the manuscript. KG helped draft the manuscript. IS participated in study design and coordination and helped to draft the manuscript. EO conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Outcome and time to SE after pilocarpine.

Figure S2. TLR2 and 7 expression levels decrease in TLR3+/+ but not TLR3−/− mice following pilocarpine treatment.