

TLR2 activation inhibits embryonic neural progenitor cell proliferation

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Abstract

Toll-like receptors (TLRs) play essential roles in innate immunity, and increasing evidence indicates that these receptors are expressed in neurons, astrocytes, and microglia in the brain, where they mediate responses to infection, stress, and injury. To address the possibility that TLR2 heterodimer activation could affect progenitor cells in the developing brain, we analyzed the expression of TLR2 throughout mouse cortical development, and assessed the role of TLR2 heterodimer activation in neuronal progenitor cell (NPC) proliferation. TLR2 mRNA and protein was expressed in the cortex in embryonic and early postnatal stages of development, and in cultured cortical NPC. While NPC from TLR2-deficient and wild type embryos had the same proliferative capacity, TLR2 activation by the synthetic bacterial lipopeptides Pam₃CSK₄ and FSL1, or low molecular weight hyaluronan, an endogenous ligand for TLR2, inhibited

neurosphere formation *in vitro*. Intracerebral *in utero* administration of TLR2 ligands resulted in ventricular dysgenesis characterized by increased ventricle size, reduced proliferative area around the ventricles, increased cell density, an increase in phospho-histone 3 cells, and a decrease in BrdU⁺ cells in the sub-ventricular zone. Our findings indicate that loss of TLR2 does not result in defects in cerebral development. However, TLR2 is expressed and functional in the developing telencephalon from early embryonic stages and infectious agent-related activation of TLR2 inhibits NPC proliferation. TLR2-mediated inhibition of NPC proliferation may therefore be a mechanism by which infection, ischemia, and inflammation adversely affect brain development.

Keywords: toll-like receptors, cerebral cortex, neuronal progenitor cells, nuclear factor- κ B, proliferation.

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The family of mammalian toll-like receptors (TLRs) includes at least 10 members that sense the presence of pathogenic microorganisms and injury-induced endogenous ligands (Wagner 2006). Activation of TLRs mediates innate immune responses through nuclear factor- κ B (NF- κ B) and interferon regulatory factor (IRF)-dependent pathways (Akira 2006). IRF5 is mainly activated following TLR2 activation while other IRFs such as IRF3 are more specific for TLR3 and TLR4 activation (Lee and Kim 2007). Although TLRs are abundant in the immune system, they are also expressed in non-immune cells including brain cells. While TLR expression within the CNS was originally believed to be restricted to microglia, recent data indicate that neurons also express functional TLRs 2, 3, 4, and 8 (Okun *et al.* 2008).

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Abbreviations used: BrDU, 5-bromo-2-deoxyuridine; DAPI, 4'-6-diamidino-2-phenylindole; FSL1, Pam2CGDPKHPKSF; HA, hyaluronan; HMW-HA, high molecular weight hyaluronan; IRF, interferon regulatory factor; LDH, lactate dehydrogenase; LMW-HA, low molecular weight hyaluronan; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; NPC, neuronal progenitor cells; PCNA, Proliferating cell nuclear antigen; PH3⁺, phospho-histone 3; TLR, toll-like receptors; TLR2KO, TLR2-deficient mice; WT, wild type.

Recent findings suggest that neuronal progenitor cells (NPC) express one or more TLRs which may play roles in normal brain development and responses of the developing brain to infection and injury. TLR4 activation restricts retinal progenitor cell proliferation during the early postnatal period (Shechter *et al.* 2008). TLR2 and TLR4 are expressed in adult NPC where they exert opposite effects on proliferation and differentiation; TLR2 enhances dentate gyrus neurogenesis, whereas TLR4 inhibits adult NPC proliferation and neuronal differentiation (Rolls *et al.* 2007). Activation of TLR2 and TLR4 in adult NPC leads to secretion of pro-inflammatory cytokines, such as tumor necrosis factor- α (Covacu *et al.* 2009). However, while TLR2 is abundant within the nervous system (Bsibsi *et al.* 2002; Kielian 2006), the effects of TLR2 activation during embryonic brain development are unknown.

TLR2 is expressed on the plasma membrane and can form heterodimers with either TLR1 or TLR6. In addition, CD14 acts as a co-receptor for TLR2/1 and TLR2/6 heterodimers, while CD36 is a co-receptor for TLR2/6 (Triantafilou *et al.* 2006). TLR2 can dimerize with TLR1 to recognize triacylated lipopeptides from bacteria, such as Pam₃CSK₄, or with TLR6 to respond to diacylated lipopeptides, such as Pam₂CSK₄ or Pam2CGDPKHPKSF (FSL1). These ligands are specific to TLR2, as in the absence of TLR2 they do not have TLR-related biological function, however, they can still induce signaling in the absence of either TLR1 or TLR6 (Buwitt-Beckmann *et al.* 2005, 2006). Increasing numbers of endogenous ligands are found to activate TLR2. For example, hyaluronic acid (HA) cleaved by hyaluronidase, generates low molecular weight (LMW)-HA, activating TLR2 and TLR4 (Jiang *et al.* 2007). Signaling through TLR2 is mediated by the Toll/interleukin-1 receptor (TIR) adaptor proteins MyD88 and Mal (also known as TIRAP) resulting in NF- κ B activation (Akira 2006).

TLR2 activation has long been known to occur during bacterial infections. Bacterial infection is common during pregnancy, including bacterial vaginosis, and can result in pre-term birth, spontaneous abortion and may have long-lasting adverse effects on brain development (Leitich *et al.* 2003; Lowe *et al.* 2008; Sorensen *et al.* 2008). TLR2 can be activated following cerebral ischemia (Tang *et al.* 2007) and may play a role in pathogenesis of brain abnormalities resulting from intrapartum hypoxia/ischemia (Graham *et al.* 2008). Here, we show that TLR2 heterodimer components are expressed in proliferating cells in the embryonic mouse telencephalon *in vivo* and NPC *in vitro*. Using TLR2-deficient mice and TLR2-specific ligands, we show that while in the absence of an exogenous ligand, TLR2 does not alter NPC proliferation in the developing embryo, TLR2 activation increases the number of phospho-histone 3 (PH3⁺) cells and reduces the number of 5-bromo-2-deoxyuridine (BrdU⁺) cells in the ventricular zone resulting in ventricular dysplasia. This suppression of

NPC proliferation is specific for TLR2, as co-administration of ligands for other bacterial-sensing TLRs (TLRs 4, 5, and 9) did not modify the inhibitory effect of TLR2 on NPC.

Materials and methods

Animals and *in utero* microinjections

Congenitally TLR2-deficient mice (TLR2KO) and wild type (WT) mice of the same genetic background (C57BL/6) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were maintained in the National Institute on Aging animal facility under pathogen-free conditions on a 12 h light/12 h dark cycle with continuous access to food and water. Embryos of different gestational ages were collected from pregnant mice for tissue analysis. NPC neurosphere cultures were prepared using brain tissue from embryos of TLR2KO and WT mice at either E12 or E15 developmental stages. For *in utero* microinjections of TLR2 ligands, timed-pregnant mice (gestational day 15) were anesthetized with isoflurane and a midline laparotomy was performed to expose the uterine horns. The lateral ventricle in the brain of each embryo was visualized with transillumination and the injections were performed using a glass capillary pipette (75–125 μ m outer diameter with beveled tip) driven by a Sutter micromanipulator (Sutter Instrument Company, Novato, CA, USA) equipped with 20 μ L Hamilton gas-tight syringe (Reno, NV, USA). A 1- μ L volume of 0.1 μ g of Pam₃CSK₄ dissolved in phosphate-buffered saline (PBS), or PBS alone, was injected. Following intrauterine surgery, the incision site was closed with sutures (4-0; Ethicon, Somerville, NJ, USA) and the mouse was allowed to recover in a clean cage. Mice were killed at 24 and 72 h after the injection. All procedures were approved by the National Institute on Aging Animal Care and Use Committee.

BrdU injections protocol

E18.5 pregnant dams were BrdU-injected (50 mg/kg) twice with a 2-h delay between injections. BrdU was freshly prepared in PBS with 0.07N NaOH. Two hours after the last injection, the dams were euthanized and embryos were collected for further analysis.

Immunofluorescence

Embryonic brains were fixed in 4% paraformaldehyde in PBS overnight at 4°C and cryoprotected at 4°C in sequential 20 and 30% sucrose solutions. The brains were embedded in O.C.T. embedding medium (Sakura, Torrance, CA, USA) and stored at -80°C until coronal sections (10 μ m) were prepared using a cryostat. For proliferating cell nuclear antigen (PCNA) staining, antigen unmasking was performed as follows: sections were rehydrated with PBS and incubated for 10 min in 10 mM sodium citrate (pH 6.5) at 80°C. Non-specific antibody binding was blocked with 10% normal goat serum (Sigma, St Louis, MO, USA) containing 0.1% Triton X-100 (Sigma). Sections were incubated overnight at 4°C with the relevant primary antibodies, washed with PBS, followed by incubation with the appropriate species-specific Alexa Fluor 488-conjugated IgG (1 : 1000; Invitrogen, Carlsbad, CA, USA) antibodies in the dark at 25°C for 1 h. Sections were counter-stained with 4'-6-diamidino-2-phenylindole (DAPI; 0.05 mg/mL solution in

PBS; Molecular Probes, Eugene, OR, USA). Slides were mounted using Permafluor mounting solution (Lab vision, Fremont, CA, USA) and kept at 4°C for volumetric analysis and cell counting. For BrdU staining, slices were washed with Tris-buffered saline (TBS) and incubated in 2N HCl at 37°C for 30 min. Slices were then transferred to 0.1 M Boric acid (pH 6.5) for 10 min at 25°C. Slices were washed in TBS and blocked for 30 min in 3% goat serum. Slices were incubated in rat anti-BrdU (1 : 50; Harlan, Indianapolis, IN, USA) for 72 h at 4°C. Slices were subsequently washed with TBS and the appropriate alexafluor goat anti-rat antibodies were applied. Slices were washed in TBS and mounted in Permafluor. For staining of NPC, E12 NPC plated on polyethyleneimine (Sigma) coated eight-well chamber slides (Nunc, Naperville, IL, USA) were maintained in culture for 10 days with the medium replenished every 3 days. NPC were then fixed with fixation solution (4% paraformaldehyde, 4% sucrose in PBS) at 25°C for 10 min, followed by three washes in PBS and incubated with primary and secondary antibodies as described above.

The following antibodies were used for immunofluorescence: anti-TLR1 (see Alexopoulou *et al.* 2002; 1 : 100; Cat# 14-9011; eBioscience (San Diego, CA, USA) [this antibody recognizes the TLR1 epitope in immunofluorescence, but not in paraffin-embedded immunohistochemistry or western blotting]); anti-TLR2 (see Dulay *et al.* 2009; 1 : 100; Cat# ab47840, Abcam); anti-TLR6 (1 : 100; Cat# sc-5661; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Sox2 (see Wang *et al.* 2009; 1 : 500; Cat# MAB-2018; R&D systems); anti-S100 β (see Markovitz *et al.* 1997; 1 : 100; Cat# Z0311; DakoCytomation); anti-Microtubule-associated protein 2 (MAP2) (see Annies *et al.* 2006; 1 : 100; Cat# ab5392; Abcam); anti-Myelin Basic Protein (MBP) (see Lattanzi *et al.* 2010; 1 : 100; Cat# MAB386; Chemicon); anti-PCNA (see Lathia *et al.* 2008; 1 : 200; Cat# ab29; Abcam); anti-Ki67 (see Wong *et al.* 2009; 1 : 100; Cat# ab15580; Abcam); and anti-PH3 (see Lathia *et al.* 2008; 1 : 100; Cat# ab5176; Abcam). Specificity of staining for TLR1, 2 and 6 was verified using RAW264 cells (Fig. S1A).

Immunohistochemistry

Embryonic brains or whole embryos were fixed in Bouin's solution (Cat# HT10132; Sigma) for 24 h at 25°C. Embryos were then transferred to a 70% ethanol solution that was replaced every day until solution became transparent. Following that, tissues were immersed at 80%, 85%, 90%, 95%, and 100% ethanol solutions for 1 h each. Fixed tissues were then paraffin-embedded and 5 μ m thick slices were mounted on poly-L-lysine coated slides. After deparaffinization and hydration through a graded alcohol series, endogenous peroxidase was quenched by incubation with 0.3% peroxide in methanol for 30 min. A Citrate buffer (pH 6.0) was applied to the tissue sections for antigen retrieval as needed, and the process was carried out before primary antibody incubations. Thereafter, the slides were incubated with antibodies against TLR2 (1 : 200 dilution; Cat# ab47840; Abcam) for 24 h at 4°C. A standard labeled streptavidin–biotin–peroxidase complex (Vector ABC kit, Burlingame, CA, USA) was used to visualize TLR1, TLR2, and TLR6 immunoreactions. Tissues were stained for 5 min with 3,3'-diaminobenzidine as the chromogen (Vector) and counter-stained with hematoxylin, dehydrated, and cover-slipped. Primary antibodies were omitted in negative controls.

Stereology

Calculations of ventricle volume and the total proliferative area were based on areas obtained by tracing contours around closely matched sections of ventricles in which cell nuclei were stained with the DNA-binding dye DAPI (20 \times objective) or around the proliferative area of sections immunostained with PCNA and Ki67 antibodies (20 \times objective). Images were acquired using an Olympus BX51 epifluorescence microscope (Olympus, Center Valley, PA, USA) modified for stereology with a computer-driven motorized stage, a microfire video camera interfaced to a PC equipped with Stereo Investigator 8 software (MicroBrightfield, Inc., Williston, VT, USA). Ventricular volume was measured in three closely matched sagittal sections (spaced 100 μ m apart) and an average ventricular volume was calculated. Measurements were performed on five animals in each experimental group. Volume measurements of the proliferative zone were performed between contours surrounding the ventricle and the proliferative area as defined by PCNA and Ki67 staining. The borders of proliferative areas were defined at the edge of the continuum of cells positively stained for PCNA or Ki67. Areas with more diffuse cellular staining (i.e., near-by cells that were not stained with Ki67 or PCNA antibodies) were considered outside the proliferative area. Measurements were performed in three closely matched sagittal sections (spaced 100 μ m apart) and an average volume of the proliferative zone was calculated. The same area was used to quantify cell number and density. Cells were counted in randomly selected 20 μ m \times 20 μ m squares within a 130 μ m \times 130 μ m grid throughout the proliferative area. These parameters were selected based on the minimum area for which cell counts yielded a confidence of variance < 0.1.

Immunoblotting

Brain tissue protein was extracted using a modified RIPA buffer (150 mM sodium chloride, 50 mM Tris-HCL, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1% Triton-X100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate) supplemented with protease inhibitor cocktail (Cat# 2714; Sigma) and phosphatase inhibitor cocktails 1 and 2 (Cat# P2850 and P5726 respectively; Sigma). Protein concentration was determined using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). Proteins in samples (40 μ g) were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (4–12% polyacrylamide gradient gel) and transferred to a nitrocellulose membrane. The membrane was blocked in 5% non-fat milk for 1 h at 25°C, followed by an overnight incubation at 4°C with primary antibodies: TLR2 (1 : 1000, Cat# ab47840; Abcam), TLR6 (1 : 400, Cat# sc-5661; Santacruz), Myd88 (1 : 1000; Cat# ab2064; Abcam), IRF3 (1 : 1000, Cat# 4962; Cell Signaling Technology, Beverly, MA, USA), IRF5 (1 : 1000, Cat# ab21689; Abcam), and α -Tubulin (1 : 10,000; Cat# T6074; Sigma) diluted in 1% non-fat milk and 1% bovine-serum albumin in PBS. The membrane was then washed and incubated with a secondary antibody diluted in 1% non-fat milk and 1% bovine-serum albumin in PBS for 1 h at 25°C. Protein bands were visualized using a chemiluminescence detection kit (Pierce Endogen, Rockford, IL, USA).

Neurosphere cell cultures

Neuronal progenitor cells from the cortex were propagated as free-floating aggregates to promote proliferation of neural stem and

progenitor cells prior to use in experiments. In brief, the dorsal telencephalon from E15 mouse embryos was isolated, mechanically dissociated, and cells were seeded at a density of 200 000 per mL in a T75 flask containing Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with B27 (1 : 50; Invitrogen), epidermal growth factor, and fibroblast growth factor 2 (both at 20 ng/ml; R&D Systems Inc, Minneapolis, Ramsey, MN, USA). After neurospheres had grown for 7 days in culture, cells were dissociated using a NeuroCult cell dissociation kit (StemCell Technologies, Vancouver, BC, Canada) and plated into 96-wells plates (Nunc) at 2000 cells per well. Neurospheres which formed were counted after 7 days in culture and the average of 10 wells was determined for each experimental condition. Neurosphere forming assays for E15 WT and TLR2KO NPC were performed in triplicate; each replicate consisted of a pool of embryos from one litter. To measure neurosphere diameter, pictures of 10 wells of each experimental condition were taken using a Magnafire camera (Optronics, Goleta, CA, USA). Images were then analyzed using ImageJ software (<http://rsbweb.nih.gov/ij/>). To induce differentiation, growth factors were withdrawn and neurospheres were plated on polyethyleneimine (Sigma) coated dishes. Immunofluorescence in NPC was performed on E12 derived NPC plated on Polyethyleneimine (PEI)-coated dishes. Differentiation of these cells was obtained by epidermal growth factor and basic fibroblast growth factors withdrawal.

Lactate dehydrogenase (LDH) assay

Conditioned medium from NPC cultures treated with Pam₃CSK₄ or FSL1 were assayed using the LDH cytotoxicity kit (Roche, Indianapolis, IN, USA). Absorbance was read at 540 nm.

Low molecular weight hyaluronan preparation

High molecular weight hyaluronan (HMW-HA) obtained from human umbilical cord (H1504; Sigma) LMW-HA was digested using hyaluronidase (H4272; Sigma) as previously described (Ikegami-Kawai and Takahashi 2002). Briefly, 5 mg/mL HMW-HA in distilled water was incubated with 500 U hyaluronidase 50 µL in Double deionized water (DDW) at 37°C for 48 h. Quality of LMW-HA preparations was examined using Alcian-Blue stained-polyacrylamide gel electrophoresis as previously described (Ikegami-Kawai and Takahashi 2002). Briefly, HMW-HA and LMW-HA were run on a 10% bis-tris gel. Samples were prepared by loading 5 µL of HA samples supplemented with 2.4 µL 2M sucrose in Tris Buffered EDTA (TBE) solution. An additional lane running 0.05% bromo phenol blue in TBE/0.3 M sucrose solution was used to indicate running stage. Gels were run in a cold room under 100 V until bromo phenol blue reached 1 cm from the bottom of the gel. Gels were then stained using 0.05% Alcian blue in 2% acetic acid in water for 30 min in the dark. HMW and LMW-HA are visualized as blue bands.

Mitochondrial activity assay

Neuronal progenitor cells were plated on 96-well plates and treated with either Pam₃CSK₄ or FSL1 for 48 h. NPC were then incubated with 10 µM Rezasurin Dye (Sigma) for 30 min. Fluorescence was measured using 540 nm excitation and 590 nm emission wavelengths.

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated using the QiaZol reagent (Qiagen, Valencia, CA, USA). RNA was then treated with DNase I to remove contaminating DNA followed by RNA cleanup using RNeasy Minelute cleanup kit (Qiagen). Reverse transcription of RNA to cDNA was carried out using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). For semi-quantitative PCR, the cDNA synthesized was subjected to PCR amplification using Platinum Blue PCR SuperMix (Invitrogen). PCR was performed using PTC-200 (MJ Research, Ramsey, MN, USA) PCR system, DNA was separated in a 1% TBE-agarose gel and the DNA was visualized by ethidium bromide staining. PCR amplification consisted of 30 cycles of 30 s denaturation at 94°C, 30 s annealing, and 30 s of elongation at 72°C. For real-time PCR reactions, SYBR GREEN PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) was used in a PTC-200 with a Chromo 4 fluorescence detector (Bio-Rad). The following primers were used throughout the study: TLR1 – Forward: 5'-ATG ATTCTGCCTGGGTGAAG-3', Reverse: 5'-TCTGGATGAAGTGG GGAGAC-3' (174 bp, annealing temperature 53°C); TLR2 – Forward: 5'-CTCCCACTTCAGGCTCTTTG-3', Reverse: 5'-AGG AACTGGGTGGAGAACC-3' (217 bp, annealing temperature 53°C); TLR6 – Forward: 5'-ACACAATCGGTTGCAAAACA-3', Reverse: 5'-GGAAAGTCAGCTTCGTCAGG-3' (128 bp, annealing temperature 53°C); CD14 – Forward: 5'-AGGACTAGACCTTA GTCACA-3', Reverse: 5'-GAGTCCAAAAGGGATTTC-3' (240 bp, annealing temperature 51°C); CD36 – Forward: 5'-GCT TGCAACTGTTCAGCACAT-3', Reverse: 5'-GAGCTATGCAAGCAT GGAACA-3' (290 bp, annealing temperature 53°C); Myd88 – Forward: 5'-ACTGGCCTGAGCAACTA-3', Reverse: 5'-CTTCTT TTCTGGGGTAGGG-3' (360 bp, annealing temperature 54°C). PCRs were normalized against β-actin (Forward: 5'-TGGAATCC TGTGGCATCCATGAAAC-3', Reverse: 5'-TAAAACGCAGCT CAGTAACAGTCCG-3', 348 bp, annealing temperature 53°C). The following three primers were used in conjunction to amplify TLR2 mRNA that exists in WT, but not TLR2KO animals (Fig. 1a, right panel): 5'-CTTCTGAATTTGTCCAGTACA-3'; 5'-GGCCAGCTCATTCTCCAC-3'; 5'-ACGAGCAAGATCA ACAGGAGA-3', 499 bp (WT), 334 bp (TLR2KO), annealing temperature 52°C.

Luciferase reporter activity assay

Primary neurospheres obtained from WT and TLR2KO mice were dissociated and plated at 4000 cells/mL. Cells were transfected with NF-κB, Activator protein-1 (AP-1), or NF-E2-related factor 2 (Nrf-2) plasmids along with renilla plasmid using Lipofectamin (Invitrogen) according to the manufacturer's recommendation. At 24 h after transfection, luciferase activity was quantified using a Dual-Luciferase-Reporter System (Promega, Madison, WI, USA) and normalized to renilla expression. All analyses were performed in triplicate.

Statistical analysis

Experimental results are expressed as mean ± SEM. Statistical comparisons were made with ANOVA, followed by Newman-Keuls *post hoc* analysis for pairwise comparisons. All statistical analyses were performed using OriginPro 8.0 software (Northampton, MA, USA).

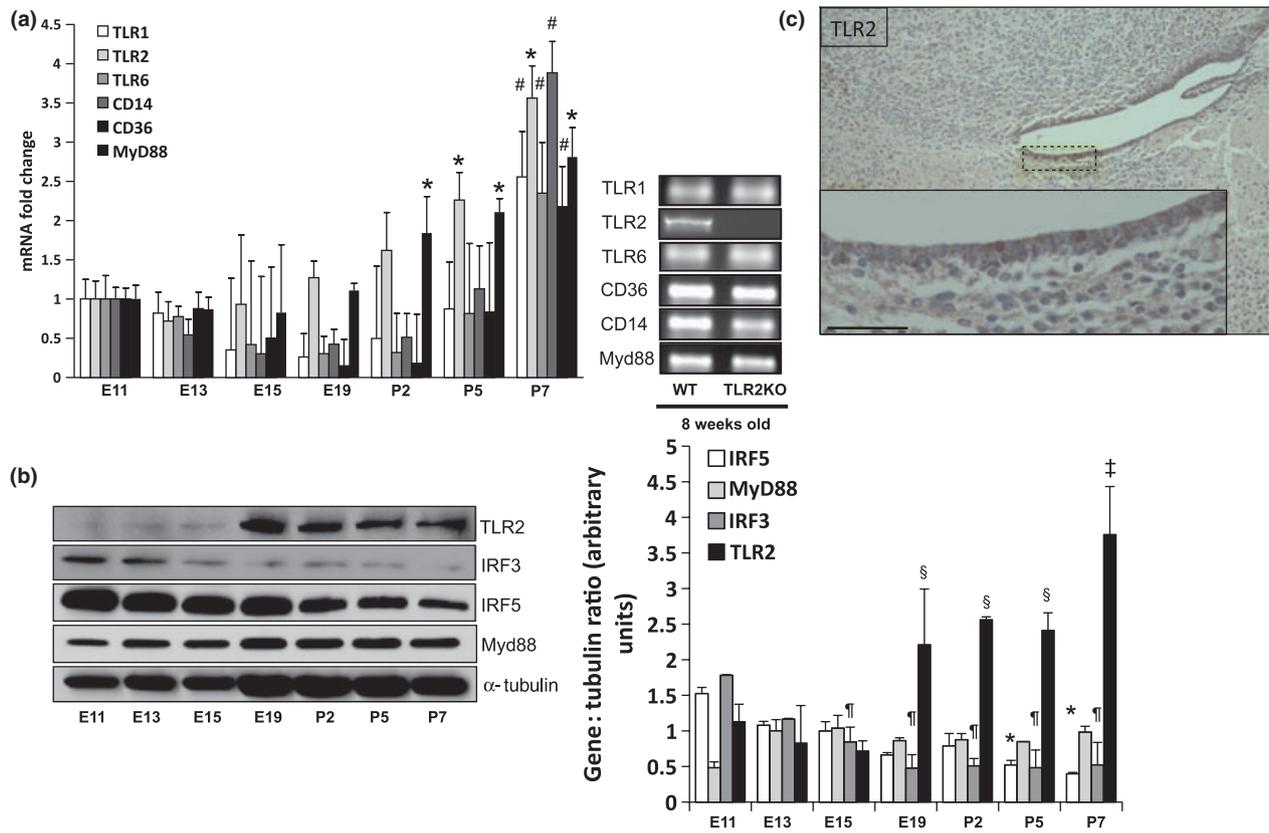


Fig. 1 Toll-like receptor 2 (TLR2) is expressed in the brain cortex during development. (a) Real-time PCR analysis of TLR1, TLR2, TLR6, CD36, CD14, and Myd88 at different embryonic and early postnatal developmental stages (left panel); PCR analysis of the same genes in adult (8 weeks old) wild type and TLR2-deficient mice (right panel). * $p < 0.05$ compared with E11–E19 developmental stages. # $p < 0.05$ compared with E11–P5 developmental stages (two-way ANOVA). (b) Immunoblots of TLR2, interferon regulatory factor 3 (IRF3), IRF5, and MyD88 proteins in the developing cortex (left panel); den-

sitometric analysis of protein levels normalized to α -tubulin levels (right panel). § $p < 0.05$ compared with E11–E15 developmental stages. ‡ $p < 0.05$ compared with E11–P5 developmental stages. * $p < 0.05$ compared with E11 developmental stage. ¶ $p < 0.05$ compared with E11 developmental stage (two-way ANOVA). For each developmental stage, $n = 3$. (c) Sagittal section showing immunostaining for TLR2 with hematoxylin counter-staining in the SVZ of an E15 embryo. Scale bar = 200 μm . Scale bar of inset images = 50 μm . V, ventricle.

Results

TLR2 receptor complex components are expressed in the telencephalon during embryonic and early postnatal development and in cultured embryonic NPC

TLR2 can function as a heterodimer with either TLR1 or TLR6. In addition, both TLR2/1 and TLR2/6 heterodimers use CD14 as a co-receptor, while CD36 is a co-receptor for TLR2/6 alone (Triantafyllou *et al.* 2006). Both TLR2/1 and TLR2/6 require the Toll/interleukin-1 receptor (TIR) adaptor protein Myd88 for intracellular signaling. We therefore asked whether the TLR2 receptor, its signaling adaptor protein Myd88 and two TLR-related transcription factors, IRF3 and IRF5 are expressed in NPC *in vitro* and *in vivo*. To examine the developmental expression of TLR2 and its signaling components, we first measured

levels of mRNAs encoding TLR1, TLR2, TLR6, CD14, CD36, and MyD88 during embryonic and early postnatal development of the cerebral cortex. The mRNAs for TLR1, TLR2, TLR6, CD14, CD36, and Myd88 were all expressed in the cortex of embryos as early as E11. mRNA levels for Myd88 were significantly elevated at P2, mRNA levels for TLR2 were significantly elevated at P5, and mRNA levels of TLR1, TLR6, CD14, and CD36 were greatest at P7 (Fig. 1a, left panel). In the adult cortex, all these genes are expressed even in the absence of TLR2 (Fig. 1a, right panel). Protein levels for TLR2 are expressed at E11–E15, and have a significant sustained increase at later embryonic and postnatal ages (Fig. 1b), a result consistent with the expression levels of TLR2 mRNA. Interestingly, the protein levels of Myd88 were highly and stably expressed during embryogenesis as well as at postnatal stages (Fig. 1b) despite a consistent and

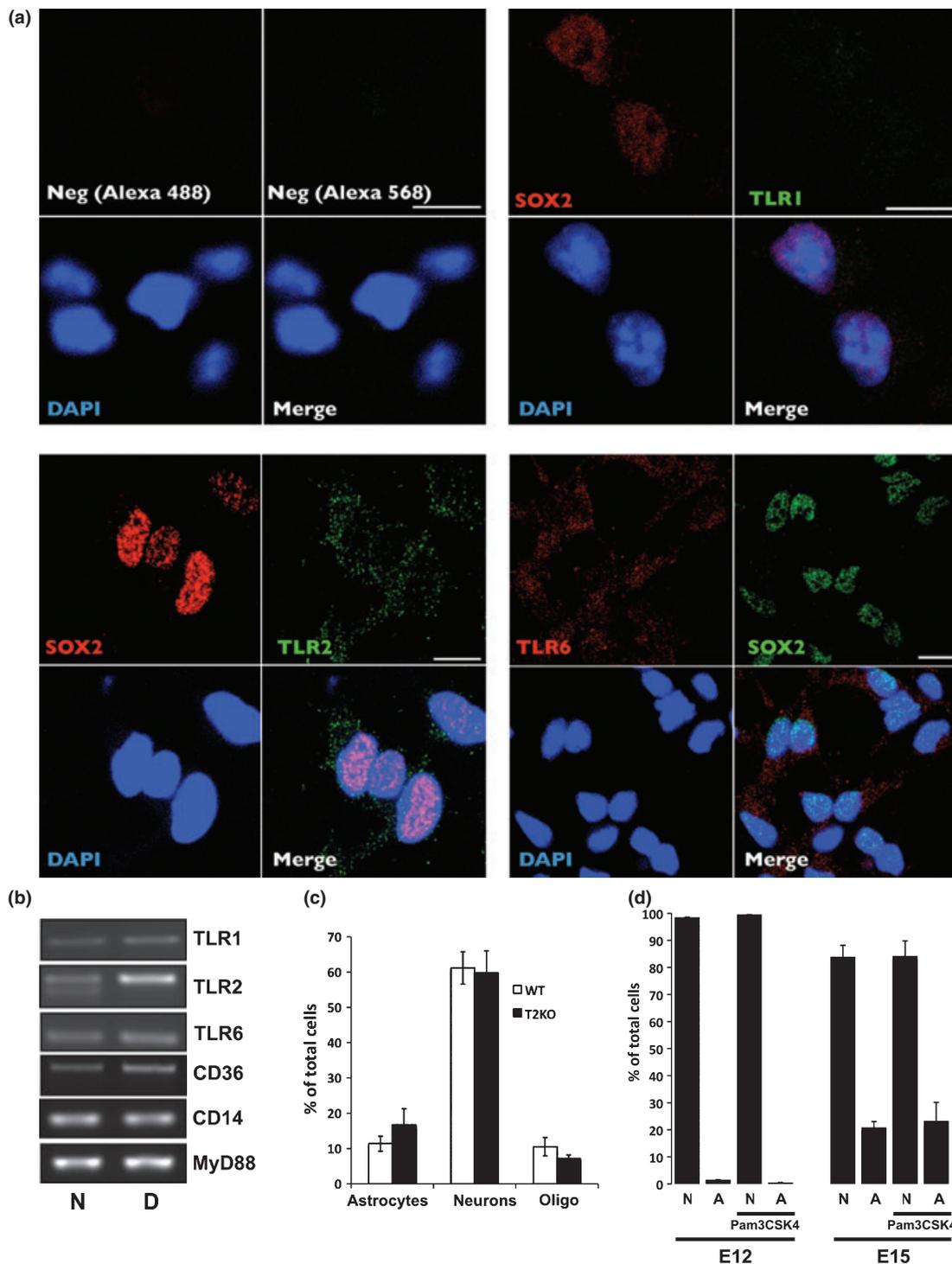


Fig. 2 Toll-like receptor 2 (TLR2) mRNA and protein is expressed in cultured embryonic neuronal progenitor cells (NPC). (a) SOX2⁺ embryonic NPC *in vitro* express TLR2 and TLR6, but not TLR1. (b) Representative PCRs for TLR1, TLR2, TLR6, CD36, CD14, and Myd88 mRNA expression in NPC (N) and differentiated NPC (D). (c) No difference is observed between E15 TLR2-deficient and wild-type mice NPC in their differentiation capacity. The graph shows percentage of astrocytes, neurons, and oligodendrocytes differentiated from

E15.5-derived NPC (values are the mean and SEM; $n = 3$ experiments). (d) While E15-derived NPC give rise to fewer neurons and more glial cells compared with E12-derived NPC, TLR2 activation using Pam₃CSK₄ does not change the differentiation ratio into neurons (N, determined by MAP2⁺ staining) or astrocytes (A, determined by S100 β ⁺ staining) in both E12 and E15 derived NPC. Scale bar = 10 μ m.

significant increase in its mRNA levels (Fig. 1a, left panel). IRF5 is a transcription factor that is recruited by MyD88 following TLR2 activation. Surprisingly, the protein levels of IRF5 consistently decrease during cortical embryogenesis and are significantly lower at postnatal stages P5 and P7 compared with earlier stages (Fig. 1b). Interestingly, the protein levels of IRF3, a transcription factor that is activated following TLR3 and TLR4 (but not TLR2) activation also decrease during cortical embryogenesis between E15 and P7 compared with E11 developmental stages (Fig. 1b), suggesting a developmental role in addition to mediating signaling via TLR2, TLR3, and TLR4. TLR2 is also expressed on proliferating cells in the embryonic Subventricular zone (SVZ) (Fig. 1c). TLR2 is broadly expressed in cells throughout the developing mouse embryo, including the brain (Fig. S2A). The level of TLR2 immunoreactivity in the SVZ (Fig. 1c) is considerable, but not as great as its levels in immunogenic tissues, such as the thymus (Fig. S2B) or the spleen (Fig. S2C). TLR2 was also relatively strongly expressed in the choroid plexus in the ventricles (Fig. S2D), and was absent in other tissues, such as cartilage (Fig. S2E).

Since TLR2 is expressed in the embryonic SVZ brain *in vivo*, we next examined its expression in telencephalic embryonic NPC. The protein expression of TLR1, TLR2, and TLR6 was assessed by immunostaining in SOX2-expressing cultured embryonic NPC. SOX2-expressing NPC were positively stained for TLR2 and TLR6, but not TLR1 (Fig. 2a). Similar to developing cortical tissue, embryonic NPC, as well as NPC-derived differentiated cells that were grown for 10 days in culture express mRNA for TLR1, TLR2, and TLR6 as well as their co-receptors, CD14 and CD36 and the adaptor protein MyD88 (Fig. 2b). Adult hippocampal NPC from TLR2KO mice give rise to a lower percentage of neurons and a higher percentage of astrocytes than their WT counterparts (Rolls *et al.* 2007). However, it is unknown whether embryonic TLR2-deficient NPC differs from their WT counterparts in their differentiation potential or whether TLR2 activation affects the fate of differentiating NPC. The ability of E15 cortical NPC to give rise to astrocytes, oligodendrocytes, and neurons was not compromised by TLR2 deficiency compared with WT NPC [WT and TLR2KO cells, respectively: astrocytes (S100 β ⁺ cells) 11.4 \pm 2.1% and 16.7 \pm 4.5%; neurons (MAP2⁺ cells) 61.1 \pm 4.5% and 59.8 \pm 6.3%; oligodendrocytes (Myelin Basic Protein (MBP⁺) cells) 10.5 \pm 2.6% and 7.2 \pm 0.088%; Fig. 2c]. TLR2 activation of both E12 or E15 derived NPC did not result in significant changes in differentiation proportions to neurons or astrocytes (E12 derived NPC: MAP2⁺ cells, 99.5 \pm 0.03% vs. 98.5 \pm 0.05% in PBS-treated cells; S100 β ⁺ cells, 0.5 \pm 0.04% vs. 1.51 \pm 0.06% in PBS-treated cells; E15 derived NPC: MAP2⁺ cells, 84.2 \pm 5.6% vs. 83.5 \pm 4.2% in PBS-treated cells; S100 β ⁺ cells, 23.22 \pm 6.9% vs. 20.8 \pm 2.1% in PBS-treated cells) (Fig. 2d).

Activation of TLR2 heterodimers inhibits NPC proliferation

The presence of TLR3 was recently reported to negatively regulate the proliferative capability of NPC in the developing embryo (Lathia *et al.* 2008). To determine whether TLR2 also alters NPC dynamics, we asked whether NPC derived from TLR2-deficient embryos exhibit differences in proliferation. No significant difference was observed between TLR2-deficient and WT NPC in both primary (Fig. S3A) and secondary (Fig. S3B) sphere formation, or in sphere diameter (Fig. S3C). In addition, no difference was observed in viability between the cells as measured by mitochondrial activity (Fig. S3D) at 7 days *in vitro*. Correlated with these *in vitro* observations, no difference is observed between WT and TLR2KO mice in ventricular size, proliferative area size, the number of proliferative cells in the SVZ, and in cell density in the SVZ of E16.5 mouse embryos (Fig. S3E).

We next examined whether activation of the TLR2 receptor complex altered proliferation of NPC. This was accomplished using the TLR2/6-specific ligand FSL1 (a synthetic lipoprotein from *Mycoplasma salivarium*; Kiura *et al.* 2006) and the TLR2/1-specific ligand Pam₃CSK₄ (a synthetic bacterial lipopeptide; Patel *et al.* 2005). NPC activated with Pam₃CSK₄ (1 μ g/mL) or FSL1 (1 μ g/mL) formed significantly fewer spheres compared with control NPC (80.11 \pm 1.43 for Pam₃CSK₄ and 79.51 \pm 1.63 for FSL1, both % of control; Fig. 3a). However, the diameter of the spheres following Pam₃CSK₄ or FSL1 treatment was not reduced (Fig. 3b). Further, the inhibitory effects of Pam₃CSK₄ and FSL1 on sphere formation in WT cells were not correlated with increased toxicity when measured 24 h following treatment using a LDH release assay (Fig. 3c). The combined treatment of Pam₃CSK₄ and FSL1 revealed an additive effect rather than a synergistic effect on sphere formation (70.42 \pm 1.64, % of control; Fig. 3a). Similar to single ligand treatments, combined administration had no effect on sphere diameter (Fig. 3b) or viability (Fig. 3c). LMW-HA, an endogenous ligand for TLR2 and TLR4 was also tested for its effects on NPC proliferation. LMW-HA produced by digestion of HMW-HA by hyaluronidase showed inhibition of sphere formation at the dose of 1 μ g/mL (Fig. S4).

During bacterial infections, not only are TLR2 ligands present in the site of infection, but also ligands for other TLRs are present, such as the TLR4 ligand lipopolysaccharide (LPS), the TLR5 ligand, flagellin, and the TLR9 ligand CpG. We therefore tested whether a combination of these four ligands exacerbates the effect of TLR2 ligands on sphere formation. While activation of TLR2 using Pam₃CSK₄ resulted in significant inhibition of sphere formation, no combination of the ligands for TLR4 (LPS, 1 μ g/mL), TLR5 (flagellin, 1 μ g/mL), and TLR9 (CpG DNA, 10 μ M) increased this effect (Fig. 3d). Similar results were observed following combined treatment with FSL1 (1 μ g/mL) and ligands for TLR4, TLR5, and TLR9 (data not shown). None of the TLR ligand combination treatments had an effect on

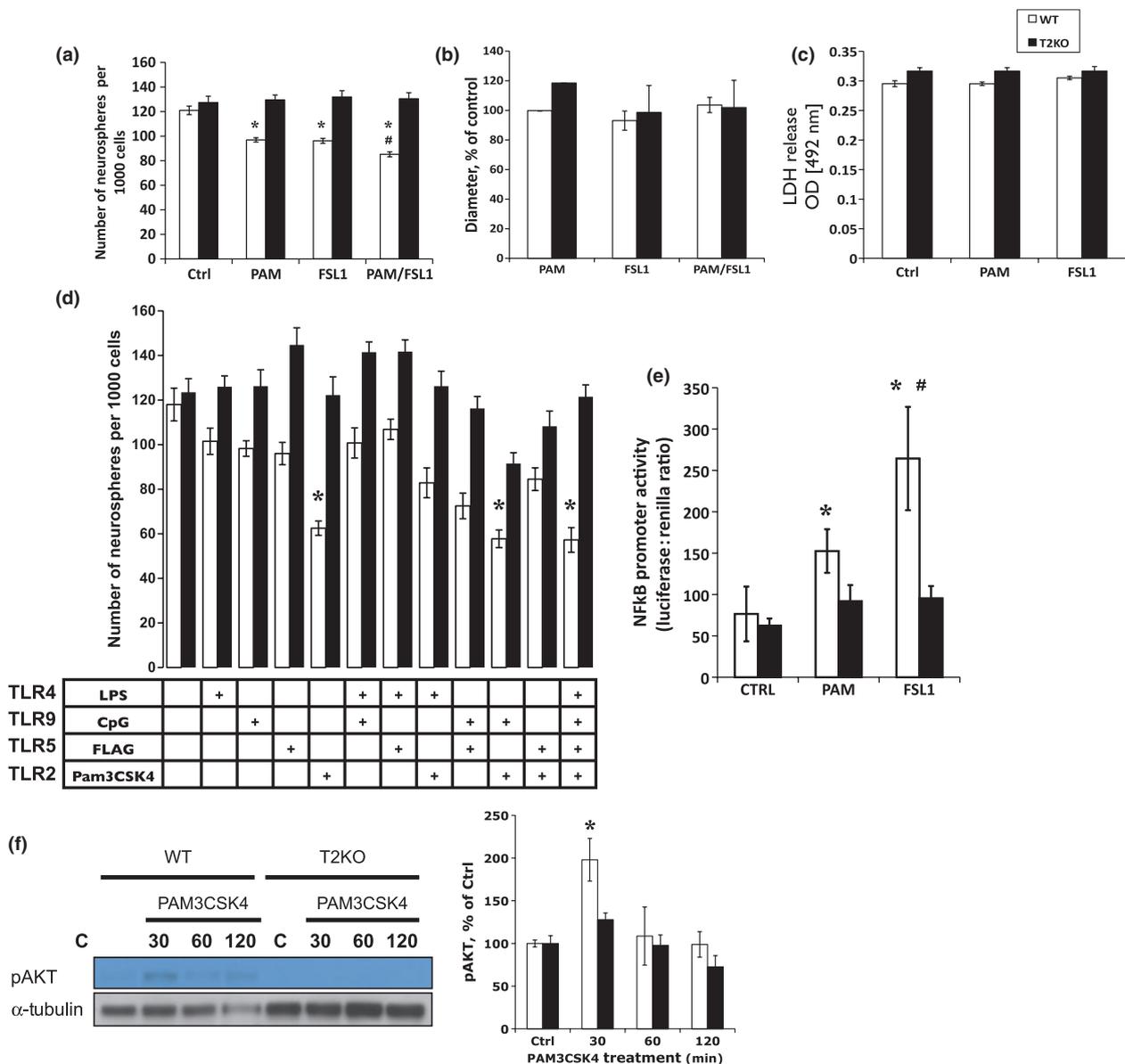


Fig. 3 Toll-like receptor 2 (TLR2) activation inhibits neurosphere formation. (a) Sphere formation is reduced following Pam₃CSK₄ and FSL1 administration, while the combined administration of both ligands results in a mild additive effect. [(a–f) open bars wild type (WT), filled bars TLR2-deficient mice (TLR2KO)]. (b and c) Pam₃CSK₄, FSL1 or their combination does not alter neurosphere diameter (b) or viability of cells (c) in the neurospheres. (d) Inhibition of neurosphere formation is specific for TLR2. Administration of lipopolysaccharide, CpG, Flagellin, Pam₃CSK₄, or any combination of these ligands did

not enhance the inhibition of neurosphere formation induced by Pam₃CSK₄ alone. (e) Pam₃CSK₄ and FSL1 significantly increase NF-κB activation in NPC. (f) Pam₃CSK₄ activation results in rapid phosphorylation of AKT at 30 min in WT but not TLR2KO cells (left panel); densitometric analysis of AKT phosphorylation (right panel). (For all graphs the values are the mean and SEM; *n* = 5 experiments). **p* < 0.05 compared with control; #*p* < 0.05 compared with Pam₃CSK₄ (two-way ANOVA). Legend refers to all graphs.

the viability of the cells when measured 48 h following treatment (Fig. S5). Depending on the cell type, TLR2 mediates its innate immune responses in part through the activation of transcription factors, such as NF-κB, AP-1, or Nrf-2. We therefore examined the ability of TLR2 agonists to activate NF-κB in NPC. Both TLR2 ligands significantly

induced NF-κB promoter activity, and FSL1 administration resulted in a significantly greater induction of the NF-κB promoter than Pam₃CSK₄ (Fig. 3e). These effects were not observed in TLR2KO NPC (Fig. 3e). Interestingly, although reported to activate AP-1 and Nrf-2 transcription factors in other cell types, TLR2 ligands failed to activate either AP-1

(Fig. S6A) or Nrf-2 (Fig. S6B) in NPC. Interestingly, sulforaphane, an Nrf-2 agonist failed to induce Nrf-2 activation in NPC but did activate Nrf-2 in SH-SY5Y cells (Fig. S6B). Consistent with other reports (Akira and Takeda 2004), TLR2 activation induces phosphorylation of Akt in NPC that was evident at 30 min and then was reduced at 60 min (Fig. 3f).

TLR2 heterodimer activation results in telencephalic dysplasia in embryonic mice

Because TLR2 activation reduced sphere formation of NPC derived from the embryonic brain *in vitro*, we next examined the effect of intracerebral injections of Pam₃CSK₄ and FSL1 on ventricular morphology and numbers of proliferating cells in the periventricular region *in utero*. When injected into the ventricles of E15 WT mouse embryos, Pam₃CSK₄ (0.1 µg in 1 µL of PBS) induced a significant increase in ventricle size, as measured at E16.5 (Fig. S7, PBS, $184 \pm 43 \mu\text{m}^3 \times 10^{-2}$, $n = 5$; Pam₃CSK₄, $283 \pm 38 \mu\text{m}^3 \times 10^{-2}$, $n = 5$), and further increased by E18.5 (Fig. 4a,b; PBS, $162.88 \pm 19.72 \mu\text{m}^3 \times 10^{-2}$, $n = 5$; Pam₃CSK₄, $487.51 \pm 30.67 \mu\text{m}^3 \times 10^{-2}$, $n = 5$). Pam₃CSK₄ administration resulted in a downward trend in the size of the proliferative area at E16.5 (PBS, $410 \pm 48.2 \mu\text{m}^3 \times 10^{-3}$; Pam₃CSK₄, $350.9 \pm 39.3 \mu\text{m}^3 \times 10^{-3}$; Fig. S7) and at E18.5, the proliferative area was significantly smaller (PBS, $319.65 \pm 8.88 \mu\text{m}^3 \times 10^{-3}$; Pam₃CSK₄, $289.19 \pm 9.1 \mu\text{m}^3 \times 10^{-3}$; Fig. 4a,b). This decrease was not observed, however, in TLR2KO mice ($p > 0.05$, $n = 5$) implying specificity for TLR2 activation in this effect. As a result, cell density in the proliferative area, as determined by Ki67 and PCNA staining was significantly higher in Pam₃CSK₄-treated mice (PBS, $0.0121 \pm 0.000213 \text{ cells}/\mu\text{m}^3$; Pam₃CSK₄, $0.0134 \pm 0.000125 \text{ cells}/\mu\text{m}^3$; Fig. 4a,b). At E18.5, no difference was observed in the total number of proliferating cells (PBS, 4313.02 ± 113.44 ; Pam₃CSK₄ 4478.5 ± 110.6), as measured using PCNA or Ki67, however, Pam₃CSK₄ treatment caused a significant decrease in the numbers of BrdU⁺ cells in the SVZ (PBS, 840.2 ± 20.44 ; Pam₃CSK₄, 690.5 ± 21.6 ; Fig. 4c). Moreover, Pam₃CSK₄ treatment caused a significant twofold increase in the number of PH3⁺ cells (cells at M-phase of the cell cycle) (PBS, 77.8 ± 10.19 ; Pam₃CSK₄, 135.73 ± 10.9 ; Fig. 4d) and an increase in the number of BrdU⁻ PH3⁺ cells compared with PBS-treated mice (Fig. 4e), indicating an increase in the number of cells arrested in their M-phase of the cell cycle prior to the BrdU injection at E18.5. Importantly, in all parameters tested following *in utero* injections both at E16.5 as well as E18.5, we did not observe differences between the ipsilateral and contralateral hemispheres (data not shown).

Therefore, activation of heteromeric TLR2 receptors during embryonic development results in increased ventricle size, decreased proliferative area, an increase in PH3⁺ cells, and a decrease in the numbers of BrdU⁺ cells. These findings

reveal an adverse effect of TLR2 activation during late embryonic development that results in a reduction in the proliferation of cells in the SVZ.

Discussion

Our data show that: (i) TLR2 is expressed in the developing mouse brain and in cultured embryonic NPC. (ii) The presence of TLR2 does not influence NPC proliferation both *in vitro* and *in vivo*, whereas its activation inhibits NPC proliferation *in vitro* and decreases cell proliferation *in vivo*. (iii) Activation of TLR2 with bacterial ligands *in utero* results in ventricular dysplasia. The lack of a difference in the proliferation rate of NPC from WT and TLR2KO mice, and the similar ventricular size and gross morphology of brains in WT and TLR2KO embryos, suggests that TLR2 signaling does not play a critical role in embryonic brain development under normal conditions. However, we cannot rule out more subtle effects of endogenous TLR2 signaling on the development of cortical cytoarchitecture, or on the behavior of NPC in the adult brain. In a study of adult hippocampal NPC, TLR2 deficiency did not alter the proliferation of NPC, but did affect their phenotypic fate by increasing the formation of astrocytes at the expense of neurons (Rolls *et al.* 2007). In contrast, in this study, the absence of TLR2 did not alter the proportion of astrocytes, oligodendrocytes, and neurons that differentiate from embryonic NPC. This apparent discrepancy may result from differences in NPC-specific developmental transcriptional changes at embryonic and adult stages, or alternatively from differences between NPC niches in the developing and adult brain. TLR2 activation in differentiating embryonic NPC derived from either E12 or E15 developmental stages does not affect differentiation proportions to neurons or astrocytes, limiting the effects of TLR2 activation in NPC to proliferation inhibition only.

Very little data exist regarding the expression and functions of TLRs in the developing nervous system. TLR8 is abundant in embryonic brain as early as E12, however, this expression is limited to sympathetic ganglia and post-mitotic migrating cortical neurons and axons and is not present in the proliferating ventricular zone (Ma *et al.* 2006). In contrast, NPC express TLRs 2, 3, and 4 from early in CNS development (Lathia *et al.* 2008). TLR3 is transiently increased from early brain development (E12.5) and begins to diminish at E17.5 (Lathia *et al.* 2008). In contrast, TLR4 maintains relatively constant expression levels throughout neuronal development and into postnatal stages (Lathia *et al.* 2008). Our data indicate that TLR2 mRNA is expressed at E11 and into postnatal ages *in vivo* as well as in SOX2-expressing NPC *in vitro*. Notably, while both mRNA and protein levels for TLR2 increase during embryogenesis and into postnatal ages, mRNA for MyD88 increases while its protein levels remain constant during embryogenesis and early postnatal stages. This could be because of reduced

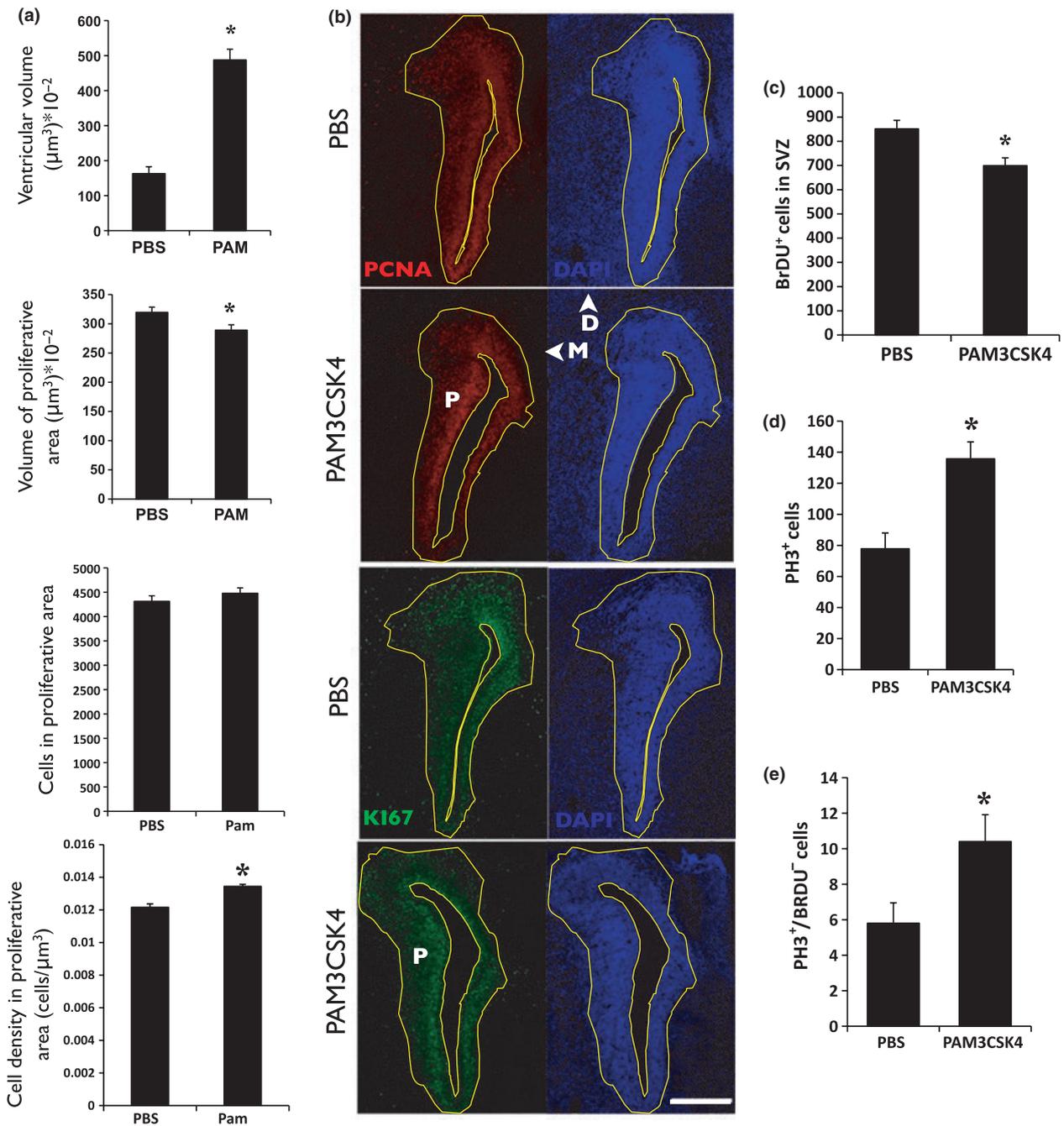


Fig. 4 Toll-like receptor 2 (TLR2) heterodimer activation *in utero* at E15 results in telencephalic dysplasia at E18.5. (a) Pam₃CSK₄ (0.1 μg in 1 μL PBS, $n = 5$) injection but not PBS ($n = 5$), significantly enlarges ventricles. The proliferative area, as visualized using Ki67 and PCNA staining significantly decreased while cell density significantly increased following Pam₃CSK₄ administration. No difference was observed in the total number of cells labeled with PCNA or Ki67. (b) Representative example of ventricular and proliferative area measurements taken from 10 μm coronal brain sections from a PBS-treated control embryo and a Pam₃CSK₄-treated embryo. Contours

were drawn around the ventricle according to DAPI staining (blue), and around the proliferative area according to the PCNA (red) or Ki67 (green) immunostaining. (c) Pam₃CSK₄ (0.1 μg in 1 μL PBS, $n = 5$) injection but not PBS ($n = 5$), significantly decreases the number of BrdU⁺ cells in the SVZ. (d) Pam₃CSK₄ (0.1 μg in 1 μL PBS, $n = 5$) injection but not PBS ($n = 5$), significantly increases the number of PH3⁺ cells in the SVZ. (e) Pam₃CSK₄ (0.1 μg in 1 μL PBS, $n = 5$) injection but not PBS ($n = 5$) increases the ration of PH3⁺/BrdU⁻ cells in the SVZ. P, proliferative zone; V, ventricle; D, dorsal; M, midline. Scale bar represents 300 μm .

stability of the mRNA for MyD88, which requires higher levels of the mRNA to maintain high protein expression levels. The increased expression of TLR2 during cortical development was contrasted by the decreased expression of IRF5, a transcription factor that is activated by TLR2, suggesting a developmental role of IRF5 that may be independent of TLR2. Further, we show that TLR2 and its heterodimer partner, TLR6, but not TLR1, are expressed in proliferating cells in the SVZ. The presence of TLR1 mRNA but not protein levels could imply lack of expression, but could also be the result of differences between *in vivo* and *in vitro* growth conditions. We further show that TLR2 is indeed functional and can be activated in response to both exogenous and endogenous TLR2-specific stimuli which results in Akt phosphorylation and activation of the transcription factor NF- κ B, in a manner similar to that shown in immune cells. While in earlier developmental stages, the percentage of proliferating cells is greater than in later developmental stages, the changes discussed above observed in embryonic cortical tissue reflect total mRNA and protein levels not only in SVZ proliferating cells, but also in neurons, glia, and to a lower extent possibly other cell types.

Emerging findings suggest that TLRs, whether activated by endogenous or exogenous ligands, often act as negative regulators of NPC proliferation and differentiation. TLR4 activation restricts retinal progenitor cell proliferation in early postnatal eye development (Shechter *et al.* 2008). TLR8 is expressed in the cortex as early as E12, where its activation restricts neurite outgrowth and induces apoptosis in differentiated neurons (Ma *et al.* 2006). Similarly, activation of TLR3 inhibits neurite outgrowth and causes growth cone collapse (Cameron *et al.* 2007). TLR3 was recently reported to negatively regulate NPC proliferation during early stages of neuronal development and that TLR3 activation with PolyI:C decreases NPC proliferation (Lathia *et al.* 2008). The absence of functional TLR3 during embryogenesis promotes proliferation of NPC suggesting that TLR3 may be activated by an endogenous ligand. In contrast, we found that elimination of TLR2 neither affects the proliferative capacity of embryonic NPC, nor alters the fate of cells during differentiation *in vitro*. However, similar to TLR3 activation, activation of TLR2/6 or TLR2/1 heterodimers with either FSL1 or Pam₃CSK₄, respectively, or with an endogenous ligand for TLR2, LMW-HA, inhibits NPC proliferation *in vitro*. Therefore, while TLR2 itself may not participate in restricting NPC proliferation, its activation by either exogenous or endogenous ligands diminishes the proliferative capacity of NPC.

Activation of TLR2 with FSL1, Pam₃CSK₄, or with LMW-HA inhibited neurosphere formation *in vitro* and caused ventricular dysplasia when injected into the ventricles of E15 mouse embryos as indicated by a significant increase in ventricle size, decrease in the proliferative area, increase in cell density in the proliferative area, an increase in the

number of PH3⁺ cells, and a decrease in the number of BrdU⁺ cells in the SVZ. This suggests that TLR2 activation interferes with the cell cycle of NPC, prevents cells from incorporating BrdU, and prevents them from exiting M-phase of the cell cycle, thereby causing telencephalic dysplasia. The mechanism for these effects could involve both direct actions on the NPC and indirect effects. Indirect effects could result from an increase in cell density followed by cellular stress leading to deleterious counter effects, or also ventricular edema induced by TLR2 signaling in ependymal cells could cause ventricular volume increase, which culminates in decreased SVZ volume. Interestingly, there were no significant changes in all the tested parameters following *in utero* injection between the ipsilateral and contralateral embryonic hemispheres. This could be because of either a rapid dispersion of Pam₃CSK₄ throughout the embryonic CNS or as suggested above, because of an extrinsic effect such as a released factor(s) that culminate in telencephalic dysplasia.

Despite the high degree of specificity of TLR2/1 and TLR2/6 heterodimers to their ligands, we did not observe any differences in the effects conferred by the two ligands. Equimolar doses of both TLR2/6 and TLR2/1 activation induced comparable rates of neurosphere formation inhibition. TLR2 stimulation results in NF- κ B activation in macrophages (Schwandner *et al.* 1999), AP-1 in gastric cells (Chang *et al.* 2005), and Nrf-2 in tracheal smooth muscle cells (Lee *et al.* 2008). Inhibition of NPC proliferation by both ligands was correlated with NF- κ B promoter activity but not AP-1 or Nrf-2. While FSL1 administration resulted in greater NF- κ B promoter activity compared with Pam₃CSK₄, FSL1 was not more potent than Pam₃CSK₄ in inhibiting NPC proliferation, suggesting that the increase evoked by Pam₃CSK₄ was sufficient to maximally inhibit NPC proliferation. In addition, the basal activity of AP-1 was not affected by either TLR2 ligands, and no promoter activity of Nrf-2 was observed in NPC even in response to sulforaphane, an Nrf-2 activator (Ellis 2007). Moreover, co-administration of Pam₃CSK₄ and FSL1 resulted in a significant but mild additive inhibitory effect, which likely results from mutual downstream signaling induced by the two ligands.

Our findings are of interest in the context of alterations in brain development caused by infectious agents, ischemia, and inflammatory states. TLR2 activation has long been known to occur in immune cells during bacterial infections. Bacterial infection is common during pregnancy, including bacterial vaginosis, and can result in pre-term birth and spontaneous abortion (Leitich *et al.* 2003; Sorensen *et al.* 2008). Prenatal infection can also have profound lasting effects on brain development. For example, maternal bacterial infection is correlated with increased risk of schizophrenia (Sorensen *et al.* 2008). Maternal infection with LPS, a TLR4 agonist, is associated with altered hippocampal

morphology and decreased synaptic transmission (Golan *et al.* 2005; Lowe *et al.* 2008). LPS administration results in cerebral death and sensitizes the developing chick brain to hypoxic damage and death (Wang *et al.* 2008). Further, prenatal LPS infection selectively reduces baseline numbers of dopaminergic neurons in the substantia nigra, thereby increasing the vulnerability of the animals to a Parkinsonian toxin (6-hydroxydopamine) later in life (Ling *et al.* 2004). Our findings suggest that *in utero* exposure of the brain to infectious agents that produce TLR2 ligands or endogenous activation of TLR2 such as those used in this study may diminish the proliferative capacity of embryonic NPC and possibly lead to lasting abnormalities of neuronal plasticity and behavior. One particularly interesting long-term effect is whether TLR2-mediated telencephalic dysplasia affects the rostral migratory stream of NPC to the olfactory bulb, and whether this culminates in behavioral abnormalities in the adult mouse.

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Supporting Information

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

Figure S1. Antibody specificity for TLRs 1, 2 and 6.

Figure S2. TLR2 expression in different embryonic tissues.

Figure S3. (A–C) The proliferative capacity of WT and TLR2KO NPC is similar.

Figure S4. LMW-HA (1 μ g/mL) but not HMW-HA (1 μ g/mL) significantly decreases neurosphere formation.

Figure S5. Co-administration of TLR ligands does not alter NPC viability.

Figure S6. Both AP-1 and Nrf-2 are not activated in NPC by TLR2 activation.

Figure S7. TLR2 activation *in utero* at E15 increases ventricle size of E16 embryos.

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