



Postnatal TLR2 activation impairs learning and memory in adulthood



Ravit Madar^{a,b}, Aviva Rotter^{a,b}, Hiba Waldman Ben-Asher^a, Mohamed R. Mughal^c,
Thiruma V. Arumugam^d, W.H. Wood 3rd^c, K.G. Becker^c, Mark P. Mattson^{c,e}, Eitan Okun^{a,b,*}

^aThe Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 5290000, Israel

^bThe Leslie and Susan Gonda Multidisciplinary Brain Research Center, Bar-Ilan University, Ramat Gan 5290002, Israel

^cLaboratory of Neurosciences, National Institute on Aging Intramural Research Program, Baltimore, MD 21224, USA

^dDepartment of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

^eDepartment of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

ARTICLE INFO

Article history:

Received 13 February 2015

Received in revised form 12 April 2015

Accepted 12 April 2015

Available online 27 May 2015

Keywords:

Toll-like receptors

TLR2

Hippocampus

Spatial learning

Morris water maze

Fear conditioning

Cognition

ABSTRACT

Neuroinflammation in the central nervous system is detrimental for learning and memory, as evident from epidemiological studies linking developmental defects and maternal exposure to harmful pathogens. Postnatal infections can also induce neuroinflammatory responses with long-term consequences. These inflammatory responses can lead to motor deficits and/or behavioral disabilities. Toll like receptors (TLRs) are a family of innate immune receptors best known as sensors of microbial-associated molecular patterns, and are the first responders to infection. TLR2 forms heterodimers with either TLR1 or TLR6, is activated in response to gram-positive bacterial infections, and is expressed in the brain during embryonic development. We hypothesized that early postnatal TLR2-mediated neuroinflammation would adversely affect cognitive behavior in the adult. Our data indicate that postnatal TLR2 activation affects learning and memory in adult mice in a heterodimer-dependent manner. TLR2/6 activation improved motor function and fear learning, while TLR2/1 activation impaired spatial learning and enhanced fear learning. Moreover, developmental TLR2 deficiency significantly impairs spatial learning and enhances fear learning, stressing the involvement of the TLR2 pathway in learning and memory. Analysis of the transcriptional effects of TLR2 activation reveals both common and unique transcriptional programs following heterodimer-specific TLR2 activation. These results imply that adult cognitive behavior could be influenced in part, by activation or alterations in the TLR2 pathway at birth.

© 2015 Elsevier Inc. All rights reserved.

1. Introduction

Toll like receptors (TLRs) are a family of type-I trans-membrane receptors best known as sensors of microbial-associated molecular patterns (MAMPs) by cells of the innate immune system (Kawai and Akira, 2007). Binding of MAMPs to TLRs typically activates signaling cascades that result in production of inflammatory cytokines/chemokines (Takeda and Akira, 2004). Activation of TLRs in the central nervous system (CNS) by MAMPs at different developmental stages (e.g.: embryonic, postnatal or adults) results in numerous effects, including impairment of various aspects of learning and memory in a neuroinflammation-dependent manner

(Okun et al., 2011). Neuroinflammation in the CNS is a detrimental process for learning and memory (Hao et al., 2010). This insight draws from epidemiological studies linking developmental defects and maternal exposure to harmful pathogens. Such exposures could be either direct or indirect, affecting the intrauterine environment (Heindel, 2006). Indeed, maternal infection, whether local within the reproductive tract or a systemic subclinical infection may lead to both maternal and/or fetal inflammatory responses. These inflammatory responses can lead to motor deficits and/or behavioral disabilities (Rees et al., 2008). TLRs are heavily implicated in CNS neuroinflammation, as numerous MAMPs and tissue damage-associated molecular patterns (DAMPs) were reported to activate TLRs during infection and tissue damage (Mallard, 2012). TLR2 forms heterodimers with either TLR1 or TLR6 and different lipopeptides are thought to activate each heterodimer (Buwitt-Beckmann et al., 2006) with no apparent differences in the transcriptional outcomes (Farhat et al., 2008). FSL-1 (Pam2CGDPKHPKSF) is a synthetic lipoprotein (LP) derived from

* Corresponding author at: The Mina and Everard Goodman Faculty of Life Sciences, Building 901, room 315, Bar Ilan University, Ramat Gan 5290002, Israel. Tel.: +972 (0)3 7384670 (Laboratory), +972 (0)3 7384671 (Office); fax: +972 (0)3 5352184.

E-mail address: eitan.okun@biu.ac.il (E. Okun).

Mycoplasma salivarium. Mycoplasmal LPs, such as FSL-1, contain a diacylated cysteine residue, whereas bacterial LP contains a triacylated cysteine residue. FSL-1 is recognized by the TLR2/6 heterodimer, whereas bacterial LPs such as Pam3CysSerLys4 (Pam3CSK4) are recognized by the TLR2/1 heterodimer (Long et al., 2009). The interaction between TLR2 heterodimers and lipoproteins is governed by 4 different interaction types (Kang et al., 2009), which dictate its heterodimer binding partner. As a result, while FSL-1 and Pam3CSK4 bind different TLR2 heterodimers with strong affinity, both are probably capable of binding other heterodimers albeit with significantly lower affinity *in vivo*. Postnatal exposure of mouse pups to TLR2 activation, between postnatal day (PND)-3 and PND11 with the synthetic lipopeptide Pam3CysSerLys4 (Pam3CSK4), displayed increased levels of interleukin (IL)-1 β , IL-6, chemokine (C-X-C motif) ligand 1, and monocyte chemoattractant protein-1 in the brain. These mice also exhibited decreased volume of cerebral gray matter, and white matter in the forebrain at PND12, but no cognitive deficit was found at PND53 (Du et al., 2011). Despite this, the impact of postnatal TLR2 activation on the various aspects of hippocampus-dependent learning and memory remains unclear. Here we provide evidence that early postnatal exposure to TLR2 activation confers long-term effects on hippocampus-dependent cognitive spatial and fear learning as well as on motor functions in a TLR2-heterodimer dependent manner during adulthood. These effects are correlated with immune- and extracellular matrix-related transcriptional changes.

2. Materials and methods

2.1. Animals

Congenitively wild-type mice (TLR2^{+/+}, B6.129) and TLR2^{-/-} mice (B6.129-Tlr2(tm1Kir)/J) were purchased from Jackson Laboratories (Bar Harbor, ME), and bred in-house to generate pups for this study. Animal care and experimental procedures followed NIH guidelines and were approved by the National Institute on Aging Animal Care and Use Committee.

2.2. Postnatal injections

For cerebral microinjections of endotoxin-free phosphate buffered saline (PBS) or the TLR2 ligands, Pam3CSK4 or FSL-1, P0 pups were placed on an ice-cooled plastic surface. 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 either by a Sutter micromanipulator (Sutter Instrument Company, Novato, CA, USA) equipped with a 20 μ l Hamilton gas-tight syringe or a nitrogen-fed microinjector

(Harvard Apparatus, PL1-100). Each pup was hand-held on top of the ice-cooled plastic surface, and a single 0.5 μ l bolus of either saline or 0.07 mg/kg of Pam3CSK4 or FSL1 (both dissolved in endotoxin-free PBS) was injected during 2 min to each hemisphere. This dose was previously determined by us to affect central neuroinflammation in late embryonic stages (Okun et al., 2010b). A total of 41 TLR2^{+/+} pups were injected with PBS, 33 TLR2^{+/+} pups were injected with Pam3CSK4, 38 TLR2^{+/+} pups were injected with FSL1, 33 TLR2^{-/-} were injected with PBS and 31 TLR2^{-/-} pups were injected with Pam3CSK4, and 26 TLR2^{-/-} pups were injected with FSL1.

3. Behavioral testing

3.1. Rotarod test

Rotarod tests were performed at 6 weeks following birth (Fig. 1) using the ENV-577M system (Med-associates, St. Albans, VT, USA). Rotarod acceleration was set to 4–40 rpm over a period of 5 min. Mice were placed on the rotarod for three 5-min trials with 15 min to rest between trials. Both the number of falls within 5 min and rpm at first fall were recorded and averaged.

3.2. Exploratory behavior

The open field tests were performed at 7 weeks following birth (1 week following completion of the rotarod tests, Fig. 1). Exploratory behavior was assessed in the open field arena using the MED-OFA-MS system (Med Associates, St Albans, VT, USA) placed inside sound-attenuating boxes (model MED ENV-022V; Med Associates, St Albans, VT, USA). Animals were placed in the center of an open field (40.6 cm \times 40.6 cm) and exploration was assessed for 15 min. Cages were cleaned with 40% ethanol following each session. The peripheral 10.16 cm of the zone were considered as the peripheral zone and the central 20.32 cm were considered as the central zone. All open field tests were conducted under a light intensity of 400 lux. Open field-testing were conducted 6 weeks following birth (Fig. 1).

3.3. Spatial learning

To evaluate spatial learning and memory, mice were tested in the Morris water maze (MWM). The MWM task was performed at 12 weeks following birth (Fig. 1). To test for spatial reference (long-term) memory, mice were trained in a water-filled pool (160 cm diameter) for 5 consecutive days, 4 trials per day, with spatial cues on the walls of the room. The cues (see (Okun et al., 2010a) for details) were black and white only, to reduce possible

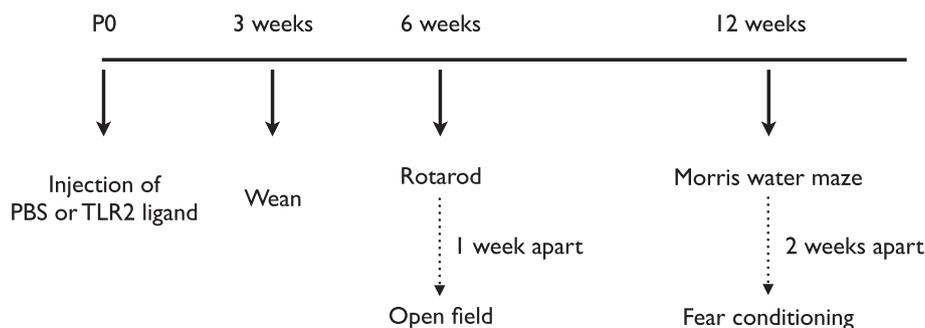


Fig. 1. Experimental design. TLR2^{+/+} mice ($n = 112$) and TLR2^{-/-} ($n = 90$) mice were injected with PBS, FSL1 (a TLR2/6 ligand) or Pam3CSK4 (a TLR2/1 ligand) immediately after birth. At 3 weeks, pups were weaned, and at 6 weeks mice were tested in the open field to assess exploratory behavior and on the rotarod to assess their motor function. At 12 weeks, mice were tested for spatial and contextual fear learning.

effects of color discrimination capabilities between the different treatments. The platform (rectangular platform, 153 cm² area) was hidden 0.5 cm below the water surface, at a constant location with mouse starting points changed every trial to avoid track memorization. When trials ended, either when the mouse had found the platform or when 60 s passed, mice were allowed to rest on the platform for 60 s. Latency to reach the platform, swimming distance, swimming speed and mean distance from the platform were recorded automatically by the AnyMaze video tracking system (Stoelting Co, Wood Dale, IL, USA). Twenty-four hours following training, mice were subjected to a probe test, to evaluate memory retention. In this test, the platform was removed, and mice were allowed to swim for 30 s, and time the mice spent in each quadrant was measured. All tests were conducted under 20 lux illumination to reduce stress to the mice. In all the MWM tests, mice that exhibited passivity or a thigmotaxic swimming pattern were excluded from analysis (Okun et al., 2012). Water temperature was maintained at 27 ± 0.5 °C and was made opaque using nontoxic white paint. MWM tests were conducted 12 weeks following birth (Fig. 1). Mice exhibiting passive behavior in the pool (determined by both swim duration >52 s, average swim speed <0.13 m/s and mean distance from the platform >0.41 m) were excluded from analysis.

3.4. Fear conditioning

The fear-learning paradigm was conducted 2 weeks following completion of the MWM spatial task. Before testing, mice were first habituated to the testing room for 3 h/day for 3 days. In the training session, mice were placed in a contextual conditioning chamber (model MED VFC-NIR-M; Med Associates, St Albans, VT, USA) placed inside sound-attenuating boxes (model MED ENV-022V; Med Associates, St Albans, VT, USA). The conditioning chambers contained a metal grid on the floor (context A) and mice were allowed to explore the chamber for 2 min. At the end of 2 min mice were subjected to three sessions of audio tone (CS, conditioned stimulus) and foot shock (US, unconditioned stimulus). Audio tone (5 kHz, 70 dB) was on for 30 s, followed immediately by a 0.5 mA, 2 s foot shock from the metal grid floor. Thirty seconds separated each session. Foot shock intensity was determined in a preliminary test on a separate cohort of animals for the minimal applicable intensity to achieve a minimal freezing threshold of 40% during contextual fear. On the following day, in the contextual fear session (context A), mice were returned to the conditioning chamber for 5 min without any shock or audio tone. The percentage of time freezing was recorded and used as an index of contextual memory. In the cued conditioning (conducted 3 h following contextual conditioning) mice were returned to the chamber but in a different context (context B). Context B was comprised of plastic triangle inserted to the cage coupled with plastic flooring placed underneath the triangular plastic insert. Mice were allowed to explore the chamber for 5 min without any audio tone. Following this, five audio tones were played for 30 s each. The percentage of time freezing until and after the audio tones was recorded and used as an index of cued memory. In all fear conditioning tests, cages were cleaned with ethanol between tests. Fear conditioning testing was conducted 12 weeks following birth (Fig. 1).

3.5. Cortical neuronal cell cultures

Dissociated cell cultures of cortical fragments were established from 18-day Sprague–Dawley rat embryos as previously described (Mattson and Kater, 1988). Neurons were plated on polyethyleneimine (PEI) (0.005%) coated 60 mm plastic dishes and were maintained with Neurobasal medium supplemented with B27 (1:50, v:v) and arabinose-c (ara-c, 1 μM, Sigma, USA) to eliminate

astrocyte contamination. In all experiments, TLR2 ligands were applied for 24 h.

3.6. Neuronal progenitor cell cultures

Neuronal progenitor cells (NPC) 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/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 (EGF), and fibroblast growth factor 2 (FGF2) (both at 20 ng/ml; R&D Systems Inc). Growth factors were replenished every 3 days in culture. After neurospheres were grown for 7 days in culture the cells were induced for differentiation. To induce differentiation, growth factors were withdrawn and neurospheres were plated on polyethyleneimine (Sigma) coated dishes with the presence of 10% fetal calf serum.

3.7. Quantitative real-time PCR

RNA from either neuronal cultures or the cerebral cortex of PO pups, was extracted using TRIzol Reagent (Ambion, Life Technologies, CA, USA). Complementary DNA (cDNA) was generated using Revert Aid H minus first strand cDNA synthesis kit (Thermo Scientific, Lithuania). The resulting cDNA was diluted 1:5 in nuclease-free water and stored in aliquots at –80 °C until used. RT-PCR reactions were performed using Fast SYBR Green Master Mix (Applied Biosystems, CA, USA) in a StepOnePlus instrument (Applied Biosystems, CA, USA). Primers were calibrated and negative control was performed for each primer pair. Samples were measured in triplicates and values were normalized according to mRNA levels of the β-Actin housekeeping gene. Quantification was assessed at the logarithmic phase of the PCR reaction using 2^{–ΔΔCT} method as described previously (Livak and Schmittgen, 2001). Gene-specific primers are listed in the following table:

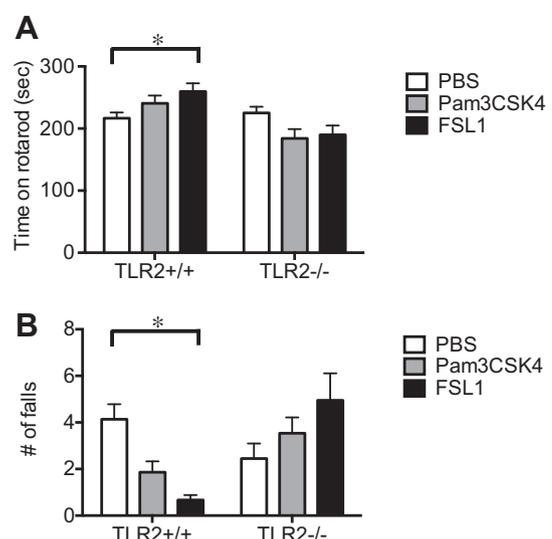


Fig. 2. Central postnatal TLR2/6 but not TLR2/1 activation enhances motor performance in adult mice. TLR2^{+/+} and TLR2^{-/-} mice were injected with either PBS ($n = 41$ and 33 respectively), the TLR2/6 ligand FSL1 ($n = 14$ and 20 , respectively), or the TLR2/1 ligand Pam3CSK4 ($n = 30$ and 31 , respectively), immediately after birth. At the age of 6 weeks, the mice were tested for motor function on the rotarod. (A) The average time mice spent on the rotating rod and (B) the number of falls from the rotarod was measured. * $P < 0.05$.

Gene	Primers	Annealing temp (°C)	Amplicon size (bp)	Species
<i>Lcn2</i>	Forward 5' GGGCTGTCGCTACTGGATCA Reverse 5' TGTACCTGAGGATACCTGTGCATATT	58.25	90	Ms
<i>Lcn2</i>	Forward 5' GACCAGTTTGCCATGGTA Reverse 5' CAGTCAGCCAGCTCA	52	216	Rat
<i>Tf</i>	Forward 5' TGGTACGTAGGCGCATTC Reverse 5' TCCGGCAAGACCTCAAATATG	56.3	90	Ms
<i>Tf</i>	Forward 5' GGTGAAGAAGGGAACAGA Reverse 5' GACACAGCTGGGGGAA	51	214	Rat
<i>Cxcl1</i>	Forward 5' TGCACCCAAACCGAAGTCAT Reverse 5' TTGTCAGAAGCCAGCGTTTAC	57.65	177	Ms
<i>Cxcl1</i>	Forward 5' ATGGCGTCTGTCTGGTGAA Reverse 5' GTCCTTTGAACTTCTCTGTCT	50	520	Rat
<i>Timp1</i>	Forward 5' GCCCTTCGCATGGACATTTA Reverse 5' ATGGTATCTCTGGTGTGTCTCTAGGA	56.9	90	Ms
<i>Timp1</i>	Forward 5' GGGTCCCCAGAAATCATCC Reverse 5' CAGTGTTTCAGGCTTCAGCTT	54	515	Rat
<i>C1r</i>	Forward 5' GACCAGCTCCAGATCTACGC Reverse 5' GGGCACTTGATGGTTTCAGT	56.45	173	Ms
<i>C1r</i>	Forward 5' GCAAAGGCCTCCAGAC Reverse 5' TCTCTCCTTCCTTTCGT	50	517	Rat
<i>Nfkbia</i>	Forward 5' TGGCCAGTGTAGCAGTCTTG Reverse 5' GACACGTGTGGCCATTGTAG	56.75	90	Ms, Rat
<i>Il17rb</i>	Forward 5' CCATCCCTCCAGATGACAAC Reverse 5' GCTCCTTCCTTGCCCTCAAGTTA	57.1	122	Ms
<i>Il17rb</i>	Forward 5' GATCTACCTAACTGGAGGCAAG Reverse 5' GTAGCCTTGAGAAGTTCTGTGT	53	525	Rat
<i>C1s</i>	Forward 5' GTGGTGACGATGCAGAGAGA Reverse 5' GCCCATTAGGTCAGTTTGA	55.75	193	Ms
<i>C1s</i>	Forward 5' AACCCAGAGCAATACTCTTG Reverse 5' TGATACTCTCCGCCTTCTTC	50	420	Rat
<i>Ccl2</i>	Forward 5' GGCTGGAGAGCTACAAGAGG Reverse 5' ATGTCTGGACCCATTCCTTC	55.8	117	Ms
<i>Ccl2</i>	Forward 5' CCCACTCACCTGCTGCT Reverse 5' ACAGAAGTGCTTGAGGT	50	330	Rat
<i>Tuj1</i>	Forward 5' CGC ATC ATG AAC ACC TTC AG Reverse 5' ACA GGC AGC CAT CAT GTT C	54	426	Ms, Rat
<i>S100b</i>	Forward 5' TGTTACTCGGACACTGAAG Reverse 5' TTCCTGCTCCTTGATTTC	51	218	Ms
<i>S100b</i>	Forward 5' CTACACTAGGTATTCCTGTG Reverse 5' GGGTGTGGGTGATAGGT	50	317	Rat
<i>Gfap</i>	Forward 5' GTGTCAGAAGGCCACCT Reverse 5' AAGGAAGGGGAGCACTG	54	217	Rat
<i>Gfap</i>	Forward 5' CAACTGCAGGCCTTGAC Reverse 5' CAGGGCTAGCTTAACGT	51	216	Ms
<i>Mbp</i>	Forward 5' GGATTCAAGGGGGCCTA Reverse 5' AGAGAAGACCCCGAGGA	53	317	Rat
<i>Mbp</i>	Forward 5' ATCTCCCATGGCGAGAC Reverse 5' GTAGGGGTGAACTTGGAAAG	52	319	Ms
<i>Tlr1</i>	Forward 5' ATG ATT CTG CCT GGG TGA AG Reverse 5' TCT GGA TGA AGT GGG GAG AC	53	174	Ms
<i>Tlr1</i>	Forward 5' TACCCTGAACAACGTGGACA Reverse 5' ATCGACAAAGCCCTCAGAGA	54	165	Rat
<i>Tlr2</i>	Forward 5' CTC CCA CTT CAG GCT CTT TG Reverse 5' AGG AAC TGG GTG GAG AAC CT	53	217	Ms
<i>Tlr2</i>	Forward 5' CGAAAAGAGCCACAAAAGTGT Reverse 5' CATTATCTTGCGCAGTTTGC	52	189	Rat
<i>Tlr6</i>	Forward 5' ACACAATCGGTTGCAAAACA Reverse 5' GGAAAGTCAGCTTCGTCAGG	50	178	Ms
<i>Tlr6</i>	Forward 5' TGGCCCCAAAACCTTCTTCAACGGA Reverse 5' GGGGCTTTCCTCTGTCTCTA	51	593	Rat
<i>Tlr10</i>	Forward 5' GAACTATCTGGCCACCA Reverse 5' CACACCTTCCCCTTGTGTCT	51	235	Rat

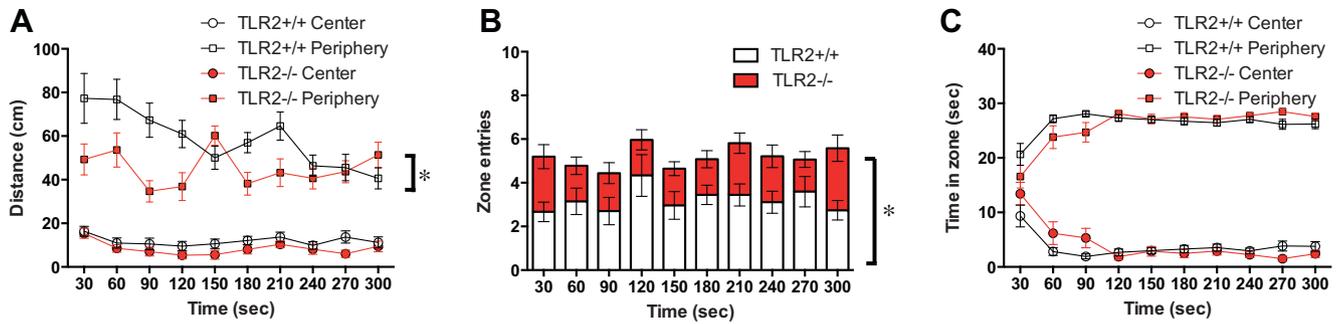


Fig. 3. TLR2 deficiency reduces exploratory behavior in adult mice. TLR2^{+/+} ($n = 42$) and TLR2^{-/-} ($n = 33$) mice were injected with PBS immediately after birth. At the age of 6 weeks, the mice were placed for 5 min in the center of an open field under light intensity of 400 lux. Mouse activity was recorded and total walking distance, zone crosses between the peripheral and the center zones, and time in each zone, were compared between TLR2^{+/+} and TLR2^{-/-} mice (A–C). * $P < 0.05$.

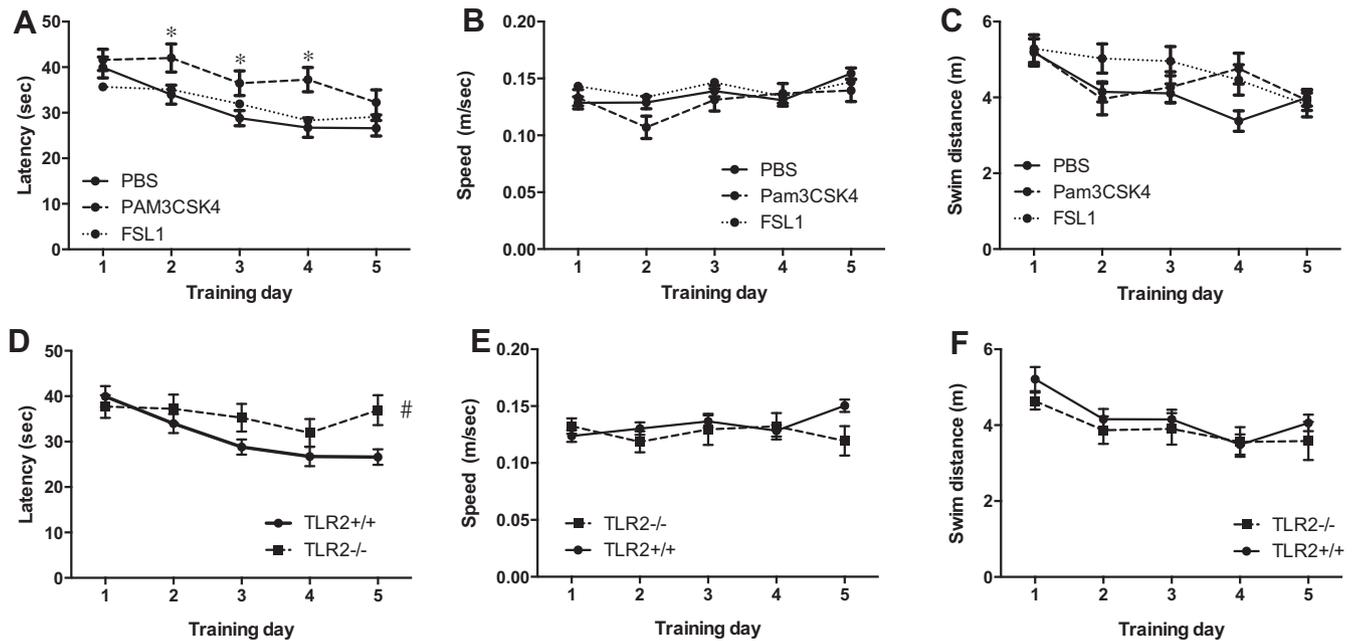


Fig. 4. Central postnatal TLR2/1 activation in TLR2^{+/+} mice and TLR2 deficiency impair spatial learning in adult mice. TLR2^{+/+} mice were injected with either PBS ($n = 39$), the TLR2/6 ligand FSL1 ($n = 37$), or the TLR2/1 ligand Pam3CSK4 ($n = 30$), immediately after birth. At the age of 12 weeks, the mice were tested for spatial learning ability in the MWM. The performance of each mouse was video-recorded and the goal latency, swim speed and swim distance were analyzed for TLR2^{+/+} mice (A–C). A Direct comparison of TLR2^{+/+} ($n = 39$) and TLR2^{-/-} ($n = 29$) mice is shown in panels (D–F). * $P < 0.05$ on days 2–4 in Pam3CSK4- vs. PBS-treated mice. # $P < 0.05$ when comparing TLR2^{+/+} and TLR2^{-/-} mice.

Gene abbreviations: *Lcn2*, lipocalin2; *Tf*, transferrin; *Cxcl1*, chemokine (C–X–C motif) ligand 1; *Timp1*, tissue inhibitor of metalloproteinase-1; *C1r*, Complement component 1R; *Nfkbia*, NF-Kappa-B Inhibitor Alpha; *Il17rb*, Interleukin-17 receptor B; *C1s*, Complement component 1S; *Ccl2*, chemokine (C–C motif) ligand 2; *Tuj1*, Neuron-specific beta-III Tubulin; *S100b*, S100 calcium binding protein B; *Mbp*, Myelin basic protein; *Gfap*, glial fibrillary acidic protein; *Tlr*, Toll-like receptor.

3.8. Illumina oligonucleotide microarray

Total RNA was used to generate biotin labeled cRNA using the Illumina TotalPrep RNA Amplification Kit (Ambion; Austin, TX, cat #IL1791). In short, 0.5 μ g of total RNA was first converted into single-stranded cDNA with reverse transcriptase using an oligo-dT primer containing the T7 RNA polymerase promoter site and then copied to produce double-stranded cDNA molecules. The double stranded cDNA was cleaned and concentrated with the supplied columns and used in an overnight in vitro transcription reaction

where single-stranded complementary RNA (cRNA) was generated and labeled by incorporation of biotin-16-UTP. A total of 0.75 μ g of biotin-labeled cRNA was hybridized at 58 °C for 16 h to Illumina's Sentrix MouseRef-8 Expression Bead-Chips (Illumina, San Diego, CA). Each BeadChip has 24,000 well-annotated RefSeq transcripts with approximately 30-fold redundancy. The arrays were washed, blocked and the labeled cRNA was detected by staining with streptavidin-Cy3. The arrays were scanned using an Illumina BeadStation 500 \times Genetic Analysis Systems scanner and the image data extracted using the Illumina BeadStudio software, Version 3.0.

3.9. Statistical analysis

Analysis of all behavioral tests included both males and females as no statistically significant differences were observed between genders. Analysis of the open field, MWM and fear-conditioning experiments were performed using two-way analysis of variance (ANOVA) repeated measures with a Bonferroni post hoc test. Analysis of rotarod experiments was done using one-way

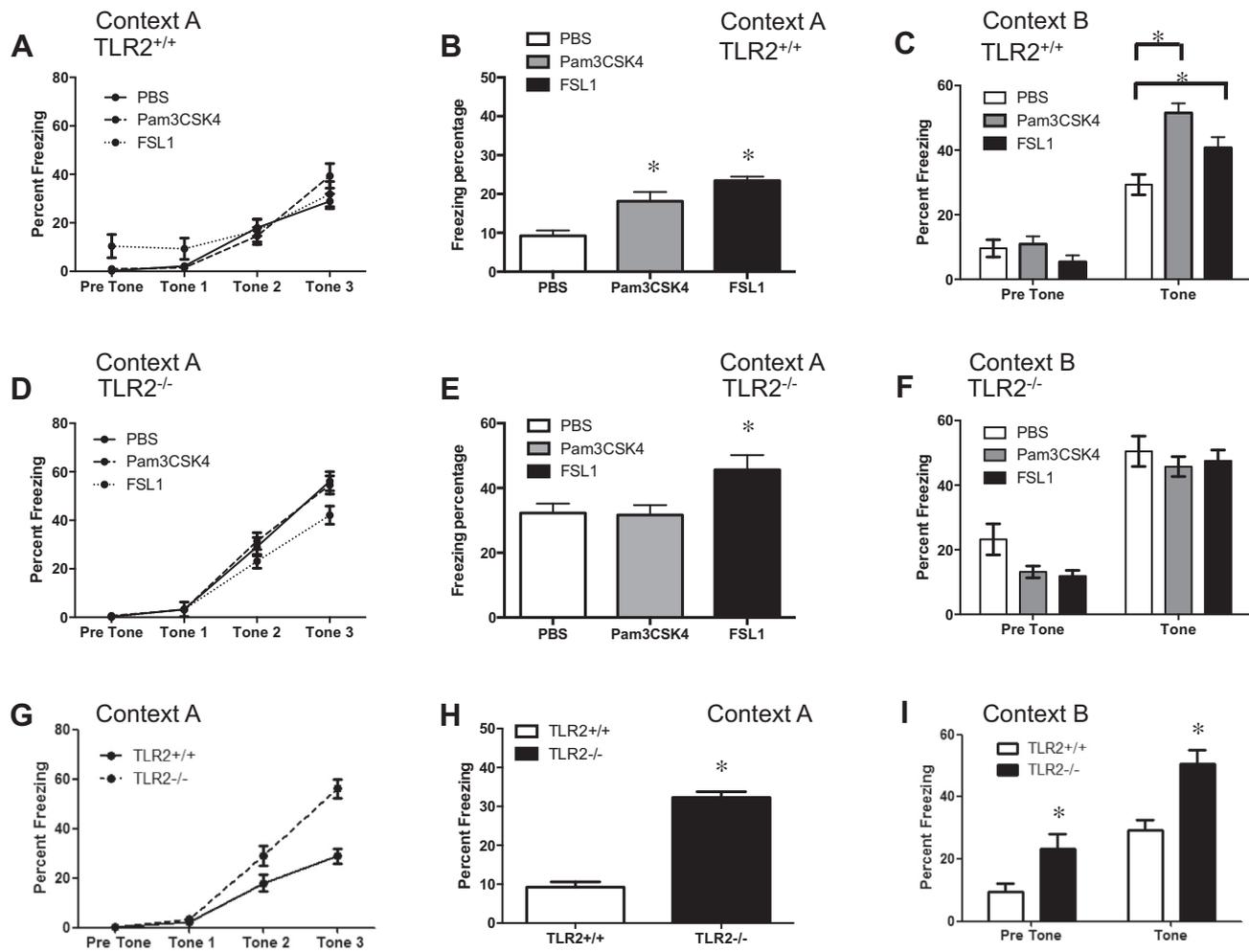


Fig. 5. Central postnatal TLR2 activation and TLR2 deficiency enhance contextual and cued fear learning in adult mice. TLR2^{+/+} and TLR2^{-/-} mice injected with either PBS ($n = 39$ and 29 respectively), the TLR2/6 ligand FSL1 ($n = 35$ and 26 respectively), or the TLR2/1 ligand Pam₃CSK₄ ($n = 30$ and 31 respectively), immediately after birth. At the age of 12 weeks, the mice were tested in the fear-conditioning paradigm. The performance of the mice during the acquisition phase, contextual fear and cued fear was analyzed for TLR2^{+/+} mice (A–C) and TLR2^{-/-} mice (D–F). Direct comparison of PBS-treated TLR2^{+/+} and TLR2^{-/-} mice is shown in (H–I). Contexts A and B indicate the context in which the mice were tested in. * $P < 0.05$.

ANOVA. Statistical analysis was performed using Prism 5 (Graphpad, USA). Results are expressed as mean \pm S.E.M.

3.10. Microarray data analysis

The data from all arrays were first subjected to background correction by GenomeStudio software (Illumina, Inc., San Diego, CA). The rest of the analysis was performed in Partek[®] Genomics Suite software, version 6.6 Copyright© 2012 Partek Inc., St. Louis, MO, USA. Normalized data from two to six biological and technical replicates of control and treated cells were analyzed to identify genes whose expression was up- or down-regulated by an arbitrary cutoff of at least 1.8-fold, and had a P -value < 0.05 in all replicates when testing for differential expression (ANOVA test).

3.11. Gene interaction analysis

We generated networks of highly interconnected proteins using the STRING (Search Tool for the Retrieval of Interacting Genes, Heidelberg, Germany) 9.1 database (Franceschini et al., 2013). The Gene Ontology (GO) biological processes and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated

with the up- and down-regulated genes were retrieved from the STRING enrichment analysis tool (P -value < 0.05).

4. Results

4.1. Postnatal TLR2/6 activation improves motor function in adult TLR2^{+/+} but not TLR2^{-/-} mice

Central postnatal TLR2/6 activation with FSL1 in TLR2^{+/+} increased the time mice spent on the rotating rod (260 ± 13 s in FSL1-treated mice vs. 217 ± 9.3 s in PBS-treated mice; $P = 0.0078$, two-way ANOVA with multiple comparisons; Fig. 2A) and decreased the number of falls from the rod (0.67 ± 0.22 in FSL1-treated mice vs. 4.1 ± 0.65 in PBS-treated mice; $P = 0.002$, two-way ANOVA with multiple comparisons; Fig. 2B). Treatment of mice with the TLR2/1 ligand Pam3CSK4 did not affect time on the rotarod or number of falls in either TLR^{+/+} or TLR^{-/-} mice (Fig. 2A and B). These data suggest that central postnatal TLR2/6 but not TLR2/1 activation improves motor learning during adulthood in TLR2^{+/+} mice. No baseline differences in motor learning or coordination were noted between TLR2^{-/-} and TLR2^{+/+} mice in either time spent on the rod or number of falls ($P > 0.05$) (Fig. 2A and B).

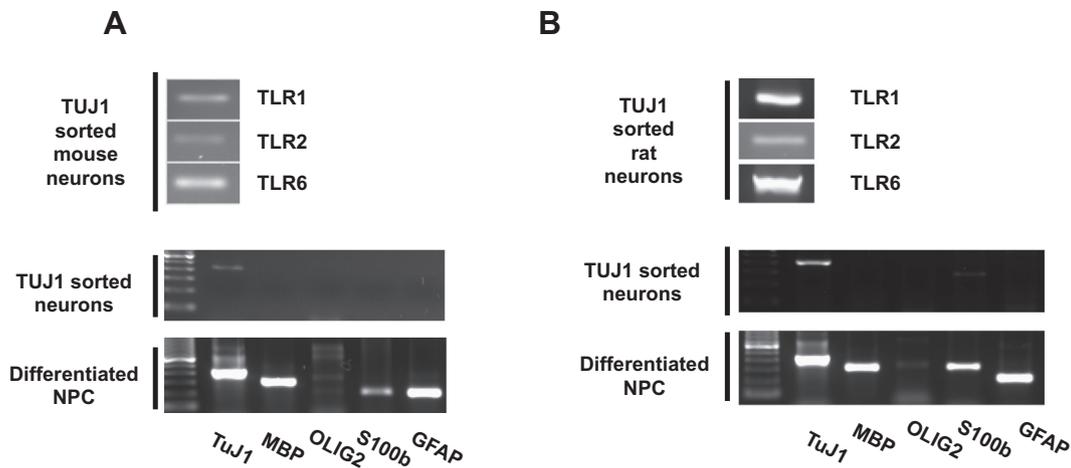


Fig. 6. Neurons express mRNA for TLRs 1, 2 and 6. NPCs derived from (A) mouse and (B) rat embryos were differentiated into neurons, astrocytes and oligodendrocytes. The cells were then sorted using FACS into TUJ1-expressing cells and analyzed for expression of TLR 1, 2 and 6 mRNAs.

4.2. TLR2 deficiency but not postnatal TLR2 activation affects exploratory behavior

In addition to motor function, another behavioral trait, which can affect the interpretation of cognitive behavior, is exploratory motivation. To test for differences in exploratory behavior, mice were placed in an open field arena, and walking distance, number of crosses between the center and peripheral zones, and time spent in each zone were measured (see methods for zone descriptions). TLR2 activation had no effect on TLR2^{+/+} mice in walking distance ($P > 0.05$, Fig. S1A), zone entries ($P > 0.05$, Fig. S1B) and time spent in each zone ($P > 0.05$, Fig. S1C). Similar results were obtained for TLR2^{-/-} mice (walking distance, $P > 0.05$ Fig. S1D; zone entries, $P > 0.05$ Fig. S1E; or time spent in each zone, $P > 0.05$ Fig. S1F). When baseline differences between TLR2^{-/-} and TLR2^{+/+} mice were compared, significant differences were observed in walking distance ($P < 0.0001$, $F_{3,1040} = 264.69$, two-way repeated measures (RM)-ANOVA; Fig. 3A) and number of center to periphery zone crosses ($P < 0.0001$, $F_{3,520} = 27.09$, two-way RM-ANOVA; Fig. 3B). Time spent in each zone was not different between the two strains ($P > 0.05$; Fig. 3C). This suggests that while TLR2^{-/-} mice are mildly less active than TLR2^{+/+} mice, they do not exhibit impaired anxiety.

4.3. Postnatal TLR2/1 but not TLR2/6 heterodimer activation impairs spatial learning in adulthood

In order to assess the long-term effects of postnatal exposure to TLR2 activation on spatial learning, we tested mice 12 weeks following postnatal exposure to PBS, the TLR2/1 ligand Pam3CSK4 or the TLR2/6 ligand FSL1 in the MWM, a dorsal-hippocampus dependent spatial learning and memory task. A significant day effect was observed in latency to reach the platform for PBS- and TLR2/6 ligand-treated TLR2^{+/+} mice ($P < 0.0001$, $F_{2,400} = 10.22$, two-way RM-ANOVA, Fig. 4A). However, compared with these mice, the latency of TLR2^{+/+} treated with the TLR2/1 ligand to reach the platform was greater on days 2–4 ($F_{2,400} = 11.58$, two-way RM-ANOVA, Fig. 4A). Swim speed did not differ between the groups ($P > 0.05$, Fig. 4B). Although a day effect was observed for the three groups in swim distance ($F_{2,400} = 6.15$, two-way RM-ANOVA, Fig. 4C), no difference was observed between groups. While PBS-treated mice exhibited memory retention in the probe trial ($P = 0.0028$, one-way ANOVA, Fig. S2A), FSL1- and Pam3CSK4-treated mice failed to exhibit such memory retention ($P > 0.05$, one way ANOVA, Fig. S2B and C).

These data suggest that TLR2^{+/+} mice exposed to postnatal TLR2/1 activation exhibited slower learning of a spatial cognitive task compared with mice receiving PBS or a TLR2/6 ligand. Neither TLR2/1 nor TLR2/6 postnatal activation had an effect on the performance of TLR2^{-/-} mice in the MWM compared with PBS-treated TLR2^{-/-} mice as measured by latency to reach the hidden platform ($P > 0.05$, Two-way RM-ANOVA, Fig. S3A), swim speed ($P > 0.05$, Two-way RM-ANOVA, Fig. S3B) or swim distance ($P > 0.05$, Two-way RM-ANOVA, Fig. S3C). As a result, TLR2^{-/-} mice treated with PBS, FSL1- or Pam3CSK4-failed to exhibit spatial memory retention ($P > 0.05$, one way ANOVA, Fig. S2D–F). In fact, TLR2 deficient mice exhibited a higher latency to reach the platform compared to TLR2^{+/+} mice ($F_{1,237} = 43$, $P < 0.0001$, Two-way-RM ANOVA, Fig. 4E). TLR2^{-/-} mice also exhibited slower swim speed ($F_{1,237} = 50$, $P < 0.0001$, Two-way-RM ANOVA, Fig. 4F) and longer swimming distance ($F_{1,237} = 8.4$, $P < 0.0001$, Two-way RM-ANOVA, Fig. 4G). Thus, performance of TLR2^{-/-} mice in the MWM task was impaired compared with TLR2^{+/+} mice. Of notice, TLR2^{+/+} mice treated with Pam3CSK4, FSL1 or PBS exhibited similar swim speed throughout training days 1–5 (Fig. 4B). As a result, swim speed did not affect their performance in this task. When compared with TLR2^{+/+} mice, TLR2^{-/-} mice also exhibited similar swim speeds (Fig. 4E). Therefore, the higher latency of TLR2^{-/-} mice in reaching the platform could not be explained by altered swim speed.

Taken together, these data suggest that both developmental TLR2 deficiency and neonatal TLR2/1 but not TLR2/6 activation impair spatial learning in adulthood, highlighting the importance of the TLR2 pathway to spatial learning.

4.4. Central postnatal TLR2 activation enhances contextual and cued fear learning in adult TLR2^{+/+} mice

The hippocampus is a central hub for multiple types of learning behaviors. As spatial learning was affected by early post-natal TLR2 activation, we sought to test whether additional hippocampus-dependent cognitive domains such as fear learning (Maren, 2008) are affected by postnatal TLR2 activation. Fear conditioning is a Pavlovian task shown to mostly involve the ventral hippocampal formation, the amygdala and the prefrontal cortex (Liu et al., 2011). To test whether TLR2 activation in the CNS of neonate pups confers long-term effects on fear learning during adulthood, we utilized the fear-conditioning paradigm. Central postnatal TLR2 activation with either Pam3CSK4 or FSL1 in

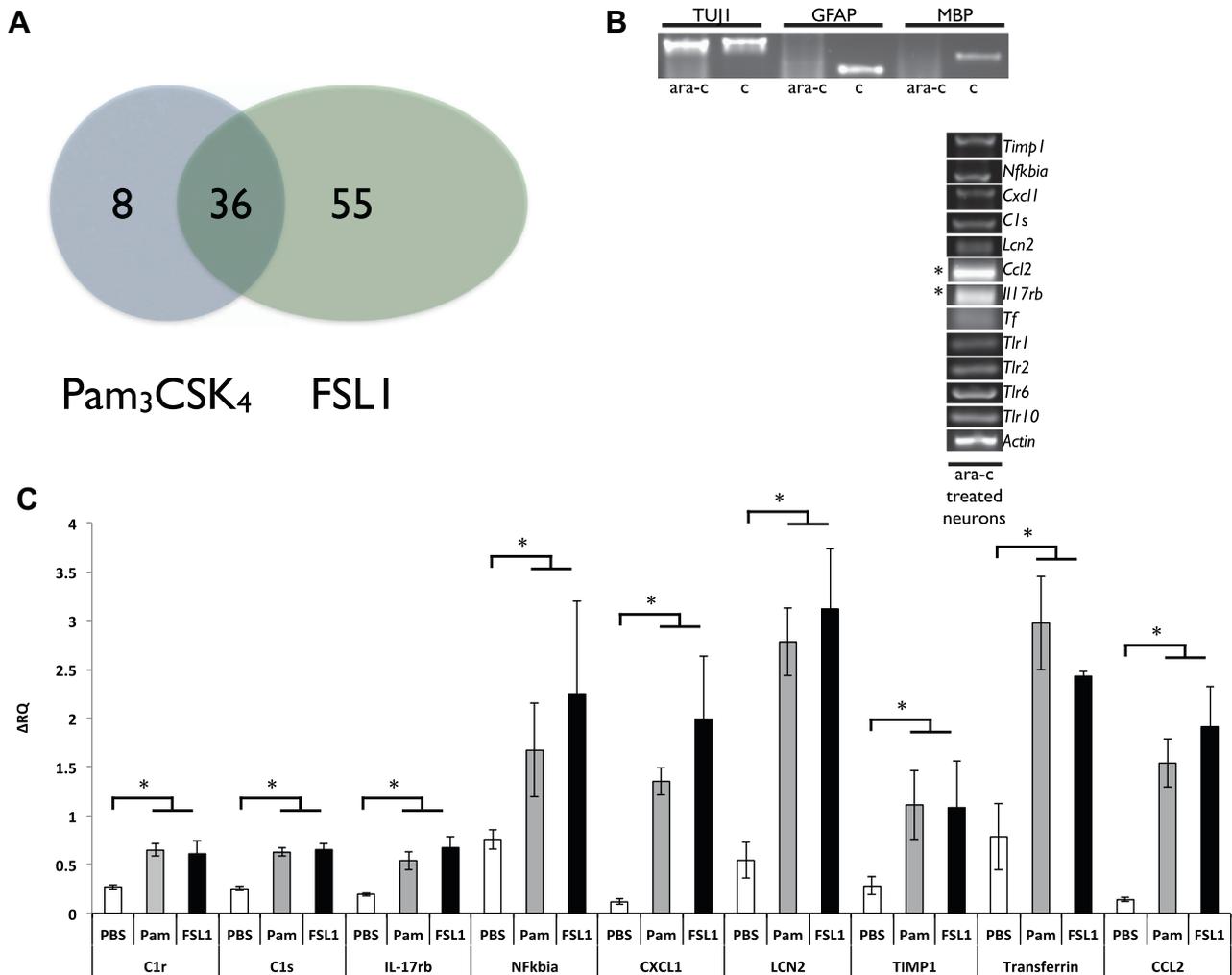


Fig. 7. TLR2/1 and TLR2/6 heterodimer activation in gliia-depleted cortical neurons in culture induces expression of immune-related genes. (A) Gene array analysis of FSL1- and Pam3CSK4-treated neurons resulted in 36 genes co-regulated by both treatments, 8 genes uniquely altered by Pam3CSK4, and 55 genes uniquely altered by FSL1 treatment. Cortical neurons derived from E18 rat embryos were treated with Ara-c to prevent growth of astrocytes in the culture. (B) Neuronal cultures exhibit high purity of neurons with negligible glial cells. These neurons express mRNA for *Timp1*, *Nfkb1a*, *Cxcl1*, *C1s*, *Lcn2*, *Ccl2*, *Il17rb*, *Tf*, *Tlr1*, *Tlr2*, *Tlr6* and *Tlr10*. (C) FSL1 and Pam3CSK4-treated cortical neurons exhibit up regulation of the above genes. * $P < 0.05$.

TLR2^{+/+} mice had no effect on the rate of acquiring the association between the tone-shock pairs ($P > 0.05$, Fig. 5A). However, both activation of TLR2/1 and TLR2/6 resulted in increased freezing behavior in response to the context of the fear ($F_{2,504} = 18.81$, $P < 0.0001$, two-way ANOVA; Fig. 5B). In addition, when the mice were exposed to the tone cue under a different context, a significantly higher freezing behavior was recorded in mice exposed to either TLR2/1 or TLR2/6 ligands compared with mice exposed to PBS ($F_{2,202} = 8.72$, $P = 0.0002$, two-way ANOVA; Fig. 5C). These data suggest that postnatal TLR2 activation does not affect fear-memory acquisition, but enhances its expression in hippocampus- and amygdala-dependent tasks. TLR2 activation resulted in minimal non-specific effects in TLR2^{-/-} mice. While no significant differences were noted in memory acquisition ($P > 0.05$, Fig. 5D), postnatal TLR2/6 but not TLR2/1 activation in TLR2^{-/-} mice resulted in higher contextual freezing ($F_{2,83} = 4.788$, $P = 0.0108$, two way ANOVA, Fig. 5E) but not cued fear ($P > 0.05$, Fig. 5F).

Similar to the impairment of spatial learning in TLR2^{-/-} mice compared to TLR2^{+/+} mice, TLR2 deficiency also resulted in significant alterations in fear learning. TLR2^{-/-} mice exhibited more freezing during the acquisition phase ($F_{1,198} = 21$, $P < 0.0001$, two-way RM-ANOVA, Fig. 5G), indicating enhanced acquisition of

the tone-shock pairs compared with TLR2^{+/+} mice. TLR2^{-/-} mice also exhibited a significantly higher freezing percentage during the contextual fear phase compared to TLR2^{+/+} mice ($F_{1,264} = 39.63$, $P < 0.0001$, Fig. 5H). In the cued fear, TLR2^{-/-} mice exhibited more baseline freezing compared to TLR2^{+/+} mice and also greater cue-induced fear ($F_{1,66} = 13.3$, $P = 0.0005$, Fig. 5I). These data suggest that TLR2^{-/-} mice are hypersensitive to fear learning, as indicated by altered acquisition rate, and fear-memory retention, as indicated by greater contextual and cued fear expression.

4.5. mRNA for the TLR2/1 and TLR2/6 heterodimers components are expressed in NPC-derived neurons

To unravel a possible underlying mechanism for the effects of postnatal central TLR2 activation on adult cognitive learning, we assessed which components of the TLR2 heterodimer receptor complex are expressed in neurons. Mouse and rat NPC were grown and subsequently induced to differentiate into mixed or pure neuronal cultures (Fig. 6A and B respectively). The mRNA of TLRs 1, 2 and 6 were expressed in TUJ1-sorted neurons differentiated from mouse or rat NPCs (Fig. 6A and B respectively).

Table 1
Genes significantly changed in both FSL1- and Pam₃CSK₄-treated cells.

Gene	Accession	Description	Fold change (treatment vs. control)	
			FSL1	Pam3CSK4
<i>Immune response</i>				
<i>Vcam1</i>	NM_012889.1	Vascular cell adhesion molecule 1	2.15536	2.38354
<i>Nfkb1a</i>	NM_001105720	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	2.54205	2.77152
<i>C3</i>	NM_016994	Complement component 3	3.44035	3.38284
<i>C1s</i>	NM_138900.1	Complement component 1, s subcomponent	4.12182	3.61322
<i>Cxcl1</i>	NM_030845.1	Chemokine (C-X-C motif) ligand 1	7.54967	7.18406
<i>Csf1</i>	NM_023981.4	Colony stimulating factor 1 (macrophage)	2.07123	2.3566
<i>Lgals3</i>	NM_031832.1	Lectin, galactose binding, soluble 3	2.66067	2.44433
<i>Ccl20</i>	NM_019233.1	Chemokine (C-C motif) ligand 20	4.40416	4.26894
<i>C1r</i>	NM_001134555	Complement subcomponent 1, r precursor	2.79518	2.64204
<i>Rt1-a1</i>	NM_001008827	RT1 class Ia, locus A1	1.88943	1.81264
<i>Icam1</i>	NM_012967.1	Intercellular adhesion molecule 1	1.95994	2.03059
<i>Ccl2</i>	NM_031530.1	Chemokine (C-C motif) ligand 2	12.4705	9.34879
<i>Lcn2</i>	NM_130741.1	Lipocalin 2	35.5416	19.3122
<i>Cfb</i>	NM_212466.3	Complement factor B	3.19197	2.02654
<i>Ccl7</i>	NM_001007612.1	Chemokine (C-C motif) ligand 7	4.48606	3.55715
<i>Il17rb</i>	NM_001107290	Interleukin 17 receptor B	2.2991	1.87533
<i>Chit3l1</i>	NM_053560	Chitinase 3-like 1	3.5555	2.27651
<i>Cd83</i>	NM_001108410	CD83 antigen	2.25055	2.48039
<i>Iron homeostasis</i>				
<i>Cp</i>	NM_012532	Ceruloplasmin (ferroxidase)	5.15266	3.27432
<i>Tf</i>	NM_001013110	Transferrin	3.7506	3.10038
<i>Response to oxygen compounds</i>				
<i>Nqo1</i>	NM_017000.2	NAD(P)H dehydrogenase, quinone 1	2.37531	2.33166
<i>Mlc1</i>	NM_001108105	Megalencephalic leukoencephalopathy with subcortical cysts 1	2.12194	2.55643
<i>Gbp2</i>	NM_133624.1	Guanylate nucleotide binding protein 2	2.68581	2.29991
<i>Abcb1</i>	NM_012623.2	ATP-binding cassette, sub-family B (MDR/TAP), member 1	2.98633	2.81256
<i>Cebpb</i>	NM_024125.3	CCAAT/enhancer binding protein (C/EBP), beta	2.45337	1.98883
<i>Timp1</i>	NM_053819.1	Tissue inhibitor of metalloproteinase 1	4.11365	2.61004
<i>Cell adhesion</i>				
<i>Fbln5</i>	NM_019153.2	Fibulin 5	-2.39974	-2.19017
<i>Gp38</i>	NM_019358.1	Glycoprotein 38	2.46865	1.85081
<i>Other</i>				
<i>Tnk2</i>	NM_001008336.1	Tyrosine kinase, non-receptor, 2	-1.93094	-1.80097
<i>Klhd7a</i>	NM_173427.2	Kelch domain containing domain 7a	1.86953	1.86771
<i>Slc16a1</i>	NM_012716.1	Solute carrier family 16 (monocarboxylic acid transporters), member 1	2.33029	2.01858
<i>Prss35</i>	NM_001008560.1	Protease, serine, 35	-1.84014	-2.06976
<i>Hnrpa2b1</i>	NM_001104613	Heterogeneous nuclear ribonucleoprotein A2/B1	2.40152	2.11111
<i>Penk-rs</i>	NM_017139.1	Preproenkephalin, related sequence	-2.37654	-2.23044
<i>Olr557</i>	NM_001000669.1	Olfactory receptor 557	2.08804	2.35568
<i>Hdac4</i>	NM_053449	Histone deacetylase 4	1.81091	1.99337

4.6. TLR2/1 and TLR2/6 activation in neurons drives an immune-related transcriptional response

Since both the mRNA for TLR1 and TLR6 were present in cortical neurons, we aimed at assessing whether activation of the two TLR2 heterodimers results in functionally different outcomes. To this end, RNA from neurons treated with the TLR2/6 ligand FSL1 and the TLR2/1 ligand Pam₃CSK₄ were analyzed using the Illumina rat gene array platform. Each experiment was repeated between two to six times (biological and technical replicates). Normalized data were used to identify genes whose expression was significantly up- or down-regulated (*P* value 0.05, ANOVA) with a magnitude exceeding a cutoff of 1.8-fold. Analysis of the degree of overlap between the genes affected by the Pam₃CSK₄ and the FSL1 groups showed that 36 genes were common to both FSL1 and Pam₃CSK₄ (Spearman correlation 0.83 with *P* value for the overlap of 1×10^6) (Fig. 7A, Table 1). Gene ontology analysis of the 36 genes indicates that 18 of the 36 genes (50%) are immune-related transcripts. Other genes are related to iron homeostasis (2 transcripts), response to oxygen compounds (6 transcripts) and cell adhesion (2 transcripts). The number of uniquely regulated genes in FSL1 and Pam₃CSK₄-treated neurons

was 55 and 8 respectively (Fig. 7A, Tables 2 and 3). FSL1 induced unique changes in transcripts related to extracellular matrix (8 transcripts), cell adhesion (7 transcripts) and cell differentiation (12 transcripts). Extracellular matrix-related transcripts were also affected by Pam₃CSK₄ treatment (3 of 8 transcripts).

As our gene-array analysis revealed that both TLR2/1 and TLR2/6 activation induce significant expression of immune-related transcripts, we initially tested whether this was due to astrocyte contamination of the neuronal cultures or whether these genes were indeed expressed by neurons. Single-cell PCR performed on RNA from isolated neurons showed that neurons express the mRNA of a select list of immune-related transcripts found to be altered in the gene array analysis: *Timp1*, *Nfkb1a*, *Cxcl1*, *C1s*, *Lcn2*, *Ccl2*, *Il17rb* and *Tf* (Fig. 7B). Since these genes are expressed in neurons, we tested whether neuronal cultures depleted of glia by treatment with Ara-c exhibit up-regulation of these genes in response to TLR2/1 or TLR2/6 activation. Indeed, these immune-related genes were up-regulated by FSL1 and Pam₃CSK₄ (Fig. 7C). To verify that this phenomenon also occurs in vivo, we injected P0 pups with Pam₃CSK₄, and extracted RNA from cortical tissues 24 h later. Of the 9 immune-related genes validated in vitro, only 5 genes were validated in vivo (*C1s*, *C1r*, *Nfkb1a*, *Lcn2* and *Tf* (Fig. 8)).

Table 2
Genes unique to FSL1-treated cells.

Gene	Accession	Description	Fold change
<i>Extracellular matrix</i>			
<i>Col11a1</i>	XM_342325.2	Procollagen, type XI, alpha 1	−2.25628
<i>Col3a1</i>	NM_032085.1	Collagen, type III, alpha 1	−2.4782
<i>Lum</i>	NM_031050.1	Lumican	−2.36492
<i>Cyp1b1</i>	NM_012940	Cytochrome P450, family 1, subfamily b, polypeptide 1	1.87075
<i>Col1a2</i>	NM_053356.1	Procollagen, type I, alpha 2	−2.04869
<i>Fbln1</i>	NM_001127547	Fibulin 1	−2.01646
<i>Col1a1</i>	NM_053304	Collagen, type 1, alpha 1	−2.40183
<i>Col5a2</i>	NM_053488.1	Collagen, type V, alpha 2	−1.98384
<i>Cell adhesion</i>			
<i>Spp1</i>	NM_012881.1	Secreted phosphoprotein 1	2.09366
<i>Ccdc80</i>	NM_022543	Coiled-coil domain containing 80	−1.82219
<i>Cd44</i>	NM_012924.2	CD44 antigen	1.93749
<i>Fam38a</i>	NM_001077200	Piezo-type mechanosensitive ion channel component 1 (Piezo1)	2.02991
<i>Cx3cl1</i>	NM_134455.1	Chemokine (C–X3–C motif) ligand 1	2.03788
<i>Dab2</i>	NM_024159.1	Disabled homolog 2 (Drosophila)	−1.89182
<i>Cd63</i>	NM_017125.2	CD63 antigen	2.03799
<i>Cell differentiation</i>			
<i>Picalm</i>	NM_053554.1	Phosphatidylinositol binding clathrin assembly protein	1.93321
<i>Dbn1</i>	NM_031024.1	Drebrin 1	1.88264
<i>Rasip1</i>	NM_001106261	Ras interacting protein 1	1.82286
<i>Nkx2-2</i>	NM_001191904	NK2 homeobox 2	−2.41304
<i>Igf2</i>	NM_031511.1	Insulin-like growth factor 2	−3.53454
<i>Ppp3r1</i>	NM_017309.2	Protein phosphatase 3, regulatory subunit B, alpha isoform (calcineurin B, type 1)	2.26484
<i>Rohn</i>	NM_001010953.1	Ras homolog gene family, member N	−2.16807
<i>Junb</i>	NM_021836.2	Jun-B oncogene	1.82868
<i>Myt1</i>	NM_001108615	Myelin transcription factor 1	−1.95865
<i>Gpc3</i>	NM_012774.1	Glypican 3	−2.06978
<i>Tnmd</i>	NM_022290.1	Tenomodulin	−2.66899
<i>Msn</i>	NM_030863.1	Moesin	1.91146
<i>Other</i>			
<i>Pcp4</i>	NM_013002.2	Purkinje cell protein 4	−2.03656
<i>Angptl4</i>	NM_199115.2	Angiotensin-like protein 4	1.8038
<i>Rarres2</i>	NP_001013445	Retinoic acid receptor responder (tazarotene induced) 2	−1.83247
<i>Acvrinp1</i>	NM_053621.1	Activin receptor interacting protein 1	1.82705
<i>Ns5atp9</i>	NM_201418.1	NS5A (hepatitis C virus) transactivated protein 9	−1.86284
<i>Tcn2</i>	NM_022534.1	Transcobalamin 2	−1.81345
<i>Rgs4</i>	NM_017214.1	Regulator of G-protein signaling 4	1.94574
<i>Setd3</i>	XM_216781	SET domain containing 3	1.82581
<i>Atp1a2</i>	NM_012505.1	ATPase, Na ⁺ /K ⁺ transporting, alpha 2 polypeptide	−2.26933
<i>Fmo1</i>	NM_012792.1	Flavin containing monooxygenase 1	−1.96393
<i>Ptgds</i>	NM_013015.2	Prostaglandin D2 synthase	−5.86134
<i>Uxs1</i>	NM_139336.1	UDP-glucuronate decarboxylase 1	1.82609
<i>Dcn</i>	NM_024129.1	Decorin	−2.84111
<i>Waspip</i>	NM_057192.2	Wiskott-Aldrich syndrome protein interacting protein	−2.08682
<i>Cjb2</i>	NM_001004099.1	Gap junction membrane channel protein beta 2	−1.80584
<i>Ap2b1</i>	NM_080583.1	Adaptor-related protein complex 2, beta 1 subunit	2.16441
<i>Tbxa2r</i>	NM_017054.1	Thromboxane A2 receptor	1.82802
<i>Ogn</i>	NM_001106103	Osteoglycin	−2.00267
<i>Aspn</i>	NP_001014030	Asporin	−1.99136
<i>Cdc2a</i>	NM_019296.1	Cell division cycle 2 homolog A (<i>S. pombe</i>)	−2.13298
<i>Lomp1</i>	NM_133404.1	Protease, serine, 15 (Prss15), mRNA	2.04288
<i>Hivep2</i>	NM_024137	Human immunodeficiency virus type 1 enhancer binding protein 2	1.89602
<i>Serping1</i>	NM_199093.1	Serine (or cysteine) peptidase inhibitor, clade G, member 1	2.00974
<i>Scrg1</i>	NM_033499.1	Scrapie responsive gene 1	−2.13317
<i>Pgd</i>	XM_003754118	6-phosphogluconate dehydrogenase, decarboxylating-like	2.02041
<i>Ccdc85b</i>	XM_003749044	Coiled-coil domain containing 85B	1.90987
<i>Zeb1</i>	NM_013164	Zinc finger E-box binding homeobox 1	−1.84333
<i>Cxcl16</i>	NM_001017478	Chemokine (C–X–C motif) ligand 16	1.80757

5. Discussion

The present study contributes a mechanistic insight into a body of evidence linking postnatal central infection and long-lasting cognitive impairments during adulthood. Our data indicates that TLR2-induced neuroinflammation in the neonatal brain differentially affects cognitive spatial and fear learning, as well as motor functions during adulthood in a TLR2-heterodimer specific manner.

TLR2 forms heterodimers with either TLR1 or TLR6. The MAMP Pam3CSK4 preferentially activates the TLR2/1 heterodimer;

however, in the absence of TLR1, Pam₃CSK₄ also activates the TLR2/6 heterodimer with a lower affinity (Buwitt-Beckmann et al., 2006). In the absence of TLR2, however, Pam3CSK4 does not activate TLR1 or TLR6 (Buwitt-Beckmann et al., 2006). While different MAMPs activate the TLR2/1 and TLR2/6 heterodimer complexes, the two-heterodimer complexes were thought to initiate an identical signaling cascade (Farhat et al., 2008). Therefore, to resolve whether a differential activation of the two heterodimers result in similar transcriptional and behavioral effects, we used Pam3CSK4 as a ligand to activate the TLR2/1 heterodimer complex, and FSL1 to activate the TLR2/6 heterodimer complex. A recent

Table 3
Genes unique to Pam₃CSK₄-treated cells.

Gene	Accession	Description	Fold change
<i>Extracellular matrix</i>			
<i>Ctgf</i>	NM_022266.1	Connective tissue growth factor	–2.06223
<i>Lox</i>	NM_017061	Lysyl oxidase	–2.38908
<i>A2m</i>	NM_012488.1	Alpha-2-macroglobulin	1.8568
<i>Other</i>			
<i>Lpd</i>	NM_134389.1	Lipidosin	1.80417
<i>Sst</i>	NM_012659.1	Somatostatin (Sst), mRNA.	–1.84121
<i>Cldn17</i>	NM_001107112.1	Claudin 17	1.8814
<i>Cyp7b1</i>	NM_019138	Cytochrome P450, family 7, subfamily b, polypeptide 1	1.94516

study (Du et al., 2011) showed that daily administration of a TLR2/1 ligand from PND 3–11 confers no effect on fear learning on PND 53. In contrast, our data indicate that exposure to TLR2/1 and TLR2/6 activation in newborn mice significantly increases contextual fear responses at 2 months after birth. These effects were not accompanied by abnormalities in open field behavior, indicating that the cognitive effects of early postnatal TLR2 activation were not the result of altered anxiety. This is further exemplified by the fact that only TLR2/1 activation impaired spatial learning while TLR2/6 had no such effect. Injecting a TLR2 ligand to TLR2^{−/−} mice did not affect exploratory behavior, motor or spatial learning. However, TLR2/6 activation did increase contextual fear learning. The increased freezing exhibited by TLR2^{−/−} mice does not seem to be dependent on context, as in both context dependent and context independent responses the baseline freezing was higher in TLR2^{−/−} mice compared with TLR2^{+/+} mice. These results suggest that in the absence of TLR2, receptors other than TLR1 or TLR6 could be activated and promote long-term effects on contextual but not cued fear learning.

Our data clearly indicates that TLR2 deficiency alters cognitive function. TLR2 deficient mice exhibit reduced exploratory behavior in the open field, blunted spatial learning capacity and an elevated fear response. Indeed, both TLR2/1 activation and a developmental deficiency of the TLR2 receptor similarly impaired spatial learning. However, the mechanisms behind the two effects probably differ. Developmental TLR2 deficiency does not seem to involve inflammatory mediators but may affect brain development during

embryogenesis (Okun et al., 2011). Park and colleagues have recently linked TLR2 deficiency to schizophrenia-like behavior in mice (Park et al., 2015). In their study, TLR2 deficiency resulted in hyperlocomotion, reduced anxiety-like behavior and impaired spatial and fear learning and memory. In contrast, our data show that TLR2 deficiency does not affect motor function, reduces exploratory behavior while not affecting anxiety levels, enhances fear learning and memory and impairs spatial memory. It is difficult to interpret these contradictory results, however, it appears that the TLR2^{−/−} mice used by Park et al. (2015) were on a mixed genetic background, whereas the wild type mice used by the authors were C57Bl/6 mice. In contrast, all the genotypes in the current study were on a congenic C57Bl/6 background. Therefore, differences in animal behavior could be result of genetic background rather than TLR2 deficiency.

It was previously suggested that activation of the TLR2/1 and TLR2/6 heterodimers results in similar transcriptional outcomes (Farhat et al., 2008). Our validated gene array data indicate that while a significant transcriptional overlap exists in neurons exposed to TLR2/1 and TLR2/6 ligands, each heterodimer also triggers a distinct transcriptional program, exemplified by extracellular remodeling, cell adhesion and cell differentiation transcriptional programs by TLR2/6 activation, while TLR2/1 activation affects several genes related to extracellular matrix remodeling. Although several important immune-related genes are differentially expressed in FSL1 and Pam3CSK4-treated neurons compared to controls (Table 1), no distinct mRNAs for

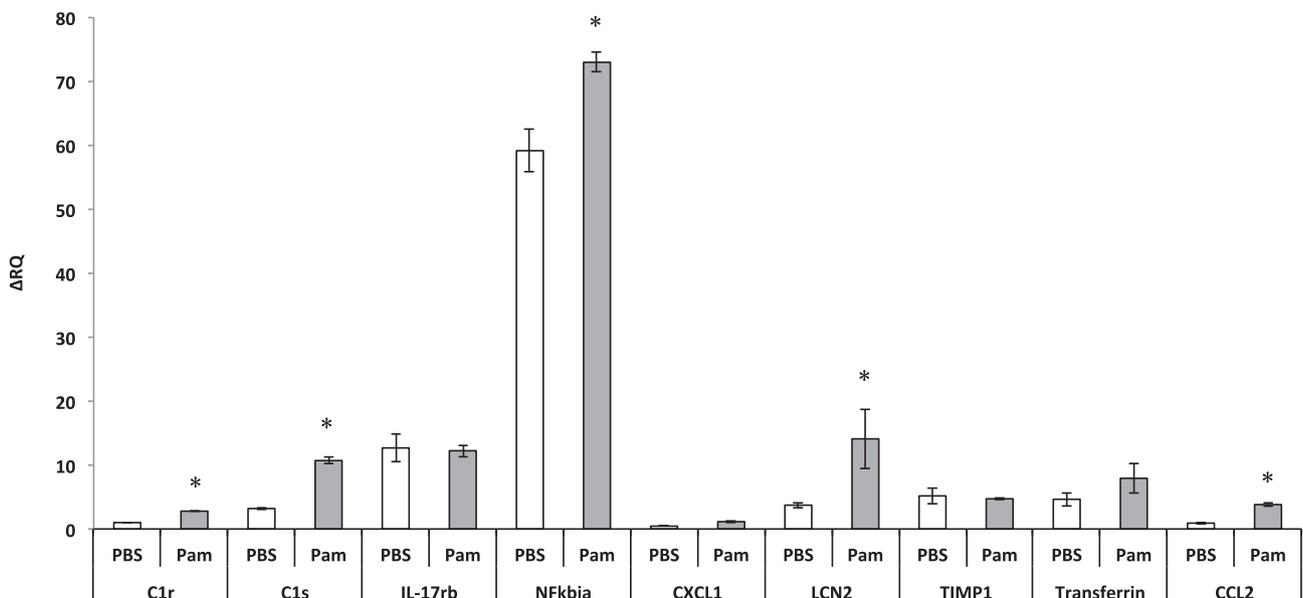


Fig. 8. TLR2/1 activation in P0 mouse pups induces the expression of immune-related genes. P0 TLR2^{+/+} mice were injected with Pam₃CSK₄ and were sacrificed after 24 h. RNA was then extracted from the cortices of these pups and mRNA for *Timp1*, *Nfkb1a*, *Cxcl1*, *C1s*, *Lcn2*, *Ccl2*, *Il17rb* and *Tf* was measured. **P* < 0.05.

pro-inflammatory cytokines appear to be driven by TLR2 activation in neurons. Although TLR2 activation is expected to drive expression of pro-inflammatory genes in immune cells, it appears that neurons exposed to PAMP-induced TLR activation, TLR2 included, do not respond in a nuclear-factor kappa-B-dependent (NFkB)-dependent manner (Okun et al., 2011). While the reason for this is unknown, the high levels of TLR2-induced *Nfkb* expression (Table 1, Figs. 7 and 8) by neurons both in vitro and in vivo could provide a possible explanation for this. *Nfkb*, Inhibits the activity of dimeric NFkB/REL complexes by trapping REL dimers in the cytoplasm through masking of their nuclear localization signals (Huxford et al., 1998). Therefore, our data raise the possibility that in neurons, expression of pro-inflammatory mediators is blunted following TLR2 activation due to *Nfkb* expression.

Of the 9 immune-related genes we observed to alter following TLR2/1 and TLR2/6 activation in vitro, only 5 genes (*C1s*, *C1r*, *Nfkb*, *Lcn2* and *Ccl2*) exhibited similar changes following TLR2/1 activation in vivo. We conclude that these 5 validated genes indicate that neurons in vivo also responded in a similar manner to TLR2 activation as did neurons in vitro. We regard the genes that failed to validate as TLR2-mediated effects that occurred solely in neurons in vitro or transcriptional changes that were negated by glial cells in vivo.

While the data herein indicate the long-term effects of postnatal central infection, several factors need to be considered. First, when a pathogen infects the brain, more than one innate immune pathway is activated. For example, in addition to TLR2, members of the NOD-like receptors (NLRs) family of innate immune receptors, could be activated by bacterial-derived PAMPs (Feldman et al., 2015). In addition, the tissue damage caused by injection can present a source of DAMPs presented to the brain, unlike in a situation in which bacterial infection compromises the neonatal brain. Lastly, this study focuses on the outcomes of central rather than peripheral induction of neuroinflammation. Indeed, peripheral neonatal infection mildly affects spatial learning in adulthood (Cronise and Kelly, 2001; Wallace et al., 2010; Williamson and Bilbo, 2014). Our conclusions on the impact of central postnatal neuroinflammation on adult cognitive learning, however, cannot be extrapolated to peripheral infection in neonates.

Overall, our findings suggest that TLR2 is an important player in the plasticity of cognitive behavior, in that activation of TLR2 impairs certain cognitive functions, while enhancing others. Importantly, our findings demonstrate that a single exposure to a TLR2 ligand in the early postnatal period can cause long-lasting alterations in cognitive function, suggesting that exposure of infants to infectious agents that activate TLR2 might affect their learning and memory ability later in life.

Acknowledgment

This work was supported, in part, by the Intramural Research Program of the National Institute on Aging.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbi.2015.04.020>.

References

- Buwitt-Beckmann, U., Heine, H., Wiesmuller, K.H., Jung, G., Brock, R., Akira, S., Ulmer, A.J., 2006. TLR1- and TLR6-independent recognition of bacterial lipopeptides. *J. Biol. Chem.* 281, 9049–9057.
- Cronise, K., Kelly, S.J., 2001. Maternal urinary tract infection alters water maze performance in the offspring. *Neurotoxicol. Teratol.* 23, 373–379.
- Du, X., Fleiss, B., Li, H., D'Angelo, B., Sun, Y., Zhu, C., Hagberg, H., Levy, O., Mallard, C., Wang, X., 2011. Systemic stimulation of TLR2 impairs neonatal mouse brain development. *PLoS One* 6, e19583.
- Farhat, K., Riekenberg, S., Heine, H., Debarry, J., Lang, R., Mages, J., Buwitt-Beckmann, U., Roschmann, K., Jung, G., Wiesmuller, K.H., Ulmer, A.J., 2008. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. *J. Leukoc. Biol.* 83, 692–701.
- Feldman, N., Rotter-Maskowitz, A., Okun, E., 2015. DAMPs as mediators of sterile inflammation in aging-related pathologies. *Ageing Res. Rev.*
- Franceschini, A., Szklarczyk, D., Frankild, S., Kuhn, M., Simonovic, M., Roth, A., Lin, J., Minguez, P., Bork, P., von Mering, C., Jensen, L.J., 2013. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res.* 41, D808–D815.
- Hao, L.Y., Hao, X.Q., Li, S.H., Li, X.H., 2010. Prenatal exposure to lipopolysaccharide results in cognitive deficits in age-increasing offspring rats. *Neuroscience* 166, 763–770.
- Heindel, J.J., 2006. Role of exposure to environmental chemicals in the developmental basis of reproductive disease and dysfunction. *Semin. Reprod. Med.* 24, 168–177.
- Huxford, T., Huang, D.B., Malek, S., Ghosh, G., 1998. The crystal structure of the IkappaBalpha/NF-kappaB complex reveals mechanisms of NF-kappaB inactivation. *Cell* 95, 759–770.
- Kang, J.Y., Nan, X., Jin, M.S., Youn, S.J., Ryu, Y.H., Mah, S., Han, S.H., Lee, H., Paik, S.G., Lee, J.O., 2009. Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. *Immunity* 31, 873–884.
- Kawai, T., Akira, S., 2007. Signaling to NF-kappaB by Toll-like receptors. *Trends Mol. Med.* 13, 460–469.
- Liu, C.C., Crone, N.E., Franaszczuk, P.J., Cheng, D.T., Schretlen, D.S., Lenz, F.A., 2011. Fear conditioning is associated with dynamic directed functional interactions between and within the human amygdala, hippocampus, and frontal lobe. *Neuroscience* 189, 359–369.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25, 402–408.
- Long, E.M., Millen, B., Kubes, P., Robbins, S.M., 2009. Lipoteichoic acid induces unique inflammatory responses when compared to other toll-like receptor 2 ligands. *PLoS One* 4, e5601.
- Mallard, C., 2012. Innate immune regulation by Toll-like receptors in the brain. *ISRN Neurol.* 2012, 701950.
- Maren, S., 2008. Pavlovian fear conditioning as a behavioral assay for hippocampus and amygdala function: cautions and caveats. *Euro. J. Neurosci.* 28, 1661–1666.
- Mattson, M.P., Kater, S.B., 1988. Isolated hippocampal neurons in cryopreserved long-term cultures: development of neuroarchitecture and sensitivity to NMDA. *Int. J. Dev. Neurosci.* 6, 439–452.
- Okun, E., Griffioen, K., Barak, B., Roberts, N.J., Castro, K., Pita, M.A., Cheng, A., Mughal, M.R., Wan, R., Ashery, U., Mattson, M.P., 2010a. Toll-like receptor 3 inhibits memory retention and constrains adult hippocampal neurogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15625–15630.
- Okun, E., Griffioen, K.J., Son, T.G., Lee, J.H., Roberts, N.J., Mughal, M.R., Hutchison, E., Cheng, A., Arumugam, T.V., Lathia, J.D., van Praag, H., Mattson, M.P., 2010b. TLR2 activation inhibits embryonic neural progenitor cell proliferation. *J. Neurochem.* 114, 462–474.
- Okun, E., Griffioen, K.J., Mattson, M.P., 2011. Toll-like receptor signaling in neural plasticity and disease. *Trends Neurosci.* 34, 269–281.
- Okun, E., Barak, B., Saada-Madar, R., Rothman, S.M., Griffioen, K.J., Roberts, N., Castro, K., Mughal, M.R., Pita, M.A., Stranahan, A.M., Arumugam, T.V., Mattson, M.P., 2012. Evidence for a developmental role for TLR4 in learning and memory. *PLoS One* 7, e47522.
- Park, S.J., Lee, J.Y., Kim, S.J., Choi, S.Y., Yune, T.Y., Ryu, J.H., 2015. Toll-like receptor-2 deficiency induces schizophrenia-like behaviors in mice. *Sci. Rep.* 5, 8502.
- Rees, S., Harding, R., Walker, D., 2008. An adverse intrauterine environment: implications for injury and altered development of the brain. *Int. J. Dev. Neurosci.* 26, 3–11.
- Takeda, K., Akira, S., 2004. TLR signaling pathways. *Semin. Immunol.* 16, 3–9.
- Wallace, K.L., Lopez, J., Shaffery, J.P., Wells, A., Paul, I.A., Bennett, W.A., 2010. Interleukin-10/ceftriaxone prevents *E. coli*-induced delays in sensorimotor task learning and spatial memory in neonatal and adult Sprague–Dawley rats. *Brain Res. Bull.* 81, 141–148.
- Williamson, L.L., Bilbo, S.D., 2014. Neonatal infection modulates behavioral flexibility and hippocampal activation on a Morris Water Maze task. *Physiol. Behav.* 129, 152–159.