EXPRESSION OF TOLL-LIKE RECEPTORS 3 and 4 (TLR3/4) ON NEURAL PROGENITOR CELLS (RADIAL GLIAL CELLS): IS DIRECT IMMUNE ACTIVATION POSSIBLE IN RAT EMBRYONIC BRAIN WITH MATERNAL IMMUNE ACTIVATION?

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Amanda Waber Nguyen

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Jamie Kneitel, Ph.D.                                    Date

Department of Biological Sciences
Abstract

of

EXPRESSION OF TOLL-LIKE RECEPTORS 3 and 4 (TLR3/4) ON NEURAL PROGENITOR CELLS (RADIAL GLIAL CELLS): IS DIRECT IMMUNE ACTIVATION POSSIBLE IN RAT EMBRYONIC BRAIN WITH MATERNAL IMMUNE ACTIVATION?

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Understanding the underlying factors that regulate early neurogenesis is essential to identifying inducers of aberrant neurodevelopment linked to autism and schizophrenia. Determination of these factors will eventually yield possible strategies for treatment and preventive measures to ameliorate negative effects of these inducers. Endogenous regulatory mechanisms ensure proper migration and proliferation of neuronal precursor cells, called neural progenitor cells, in the proliferative zone during embryonic cortical neurogenesis. Although all regulatory factors have yet to be determined, microglia in the developing brain have been shown in previous studies to exhibit regulatory function, engulfing neurons and neural progenitor cells to ensure proper developmental patterning. In response to antigen exposure, the maternal immune response (MIA) increases Interleukin-6 levels through binding of Toll-like Receptor 3 for viral antigens and Toll-like Receptor 4 for bacterial endotoxins. The fetus is then exposed to the resulting increased inflammatory cytokines in the mother, which can pass through the placenta and
into the fetus. Increased cytokines correlated with an increased propensity for schizophrenia in human studies, especially in cases with a family history of the disease. These clinical observations are supported by rat and mouse studies that indicate MIA negatively impacts neural proliferation and promotes microglial cell activation, resulting in behavioral deficits in offspring. Our hypothesis is that MIA activates microglia and increases cytokines, altering fetal neuroproliferation and migration patterning. Results from in utero electroporation and intraventricular injection labeled radial glial cells indicated expression of TLR3 and TLR4 in the embryonic rat proliferative zone during neurogenesis. An ELISA method was developed for analysis of IL-6 cytokine levels that indicates an inflammatory response, as is seen in maternal immune activation. This evidence would help clarify the observed differences in susceptibility of autism and schizophrenia in human clinical studies and MIA effects on early neurogenesis. The project was conducted in the Noctor Laboratory, MIND Institute, UC Davis College of Medicine.

_______________________, Committee Chair
Rosalee Carter, PhD.

_______________________
Date

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INTRODUCTION

Understanding the underlying factors that regulate early neural development is essential to identify inducers of aberrant embryonic neurogenesis. Determination of these factors will help develop effective prevention and treatment for behavioral disorders autism and schizophrenia caused by alterations in the pattern of neurodevelopment. Autism is a spectrum disorder characterized by repetitive behaviors, reduced social interactions and communication abilities, often with reduced mental capacity. Autism prevalence is approximately 1 in 110 children in the United States, and there is currently no cure or effective treatment. Treatments for symptoms of Autism include primarily social and behavioral training from a young age (Lounds et al, 2012). Some clinical trials for drug therapy show some symptomatic improvement using agonist of the gamma aminobutyric acid receptor type B, arbaclofen (Frye, 2014). Schizophrenia is a debilitating behavioral disorder affecting 1% of the population. Schizophrenic individuals often exhibit depressed emotional expression and neural developmental disturbances early in life, then develop paranoid delusions and hallucinations as a young adult. Current therapies for schizophrenia are anti-psychotic drug treatments that decrease hallucinations, but have severe side effects (Hagberg et al, 2012). New atypical anti-psychotic drugs like 9-hydroxrisperidone have improved efficacy and can be injected once a month intramuscularly, improving symptom treatment but not curing disease (Carter, 2012). Altered patterning of embryonic brain development are evident in both schizophrenia and autism, however the triggers of this aberrant neurogenesis patterning are not known.
Embryonic neurogenesis is rapid, producing in the span of a few days billions of neurons from the multipotent neural progenitor cells, also known as the radial glia cells (RG). Therefore, multifaceted regulatory factors ensure the proper neuroproliferation and neuronal migratory patterning essential for neurodevelopment. During neural development, neurons are borne of asymmetric and symmetric divisions of RG cells. When RG cells were first observed in 1888 by Magini, he described them as “radial neuroglial cells” due to their long radial processes. Magini also identified small varicosities along the fibers of RG cells as newborn neurons. As microscopic techniques improved, RG were described as only a scaffold and support cell for newborn neurons to migrate along. In 1972 Rakic hypothesized neurons were born from a separate population of neural precursor cells yet to be identified. Currently, RG cells are observed to be both a supportive cell and a multipotent neuronal precursor cells, giving rise to neurons and then glial cells within the developing embryonic cortex (reviewed in Bentivoglio and Mazzarello, 1999; Noctor et al, 2002). Within the embryonic forebrain, this cortical neurogenesis occurs in the proliferative zone, which is comprised of the primary Ventricular Zone (VZ) and the secondary Sub-Ventricular Zone (SVZ). The neurons develop directly in the SVZ from the RG cells and indirectly from the secondary intermediate progenitor cells in the VZ. Proper neurogenesis patterning requires balance of factors which promote and quell neuroproliferation and migration. All factors and cells which influence this early embryonic neurogenesis are not yet completely understood, but include microglia and neural growth factors (Noctor et al, 2004). Neural growth factors include epidermal growth factor (EGF). Microglia are immunologically active phagocytic cells related to macrophages, and can become activated with exposure to infection, phagocytosing infected or damaged cells. Activated microglia release multiple inflammatory factors, such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα). In the developing brain, microglia are observed to phagocytize some neurons and neural progenitor cells within the proliferative
zone which are not damaged. If microglia are eliminated \textit{in vivo} or \textit{ex vivo}, excessive neuproliferation is observed. Elimination or activation of microglia, or exposure to inflammatory cytokines which can occur due to infection, influences neuronal proliferation and migration, altering the patterning of the developing cortex (reviewed in Breunig et al, 2011; Cunningham et al, 2013).

An additional factor recently implemented as influencing early neurogenesis is expression of toll-like receptors (TLRs) in the developing cortex. The primary function of TLRs are as pattern recognition of antigens, both endogenous and exogenous, to illicit an immune response to pathogens, and cells, like microglia, expressing TLRs can become activated when bound to an antigen initiating an inflammatory cascade response. Although multiple TLRs expression in the brain has been observed, TLR3 and TLR4 are important due to recognition of common antigens and increased cortical expression during embryonic neurogenesis. TLR3 expression levels in mice brains peak during early neurogenesis, at Embryonic Day 12.5 (e12.5), then decreases until birth. TLR4 expression was at a constant level during the same period. The proliferating region in the developing cortex showed diffuse TLR3 expression, although specific expression on neural progenitor cells was observed \textit{in vitro} (Lathia et al, 2008; De Miranda et al, 2010). Developed murine neurons in culture expressed TLR3 and TLR4. The neurons were also responsive to cytokine inflammatory signal interferon gamma. When derived from TLR4 deficient mice, cortical neurons were resistant to glucose deprived cell death (Tang et al, 2007). Dissociated murine neurospheres, which contain RG cells, expressed TLR3 and TLR4 (Yaddanapudi et al, 2011). The expression and activation of TLR3 decreases neuroproliferation and axonal growth during neurogenesis (Lathia et al, 2008). In mice with TLR3 deficiency, spatial memory decreased and anxiety response increased (Okin et al, 2012). Mice with TLR4
deficiency have decreased proliferation of neuronal progenitor cells and hippocampal neurogenesis (Rolls et al, 2007). Memory acquisition and fear learning were impaired in TLR4 deficient mice (Okun et al, 2012). Epidermal Growth Factor (EGF) promotes neurogenesis, but inflammation increased TLR3 activation decreases neuroproliferation. The sonic hedgehog-signaling pathway promotes neurogenesis through activation of downstream zinc finger transcription factors Gli1-3, which induce transcriptional changes of the cell cycle regulators cyclin-dependent kinases. In dissociated mice neurospheres, the induction of the TLR3 inflammatory cascade interferes with sonic hedgehog signaling by inhibiting the intermediate Erk phosphorylation, thereby decreasing neuron proliferation (Yaddanapudi et al, 2011). Although specific mechanisms and targets are not yet delineated, TLR3 and TLR4 activation has direct influence on embryonic neurogenesis.

The proper balance of regulatory factors, which include endogenous TLRs expression and microglia activity, is important for proper embryonic neurogenesis patterning although not yet completely understood. Inflammation can upset that balance in the developing brain. MIA is the activation of the immune response of a pregnant mother, potentially exposing the developing fetus to inflammatory cytokines and inducing inflammation, an innate immune response marked by increased vascular permeability and release of cytokines (Smith et al, 2007). The pathway of inflammation in the mother begins when TLRs recognize exogenous or endogenous antigens. TLR3, located in the endosomes, detects double stranded viral RNA (Choe et al, 2005; reviewed in Mallard et al, 2012). Polyinosinic:polycytidylic acid (Poly-IC) exposure models viral infection, with the two polymer compound mimicking viral double stranded RNA and binds TLR3 (Fortier et al, 2004). When TLR4 binds the gram-negative bacterial endotoxin lipopolysaccharide (LPS), a heteromeric complex with MD-4 forms, eliciting an inflammatory
cascasde (Poltorak et al, 1998; Park et al, 2009). Activation and dimer formation for both TLR3 and TLR4 initiates downstream signaling and inflammatory cytokine production. When the antigen binds to TLR3, an activated homodimer forms, initiating a downstream inflammatory cascade (Choe et al, 2005). TLR4 is located in the outer cell membrane and binds the endotoxin bacterial lipopolysaccharide (LPS) produced by gram-negative bacteria. Endogenous inflammatory factors released by damaged tissue can also activate TLRs (Kaul et al, 2012).

Activation of TLR3 and TLR4 initiates downstream signaling and cytokine production, including the cytokine IL-6. This cytokine production is mediated by Janus Kinase 2 (JAK2), a tyrosine kinase which activates the Signal-Transducers and Activators of Transcription (STATs) and induces inflammatory gene transcription (Minogue et al, 2012; Takeuchi and Akira, 2010).

Primary microglia cells release Interleukin-6 (IL-6), TNFα, and IL-1β in response to LPS (Aguirre et al 2013). Inflammatory cytokines of the mother pass through placenta and across the fetal blood-brain during MIA in mice (reviewed in Mallard et al, 2012). IL-6 production is mediated by JAK2, a tyrosine kinase that activates signal-transducers and activators of transcription (STATs). Downstream inflammatory gene transcription is promoted, including induction of IL-1β (Minogue et al, 2012; Takeuchi and Akira, 2010). The downstream cytokine IL-6 alone can elicit the same MIA induction response as PolyIC and LPS (Smith et al, 2007). Although the immunogens LPS and Poly IC did not pass across the placenta during MIA in mice, cytokines directly transferred from mother to fetus (Smith et al, 2008). Increased levels of cytokines in some studies were not observed in the fetal brain tissue, but fetal plasma levels of the cytokine IL-1β were increased (Ashdown et al, 2006). One theory of MIA is that the increased inflammatory cytokines in maternal blood pass through the placenta and induce brain inflammation in the fetus (reviewed in Mallard, 2012). Some inflammatory cytokines, such as
neurotensin and IL-6, can increase permeability of the blood-brain barrier epithelial cells, thereby exposing the fetal brain to external factors (reviewed in Theoharides et al, 2013). IL-6 levels increased in fetal rat brain with LPS induced MIA (Oskvig et al, 2013; reviewed in Mallard, 2012). In rat models, one study observed only increased fetal plasma levels of IL-1β, while another observed mice fetal brain levels with increased in IL-6 with LPS and TNFα with PolyIC (Ashdown et al, 2006; Bachstetter et al, 2011). IL-6-induced MIA increased by 16-fold the levels of inflammatory cytokines in the placenta (Hsiao and Patterson, 2011). Cells within the pregnant mother and placenta express TLRs, with TLR4 increased expression and activation in the placentas from premature birth clinically. TLR3 is the highest expressed TLR in the placenta (reviewed in Amirchaghmaghi et al, 2013). The activation of TLRs with inflammation, such as MIA, can impact embryonic neurodevelopment.

With the imbalance of regulatory mechanisms during neurogenesis, embryonic brain neurodevelopmental deficits can result. In rat models, MIA increased microglia activation and decreased neural progenitor proliferation (Cunningham et al, 2013; de Miranda et al, 2010; reviewed in Mallard, 2012). Synaptic formation and receptors abnormalities develop after MIA in some rat and mice studies (Naviaux et al, 2013; Smith et al, 2007; reviewed in Boksa, 2010). In mice, placenta size, functionality decreased and permeability increased with MIA, possibly contributing to aberrant embryonic brain development as well (Hsiao and Patterson, 2011). The levels of neurodevelopment proteins including GluN1 of NMDA receptor and VAMP-1 decreased in fetal rats exposed to MIA (Forrest et al, 2012). Recent microarray transcriptome data indicated gene expression changes with exposure of mother mice to influenza, PolyIC, or IL-6 in offspring. Genes with the most expression changed across the three conditions from control group included five crystalline genes, and these expression level changes were
acute, and correlated with level of MIA. However, expression of inflammatory genes such as IL-6 and TLRs was not increased (Garbett et al, 2012). No significant changes in gene expression in cortex was observed in adult mice exposed to MIA \textit{in utero}, or significant histone modifications (Connor et al, 2012). LPS resulted in an increase in IL-1b production by microglia cells, while PolyIC increased TNF\textsubscript{a} (Bachstetter et al, 2011). In individuals with a genetic susceptibility for schizophrenia or autism, MIA could increase the risk of having these disorders. Incidence of autism and schizophrenia have been correlated to exposure of inflammatory factors \textit{in utero}-by maternal immune activation (MIA), potentially triggering the altered patterning in embryonic brain development (Patterson, 2002).

Exposure of developing rats and mice fetuses to MIA yields adult offspring with behavioral deficits in tests of social preference and sensorimotor coordination, which seen in autism. In clinical evaluations, a relationship between maternal infections during pregnancy has been associated with increased incidence of behavioral abnormalities in children. When there is a family history of the disease, then the strength of the correlation increases (Patterson et al, 2002; reviewed in Boksa, 2010; reviewed in Hagberg et al, 2012). Epidemiological studies indicate a three-fold increase with the first trimester exposure, and a seven-fold increase with early second trimester exposure to influenza \textit{in utero} (Brown, 2006). Increased cytokines correlated with an increased propensity for schizophrenia in human studies (Brown et al, 2004). Current research in autism links many genetic mutations with the disorder without causality, besides Fragile X Syndrome (reviewed in Theoharides et al, 2013). In offspring exposed to MIA behavioral abnormalities, primary in social interactions and also working memory, are observed in rat and lower primate studies (Ashdown et al, 2006; Conner et al, 2012; de Miranda et al, 2010;
Zuckerman et al, 2005). PolyIC-induced MIA was also associated with behavioral abnormalities in offspring in mice (De Miranda et al, 2010; Smith et al, 2007; Meyer et al, 2006).

Determining which specific neurogenesis regulatory factors MIA alters and activate microglia cells in the developing brain will lead to a more comprehensive understanding of the mechanisms contributing to aberrant neural development in autism and schizophrenia (Noctor et al, 2004; Cunningham et al, 2013). My project was to explore specific TLR3 and TLR4 expression pattern in embryonic rat brains in vivo, then evaluate MIA influences on expression patterns. An ELISA for measuring the inflammatory cytokine IL-6 levels was developed and tested to evaluate MIA activation in the rat dam and embryos and to correlate with IHC fetal brain protein expression patterns of TLR3, TLR4, and markers for cell types.
METHODS

Animals

Timed pregnancy Sprague-Daley rat dams were utilized in the procedures. Rat dams were on 12-hour light, 12-hour dark cycle, in individual cages, and fed food and water *ad libitum*. Animal procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee, Protocol 16386.
Figure 1. The vector and plasmid constructs injected in utero intraventricularly into embryonic rat brain have mammalian specificity and low copy number for retrovirus. a. pCMV-I-GFP b. pVSV-G (Clontech Laboratories, Mountain View, CA) c. BLBP-dsRed2 in pCAT3 (gift of Anton Lab).

**In utero Intracerebral Electroporation**

As seen in figure 2, according to established protocols, to allow whole cell visualization, neural progenitor cells in E19 rat embryos were labeled through in utero intracerebral electroporation of the reporter gene expressing plasmid, pCAG-DsRed. CAG is a promoter and enhancer that contains Cytomegalovirus early enhancer element (C), the first exon and intron of the chicken β-actin gene, which is highly conserved in vertebrates (A), and the rabbit β-globin gene splice acceptor (G). Within the plasmid, the CAG promoter is upstream from the reporter gene, which is DsRed, specific to mammalian cells, vector is shown in figure 1 (Niwa et al, 1991). The rat dam was anesthetized, and respiration assisted during procedure with oxygen and reduced
respiration of anesthetic isofluorane. A pinch of the dam’s toe was employed to ensure that she was completely non-responsive. She was placed on a heating pad, ventral side up, and then her abdomen is shaved and cleaned with alcohol and then betadine. A laparotomy with a small, approximately 10 cm incision near the midline was made through the skin and muscle layer. The uterine horns were carefully extracted to not rupture amniotic sacs, and all embryos were counted. One microliter of pCAG-DsRed plasmid (1mg/ml) was mixed with 1 microliter of blue dye visualization and then was injected into the lateral ventricle of E19 embryos with a pulled, beveled glass micropipette. The micropipette was pulled from 75mm glass capillary tubes using a puller P-97 with conditions of heat 800, pressure 500, pull 30 velocity 40 until the external diameter is approximately 60µm, then tip was beveled (Drummond Scientific, Broomall, PA) (Siato and Nakatsuji, 2001). Next, the embryonic brain was kept moist with warmed sterile saline. Next, the brain was grasped gently with electrode forceps with 1 cm plates on lateral sides of the brain parallel to the anteroposterior axis, as seen in figure 2a. Higher voltage increased the uptake of plasmid, but decreased the survival of embryos. Therefore, multiple pulses at lowest voltage possible to still have plasmid uptake and expression was utilized during electroporation of embryo brains, conditions were 5 pulses, 50 V, 50 msec pulse length, 1 sec interpulse interval ; BTX (Saito and Nakasuji, 2001; Noctor et al, 2002). The embryos in uterine horns were placed back into the dam abdominal cavity, and 3ml sterile saline was added directly into the interperitoneal (IP) cavity. After the abdominal wall layer was sutured, the skin layer was stapled. Pain relief was provided by subcutaneous injection of bupernex (0.05mg/100g body weight)
into dam, then twice a day for 3 days post-surgery. The embryos were allowed to further develop for two days, then sacrificed for evaluation (Saito and Nakatsuji, 2001; Noctor et al, 2002; Cunningham et al, 2013).

**In utero Intra-cerebral Injections**

As seen in figure 1, a retrovirus that is replication incompetent expressing an enhanced GFP was produced in 293gp NIT-GFP stably transfected for packaging cell line (gift from Dr. Fred Gage, Salk Institute, La Jolla, CA) (Noctor et al, 2002). The NIT-GFP is a MoMLV based package with retrovirus elements, neomycin phosphotransferase (neo), tetracycline transactivator protein (tet) as well as the enhanced GFP. As shown in figure 1, GFP expression vector suppressed with tetracycline, and has G418 resistance (Palmer et al, 1999). Then the cells were transiently transfected with Vesicular Stomatitis Virus Glycoprotein (pVSV-G) using the Calphos Mammalian Transfection Kit (Clontech, Palo Alto, CA) (Palmer et al, 1999). VSV-G was chosen due to increased mammalian transfection efficiency (Okimoto et al, 2001). The supernatant containing retrovirus was harvested after 48 hours, filtered for cell debris using a 0.45µm filter, then concentrated after centrifuging at 35,000rpm at 4°C for 1.5 hours. The final titer of the resolublized pellet was $1 \times 10^7$ colony-forming units/ml$^{-1}$. As seen in figure 2, 1µl of retrovirus GFP was injected into the lateral ventricle of the embryo via *in utero* intra-cerebral injections. The embryos were then returned into the dam and allowed to further develop for two days, then were sacrificed for evaluation (Saito and Nakatsuji, 2001; Noctor et al, 2002; Cunningham et al, 2013).
Maternal Immune Activation induction in rat

To determine differences in Maternal Immune Activation (MIA) response, timed pregnant rats were given with interperitoneal injections (IP) with either 100 µg/kg lipopolysaccharide (LPS; *E. coli* 0111:B4, Sigma) or sham injection (0.9% saline) on embryonic development day 15 (E15) and E16. To test LPS induction in the presence of the microglial inhibitor doxacycline (Dox), dam received an LPS injection on E15 and E16, and fed Dox containing chow *ad libitum* starting on day E15 until sacrifice on day E19. Control condition dams received Dox *ad libitum* starting on day E15. To determine how much food containing Dox the dams ingested, the food was weighed daily (Cunningham et al, 2013). On day E19, dams received an injection of 100mg/kg of Bromodeoxyuridine (BrdU) to label dividing cells. BrdU is a thymidine analog that integrates in cells that are undergoing synthesis. These proliferating cells were identified through Immunohistochemistry (IHC) using BrdU antibody. BrdU is a commonly used proliferation marker for neurogenesis since the 1960s, and the radioactive thymidine analog since the 1950s, though only labeling cells undergoing DNA synthesis not cell division. Since BrdU introduces a foreign component into DNA, it results downstream effects, such as altering RNA then protein expression, and toxic effects on cell differentiation, cell cycle, cell migration and neural progenitor cell survival. Effects are decreased when exposure time decreases (Duque and Rakic, 2011). Therefore in all experiments, BrdU was only administered by a single injection 2 hours before sacrifice of embryos and dam (Noctor et al, 2002).
Protein Extraction from Maternal Serum

For cytokine analysis indicative of immune activation, maternal blood was collected by cardiac puncture in tubes with 20U/ml heparin. Next, blood samples were centrifuged at 600g for 15 minutes, and then supernatant stored at -80°C (Bachstetter et al, 2011; Forrest et al, 2012).

Protein Extraction from Rat Brain Tissue

The embryos were removed from the dam on e19. To determine if perfusion of tissue impedes antigen retrieval, some embryos were only flushed with Phosphate Buffered Saline (PBS), some were only decapitated, and others were flushed with PBS and then perfused with 4% paraformaldehyde (PFA) for fixation of tissue. Brain tissue was micro dissected from the skull cavity after decapitation of the embryo and quickly rinsed with ice-cold, sterile PBS for unfixed tissue. To compare the histological to protein analyses, the left hemisphere was dissected from the right hemisphere. The left hemisphere was isolated, then prepared for fresh tissue histology. Using a 30-gauge needle with tip bent, the fresh brain tissue was secured through the cerebellum, and slowly lowered into a 50ml conical tube filled with isopentane. The tube was placed in a foam box of crushed dry ice to flash freeze the tissue and minimize cracking or crystal formation within the tissue. Tissue was immediately frozen, placed in tubes and stored in -80°C freezer. Then tissue was cryosectioned in 30um sagittal slices. Some slices remained fresh. Other slices were fixed onto the slide using 50ul of 4% paraformaldehyde for 20 minutes at -20°C. Then the right hemisphere was flash-frozen in a dry ice and ethanol solution, and stored at -80°C until further processing and
analysis. Initially control embryonic brain tissue was subjected to different conditional buffers to ensure maximum protein recovery and minimal ELISA interference. The higher yield protein extraction method was from Barichello et al, in which the brain tissue was homogenized using a mortar and pistol on ice in extraction solution (0.4mol/L NaCl, 0.05% Tween 20, 0.5% BSA, 0.1mmol/L Phenylmethyl sulfonil fluoride, 0.1mmol/L benzethonium chloride, 10mmol/L EDTA and 20KI aprotinin, using Ultra-Turrax (Fisher Scientific, Pittsburgh, PA)). The ratio of tissue to buffer was 100mg/ml. To improve isolation of proteins, brain tissue homogenates were spun at 15000 rpm for 30 min at 4°C and the supernatants were collected and stored at -80°C until analysis (Barichello et al, 2010).

The placentas were dissected from the uterine horn, quickly rinsed with ice-cold, sterile PBS, then flash-frozen in an ethanol and dry ice solution, and stored at -80°C until analysis. To generate cell lysates, each placenta was placed in 1 ml of cell lysis buffer (50 mM Tris-HCl (pH 7.4) with 0.6 M NaCl, 0.2% Triton X-100, 1% BSA, and 1 EDTA-free protease inhibitor cocktail tablet/10 ml buffer) (Roche Applied Sciences; Indianapolis, IN). The placental tissues were then homogenized on ice using a syringe fitted with an 18G needle and then crushed with mortar and pistol. The homogenates were centrifuged at 4°C at 13,000 rpm for 30 minutes. Next, the supernatants were recovered, then frozen at -80°C until assayed.
For storage and stability, most current databanks of tissue and samples were fixed for preservation, then histological assessment. Fixed protein retrieval removes crosslinking of paraformaldehyde during fixation, so protein structure is accessible and correlated with histochemistry findings. Fixed e19 rat brains were immersed in low-melt agarose and then sliced coronally in 500μm sections on a cryostat. Then as seen in figure 10a, cortex containing slices were microdissected for inclusion of the subventricular zone region, and reserved. To extract protein from this fixed section of the brain, an extraction buffer containing TBS, 2%SDS, and .2M Glycine was added in a 1:10v/v. The tissue was then crushed using a cold mortar and pistol, and placed in a microcentrifuge tube. The sample was boiled at 100°C for 20 minutes, then incubated for 2 hours at 80°C. The samples were allowed to cool to room temperature and spun at 20,000g for 10 minutes at 4°C. The lysate containing extracted proteins was collected, and stored at -80°C (Tibes et al, 2006). The protein concentration was determined by a BCA assay, then protein quality and presence of specific proteins were evaluated using a western blot analysis as depicted below.

BCA Protein Concentration Assay

A Pierce BCA (bicinchoninic acid) Protein Assay was conducted on all samples after homogenization according to manufacturer’s instructions (Thermo Scientific, Rockford, IL). Briefly, a standard curve of bovine serum albumin (BSA) was prepared from 0 to 4 mg/ml in PBS with volume of 10μl per well in duplicate, and a serial dilution
of sample (1, 1:2, 1:4) in PBS. Next added to each well was 200 µl of 1:50 mixture of Reagent B:A, mixed right before use. Plates were covered and incubated at 37°C for 30 minutes, then results read on plate reader at 562nm. Sample protein concentrations were estimated based on the BSA standard curve.

ELISA

The IL-6 levels were compared using a DuoSet sandwich Enzyme-Linked ImmunoabSorbant Assay (ELISA) construction kit, with plates prepared per manufacturer directions (Antigenix, Huntington Station, NY). Briefly, the capture antibody mouse anti-rat IL-6 (4.0 µg/ml) diluted in sterile PBS is coated onto a 96-well microplate and incubated overnight at room temperature. Antibodies were diluted from stock concentration (720 µg/ml) to the working concentration in reagent diluent (2% heat-inactivated Normal Goat Serum, 1% BSA, in sterile PBS). Heat-inactivation of serum is necessary so as to not interfere with the assay by inactivating complement components (Soltis et al, 1978). All steps require covering with adhesive strip during incubation. The solution was aspirated from the wells and then washed with wash buffer (PBS, .05% Tween 20). The plate was next incubated with reagent diluent (1% BSA in PBS) for 1 hour at room temperature. To conduct the assay, 100µl of samples per well in duplicate were incubated for 2 hours at room temperature, 50 µg/100µl. In the initial assay assessment, each sample was assessed with a serial dilution (1, 1:2, 1:4, 1:8) to ensure IL-6 levels are within the standard curve range. Additionally, a serial dilution of the sample spiked with the same concentration of IL-6 standard (200pg/ml) was also
assayed to determine if sample buffer conditions interfered with detection of a known concentration of IL-6. Then the plate was washed three times with wash buffer, followed by incubation with detection antibody biotinylated goat anti-rat IL-6 (400ng/ml) for 2 hours at room temperature. After an additional 3 washes with wash buffer, 100µl of streptavidin-HRP was added per well and incubated covered for 20 minutes at room temperature.

Embryonic Transcardial Perfusion

For fixed slices, embryonic rats of either sex were removed from the dam and were transcardially perfused with 4% PFA. The dam was anesthetized with interperitoneal injection of ketamine and zylazine (1:3) (.35ml). The dam was kept on a warming pad, and a midline incision through the skin and abdominal lining is made approximately 2 inches from base of tail dorsally. The embryos were removed individually, beginning at the top of the uterine horn. Embryos were placed in a glove on ice to anesthetize. Then moved onto a dissecting dish with arms and legs pinned under dissection microscope. A ‘u-shaped’ incision was made with microscissors under arms and through ribcage to expose heart. The right atria is snipped with scissors, and needle injected into left ventricle. Approximately 5 ml of PBS was flushed through embryo transcardially, then approximately 5 ml of cold 4% paraformaldehyde over the course of 3 minutes with constant pressure. Brains were then removed from skulls and processed (Martinez-Cerdeno et al, 2012). After fixation in 4% PFA for two hours, brains are cryoprotected overnight in 30% sucrose. The fixed brains were sectioned along the
coronal plane on a cryostat, then dry mounted on Super frost Plus slides (Fisher) and stored at 4°C until used for imaging (Martinez-Cerdeno et al, 2012).

**Immunohistochemistry**

The antibody used to identify Primary Neural Progenitor Cells (Radial Glial Cells) was transcription factor Tbr2, polyclonal chicken anti-Tbr2 (1:500, Abcam), and for intermediate progenitor cells, the marker was Pax-6 transcription factor, polyclonal rabbit anti-Pax6 (1:50, Abcam). The polyclonal antibody rabbit anti- NeuN, a neuron specific nuclear protein in vertebrates, was used as marker for mature neurons (1:200, Milipore). The anti-ionized calcium-binding adaptor molecule 1 (Iba1) was used as a marker for microglia cells, in polyclonal rabbit anti-Iba1 (1:250, Wako) and polyclonal goat anti-Iba1 (1:100) (Noctor et al, 2004; reviewed in Breunig et al, 2011).

Polyclonal rabbit anti-rat Toll-like Receptor 3 (TLR3) (1:50-1:250) (Abcam) and monoclonal mouse anti-human TLR3 IHC-plus (1:50-1:1000) (Millipore) were used. For TLR4 staining, antibodies evaluated were rabbit anti-mouse TLR4 and mouse anti-rat TLR4 (Abcam) (1:50-1:500). Secondary antibodies were conjugated to Dylight 405, Cy2/Dylight 488, Cy3/Dylight 549, or Cy5/Dylight 649 (Jackson ImmunoResearch), including secondary antibodies: donkey anti-mouse, donkey anti-rabbit, donkey anti-chicken, donkey anti-goat, and donkey anti-rat (1:200). A range of antibody concentrations were tested, as well as negative and positive controls for both TLR3 and TLR4 antibodies, since the manufacturer did not yet test immunohistochemistry conditions specific for brain tissue. Dapi stain was used to label the nuclei of all cells within the proliferative region. Positive results were indicated by co-staining of TLR3 or
TLR4 with Tbr2 and Pax6 for neural precursor cells. Co-staining with Iba1 would indicate labeling of microglia with TLR3 or TLR4, respectively. Additional brain tissue samples of different ages was conducted due to the lack of information on antibody use in these conditions. Initially, since neural precursor cells of adult hippocampus are a positive control for TLR3 and TLR4 based on previous immunohistochemistry studies, albeit utilizing different antibodies, adult brain tissue containing hippocampus was utilized for positive control (Rolls et al, 2007). The standard immunohistochemistry (IHC) method was implemented initially, with serial dilutions of antibody. Briefly, brain tissue that was cryosectioned into 30um sections and frozen at -20°C onto slides were warmed to room temperature, washed 3 times in 0.1M PBS, then subjected to antigen retrieval by submersing in tray with citrate buffer in microwave for 15 minutes to boil. The slides were cooled to room temperature, and then carefully washed 3 times for 5 minutes each wash in PBS to remove citrate buffer. The slices on the slide were circled with a PAP pen to form hydrophobic barrier wells, then blocked directly on the slide in 10% donkey buffer (donkey serum with 0.01%Triton-X) for 1 hour at room temperature, with a humidification chamber so tissue does not dry out and become damaged. Block solution was removed, and primary antibodies were added to each well, diluted to concentration in 2% donkey buffer. Another method with no antigen retrieval was also tested to determine if the antigen retrieval method damaged antibody binding. The background increased significantly, also with free-floating 30um brain tissue slice without antigen retrieval in antibody and 2% donkey buffer solution in a well of a 24 well microplate. After overnight incubation at room temperature in primary antibody, brain
tissue on wells were rinsed carefully 3 times for 5 minutes each with PBS rocking at room temperature. The fluorescently labeled secondary antibody were added at 1:200 in 2% donkey buffer with 1% triton-x to the brain slices according to species of primary antibody (donkey) for 2 hours at room temperature in a humidification chamber.

**BV2 cells**

To isolate the microglia and assess expression of TLR3 and TLR4 as well as the effects of immune activation and microglial suppression directly on microglia was also assessed *in vitro*. The microglial cell line BV2 expresses markers, and undergoes phagocytosis similar to microglial cells *in vivo* (Blasi et al, 1990). Cells were grown in a sterile environment incubator in DMEM with 10% fetal bovine serum albumin (FBS). BV2 cells were treated with LPS, LPS and the microglial inhibitor minocycline, minocycline alone, or control, for increasing time points (0, 12, 24 hours). Serum for cytokine production was also collected. The cells were then fixed with 4% PFA and wet mounted onto glass slides. Fixed BV2 cells were evaluated for protein expression using immunocytochemistry. Antibodies evaluated were used at same concentrations as IHC.

**Confocal Microscopy Imaging**

Sections were imaged on an inverted Olympus Fluoview laser-scanning confocal microscope. Excitation/emission wavelengths were 488/515 nm (GFP), 568/590 nm (Texas red) and 685/690 nm (Cy5). Images were contrast-enhanced, assembled and false color added using Adobe Photoshop (Noctor et al, 2004).
Western Blot

After samples are homogenized and protein assayed with BCA assay, Laemmli Buffer (10% β-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl) was added 1:4 to 10µg of sample and boiled at 95°C for 5 min, cooled to room temperature on ice, then centrifuged at 16,000g in microcentrifuge for 1 min. Then samples were electrophoresed on SDS-PAGE gels at 50V for 5 min, then 100V approximately 1 hour, until dye front reached the bottom of the gel. Next, the proteins were transferred from the gel onto a nitrocellulose membrane overnight at constant current of 10mA. Transfer efficiency was assessed by Coomassie gel stain and destaining until distinct bands were observed. Membrane was blocked in 3% BSA in TBST (20mM Tris, 150mM NaCl, 0.15% Tween 20) for 1 hour at room temperature. Primary antibodies diluted in blocking buffer were at following concentrations: polyclonal rabbit anti-rat TLR3 (1:500), monoclonal mouse anti-rat GAPDH (1:2000), monoclonal mouse anti-rat TLR4 (1:1000), and polyclonal rabbit anti-γ tubulin (1:1000) as per manufacturer recommendations. Primary antibody incubation was performed overnight with rocking at 4°C. Membrane was washed 4 times with TBST, and then incubated with secondary antibodies. Membrane for chemiluminescent detection incubated with donkey anti-rabbit-HRP(1:10,000) (Cell Signaling, gift of Dr.Tassone Lab, Sacramento,CA) and donkey anti-mouse-HRP (Bio-Rad, gift of Dr.Tassone Lab, Sacramento, CA) diluted in blocking buffer, 1 hour at room temperature, covered. Membrane for infrared (IR) detection, incubated with IR dye 800 CW goat anti-rabbit
(1:15,000) (Li-Cor) and IR dye 680LT (1:15,000) (Li-Cor) in Li-Cor blocking buffer. Both membranes were washed 3 times in wash buffer (TBST). IR Li-Cor membrane was then visualized on a Li-Cor IR scanner for presence of bands. Chemiluminescent detection membrane was then rinsed with 2 times with ddH2O rocking for 3 min at RT before addition of ECL (1:1 A:B solution). Detected bands were imaged on film.

Statistics

Qualitative immunohistochemistry analysis will observe if there is co-localization of TLR3/4 and markers for neural precursor cells, neurons, microglia, or astrocytes. Analysis of ELISA was determined initially if best fit standard curve, R^2 value > .70. Then compare samples to curve to estimate IL-6 level in samples, as averages of 3 or more replicates of each condition. Degree of individual variability within groups and between groups was assessed.
RESULTS

*In Utero* Intraventricular injection labels radial glia cells

Initial studies with dual electroporation and intraventricular *in utero* injections of both plasmid pCAG-dsRED and RV-GFP into rat embryos resulted in reabsorption of embryos in two out of three litters, likely due to maternal stress. In the litter that survived until extraction at day e21, five embryos expressed both plasmid-dsRED and RV-GFP, three expressed RV-GFP alone, and one was not successful. Figure 2b-e shows images depicting the cortex of the embryos that expressed both plasmid and RV vector expression pattern, location in subventricular zone, and cellular morphology that both methods label RG cells. However, dsRED plasmid expressing cells were more localized at site of injection, while RV-GFP positive cells were more dispersed beyond initial injection site. Processes extend from the ventricular surface into the proliferative zone, progeny daughter neurons also are expressing RV-GFP, co-localization of both dsRED and GFP expression indicated labeling of the same cell population. In figure 2f, an inset of an RV-GFP positive cell exhibits RG morphology, with a cell soma near ventricular surface, and long process with visible varicosities (small thickening) of process. RV-GFP injection had a more dispersed and robust expression, so progressed utilizing this method for identifying RG cells for IHC.
Figure 2. In utero intraventricular injection and electroporation into rat embryonic brain for identification of radial glial cells. a. Diagram of rat dam laparotomy surgery and in utero embryo intraventricular injection (1ml of 1mg/ml dsRed plasmid and RV-GFP) and electroporation site on embryonic brain through amniotic sac and embryo head. b-e. Expression of plasmid and RV in subventricular zone (SVZ) of e21 embryonic rat brain (150um coronal slice, 20x) (RVEP278 individuals L2,L3,R2,R3 respectively). Diagram labeling location of image in embryonic rat brain slice (V ventricle). c. Radial glial cell expressing RV-GFP (150um coronal slice, 40x). Inset figure of rat brain coronal slice for approximate location of image (Ventricle (V) subventricular zone (SVZ)).

Determining antibody conditions to identify TLR3 and TLR4 in immunohistochemistry

As noted in Table 1, multiple concentrations of monoclonal mouse anti-TLR3 (Milipore), monoclonal mouse anti-TLR4 (Abcam), polyclonal rabbit anti-TLR3 (Abcam) were tested, in the range of 1:50-1:500 (originally 1mg/ml antibody
concentration). Secondary alone was observed as in Figure 4d and 6c, to determine background non-specific staining. High background was observed with diffuse staining pattern under standard IHC conditions, which made identification of positive cells not clear. Additional IHC conditions were tested (data not shown). Without antigen retrieval, the background increased, impeding identification of specific cells of interest and co-localization was not clear. Some overlap of the fluorophores was apparent due to the diffuse nature of the antibody. As in Table 1, biotin labeling was used to amplify the signal, without confounding fluorophore overlap. However, no distinguishable biotin label was observed, indicating necessity of additional antibodies to be tested to accurately identify cells positive for TLR3 and TLR4 (data not shown).

<table>
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<td>rabbit anti-TLR4</td>
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<td>rabbit anti-TRAIL</td>
<td>Santa Cruz</td>
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<td>(Citrin buffer 15 min microwave)</td>
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<td>IHC standard condition varied concentration antibody [1:50, 1:100, 1:500]</td>
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<td>tested a11-P116 Rat</td>
<td>tested a11-P116 Rat</td>
<td>tested a11-P116 Rat</td>
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<tr>
<td>IHC standard condition concentration [1:50] multiple ages of fixed rat cortex</td>
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<td>tested a11-P116 Rat</td>
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<tr>
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<td>tested high background</td>
<td>not tested</td>
<td>tested negative</td>
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<td>tested high background</td>
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Table 1. TLR3 and TLR4 antibody testing conditions for fixed embryonic rat brain tissue.
After contacting antibody companies for specific region antibody was raised against, a BLAST was conducted to determine if these regions were possibly bound and not available for antibody in native state, although effective for denatured protein as in western blot. Although the exact epitopes of the antibody were not delineated, especially in polyclonal rabbit anti-TLR3, results indicated that the mouse anti-TLR3 epitope is in a possible binding site. This may have interfered with antibody binding in tissue conditions, in which proteins are in a native conformation state. Therefore, additional polyclonal rabbit-anti TLR3 (Abcam) and polyclonal rabbit-anti TLR4 (Santa Cruz) were tested as well, as observed in Table 1. To determine if tissue conditions were impeding the observation of positive TLR3 and TLR4, the antibodies were also tested in BV2 cells, with immunocytochemistry.
Figure 3. Differences in pattern of expression of TLR3 in embryonic and adult rat cortex.


TLR3 expression pattern is diffuse in embryonic rat cortex
As seen in figure 3, although expression detection is low, a trend for age related expression pattern differences was observed for TLR3. Positive cells were diffuse within the embryonic rat brain. Observed TLR3 positive cells are more intense at the early e14 and e15 and decreases in adult. In figure 3a, punctate TLR3 staining was seen throughout the cortex, including the proliferative zone in the e14 rat during early neurogenesis. By e19, the expression was decreased in intensity, with a trend for less positive staining (Figure 4d). After birth, the expression of TLR3 was still observed in Figure 3b, with decrease in TLR3 positive cells, and change in expression pattern. There was a higher expression the outer cortex layers, with less intense observed in the ventricular region. In adult rat cortex, TLR3 expression changes, with some cells still highly expressing TLR3, as in Figure 3c and e, in a pattern similar to that observed with neuronal processes of intermediate neurons.

NeuN neuronal marker co-localized with TLR3 in P4 rat cortex, although additional samples will be needed for validation. Although microglia in rat express TLR3 at e14, evident by co-expression of Iba1 and TLR3 in figure 1a and 1b, this was not evident in later ages, as in figure 4c-e.
Figure 4. TLR3 expression and co-localization within embryonic rat brain. a. e14 rat rabbit-TLR3 (1:50), goat anti-Iba1 (1:100), chicken-tbr1 (1:100) (20x, 30um slice, z-stack)
b. e14 rat dapi (1:200), rabbit-TLR3 (1:50), goat anti-Iba1 (1:100)(40x, 30um slice, z-stack).c. e15 rat rabbit-TLR3 (1:50), goat anti-Iba1 (1:100), chicken-tbr2 (1:100) (100x, 30um slice) d. e19 rat rabbit-TLR3 (1:50), goat anti-Iba1 (1:100), chicken-tbr2 (1:100) (100x, 30um slice, z-stack) e. e19 rat rabbit-TLR3 (1:50), goat anti-Iba1 (1:100), chicken-tbr2 (1:100) (100x, 30um slice, z-stack).f. P4 Rat dapi (1:200), rabbit-TLR3 (1:50), rat-NeuN (1:100) (20x, 30um coronal slice z-stack depth) Inset figure of rat brain coronal slice for approximate location of image (Ventricle (V) subventricular zone (SVZ)).
Expression of TLR3 and TLR4 in BV2 Cells

BV2 microglial cells expressed TLR3, as seen in figure 9a. Precursor cells express punctate TLR3, as in e15 rat, noted by positive stain of Tbr2 cells in figure 4c. In e19 and e15 rat, activated microglia are near cells positively expressing TLR3, in figure 4c-e.

Figure 5. TLR3 expression in embryonic rat brain is diffuse and low signal without antigen retrieval. a. e19 embryonic rat TLR3 expression (30um coronal slice, 40x). b-c. e19 RV in utero injection, chicken-gfp-FITC(1:200),rabbit-TLR3(1:50)(Abcam),goat-Iba1(1:100) (30um coronal slice, 40x, IHC no antigen retrieval, 0.1% triton in blocking buffer).d-e.RV-GFP RG cell (30um slice, 40x). Inset figure of rat brain coronal slice for approximate location of image (Ventricle (V) subventricular zone (SVZ).
Expression of TLR3 and TLR4 on RG cells identified by RV-GFP expression was not clear, as conditions for maintaining GFP expression in IHC resulted in high background for TLR3 antibody, as seen in Figure 5b-d, TLR4 had a similar high background (TLR4 expression data not shown). To observe GFP expression in fixed embryonic brain tissue with IHC, no antigen retrieval was used, and blocking buffer permeabilization component triton x was decreased to 0.1%, from 1%. As TLR3 and TLR4 antibodies labeling was not clear without antigen retrieval, an additional antigen retrieval method, incubation at room temperature in hydrogen peroxide, was used to evaluate RG expression of TLR3 and TLR4.

Figure 6. Patterns of expression of TLR4 in embryonic and adult rat brain. a. e19 rat, goat-ibal(1:100),rabbit-TLR4(1:100)(Santa Cruz)(40x,30um coronal slice) b. P116 female rat no antigen retrieval dapi(1:200), goat-Iba1(1:100) and rabbit-TLR4(1:50)(Abcam) (30um coronal section, 100x).c. P116 female rat, no antigen
retrieval dapi (1:200), secondary alone (30um coronal section, 100x). d. P116 female rat, dapi(1:200), goat-Iba1(1:100) and rabbit-TLR4(1:50)(Abcam) (30um coronal section, 40x). Inset figure of rat brain coronal slice for approximate location of image (Ventricle (V) subventricular zone (SVZ).

Expression TLR4 is diffuse in embryonic and adult rat cortex

As seen in Figure 6a-d, although additional samples were needed for verification, expression of TLR4 is diffuse within the rat cortex across different ages. Co-localization with microglial marker Iba1 was not observed in tissue; however, BV2 microglial cells expressed TLR4, as seen in figure 7b.

![Image of BV2 cells expression of proteins in response to immune activation with LPS. a. BV2 cells exposed to LPS, rabbit-TLR3 (1:100), goat-Iba1 (1:100), mouse-PH3 (1:100) (40x). b. BV2 cells exposed to LPS rabbit-TLR4 (1:100) c. BV2 cells exposed to LPS dapi (1:200), goat-Iba1 (1:100) mouse-PH3 (1:100) (100x).]
TLR3 expression pattern in \textit{in utero} LPS MIA e19 rat cortex

TLR3 expression in e19 rat cortex is faint and diffuse, and increased with \textit{in utero} LPS MIA exposure, as in figure 8a-d. With MIA, increased expression of TLR3 was seen throughout the e19 cortex, as in figure 8c and 8e. This observation supports the Co-expression of TLR3 and Iba1 microglial marker is noted with LPS MIA exposure, figure 8b, indicating either TLR3 is potentially always expressed in the fetal microglia but at levels too low to detect by IHC, or TLR3 is up regulated in fetal microglia only during inflammation. The expression of TLR3 on BV2 microglial cells \textit{in vitro} with LPS exposure in figure 7 supports this finding.
Figure 8. Expression pattern of TLR3 in embryonic rat brain with MIA activation with LPS compared to no activation. a. e19 rat LPS in utero exposure, dapi (1:200), goat-iba1(1:100), rabbit-TLR3(1:50)(Abcam). Co-localization with iba1 seen in depth image by yellow color (30um coronal slice, 20x) b. e19 rat, depth image z-stack (30um coronal slice, 40x) (Fluoview). c. e19 embryonic rat TLR3 expression dapi (1:200), goat-iba1(1:100), rabbit-TLR3(1:50)(Abcam) (30um coronal slice, 20x). Inset figure of rat brain coronal slice for approximate location of image (Ventricle (V) subventricular zone (SVZ)).

Pattern of expression of TLR4 in in utero LPS MIA e19 rat cortex

Proliferating Cell Nuclear Antigen (PCNA) is a marker for mitotic cells, and co-localized with TLR4 expression in cells within the SVZ proliferative zone in e19 rat exposed to LPS MIA and the microglial inhibitor Doxycycline, as seen in figure 9a and
9b. When observing TLR4 expression pattern and intensity, potential subtle differences between LPS, LPS and Doxacycline, and control e19 brains are only evident, as in Figure 9. Further analysis with cell counting and additional samples would be necessary to determine if the differences between TLR4 patterning is significant with MIA. The cortical expression pattern of TLR4 in e19 rat is diffuse, with expression of TLR4 in proliferative zone region of high Tbr2 expression lower than in more ventral cortical regions containing mature neurons, as in Figure 9c-e.
Figure 9. TLR4 expression with exposure to MIA induced by LPS and treated with Doxycycline and LPS compared to control.  

a. e19 rat lps/dox in utero exposure, goat-Iba1 (1:100), rabbit-TLR4 (1:100) (Santa Cruz), mouse-PCNA (1:100) (30um coronal slice, 100x).  
b. e19 rat LPS in utero exposure, dapi (1:200), rabbit-TLR4 (1:100) (Santa Cruz)  
e. depth image of z-stack no co-localization with microglia 40x.  
e-f. e19 rat dapi (1:200), goat-iba1 (1:100), rabbit-TLR4 (1:100) (Santa Cruz) (40x, 30um slice)  
g-h. e19 rat in utero LPS exposure, dapi (1:200), goat-iba1 (1:100), rabbit-TLR4 (1:100) (Santa Cruz) (40x, 30um slice).  
Inset figure of rat brain coronal slice for approximate location of image (Ventricle (V) subventricular zone (SVZ).
Recovery of proteins from fixed samples

Protein assay indicated that protein was recovered from fixed samples, and the standard curve of BSA indicated accuracy with $R^2=0.997$. Western blot transfer efficiency was good, as distinct but faint bands are apparent on the gel with Coomassie stain.

Figure 10. Western blot analysis for TLR3 and TLR4 in SVZ region with microdissection in fixed-recovered rat embryonic brain tissue. a. diagram of region of tissue analyzed outlined in purple, slice from vibratome of e19 rat fixed brain, slice image from Li-cor whole tissue IR scanner. Red indicates BrdU positive cells in SVZ region of control e19 rat cortex. b. Li-cor western blot result indicates presence of TLR4 detected...
in samples with rabbit anti-TLR4 antibody (Abcam), and high degree of background banding.

Preliminary TLR4 protein expression in embryonic rat cortex by western blot

To determine if TLR3 and TLR4 antibodies detected denatured protein in the sample, a western blot was conducted. As in figure 10, tissue used for initial western blot contained fresh-frozen embryonic cortex, placenta, BV2 cells (microglial immortalized mice cell line) and fixed embryonic tissue after protein recovery method. Results from western blot contained multiple bands, with high background, higher with fixed-recovered cortex samples. Detected bands in fixed samples had smearing, which indicated some protein degradation in sample, as would be expected. In previous studies protein retrieval from fixed samples recovered approximately 40% of protein from sample (Tibes et al, 2006). Concentration of IR secondary antibody will be decreased in future studies to decrease background.
Figure 11. ELISA detects IL-6 protein in tissue sample. a. Standard curve of IL-6 protein is a good fit and linear (r²=0.9824, between replicates standard curve). b. E18 rat brain IL-6 protein detected with ELISA with higher spiked levels indicating not interference by tissue and buffer conditions (spiked sample has 200pg/ml IL-6 added). c.
Addition of sample in buffer decreases detection of IL-6 protein in ELISA significantly with SDS high fixed retrieval buffer compared to lysis buffer b.

ELISA detection of IL-6 in fresh tissue conditions

In figure 11c, preliminary ELISA indicated that IL-6 was detected in samples, and the buffer conditions did not interfere with detection of spiked IL-6 standard into samples. Standard curve of IL-6 standard has a $R^2 = 0.982$ value, indicating good fit, in figure 11a. This standard curve equation was then used to estimate concentration of IL-6 in samples. To improve precision of concentration estimate since the samples contained low levels of IL-6 protein, in future assays an increase in lower concentration of IL-6 standard will be used. In addition, the samples will be allowed to incubate longer than 20 minutes with streptavidin-HRP, noting maximum colormetric saturation in highest concentration of standard, to better delineate the lower level concentrations. Importantly, as seen in earlier MIA studies in mice, LPS induction increases the IL-6 levels dramatically, therefore care to contain the range necessary for all samples is essential for evaluation. A dilution series of samples was implemented in this preliminary ELISA, as in figure 11b, which allowed for detection of samples with high and low IL-6 levels. Preliminary results from figure 12 indicated that buffer and sample conditions did not interfere significantly with ELISA assay. When fixed tissue was processed to remove cross-linking, interference with assay were detected, with significant reduction in spiked IL-6 detection within sample, due to the high percent SDS buffer.
DISCUSSION

Multiple factors regulate embryonic neurodevelopment. The TLRs expressed on neurons, microglia, and potentially RG cells influence neurogenesis in the developing brain. In BV2 microglial cell line cells were observed positive by immunocytochemistry for Iba1, TLR3 and TLR4. This observation supports previous studies which noted the expression of TLR3 and TLR4 on microglia cells in dissociated culture based on mRNA expression and western blot. Immune competent cells, such as microglia, contain multiple receptors to identify pathogenic immunogens. However, in tissue immunohistochemistry, co-labeling of microglia positive marker Iba1 or cd11b and the TLR3 and TLR4 markers was difficult to identify. Previous studies did not observe positive co-labeling of TLR3 on microglia in embryonic brain ( ). Since expression may be low on microglia, although evident in mRNA expression, and the native conformation may be masking multiple epitopes for antibody binding in immunohistochemistry. Qualitative differences in TLR3 expression pattern observed by IHC support the trend observed in previous studies by western blot, that in early embryonic development expression of TLR3 levels were highest in the cortex (Lathia et al, 2008).

The TLR4 IHC results did not indicate qualitative difference between ages in pattern of expression, or with exposure to LPS induced MIA. Interestingly, as TLR4 bind to LPS antigen, an increase in the TLR4 would be expected if brain tissue was exposed to the antigen. This finding supports the theory that the adverse effects on neurogenesis with MIA result from exposure of inflammatory cytokines from the mother,
or placenta (reviewed in Mallard, 2012). Additionally, it is important to note that all adult cortex observed were adult, pregnant female rats. Expression of TLRs were seen to change in the mother during stages of pregnancy in rats and clinically. TLR expression pattern indicates importance in stages of pregnancy beginning with implantation through labor. Differences in TLR expression were observed in genital tissue, uterine tissue, and placental tissue, most notably TLR4. Therefore, differences observed in adult IHC TLR expression may differ from adult rats that were not pregnant (reviewed in Amirchaghmaghi et al, 2013). Comparison of pregnant dams to non-pregnant female rats would reveal any differences due to pregnancy in TLR3 and TLR4 expression with immunohistochemistry within the rat brain and uterus. While comparison to adult male rats would indicate sex differences in TLR3 and TLR4 expression. Similarly, to evaluate the specific interactions on a cellular level in the embryonic brain, the cortex of embryos exposed to MIA should be compared to the cortex of control embryos.

Understanding the regulatory mechanisms of embryonic neurogenesis is essential the process of neurodevelopment, and eventually prevent or treat neurodevelopmental diseases of autism and schizophrenia. The presence of TLRs in the developing brain is important for balanced neurogenesis.

Future studies will be to analyze the embryonic brain samples from the maternal immune activation study for IL-6 levels with ELISA. In addition, protein expression of TLR3 and TLR4, microglial marker iba1 and precursor cell marker tbr2 by western blot and immunohistochemistry. Results will be compared with previous immunohistochemistry data that indicated maternal immune activation increased
microglia activation and decreased number of neural precursor cells in vivo and ex vivo tissue culture (Cunningham et al, 2013). The number of cells expressing TLR3 and TLR4 would expected to increase in the embryonic brain with MIA exposure, while depletion of microglia with MIA exposure would not increase expression of TLR3 and TLR4, if microglia are the modulators of the change in expression. If TLR3 and TLR4 expression on microglia, neurons and NG cells sufficiently altered microglia activation, these receptors would contribute in the changes in patterns of neural development in MIA response observed in mice and rat models. Dissociated microglia studies are necessary to evaluate gene level expression changes with activation.

Abberant microglia activation has been implicated in both neurodevelopmental and neurodegenerative disease. Immune activation of microglia illicits cytokine production effecting expression of multiple immune related proteins such as TLR3 and TLR4. Microglia activated with LPS also increase production and release of pro-neural growth factor (proNGF). Imbalance of proNGF and NGF levels can cause neuron cell death, as seen in Alzheimer’s Disease and ALS (Duan et al, 2013; Aguirre et al, 2013). Therefore future studies will also explore changes in NGF and proNGF levels with ELISA and immunohistochemistry in MIA rat dam serum as well as embryonic rat cortex.

Activated microglia are prevalent the SVZ during embryonic neurodevelopment, and neurogenic niches in the postnatal rat brain (Cunningham et al, 2013; Sato et al, 2013). Stress on the CNS through insults recognized by mast cells, TNFα and microglia elicit release of cytokines, opening connexin hemichannels and pannexin channels in
microglia while increasing blood-brain barrier permeability allowing the entry of T cells into the CNS. Mast cells are along blood-brain barrier, and respond first to inflammatory inducers, degranulating and releases cytokine and factors, IL-6, monocyte chemoattractant protein-1, nitric oxide, and IL13, which can modulate microglia activity. Microglia then produce cytokines and microglia proteinase-activated receptor-2, which promotes the release of brain-derived neural growth factor, and release ATP and glutamate (reviewed in Aguirre et al, 2013).

The sensitization of microglia to a stimulus such as MIA is not completely understood, however application of an antagonist to glucocorticoid receptors inhibited the release of cytokines. Prenatal stress, such as MIA, increased maternal corticosterone levels and activated microglia in the embryonic brain (reviewed in Aguirre et al, 2013). The prevalence of microglia within the developing embryonic cortex neurogenesis region of the SVZ, and the observation that depletion of microglia altered the neuronal patterning and distribution indicating the importance of microglia as regulators in neurogenesis, as observed in rat (Cunningham et al, 2013). This indicates that activation of microglia during MIA, potentially through exposure to maternal cytokines, potentially functions as an indirect effector on proper neurogenesis patterning in the developing embryonic cortex. In future studies mice deficient in TLR3 or TLR4 respectively, can be utilized to evaluate if TLR expression is a primary player in the negative changes in neurodevelopment observed with MIA.

Determination of gene level expression in both isolated RG cells and primary microglia in response to MIA would indicate how TLR3, TLR4 and other possible factors
contribute to the aberrant neurogenesis and resulting behavioral deficits in offspring in animal studies as well as clinically in autism and schizophrenia.

Future Directions

Further quantitative assessment will be to count the number of TLR3 positive cells in the neuroproliferative zone (VZ, SVZ) and then to determine the ratio with respect to total number of cells. Co-expression of TLR3 with Tbr2 precursor cell marker, Iba1 microglia marker, and neuronal markers, NeuN and MAP2 will also be quantified to evaluate specific variability in patterns of expression in different cell types. Comparisons will then be made of rat cortex at different developmental ages for differences in expression, as the trend observed in IHC results for TLR3.
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