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Intrauterine inflammation, insufficient to induce parturition, still evokes fetal and neonatal brain injury

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1. Introduction

Abnormal development of the fetal brain *in utero* is now thought to contribute to the etiology of many functional and behavioral disorders that manifest throughout life. While differences in genetic makeup contribute to this, an ‘adverse’ intrauterine environment is a strong modulator of abnormal development. Maternal bacterial and viral infections during pregnancy represent a significant risk factor in several neuropsychiatric disorders with a presumed neurodevelopmental origin, including schizophrenia, autism, and cognitive delay. (Abecasis *et al* 2002; Brown *et al* 2004; Brown *et al* 2009; Ellman *et al* 2009; Fatemi *et al* 2002; Rantakallio *et al* 1997) However, specific infectious agents in this pathogenesis have not been demonstrated. It appears that prenatal inflammation is the greatest determinant of subsequent adverse outcomes for the offspring. While systemic infections during pregnancy are of concern, perhaps of greater concern, is the risk of *in utero* exposure to localized inflammation. A fetus is exposed to intrauterine inflammation in a woman with preterm labor and/or preterm birth or at any point in gestation when there evidence of chorioamnionitis (infection/inflammation of the fetal membranes). Inflammation is believed to be a leading cause of preterm birth which is defined as delivery at less than 37 weeks of gestation.(Andrews *et al* 2008; Romero 2007) Intrauterine inflammation, documented by histological examination of the placenta, occurs in approximately 20% of all pregnancies. However, the prevalence of histological chorioamnionitis is dramatically increased in preterm births with approximately 85% of very preterm births demonstrating this finding. (Yoon *et al* 2000b)

There are now several studies supporting an association between inflammation in preterm birth and cerebral palsy. (Bashiri *et al* 2006; Bracci and Buonocore 2003; Dammann *et al* 2003; Grether *et al* 2003; Hagberg *et al* 2005; Jacobsson and Hagberg 2004; Neufeld *et al* 2005; Wu 2002). These studies, along with animal studies,(Bell and Hallenbeck 2002; Borrell *et al* 2002; Cai *et al* 2000; Debillon *et al* 2000; Duncan *et al* 2002; Elovitz and Mrinalini 2004; Fatemi *et al* 2002; Hagberg *et al* 2002; Ito *et al* ; Kannan *et al* 2007; Meyer *et al* 2006; Ozawa *et al* 2006; Rousset *et al* 2006; Saadani-Makki *et al* 2008) suggests that inflammation is causal in adverse neurological outcomes for ex-preterm children. Further

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demonstrating a causal relation for prenatal inflammation in brain injury, ex-preterm children are at significant risk for other adverse neurobehavioral outcomes, aside from cerebral palsy.(Davis *et al* 2007; Johnson *et al* ; Johnson 2007; Kaukola *et al* 2006; Wood *et al* 2005) Chorioamnionitis has been reported to be an independent risk factor (with an odds ratio of 16) for a positive screening for early autistic features.(Pinelli and Zwaigenbaum 2008) To that end, emerging data has pointed to an astonishing prevalence of higher order neurodevelopmental impairment by the time these children reach school age.(Adams-Chapman and Stoll 2006; Hansen-Pupp *et al* 2008; Wood *et al* 2000; Wood *et al* 2005) In some studies, up to 50% of ex-preterm infants experience difficulties in executive functioning, as well as in the areas of attention and socioemotional behaviors, often requiring special academic support.(Bayless and Stevenson 2007; Davis *et al* 2007; Marlow *et al* 2007; Msall and Park 2008) Noting this growing body of evidence, it has been difficult to tease out the contribution of prematurity versus inflammation to long-term adverse outcomes for ex-preterm children.

In an effort to elucidate the mechanisms by which prenatal inflammation adversely affects the developing fetal brain, several types of animal models have been created.(Bell and Hallenbeck 2002; Elovitz and Mrinalini 2004; Gilles *et al* 1976; Gravett *et al* 1994; Hagberg *et al* 2002) While all of these models are useful in unraveling pathogenesis, it is crucial to understand the clinical and biological significance of each model.(Elovitz and Mrinalini 2004) The systemic models mimic the clinical scenario of a pregnant woman who has a bacteria (e.g. pyelonephritis, sepsis) or viral (e.g. influenza or CMV) infection. In contrast, the *in utero* or local models most aptly mirror inflammation in preterm birth or with chorioamnionitis at term; hence, these models may be more relevant in regards to the more common clinical exposure of a fetus to an inflammatory process.

Prior work from our laboratory has demonstrated that local intrauterine inflammation, sufficient to induce preterm birth, also causes significant fetal brain injury including loss of prooligodendrocytes, a reactive astrogliosis and a significant disruption in neuronal development.(Burd *et al* 2010a; Burd *et al* 2010b; Elovitz *et al* 2006; Ernst *et al* 2009) We have further demonstrated that neurons injured *in utero* by inflammation can continue to induce injury in other neurons in a cytokine-independent fashion.(Burd *et al* 2009) The question remains whether intrauterine inflammation, insufficient to induce preterm birth, can still evoke fetal brain injury. The fetus is most likely to be exposed to bacteria or bacterial byproducts through colonization of the uterine cavity; consequently, it is crucial to understand how infection and/or inflammation of the uterine cavity can evoke fetal brain injury and contribute to long term adverse neurological outcomes. Therefore, the objective of this study was to determine if a low-dose intrauterine inflammation, that was insufficient to induce preterm parturition, results in fetal and neonatal brain injury. We also sought to assess if the maternal and fetal response to intrauterine inflammation was dependent on gestational age of exposure. Using an established mouse model of intrauterine inflammation, we explored the effect of exposure to intrauterine inflammation in the preterm and term period on fetal brain development.

2. Methods

2.1 Animal model

A mouse model of intrauterine inflammation was used as previously reported. (Burd *et al* 2010a; Burd *et al* 2010b; Elovitz *et al* 2003; Elovitz *et al* 2006; Gonzalez *et al* 2009a; Gonzalez *et al* 2009b) CD-1 out-bred mice were used for these studies; this strain of mice has a gestational period of 19 days. We defined the preterm period as −70% of gestation which would be embryonic day 15 (E15) in this strain of mice. We defined the term period as the 12 hours prior to delivery which occurs on E18.5. We created the model of

intrauterine inflammation in both preterm (E15) and term (E18.5) timed-pregnant CD-1 mice. For all experiments outlined, different sets of pregnant mice were utilized at each gestational time point. To induce intrauterine inflammation, at either E15 or E18.5, timed-pregnant CD-1 dams were placed under isoflurane anesthesia. A mini-laparotomy was performed and the lower right uterine horn was identified. Lipopolysaccharide (LPS) (Sigma, St Louis, MO), 50 μg in a hundred μL volume, or sterile saline (100 μL) was infused between two gestational sacs, with care not to inject into the amniotic cavity. Different experimental groups of mice, at each gestational time period, were used to assess our outcomes of interest 1) preterm birth and maternal morbidity and mortality; 2) immune response in the mother, placenta and fetus; 3) neuronal injury during fetal life, and 3) effect of exposure of prenatal inflammation on gene expression in the postnatal brain (P7).

2.2. Tissue collection (prenatal)

To assess the short term effect of intrauterine inflammation on the mother and fetus in both the preterm and term period, the following tissues were collected 6 hours after intrauterine infusion of LPS or saline: maternal serum, amniotic fluid, placentas, and fetal brains. For each gestational age and each treatment group, 4-6 dams per treatment group were used. Six hours after infusion, dams were euthanized with CO2 and a laparotomy was immediately performed. Maternal serum was obtained using a 22 gauge needle from the aorta with 0.8 to 1.0 mL collected per dam. Maternal blood was allowed to separate on ice and then serum was removed and centrifuged to remove debris. Serum was then placed in liquid nitrogen and stored at −80 degrees Celsius. To collect amniotic fluid, the lower 2-3 gestational sacs in both the left and right horn were identified. An 18 gauge needle on a 1 mL syringe was used to extract amniotic fluid from each sac and pooled as one sample. The sample was placed on ice, centrifuged to remove debris, and then placed in liquid nitrogen and stored at −80 degrees Celsius. The same gestational sacs used to collect amniotic fluid were used to collect placentas and fetal brains. The placentas were identified and fetal membranes were dissected off. Placentas were rinsed in sterile saline. The brain was removed from the cranium and placed in microfuge tubes. Both placenta and fetal brains placed in liquid nitrogen and stored at −80 degrees Celsius.

2.3 Tissue Collection (postnatal)

To determine the effect of prenatal (intrauterine) inflammation in the postnatal period, a separate group of timed-pregnant CD-1 mice at each gestational time period were used. Intrauterine LPS (LPS, 50 micrograms/dam) or saline was administered as described above on either E15 or E18.5 (N=8/group). Dams were observed in their cages every 12 hours for any sign of maternal morbidity or preterm birth. Preterm birth was defined as delivery of at least one pup. Morbidity was classified in a 1-4 scale with 1 being no signs of morbidity, 2 being minimal lethargy or piloerection, 3 was significant lethargy and/or piloerection and 4 being when a dam appear moribund. Dams were observed until after delivery of the litter at term. After delivery, pups were observed in their cages on a daily basis until collection on postnatal day (P7). Each dam and her litter were housed in separate cages so that there was no interaction between litters or mothers. The dam and litter were left undisturbed until P7.

Number of pups at postnatal day 7 (P7) was recorded. On P7, from each litter, one pup was selected at random to represent that litter. Pups were euthanized and the brain removed. The brain was dissected out from the cranium and overlying meninges. Three brain regions were collected: prefrontal cortex (PFC), cerebellum (CBL) and hippocampus (HC). Tissues were rinsed in sterile saline and placed in liquid nitrogen and stored at −80 degrees.

2.4 Measuring a Cytokine Response in Maternal Serum and Amniotic Fluid

To assess a cytokine response in the maternal serum and amniotic fluid, ELISAs were performed per manufacturer's instructions ((R&D Systems, Minneapolis, MN)). Each sample was run in duplicate and averages obtained. Five –six samples per treatment group (representing 5-6 dams as detailed in 2.2 above) at each gestational age were used. Mean and standard deviations were generated from the data and statistically analyzed using Sigma Stat software. T-test or Mann Whitney Rank Sum was utilized depending if the data was normally or non-normally distributed respectively.

2.5 Assessing Gene Expression in the Fetal and Neonatal Brain

To assess message expression in the placenta and brain (fetal and neonatal), quantitative PCR (QPCR) was performed as previously reported.(Burd *et al* 2010a; Burd *et al* 2010b; Elovitz *et al* 2006; Elovitz and Gonzalez 2008; Gonzalez *et al* 2009b; Xu *et al* 2008) Briefly, total RNA was extracted from placenta or brain tissues with trizol (Invitrogen) and complementary DNA (cDNA) was generated using high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). TaqMan Gene Expression Assays consisting of a 20X mix of unlabeled PCR primers and TaqMan minor groove binder probe, were purchased from Applied Biosystems (Foster City, CA). All TaqMan Gene Expression Assays were performed using TaqMan Universal PCR Master Mix from Applied Biosystems (Foster City, CA). QPCR reactions were carried out using equivalent dilutions of each cDNA sample on the Applied Biosystems Model 7900 sequence detector PCR machine. Target mRNA was normalized to the amount of 18S ribosomal RNA in each sample. The relative abundance of the target of interest was divided by the relative abundance of 18S in each sample to generate a standardized abundance for the target transcript of interest. All samples were analyzed in duplicates. Mean target mRNA expression was compared by Student T-test (for parametric data) or Mann Whiteny rank sum (for non-parametric data).

For placental tissues and fetal brains, 6 specimens from (representing 6 different dams) from each treatment group at each gestational time point were used. In placental and fetal brains, cytokine mRNA expression was assessed using QPCR as described above. For neonatal brains, 3-6 specimens (each specimen originating from a different dam) from each treatment group at each gestational age were used. For each brain region, gene expression in the following pathways was investigated: 1) white matter damage 2) neurobehavioral targets 3) glutamate-glutamine (GLU-GLN) cycle and regulation. (Table I)

2.6 Assessing fetal neuronal injury: primary cortical neuronal cultures and quantitative assessment of dendritic processes

To assess neuronal injury (acutely) from exposure to intrauterine inflammation, primary cortical cultures were established as previously reported.(Burd *et al* 2010a; Burd *et al* 2010b; Burd *et al* 2009) For both gestational time points investigated, ix hours after intrauterine infusion of LPS or saline, fetal brains were collected. From each dam, 3-4 fetal brains were collected and treated as one sample (n=1). Using sterile technique, E15 and E18.5 fetal brains were harvested and placed into Petri dishes containing cold Ca^{++}/Mg^{++}-free Hanks Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA), pH 7.4. The fetal cortex, was separated from meninges, olfactory bulbs, brain stem and cerebellum. Each cortex was minced, placed in 4 ml neurobasal medium (NBM; Invitrogen, Carlsbad, CA) containing 0.03% Trypsin (Invitrogen, Carlsbad, CA) and incubated for 15 minutes at 37°C and 5% CO_2. Brain tissue was removed and placed in 4.5 ml NBM containing 10% fetal bovine serum (FBS) and allowed to settle to inactivate the trypsin. The medium was decanted and replaced with NBM supplemented with B-27 vitamin (Invitrogen, Carlsbad, CA) and 0.5mM L-glutamine and cells were dissociated by trituration. This media

combination, NBM in the absence of fetal bovine serum, allows for the selective growth of neurons and not glia (astrocytes or microglia).(Burd *et al* 2009),(Monnerie *et al* 2003; Monnerie and Le Roux 2006; Monnerie and Le Roux 2007) Cells were plated at 4×10^4 cells/ml density on poly-L-lysine (1 mg/ml; Sigma-Aldrich, St. Louis, MO) coated glass coverslips, using 6- and 12-well culture plates. Groups were plated to equal density for each experiment. For each experiment, 4 fetal brains from one dam constituted one culture. Four dams from each treatment group at each gestational age were utilized for the analysis and the comparison of the dendritic counts. All experiments were performed in triplicate to assure the consistency of the results. To determine if intrauterine inflammation objectively altered neuronal morphology, quantitative analysis of dendritic processes was performed as previously reported.(Burd *et al* 2010a; Burd *et al* 2010b ; Burd *et al* 2009) Dendrite processes were analyzed at DIV 3, using previously described techniques. (Monnerie *et al* 2003; Monnerie and Le Roux 2006; Monnerie and Le Roux 2007) Briefly, cells were selected at random using at least 3 coverslips for each condition. One coverslip represented 4 fetal brains from 1 dam and three different dams were used for each condition in order to quantify processes emanating from each cell body. Neurons from each treatment group were evaluated at a final image magnification of 400X. Individual neurons were selected at random if they were clearly defined and not overlapping with other neurons. Approximately 10 neurons were evaluated from each coverslip. Each coverslip represented 1 dam (with pooled cultures from 4 fetal brains from that same dam). This experiment was repeated 3 times. Fluorescent images were recorded and analyzed using a Dell Latitude D620, using an image processing program (Image J 1.37v).

2.7 Statistical Analysis

Statistical analysis was performed using SigmaStat 3.5 (Aspire Software International, Asburn, VA). Data are expressed as the mean standard deviation of the mean (SD) or as the median where the data is not normally distributed. Statistical significance was defined as a two-sided $p < 0.05$. For each outcome measure, statistical analyses were performed comparing saline-exposed to LPS-exposed for each gestational time period as delineated in each section above.

3. Results

3.1 Preterm birth, maternal morbidity and pup survival

As previously reported, intrauterine infusion of a higher dose of LPS (250 micrograms per dam) consistently results in greater than 90% preterm birth rate.(Elovitz and Mrinalini 2006; Elovitz *et al* 2003) In contrast, on E15, low dose LPS resulted in a 30% preterm birth rate. Exposure to low dose LPS on E18.5 resulted in no preterm birth; all dams exposed delivered at term on E19. There was no evidence of maternal morbidity (piloerection, decreased movement) or mortality at either gestational time point. All dams (saline and LPS-exposed) had a score of 1. After exposure on E15, mean litter sizes on P7 were not significantly different in saline-exposed dams (9.8+/−4.8) and LPS-exposed dams (9.8+/−2.5). Similarly, after E18.5 exposure, mean litter size on P7 was not significantly different in dams who were exposed to saline (10.8 +/− 2.7) or LPS (9.7 +/−3.9).

3.2 Cytokine response in mother, placenta and amniotic fluid

A cytokine response was assessed in the dam, placenta and amniotic fluid six hours after exposure to intrauterine inflammation on either E15 or E18.5. In maternal serum, intrauterine infusion of low-dose LPS resulted in a mild elevation of IL6 levels on E18 but did not alter levels after exposure to inflammation on E15 compared to saline-exposed (Figure 1). In the amniotic fluid, low-dose LPS induced a non-significant increase in IL6 levels on E15 and a 27-fold increase in IL6 on E18 (Figure 2). Exposure to low dose LPS

increased cytokine mRNA expression in the placenta in both the preterm (E15) and term (E18.5) period. (Figure 3)

3.3 Immune response in the fetal brain

In the preterm (E15) period, compared to saline, exposure to intrauterine low dose LPS resulted in a significant increase in mRNA expression of IL1β, TNFα and cyclooxygenase-2 (COX-2) (Figure 4). On E18.5, compared to saline, exposure to intrauterine low dose LPS significantly increased TNFα and COX2 (1.6-fold, P=0.04). IL6 mRNA expression was not significantly different in brains exposed to saline compared to LPS. (Figure 4)

3.4 Acute neuronal injury

Neurons exposed to intrauterine saline demonstrated abnormal cell bodies, decreased aggregation, and abnormal processes, similar to our prior reports with a higher dose of LPS. (Burd *et al 2010a* ; Burd *et al* 2010b; Burd *et al* 2009) To provide objective measures of neuronal injury, we assessed the number of dendritic processes in each treatment group at each gestational age. Exposure to intrauterine inflammation on either E15 or E18.5 resulted in a decreased number of dendritic processes (Figure 5) The number of dendritic processes in the saline-exposed cortical cultures is similar to what we have previously reported for saline-exposed as well as for non-infectious induced preterm birth.(Burd *et al* 2010a)

3.5 Gene expression changes in the neonatal brain

Exposure to low dose intrauterine inflammation significantly altered gene expression in all three pathways at both gestational age periods. (Table 1) 1) *White matter damage:* Proteolipid protein (PLP) and myelin basic protein (MBP) mRNA were differentially expressed in a region-specific manner after exposure to LPS at both gestational time periods. Glial fibrillary acidic protein (GFAP) was not increased in any brain region after E15 or E18 exposure. 2) *Neurobehavioral targets:* In a region-specific manner and dependent on gestational age of exposure, AUTS2, MECP2, Oxytocin receptor (OXTR), reelin (RLN) and neurexin (NRXN) mRNA expression were differentially expressed after after exposure to intrauterine inflammation. 3) *Glutamate-glutamine cycle:* Exposure to intrauterine inflammation on E15 and E18.5 increased mRNA expression of VGLUT1, VGLUT2 and VGLUT3 in a region specific in P7 neonatal brains (Table I). Similarly, EAAT1-4 were differently expressed in P7 neonatal brains exposed to intrauterine inflammation during the prenatal period (both preterm and term).

4. Conclusions

Exposure to prenatal inflammation is believed to be an important causal factor in adverse neurological outcomes for children born preterm or at term. While there are conceptual paradigms of how prenatal inflammation may affect the developing fetal brain, the precise mechanisms by which prenatal inflammation results in fetal brain injury and how the ‘injured’ fetal brain contributes to long-term adverse neurobehavioral outcomes remains to be fully elucidated. The studies presented herein advances our understanding of this pathogenesis by revealing the following: 1) intrauterine inflammation, that is insufficient to cause parturition, is sufficient to induce fetal brain injury in both the preterm and term period; 2) a maternal immune response (IL6) does not appear necessary for fetal or neonatal brain injury to occur; 3) exposure to prenatal inflammation alters gene expression in the brain of the exposed offspring; 4) the gestational age of the fetus at the time of exposure to prenatal (intrauterine) inflammation dictates gene expression patterns in the brain; 5) exposure to intrauterine inflammation has a differential effect on brain regions and this is also dependent on gestational age of exposure; 6) exposure to prenatal inflammation in either the preterm or term period has significant effects on several gene pathways in the

brain that are likely involved in the diverse neurobehavioral, motor and psychosocial deficits observed in children exposed to inflammation during fetal life.

Clinical studies support the association between prenatal inflammation and adverse neurobehavioral outcomes. Animal models have been utilized to investigate if there is a causal relationship between prenatal inflammation, brain injury and adverse outcomes. Animal models that utilized the systemic administration of inflammatory agents (Poly I:C, LPS) or bacteria demonstrate that exposure to such agents alters neurobehavioral function in the offspring.(Bakos *et al* 2004; Golan *et al* 2005; Meyer *et al* 2006) While these models clearly demonstrate a link between exposure to adverse neurobehavioral outcomes and exposure to prenatal inflammation, they do not address the most common clinical scenario in which a fetus is exposed to inflammation. Infection and/or inflammation of the uterine cavity and/or amniotic sac, referred to clinically as chorioamnionitis, is a much more prevalent event that women presenting with systemic illness. Intrauterine inflammation, defined clinically or histologically as chorioamnionitis affects greater than 85% of pregnancies ending in very preterm births and is present in approximately 20% of all pregnancies. (Edwards 2005; Yoon *et al* 2000b) Thus, exposure to intrauterine inflammation is a common event in human pregnancy. Consequently, understanding the effect of intrauterine inflammation to fetal brain development and subsequent adverse outcomes is of great clinical significance. Our mouse model serves to aptly mimic the human condition of inflammation/infection the uterine cavity. Hence, the studies described herein can provide insight into adverse outcomes from exposure to intrauterine inflammation.

Regardless of the route of inflammation, the mechanisms linking prenatal inflammation to fetal injury must be understood if therapeutic strategies are our goal. Prior work suggests that maternal IL6 is an essential mediator linking prenatal inflammation to adverse outcomes for the offspring.(Smith *et al* 2007) Our work demonstrates that maternal IL6 levels are, in fact, not altered in the setting of intrauterine inflammation in the preterm period. Yet, despite no change in maternal IL6 levels, we observed a significant cytokine response in the placenta, fetus (amniotic fluid) and fetal brain suggesting that maternal IL6 is not the critical link to fetal injury in the setting of intrauterine inflammation. Differences between our study and that of Smith et al include 1) different inflammatory agent (LPS vs PolyI:C and 2) a local versus a systemic model of inflammation. It is possible that maternal IL6 dictates fetal injury in the settting of systemic but not local (intrauterine) inflammation.

There has been little research exploring the neonatal outcomes after exposure to intrauterine inflammation in animal models. Recent work in rabbits suggest that intrauterine inflammation leads to changes in white matter development, alters cortical serotonin and disrupts serotonin-regulated thalamocortical development in the neonatal brain (Kannan *et al* ; Saadani-Makki *et al* 2009); this same model demonstrated that exposed offspring manifest motor deficits, similar to what is observed in cerebral palsy.(Saadani-Makki *et al* 2008). These studies begin to demonstrate a definitive causal relationship between exposure to intrauterine inflammation and adverse neurological outcomes.

Similarly, our work suggests proposed mechanisms for how intrauterine inflammation may lead to a spectrum of neurobehavioral disorders. Most importantly, we have found, similar to what we report with a higher dose of intrauterine LPS, that low dose intrauterine LPS results in a specific neuronal insult.(Burd *et al* 2010a; Burd *et al* 2009) A method to objectively measure neuronal injury is to assess the number of dendritic processes; a decrease in the number of processes suggest aberrant arboritization and can lead to disrupted synaptic communication. (Esquenazi *et al* 2002; Labelle and Leclerc 2000) Abnormalities in cytoskeletal structure and neuronal aboritization have been demonstrated to alter synaptic connectively and implicated in diverse neurobehavioral disorders.(Moretti *et al* 2006; Snow

et al 2008) These studies demonstrate that low dose inflammation elicits neuronal injury and that this injury is *independent* of gestational-age. These findings along with our prior work suggest that neuronal dysfunction may underlie several neurobehavioral disorders observed after exposure to prenatal inflammation.(Burd *et al* 2010a; Burd *et al* 2009) Our findings in the P7 neonatal brains further support the concept that neuronal dysfunction may be a common mechanism in adverse neurobehavioral outcomes after exposure to prenatal inflammation. Our data demonstrates that expression of several genes in the GLU-GLN cycle is altered in the P7 brain. Exploration of regulators and transporters (VGLUT and EAAT) is a novel part of our work; these mediators are essential for glutamate homeostasis in the brain and, specifically, are involved in glial-neuronal and neuronal-neuronal communication.(Bak *et al* 2005; Broer and Brookes 2001) We also found that reelin, neurexin and MECP2 were differently expressed after exposure to inflammation; these three genes have been implicated in 1) neuronal development and synaptic communication and 2) specific neurobehavioral disorders that have been reported to be associated with prenatal inflammation. Reelin is a candidate gene for autism spectrum disorder and is involved in neuronal migration.(Fatemi *et al* 2005; Gong *et al* 2007; Qiu *et al* 2006) Neurexin plays a role in dendrite formation and synapse formation(Chen *et al* ; Dean and Dresbach 2006) has also been implicated in autism spectrum disorder (Arking *et al* 2008). MECP2 is associated with neuropsychiatric phentoypes, specifically Rett's syndrome.(Adegbola *et al* 2009; Amano *et al* 2000; Bienvenu *et al* 2000). On a molecular level, MECP2 is involved in neuronal architecture, synaptogenesis and regulates glial-neuronal crosstalk.(Ballas *et al* 2009; Fukuda *et al* 2005) Whether the observed gene expression changes in the postnatal brain from exposure to prenatal inflammation contribute to specific neurobehavioral phenotype requires further investigation.

Prior work using systemic models of inflammation have suggested that the gestational age of exposure dictates the presence and type of neurobehavioral phenotype in the exposed offspring. (Aguilar-Valles and Luheshi ; Meyer *et al* 2006) The studies herein confirm that are gestational age dependent and independent events in terms of the ‘type’ of brain injury that occurs after exposure to intrauterine inflammation. Indeed, our findings of neuronal injury—*with exposure at either gestational time point*—and our prior work demonstrating this finding at higher doses of inflammation,(Burd *et al* 2010a; Burd *et al* 2009) suggest that neuronal injury and/or dysfunction is a central mechanism leading to adverse outcomes. Our work also suggests that the immune response in the fetal brain is more potent in the preterm period; whether this heightened immune response dictates specific changes in gene expression observed on P7 or would correlated to different behavioral phenotypes is not yet known. The different patterns of gene expression in the P7 brain do appear to be a gestational age dependent event suggesting a differential response of the fetal brain to an immune challenge. Yet, the selected pathways appear to be disrupted from both gestational time points with the expression changes differing by gene and by brain region. It is likely that gene expression changes in one region (i.e cerebellum versus prefrontal cortex) will manifest in a different behavioral phenotype.

Our study has limitations. We are using a rodent model and hence brain development does not parallel human fetal development specifically in regards to myelination. Yet, these types of studies are not feasible in humans and quite costly in non-human primates. While it is accepted to correlate E15 with the ‘preterm period’ and E18.5 with ‘term’, these designations may be most appropriate in regards to parturition pathways. Glial development, specifically the oligodendrocyte lineage, is a postnatal event in the mouse as compared to the human. These limitations of the mouse model are acknowledged and must be understood when interpreting results. We did not look at a panel of cytokines in the maternal serum or amniotic fluid but instead choose to focus on IL6. Systemic models of inflammation have suggested that IL6 is essential for adverse outcomes while clinical studies suggest IL6 levels

in the amniotic fluid predict development of cerebral palsy.(Smith *et al* 2007; Yoon *et al* 1996; Yoon *et al* 1997; Yoon *et al* 2000a) Despite these reports, we did not find IL6 to be altered in maternal serum or in the fetal brain. While IL6 was increased in maternal serum on E18.5, this did not reach significance. In addition, the level of IL6 remained much lower than demonstrated in our high dose model of LPS.(Gonzalez *et al* 2009a) In comparing prior studies with our findings, we propose that IL6 may play a role in causing brain injury from a systemic or high dose intrauterine inflammatory exposure but that it is not necessary for brain injury to occur. It is possible that other cytokines may be altered in the mother after low dose intrauterine inflammation and that these cytokines can contribute to adverse outcomes. Another limitation of this work is that we choose to investigate only certain genes in each pathway in the postnatal brain and thus can only provide limited information regarding gene expression patterns. However, genes were picked based on 1) knock-out studies that suggest certain proteins are critical for neuronal development or lead to a specific neurobehavioral phenotype;(Bakker *et al* 1994; Bouwknecht *et al* 2001; Chang *et al* 2006; Cheh *et al* 2006; Comery *et al* 1997; Degano *et al* 2009) 2) genetic association studies have demonstrated that a gene is associated with a neurobehavioral disorder(Adegbola *et al* 2009; Alarcon *et al* 2002; Han *et al* 2004); or 3) clinical studies demonstrate changes in these pathways (i.e white matter development) in children born preterm or with specific neurobehavioral disorders common to preterm infants or those exposed to chorioamnionitis. (Dammann *et al* 2009; Jacobsson and Hagberg 2004; O'Shea *et al* 2008; O'Shea *et al* 2009; Polam *et al* 2005; Wu *et al* 2003; Yoon *et al* 2003) While our method of gene selection provides is based on biological plausibility, other unstudied gene pathways may also play a role in adverse neurobehavioral phenotypes. Numerous reports demonstrate an association between aberrations in gene expression (of pathways studied herein) and neurobehavioral phenotypes in both animal models and humans.(Abdolmaleky *et al* 2005; Adachi *et al* 2009; Adegbola *et al* 2009; Dean and Dresbach 2006; Fatemi *et al* 2005; Hung *et al* 2008; Jugloff *et al* 2008; Kaufmann *et al* 2000; Medrihan *et al* 2008; Samaco *et al* 2008) Yet, while these studies confirm the probably importance of these gene expression changes in the P7 brain, further work demonstrating a link between these gene expression patterns on P7, in this model, and behavioral phenotypes will be necessary.

Understanding this limitation, this study and our prior work begin to ascribe specific mechanisms by which exposure to prenatal inflammation (intrauterine) results in adverse neurological outcomes. Specifically, activation of the immune system in the placenta and fetus (even in the absence of significant maternal response) is sufficient to ‘compromise’ the fetal brain. The pathways activated in the fetal brain result in permanent gene expression changes in the offspring. Whether there are epigenetic modifications, resulting from an inflammatory exposure, requires further investigation. There are emerging data to suggest that inflammatory pathways can result in epigenetic modifications leading to altered gene expression.(Angrisano *et al* 2010; Lyn-Kew *et al* 2010). A recent opinion paper hypothesize that epigenetic modifications contribute to adverse outcomes in neonatal brain injury. (Kumral *et al* 2009) While our data support some epigenetic modification occurring in the fetal period, future work will need to explore this possible mechanism. Important for extrapolation to the clinical realm, our results do suggest that ‘surviving’ an inflammatory insult is not protective again future brain injury and perhaps, more importantly, these studies demonstrate that the insult occurred in the fetal period continues to propagate after birth.

Our prior work used a higher dose of LPS and demonstrated significant disturbances in glial and neuronal development with this dose.(Burd *et al* 2010a; Burd *et al* 2009; Elovitz *et al* 2006) This higher dose of LPS in the preterm period resulted in nearly 100% of preterm birth.(Elovitz *et al* 2003) As preterm birth in mice results in neonatal death, the long term effect of intrauterine inflammation on the fetus could not be studied. What we now demonstrate is that intrauterine inflammation—*that is insufficient to cause parturition*—still

evokes injury to the developing fetal brain. This is a critical finding. The conceptual paradigm for brain injury in preterm birth has assumed that if inflammatory pathways were insufficient to induce parturition than they were insufficient to cause fetal harm (or were not present). Our study suggests that this paradigm is incorrect. Our findings argue that small doses of inflammation in the uterus—that do not cause a maternal systemic immune response as measured here by IL6—are still quite capable of inducing a potent immune response in the placenta, fetus (amniotic fluid) and fetal brain. If we begin to extrapolate these findings to the clinical realm, these findings might suggest that women with preterm labor—who do not have a preterm birth—may be at significant risk for adverse neurological outcomes for that infant despite the absence of prematurity. Thus, clinically, our challenge will be, if a maternal immune response is not required for this pathogenesis to occur, than identifying fetuses at risk antenatally will be a daunting task. Exploring mechanisms how acute events lead to chronic changes in the neonatal brain may be more realistic targets in preventing adverse outcomes.

In conclusion, these studies demonstrate that intrauterine inflammation, which evokes a limited or no maternal response and does not induce preterm birth, can induce acute injury in the fetal brain and result in permanent changes in gene expression in the offspring. Collectively, our data support that inflammatory pathways activated in the uterus and placenta are sufficient to induce fetal neuronal injury *in utero* and may be a causative factor in adverse neurobehavioral outcomes in children exposed to prenatal inflammation.

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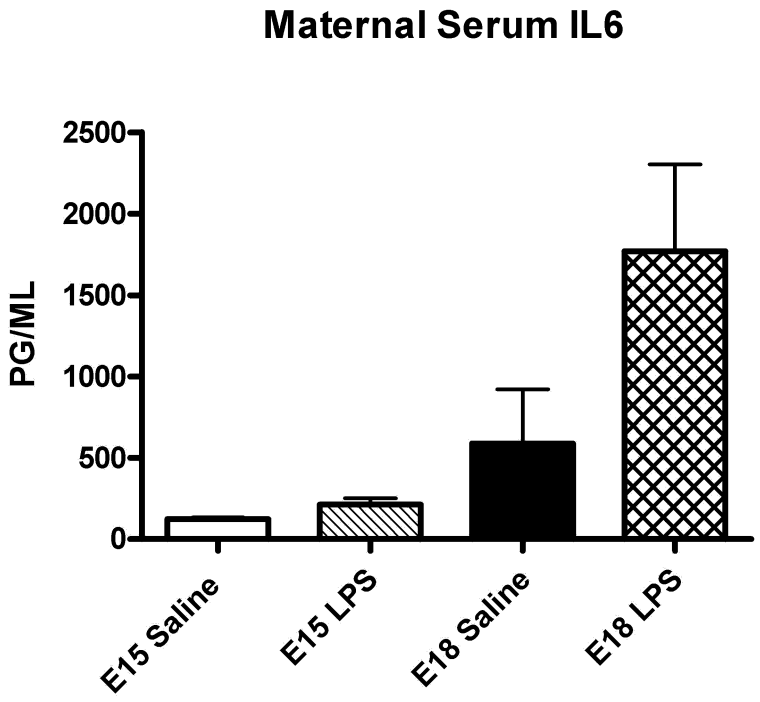

Figure 1.
IL6 levels in maternal serum. Bar graph depicting means and standard deviations of IL6 levels in maternal serum from preterm (E15) and term (E18.5) exposure to low dose intrauterine LPS. Low dose intrauterine LPS did not significantly alter maternal IL6 levels in the preterm period. At term (E18.5), IL6 levels increased 3-fold but this was not statistically significant (P=0.09, T-test).

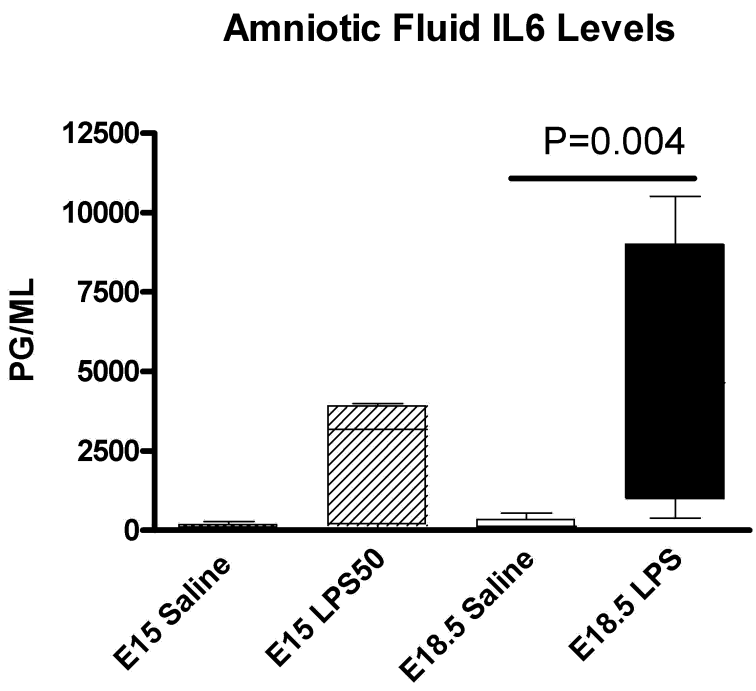

Figure 2.

IL6 levels in amniotic fluid. Bar graph depicting means and standard deviations of IL6 levels in amniotic fluid from preterm (E15) and term (E18.5) exposure to low dose intrauterine LPS. There was wide variation in IL6 levels between the dams; the data were not normally distributed. IL6 levels were increased in the amniotic fluid in the preterm period but this did not reach statistical significance (Mann Whitney Rank Sum). In the term period, intrauterine inflammation significantly increased IL6 levels 27-fold compared to saline-exposed (P=0.004, Mann Whitney Rank Sum)

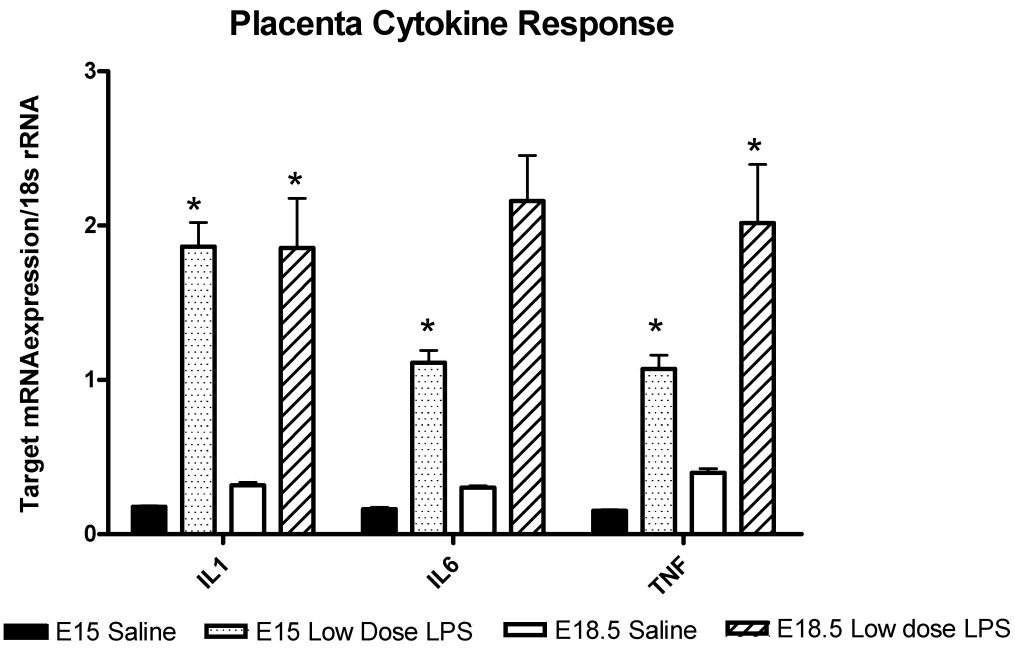

Figure 3.

Cytokine expression in the placenta. Bar graph representing mean mRNA expression normalized to the amount of 18s rRNA in placentas exposed to saline or low dose LPS at both gestational time points (E15 and E18.5). Exposure to low dose LPS increased placental IL1β 12-fold (*P=0.008) on E15 and 5.8-fold on E18.5 (*P=0.009). Low dose LPS increased IL6 mRNA expression 8.6-fold (P=0.008) on E15 and 87-fold (P=0.06) on E18.5. Low dose LPS increased TNF 7-fold (P=0.002) on E15 and 5-fold (P=0.04) on E18.5. MRNA expression was compared using T-test when data was normally distributed and Mann Whitney Rank Sum test when the data was non-parametric.

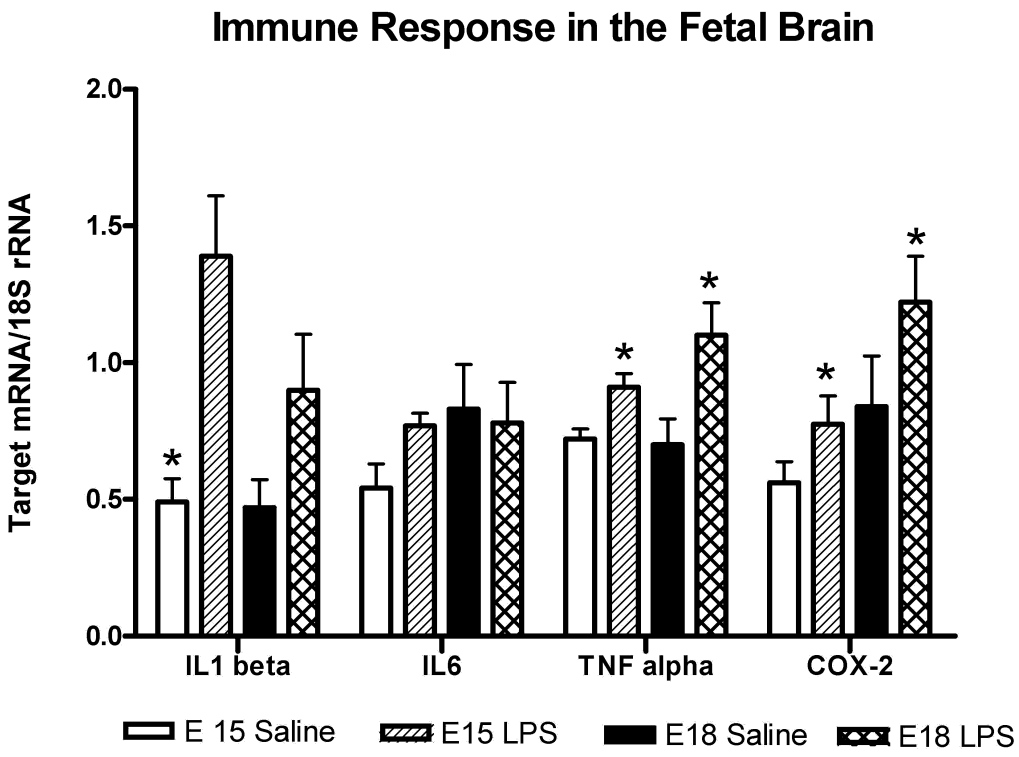

Figure 4.

Immune response in the fetal brain. Bar graph representing mean mRNA expression normalized to the amount of 18s rRNA in fetal brains exposed to saline or low dose LPS at both gestational time points (E15 and E18.5). In the preterm (E15) period, compared to saline, exposure to intrauterine low dose LPS resulted in an 2.8-fold increase in IL1 beta (P=0.007), a 1.4-fold increase in TNF (P=0.018) and 1.6-fold increase in COX-2 (P=0.02) mRNA expression. On E18.5, compared to saline, exposure to intrauterine low dose LPS increased IL1 beta 1.9-fold (P=0.13), TNF 1.8-fold (P=0.04) and COX2 (1.6-fold, P=0.04). IL6 mRNA expression was not significantly different in brains exposed to saline compared to LPS. Mean mRNA expression was compared using T-test for each gestational time period.

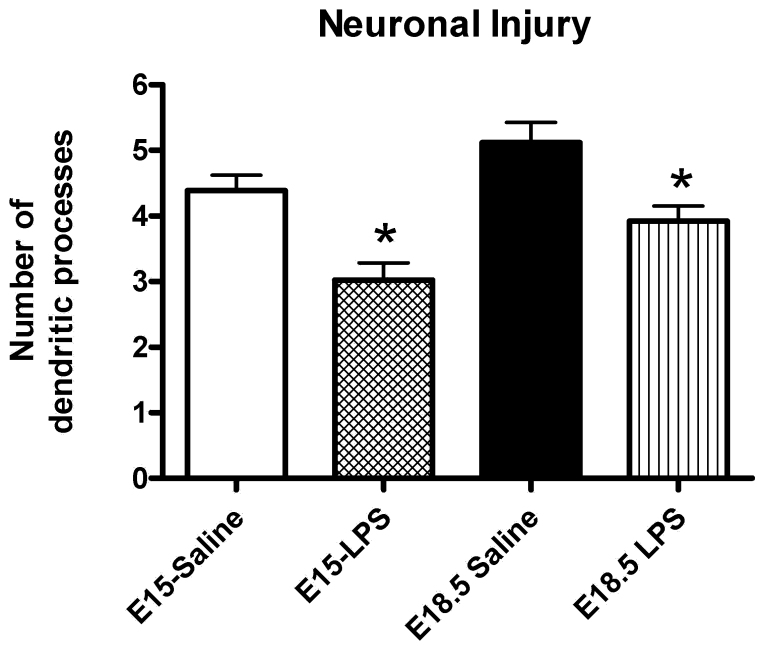

Figure 5.
Fetal neuronal injury. Bar graph representing means and standard deviations of dendritic counts in the preterm and term period after exposure to low dose intrauterine inflammation or saline. Exposure to low dose LPS significantly decreased dendritic counts on E15 and E18.5 compared to saline-exposed ($P<0.001$ for both gestational time periods).

Table I

Fold Change in Gene Expression in Brain-Specific Regions on Postnatal Day 7 After Exposure to Low-Dose Intrauterine Inflammation

Pathway	*Gene*	Exposure in Preterm Period (E15)			Exposure at Term (E18.5)		
		PFC	CBL	HC	PFC	CBL	HC
White Matter Targets	Gfap	NS	NS	NS	NS	NS	NS
	Plp1	**3.1 (P=0.006)**	**2.2 (P=0.028)**	NS	**1.6 (P=0.05)**	**1.1 (P=0.047)**	NS
	Mbp	**2.1 (P=0.021)**	NS	NS	NS	NS	NS
Neuro-behavioral Targets	MeCP2	NS	NS	NS	**1.3 (p=0.005)**	**1.2 (P=0.029)**	NS
	Auts2	**1.5 (P=0.004)**	NS	NS	**1.3 (P=0.002)**	NS	**1.2 (P=0.026)**
	Auts2	**1.5 (P=0.004)**	NS	NS	**1.3 (P=0.002)**	NS	**1.2 (P=0.026)**
	TH	NS	NS	NS	NS	NS	NS
	Oxtr	**1.3 (P=0.019)**	NS	NS	**1.4 (P=0.02)**	NS	NS
	Nrxn1	NS	NS	NS	NS	NS	**1.1 (P=0.03)**
	Rein	NS	**1..6 (P=0.028)**	NS	NS	NS	**1.2 ([=0.021)**
Glutamate-Glutamine cycle	Vglut1	**1.4 (P=0.02)**	**2.2 (P=0.004)**	NS	**1.3 (P=0.05)**	NS	NS
	Vglut2	**1.3 (p=0.03)**	NS	NS	NS	**1.1 (P=0.012)**	**1.2 (P=0.007)**
	Vglut3	NS	NS	NS	NS	NS	NS
	Eaat1	**1.5 (P=0.02)**	**1.8 (P=0.05)**	NS	**1.4 (P=0.049)**	NS	**1.2 (P=0.003)**
	Eaat2	NS	NS	NS	NS	NS	NS
	Eaat3	NS	NS	NS	NS	NS	**1.2 (P<0.001)**
	Eaat4	NS	**1.2 (P=0.03)**	NS	**0.73 (P=0.012)**	NS	**1.2 (p=0.009)**

PFC-prefrontal cortex; CBL-cerebellum; HC-hippocampus; GFAP=glial fibrillary acid protein; PLP-proteolipid protein; MBP-myelin basic protein; TH-tyrosine hydroxylase; OXTR-oxytocin receptor, NRXN1-neurexin 1; RELN-reelin; VGLUT 1-3-glutamine transporter P value was calculated by T-test; NS=non-significant with P value >0.05