Neuromolecular changes in developing offspring following maternal infection; implications for schizophrenia

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Abstract:

Environmental and genetic factors contribute to the development of schizophrenia. For example, epidemiological evidence has linked infections during pregnancy with increased incidence of schizophrenia in the adult offspring. At the same time mutation to DISC1, a protein involved in neuronal migration and synaptic plasticity, is an important genetic risk for the disorder. Accordingly, the aim of this project was to determine if these environmental and genetic influences converge along a common pathogenic pathway leading to schizophrenia. Using a model of prenatal infection by bacterial endotoxin in rodents, we demonstrated a 50% reduction in DISC1 protein expression in the hippocampus and cortex of juvenile offspring. In addition, we found a significant induction of prostaglandins (final mediators of the inflammatory process) in the fetal brain while many cytokines remained unaltered. Taken together our results identify prostaglandins as potential mediators of the teratogenic effects of prenatal infection and show that prenatal infection itself can affect systems related to genetic risk factors for schizophrenia, in this case DISC1.

Résumé:

Des facteurs environnementaux et génétiques contribuent à la schizophrénie. Par exemple, plusieurs études démontrent un lien entre les infections pendant la grossesse et une incidence accrue de la schizophrénie chez la progéniture. D'un autre côté, certaines mutations héréditaires, comme à DISC1, une protéine impliquée dans la migration neuronale et la plasticité synaptique, sont reconnues comme un facteur de risque important de cette maladie. Le but de la présente étude est de déterminer si ces influences environnementales et génétiques convergent vers des mécanismes communs menant à la schizophrénie. En utilisant un modèle d'infection prénatal par endotoxines bactériennes chez le rat, nous avons observé une réduction de 50% des niveaux d'expression de la protéine DISC1 dans l'hippocampe et le cortex de la progéniture. De plus, une augmentation des prostaglandines a été observée dans le cerveau embryonnaire tandis que plusieurs cytokines étudiées restent inchangées. En conclusion, nos résultats suggèrent une implication des prostaglandines comme médiatrices des effets tératogènes de l'infection prénatale et démontrent que l'infection prénatale affecte des systèmes communs à certains facteurs de risque génétiques, dans le cas présent la protéine DISC1.

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Introduction:

Schizophrenia is a severe and debilitating psychiatric disorder characterized by heterogeneous symptoms including hallucinations and delusions, social dysfunction and cognitive deficits (Ross *et al.*, 2006). The disease is multi-factorial in nature, and although highly heritable it involves important environmental (Gray & Hannan, 2007) and social aspects (Allardyce & Boydell, 2006). Lacking features of a neurodegenerative disorder such as reactive gliosis or inclusion bodies (Arnold et al., 2005), schizophrenia is instead described as a subtle disorder of neurodevelopment (Harrison & Weinberger, 2005), one that robustly affects the synapse and thus has deleterious effects throughout the brain. Indeed, defective connectivity is observed between the prefrontal cortex, thalamus, limbic systems, and dopaminergic regions (midbrain and nucleus accumbens). Added support for a neurodevelopmental theory of the disorder is provided by association of susceptibility genes such as *Disrupted-in-Schizophrenia 1* (DISC1) that play an important role in development. DISC1 is a multi-isoform protein that interacts with several other proteins linked to schizophrenia (PDE4B, LIS1) and developmental processes (Ndel1) to participate in neuronal migration, neurite outgrowth and synaptic plasticity (Ross *et al.*, 2006).

Epidemiological studies have revealed links between several prenatal environmental events and an increased incidence of schizophrenia (Cannon & Clarke, 2005;Clarke *et al.*, 2006), including obstetric complications, prenatal maternal stress, and viral or bacterial infections during pregnancy (such as rubella, influenza, herpes simplex virus-2, toxoplasma, measles, diphtheria, pneumonia and other respiratory

infections) (Brown *et al.*, 2000b;Brown *et al.*, 2000a;Brown *et al.*, 2004;Brown *et al.*, 2005;Buka *et al.*, 2008). Substantial evidence supports the hypothesis that activation of the maternal immune response during critical periods of gestation increases the susceptibility of the fetus to exhibit neurodevelopmental defects as an adult (Fatemi *et al.*, 2008;Meyer *et al.*, 2007b). As both prenatal infection and disruptions of DISC1 function are associated with developmental problems leading to the disease, the aim of the proposed research project is to determine if these influences converge along a common pathogenic pathway (Caspi & Moffitt, 2006) to produce changes resembling those in schizophrenia. Using a well-established animal model of maternal infection by bacterial endotoxin lipopolysaccharide (LPS), brains will be analysed both in prenatal periods, during maternal infection, and in postnatal periods to monitor alterations in DISC1 expression. This analysis also aims to establish mechanistic candidates of such changes, such as mediators of the immune system including cytokines and prostaglandins.

Background:

Relating Psychopathology and Prenatal Infection: Evidence and Modelling

Epidemiological studies have revealed links between several prenatal environmental events and an increased incidence of schizophrenia (Cannon & Clarke, 2005;Clarke *et al.*, 2006), including obstetric complications, prenatal maternal stress, and viral or bacterial infections during pregnancy (such as rubella, influenza, herpes simplex virus-2, toxoplasma, measles, diphtheria, pneumonia and other respiratory

infections) (Brown *et al.*, 2000b;Brown *et al.*, 2000a;Brown *et al.*, 2004;Brown *et al.*, 2005;Buka *et al.*, 2008;Sorensen *et al.*, 2008). Epidemiological evidence suggests a connection between infection during second or first trimester pregnancy and psychiatric disorders, including autism (Juul-Dam et al., 2001) and schizophrenia (Brown, 2006;Buka *et al.*, 2008;Sorensen *et al.*, 2008). Although original epidemiological studies have relied on 'imperfect' proxies of infection during pregnancy such as maternal recall of infection or timing of an epidemic, prospective studies are now being published containing more reliable and quantifiable confirmation of infection from maternal serum. This research suggests that influenza infection confers as much as 3-7 fold increased risk of schizophrenia in offspring (Brown *et al.*, 2004), while similar serological results have also been collected for rubella (Brown *et al.*, 2000a), toxoplasmosis (Brown *et al.*, 2005) and herpes simplex virus-2 (Buka *et al.*, 2008). Since a wide variety of infectious agents show association to schizophrenia, it may be common factors to infection such as cytokine induction, fever, or activation of the HPA-axis that are imparting the increased risk to the fetus.

Modelling prenatal infection in animals allows for experimental characterization of the mechanisms involved in the association with psychiatric disease, providing targets of prevention in humans (Boksa, 2004). Indeed, injection of viral pathogens into pregnant rodents leads to behavioural traits (endophenotypes) in the offspring that resemble those of schizophrenia (Shi et al., 2003). Since no viral components are detected in the fetus itself, it has been suggested that the neurobiological changes seen are due to maternal factors (Shi et al., 2005). A more controlled method of stimulating

the maternal immune system is to inject either the bacterial mimic lipopolysaccharide (LPS) or viral mimic polyriboinosinic-polyribocytidilic acid (poly-I.C.) which act through toll-like receptors 4 and 3, respectively (Sabroe et al., 2008), and induce a maternal immune response involving a cascade of pro-inflammatory and anti-inflammatory cytokine production. In adulthood the offspring exhibit several schizophrenia-like behaviours. These include deficits in sensori-motor gating (as measured by pre-pulse inhibition to acoustic startle or PPI), working memory, social interaction, and increased anxiety and responsiveness to amphetamine. (Fortier *et al.*, 2004;Fortier *et al.*, 2007;Hava *et al.*, 2006;Shi *et al.*, 2003;Smith *et al.*, 2007).

Whereas behavioural effects of prenatal infection are highly characterized in adulthood, morphological effects in the offspring brain are often observable at younger ages. The hippocampus, a region in which the most striking effects are often detected, shows decreased myelination and axonal diameter during development (Makinodan et al., 2008), neuronal atrophy (Fatemi *et al.*, 2002;Fatemi *et al.*, 2008), reductions in markers of the GABAergic system such as reelin and parvalbumin and changes in NMDA receptor expression (Meyer et al., 2007a). Abnormalities in gene expression and brain atrophy are also seen in other areas affected in human schizophrenics, such as the prefrontal cortex and cerebellum (Fatemi et al., 2008), confirming the relevance of maternal infection in rodents as a model of the neurodevelopmental effects associated with the disorder and making it a useful tool for determining the underlying mechanisms involved in its development.

Sickness Response Factors Affecting Fetal Development

An important aspect that is just starting to be explored is whether maternal immune factors such as cytokines, the physiological responses they mediate such as fever and stress, or direct infection of the fetus are disrupting normal fetal development. See Figure 1.

Cytokines

Cytokines are a family of molecules involved in mediating the innate immune response to infection and injury. The levels of these mediators increase dramatically in the circulation and often in the brain following a systemic inflammatory stimulus and often correlate with the physiological responses associated with 'sickness' such as fever (Dantzer, 2001). Thus the administration of either LPS or poly-I.C., as bacterial and viral mimics, each initiate the elaboration of pro-inflammatory [most prominently interleukin-1β (IL-1β), IL-6 (considered to be the main circulating pyrogen), tumor necrosis factor-α (TNFα)] and anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1ra). These molecules are logical candidates for causing CNS abnormalities in the fetus as they are also strongly implicated in the neurodevelopment process. For example, IL-1β, TNFα and other cytokines have been shown to impair survival of embryonic dopaminergic or serotonergic neurons to LPS *in vitro* (Jarskog *et al.*, 1997;McGuire *et al.*, 2001) and to affect differentiation of progenitor cells into dopaminergic neurons (Potter et al., 1999). Several fetal tissues, including the placenta and liver, and the fetal circulation do indeed show increased cytokine expression during maternal immune stimulation despite the fact that LPS, poly-I.C. or live viruses themselves do not cross into the fetal compartment

(Ashdown *et al.*, 2006;Goto *et al.*, 1994;Shi *et al.*, 2003). There is however significant variation between studies investigating immunogen modulation of fetal brain cytokine content with some showing no change (Ashdown *et al.*, 2006;Rounioja *et al.*, 2003), an increase (Cai *et al.*, 2000;Ning *et al.*, 2008;Xu *et al.*, 2007), or a decrease (Urakubo *et al.*, 2001;Xu *et al.*, 2007) in pro-inflammatory cytokine expression.

Despite the inconsistencies found for the immune response in the fetal brain, evidence is now accumulating which points to maternal IL-6 as a key effector molecule of the observed behavioural/neurological abnormalities. This pyrogenic cytokine has been shown to cross the placenta, although more efficiently at earlier than later stages of gestation (Dahlgren et al., 2006). Maternal IL-6 injection alone, but not injection of other cytokines like IL-1β, can cause similar behavioural abnormalities in offspring as poly-I.C.(Samuelsson et al., 2006), and poly-I.C.-induced behavioural alterations are inhibited by co-injection of specific IL-6 antibody (Smith et al., 2007). It has also been suggested that instead it is the disrupted balance between pro-inflammatory and anti-inflammatory signalling that may be mediating the effects on the fetus (Meyer *et al.*, 2008a;Meyer *et al.*, 2008b).

Fever

Other sickness response elements that are downstream of cytokine induction may instead be imparting the risk to the fetus, as both fever and activation of the HPA-axis during pregnancy are also associated with the development of schizophrenia in offspring (Clarke *et al.*, 2006;Fuller *et al.*, 2000). Fever, a hallmark response to injury or infection, is the elevation of body temperature induced and regulated by cytokines,

particularly IL-6 . Pro-inflammatory/pyrogenic and anti-inflammatory/cryogenic cytokines act on the preoptic area of the hypothalamus to orchestrate the thermoregulatory response (Conti et al., 2004). Although deemed an adaptive sickness response against pathogens, fever can also have negative consequences during pregnancy. In extreme cases fever may lead to abortion, but in surviving fetuses can affect CNS development by disturbing neuronal survival and migration (Edwards, 2007). Interestingly fever is suppressed in later stages of pregnancy (Aguilar-Valles et al., 2007), presumably as an inherent mechanism for protecting the developing fetus. This is reflected by a decrease in maternal circulatory cytokines such as IL-6 (Fofie et al., 2005) and a CNS decrease in the final fever-mediating molecule, prostaglandin E_2 (PGE_2) (Mouihate et al., 2008). Whether fever itself plays a causal role in the pathogenesis of schizophrenia, or merely an associative one due to correlating sickness response factors, has yet to be determined.

HPA-Axis Activation

Maternal stress is another obstetric complication associated with increased risk for schizophrenia (Clarke *et al.*, 2006;Fuller *et al.*, 2000). Moreover, several similarities between prenatal stress and prenatal infection models of schizophrenia have been considered (Boksa, 2004). For example, animal models in rodents and non-human primates have shown that prenatal maternal stress is associated with elevated glucocorticoid release in offspring, enhanced responsiveness to stressors, hippocampal abnormalities such as attenuated LTP, and alterations in serotonergic and dopaminergic systems (Kofman, 2002;Weinstock, 2008;Yaka *et al.*, 2007). These results correspond

with later abnormalities in learning, attention, anxiety and social behaviour in the adult offspring. The similar outcomes between prenatal infection and stress may be connected by the fact that cytokines induced during the sickness response also stimulate the secretory activity of the stress axis (Turnbull & Rivier, 1999). Determining which of these or other aspects of the sickness response have a primary role in disturbing the fetus is crucial for developing clinical preventative interventions. However, one must not disregard the fact that environmental factors related to infection are only one influential aspect, with genetic factors contributing to neurodevelopmental defects as well.

DISC1 in the Pathology of Schizophrenia

Schizophrenia is a polygenic disorder and despite being highly heritable, individual associated genes have shown low relative risk scores (Harrison & Weinberger, 2005). However, a few genes are gaining recognition as key susceptibility factors because of growing linkage, association, clinical and biological evidence implicating them in the disorder. One such gene is DISC1, which was identified originally from a disruption by chromosomal translocation in a large Scottish pedigree that segregated with a broad phenotype of major mental illnesses (Millar et al., 2000). Since its discovery the DISC1 locus has been associated with schizophrenia and affective disorders in several populations (Craddock et al., 2005; Hamshere et al., 2005, Hennah et al., 2006; Millar et al., 2003; Thomson et al., 2005; Porteous and Millar, 2006) and is now being deemed a more general risk factor. Indeed, DISC1 is emerging as one of the strongest risk genes that is associated with schizophrenia (Porteous *et al.*, 2006;Wang *et al.*, 2008).

As schizophrenia is a disorder that grossly affects human cognition it is notable that DISC1 single nucleotide polymorphisms (SNPs) and haplotypes are also related to brain structure and function in the healthy population. A Ser704Cys mutation within DISC1 has been associated with reduced hippocampal grey matter volume and fMRI assessment shows abnormal hippocampal function during various cognitive tasks (Callicott et al., 2005). DISC1 haplotypes have also been associated with reduced grey matter in the prefrontal cortex, as well as deficits in short- and long-term memory (Cannon et al., 2005). Compellingly, family members who are carriers of the original Scottish DISC1 disruption but do not show psychiatric symptoms still maintain endophenotypes of the disease, such as reduced event-evoked P300 potential amplitude and latency (Blackwood et al., 2001). Indications that DISC1 plays a role in behavioural aspects of schizophrenia are growing. More importantly, DISC1's emerging neurobiological roles seem to fit a framework that makes this link plausible.

The expression of DISC1 correlates well with both neurodevelopmental and psychiatric involvement. It is expressed in the brain throughout neuronal development, peaking at E13.5 and P35, ages corresponding with active neurogenesis and puberty, respectively, and continuing into adulthood (Schurov *et al.*, 2004). Expression is highest in areas relevant to cognitive dysfunction, including the cerebral cortex and hippocampus (Austin et al., 2004). Studying the role of DISC1 has been slowed by its inherent complexity, as the *disc1* gene includes at least 13 exons which produce several splice variants (Ishizuka et al., 2006) and these numerous isoforms seem to undergo further post-translational modifications. Additionally, different DISC1 isoforms localize

to different subcellular compartments within cortical neurons (James *et al.*, 2004), most notably mitochondria (Millar et al., 2005a), spines and the post-synaptic density (Kirkpatrick et al., 2006).

Through its coiled-coiled domain, DISC1 interacts with numerous proteins that reveal functions in both development and adulthood. Using yeast two hybrid screen methods (Camargo et al., 2007) interactors such as PDE4B, Ndel1, Lis1, Citron and kinesin motor proteins have been identified. This initiated research that shows DISC1 involvement in cellular processes including neurite outgrowth, neuronal migration, axonal transport, cAMP signalling and synaptic plasticity. The interaction with PDE4B, a cAMP hydrolyzing enzyme, has gained a lot of attention recently because direct genetic associations with schizophrenia have been found (Millar *et al.*, 2005b). The synaptic role of DISC1 is supported not only by localization studies but also by interactions with Citron, a postsynaptic protein that interacts with PSD-95, and this may indicate functions in synaptic plasticity (Ross *et al.*, 2006).

The most thoroughly studied interaction is the DISC1-Ndel1 complex that is required for neurite outgrowth, nucleokinesis, and neuronal migration, all of which are important aspects of embryonic cortical development. This complex also stabilizes the centrosome (Kamiya *et al.*, 2005), and interacts with motor proteins to regulate anterograde axonal transport (Taya et al., 2007). Although there is debate on whether the original DISC1 translocation yields disorder through haploinsufficiency (Millar et al., 2005b), or through a dominant-negative mechanism (where production of a truncated protein disrupts the normal function of the full-length DISC1 (Kamiya *et al.*, 2005), both

scenarios result in a loss of function of the protein and notably diminished DISC1-Ndel1 interaction. The neuropathological importance of this interaction is highlighted by new evidence that forebrains of schizophrenics are more likely to exhibit insoluble DISC1 aggregates which fail to interact with Ndel1 (Leliveld et al., 2008).

Considering the involvement of these proteins in neural migration it is not surprising that Ndel1 and DISC1 participate in neurogenesis, the proliferation and integration of new neurons, throughout both development and adulthood (Dranovsky & Hen, 2007;Duan *et al.*, 2007). Knock-down studies reveal divergent effects of DISC1 at different ages, with DISC1 enhancing neuronal sprouting and migration in the developing brain (Dranovsky & Hen, 2007), and inhibiting these processes in adulthood (Duan *et al.*, 2007). The connection becomes apparent when we consider that alterations in neurogenesis have been observed in psychiatric pathology, including both mood disorders and schizophrenia (Kempermann *et al.*, 2008;Reif *et al.*, 2007).

There is now recognition that animal models based on DISC1 genetic modification could provide better models of psychiatric disorders such as schizophrenia than currently exist (Wang *et al.*, 2008), and less than a decade after DISC1's discovery reasonable progress has been made. No knock-outs have been successfully produced to date, probably due to the complexity of exon usage in the DISC1 gene, but mice with either missense mutations in the DISC1 locus (Clapcote et al., 2007) or transgenics that express truncated DISC1 have both been generated (Hikida *et al.*, 2007;Li *et al.*, 2007;Pletnikov *et al.*, 2007). Although these mutants only show minor changes in endogenous DISC1 expression, neurobiological and behavioural attributes resembling

schizophrenia are observed such as reduced lateral ventricle size, reductions in cortical interneurons, and deficits in PPI and working memory.

Rationale

Despite a number of significant advances in this area of research, it is still not clear which factors are acting on the fetal brain during prenatal infection. Previously our group found induction of IL-1β in the fetal circulation but did not observe cytokine induction in the fetal brain when LPS was given to rodent dams during late pregnancy (Ashdown et al., 2006). However, inflammatory changes may be transient and thus several other time points and additional inflammatory mediators downstream of pro-inflammatory cytokines, such as transcription factors and prostanoid induction, should be analysed. Knowledge of what is occurring in the fetus at times close to infection can begin to clarify how later neurological effects occur as a result of maternal infection.

Intriguingly, maternal infection and DISC1 disruption (through mutation or truncation) share common consequences, namely schizophrenia-relevant brain changes later in life. Several have described the pertinence of studying these risk factors in combination to more closely understand the disorder's genetic and environmental components and to see how they interact (Sawa & Kamiya, 2003). One possibility is that these factors share a common pathway in the pathogenesis of schizophrenia, for example both modifying DISC1 neuronal expression and thus affecting processes such as neuronal migration. Analysing the effect of maternal infection on the DISC1-system in offspring is one step in elucidating how these risk factors are related.

Hypothesis and Aims

Our working hypothesis is that after maternal systemic LPS the induction of immune molecules within the fetal brain directly or indirectly affects fetal neuronal development. Secondly, we hypothesise that through this mechanism maternal LPS subsequently disrupts neuronal DISC1 in the offspring, disturbing normal DISC1 expression in schizophrenia-relevant areas such as the hippocampus and prefrontal cortex. These hypotheses will be tested through the following two aims:

AIM 1: To examine how LPS-induced maternal inflammation during late pregnancy affects the fetus:

a. Determine if there are changes in the immune mediators in the fetal tissues, particularly in the brain, by assessing pro-inflammatory cytokine responses and downstream signalling molecules such as prostaglandins at varying time points after maternal LPS
b. Correlate any changes with the immune or febrile response of the dam

AIM 2: To determine if maternal infection affects DISC1 expression in the offspring's brain during postnatal development:

a. Analyze DISC1 levels in the brain (at the levels of protein and messenger RNA) in offspring at various stages of development, especially in areas relevant to schizophrenia (hippocampus, prefrontal cortex)
b. Correlate any changes with the immune or febrile response of the dam as a step towards determining the mechanism of neurodevelopmental changes.

Materials and Methods

General Procedures:

Timed-pregnant Sprague-Dawley rats (Charles River, Saint Constant, Quebec, Canada) weighing 300-400g at gestational day (GD) 18 and 19 were used for these studies. This time point was chosen because GD18 and 19 of the rat corresponds roughly to late second trimester of pregnancy in humans (Meyer *et al.*, 2006), one of the gestational periods where maternal infection is can impart risk to the offspring for the development of schizophrenia (Boksa, 2004). Pregnant females were housed individually in a controlled environment with an ambient temperature of 21 ± 2°C, a 12h:12h light-dark cycle (with lights on from 8h00-20h00), and were given free access to food and water. Animals were each handled for 4-5 days before GD18 to reduce stress on the day(s) of injection. Concurrently these animals were habituated daily to a rectal thermometer (Physitemp Instruments Inc., NJ, USA) to reduce stress-induced temperature changes that can confound experimental temperature measurements. In all studies pregnant dams received intraperitoneal injections of either 50ug/kg LPS (Sigma) or 1ml/kg sterile physiological saline (SAL). These injections were administered between 10h00 and 12h00 on GD18 (Aim 1) or on both GD18 and 19 (Aim 2). Whereas dams from single injection studies were sacrificed from 1-4 hours after injection for analysis prenatal immune mediators, dams in dual injection studies were allowed to give birth so that postnatal effects of maternal infection could be studied.

All procedures were performed in accordance with protocols and guidelines approved by the Canadian Council on Animal Care and the Animal Committee of McGill University.

Procedures for Prenatal Analysis of Maternal Infection

The immune mediators in pregnant animals and their fetuses on GD18 were examined at 1, 2, 3 and 4 hours after LPS injection and compared to SAL-injected animals as control. This time course range was chosen based on previous studies in our lab which show a peak of pro-inflammatory cytokines in maternal and fetal plasma from 2-4 hours after LPS (Ashdown et al., 2006)

Maternal Temperature and Tissue Collection:

Using a rectal probe for rat (Physitemp Instruments Inc., NJ, USA), baseline body temperatures of GD18 dams were taken immediately before LPS or SAL injection and at each hour until time of sacrifice. Animals were sacrificed by live decapitation at these time points to avoid the attenuating effects of anaesthesia on cytokine responses (Flondor et al., 2008). After decapitation, trunk blood was collected in sterile tubes on ice containing pyrogen-free heparin (10 U/ml) to prevent coagulation. Blood samples were then centrifuged for 10min at 4°C and the plasma aliquoted on ice and frozen at -80°C until analysis.

Fetal Tissue Collection:

Immediately after the maternal sacrifice the uterine horn was removed and placed on ice. Eight fetuses were randomly selected from each dam and amniotic fluid, placenta, and fetal brain were collected, snap-frozen and stored at -80°C.

Procedures for Postnatal Analysis of Maternal Infection

Monitoring Maternal Response to LPS:

Dams were injected on both GD18 and 19 with LPS or SAL and body temperatures were monitored by a rectal probe at 0, 4 and 6 hours after each injection. As no blood was taken from the dam after LPS to reduce stress exposure during pregnancy, a preliminary study was performed which determined that temperature changes at this stage of pregnancy correlate well with maternal cytokines (specifically IL-1β and IL-6) and is a good indication of maternal response to LPS. Pregnant dams were monitored daily until parturition.

Cross-fostering of Offspring:

To remove confounding effects of immune system stimulation on maternal behaviour (Meyer *et al.*, 2008c), pups were fostered to non-treated surrogate mothers. At postnatal day (PD) 0, the day of birth, pups were sexed, weighed, tattooed (by a subcutaneous paw injection with indelible non-toxic ink), and fostered to surrogates who had given birth the day before. Each of the new foster litters contained a total of 12 pups including pups from both experimental and control groups.

Offspring Brain Collection and Preparation:

To determine molecular effects of prenatal infection later than fetal life offspring were analysed at two weeks of age (PD14), a stage close to when the functionally important Ndel1-DISC1 protein interaction peaks (Brandon *et al.*, 2004). At this age male pups were sacrificed by an intraperitoneal overdose of anaesthetic mix (xylazine and ketamine diluted in physiological saline). Depending on if brains were needed for western blot, PCR or immunohistochemistry, decapitation or transcardial perfusion was performed (see below) and then brains were extracted, frozen on dry ice and kept at -80°C until further processing. For western blot, animals were sacrificed by live-decapitation and brains were harvested fresh. Animals designated for mRNA analysis were perfused by cardiac puncture with phosphate-buffered saline (PBS) and those for immunohistochemistry were subsequently perfused with 4% paraformaldehyde in PBS, post-fixed at 4°C overnight and cryoprotected in 30% sucrose until snap-frozen at -80°C.

Postnatal Brain Region Extraction:

For regional western blot and PCR analysis PD14 male pup brains were dissected by cutting 400µm cryostat sections at -15°C and extraction of prefrontal cortex, motor and sensory cortex, and whole hippocampus was performed using sample corers (Fine Science Tools Inc., North Vancouver, BC, Canada). Samples were stored at -80°C.

ELISA

Aliquoted maternal plasma samples were analysed by rat-specific sandwich enzyme-linked immunosorbent assays (ELISA) for their concentrations of rat tumor

necrosis factor (TNF)α, interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra) and IL-6 (NIBSC, Potters Bar, UK) as described previously (Rees et al., 1999). All plasma samples were diluted 1:10 (except IL-1 β which was diluted 1:5) and were assayed in duplicate.

PCR

RNA Extraction and Semi-Quantitative RT-PCR:

To determine if LPS leads to changes in gene transcription of IL-1β, TNFα and DISC1 mRNA in fetal tissues RNA extraction, reverse transcription and semi-quantitative polymerase-chain reaction (PCR) were performed as previously described (Ashdown et al., 2006). Inhibiting factor (I)κBα mRNA levels are an activation marker of the transcription factor Nuclear factor (NF)κB , and thus a marker of inflammation, and were also assayed by PCR. In brief, RNA was extracted from tissues by sonication in TRIzol reagent (Invitrogen, Burlington, Ontario, Canada) by the manufacturer's instructions and 1ug of total RNA was transcribed into DNA. The cDNA product (1.8μl) and 6pmol of gene-specific primers for IL-1β, TNFα, IκBα, DISC1 and β-actin (Alpha DNA, Montreal, Quebec, Canada) underwent reactions with ReadyMix Taq PCR (Sigma) in a Gene Amp PCR system 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA). The cycling parameters for all primers were: 1) denaturation at 95 °C for 5 min, (2) 95 °C for 30 sec, annealing at 60°C for 30 sec and 72 ºC for 1 min for a set number of cycles and (3) final extension at 72 ºC for 10 min. For each set of primers the gene accession numbers, forward and reverse primer sequences, and cycle numbers that were used are listed as

follows: IκBα (NM_01105720; forward, 5'-AACAACCTGCAGCAGACTCC-3', reverse, 5'-GTGTGGCCGTTGTAGTTGG-3'; 24cycles), IL-1β (NM_031512; forward, 5'-CCCAAGCACCTTCTTTTCCTTCATCTT-3', reverse, 5'-CAGGGTGGGTGTGCCGTCTTTC-3'; 36 cycles), TNFα (NM_012675; forward, 5'-GGGCTCCCTCTCATCAGTT-3', reverse, 5'TGGAAGACTCCTCCCAGGTA-3'; 44 cycles), β-actin (NM_031144; forward, 5'-GCCGTCTTCCCCTCCATCGTG-3', reverse, 5'-TACGACCAGAGGCATACAGGGACAAC-3'; 18 cycles) and DISC1 (NM_175596; forward, 5'-CAAGATGCTGGCCCTATCAG-3', reverse, 5'-CCGTGCACTTCACAGTGTTT-3'; 30 cycles). Primers were designed to span a sequence derived from different exons (separated by at least one intron in the genomic DNA sequence) in order to minimize amplification of non mRNA-derived templates. Preceding sample analysis, it was confirmed that primer products do not result from genomic DNA amplification. In pilot experiments for each primer, the amount of PCR product (on a log scale) vs. the number of cycles was plotted, and the linear range of template amplification was determined for two samples from SAL and LPS treatment groups. The cycle numbers were determined to be within the exponential phase of amplification for both treatment groups. The final PCR products were separated by electrophoresis in a 2% agarose gel, visualized with ethidium bromide staining, and the band optical densities were then quantified by GeneTools image analysis software (Syngene, Frederick, MD, USA). The band optical density of gene X from each sample was normalized by the band optical density of a housekeeping gene, β-actin.

Real-time qRT-PCR:

Relative changes in DISC1 mRNA in the PD14 hippocampus, cortex or prefrontal cortex were analyzed quantitatively using real-time quantitative PCR (qRT-PCR). RNA was extracted and reverse transcription was conducted using the same protocol as listed above. qRT-PCR was conducted using a 7500 Standard Real-Time PCR System (Applied Biosystems, USA) in which sample cDNA, Taqman probes with primer, and enzyme mix were loaded onto a plate and the reaction processed using thermal cycling conditions of 10 min at 95 °C, 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min (60°C annealing conditions. Pre-tested primers for DISC1 (Rn00598264_m1) and β-Actin (Rn01424440_s1) were obtained from Applied Biosystems (USA). The relative amount of DISC1 mRNA in LPS vs. SAL groups was quantified using β-Actin as the internal control and the delta-delta-CT technique, as has been thoroughly described (Livak & Schmittgen, 2001).

Prostanoid Analysis:

All tissues collected on GD18 (maternal plasma, amniotic fluid, placenta and fetal brain) were analyzed for prostanoid content, including PGE_2, $PGF_{2\alpha}$, PGD_2, 6-keto-$PGF_{1\alpha}$ and thromboxane (TX) B_2 at 2, 3 and 4 hours after maternal injection. Protein extracts were analyzed by Liquid Chromatography-Mass Spectrometry (LC-MS) in collaboration with Dr. Denis Riendeau at the Merck Frosst Centre for Therapeutic Research (Kirkland, Montreal) using a previously described protocol (Guay et al., 2004). Briefly, protein was extracted from the tissues by brief sonication in 5 volumes (5 ml / g) of cold PBS

containing 10 μM indomethacin and 1x Roche Complete protease inhibitors. Homogenates were spun at 1,000 x g for 20 min (4 °C) and resulting supernatants were transferred in 96-well plates and frozen at -80°C until analysis. Prostaglandin values were presented as a ratio to the amount of total protein per sample, as measured in a separate aliquot of the same samples using Bradford's Reagent (Sigma-Aldrich) and following the manufacturer's instructions.

Protein Analysis by Western Blot:

Any regional brain changes in DISC1, Ndel 1 and LIS1 protein expression in the offspring due to prenatal infection were assessed semi-quantitatively using Western Blotting techniques. PD14 brain tissue was disaggregated by brief sonication in lysis buffer (50 mM Tris-HCl, 2 mM EDTA and 1% Nonidet) with 1x protease inhibitor cocktail and 1x phosphatase inhibitor cocktail (Sigma-Aldrich). Protein content in the soluble fraction was quantified using Bradford reagent (Sigma-Aldrich) following the manufacturer's instructions, and then aliquoted and frozen at –80°C until use. On the day of procedure, proteins were denatured by boiling 50ug sample aliquots diluted 1:1 with sample buffer (5 % β-mercaptoethanol, Sigma, USA; 95 % Laemmli buffer, Bio-Rad Laboratories, Mississauga, ON, Canada) at 95°C for 5 minutes and then loaded into precast 4-20% acrylamide gels (Invitrogen) and run at 125V for two hours. Proteins were transferred at 15V overnight onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences Corp., Piscataway, NJ, USA) in an XCell II Blot Module (Invitrogen) by the manufacturer's instructions.

Immunodetection was then performed on these membranes, and to reduce non-specific detection membranes were blocked with 10% non-fat dry milk (Bio-Rad Laboratories) in Tris-buffered Saline with 0.1% Tween-20 (TBS-T) for 3 hours. Membranes were then incubated overnight at 4°C with a primary antibody at the appropriate dilution in TBS-T and 5% milk. Antibodies for target proteins (polyclonal anti-rabbit) were kindly obtained in collaboration with Dr. Minh Dang Nguyen (University of Calgary, Canada) and included DISC1 (1:200), Ndel1 (1:10 000) and LIS1 (1:200). The antibodies used for detection of control genes (Santa Cruz Biotechnology, Santa Cruz, CA, USA) included Actin (1:100 000) and GAPDH (1:10 000). After washing thoroughly with TBS-T, membranes were incubated with appropriate detection antibodies conjugated to horseradish peroxidase in TBS-T and 1% milk for an hour at room temperature. Visualization of bands was performed by applying ECL western blotting detecting reagents (Amersham Biosciences Corp.) for 1 min and then exposing the membrane to a chemoluminescence sensitive film (Hyperfilm ECL, Invitrogen) in a darkroom. After digitizing the films, band optical density was analyzed by GeneTool image analysis software (Syngene, Frederick, MD, USA). As a control for gel loading errors, the value of each sample was presented as an optical density ratio of the target protein to an endogenous control protein (Actin or GAPDH depending on the size of the target protein) from the same membrane.

Immunohistochemistry:

This technique adds support to any protein expression changes observed for DISC1 and more narrowly determines regional changes of DISC1 in the brain (for example in the hippocampus, where the dentate gyrus has much higher levels of DISC1 than other hippocampal areas (Schurov *et al.*, 2004)). Briefly, free-floating coronal sections (40μm) were washed with phosphate buffered saline (PBS), incubated with 0.3% H_2O_2 and then blocked with normal goat serum. The antibody against DISC1 (Zymed, distributed through Invitrogen) at a 1:250 dilution were then incubated with slices overnight at room temperature, followed by 2 hour incubation with biotinylated goat anti-rabbit IgG (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). DISC1 was visualized with fluorescence by incubating samples with steptavidin-cy3, mounting on slides with VectaShield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA), and images captured with a fluorescent Zeiss microscope with a 20x objective. Specificity of the DISC1 antibody was determined by co-incubating it with its immunizing peptide (obtained by special request from Invitrogen, USA) at a ratio of 1:100 at 4 °C overnight to observe elimination of the signal. It is notable that the DISC1 antibody used for Western Blot studies (obtained in collaboration with Dr. Minh Dang Nguyen, University of Calgary) did not stain well with immunohistochemical protocols, and thus a commercial antibody (Zymed) made against the same region of the protein (C-terminal) was utilized.

Quantification of staining was done by regional cell-counting in the polymorphic layer of the dentate gyrus. Using Image J software (Image J 1.40g, National Institute of

Health, USA) a 350µm x 200µm rectangular region within the hilar region of each dorsal hippocampal slice was distinguished (Figure 9A). Cell-counting was then done manually in 6-8 hilar regions per animal. The experimenter was blind in respect to which experimental group the slices belonged to.

Double staining was conducted to compare the commercial antibody staining with that described in the literature with other DISC1 antibodies, and to more narrowly determine which cell-types DISC1 is found in. Specific antibodies were used to identify neurons (by NeuN), astrocytes [by glial fibrillary acidic protein (GFAP)], and microglia (by the cell-surface microglial marker CD11b) under the same conditions described above for the DISC1 immunohistochemistry alone. In wells already containing DISC1 antibody, either NeuN (1:200; Chemicon) or CD11b (1:200; Biosource International, Camarillo, CA, USA) antibodies were added and visualized with FITC anti-mouse (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). For astrocyte dual staining, a GFAP antibody (1:8000, Sigma-Aldrich) conjugated directly to cy3 was added to DISC1-containing wells and DISC1 was instead visualized using FITC anti-mouse (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA , USA).

Statistical Analysis:

All data are presented as mean values ± standard error of the mean (SEM) and were analysed using GraphPad Prism software (Version 4.00, GraphPad Software Inc., La Jolla, CA, USA). Maternal cytokine ELISA results were analysed using two-way analysis of variance (ANOVA) and Bonferroni post-hoc tests where necessary. A student's t-test was

used to analyze data from studies where ELISA data was only available for a single time point. The relationship between these cytokines and maternal body temperature changes was examined using linear regression methods. For all fetal experiments, tissues were analysed in pooled sets per dam for LPS and saline groups. Fetal brain cytokine or prostaglandin levels were subjected to a student's t-test and were correlated with maternal cytokine levels. Analysis by repeated measures ANOVA was not possible due to the inter-plate variability during data collection for each time point. For all postnatal experiments, mean values from LPS and Saline groups were analysed by a student's t-test. The mean values from western blot studies are a ratio of the target protein to an endogenous control. Only one PD14 pup was analysed per dam, with n referring to the number of dams. Significance was defined in all experiments by $p<0.05$

Results

Early Prenatal and Maternal Response to LPS

Maternal Plasma Cytokines in Relation to Body Temperature Changes:

Maternal plasma samples were assayed for cytokines by ELISA to characterise the response of GD18 pregnant dams to the intraperitoneal injection of 50ug/kg LPS. IL-1β, IL-6, TNFα and IL-1ra levels were all tested in plasma samples from 4 hours after injection, but due to limited sample volume only IL-1β and IL-6 were analysed at the 2 and 3 hour time points. A two-way ANOVA was used to explore IL-1β (Figure 2A) and IL-6 (Figure 2B) protein differences between groups over time. Dams injected with LPS had significantly more IL-1β and IL-6 at 2 hours than those injected with saline ($p<0.01$), with no significant interactions between time point and group. Figure 2C shows that the trend for an increase in TNFα (SAL: 35.33 ± 3.734 pg/ml, LPS: 99.53 ± 39.13 pg/ml) and IL-1ra protein (SAL: 180.6 ± 50.49 pg/ml, LPS: 4271 ± 2148 pg/ml) at 4 hours was not significant (n=4).

Whereas dams at earlier stages of pregnancy show a characteristic febrile response to LPS (Fofie & Fewell, 2003), dams at GD18 in this study most often exhibited hypothermic responses to this dose of endotoxin. At 4 hours post-LPS absolute changes in maternal body temperature (including both hypothermic and hyperthermic responses) correlated significantly with IL-6 (Figure 3A; $r^2=0.57$, $p<0.01$), IL-1β (Figure 3B; $r^2=0.47$, $p<0.05$) and the anti-inflammatory cytokine IL-1ra (Figure 3C; $r^2=0.41$,

$p<0.05$). There was, in addition a non-significant trend for a correlation with TNFα (Figure 3D; $r^2=0.33$, $p=0.052$).

Messenger RNA Levels in Fetal Brains:

As dams treated with LPS exhibit pro-inflammatory cytokine production at early stages of infection, independent t-tests were employed to determine if gene transcription related to inflammation was occurring in the fetal brain after maternal LPS. Semi-quantitative RT-PCR did not reveal any significant changes in IL-1β or TNFα mRNA in whole fetal brains at 1-4 hours (n=5-7 at 1 and 2 hours, n=1-3 at 3 and 4 hours; Figure 4A and 4B), nor were there significant differences in IκBα mRNA (Figure 4C). For all comparisons $p>0.05$.

Prostanoid Content of Fetal Brains:

Prostanoid content of fetal brains was analysed by LC-MS at 2, 3 and 4 hours after maternal LPS (Figure 5). Although many individual fetal brains were studied per time point, the results from the studies at 2 and 3 hours should be deemed preliminary as the fetuses came from low numbers of dams per group (ranging from 1-3 dams/group for 2 and 3 hour time points). Corresponding with peak maternal circulatory pro-inflammatory cytokines, pilot studies show that at 2 hours after LPS there is induction of PGE_2, $PGF_{2\alpha}$ and PGD_2 in the fetal brain (data not shown).

At 3 hours (Figure 5A) there was 3.3-fold increase in fetal brain PGD_2 content, from 2.977 ± 0.08017 to 9.875 ± 0.4421 pg/mg total protein in SAL vs. LPS animals

(t(3)=12.00, p<0.01). Similarly, maternal LPS increased $PGF_{2\alpha}$ content from 86.68 ± 3.673 to 139.3 ± 9.387 pg/mg total protein (t(3)=4.232, p<0.05). Although not reaching significance possibly because fetal brains came from a low number of dams, PGE_2 content was the most dramatically induced prostaglandin by maternal endotoxin treatment, in fact showing 25 times more in fetal brains from dams treated with LPS (4.568 ± 1.995 pg/mg total protein) than controls (0.1847 ± 0.004151 pg/mg protein).

Figure 5B illustrates that this induction seems fairly transient, as SAL and LPS fetal brains show comparable prostanoid content at 4 hours (n=4, 2-6 fetuses per dam). There were no significant differences between groups in any of the prostaglandins PGE_2 (SAL: 3.047 ± 1.221, LPS: 3.055 ± 1.957, t(6)=0.003470, p>0.05), PGD_2 (SAL: 110.4 ± 9.758, LPS: 92.29 ± 20.02, t(6)=0.8112, p>0.05), or $PGF_{2\alpha}$ (SAL: 207.2 ± 25.05, LPS: 179.5 ± 6.228, t(6)=1.075; p>0.05).

At each of the time points assayed (2-4 hours) no significant differences were detected in the 6-keto-$PGF_{1\alpha}$ or thromboxane $(TX)B_2$ content of fetal brains from LPS or SAL-treated dams (data not shown, p>0.05).

Postnatal Response to Maternal LPS:

Maternal and Litter Effects of LPS:

Table 1 outlines some of the responses pregnant dams had to sequential saline or LPS injections on GD18 and GD19. Most prominent is the effect of LPS on dam survival, with 88% surviving the injections compared to 100% of saline injected animals. Notably, these dams died from the first LPS injection on GD18. At GD18 the surviving dams exhibited significant change in body temperature at 4 hours after injection

compared to saline-injected animals. The second injection of LPS, at GD19, had no outward measureable effect on the core body temperature, most likely due to the a desensitisation after the first injection, a phenomenon that is well described for multiple injection of this exogenous pathogen (Mengozzi & Ghezzi, 1993). Although body temperature responses to LPS were quite variable (including both hypothermic and hyperthermic responses), dams injected with LPS consistently gained less weight between GD18 and GD19 than saline injected dams ($p<0.001$). This did not affect the litter size, nor did it affect the male: female ratio of the litter. However LPS-injected dams were more likely to lose their pregnancy (data not shown).

DISC1 mRNA Levels:

To ascertain how DISC1 mRNA levels are affected by maternal infection, regional qRT-PCR analysis was performed. For both hippocampal (Figure 8A) and cortical (Figure 8B) areas of the PD14 brain, t-tests were performed comparing the ddCt levels of DISC1 from both groups. For the hippocampus, means did not differ significantly (SAL: 0.1895 ± 0.1614, N=4; LPS: 0.1620 ± 0.08174, N=5; $t(7)=0.1624$, $p>0.05$). DISC1 mRNA levels in the cortex (SAL: 0.1540 ± 0.1160, N=4; LPS: 0.2870 ± 0.2012, N=5) also did not show a significant difference between groups, with $t(7)=0.5322$ and $p>0.05$.

Protein Expression of DISC1 and its Binding Partners:

Protein content assays of DISC1 and its binding partners Ndel1 and LIS1 were performed for the prefrontal cortex, hippocampus, and sensory and motor cortices of developing PD14 offspring from the maternal infection model.

DISC1

Preliminary experiments focused on the changes in the 'principal' 100kDa DISC1 isoform in LPS pups and controls. A significantly lower expression of DISC1 protein in both the hippocampus (Figure 6A) and cortex (Figure 7A) was detected in pups from LPS-treated versus SAL-treated dams (SAL: n=3 or 4, LPS: n=4; $p<0.05$). DISC1 levels were compared to the expression levels of an endogenous control, Actin, in the same samples.

This experiment was replicated to both include a larger number of animals and analyses of other important DISC1 isoforms. Previous studies using Western Blot techniques on protein extracts from developing rodent brains have demonstrated the expression of three major DISC1 isoforms, namely a 70kDa protein, a 100kDa protein, and a large isoform over the size of 120kDa (>120kDa), which is most likely a congregation of smaller isoforms (Brandon *et al.*, 2004). Table 2A outlines the results obtained comparing LPS and SAL pups for the expression levels of the three major isoforms in the hippocampus and cortex, and also the prefrontal cortex. Hippocampal DISC1 expression was drastically lower in LPS pups than saline pups for all detected major isoforms (Figure 6B). The 70kDa, 100kDa and >120kDa isoforms were 46% ($p<0.001$), 45% ($p<0.05$) and 62% ($p<0.001$) lower, respectively, in pups born to LPS- vs.

SAL-treated dams. Figure 7B shows that in the cortex of LPS pups there was significantly less 70kDa DISC1 ($p<0.05$) but no significant disparity between expression levels of the >120kDa isoform ($p>0.05$). Replication data for the 100kDa isoform was not collected for the cortex due to a technical error during the analysis procedure. The detectible differences DISC1 protein expression in the prefrontal cortex were not significant for any of the quantified DISC1 isoforms, however, there was a trend for diminished levels of the 70kDa isoform in this region for LPS animals compared to SAL ($p=0.057$).

DISC1 Binding Partners LIS1and Ndel1

Initial experiments also compared expression levels of the 49kDa protein Ndel1 and the 45kDa protein LIS1 in the cortex and hippocampus (Figure 6A and B). T-tests revealed no differences in either Ndel1 or LIS1 expression between LPS and SAL groups in these areas ($p<0.05$) for this initial study or in a follow up study using a new cohort of animals. Table 3 outlines the data for Ndel1 levels in the prefrontal cortex, cortex and hippocampus for the follow-up study.

DISC1 Immunohistochemistry in the Hippocampus:

Qualitative Analysis of DISC1:

Qualitative analysis of DISC1 staining in the hippocampus by immunohistochemistry narrowed down areas potentially involved in the diminished DISC1 levels seen by Western blot analysis. DISC1 staining in the PD14 hippocampus was robust in the cellular layers of CA1-3 and throughout the dentate gyrus (Figure 9A). In

other areas of the hippocampus DISC1 staining was sparser, but could be observed primarily in cell bodies.

Quantitative DISC1-Positive Cell Concentration:

To determine whether regional hippocampal differences in quantifiable DISC1 levels exist between offspring from LPS and control dams, the concentration of DISC1-positive cells from the hilus was compared between groups by an unpaired t-test. Within a designated area (Figure 9A) LPS offspring had significantly fewer DISC1-positive cells (Figure 9B; LPS, 12.59 ± 0.9035) compared to saline (SAL, 16.11 ± 0.9023). This represents a 12% decrease in DISC1 cells within that area ($t(8) = 2.758$, $p=0.0247$). The cellular layer of the dentate gyrus was not quantified as the concentration of DISC1-positive cells was too high for accurate counting.

DISC1-Positive Cell Identification:

Co-immunofluorescence with DISC1 and other cellular markers determined which hippocampal cell types might be involved in the DISC1 decrease within the hilus or elsewhere. Double-staining with DISC1 and neuronal nuclei (NeuN), a marker of mature neurons, showed significant co-localisation between the two (Figure 10A). Some DISC1 staining was also observed in microglial cells in the same brain region (Figure 10C) but there was no co-localisation between DISC-1 and astrocytes (Figure 10B).

Discussion:

Prenatal exposure to maternal infection in experimental animals is now recognised as an effective and useful model for studying developmental defects in the offspring that are related to number of mental disorders such as schizophrenia. Using this approach the studies outlined in this thesis have produced results showing that expression of DISC1, a schizophrenia-risk gene, can be modified by maternal infection, possibly through the induction of immune mediators in the fetal brain. These observations support a gene/environment interaction in the aetiology of psychiatric disorders including schizophrenia.

Maternal Response to LPS

As immune stimulation by LPS in dams during late pregnancy was used to mimic maternal infection, confirmation of an induced sickness response was gained by observing febrile and hypothermic responses, cytokine induction, weight loss, and reduced responsiveness to a second LPS injection. Indeed these dams responded with significant changes in body temperature which correlated significantly with IL-1β, IL-6 and IL-1ra plasma levels at 4 hours after infection. Notably, the peak induction of IL-1β and IL-6 was observed at 2 hours, occurring 2 hours earlier than that reported by Ashdown and others (2006) using the same model. By having knowledge of the induced body temperature and cytokine changes in these pregnant animals, it was possible to narrow down which maternal immune factor might be conveying changes in the prenatal or postnatal CNS.

Fetal Brain Changes During Maternal Infection

We hypothesised that pro-inflammatory cytokines, key inflammatory mediators, would be produced in the fetal brain during maternal immune stimulation. Expanding a time-course of previous results from our group (Ashdown *et al.*, 2006), we detected no change in the fetal brain content of pyrogenic cytokine mRNA levels, specifically TNFα and IL-1β, from one to four hours after maternal LPS injection. Correspondingly, no changes in mRNA of the inflammatory marker IκBα were detected in these fetal brains. It remains to be confirmed whether inflammatory changes at the mRNA level were undetected due to their transient nature, or whether regional cytokine signalling was concealed by dilution effects due to studying the whole brain.

Despite cytokine mRNA results, this study revealed the novel finding that prostaglandins, final mediators of the inflammatory process, are significantly induced in the whole fetal brain in response to maternal inflammatory stimuli. This induction of PGE_2, $PGF_{2\alpha}$ and PGD_2 occurs quite early after maternal LPS injection (at 2 and 3 hours post-injection) but seems to be transient, with levels decreasing to that of controls by the fourth hour. It is notable that augmented prenatal prostaglandin levels occurred during the same period that maternal plasma showed significant increases in IL-6 and IL-1β, while both fetal and maternal measures of inflammation were similar to that of controls by 4 hours.

The cytokine IL-1β is a likely candidate for inducing the prostaglandins we observed in the fetal brain, since IL-1β levels are largely increased in the fetal circulation soon after maternal LPS injection (Ashdown *et al.*, 2006) and this cytokine has the ability

to stimulate further inflammatory molecules. One hypothesis is that IL-1β in the fetal circulation passes through a more permeable fetal blood brain barrier (BBB), allowing it to act directly in the brain. At GD18 the BBB is not fully developed, and much debate exists over whether the fetal blood brain barrier is thus more "leaky" than that of the adult (Engelhardt, 2003). Evidence, for example, that the ovine fetal brain has higher permeability to some molecules at earlier stages of gestation (Stonestreet *et al.*, 1996) supports the idea that cytokines in the circulation, such as IL-1β detected in the fetal blood in response to LPS, could more readily pass into the fetal brain.

An alternative and more likely hypothesis is that cytokines in the fetal circulation stimulated by the maternal inflammatory response act on the endothelial cells of the fetal BBB to cause cyclooxygenase (COX)-2 to be released into the fetal brain. This enzyme is responsible for prostaglandin production in the context of inflammation (Liang *et al.*, 2007) and although it is not yet characterised in the prenatal system, it is known that COX-2 is induced in the adult brain by pyrogenic cytokines or LPS, in areas such as the endothelial cells lining the blood vessels of the hypothalamus (Cao *et al.*, 1998;Cao *et al.*, 1999;Cao *et al.*, 2001;Ek *et al.*, 2001). Further studies analysing if there is COX-2 release into the fetal brain during early stages of maternal inflammation could help explain the large induction of prostaglandins observed there.

Determining the specific effects of PGE_2, $PGF_{2\alpha}$ and PGD_2 induction on the developing prenatal brain is complicated by the varying roles each prostanoid subserves. Prostaglandins are small lipophilic molecules that are produced by a wide variety of cell types, including neurons and glia in response to physiological and pathological stimuli

such as neurotoxicity or inflammation (Simmons *et al.*, 2004). The roles of prostaglandins in the adult brain are quite numerous and range from general housekeeping functions to contributions in pain, inflammation, fever, and sleep depending on the specific prostaglandin and the receptor it acts on (Simmons *et al.*, 2004). Similarly to the effect of cytokines on neuronal viability, prostaglandins seem to take both protective and damaging roles, for example PGE_2, $PGF_{2\alpha}$ and PGD_2 have been shown to both cause or intensify neuronal apoptosis (Li *et al.*, 2008;Takadera *et al.*, 2004;Takadera & Ohyashiki, 2006), but can also attenuate it (Liang *et al.*, 2007). More specifically, PGE_2 can be protective if it acts on its receptors EP2 or 4 (Ahmad *et al.*, 2005;Ahmad *et al.*, 2006b;Takadera & Ohyashiki, 2006), or can contribute to neuronal damage if acting on the EP1 receptor(Ahmad *et al.*, 2006a). To add to the complexity, neuroprotective or damaging roles can reverse depending on the type of stimulus that initiated the prostaglandin production (Liang *et al.*, 2007;Wu *et al.*, 2007). With such a wide range of possible effects of prostaglandin action, further experimentation is needed to narrow down how specific prostaglandins may be affecting neuronal development during maternal infection. However, with the awareness that prostaglandins within the fetal CNS have the ability to disturb the normal course of neurodevelopment they are plausible candidates for causing postnatal neurological effects of maternal infection and should be explored further.

Postnatal Effects of Maternal Infection on DISC1

To the best of our knowledge, this is the first study to demonstrate a biochemical link between two factors that share common schizophrenia-relevant neurological consequences, namely maternal infection and alterations to the DISC1-system. Our studies revealed that maternal infection effectively suppresses three major hippocampal DISC1 protein isoforms to the extent of nearly 50% that of controls. The amount of DISC1 suppression observed here is remarkable considering that a simple prenatal manipulation like immune stimulation causes similar decreases in protein expression as advanced manipulations aimed specifically at DISC1, such as mutation or knockdown (Pletnikov *et al.*, 2007). Other brain regions affected included the sensory and motor cortex which exhibited significant attenuation of two of these isoforms, sized at 70 and 100kDa. These changes may be regional in nature, as the prefrontal cortex was not significantly altered in respect to DISC1 expression (although there was a trend for a decrease).

Surprisingly maternal infection did not affect the expression of two major DISC1-binding proteins, LIS1 and Ndel1 in the hippocampus or cortex, areas that had shown such extensive disruptions to DISC1. Although we did not specifically determine if there was a functional effect of the attenuated DISC1 levels in this model, it has been shown previously that merely reducing the amount of this protein through knockdown or mutation can have sizeable consequences for an animal's development, neurological function and behaviour. For example, diminished DISC1-Ndel1 interaction because of DISC1 knockdown in culture has been shown to be extremely disruptive to the cell, as

this interaction is required for the crucial process of neurite outgrowth (Kamiya *et al.*, 2006).

Theories of functional consequences of this DISC1 suppression after maternal infection must not only recognize DISC1's status as a hub protein that interacts with many others but also that its different isoforms have different subcellular locations and thus different cellular roles. For example, although these proteins are found at many sites within a neuron, many DISC1 isoforms have a predominantly mitochondrial localization (James *et al.*, 2004), and disruption of these may have important consequences for normal brain function. Among other roles, mitochondria provide an abundant supply of energy in the form of ATP that is necessary for normal neuronal functioning, and also play key roles in apoptosis (Suen *et al.*, 2008). DISC1 isoforms should also be thought of independently because of their different roles regardless of cellular location; this is illustrated by the fact that neuronal cAMP levels are regulated differently depending on the DISC1 isoform that PDE4B is interacting with (Honda *et al.*, 2004;Murdoch *et al.*, 2007).

Despite the strong differences in DISC1 protein levels between LPS and control groups, our studies of mRNA levels in the hippocampus and cortex do not show the same relationship. In fact this situation is not a rare occurrence; several studies have shown that in general the correlation between protein and mRNA abundance can be quite low, varying up to 20-30 fold, and making one an insufficient predictor of the other (Gygi *et al.*, 1999;Washburn *et al.*, 2003). Possible reasons for these miscorrelations include translational regulation, experimental conditions and errors, and

differences in protein and mRNA decay rates (Beyer *et al.*, 2004;Nie *et al.*, 2006). This analysis becomes even more complicated in the study of DISC1 because of the vast number of splice variants and protein isoforms in existence (James *et al.*, 2004;Murdoch *et al.*, 2007;Taylor *et al.*, 2003). Although our mRNA analysis used primers designed to amplify the largest spliceform, thought to encode for the 100kDa protein (James *et al.*, 2004), transcriptional and post-translational modifications make it difficult to associate these results with those of the protein. Obviously levels of the downstream product, in this case the functional DISC1 protein, typically have the greatest implications.

Immunohistochemical analysis of DISC1 expression was performed to narrow down areas involved in the DISC1 decrease observed during western blot experiments. This was done with particular focus on the hippocampus, where abnormalities in structure and function are known to occur in schizophrenia (Chew *et al.*, 2006;Harrison & Weinberger, 2005;White *et al.*, 2008). Our qualitative studies of DISC1 in the rat hippocampus correspond with what has been previously described in the literature regarding protein distribution of the mouse orthologue Disc1, both during development (Meyer & Morris, 2008) and in adulthood (Schurov *et al.*, 2004). We observed the highest DISC1 expression to be in cells of Ammon's horn (CA1-3) and in the dentate gyrus, with staining seen mostly in the cellular layers but also sparsely throughout the hippocampus.

In a similar tendency to that observed during western blot experiments, we found a 12% decrease in density of DISC1-positive cells in the hilus of the hippocampus, the polymorphic layer of the dentate gyrus. This quantitative study pinpoints the hilus as

one specific hippocampal area where alterations are occurring, as a step towards linking known functional consequences with the observed DISC1 attenuation. The hilus contains a multitude of cell types, including several types of interneurons, migrating immature granule cells involved in neurogenesis, as well as astrocytes and microglia (Anderson *et al.*, 2006). Unfortunately identities of DISC1-positive cells in the hilus or other regions of the hippocampus had not previously been thoroughly examined. To help shed light on how DISC1 is attenuated in developing brains from the maternal infection model, it first needs to be determined which hippocampal cell-types contain DISC1 and which are affected by maternal infection. This will address if lower DISC1 levels are either (i) a by-product of a decrease in specific types of cells (i.e. due to decreased numbers of microglia or decreases in neurogenesis), or (ii) a more general disruption in DISC1 expression which is not cell-type specific but could affect cellular structure or function (Kamiya *et al.*, 2006).

In an effort to identify whether cell types other than neurons express DISC1 we performed immunohistochemical studies using cell specific markers for astrocytes and microglia. Confirming previous reports (Austin *et al.*, 2004;Schurov *et al.*, 2004), we found that astrocytes do not contain DISC1, with no co-localisation observed between the astrocyte marker GFAP and DISC1 throughout the entire hippocampus. With this in mind other groups, with the exception of Ma et al.(2002), have concluded that DISC1 may solely be a neuronal protein. Our preliminary data on microglia would suggest otherwise. In the current study we have demonstrated for the first time that cd11B-positive microglia co-localise with DISC1 protein. In the context of maternal infection

this finding may be particularly significant, as microglia are the resident immune-competent cells of the brain and integral to the inflammatory process of the CNS (Muller & Ackenheil, 1998).

Before speculating about the roles of microglia within this model of maternal infection, it must first be acknowledged that the role of microglia in the normal developing brain itself is not yet clear. Indeed there is still debate on the developmental timeline of microglial emergence, the function of microglial cell types in the developing versus mature CNS, and whether their actions are primarily damaging or beneficial (Jonakait, 2007). Amoeboid microglia (in the round and presumably activated state) and ramified microglia (in the branched and quiescent antigen-detecting state) begin to take residence in the embryonic rat brain as early as GD11-14 (Ashwell, 1991;Jonakait, 2007) and in the primordial hippocampus from GD14 and onwards (Dalmau *et al.*, 1997), and thus are present in the developing brain during the time points of this study. Once established they account for almost 10% of the overall cell number in the brain throughout development (Chew *et al.*, 2006). When activated by, for example, LPS or pro-inflammatory cytokines in culture, they propagate the immune response by producing pro-inflammatory cytokines, prostaglandins and reactive oxygen species such as nitric oxide (NO), each of which can be potentially toxic to neurons and oligodendrocytes (Chew *et al.*, 2006;Jonakait, 2007). For example, NO from LPS-activated microglia can kill cholinergic neurons (McMillian *et al.*, 1995), and microglia-derived TNFα reduces the survival of dopaminergic neurons (McGuire *et al.*, 2001). On the other hand, microglia also release salutary factors such as brain-derived

neurotrophic factor (BDNF) (Elkabes *et al.*, 1996;Nakajima *et al.*, 2002) and nerve growth factor (NGF) (Elkabes *et al.*, 1996;Nakajima *et al.*, 2007), highlighting the multidimensional role of these cells.

Interestingly, Cai and others (2000), who employed a similar model of maternal infection to the one used in the current study, observed postnatal changes in microglia at PD8. Pups from LPS-injected dams showed either fewer microglia or altered microglial immunoreactivity to some cellular markers (Cai *et al.*, 2000;Pang *et al.*, 2005). Similar changes have not been detected prenatally, closer to the time of infection (Bell & Hallenbeck, 2002). It has been hypothesised that offspring subjected to prenatal infection may profit from attenuated microglial activation (or number) because the CNS is protected from further injuries induced by excessive inflammation (Jonakait, 2007;Pang *et al.*, 2005). Although plausible, it has yet to be established whether an alteration in microglia contributes to the decreased DISC1 levels we detect in the postnatal brains after maternal infection.

Our double-staining experiments confirm that most mature neurons contain DISC1, but these do not account for all DISC1-positive cells. Despite previous DISC1 *in situ* hybridization results (Honda *et al.*, 2004), DISC1 protein is not ubiquitously expressed by all neurons. Indeed other groups have determined that DISC1-positive cells can be both glutamatergic (Schurov *et al.*, 2004) and GABAergic neurons (Meyer & Morris, 2008;Schurov *et al.*, 2004). Fittingly both these neuronal systems are involved in the pathophysiology of schizophrenia (Harrison & Weinberger, 2005) and are also

affected by an offspring's exposure to infection during pregnancy (Lowe *et al.*, 2008;Nyffeler *et al.*, 2006).

The reduction in DISC1 expression and concentration of DISC1-positive cells we have observed in the hippocampus may be indicative of a decrease in neurogenesis, the proliferation and integration of new neurons. In fact a change in hippocampal neurogenesis has already been deemed to be a consequence of maternal infection. The number of newly born neurons in the dorsal dentate gyrus is reduced in juvenile offspring born to Poly I:C-treated dams, independent of prenatal treatment times (Meyer *et al.*, 2006). Our group also showed that maternal stimulation by LPS not only reduces the offspring's cell proliferation in the hilus but also decreases cell survival (Cui, Luheshi & Boksa, unpublished data), observations mirroring what is seen in the psychiatric pathology of mood disorders and schizophrenia (Kempermann *et al.*, 2008;Reif *et al.*, 2007). With DISC1's established roles in neuronal migration and neurite outgrowth it is not surprising that this protein is involved in the neurogenesis process as well. Recently Meyer and Morris (2008) showed that DISC1 co-localises with cells involved in this process, such as KI-67-positive proliferating cells of the hippocampal subgranular zone, and calretinin-positive early post-mitotic granule neurons. Despite divergent roles in adulthood, DISC1 has been shown to enhance neuronal sprouting and migration in the developing brain (Dranovsky & Hen, 2007). Although the significant decrease in DISC1 due to maternal infection exposure could merely be a consequence of lower neuron numbers due to less neurogenesis, it is also likely to be involved in the inhibition of these processes.

Conclusions

The results from this thesis are the first to show that DISC1, a genetic risk factor for schizophrenia, is also modified in the developing brain by maternal infection, an environmental event associated with the disease. At the same time we have discovered that prostaglandins are induced in the fetal brain soon after maternal infection. Although prostaglandins have the ability to affect neurodevelopment, at this time they are only correlative factors to postnatal DISC1 suppression detected after exposure to maternal immune stimulation. Some theories have been put forward on how immune factors in the developing brain could interact with DISC1 (Carter, 2007;Carter, 2008) but no direct experimental evidence yet exists of a modulatory mechanism.

Caspi and Moffitt (2006) propose that genetic and environmental factors may share common pathways, and that genetic risk factors are a means to determine which molecules, cells, regions an environmental pathogen may be acting on. We suggest that DISC1 represents a commonly affected factor in the context of risk factors for schizophrenia. Indeed features of schizophrenia may develop from disruptions of DISC1, a protein involved in such developmentally important functions such as neural migration, neurite outgrowth, intracellular transport, and synaptic plasticity. In combination with what is known from the literature and what we have seen from our studies, this protein can be affected by genetic disruptions in the human schizophrenic population and in animal models, as well as by prenatal infection. Some suggest that environmental and genetic factors could function in an additive fashion to produce psychiatric disease (Gray & Hannan, 2007). Studies involving a combination of maternal

infection and DISC1 genetically-modified animal models may help clarify how these factors interact. This interaction is likely to be complex, as schizophrenia is a polygenic psychiatric disorder with several environmental risk factors associated with it. However, this study expands our understanding of the aetiology of mental disorders such as schizophrenia by providing the first tangible link between environmental influences and the expression of an associated risk gene.

Figures

Figure 1.

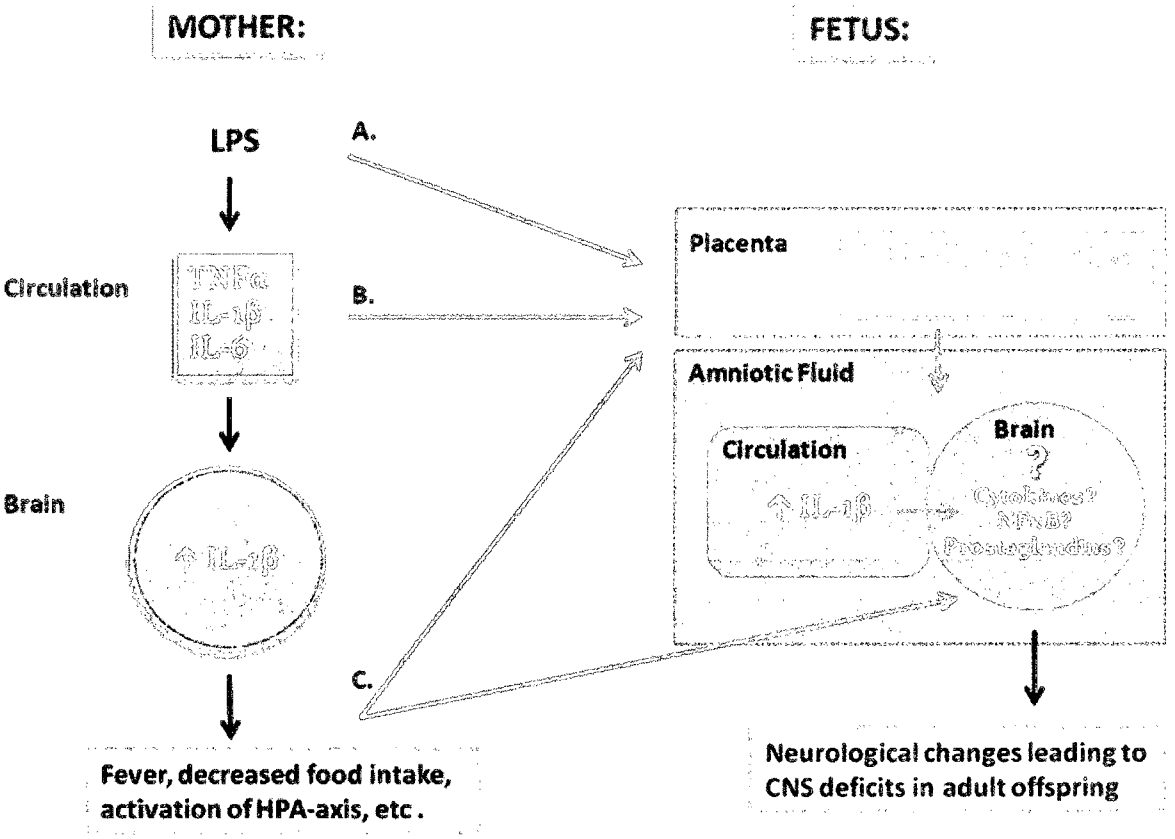

Figure 1: *Schematic diagram outlining possible factors affecting the fetal compartment during maternal infection.* **A.** LPS may directly affect the fetal compartment by acting on toll-like receptors of the placenta, or **B.** indirectly by inducing cytokines in the maternal circulation that can act on or pass through the placenta. **C.** Downstream maternal reactions to LPS related to the sickness response (fever, nutritional changes due to decreased food intake, or activation of the HPA axis) may also affect the fetal compartment and thus brain development. Tissue cytokines listed are those observed previously in this model by Ashdown *et al.* (2006).

Figure 2.

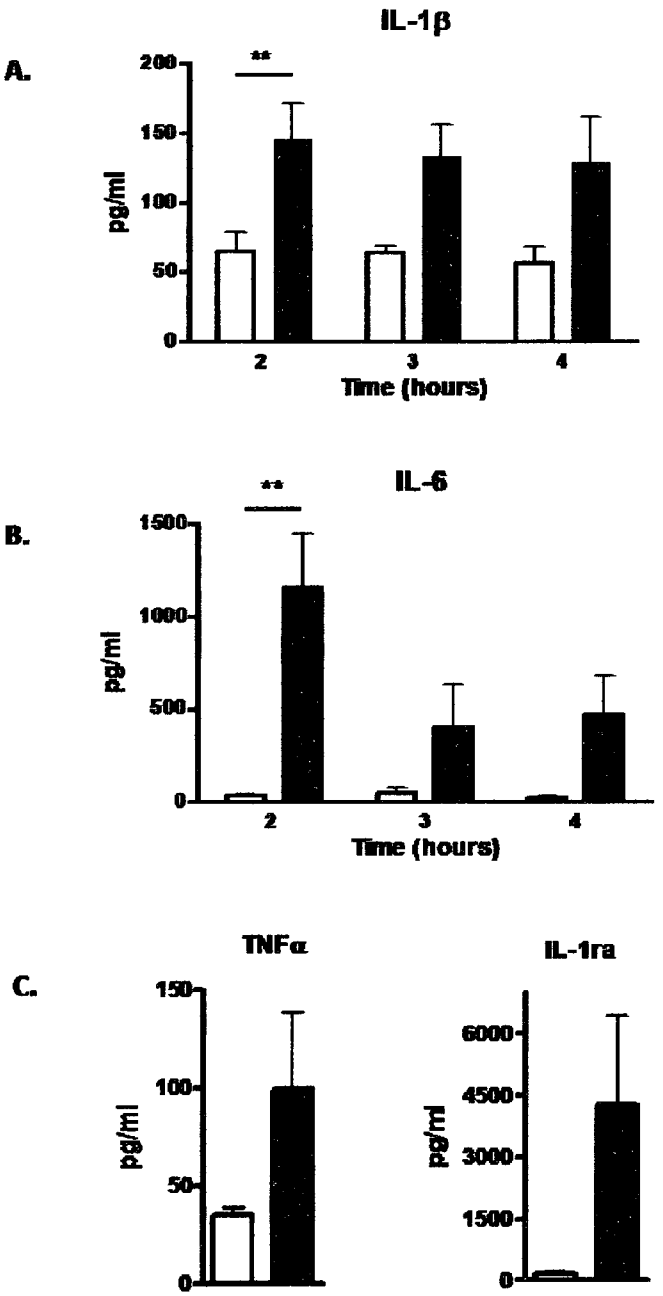
A.
IL-1β
**
200
150
100
50
0
pg/ml
2
3
4
Time (hours)
B.
IL-6
**
1500
1000
500
0
pg/ml
2
3
4
Time (hours)
C.
TNFα
150
100
50
0
pg/ml
IL-1ra
6000
4500
3000
1500
0
pg/ml

Figure 2: *Time-course of maternal plasma cytokine protein levels.* Data shows IL-1β (**A.**) or IL-6 (**B.**) protein levels in the plasma of pregnant dams at 2, 3 or 4 hours after LPS (filled bars) or saline (open bars) injection. Similarly in **C.** TNFα and IL-1ra protein levels are shown at 4 hours after LPS injection. Data graphed are means ± SEM. N=3-6 dams per treatment group at each time point. **p<0.01.

Figure 3:

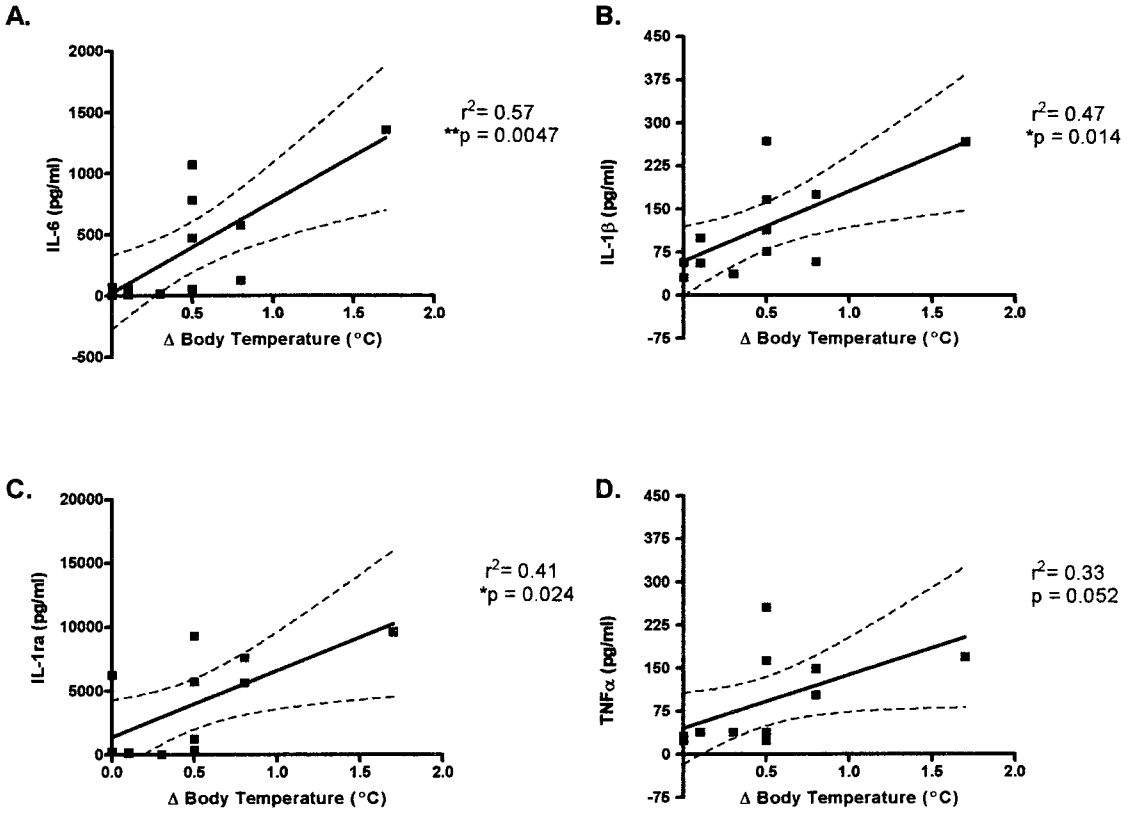

Figure 3: *Correlation of LPS-induced body temperature changes and cytokines.* Data shown are correlations between plasma cytokine levels with the absolute change in body temperature (including both hypothermic and hypothermic responses) of dams 4 hours after injection of LPS. Values shown are **A.** IL-6 **B.** IL-1β **C.** IL-1ra and **D.** TNFα levels versus body temperature changes from the same dam. R^2 values were measured using linear regression analysis. *p<0.05, **p<0.01.

Figure 4.

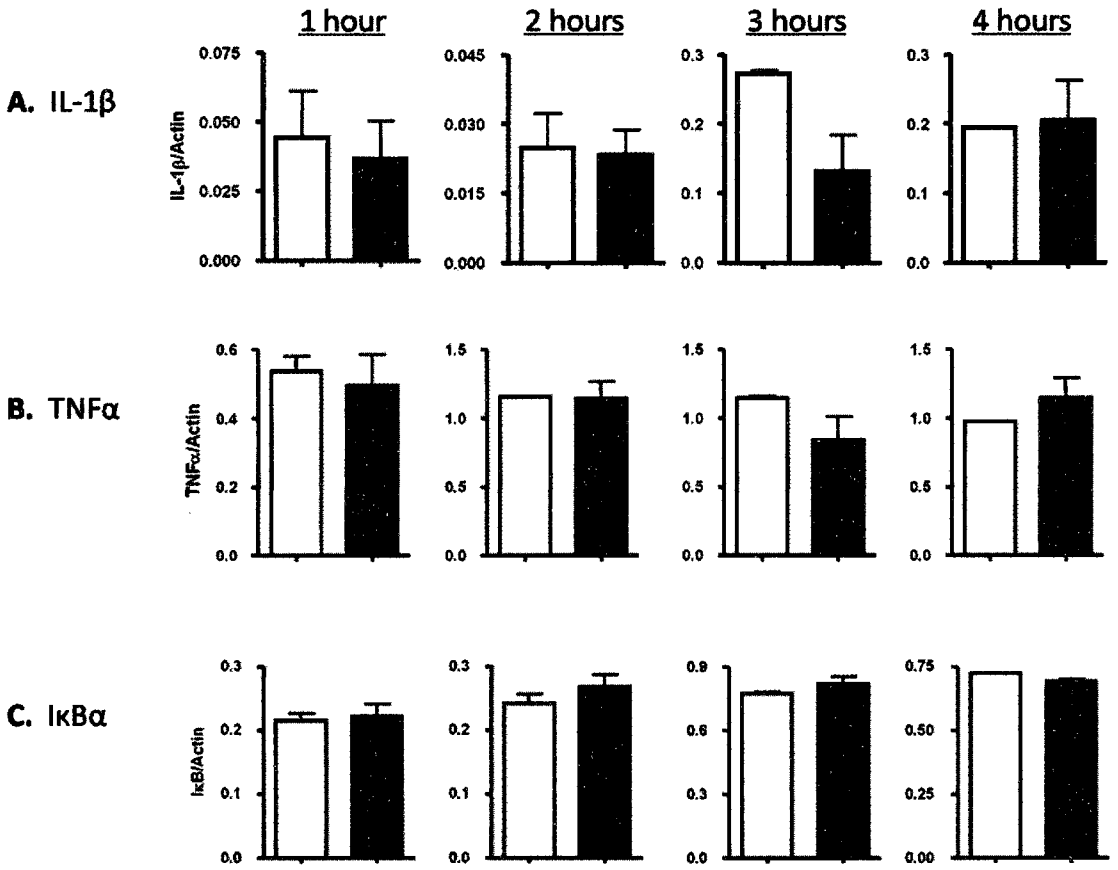

Figure 4: *RT-PCR time course of the genes IL-1β, TNFα and IκB in fetal brains after maternal LPS.* Values shown are the ratio between the target gene (**A.** IL-1β, **B.** TNFα or **C.** IκBα*)* and an endogenous housekeeping gene, β-actin, as a control. Data is graphed as the mean ± SEM. mRNA data is from four pooled fetal brains per pregnant rat dam. At 1 and 2 hours N=5-7, and preliminary data is shown at 3 and 4 hour time points, with n=1-3. No significant differences were found at any time-point for the IL-1β, TNFα or IκB genes between SAL (open bars) and LPS (filled bars) animals ($p>0.05$).

Figure 5.

Figure 7.

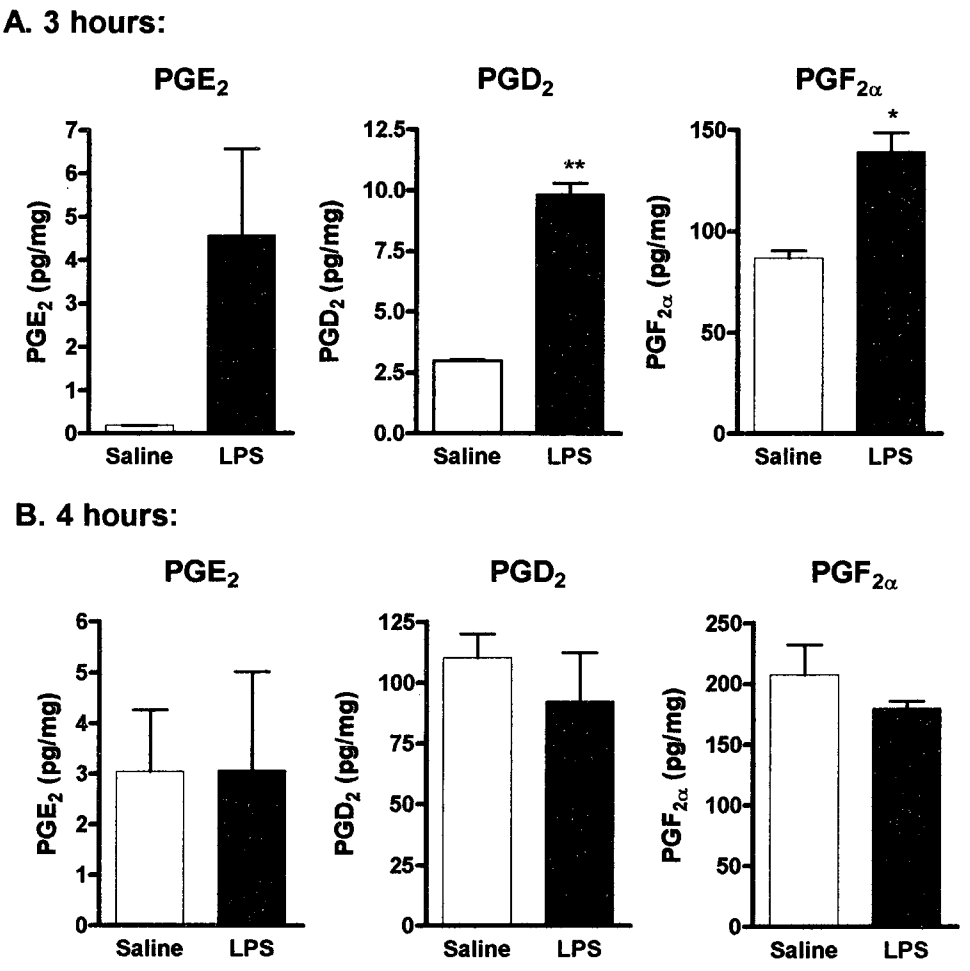

Figure 5: *Prostaglandin measurements in fetal brain after maternal LPS injection.* LC-MS analysis shows that maternal LPS (filled bars) induces significantly more PGD_2 and $PGF_{2\alpha}$ in the fetal brain than Saline treatment (open bars). This effect can be observed at **A.** 3 hours (n=2-3, 4 fetal brains per treated dam) but not **B.** at 4 hours (n=4, 2-6 fetal brains per treated dam). All data are presented as mean values ± SEM. *p<0.05 and **p<0.01.

Figure 6.

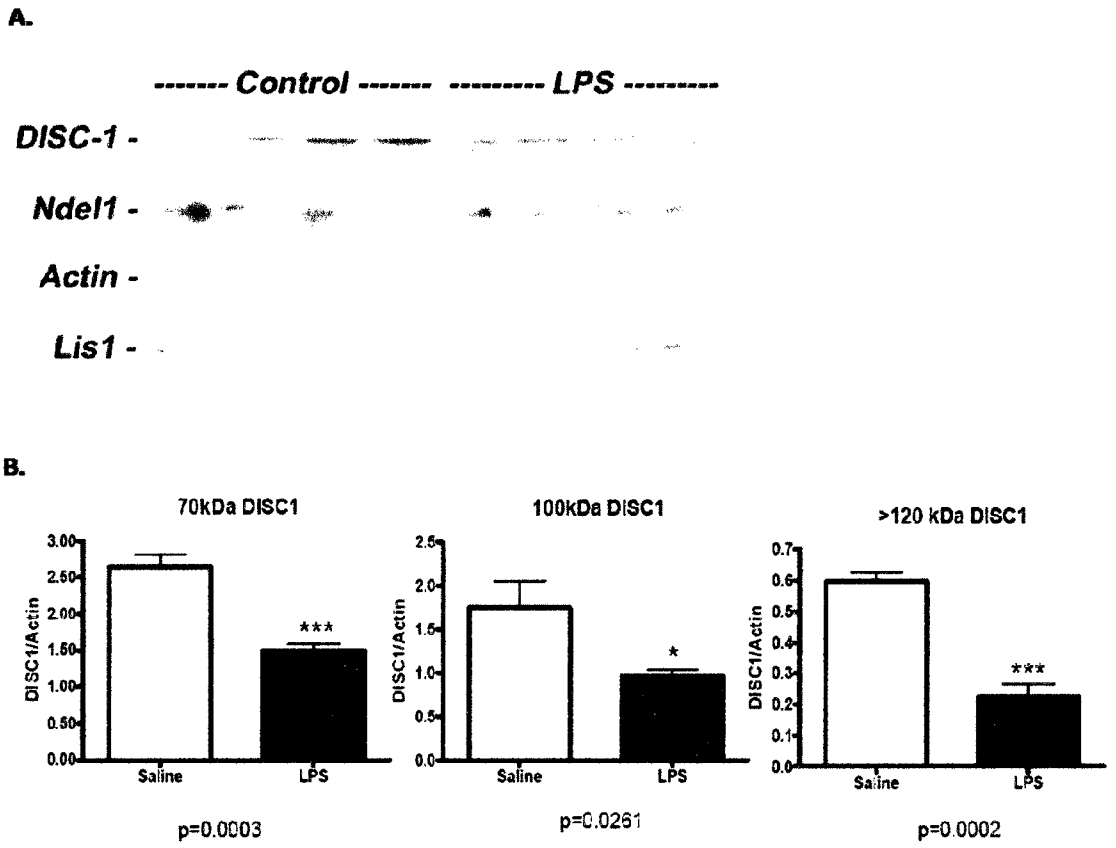

Figure 6: *Protein expression of DISC1 and its binding partners in the hippocampus of juvenile offspring of the maternal infection model.* **A.** Western blot images from PD14 hippocampal tissue from mothers exposed to saline (control) or LPS during pregnancy. DISC1, Ndel1 and LIS1 were probed and compared to Actin as a control (n=4). A significant decrease in DISC1 (100kDa protein; $p<0.05$), but not Ndel1 or LIS1 was observed. **B.** Western blot data of a new cohort of animals for replication and quantification of other important DISC1 isoforms (70kDa, 100kDa and greater than 120kDa). Hippocampal DISC1 levels were less for all detected isoforms (*$p<0.05$;***$p<0.001$) in LPS (filled bars; n=5) versus saline offspring (open bars; n=4).

Figure 7.

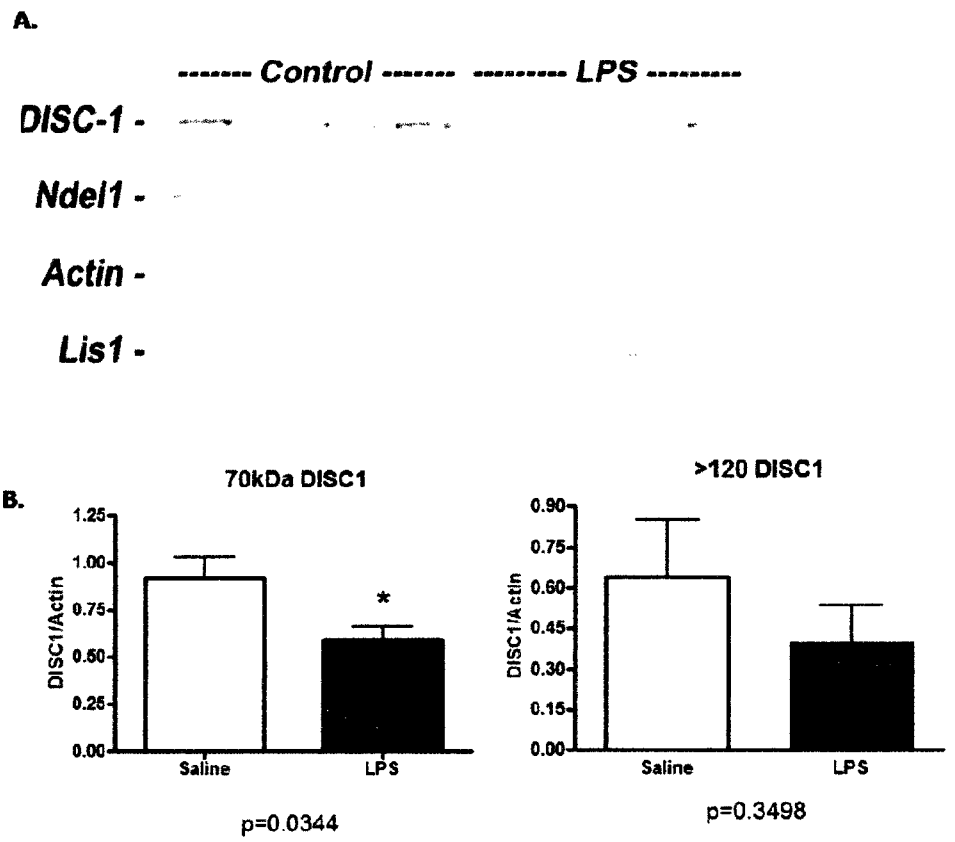

Figure 7. *Protein expression of DISC1 and its binding partners in the cortex of juvenile offspring of the maternal infection model.* **A.** Western blot images from PD14 cortical tissue from mothers exposed to saline (control) or LPS during pregnancy. DISC1, Ndel1 and LIS1 were probed and compared to Actin as a control (LPS: n=4; SAL: n=3, sample excluded due to protein degradation). A significant decrease in DISC1 (100kDa protein; $p<0.05$) but not Ndel1 or LIS1 was observed. **B.** Western blot data of a new cohort of animals for quantification of other important DISC1 isoforms (70kDa and greater than 120kDa) in the cortex. DISC1 levels for the 70kDa isoform were less (*$p<0.05$) in LPS (filled bars; n=5) versus saline offspring (open bars; n=4).

Figure 8.

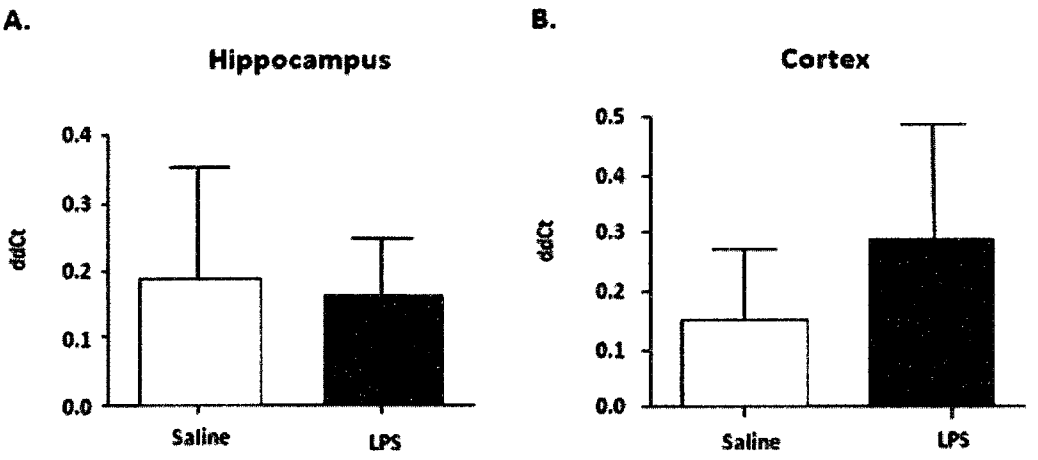

Figure 8: *Regional DISC1 mRNA analysis of juvenile pups exposed to prenatal infection.* DISC1 mRNA measurements using quantitative real-time PCR of the **A.** hippocampus and **B.** motor and sensory cortex from PD14 animals whose mothers had been exposed to saline (unfilled bars) or LPS (filled bars) during pregnancy. Data shown are the relative DISC1 levels ± SEM, determined using the delta-delta-C technique (ddCt) with Actin as the endogenous control. For both **A.** and **B.** n=5 for LPS, n=4 for saline and $p>0.05$.

Figure 9.

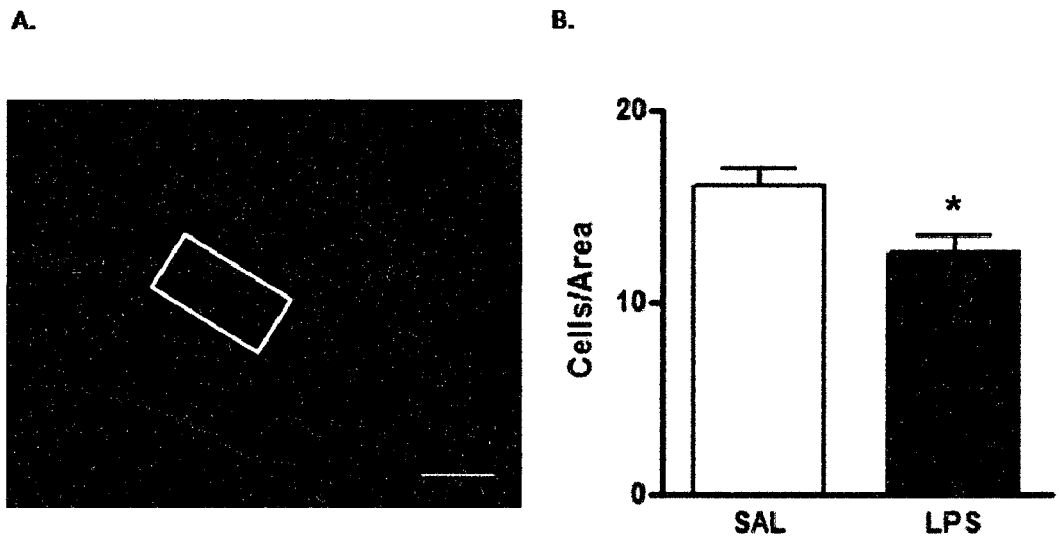

Figure 9: *Effect of maternal infection on DISC1-positive cell concentration in the dentate gyrus region of developing offspring.* **A.** Example of cell-counting region. A standardized rectangular area was designated within the polymorphic layer of the dentate gyrus (hilus) for each PD14 hippocampal slice. Scale (white line) = 200 μm. **B.** Number of DISC1-positive cells within these areas from PD14 offspring. Significantly fewer (12%) DISC1-labelled cells per area were observed in offspring from LPS (filled bar) versus saline (SAL; open bar)-treated dams. 6-8 hippocampal slices (40 μm thick) per animal were hand counted in a blind fashion, n=5 animals per group. Values shown are the mean values ± SEM. *$p<0.05$.

Figure 10.

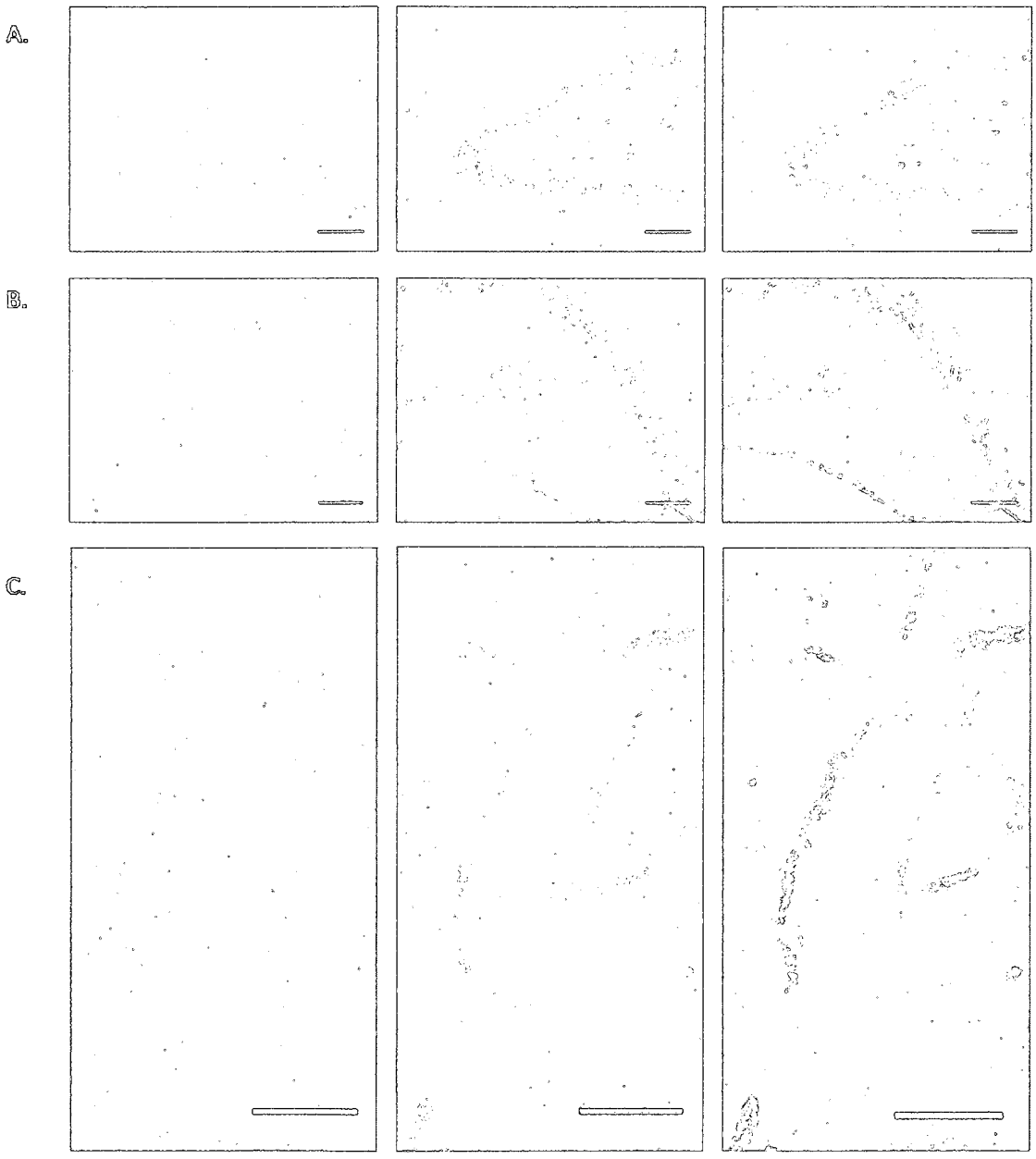

Figure 10: *Determination of DISC1-positive cell type in the hippocampus.* Double-staining was done in PD14 rat hippocampal slices with DISC1 and cellular markers. Images were taken by a fluorescent (**A.** and **B.**) or confocal (**C.**) microscope. **A.** Separate image pairs and merged images of DG sections double-stained for DISC1 (red) and the mature neuronal marker NeuN (green). DISC1 was found in mature neurons (yellow) but does not account for all DISC1-stained cells. Scale (white bars) = 100μm. **B.** Separate image pairs and merged image of a DG slice dual-stained for DISC1 (red) and the astrocyte marker GFAP (green). GFAP positive cells were not immunopositive for DISC1. Scale (white bars) = 200μm. **C.** High magnification confocal image pairs and merged image of DG slice with dual-staining for DISC1 (red) and cd11B (green), a marker of microglia. cd11B positive microglia were immunopositive for DISC1 (yellow). Scale (white bars) = 10μm. DG = dentate gyrus.

Tables

Table 1.

	Survival rate (%)	Absolute Δ T at 4 hours GD18 (°C)	Absolute Δ T at 4 hours GD19 (°C)	Weight GD18 (g)	Δ Weight from GD18-19 (g)	Litter size (# pups)	Litter male:female ratio
Saline Treatment:	100%	0.2769 ± 0.05565	0.3118 ± 0.06056	313.3 ± 3.344	10.86 ± 1.611	9.824 ± 0.8100	1.670 ± 0.4282
LPS Treatment:	88%	0.8158 ± 0.1833	0.3636 ± 0.06980	320.1 ± 4.450	-1.243 ± 2.454	8.409 ± 0.8917	1.095 ± 0.1700
Significance:		*	n.s.	n.s.	***	n.s.	n.s

Table 1: *Summary of maternal response to LPS.* Statistics of pregnant dams exposed to saline or LPS on GD18 and GD19. Survival rate refers to the percentage of dams surviving the injections. Other values shown are means ± SEM. Saline: N=17, LPS: N=23. Significance results refer to unpaired t-tests comparing LPS and Saline. n.s = $p>0.05$, *$p<0.05$, ***$p<0.001$.

Table 2.

Tissue	Isoform	Saline Mean ±SEM	LPS Mean ±SEM	t value	p value	p value summary
CX	70kDa	0.9224 ± 0.1112	0.5967 ± 0.06782	t(7)=2.619	0.0344	*
CX	>120kDa	0.6412 ± 0.2107	0.3959 ± 0.1413	t(7)=1.002	0.3498	n.s.
PFC	70kDa	0.8773 ± 0.06146	0.7033 ± 0.04760	t(7)=2.280	0.0566	n.s.
PFC	100kDa	1.130 ± 0.1618	0.9750 ± 0.05575	t(7)=0.9970	0.3520	n.s.
PFC	>120kDa	0.3481 ± 0.2169	0.1412 ± 0.03527	t(7)=1.063	0.3231	n.s.
HC	70kDa	2.646 ± 0.1613	1.493 ± 0.09305	t(7)=6.527	0.0003	***
HC	100kDa	1.749 ± 0.3026	0.9618 ± 0.07746	t(7)=2.811	0.0261	*
HC	>120kDa	0.5972 ± 0.03023	0.2252 ± 0.03928	t(7)=7.172	0.0002	***

Table 2: *DISC1 western blot results.* Values shown are the means ± SEM for the ratio of DISC1 protein level detected (70kDa, 100kDa or >120kDa isoform) to the endogenous control Actin. Data of the 100kDa isoform from the CX is not available. Statistical results from unpaired t-tests are shown. N=4 for Saline and N=5 for LPS. n.s = $p>0.05$, *$p<0.05$, ***$p<0.001$. CX = sensory and motor cortex; PFC = prefrontal cortex; HC = hippocampus.

Table 3.

Tissue	Saline Mean ± SEM	LPS Mean ± SEM	t value	p value	p value summary
CX	1.419 ± 0.05905	1.565 ± 0.1030	t(7)=1.146	0.2896	n.s.
PFC	0.8899 ± 0.03969	0.8129 ± 0.06067	t(8)=0.9981	0.3515	n.s.
HC	0.7340 ± 0.07619	0.7863 ± 0.1136	t(10)=0.3829	0.7098	n.s.

Table 3: *Ndel1 western blot results.* Values shown are the means ± SEM for the ratio of Ndel1 protein levels to the endogenous control GAPDH. Statistical results from unpaired t-tests are shown. N=4 for Saline and N=5 for LPS in CX and PFC tests. N=6 in HC test. CX = sensory and motor cortex, PFC = prefrontal cortex, HC = hippocampus.

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