Comprehensive exam grant proposal

Determining the effects of influenza infection and interleukin-6 on fetal neural stem cells, microglia, and long-term DNA methylation in immune-related neurodevelopmental risk genes.

Lay abstract

Maternal influenza infection during early pregnancy is associated with an increased risk of offspring developmental brain disorders such as autism spectrum disorder (ASD) and schizophrenia. This hypothesis is backed up by rodent research showing brain and behavioural impairments related to ASD and schizophrenia in offspring exposed to maternal influenza infection during pregnancy. Interestingly, both disorders also show abnormalities in immune system function, linking them to an immune causal agent such as maternal infection.

Although the association between maternal infection and aberrant brain development is strong, little is known about the precise changes occurring in the womb that alter the brain, and such changes will be the focus of this research proposal. Specifically, I will highlight the impact of maternal infection on two important cell populations that inhabit the fetal brain and are essential in normal brain development: Neural stem cells (NSCs), the cells that divide and give rise to newborn neurons, and microglia, the brain's resident immune cells that protect it from infection.

Using a variety of experiments performed with cells isolated from fetal brains exposed to maternal infection during pregnancy, I will attempt to uncover how NSCs and microglia are affected shortly after infection and whether these effects persist into postnatal life. Results from this study will contribute to our understanding of brain development in both normal and disease conditions and provide evidence to inform the causes, treatment and prevention of developmental brain disorders linked with maternal infection during pregnancy such as ASD and schizophrenia.

Proposal Summary

Background

Maternal influenza infection during early pregnancy is associated with an increased risk of offspring developmental brain disorders such as autism spectrum disorder (ASD) and schizophrenia. Maternal infection confers the highest risk during the first and second trimesters of pregnancy and may trigger preexisting genetic predispositions for such disorders. Preclinical rodent models have confirmed the detrimental effects of maternal infection on offspring brain and behaviour in relation to ASD and schizophrenia. Despite consistent evidence of relevant impairments, little is known about the prenatal changes that alter the brain *in utero*. Furthermore, there are currently few direct links between maternal infection and the deficits that manifest much later in postnatal life.

Aims

The main goal of this proposal is to clarify the prenatal changes that occur following maternal influenza infection in the context of two fetal brain cell populations: Neural stem cells (NSCs) and microglia. Beyond the womb, another main goal of this proposal is to discover whether prenatal changes in NSCs and microglia persist into postnatal life through alterations in their epigenetic and gene expression profiles for immune-related neurodevelopmental risk genes. The specific aims of the proposal are:

1. Determine how maternal influenza infection alters prenatal NSC proliferation and differentiation into neurons and astrocytes.
2. Explore whether maternal influenza infection hyperactivate prenatal microglia.
3. Determine how maternal influenza infections alters pre- and postnatal epigenetic markers and gene expression profiles associated with immune-related neurodevelopmental risk genes.

Methods

Throughout the proposal, a special focus is directed towards interleukin-6 (IL-6), a proinflammatory cytokine which activates downstream signaling pathways heavily implicated in NSC proliferation and differentiation. Similarly, Microglia can produce IL-6 or respond to it in conditions of inflammation or immune stimulation. Blocking IL-6 during maternal immune activation in rodents has been shown to prevent the manifestation of several offspring deficits.

This proposal will utilize well-established protocols of rodent maternal infection and *in vitro* assays for culturing NSCs and microglia. Pregnant rats will be exposed to influenza infection during a developmental stage that corresponds to the first half or pregnancy in humans. Following exposure, fetal brains will be isolated and used to establish NSC neurosphere assays or microglial cell cultures. Cells will be followed and tested in culture, including tests for NSC proliferation, NSC differentiation, microglial activation and IL-6 signaling in both cell types. Lastly, DNA methylation will be measured in both cell types shortly after exposure or in adolescent or adult offspring brains to track the long-term epigenetic and gene expression impact on immune-related neurodevelopmental risk genes.

Expected Results

I expect maternal infection to hyperactivate microglia and push NSCs towards an astrocytic cell fate, reducing the proportion of neurons in neurospheres. Some changes may be associated with long-lasting alterations in DNA methylation and associated gene expression.

Significance

This project will clarify the pathophysiological mechanisms of maternal infection in relation to NSCs and microglia. This will aid in understanding complex developmental disorders such as ASD and schizophrenia and provide avenues for developing better treatments and preventions for these devastating disorders.

Introduction

Maternal infection during pregnancy is a known risk factor for neurodevelopmental disorders in the offspring. While extreme examples such as Zika-associated microcephaly serve as stark reminders of this risk[1,2], the link between common infections and abnormal neurodevelopment has accumulated considerable support over the past few decades. Epidemiological studies have associated several bacterial and viral infections with an increased risk of schizophrenia and autism spectrum disorder (ASD) in the offspring. Influenza infection, particularly during the first and second trimesters, is associated with an elevated risk of schizophrenia and ASD in the offspring[3,4]. Whether maternal infection confers this risk on its own or in combination with other genetic or environmental risk factors, the ramifications are enormous, especially considering that pregnancy may increase susceptibility to infection[5,6]. Brown et al. have estimated that the combined effect of multiple types of maternal infection may be involved in approximately one third of schizophrenia cases[4].

The etiologies of schizophrenia and ASD remain elusive, as single gene causes only account for a small percentage of total cases[7–9]. Behaviorally, schizophrenia is characterized by delusions, hallucinations and disorganized thought[10]. In contrast, ASD is characterized by deficits in social interaction, communication, and increased repetitive behaviours. Despite their distinct behavioural symptoms, schizophrenia and ASD share many characteristics such as risk genes, comorbidities, higher male preponderance and underlying molecular phenotypes. For example, the brains of individuals with ASD and schizophrenia exhibit abnormal gray and white matter distribution, as well as an excitation-inhibition imbalance[11–13]. Such cellular and molecular changes often correlate with and may underlie behavioural impairments. Furthermore, an immune association is strongly supported by consistent evidence of impaired immune signaling in the brain and periphery of patients with schizophrenia and ASD[14–17]. Since immune signaling plays an essential role in normal brain development[18], an immune stimulus such as maternal infection during a sensitive period of fetal development can explain immune and nervous system changes in both disorders.

Over the past 2 decades, many studies have investigated the effects of maternal infection or pathogen-free immune stimulation during pregnancy on offspring brain and behaviour. Rodent and non-human primate models have shown considerable face and predictive validity in replicating schizophrenia and ASD-related phenotypes. For example, neonatal rats exposed to influenza infection on the 9th day of gestation —roughly equivalent to the first trimester of human pregnancy— exhibit changes in cortical cell density and immunoreactivity for Reelin, a neurodevelopmental glycoprotein linked to both schizophrenia and ASD[19–21]. Similarly, Short et al. exposed Rhesus monkeys to influenza infection in the third trimester of pregnancy and observed reductions in cortical gray matter volume in the offspring at 1 year of age[22]. Alternatively, the effects of maternal immune stimulation in the absence of an infectious agent has been thoroughly studied in maternal immune activation (MIA) models. MIA offspring exhibit schizophrenia and ASD-related brain and behavioural deficits that are highly dependent on the timing of MIA exposure and maybe exacerbated by other environmental or genetic risk factors[23–26]. Despite their strong validity, MIA models do not fully replicate the human condition as the immune stimulants used only activate a fraction of the normal immune response to pathogens.

The phenotypes observed in maternal infection and MIA offspring are usually attributed to the maternal immune response elicited against infection. Proinflammatory cytokines produced by the mother's immune system such as Interleukin-6 (IL-6) are thought to disrupt fetal brain maturation. Blocking IL-6 during the maternal immune response prevents some of the offspring's behavioural and molecular phenotypes[27,28]. This is unsurprising, given the role of IL-6 signaling in neurodevelopment, particularly in the proliferation and differentiation of neural stem cells (NSCs)[18]. Additionally, proinflammatory cytokines can activate microglia, the brain's resident immune cells, to disturb immune signaling in the brain, which can indirectly impact NSCs and newborn cells[29]. Microglia also have direct roles in neurodevelopment, including phagocytosis of apoptotic neurons and pruning of synaptic connections at later stages of development[30,31]. Taken together, alterations in prenatal NSCs and microglia may underlie the gray/white matter and neuroinflammation alterations seen in individuals with ASD and schizophrenia.

This proposal aims to uncover the prenatal mechanisms underlying maternal infection's effects on the fetal brain. Much focus has been directed towards replicating postnatal deficits, and little towards understanding the prenatal changes that directly influence neurodevelopment. In particular, very few studies have investigated prenatal microglia and NSCs following exposure to maternal infection, making it difficult to directly link prenatal changes with postnatal phenotypes. I plan to thoroughly characterize these two key cell populations *in vitro,* provide a direct link to postnatal phenotypes through the cells' epigenetic profile. Moreover, I aim to shine the spotlight IL-6 signaling, which is heavily involved in both the immune response to infection and in normal fetal brain development. This IL-6 focus will provide a specific target for developing biomarkers, treatments and preventions for schizophrenia and ASD.

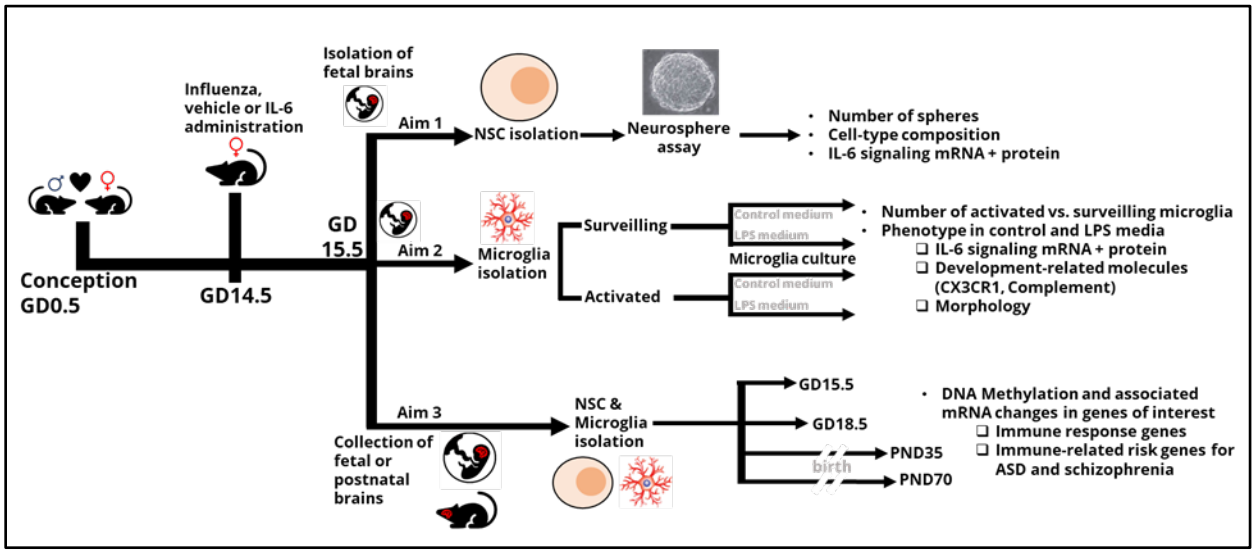

Figure 1. Proposal outline. Pregnant rats will be exposed to maternal infection or administered a dose of IL-6 on Gestation Day (GD) 14.5. In aims 1 and 2, Fetal or offspring brains will be harvested, and NSCs and microglia will be isolated on GD15.5 for culture and analysis. In aim 3, fetal, adolescent and adult brains will be harvested, and DNA methylation will be measured in NSCs and microglia. Refer to text for rationale, details on experimental design, expected results and alternative approaches.

Aim 1: Determine how maternal influenza infection alters prenatal Neural Stem Cell proliferation and differentiation.

Background and Rationale: NSCs are specialized stem cells capable of proliferating or differentiating into neurons, astrocytes or oligodendrocytes[32,33](Figure 2[34]). Neurons and oligodendrocytes make up the bulk of the brain's gray and white matter, respectively, and their proper distribution and function underlies the brain's immense processing capabilities. In schizophrenia, there are consistent reports of reductions in the brain's gray matter which are correlated with worse behavioural outcomes and response to medication[13,35]. On the other hand, individuals with ASD exhibit larger gray and white matter than typically developing individuals, although these differences become less pronounced and potentially reverse direction with age[11]. Disrupting NSC proliferation and differentiation at distinct timepoints during development may alter the number of proliferating NSCs, neurons, astrocytes and oligodendrocytes, leading to long-term changes in gray and white matter seen in ASD and schizophrenia.

Cytokine signaling has been shown to play an important role in NSC proliferation and differentiation, but not within the context of maternal infection. For example, IL-6 family cytokines, which signal through a common receptor subunit and downstream signaling, are expressed during embryonic development[36–39]. Leukemia Inhibitory Factor (LIF) and Ciliary Neurotrophic Factor (CNTF) are both members of the IL-6 family and have the ability to increase NSC proliferation in culture[38,40,41]. However, these effects are dependent on developmental stage, as IL-6, LIF and CNTF also promote premature astrocyte differentiation in cell cultures derived from mouse embryos during late but not early gestation[29,42–44]. Similarly, the proinflammatory cytokine IL-1 has shown potential as an astrocyte growth factor, highlighting the importance of accounting for all aspects of the maternal immune response[45].

While cytokine signaling is clearly implicated in neurodevelopment, it is unknown whether the cytokine response to maternal influenza infection during pregnancy can have similar effects on NSCs. Direct stimulation with a few cytokines or innate immune stimulants has been attempted, but it is too simplistic to model a complex response involving various organs, tissues and cell types. A typical viral immune response involves the production of multiple different immune factors besides IL-1 and IL-6, some of which are anti-inflammatory in nature like IL-10. Additionally, the placenta may act as a barrier and regulator of the maternal immune response before it reaches fetal tissue[46,47]. Finally, the maturation of the fetus's immune system and the fetal blood brain barrier can dictate how much of the immune

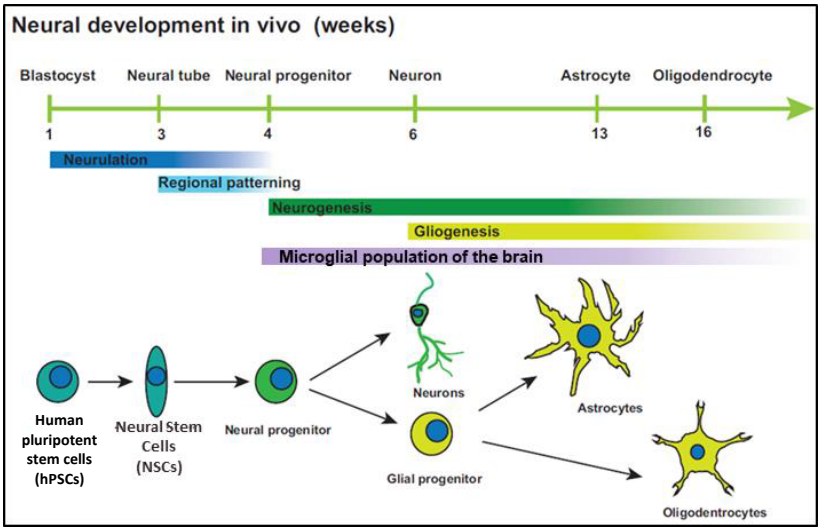

Figure 2. *In vivo* timeline for the differentiation of NSCs and microglial population of the CNS, adapted from Tao and Zhang: Neural Subtype Specification from Human Pluripotent Stem Cells[33]

response reaches the fetal brain. Overall, previous maternal infection and MIA research has given little attention to NSCs, except in cases where adult neurogenesis was investigated[48–51].

Hypothesis: Maternal infection alters the proliferation and differentiation characteristics of fetal NSCs. **The goal of aim 1** is to contextualize cytokine involvement with NSC function in a maternal influenza infection model. Combining well-established procedures of maternal infection and NSC cell cultures may help uncover etiological mechanisms behind the gray and white matter abnormalities seen in ASD and schizophrenia.

Experimental Design: Wild-type Sprague Dawley female rats will be mated with wild-type males, and the day following successful copulation will be designated as *Gestation Day (GD)0.5*. Two weeks into gestation, on GD14.5, the dams will be administered an intranasal dose of Influenza A/NWS/33 (H1N1) or vehicle (n=5 per group) under anesthesia. The precise dose that produces a robust immune response without influencing maternal or fetal viability will be determined using pilot experiments using pregnant rats as previously described[20]. Following inoculation, the rats' immune response will be monitored and measured through body temperature, weight gain, home cage behaviour and serum cytokine response[52].

GD14.5 was chosen for several reasons. Firstly, it corresponds to a developmental equivalent of first-second trimester in humans, the period for which epidemiological associations show the most consistent evidence[3,4]. Secondly, this timepoint falls within the peak of fetal neurogenesis (Figure 2), when NSC numbers and proliferation are highest[53]. Methodologically, fetuses are larger at this time, allowing for easier isolation of fetal brains. Lastly, numerous studies have thoroughly characterized the effects of immune activation at GD14.5 and have consistently shown ASD and schizophrenia-related deficits in the offspring[54–57].

On GD15.5, fetuses will be extracted (n=9 per dam, if possible). Three brains will be used to measure the level of proinflammatory cytokines and test for the presence of influenza virus. Previous studies have been inconsistent in determining whether influenza virus can reach the fetal brain, which has major implications for the interpretation of experimental results[58,59]. The remaining brains will be used to establish neurosphere assays[33,60], following sex determination using an established procedure[61]. Neurosphere assays —NSCs form spheres when grown in suspension— will be developed from a maximum of 3 fetuses per sex per pregnancy to avoid litter effects and explore sex specificity, which is present in normal brain development and in both schizophrenia and ASD[62–64]. In all aims, tests will be done on cells collected from whole brains, without focusing on any specific subregions. Most brain structures are not fully developed at GD14.5 and it is expected that cells across the entire brain are disrupted by the exposure.

For the neurosphere assay, whole brains will be homogenized, homogenates will be dissociated and cells will be grown in suspension, as described previously, for 5-7 days until the neurospheres are observed[33,60]. Cells can be passaged every 7 days and used for further investigations, although all experiments described here will be performed using primary cultures. The number of neurospheres and their size will be measured as an estimate of NSC proliferation capacity. Moreover, the cell composition of these spheres will be determined using immunostaining for cell markers specific for NSCs (Nestin), neurons (β-tubulin), astrocytes (Glial Fibrillary Acidic Protein; GFAP) and oligodendrocytes (Myelin Basic Protein; MBP). Cell composition will be measured in baseline culture conditions and in differentiating culture conditions whereby NSC differentiation is driven by the absence of proliferative factors or the addition of differentiating factors[34,60]. Using western blots and quantitative reverse-transcriptase

polymerase chain reaction (qRT-PCR), the mRNA and protein levels of IL-6 downstream signaling molecules Signal Transducer and Activator of Transcription 3 (STAT3), Suppressor Of Cytokine Signaling 3 (SOCS3), Protein Kinase B (AKT), and extracellular signal-related kinase (ERK) will be measured (Figure 3[65], red stars). A separate group of dams will be injected with IL-6 recombinant protein on GD14.5 and tested in a similar manner. IL-6 has previously been shown to cross the rat placenta into fetal tissue[66]. This group will model peripheral cytokine elevations and identify the effects of IL-6 alone and how they compare to influenza infection's effects.

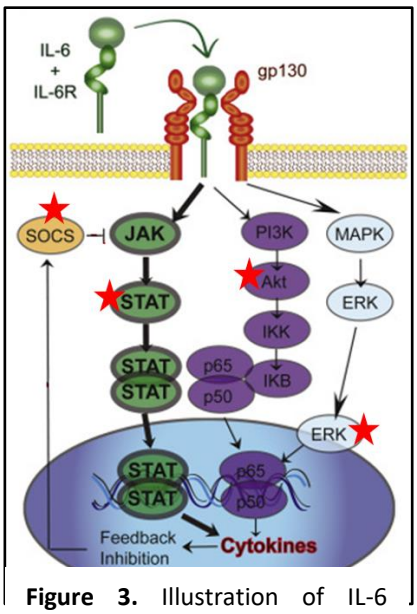

Figure 3. Illustration of IL-6 signaling, highlighting in red stars the molecules used as surrogates for activation of IL-6 signaling in NSCs and microglia. Adapted from Hodes et. al: Integrating Interleukin-6 into depression diagnosis and treatment[64]

Expected Results: I expect to find more neurospheres in infection-exposed NSC cultures and that these spheres will be bigger in size, indicative of increased proliferation, would be expected by the role of IL-6 signaling in NSC proliferation[38]. Additionally, I expect that neurospheres from infection-exposed offspring brains to exhibit a change in cell-type composition shown by an increase in glial cells (astrocytes and oligodendrocytes) and a decrease in neurons. I also expect to find increased gene expression and protein levels of downstream IL-6 signaling molecules. However, I do not expect maternal IL-6 injections to exactly replicate the effects of influenza infection, given the involvement of other immune response factors that may amplify or attenuate the effects of IL-6, in addition to having individual effects of their own. By contrasting results between IL-6 and influenza groups, more specific investigations could be planned, but specific predictions cannot be made at this point. Cytokines or cytokine inhibitors may be added to neurosphere assays in the same passage or in different passages to elucidate relevant mechanisms. Sex-specific effects for this aim and other aims are difficult to propose and are dependent on the length of the immune response, since fetal testosterone production peaks around GD18 and its concentration may be too low to have an impact prior to that stage[64].

Significance: Results from aim 1 will provide a direct association between maternal influenza infection and NSC function during prenatal development. These results will inform the hypothesis that changes in early NSC function are related to gray and white matter abnormalities seen in ASD and schizophrenia. Focusing on IL-6 signaling will provide a more mechanistic understanding of these effects, informing future investigations or development of treatments and interventions.

Alternative approaches: The neurosphere assay allows for NSCs to be easily manipulated through precise control of the culture medium, providing a more thorough characterization of the effects of maternal infection on NSCs. It also allows for monitoring of NSC activity over a long period of time to see whether changes from an acute environmental insult *in utero* persist in the same cells after one or two weeks in culture. However, if the neurosphere assay could not be performed, measuring proliferation and differentiation can still be done directly in the isolated fetal brains. In that case, the readout would provide a snapshot into the number of NSCs, neurons, astrocytes and oligodendrocytes present at the time of sacrifice. This can be done by

immunostaining for the same proposed cell-specific markers to measure cell-type composition and by performing 5-bromo-2'-deoxyuridine (BrdU) staining to measure proliferation.

Aim 2: Explore whether maternal influenza infection hyperactivates prenatal microglia.

Background and Rationale: Inflammation of the brain and periphery is consistently observed in both schizophrenia and ASD, further implicating maternal infection. For example, individuals with autism or schizophrenia show increased microglial activation in the prefrontal cortex[15,67,68]. Beyond the brain, patients with either disorder exhibit an inflammatory profile as measured by circulating cytokines[14,69] and, in some cases, cytokine levels are associated with worse behavioural outcomes[14,16]. Since maternal infection influences the whole fetus and not just the brain, it may explain both central and peripheral immune changes seen in ASD and schizophrenia.

Out of all the immunological evidence, microglial activation must be emphasized, given their role in neurodevelopment and their ability to respond to immune signaling produced by maternal infection[70,71]. Although microglia originate outside the brain and migrate into the brain during early gestation[72], they are distinct from their peripheral macrophage counterparts, exhibiting self-renewal within the brain and a distinct gene expression profile[73,74]. Their role in both early and late neurodevelopment has gained much attention in recent years[75]. In prenatal development, microglia have been shown to regulate the number of neural precursors through phagocytosis, influence neural precursor cell fate through cytokine secretion, and promote white matter development[76–78]. In postnatal development, microglia are major players in synaptic pruning, a process by which unnecessary neuronal connections are removed to refine the brain's circuitry[30]. Microglia can also exert indirect effects by their contribution to the central nervous system environment. Indeed, microglia's cytokine secretions in response to immune stimulation are in prime position to affect NSCs, neurons, astrocytes and oligodendrocytes[29].

One hypothesis concerning maternal infection's neurodevelopmental effects posits that maternal infection primes microglia to take on an activated state throughout the individual's lifetime. As a result, microglia's developmental functions are chronically altered, causing them to phagocytose more cells or prune more synapses than they normally would. This also supports the multiple-hit hypothesis of neurodevelopmental disorders in which an early insult leaves the brain more susceptible to future insults that eventually culminate into the full disorder.

Similar to human studies on individuals with schizophrenia and ASD, findings from MIA studies support the notion that microglia are activated following maternal exposure to an immune stimulus[79–84]. However, most of these studies have focused on adult offspring and little focus have been given to how microglia change prenatally or in early postnatal life. Therefore, the question remains whether changes in microglia are present during prenatal life and early postnatal life during which their developmental roles are most striking.

Hypothesis: Maternal infection hyperactivates prenatal microglia. **The goal of aim 2** is to thoroughly characterize prenatal microglia following exposure to maternal influenza infection**.** This aim will use isolation procedures that distinguish microglia based on their activation state. Microglia will then be cultured and tracked *in vitro*. Various tests will be performed to probe microglia's gene/protein expression, particularly in the context of IL-6 signaling, at baseline conditions and in response to further immune stimulation.

Experimental Design: Timed-breeding and maternal infection will be performed as in Aim 1, with a similar number of dams per group and cultures established per sex per litter per pregnancy. On GD15.5, fetal brains will be extracted. Some brains will be used to measure the

level of proinflammatory cytokines and test for the presence of influenza virus as in aim 1, while the remaining brains will be used to isolate microglia and establish microglial cultures. The latter will be performed according to previously outlined protocols using density gradient separation of brain tissue followed by fluorescent activated cell sorting (FACS) for microglial markers CD11b and CD45[85]. Based on the intensity of CD11b and CD45 staining (CD11b+CD45^{low} or CD11b+CD45$^{medium\text{-}high}$), microglia from each fetal brain will be separated into 'activated' and 'surveilling' microglia prior to culture. Each population will be cultured as previously described[85]. Using western blots and qRT-PCR, mRNA and protein expression will be measured for IL-6 protein, IL-6 downstream signaling molecules mentioned in aim 1, as well as molecules involved in microglial phagocytosis and synaptic pruning such as fractalkine receptor CX3CR1 and complement proteins[30,86]. To test the priming of microglia and whether activation persists several days following exposure to maternal infection, these measures will be performed at baseline and in response to administration of the bacterial endotoxin lipopolysaccharide (LPS) 3 and 7 days in culture. Microglia will also be immunostained with Ionized calcium binding protein 1 (Iba1) to visualize and measure different aspects of their morphology such as overall shape and number of processes[87]. As in aim 1, a separate group of dams and their corresponding fetuses will undergo similar testing but following injection to IL-6 instead of exposure to maternal infection.

Expected Results: I expect brains exposed to maternal infection to contain a higher number of activated microglia when separated by FACS. Moreover, these microglia should exhibit elevated IL-6 signaling, increased expression of CXC3CR1 and complement proteins and an elevated response to immune stimulation by LPS. Based on findings from MIA studies, the priming effect should persist throughout the cells' time in culture and still be detected 7 days in vitro. I also expect microglia from brains exposed to maternal infection to exhibit increased activated morphology as indicated by an amoeboid shape and reduced number of processes extending from their cell body. Similar to aim 1, it is expected that IL-6 maternal immune activation produces different effects on prenatal microglia compared to maternal infection, although a precise prediction cannot be made due to the complexity of the response to influenza.

Significance: Results from aim 2 will provide information about the prenatal activation state of microglia following maternal infection. By measuring molecules specifically related to microglia's developmental roles, changes can be directly linked to their role in neurodevelopment. Expression levels of IL-6 can also be related to neurodevelopment, given its role in dictating NSC proliferation and differentiation. Furthermore, measuring the response to further immune stimulation at various times in culture will provide more information on microglial priming and the potential for double-hit interactions with immune-related risk factors.

Alternative approaches: Growing microglia in culture allows for investigation over time and direct testing of response to further immune stimulation without a changing developmental environment. A disadvantage of microglial cultures is that the culture conditions may themselves activate microglia, making it more difficult to detect changes in activation-related markers. In that case, immunostaining can be performed *in vivo* following isolation and fixation of fetal brains, whereas gene and protein expression analysis can be performed directly following FACS.

Aim 3: Determine how maternal influenza infections alters pre- and postnatal epigenetic markers and gene expression profiles associated with immune-related neurodevelopmental risk genes.

Background and Rationale: Despite the strong face validity of maternal infection models in recapitulating postnatal phenotypes related to schizophrenia and ASD, few direct causal links have been made with changes occurring shortly after infection. For instance, it is widely accepted that IL-6 is an important component of the immune response that disrupts neurodevelopment[27,28]. However, IL-6 signaling is rarely investigated in offspring exposed to maternal infection. Another important consideration is how long infection's effects persist and whether they disrupt developmental processes acutely or throughout an individual's lifetime.

Early life environmental disruptions can exert long-term effects by altering the epigenetic profile of affected cells. Epigenetics is the study of gene expression changes that are not caused by changes in DNA sequence. Briefly, changes in the packing of DNA into chromatin or the addition of methyl groups to certain DNA sequences influences how easily a stretch of DNA can be accessed by transcriptional machinery[88]. Epigenetic changes can persist for a lifetime and can be considered drivers of environmental adaptation[89,90]. Importantly, epigenetics provide a link between genes and the environment, with a prime example being cancer tumor suppressor genes, whereby epigenetic changes lead to the same outcome as a loss of function mutation[91]. In the context of maternal infection, epigenetic changes that persist for a long time and may tip genetic predispositions 'over the edge' to lead to the manifestation of a disorder.

Changes in DNA methylation have been reported in both ASD and schizophrenia[92,93]. Previous maternal infection and immune activation studies have shown changes in the gene expression and profiles of exposed adolescent and adult offspring brains[28,94–101]. Indeed, other studies have shown alterations in DNA methylation and histone acetylation in offspring exposed to maternal immune activation[102–106]. However, little attention has been directed towards linking prenatal and postnatal epigenetic profiles, or with regards to localization of epigenetic changes to DNA sequences involved in immune signaling or immune-related risk genes.

Hypothesis: Maternal infection changes the epigenetic DNA methylation profile of fetal and postnatal NSCs and microglia. **The goal of aim 3** is to study NSC and microglial DNA methylation changes in specific promoter regions of genes involved in the immune response to influenza or immune-related genes associated with an increased risk of schizophrenia or ASD at various stages of development.

Experimental Design: Timed-breeding and maternal infection will be performed as in Aims 1&2. On GD15.5, GD18.5, Postnatal day (PND) 35 and PND 70, offspring brains will be collected and NSCs and microglia will be isolated using FACS. NSCs will be isolated using the NSC marker Nestin, whereas microglia will be isolated using the same markers described in Aim 2. Cell extracts will be split into two batches. The first batch will undergo bisulfite sequencing to measure DNA methylation in promoter regions of genes of interest listed in **Table 1**[88]. The second batch will be used to measure mRNA expression of all the genes of interest using qRT-PCR. Gene expression data will be compared with DNA methylation results to determine whether a correlation exists between the extent of methylation and the change in gene expression. Some adolescent and adult offspring will also undergo behavioural testing for previously described ASD and schizophrenia-related phenotypes[54–57].

Expected Results: I expect to find decreased DNA methylation, which typically indicates increased gene expression, in promoter regions of genes involved in inflammation and immune function such as IL-6 downstream signaling molecules. In promoter regions of ASD and schizophrenia risk genes, I expect that DNA methylation will change according to the change of function found in these genetic associations. For example, I expect to find increased DNA methylation, which typically indicates decreased gene expression, in the complement 4 (C4) promoter region as the C4 allele associated with autism is a loss of function allele. I expect gene expression data to reflect the change in DNA methylation, although a lack of association may still be possible given the role of other epigenetic changes like histone acetylation. Finally, I expect some differences in DNA methylation and gene expression to persist into adolescence (PND35) and adulthood (PND70). This should be particularly noticeable in microglial immune-related genes such as IL-6 and IL-1, given findings of activated microglia in MIA offspring[79–84] and their brain's susceptibility to early life immune-related challenges such as adolescent stress[23,24].

Genes	Role, Disorder(s) implicated and References
IL6ST, STAT3, SOCS3, AKT, MAPK1	IL-6 signaling cascade[65]
RELA, NLRC5, PRKCB	Immune response regulation, ASD & Schizophrenia[107–109]
IL-2, IL-3, IL-4, IL-6 IL-10, IFNR	Response to infection, Schizophrenia[110–114]
HLA-DR(4, B1), HLA-A2, C1q, C3, C4	Major Histocompatibility Complex (MHC), ASD & Schizophrenia[113,115–124]
IL1R2, IL1RAPL1	Cytokine receptors, ASD[125–127]

Table 1. Potential targets for DNA methylation investigation in aim 3 including IL-6 signaling proteins and immune-related ASD and schizophrenia risk genes

Significance: Results from aim 3 can provide a direct link between maternal infection exposure and postnatal outcomes. By investigating changes at various stages of development, these results will clarify whether one must consider acute changes occurring shortly after infection or chronic changes occurring for multiple days, weeks or months following exposure in maternal infection models. Additionally, by linking these effects with schizophrenia and ASD risk genes, these results will group disparate risk factors under similar etiologies and may identify patient subpopulations that are more vulnerable to maternal infection, the effects of which may be exacerbated by relevant genetic predispositions.

Alternative approaches: DNA methylation was initially chosen as the epigenetic marker of interest since it provides high resolution and allows the investigation of relatively short sequences in promoter regions of interest. Alternatively, the isolated cells can be probed for changes in histone acetylation, another epigenetic marker that is closely linked with regulating gene expression. Despite having lower resolution, these changes can be interpreted in a similar way as with DNA methylation and linked to gene expression data.

Overall Significance and Implications: The combined results of this study will increase the mechanistic understanding of the detrimental effects of maternal infection during pregnancy on brain development. Investigating prenatal NSCs and microglia may uncover common causal mechanisms for widespread gray/white matter abnormalities and neuroinflammation found in ASD and schizophrenia. The focus on IL-6, on the other hand, may provide a tangible target for developing novel, immune-centered therapeutics.

Both ASD and schizophrenia remain largely idiopathic, since environmental risk factors are not predictive of the disorder while single gene causes only contribute to a small proportion of cases. By investigating the effects of an environmental risk factor on immune-related neurodevelopmental risk genes and their expression in two important cell types, results from this study can be used to develop hypotheses that combine both genetic and risk factors, which may be better suited to explain the occurrence of ASD and schizophrenia.

Since maternal infection is an early-life insult, it is difficult to decipher precisely which developmental stages are most impacted. By investigating epigenetic change *in utero* and postnatally, results from the study will more thoroughly determine if the effects of maternal infection are driven by acute changes occurring shortly after exposure or by chronic changes that persist into later prenatal development, adolescence and adulthood.

Feasibility: Infection and cell culture protocols are all well-established in many previous studies, with extensive detail about the impact of different variables on the experimental outcomes. Expertise and techniques are available within the lab for efficiently carrying out multiple simultaneous timed-breeding experiments, for administering biohazardous treatments such as influenza and for collecting fetal tissue following infection. Based on previous studies, experimental outcomes (e.g cell number, gene expression, morphological changes) can be predetermined and used to develop a priori hypotheses and power calculations. In case of methodological difficulties, less technical alternative approaches *in vivo* can help answer the same questions, albeit less comprehensively, since they would provide a snapshot of the brain without the ability to closely alter the environment in which NSCs and microglia operate.

Future Directions: Results from this project will strengthen the mechanistic understanding of influenza infection's detrimental effects on brain development, which can be easily adapted in other experimental settings. For example, the same experimental design may be replicated with a different infection such as cytomegalovirus. Just like influenza, cytomegalovirus infection during pregnancy is associated with offspring brain disorders. In the field of maternal infection, inter-pathogen differences have long been understudied in favor of the maternal immune activation model, which uses a handful of innate immune stimulants that may not be representative of the variety of viral and bacterial pathogens that infect humans. By studying multiple infection models, I can better address the role of pathogen-specific effects compared to immune response-specific effects.

This project also shares some similarities with human iPSC models of brain disorders using patient-derived cells. Mechanistic understanding of infection's effects on brain development can inform iPSC models on relevant signaling pathways (e.g IL-6) to incorporate into their research questions regarding autism and schizophrenia.

Direct links with known immune and developmental risk genes targeted in aim 3 have great potential for gene-environment interaction studies. The information can be used to develop double-hit rodent models with high construct validity. For instance, the effects of maternal influenza infection can be studied in animals with a heterozygous mutation in a complement protein necessary for microglial function to see if a mild genetic predisposition can be exacerbated by an environmental stimulus such as maternal infection. A similar approach can be used in iPSC models to study the characteristics of cells derived from patient subpopulations harboring risk genes of interest, providing highly direct evidence for a gene-environment interaction.

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