DEVELOPMENT OF ANIMAL MODELS TO
STUDY EFFECTS OF MATERNAL INFECTION
DURING PREGNANCY ON OFFSPRING
BEHAVIOR

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Abstract

Maternal infections during pregnancy are thought to be an important environmental risk factor for psychiatric illnesses of neurodevelopmental origin such as schizophrenia and autism. It has been suggested that the mother’s immune response may be affecting the offspring’s neurodevelopment and later behavior. In this thesis, we aimed to develop animal models to study the effects of maternal immune activation (MIA) on offspring behavior. In a first experiment, pregnant rats were injected with bacterial endotoxin (lipopolysaccharide, LPS) on gestational day (E) 18 and 19. The exposed offspring displayed increased amphetamine-induced locomotion, a behavioral indicator of mesolimbic dopamine activity. In a second experiment, LPS was administered in a chronic manner by using osmotic pumps from E18 to birth. Adult offspring displayed decreased prepulse inhibition (PPI), indicating disrupted sensorimotor gating. Prior to studying the effects of MIA elicited by the viral mimic polyinosinic:polycytidylic acid (poly I:C), we characterized the immune reaction it triggered in adult male rats. We demonstrated that poly I:C injection induced a robust febrile response, decreased food intake and body weight, and increased pro-inflammatory cytokines in the circulation. In another study, we compared the disruptive effects of MIA induced by the immunogens poly I:C or LPS to that induced by local inflammation, elicited by intramuscular injection of turpentine. In the same study, we also investigated the existence of gestational windows of susceptibility to the disruptive effects of MIA on offspring PPI. MIA induced by LPS or turpentine significantly decreased offspring PPI only when administered at certain times during gestation. Poly I:C had no effect at the dose administered. Thus our results demonstrated that selection of the immune agent and gestational time of administration are both critical factors in MIA models. Finally, we investigated if gestational MIA affected offspring immune function. Our results indicated no change in pyrogenic and cytokine responses to an immune challenge in adult MIA-exposed rats.
In conclusion, the work in this thesis provides evidence that gestational MIA can cause lasting alterations in offspring behavior. This lends support to the idea that the association between prenatal infection and increased schizophrenia in human epidemiological studies reflects a causal relationship.
Résumé

On considère les infections maternelles durant la grossesse comme étant un facteur de risque environnemental important des maladies psychiatriques d'origine neurodéveloppementale telles la schizophrénie et l'autisme. On a attribué de telles altérations sur le développement neurologique de la progéniture à la réponse immunitaire de la mère. Dans ce projet de thèse, nous envisagions de créer un modèle animal nous permettant d'observer les effets de l’activation de l’immunitaire maternelle (AIM) sur le comportement de la progéniture. Au cours d'une première expérience, les rates étaient injectées d'une endotoxine bactérienne (lipopolysaccharide, LPS) aux jours (E) 18 et 19 de la gestation. La progéniture soumise à ce traitement exhibait une élévation dans la locomotion liée aux amphétamines, un indicateur comportemental de l'activité du système dopaminergique mésolimbique. La deuxième expérience consistait à administrer la LPS de manière chronique à l'aide de pompes osmotiques, depuis E18 jusqu'à la naissance. À l'âge adulte, la progéniture présentait une baisse de l'inhibition du réflexe de sursaut acoustique (PPI), indiquant un dérèglement du filtrage sensoriomoteur. Préalablement à l'étude des effets de l’AIM provoquée par la mimique virale acide polynosinique:polycytidylique (poly I:C), nous avons analysé la réponse immunitaire qu'elle provoquait chez le rat adulte mâle. Nous avons démontré que l'injection du poly I:C entraînait une réponse fébrile considérable, une diminution de l'appétit et du poids des animaux et une élévation du taux de cytokines pro-inflammatoires dans le sang. Au cours d'une autre étude, nous avons comparé les effets perturbateurs de l’AIM causée par les immunogènes poly I:C ou LPS, et celle causée par l'inflammation locale par injection intramusculaire de térébenthine. Dans cette même étude, nous avons analysé la présence de périodes de susceptibility aux conséquences de l’AIM durant la gestation sur le PPI de la progéniture. L’AIM provoquée par l'injection de LPS ou de térébenthine diminuait de façon significative le PPI uniquement lorsqu'elle administrée à des moments précis durant la gestation. Le poly I:C n'a eu aucun effet à la dose administrée. Ainsi, nos résultats démontrent que l'agent immunitaire
sélectionné et le moment durant la gestation où il sera utilisé sont des facteurs cruciaux dans la création de modèles d’AIM. Finalement, nous avons examiné la relation entre l’AIM durant la gestation et la fonction immunitaire de la progéniture. Nos résultats n'indiquent aucune altération de la réponse pyrogénique ou celle des cytokines suite à l’injection d’un immunogène chez les rats adultes exposés à une AIM in utero.

En conclusion, les travaux effectués durant ce projet de thèse démontrent que l’AIM durant la gestation peut causer des modifications permanentes sur le comportement de la progéniture. Ce constat vient supporter la notion selon laquelle le lien établi entre l'infection prénatale et le risque élevé de schizophrénie relève d'une relation causale.
Contribution of the authors on co-authored papers

Three manuscripts will be presented in this dissertation, as a partial fulfillment of my PhD degree in the Neurological Sciences Program at the Department of Neurology and Neurosurgery of McGill University. I am first author for each of the three manuscripts. In the first manuscript entitled "Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring", I collected the data, performed statistical analyses and wrote the methods and results sections. The introduction and discussion were written by Dr. Patricia Boksa. In the second manuscript entitled "The viral mimic, polyinosinic:polycytidylic acid, induces fever in rats via an interleukin-1-dependent mechanism", I conducted a literature review, collected some of the data, performed statistical analyses and wrote the first draft. The dose-response and the body weight/food intake experiments were performed by Dr Stephen Kent, and some ELISAs and the PCR were performed by Dr Helen Ashdown, who were both of great help. Dr Stephen Poole kindly provided us with the IL-1ra antiserum and the different recombinant antibodies for the ELISAs. In the last publication "Effects of prenatal infection on prepulse inhibition in the rat depend on the nature of the infectious agent and the stage of pregnancy", I conducted a literature review, collected the data, performed statistical analyses and wrote the manuscript. Drs. Patricia Boksa and Giamal Luheshi closely supervised me at each level of the studies, and particularly made the appropriate revisions during the writing of the manuscripts.
Abbreviations

ANOVA: Analysis Of Variance
CD-14: cluster of differentiation 14
COMT: catecholamine-O-methyl transferase
COX: CycloOxygenase
DISC1: disrupted in schizophrenia-1
DTNBP1: dysbindin
E: embryonic day
GFAP: glial fibrillary acidic protein
HPA: Hypothalamic-Pituitary-Adrenal
IL: InterLeukin
IL-1ra: InterLeukin-1 receptor antagonist
Im: Intramuscular
Ip: IntraPeritoneal
Iv: IntraVenous
LBP: LPS binding protein
LI: latent inhibition
LIF: leukemia inhibitory factor
LPS: lipopolysaccharide
MBP: myelin basic protein
MIA: maternal immune activation
Nacc: nucleus accumbens
NFκB: Nuclear Factor κ B
nNOS: neuronal nitric oxide synthase
NRG1: neuregulin-1
PET: positron emission tomography
PGE2: prostaglandins
poly I:C: polyinosinic: polycytidylic acid
PPI: prepulse inhibition
SCZ: schizophrenia
SEM: Standard Error of the Mean
SNAP-25: synaptosome-associated protein 25 kDa;
TLR: Toll-Like Receptor
TNF: Tumor Necrosis Factor
USPEE: unconditioned stimulus pre-exposure effect
VTA: ventral tegmental area
Preamble

Schizophrenia (SCZ) is a severe mental disorder that carries a high cost to the individual, his family, and society as a whole. Even after decades of research, the underlying causes of this devastating illness are far from understood. Family and adoption studies have demonstrated that there is a strong genetic component to SCZ, but they also show that genetics is not the sole determinant of the illness; environmental factors also play a role in the origin of the disease. Epidemiologists have identified a number of environmental factors that are associated with increased risk of schizophrenia. One of the better documented is the association between maternal infection during pregnancy and increased risk of schizophrenia in the offspring. However, epidemiological studies being purely associative in nature, they cannot be used to establish the presence of a causal relationship underlying this association. Animal models constitute a logical strategy to evaluate the plausibility of this relationship, as well as to investigate the mechanisms involved. Although it is obviously impossible to mimic schizophrenia as a whole in laboratory animals, some specific alterations present in schizophrenic patients can be modeled in animal, such as sensorimotor gating deficits and cognitive deficits, over-sensitivity of the dopaminergic system to amphetamine, and so on. The aim of the current project is therefore to develop an animal model allowing us to study the effects of maternal infection on offspring behavior in relation to SCZ.

This thesis is organized into seven chapters, in which chapter II, IV and V have been published. Chapter I (introduction and literature review) covers the following issues: 1) short overview of the pathophysiology and epidemiology of SCZ; 2) Maternal infection during pregnancy as a risk factor for SCZ 3) Fever, cytokines and the innate immune response; 4) Animal models of maternal infection; 5) Hypothesis and aims of the thesis; 6) General methodology. In the following two chapters, the use of the molecular immunogen bacterial endotoxin (lipopolysaccharide; LPS) to mimic maternal infection is explored.
Chapter II consists of a published manuscript investigating the effects of prenatal LPS exposure, with or without an added period of anoxia, on selected behavioral measures (prepulse inhibition, amphetamine induced locomotion) in offspring. Chapter III details a series of unpublished experiments examining the effects of continuous LPS exposure using osmotic pump delivery. In the first part of the chapter, the febrile response to chronic LPS administration by minipump is compared to that of repeated acute LPS injection. The second part of the chapter examines the effects of prenatal chronic LPS exposure on offspring behavior. Chapter IV consists of a published manuscript investigating the immune reaction to polyinosinic: polycytidylic acid (poly I:C), a molecular immunogen representative of viral infection. Very few in vivo studies had been performed using poly I:C, so the immune response to poly I:C in rats had to be characterized prior to its use in a model of maternal infection. Chapter V addresses the issues of windows of sensitivity and specificity of the immune challenge in animal models of maternal infection. This published study compares the effects of prenatal challenge with different immune agents (LPS, poly I:C, turpentine) given at three distinct times during gestation on prepulse inhibition in adult offspring. Finally, in chapter VI, as long-term dysregulation of the immune system may affect brain function, we investigated if MIA affected immune function in adult offspring. Chapter VII constitutes the conclusion of the thesis.

In summary, the overall aim of the current PhD project is to develop an animal model allowing the study of behavioral alterations induced by maternal immune activation in the offspring, and investigate the presence of gestational windows of vulnerability for disruption of a specific behavior.
Chapter I: Introduction and Literature Review
1 **BRIEF OVERVIEW OF SCHIZOPHRENIA**

1.1 **Symptoms and prevalence of schizophrenia**

Schizophrenia (SCZ) is a brain disorder that is expressed in the form of abnormal mental functions and disturbed behavior, and is frequently associated with a lifetime of disability. Its symptoms predominantly appear in late adolescence or early adulthood, and are generally classified into two categories: (1) positive symptoms, which are abnormal by their presence, and include delusions (fixed false beliefs), hallucinations, incoherent speech and disorganized behavior; (2) negative symptoms, which are abnormal by their absence, and include emotional flattening, low motivation, and social withdrawal (Frith and Johnstone 2003). Though not SCZ symptoms per se, a cluster of cognitive impairments has been observed in patients, more specifically deficits in attention, executive functions and working memory. Some researchers now believe cognitive deficits to be the core features of the disorder (Elvevag and Goldberg 2000; Heinrichs 2005) but others disagree (Frith and Johnstone 2003).

SCZ has generally been regarded as an ‘egalitarian’ disorder, with a fairly consistent prevalence across country, racial groups and gender. This view was mostly based on a large study by the World Health Organisation (Sartorius et al. 1986; Jablensky et al. 1992). Recent work by McGrath et al (2004) challenged this long-standing belief, stating that the disease incidence varies significantly across location, with conservative rate estimates between 7.7 and 43.0 per 100,000 and a median of 15.2 per 100 000. Based on their and other researchers’ work, men also appear slightly but significantly more at risk than women, with a risk ratio of 1.4:1 (Aleman et al. 2003; McGrath et al. 2004). This parallels the long-standing observation that men tend to have more severe manifestations of the disorder, including an earlier age of onset, poorer response to treatment, and less favorable outcome (Szymanski et al. 1995; Hafner et al. 1998).

1.2 **Neurodevelopmental origin of schizophrenia**

The majority of current theories on the etiology of schizophrenia posit that the illness is of neurodevelopmental origin. Several observations support this neurodevelopmental hypothesis (Pearlson 2000): 1) In epidemiological studies,
one of the environmental factors most strongly associated with increased risk of schizophrenia is adverse perinatal events (Fuller Torrey et al. 2000); 2) Long before the age of onset of the disorder, schizophrenic patients frequently exhibit mild motor (Walker et al. 1994) as well as social and cognitive abnormalities (Jones et al. 1994; Davidson et al. 1999); 3) Neuropathological and neuroimaging abnormalities (e.g. decreased cortical thickness, increased neuronal density, ventricular enlargement) are consistent with a neurodevelopmental origin of the disease (Harrison 1999); 4) There is no clear evidence of glial proliferation (i.e. gliosis) in post-mortem brains of schizophrenic patients (Harrison 1999), which is a hallmark of “classic” neurodegenerative disorders. However, the absence of gliosis does not rule out a more limited degenerative process (Jarskog et al. 2007). In fact, recent longitudinal neuroimaging studies have reported progressive reduction of cortical and subcortical grey matter volume at the early stages of the illness (Pantelis et al. 2003; Kasai et al. 2003), possibly reflected in the functional deterioration observed in patients.

1.3 Dopamine involvement in schizophrenia

Although still poorly understood, the pathophysiology of SCZ is thought to involve disrupted synaptic connectivity affecting both excitatory and inhibitory circuits, with more than one neurotransmitter system involved. Research into this aspect suggests that a dysregulation of several neurotransmitter systems could account for the symptoms of schizophrenia, including the dopaminergic, glutamatergic, GABAergic and serotonergic systems. There is a large body of evidence involving dopamine (DA) transmission in some aspects of the disease. The traditional DA hypothesis of SCZ stated that symptoms originated from subcortical hyperdopaminergia. Recent evidence has prompted researchers to refine this concept. It is now believed that a pathological sensitization or a hyper-reactivity of the mesolimbic DA system is inducing positive symptoms, whereas cortical hypodopaminergia is underlying the negative and cognitive symptoms (Abi-Dargham 2004). Different types of experimental evidence support DA involvement in the illness, particularly in positive symptoms: 1) Pharmacological studies have demonstrated that classical antipsychotic drugs, like haloperidol,
possess dopamine D₂ receptor antagonist properties, and atypical antipsychotics such as clozapine bind with high affinity to the D₄ receptor, a member of the D₂-like DA receptor family (Seeman 1992), though these medications also interact with other neurotransmitter systems; 2) Amphetamine (AMPH), which increases DA release, elicits hallucinations and delusions reminiscent of schizophrenia in normal subjects, and exacerbates the symptoms of schizophrenic patients (Laruelle et al. 1996). Long term abuse of this substance can also induce a permanent schizophrenia-like state in addicted subjects (Flaum and Schultz 1996); 3) The most compelling evidence for DA involvement comes from in vivo imaging studies using D₂-specific radiotracers to quantify DA release. In this series of experiments, changes in DA release following an AMPH challenge were assessed by measuring the reduction in radiotracer binding following AMPH administration. It was shown that AMPH administration provoked a greater increase in striatal DA release in schizophrenic patients compared with controls (Laruelle et al. 1996; Breier et al. 1997). This increase was correlated with the worsening of patients’ symptoms (Laruelle et al. 1999). A similar protocol allowed the quantification of baseline D₂ receptor occupancy, which was shown to be higher in non-medicated schizophrenic patients than controls (Abi-Dargham et al. 2000).

Concomitant with this hyperdopaminergia in the mesolimbic pathway, researchers have obtained evidence for hypodopaminergia in the mesocortical DA pathway in SCZ. An excess of dopamine D₁ receptor levels was detected in the dorsolateral prefrontal cortex (DLPFC) of schizophrenic patients, suggesting receptor upregulation in response to decreased DA transmission; this was associated with a lower performance on memory tasks (Abi-Dargham et al. 2002). Studies in normal subjects have shown the DLPFC to be crucial in working memory (McCarthy et al. 1994; Jansma et al. 2000), which is impaired in SCZ (Carter et al. 1996; Conklin et al. 2000). Additionally, in imaging studies, schizophrenic patients fail to activate their DLPFC during memory tasks (Callicott, 1998; Barch, 2001). According to Abi-Dargham, this D₁ up-regulation may represent a compensatory effect of the DA deficit, but this compensatory
mechanism would not be functional, thus working memory performance remained deficient (Abi-Dargham et al. 2002).

Recently, improvements in positron emission tomography (PET) resolution has allowed researchers to look into more detail at the locus of the DA anomalies in SCZ, and refine their theories. Although the striatum is likely implicated in the pathophysiology of the disease, the anomaly may lie in the striatal region linked to associative cortical areas, often referred to as the “associative striatum”, rather than in the limbic part of the striatum, as previously thought. In support of this hypothesis, two recent imaging studies reported that, DA synthesis and storage (Howes et al. 2009) and synaptic DA levels following release (Kegeles et al. 2010) were elevated in the associative striatum of schizophrenic patients compared to controls. Individuals with prodromal symptoms also exhibited increased DA activity in this area, suggesting that DA anomaly precedes SCZ onset (Howes et al. 2009). No differences were observed in the limbic striatum between patients and controls. Furthermore, greater elevation in dopamine synthesis capacity in the associative striatum was linked to poorer performance on a task of executive function and to altered prefrontal activation during the same cognitive task in people with individuals in a “at risk mental state” (Fusar-Poli et al. 2009).

Disturbances in DA transmission are not necessarily the “primary pathology” of the disease. This primary pathology has yet to be identified with certainty. Some studies suggest it might be related to alterations in the GABAergic and/or glutamatergic systems (Gaspar et al. 2009; Benes 2010; Marek et al. 2010), but a review of these hypotheses is outside the scope of the present study.

2 MATERNAL INFECTION AND SCHIZOPHRENIA

2.1 Risk factors: genes and environment

The precise etiological factors responsible for these alterations in neurodevelopment and neurotransmitter systems in SCZ have not been identified, but evidence suggests that both genetic and environmental factors contribute to the disease. It is hypothesized that the interaction between genetic vulnerability
and early neurodevelopmental insults results in defective connectivity between a number of brain regions (midbrain/nucleus accumbens (Nacc) DA systems, thalamus, temporo-limbic and prefrontal cortices) (Selemon and Goldman-Rakic 1999; Lewis and Levitt 2002). This defective neural circuitry would then be vulnerable to dysfunction when unmasked by the developmental processes of adolescence (when myelination, synaptic pruning, and hormonal changes occur) and exposure to stressors as the individual moves toward adulthood (Lewis and Lieberman 2000).

2.1.1 Genetic risk factors

Family and adoption studies have clearly established that vulnerability to schizophrenia has a strong genetic component. For instance, first degree relatives of schizophrenic patients have an increased risk for the disorder (6-17%), as do second degree relatives (e.g. grand-parents, aunts, uncles; 2-6%), whereas the lifetime prevalence of SCZ in the general population is 0.30–0.66% in the strictest definition of the disease (McGrath et al. 2008), to 2.3% if the diagnostic criteria are broader (Castle et al. 1993). In addition, about 4.1% of affected dizygotic twins pairs and 50% of affected monozygotic twin pairs are concordant for the disease, yielding heritability estimates between of approximately 88% (Cardno and Gottesman 2000). Furthermore, adoption studies demonstrated that the risk for schizophrenia is related to the presence of the disease in the biological parents rather than in the adoptive ones (Gottesman et al. 1982). SCZ is a multi-factorial polygenetic disease, i.e. it is caused by several different genes, each one accounting for a small portion of increased risk, interacting with environmental risk factors. Though no associated gene or chromosomal locus has been identified with certainty, there are now several strong candidates. For instance, association between SCZ and neuregulin-1 (NRG1), dysbindin (DTNBp1), disrupted in schizophrenia-1 (DISC1), and catecholamine-O-methyl transferase (COMT) genes have all been replicated in different cohorts and have biological plausibility [reviewed in Di Forti et al (2007)]. Nevertheless, the fact that 50% of the affected monozygotic twin pairs are discordant for the disorder, and about 60% of patients do not have a first or second-degree affected family member (Gottesman and
Erlenmeyer-Kimling 2001) clearly illustrates that genetic factors alone cannot account for schizophrenia. Thus, additive or interactive environmental effects must also contribute to emergence of the disease.

2.1.2 Environmental risk factors

Epidemiological studies have documented several environmental risk factors for SCZ: early adverse events [e.g. maternal infection during pregnancy (Brown 2006), prenatal malnutrition and obstetrical complications (Cannon et al. 2002)], urban birth and upbringing (Marcelis et al. 1998; Pedersen and Mortensen 2001), migrant status (Boydell et al. 2001), cannabis use (Henquet et al. 2005), and cat ownership in childhood, potentially a proxy for exposure to toxoplasmosis and other zoonotic infections (Fuller Torrey et al. 2000). Although the magnitude of increased risk these factors confer is often considered limited, it is in the same range of that associated with most putative risk genes (relative risk of 1.5 to 3) (Risch 1990). Among environmental risk factors, maternal infection is now a prime candidate, due to the wealth of epidemiological data and the recent work on detection of immune biomarkers in the maternal plasma sampled during affected individuals’ gestation.

2.2 Prenatal infection as a risk factor for schizophrenia

The higher number of winter and spring births among schizophrenic patients, although moderate, is well documented (Kinney et al. 2000; Suvisaari et al. 2001; Davies et al. 2003). The higher odds of the mother contracting a seasonal illness, such as respiratory infections, at certain periods during her pregnancy, could contribute to that distorted birth ratio (O'Callaghan et al. 1991a). Since the pioneering work of Mednick’s group in the 1980’s, several groups have reported an association between influenza at time of pregnancy and increased incidence of SCZ among the offspring (Mednick et al. 1988; Barr et al. 1990; O'Callaghan et al. 1991b; Mednick et al. 1994; Takei et al. 1996; Munk-Jørgensen and Ewald 2001). Not all studies have replicated this association (Kendell and Kemp 1989; Crow and Done 1992; Cannon et al. 1996; Battle et al. 1999; Selten et al. 2009), although factors such as timing, virulence and strain of infection and methods of retrospectively assessing maternal infection may
confound these studies (Brown and Susser 2002). Furthermore, the statistical power of these negative studies being somewhat limited, they do not positively exclude a link between prenatal infection and SCZ. Using a different design, a recent case-control study based on Danish longitudinal registers found a significant increase in risk of SCZ associated with maternal influenza infection (Incidence Rate Ratio 8.2) after adjusting for obstetric complications, family psychiatric history, socio-economic and demographic factors (Byrne et al. 2007). Other viral infections during pregnancy have been linked with schizophrenia including measles (Torrey et al. 1988), varicella-zoster (Torrey et al. 1988), rubella (Brown et al. 2001), polio (Torrey et al. 1988; Suvisaari et al. 1999). Maternal infections of bacterial origin have also been associated with SCZ, such as diphtheria (Watson et al. 1984), pneumonia (Watson et al. 1984; O'Callaghan et al. 1994) and other respiratory infections (Brown et al. 2000) often caused by bacteria (Goodnight and Soper 2005). Two prospective studies also observed a link between increased risk of SCZ and maternal bacterial infection, genital/reproductive infections (Babulas et al. 2006) and first-trimester respiratory tract and gonococcal infections (Sorensen et al. 2009). A British longitudinal study found a significant association between presence of psychotic symptoms at age 12 and maternal infection of different origins during pregnancy (Zammit et al. 2009). Finally, a revealing study by Clarke et al. reported no increase in schizophrenic births among women who were diagnosed with urinary tract infection (UTI) in the general population. However, when the sample was restricted to women who had a positive family history of SCZ, a significant risk associated with maternal UTI emerged (Clarke et al. 2009). This study suggests that a synergistic gene x environment interaction is part of the causative mechanisms underlying SCZ, and may partly explain why not all epidemiological studies observed a significant relationship between maternal infection and SCZ risk.

Nonetheless, epidemiological studies suffer from some limitations, the most frequent being that there is often no direct evidence that the subjects actually suffered from an infection, since evidence of infection is generally based on
prevalence of the disease in the population at the time of pregnancy, maternal recall or hospital records. However, it is now possible to detect the presence of antibodies in archived maternal serum sampled during the patient’s prenatal life, allowing researchers to directly confirm whether the mother was exposed to pathogens. Using this method, Brown examined the presence of influenza antibodies in pregnancies giving rise to schizophrenic offspring and found a seven fold increased in risk of SZC deriving from influenza exposure during the first trimester (2004a). Presence of antibodies to toxoplasmosis (Brown et al. 2005; Xiao et al. 2009) and herpes simplex virus type 2 (Buka et al. 2001a; Buka et al. 2008) in maternal plasma has also been linked to higher risk of adult psychotic illness, although the latter association was not replicated in another large cohort (Brown et al. 2006). In a prospective sample, Brown et al. observed that SCZ patients who were prenatally exposed to maternal influenza or toxoplasmosis infection confirmed by stored maternal plasma examination, scored significantly lower on executive functioning tests compared to non-exposed patients (Brown et al. 2009). In another study, the verbal IQ of SCZ patients measured at 7 years old was lower in subjects who were exposed to maternal influenza during gestation compared to SCZ patients who were not exposed (Ellman et al. 2009). This effect of maternal infection was not observed in a group of exposed and non-exposed normal individuals. This suggests that prenatal viral exposure in the presence of other genetic/environmental factors may lead to poorer cognitive function even before the onset of clinical symptoms. Cytokine content of archived maternal plasma was also examined in relation to SCZ. Levels of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α (Buka et al. 2001b) and interleukin (IL)-8 (Brown et al. 2004b) were found to be increased in the plasma of mothers of schizophrenic offspring, suggestive of a prenatal inflammatory exposure.

A sizeable proportion of the epidemiological studies linking SCZ and prenatal infection found the relationship significant when the infection happened during the second trimester of pregnancy (Mednick et al. 1988; Barr et al. 1990; O'Callaghan et al. 1991b; O'Callaghan et al. 1994). However, several issues may
confound the identification of the sensitive period, in particular when retrospectively assessing the timing of maternal infection (i.e. based on maternal recall and/or peak of the epidemic in population), as the mother may not recall accurately or may have been infected outside the peak of the epidemic, and infants may be born preterm or post-term. In recent years, however, associations with infections during the first trimester have been more frequently reported, for instance with maternal bacterial infections (Sorensen et al. 2009) and rubella (Brown et al. 2001). Finally, Brown and colleagues’ study showing serological evidence of actual maternal infection with influenza (2004a) strongly implicated the first trimester.

The wide variety of infectious agents associated with increased risk of SCZ, including virus, bacteria and parasites, suggests there is a factor common to various prenatal infections which may be an integral part of the maternal immune response that affects fetal neurodevelopment. Fever and increased levels of cytokines are considered two likely candidate mechanisms.

2.3 **Possible mediators between prenatal infection and altered fetal development**

2.3.1 *Fever as a possible mediator*

Fever during pregnancy is considered to be a good candidate as a possible mediator between prenatal infection and altered fetal neurodevelopment, because it is a risk factor for schizophrenia in itself (Fuller Torrey et al. 2000), and is also associated with increased risk of major birth defects, including neural tube malformations (Erickson 1991; Graham, Jr. et al. 1998). Elevated body temperature per se was shown to have teratogenic effects in laboratory animals. For instance, environmental heat stress during gestation in rodent induced gross brain anomalies such as microcephaly and cerebral edema (Arora et al. 1979; Mottola et al. 1993; Yitzhakie et al. 1999). A study by Kahn and Brown indicated that elevated body temperature can also cause more subtle anomalies. In this experiment, keeping pregnant rats at 42°C for 45 min on gestation day 17 was sufficient to increase apoptosis in the fetal cerebral cortex, but not in mature, postmitotic cells of the adult cerebellum (Khan and Brown 2002). This indicates
that proliferating neural populations may be more sensitive to hyperthermia than postmitotic neurons.

2.3.2 Cytokines as possible mediators

Cytokines, in particular pro-inflammatory, are prime candidates as mediators between maternal infection and altered fetal development. Cytokines are signalling polypeptides secreted during the immune response that act in autocrine, paracrine, and endocrine ways to orchestrate the host defence response to infection. As their name imply, pro-inflammatory cytokines principally act as mediators of inflammation and fever, but they also play a key role in normal brain development (Zhao and Schwartz 1998). Elevated cytokine concentration can alter the differentiation and survival of neuronal progenitor cells (Ling et al. 1998; McGuire et al. 2001) and inhibit neurite growth in vitro (Gilmore et al. 2004). Cytokines have been implicated in neuronal cell death following traumatic injury, asphyxiation and chronic disease in the adult brain (Allan and Rothwell 2003). The neurotrophic and neurotoxic properties of cytokines will be reviewed in greater details after a brief introduction to their primary role in innate immunity.

3 THE IMMUNE RESPONSE, FEVER AND CYTOKINES

3.1 Regulation of innate immunity and fever

The mammalian immune defences against pathogens are typically divided into two sets of responses. The first line of defence is the innate immune response, a stereotyped set of defence mechanisms that is triggered by almost all types of pathogens. Phylogenetically ancient, it is present in some form in both vertebrate and non-vertebrates (Kluger et al. 1998). The innate immune response consists primarily of fever, inflammation, complement cascade activation, hypothalamic-pituitary-adrenal (HPA) axis activation and sickness behavior. Sickness behavior refers to an organized behavioral strategy that facilitates the role of fever in combating infections and maximizes survival (Hart 1988). It consists of anorexia, adipsia, increased sleep and reduced physical activity.

The second line of defence, termed adaptative or humoral immune response, has evolved in higher vertebrates. Its most important feature is pathogen
specificity. Lymphocytes and antibodies, the main effectors of the adaptive immune response, allow for “immunological memory”. However, it takes longer to be effective. Since this study is mainly concerned with elements that are common to many types of infectious agents, only the innate immune response is reviewed here.

3.2 Role of fever in immunity

Fever is a metabolically costly response to infection; still it has been evolutionarily conserved among ectothermic and endothermic vertebrates, which strongly suggests it has a beneficial role in defending the infected organism (Kluger et al. 1998). Laboratory evidence tends to corroborate this assumption. For instance, when laboratory animals were inoculated with live bacteria or viruses, fever maintained within a 2°C range was correlated with improved survival, recovery, and decreased viral count (Toms et al. 1977; Kluger and Vaughn 1978). Conversely, fever inhibition with antipyretic drugs in infected vertebrates led to increased mortality and slower recovery time (Bernheim and Kluger 1976; Vaughn et al. 1980; Husseini et al. 1982). Clinical studies also demonstrated a correlation between moderate fever in humans and decreased morbidity and mortality rate following a variety of infections (Bryant et al. 1971; Weinstein et al. 1978). A controlled rise in body temperature may improve host defences in many ways, for example by facilitating neutrophil migration, increasing T-cell proliferation and diminishing bacterial ability to chelate iron for their growth (Kluger 1991). However, fever may be maladaptive in circumstances where cytokines and other inflammatory mediators are overproduced (Kluger et al. 1998).

Although fever is often regarded as a peripheral response to infection, the rise in body temperature is in fact tightly regulated by the central nervous system in response to a signalling cascade that is summarized below.

Initially, effector cells of the innate immune system (macrophages and dendritic cells in the periphery, microglia in the CNS) detect the presence of pathogens through their toll-like receptors (TLRs) (fig. 1). TLRs recognize molecular patterns that are typical of a pathogen family but that are not present in
the mammalian body. One example of such a molecule is lipopolysaccharide (LPS), a membrane component of gram negative bacteria cell wall. Binding of LPS to its TLR triggers a signal transduction cascade that leads to the activation of transcription factors such as nuclear factor kappa B (NFκB). These transcription factors induce the transcription of pro-inflammatory genes implicated in the innate immune response, such as cytokines, chemokines, and proteins of the complement system (Aderem and Ulevitch 2000). The term cytokine refers to a group of small hydrophilic signalling molecules (between 8 and 30 kDa) that are primarily secreted during the immune response. Cytokines bind to specific membrane receptors on their target cells, triggering signal transduction pathways that alter gene expression and influence each others production. This creates a complex signalling network termed the cytokine cascade that is essential in regulating different aspects of the immune response.

**Figure 1. Lipopolysaccharide (LPS) signalling cascade.** LPS binds to LPS binding protein (LBP), then triggers a signal transduction cascade through binding the co-receptor Cluster of Differentiation 14 (CD-14) and activating toll-
like receptor-4 (TLR4). Activation of the transcription factor nuclear factor kappa B (NFκB) leads to changes in gene expression such as production of cytokines.

Interaction between the peripheral immune system and the CNS is essential for the development of systemic responses to peripheral infections such as fever. The principal mediators of those neuroimmune interactions are pro-inflammatory cytokines, in particular interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α). Following detection of bacterial products by macrophages, IL-1 and TNF-α are produced locally and act on fibroblast and endothelial cells to induce their own synthesis as well as that of IL-6 and other cytokines (Luheshi 1998). Circulating IL-6 then brings the inflammatory signal to the brain across specific areas devoid of a tight blood-brain barrier, such as the sensory circumventricular organs. Pro-inflammatory cytokines are then produced in the brain, and induce the synthesis of prostaglandins (PGE2) via cyclooxygenase-2 (COX-2) in the hypothalamus. Prostaglandins act on the thermosensitive neurons of the preoptic area of the anterior hypothalamus to rise the thermoregulatory set point of the organism (Conti et al. 2004). The role of the three principal pro-inflammatory cytokines in peripheral and CNS inflammation is summarized in the next paragraphs.

3.3 Pro-inflammatory cytokines

3.3.1 Interleukin (IL)-1

The IL-1 cytokine family consists of three related proteins: IL-1α and IL-1β, which have a pro-inflammatory role, and a naturally occurring receptor antagonist (IL-1ra), which is anti-inflammatory (Dinarello 1991). IL-1β acts both locally at the site of injury and centrally to mediate the immune central responses like fever, sickness behavior, and HPA axis stimulation (Luheshi and Rothwell 1996). As such, peripheral administration of IL-1β induces fever, whereas peripheral neutralisation of endogenous IL-1β inhibits it (LeMay et al. 1990; Luheshi et al. 1996). IL-1 is also thought to play a role in local brain injury in the adult CNS; IL-1 exacerbates damage induced by brain ischemia or trauma in the rat, as opposed to IL-1ra which attenuates it (Toulmond and Rothwell 1995;
Loddick and Rothwell 1996). Brain IL-1 immunoreactivity is also increased in neurodegenerative conditions like Alzheimer’s disease (Griffin et al. 1989). However, IL-1 may also have some neurotrophic properties, for example by increasing neurotrophic factor production (Heese et al. 1998).

3.3.2 Interleukin (IL)-6

IL-6 acts as an immune and hematopoietic factor in the periphery. IL-6 is considered to be the major endogenous pyrogen. Its increase in the circulation of febrile animals correlates with the magnitude and duration of the fever response to immunogens (LeMay et al. 1990). IL-6 is present at low concentration in the normal CNS, but its level is increased following infection and brain injury (Van Wagoner and Benveniste 1999) (Campbell et al. 1993b). IL-6 seems to have a dual role in brain injury; studies of cerebral ischemia in rodents (Loddick et al. 1998) and transgenic mice (Hirota et al. 1996) show that IL-6 promotes neuronal recovery in some instances, while other studies demonstrate that the molecule can be injurious to the brain at high concentrations (Campbell et al. 1993a).

3.3.3 Tumor necrosis factor (TNF)-α

In the periphery, low doses of TNF-α participate in the physiology of normal tissue remodelling and inflammation, whereas high quantities produced in disease states may lead to lethal tissue injury and septic shock. The role of TNF-α in fever remains unclear. A number of studies by Kluger (Kozak et al. 1995; Klir et al. 1995), including work on receptor knock-out mice (Leon et al. 1997), suggest it plays an antipyretic role in rodents, at least in situations where a strong inflammatory stimulus is used. Other studies suggest it has a pyrogenic action (Kettelhut and Goldberg 1988; Luheshi et al. 1997). TNF-α is present in the brain of healthy rats, but its level highly increases following infection and traumatic brain injury. Similar to IL-6, TNF-α seems to have a dual role in the adult CNS: neuroprotective at low levels (Bruce et al. 1996), neurotoxic when overproduced (Probert et al. 1995).

3.4 Pro-inflammatory cytokines and developing neurons

LPS and pro-inflammatory cytokines can influence the survival and differentiation of developing neurons, even at low concentrations. Mesencephalic
DA neurons being particularly relevant to SCZ research, their interactions with cytokines will be reviewed in greater detail. *In vitro* experiments have shown that fetal mesencephalic neurons are selectively injured by low doses of LPS, doses which have no effect on fetal hippocampal or cortical neurons (Kim *et al.* 2000). The toxic effects of LPS on DA neurons are at least partly mediated by pro-inflammatory cytokines, as demonstrated by the fact that IL-1β and TNF-α neutralizing antibodies, when added to the culture media, reduced LPS-induced cell death by half (Gayle *et al.* 2002). Cytokines can also influence cell differentiation; IL-1 was found to induce the differentiation of fetal mesencephalon progenitor cells into DA neurons (Ling *et al.* 1998). However, it is notably difficult to assign a clear neurotoxic or neurotrophic role to pro-inflammatory cytokines, as their effect on neuronal survival are highly dependent on concentration, duration of exposure and cell types present. For instance, moderate doses of IL-1β, IL-6 and TNF decrease the survival of fetal DA neurons *in vitro*, but have little effect on serotonin neurons and other cell types (Jarskog *et al.* 1997; McGuire *et al.* 2001). Reduction in DA neuron survival with IL-6 is observed only when the exposure is short in duration (< 2 days), whereas longer IL-6 exposure (> 4 days) appears to promote survival (Kushima *et al.* 1992). This dependency on cytokine specificity and dose/length of exposure might offer a partial explanation as to why some alterations in offspring neural function and/or behavior are observed in some maternal infection models but are absent in others where conditions are slightly different (Meyer *et al.* 2007).

4 **ANIMAL MODELS OF MATERNAL INFECTION IN RELATION TO SCZ**

4.1 **ANIMAL MODELS AS INVESTIGATIVE TOOLS**

Epidemiological studies linking SCZ and maternal infection are associative. Thus, they do not demonstrate a causal relationship between the two events, and provide little information about the mechanisms that would underlie a causal link. However, experimentally inducing an infection in a gravid rodent allows scientists to study the impact of maternal infection on offspring neural function, and the mechanisms by which neurodevelopment could be affected. Obviously, one cannot expect to model the whole complexity of schizophrenia in laboratory
animals. Nevertheless, some specific symptoms, deficits and neuropathological features observed in patients can be modeled in rodents. Work on animal models of disrupted neurodevelopment in relation to SCZ was pioneered by Barbara Lipska and colleagues. They observed that adult rats who had been subjected to ventral hippocampal lesions as neonates displayed behavioral changes reminiscent of SCZ symptoms in humans, including increased responsiveness to stress and psychostimulants (Lipska et al. 1993; Al Amin et al. 2000), disrupted latent inhibition (Grecksch et al. 1999), and deficits in prepulse inhibition (PPI) (Lipska et al. 1995) and working memory (Lipska et al. 2002). Although such widespread lesions of the hippocampus are not present in schizophrenic patients, Lipska’s hallmark studies opened the door to investigations of more subtle developmental insults thought to be implicated in some psychiatric illnesses, including Cesarean section birth, perinatal anoxia and, more recently, maternal infection.

4.2 Consequences of neonatal infection on neural development

A simple way to study the effects of infection on brain development is to directly inoculate rodent neonates with live viruses. This type of insult leads to observable behavioral and neuronal changes such as hyperactivity, deficit in behavioral inhibition (Crnic and Pizer 1988), decreased PPI (Engel et al. 2000), gradual loss of hippocampal cells and neuronal hyperexcitability in pups (Pearce et al. 2000). A similar model consists in administering cytokines directly to neonates. Subcutaneous injection of IL-1α from postnatal day 2 (P2) to P10 to mice pups resulted in some behavioral changes including decreased PPI, increased baseline startle and increased social interaction (Tohmi et al. 2004). Behavioral effects of other cytokines were more limited; leukemia inhibitory factor (LIF) produced a decreased PPI in juveniles and young adults which subsequently normalized as the animals matured (Watanabe et al. 2004). Neonatal IL-2 and interferon (INF)-γ administration only had a transient effect on baseline locomotion in juveniles. Though a potent pro-inflammatory cytokine, IL-6 administered to neonates induced no detectable behavioral changes (Tohmi et al. 2004).
These limited effects suggest that injecting neonates with immunogens or cytokines may not accurately model the effects of maternal infection on brain development, possibly it does not take into account the immune system’s immaturity and maternal/fetal interactions. For instance, rodent neonates cannot regulate their own body temperature and therefore cannot respond to infection with an adult’s febrile response. In addition, the placenta constitutes an important aspect of maternal/fetal interactions. It forms an active barrier that prevents most common pathogens and toxins from coming into direct contact with fetal tissue, while still allowing specific immune signals to cross. Our laboratory has shown that radiolabeled bacterial endotoxin injected in a pregnant rat can be detected in maternal internal organs as well as in the placenta, but does not penetrate the fetal cavity (Ashdown et al. 2006a). It remains unclear whether live virus can cross the placenta and directly affect the fetal brain. In one experiment, viral RNA and proteins were detected in the fetal brain and mature offspring following intranasal inoculation of influenza virus to pregnant mice (Aronsson et al. 2002). However, this finding was not replicated in another study (Shi et al. 2005).

The fetus is not entirely protected from inflammatory stimuli, as specific cytokines can transfer through the placental barrier. In vitro studies have shown that IL-6 can cross the human placenta at relatively high rate (8-17%) (Kent et al. 1994), whereas other cytokines like IL-1α, IL-1β, IL-8 and TNF-α can only cross at very low rates or not at all (Kent et al. 1994; Reisenberger et al. 1996; Zaretsky et al. 2004). In rats, IL-6 also crosses the placenta, but does so in far greater amounts in mid-gestation (embryonic day (E)11-13) compared to late gestation (E17-19) (Dahlgren et al. 2006). In summary, animal and human studies suggest that among the cytokines studied to date, IL-6 is the most likely to cross the placenta and harm the fetus.
Table 1. Animal models of maternal infection in relation to psychiatric illness: studies before 2001.

<table>
<thead>
<tr>
<th>Changes in offspring</th>
<th>Species, Infectious Agent</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>• ↑ incidence of fetal anomalies</td>
<td>Rat, LPS</td>
<td>(Ornoy and Altshuler 1976)</td>
</tr>
<tr>
<td>• ↑ neuronal necrosis, periventricular leukomalacia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Rarefaction and disorganization of white matter with apoptosis, similar to what is observed in cerebral palsy</td>
<td>Rabbit, <em>E. Coli</em> intra-uterine inoculation</td>
<td>(Yoon <em>et al.</em> 1997; Debillon <em>et al.</em> 2000)</td>
</tr>
<tr>
<td>• Regional alterations in nNOS and SNAP-25 expression</td>
<td>Mice E9, Human influenza intranasal inoculation</td>
<td>(Fatemi <em>et al.</em> 1998a; 1998b; 1999; 2000)</td>
</tr>
<tr>
<td>• ↓ reelin expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• ↓ thickness of cortex and hippocampus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Fetal brain: ↑ IL-1β mRNA and TNF-α protein</td>
<td>Rat E18-19, LPS 500µg-4mg</td>
<td>(Cai <em>et al.</em> 2000)</td>
</tr>
<tr>
<td>• Post-natal day(P)8: ↓ MBP and ↑ GFAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• ↑ pro-inflammatory cytokines in placenta and amniotic fluid after high &amp; low dose LPS</td>
<td>Rat E16, LPS 500µg or 2.5mg</td>
<td>(Urakubo <em>et al.</em> 2001)</td>
</tr>
<tr>
<td>• Fetal brain: ↓ TNF-α after high dose</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GFAP: glial fibrillary acidic protein; MBP: myelin basic protein; nNOS: neuronal nitric oxide synthase; SNAP-25: synaptosome-associated protein 25 kDa;

4.3 Animal models of maternal infection; before 2001

When this study was initiated in 2001, very few experimental studies investigating maternal infection in relation to psychopathology using animal models had been published. Studies of maternal infection during rodent gestation mainly focused on severe outcomes, such as pre-term delivery, miscarriage and severe malformations. In schizophrenia, however, there is no evidence of gross abnormalities. Instead, the neuropathological alterations are subtle, and are not diagnostic features of the disease. Subtle alterations in offspring
neurodevelopment following maternal infection remained largely undocumented at that point. In particular, there was no study of behavioral alterations, which constituted an important gap in the field as all SCZ diagnostic criteria are behavioral symptoms and deficits.

Most of the early investigations used inoculation with live pathogens to induce maternal infection (Table 1). Among those relevant to my experimental study, some examples can be cited. Intrauterine infection with live E. coli was used as a model of cerebral palsy, and caused white matter lesions, programmed cell death and increased incidence of fetal death in rabbit (Yoon et al. 1997; Debillon et al. 2000). In studies more directly related to SCZ, pregnant mice who had been infected with influenza virus on E9 gave birth to pups with a thinner cortex (Fatemi et al. 1999), abnormal synaptogenesis (Fatemi et al. 1998b) and long-lasting changes in brain nitric oxide synthase levels (Fatemi et al. 2000). To gain a better control over dose and timing of the immune challenge, other researchers used bacterial LPS injection as an alternative to live pathogen inoculation. An early study reported gross brain abnormalities and neuronal death in offspring of LPS-treated pregnant rats (Ornoy and Altschuler 1976). In 2000-2001, two groups used LPS administration to investigate the presence of cytokines in the fetal compartment after maternal LPS injection. In one study, pregnant rats injected with LPS on E16 increased IL-6 and TNF-α levels in the amniotic fluid and IL-1β, TNF-α and IL-6 in the placenta. TNF-α alone was marginally altered in the fetal brain following maternal LPS treatment (Urakubo et al. 2001). Another group detected an increase in IL-1β and TNF-α mRNA in fetal brains from LPS-treated mothers, as well as decreased myelin basic protein staining in the cortex and hippocampus of the pups (Cai et al. 2000). Taken together, these studies supported the idea that LPS administration could alter fetal brain development, thus inducing long term behavioral changes.

4.4 Animal models of maternal infection; from 2002 to present

4.4.1 Behavioral effects of maternal immune activation

In recent years, interest in this field has grown tremendously, as shown by the increasing number of articles published between 2002 and 2009 (see Table 2).
The use of molecular immunogens, in particular poly I:C, has gained in popularity, whereas live viruses and bacteria are now rarely used. Studies published during this period have sought to establish reliable and practical models demonstrating that maternal immune activation (MIA) using systemic administration of LPS, poly I:C or live virus can induce alterations relevant to SCZ in offspring. Rodent offspring exposed to MIA show behavioral changes relevant to positive symptoms of schizophrenia, such as increased locomotor activity in response to psychostimulants (Zuckerman et al. 2003; Ozawa et al. 2006), and changes related to negative symptoms of the disease, such as deficits in PPI (Meyer et al. 2005; Ozawa et al. 2006) and social interaction (Shi et al. 2003). They also display cognitive deficits that parallel those seen in SCZ patients. Thus, learning and memory deficits were observed when MIA exposed animals performed the water maze (Meyer et al. 2005) and object recognition tasks (Ozawa et al. 2006). Although their ability to perform simple associative learning, like choosing the right arm in a T-maze, remained unchanged (Meyer et al. 2006b), their capacity to perform more complex tasks was challenged, particularly when it involved changing a set of previously learned rules (Meyer et al. 2006b). Tests were also performed to evaluate the animals’ ability to ignore irrelevant stimuli, which is thought to play a role in SCZ symptomatology (Anscombe 1987). In tests of latent inhibition (LI) and unconditioned stimulus pre-exposure effect (USPEE), pre-exposure to a stimulus significantly slows down associative learning in normal animals. However, animals gestationally exposed to poly I:C displayed faster learning than controls in both paradigms (Zuckerman et al. 2003; Meyer et al. 2005; Meyer et al. 2006a), indicating that they had not previously learned that the stimulus was “irrelevant”.

Another argument supporting the relevance of the MIA model to SCZ is the delayed appearance of deficits. The emergence of symptoms in late adolescence and early adulthood is a characteristic feature of SZC. Accordingly, investigators asked whether this delayed onset of deficits is present in maternal immune activation models. A detailed study by Ozawa and colleagues showed that adult but not juvenile offspring born to poly I:C-treated mothers displayed alterations in
exploratory behavior, methamphetamine-induced locomotion and PPI (Ozawa et al. 2006). Another group showed a delayed emergence of LI deficits in MIA rat offspring (Zuckerman et al. 2003).

4.4.2 Effects of maternal immune activation on the dopaminergic system

SCZ symptoms are thought to partially arise from a dysregulation of the dopaminergic system. This aspect of the pathophysiology is also reflected in the MIA model. Two studies, by the same group, of adult animals prenatally exposed to LPS showed an increased DA content in the nucleus accumbens (Borrell et al. 2002; Romero et al. 2008). When DA concentration was measured in the whole striatum, a reduction in baseline DA level was observed. However, there was an increased ratio of DA metabolites relative to DA itself, which is indicative of an increased DA transmission (Ling et al. 2004; Ozawa et al. 2006). These results were supported by in vitro DA release measurements (Zuckerman et al. 2003). Alterations in DA receptors were also found, such as a decrease in striatal D2 receptor binding (Ozawa et al. 2006) and in prefrontal D1 receptors (Meyer et al. 2008c).

As is the case with SCZ, the physiological causes behind these behavioral and dopaminergic alterations are not yet well-defined. However, animal models offer many possible approaches to dissect the mechanisms underlying them. The prime suspects are cytokines, and current data seems to support this hypothesis, though much investigation remains to be done.

4.4.3 Role of cytokines in the effects of maternal immune activation

To investigate the implication of cytokines in MIA, Meyer and colleagues injected poly I:C during gestation to wild-type mice and in mice overexpressing the anti-inflammatory cytokine, IL-10, in macrophages (Meyer et al. 2008b). The offspring of the treated transgenic mice did not exhibit the pathologic features normally associated with MIA. However, transgenic mice that were not given an immune challenge gave birth to offspring with behavioral alterations of their own. These observations led the authors to conclude that normal brain development requires a balance between pro- and anti-inflammatory cytokines. The role of cytokines in the consequences of MIA is directly supported by another experiment
by Patterson and colleagues; co-administration of anti-IL-6 antibodies with poly 
I:C prevented the deficits associated with MIA in offspring, including deficits in 
PPI, LI, exploratory and social behavior (Smith et al. 2007). Similarly, IL-6 
knock-out mice exposed to MIA did not differ from non-exposed controls. 
Furthermore, administration of IL-6 to pregnant wild-type mice induced some of 
the same changes observed in MIA offspring, i.e. PPI and LI deficits (Smith et al. 
2007). This series of experiments demonstrates that cytokines, in particular IL-6, 
may be crucial intermediates in the neurodevelopmental alterations caused by 
MIA.

4.4.4 Windows of vulnerability

It has long been established that “susceptibility to teratogenesis varies with 
the developmental stage at the time of exposure to an adverse influence” (Wilson 
1973). Each population of developing cells has specific periods when they are 
responsive to some molecular cues. Interference with developmental signals 
during that period can alter cell fate dramatically, whereas exposure at another 
time may have no effect. From in vitro studies we know that cytokines can 
influence the fate of developing neurons. For instance, the gestational age at 
which LPS was injected to pregnant rats had a significant effect on the number of 
DA neuron precursors found in mesencephalic cultures taken from the fetuses 
(Ling et al. 2002). In view of these observations, the existence of windows of 
susceptibility to the effects of MIA exposure can be presumed. Meyer and 
colleagues did an extensive series of experiment on the subject. They observed 
that poly I:C exposure earlier in gestation lead to a different cluster of behavioral 
impairments than exposure in late gestation (reviewed in (Meyer et al. 2008a)). 
Hence, poly I:C exposure on E9 produced changes in exploratory behavior, 
sensory gating (PPI) and latent inhibition, that were not present when poly I:C 
was administered on E17. Conversely, E17 poly I:C exposure causes 
perseverative behavior (delayed reversal learning) that was not present with E9 
exposure (Meyer et al. 2006b). Other behavioral changes were common to both 
exposure periods (e.g. altered AMPH-induced locomotion and USPEE) (Meyer et 
al. 2008a). These differences in behavioral outcome are possibly brought on by
some proliferating cells being sensitive only to developmental cues (i.e. cytokines) during specific developmental periods. Another potential contributing factor is differences in the cytokine responses to immune challenge in middle compared to late gestation in rodents, both in the maternal and fetal compartment (Meyer et al. 2006b). Finally, it has been observed that some cytokines, notably IL-6, cross the rat placenta in greater amounts in middle compared to late gestation (Dahlgren et al. 2006). None of these mechanisms are mutually exclusive, yet current experimental evidence prevents us from teasing them apart.

5 Hypothesis and Aims of the Thesis

As discussed earlier in the chapter, the rationale of our study is based on observations from epidemiological research indicating that maternal infection during pregnancy increases the offspring’s risk of developing schizophrenia (SCZ) later in life (Brown 2006). Epidemiological studies linking maternal infection to SCZ in offspring are inherently associative and thus cannot be used as direct evidence of a causal relationship between the two events. One of the best ways to investigate whether such a relationship is plausible, and to study the potential mechanisms underlying it is to turn to animal models. The variety of viral and bacterial infections suggest that an element common to all pathogens, such as the activation of the maternal immune system, rather than the direct action of the pathogen on the fetus, is responsible for the neurodevelopmental changes observed in the offspring. Therefore, the principal aim of my thesis was to establish an animal model to test the following hypothesis: activation of the maternal immune system during gestation induces behavioral alterations in the offspring. More specifically, this project aims to:

1) Test whether maternal immune activation by a molecular immunogen (i.e. bacterial endotoxin) leads to behavioral changes in the offspring. This is an essential step to relate activation of the maternal immune system during gestation to SCZ, a mental illness in which all diagnostic criteria are behavioral in nature. At the time the study was undertaken, offspring behavior following maternal infection had never been investigated.
2 (a) Characterize the immune reaction to polyinosinic: polycytidylic acid (poly I:C), a molecular immunogen that mimics viral infection. Special attention was paid to the febrile response, feeding and the cytokine cascade induced, all of which could potentially affect fetal development. This was a prerequisite to (b) investigate whether the effects of maternal immune activation on offspring development could also be observed in a model of viral infection, which was predominant in the epidemiological literature linking SCZ to maternal infection.

3) Substantiate the hypothesis that the deleterious consequences of maternal infection are mediated via circulating cytokines rather than the immunogen acting directly on the fetuses. This can be accomplished by using intramuscular (im) turpentine injection in the model, since unlike LPS and poly I:C, this substance does not enter the systemic circulation.

4) Investigate the existence of windows of vulnerability to the behavioral effects of infection during gestation by inducing MIA at different gestational periods, and comparing its effects at different gestational periods using PPI as the outcome measure.

5) Because long-term dysregulation of the immune system may affect brain function, determine if adult immune response is altered MIA-exposed offspring.

6  GENERAL METHODOLOGY

6.1 Models of infection and inflammation in rodents

In our studies, peripheral administration of molecular immunogens was preferred as an experimental model of infection over live pathogen inoculation. The principal advantage of using molecular immunogens is that they allow greater control over the dose and timing of administration. This generates inflammatory
responses that are more easily reproducible and allows for the investigation of
temporal windows of sensitivity. Two of them are used in this study: LPS and
poly I:C. In addition, injection of the organic solvent turpentine was used as a
model of local inflammation.

6.1.1 Lipopolysaccharide (LPS)

Peripheral LPS administration is routinely used as an experimental model of
infection as it induces the different events of the innate immune response,
including fever, inflammation, HPA axis activation, sickness behavior and release
of pro-inflammatory cytokines (Kluger 1991; Dantzer 2001). The immune
response to LPS has been characterized in great detail over the last three decades,
which is why it was selected as the immunogen for our first study.

LPS is a structural component of Gram-negative bacteria cell walls. The
LPS molecule consists of three regions; a lipid A, a polysaccharide core (R) and a
polysaccharide side chain (O). The lipid A is the hydrophobic, membrane-
anchoring region of LPS. The structure of Lipid A is highly conserved among
Gram-negative bacteria, and is thought to mediate the physiological (‘toxic’) LPS
response in vertebrates (Galanos et al. 1985). The core (R) polysaccharide
consists of a short chain of sugars common to all members of a same bacterial
genus. Finally, the ‘O’ side chain consists of repeating oligosaccharide units and
maintains the hydrophilic domain of the LPS molecule. The composition of the
side chain is highly variable between species and even strains of Gram-negative
bacteria. Although the toxic effects of LPS are mediated by the lipid A, the O
side chain may also contribute to variations in strain virulence, possibly by
influencing the hydrophilic properties of the whole molecule (Todar 2008). This
structural variation in O side chain may partially account for the difference in
pyrogenicity between various LPS serotypes, hindering the comparison between
studies done in different laboratories.

6.1.2 Polyinosinic: polycytidylic acid (poly I:C)

Compared to bacteriological or parasitic infections, maternal infections of
viral origin have been more frequently associated with SCZ in offspring. Now,
we are looking to determine if offspring behavioral changes occurred after
maternal exposure to a viral agent in our model. To ensure control over dose and time of administration and improve reproducibility of the immune response, use of a molecular immunogen was again preferred. We selected the viral mimic polyinosinic: polycytidylic acid (poly I:C), a synthetic double-stranded RNA that stimulates antiviral activities of the innate immune system. Similar to LPS, central or systemic administration of poly I:C results in the induction of the acute-phase reaction, fever, and sickness behavior in a variety of species, including mice, rabbits and guinea pigs, (Homan et al. 1972; Cooper et al. 1988; Kimura et al. 1994a; Kimura et al. 1994b; Toth 1996). Poly I:C was initially developed as an inducer of interferon (Magee and Griffith 1972; Manetti et al. 1995) and until the last decade, was rarely used outside this context. Thus, in contrast with infection models using LPS, in which the cytokine cascade leading to fever has been extensively studied (Luheshi and Rothwell 1996; Dantzer 2001), the immune response to poly I:C was poorly characterized, particularly in terms of febrile response and circulating cytokines. As the latter two factors are suspected to be part of the mechanism linking maternal infection and fetal alteration, it was crucial to characterize the pyrogenic and cytokine response to poly I:C before introducing it in our model.

6.1.3 Turpentine

The last immune stimulus used in my experiments was intramuscular administration of turpentine, which is a well characterized model of sterile inflammation. Even in small quantities it causes local tissue damage that activates the innate immune response through the action of cytokines (Kozak et al. 1998). Like LPS and poly I:C administration, a turpentine-induced abscess elicits a dramatic increases in body temperature, HPA axis activation, and sickness behavior (Cooper and Rothwell 1991). However, the pyrogenic response it induces is much more pronounced and the cytokine cascade that underlies it is slightly different (Luheshi et al. 1997; Kozak et al. 1998) (discussed in greater detail in chapter 4). In addition, contrary to molecular immunogens, it does not enter the systemic circulation. In a model of maternal infection, it could not affect
the fetus directly; hence, any effect observed in the offspring would be through an indirect mechanism, such as fever or circulating cytokines.

6.2 Behavioral outcomes

Trying to model every aspect of a disease as complex and human as SCZ in a simple rodent is an impossible task. The complex nature and heterogeneity of SCZ and other psychiatric disorders has prompted psychiatric geneticists to look for specific aspects of diseases, termed endophenotypes, that are more easily observed and quantifiable. This concept has been integrated into behavioral neuroscience. Thus, although it is not possible to mimic the complete disorder of schizophrenia in a rodent, it is possible to emulate some features of the disease in animals, such as deficits in PPI, working memory and social interaction. This comparison between animal and human behavior is based on the principle that the basic neural circuitry underlying these behaviors is conserved across species, though we must keep in mind that they are modulated by numerous brain regions (Powell and Miyakawa 2006). This fruitful approach to animal modeling allows us to test the plausibility of some environmental and genetic hypothesis on the etiology of the disease, as well as explore the pathways by which they could act. It also suffers from some limitations, the principal being that no behavioral phenotype is specific to a single psychiatric disorder. In our model, two of these behavioral phenotypes were selected to be the principal outcome measures: prepulse inhibition of acoustic startle (PPI) and amphetamine (AMPH)-induced locomotion.

6.2.1 Prepulse inhibition of acoustic startle (PPI)

Among behavioral outcomes investigated after prenatal immune treatment, prepulse inhibition of acoustic startle is particularly relevant to schizophrenia as it reveals deficits in sensory information gating believed to be an important feature of the illness in humans. PPI is a well-established sensorimotor gating paradigm defined as “a profound decrease in startle magnitude when the startling pulse is preceded by a weak prepulse” (Swerdlow et al. 1999). PPI deficits in schizophrenic patients are well documented (Braff et al. 2001; Ludewig et al. 2003; Kumari et al. 2004). However, they are not a diagnostic feature of SCZ,
since they might also be present in patients suffering from other psychiatric
diseases, such as obsessive compulsive disorder (Hoenig et al. 2005) and
Tourette’s syndrome (Swerdlow et al. 2001).

A major advantage of studying PPI in psychopathology research is that this
very robust phenomenon occurs in virtually all mammals and primates (Swerdlow
et al. 1999) and thus can be investigated in different species using similar
methods (Swerdlow et al. 2002). The primary mammalian acoustic startle circuit
consists of three synapses linking the auditory nerve with the spinal motor neuron
(Davis et al. 1982; Koch and Schnitzler 1997). The inhibitory effect of the
prepulse on startle reactivity is effected within the pons, where descending limbic
cortico-striato-pallido-pontine circuitry regulates the degree to which the prepulse
inhibits the subsequent startle response (Koch and Schnitzler 1997; Swerdlow and
Geyer 1998). PPI disruption by DA agonists has been extensively studied, and it
was shown that DA exerts its regulatory effects on PPI at least in part via the
ventral striatum and the frontal cortex (Koch and Hauber 1998; Swerdlow and
Geyer 1998), a brain circuitry that has also been implicated in the
pathophysiology of schizophrenia. However, the dopaminergic system is not the
sole neurotransmitter system regulating PPI, as administration of 5-HT\textsubscript{2} agonists
or NMDA antagonists can also affect this behavior (Geyer et al. 2001)

6.2.2 Amphetamine (AMPH)-induced locomotion

In order to investigate the relevance of our prenatal infection model to
positive symptoms of SCZ, the second behavioral measure chosen was AMPH-
induced locomotor activity. AMPH stimulates DA activity by increasing the
concentration of DA in the synaptic cleft through two possibly co-occurring
mechanisms: 1) redistribution of DA from synaptic vesicles to cytosol; 2) DA
reverse transport by the dopamine transporter (Sulzer et al. 1995; Sulzer et al.
2005). In healthy volunteers, high doses of AMPH can induce psychotic
episodes, whereas much lower doses are sufficient to produce the same effect in
schizophrenic patients (Lieberman et al. 1987). This may be due to the fact that
AMPH induces a greater DA release in the striatum of schizophrenic patients
compared to controls (Breier et al. 1997; Laruelle and Abi-Dargham 1999). An
increased locomotor response to AMPH in animals is viewed as a behavioral indicator of activity in the mesolimbic DA pathway (Porrino et al. 1984; Flagstad et al. 2004) and is thus an interesting parallel to clinical observations. A related measure is behavioral sensitization, in which repeated administration of AMPH or other psychostimulants in a rodent leads to an augmentation of its behavioral effects, which is termed behavioral sensitization. The amphetamine-sensitized state is believed to model some of the positive symptoms observed in SCZ patients. There is also increasing evidence for cognitive deficits in sensitized animals, especially in the area of attention and cognitive flexibility (Featherstone et al. 2007). Sensitization requires adaptations within mesocorticolimbic DA neurons originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens and prefrontal cortex (Vanderschuren and Kalivas 2000), regions thought to be involved in SCZ.
Table 2. Summary of the behavioral and neurochemical alterations observed in offspring following gestational immune activation. Studies published from 2002 to the present. Changes were observed in adult offspring (P60 to 400) unless specified otherwise.

<table>
<thead>
<tr>
<th>Postnatal changes</th>
<th>Species</th>
<th>Immunogen, Dose, Route</th>
<th>Time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exploratory behavior</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>↓ exploration in response to novelty</td>
<td>Mouse</td>
<td>Influenza, in</td>
<td>E9</td>
<td>(Shi et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Poly I:C, 5 mg/kg, ip</td>
<td>E12</td>
<td>(Smith et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Poly I:C, 2 or 5 mg/kg, iv</td>
<td>E9</td>
<td>(Meyer et al. 2005; Meyer et al. 2006b)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>LPS, 8 µg/kg, ip</td>
<td>E8-E15, daily</td>
<td>(Wang et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>LPS, 1 mg/kg, sc</td>
<td>E1-birth, alternate days</td>
<td>(Liu et al. 2004)</td>
</tr>
<tr>
<td>↓ rearing activity in open field</td>
<td>Mouse</td>
<td>Poly I:C, 5 mg/kg, ip</td>
<td>E12-E17</td>
<td>(Ozawa et al. 2006)</td>
</tr>
<tr>
<td>↑ entry in the center of enclosure</td>
<td>Mouse</td>
<td>LPS, 120µg/kg</td>
<td>E17</td>
<td>(Golan et al. 2006b)</td>
</tr>
<tr>
<td>↑ exploration of familiar vs novel object</td>
<td>Mouse</td>
<td>LPS, 300µg/kg, sc</td>
<td>E9</td>
<td>(Coyle et al. 2009)</td>
</tr>
<tr>
<td><strong>Response to psychostimulants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ locomotor activity in response to psychostimulant</td>
<td>Mouse</td>
<td>Poly I:C, 2 or 5 mg/kg, iv</td>
<td>E9</td>
<td>(Meyer et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Poly I:C, 4 mg/kg, iv</td>
<td>E15</td>
<td>(Zuckerman et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Poly I:C, 5 mg/kg, ip</td>
<td>E12-E17</td>
<td>(Ozawa et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>LPS, 50µg/kg, ip</td>
<td>E18-19</td>
<td>(Fortier et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>LPS, 1 mg/kg, ip</td>
<td>E10</td>
<td>(Ling et al. 2009)</td>
</tr>
<tr>
<td>↓ locomotor activity in response to psychostimulant in old animals (P480)</td>
<td>Rat</td>
<td>LPS, 1 mg/kg, ip</td>
<td>E10</td>
<td>(Ling et al. 2009)</td>
</tr>
<tr>
<td><strong>Sensorymotor gating</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>↓ PPI</td>
<td>Mouse</td>
<td>Influenza, in</td>
<td>E9</td>
<td>(Shi et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Poly I:C, 20 mg/kg, ip</td>
<td>E9</td>
<td>(Shi et al. 2003)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Poly I:C, 2 or 5 mg/kg, iv</td>
<td>E9 or E17</td>
<td>(Meyer et al. 2005; Meyer et al. 2008)</td>
<td></td>
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<tr>
<td>---------------</td>
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<td>-------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Poly I:C, 5 mg/kg, ip</td>
<td>E12-E17, daily</td>
<td>(Ozawa et al. 2006)</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Poly I:C, 60 mg/kg, ip</td>
<td>E9</td>
<td>(Makinodan et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Poly I:C, 5 mg/kg, ip</td>
<td>E12</td>
<td>(Smith et al. 2007)</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>IL-6, 9 µg/kg, ip</td>
<td>E12</td>
<td>(Smith et al. 2007)</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>LPS, 1 mg/kg, sc</td>
<td>E1-birth, alternate days</td>
<td>(Borrell et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>LPS, 2 mg/kg, sc</td>
<td>E1-birth, daily</td>
<td>(Romero et al. 2007; Romero et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>LPS, 50 and 100µg/kg, ip</td>
<td>E18-E19/E15-E16</td>
<td>(Fortier et al. 2007)</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Poly I:C, 4 mg/kg, iv</td>
<td>E15</td>
<td>(Wolff and Bilkey 2008)</td>
<td></td>
</tr>
</tbody>
</table>

**Improvement of PPI deficit following anti-psychotic administration**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Influenza, in</th>
<th>E9</th>
<th>(Shi et al. 2003)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>LPS</td>
<td>E1-birth, alternate days</td>
<td>(Borrell et al. 2002)</td>
</tr>
</tbody>
</table>

**Sensorimotor coordination**

<table>
<thead>
<tr>
<th>Rat</th>
<th>LPS, 200 µg/kg, ip</th>
<th>E17-birth, twice daily</th>
<th>(Girard et al. 2009)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>LPS, 8 µg/kg, ip</td>
<td>E17-birth, twice daily</td>
<td>(Wang et al. 2010)</td>
</tr>
</tbody>
</table>

**Learning and memory**

<table>
<thead>
<tr>
<th>Rat</th>
<th>Poly I:C, 4 mg/kg, iv</th>
<th>E15</th>
<th>(Zuckerman et al. 2003)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Poly I:C, 5 mg/kg, iv</td>
<td>E6, E9 or E13</td>
<td>(Meyer et al. 2005; Meyer et al. 2006a)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Poly I:C, 20 mg/kg, ip</td>
<td>E12</td>
<td>(Smith et al. 2007)</td>
</tr>
<tr>
<td>Mouse</td>
<td>IL-6, 9 µg/kg, ip</td>
<td>E12</td>
<td>(Smith et al. 2007)</td>
</tr>
<tr>
<td>Rat</td>
<td>Poly I:C, 4 mg/kg, iv</td>
<td>E15</td>
<td>(Zuckerman et al. 2003)</td>
</tr>
<tr>
<td>Condition</td>
<td>Species</td>
<td>Treatment</td>
<td>Time Points</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>---------</td>
<td>------------------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Impaired unconditioned stimulus pre-exposure effect (USPEE)(^1)</td>
<td>Mouse</td>
<td>Poly I:C, 2 or 5 mg/kg, iv</td>
<td>E6, E9, E13 or E17</td>
</tr>
<tr>
<td>Deficit in reversal learning</td>
<td>Mouse</td>
<td>Poly I:C, 5 mg/kg, iv</td>
<td>E17</td>
</tr>
<tr>
<td>Impaired object recognition</td>
<td>Mouse</td>
<td>Poly I:C, 5 mg/kg, ip</td>
<td>E12-E17</td>
</tr>
<tr>
<td>Impaired spatial memory:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- in water maze on P28</td>
<td>Rat</td>
<td>LPS, 500 µg/kg, ip</td>
<td>E19</td>
</tr>
<tr>
<td>- in water maze as adults</td>
<td>Rat</td>
<td>IL-6, 9 µg/kg, ip</td>
<td>E8,10,12 or</td>
</tr>
<tr>
<td>- in cued water maze</td>
<td>Mouse</td>
<td>Poly I:C, 5 mg/kg, iv</td>
<td>E16,18,20</td>
</tr>
<tr>
<td>- in radial arm maze</td>
<td>Mouse</td>
<td>LPS, 120µg/kg, ip</td>
<td>E9 or E17</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>LPS, 8 µg/kg, ip</td>
<td>E17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E8-E15, daily</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ ethanol preference and consumption</td>
<td>Rat</td>
<td>LPS, 1 mg/kg, sc</td>
<td>E1-birth, alternate days</td>
</tr>
<tr>
<td>↓ social behavior</td>
<td>Mouse</td>
<td>Influenza, in</td>
<td>E9</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>LPS, 120µg/kg, ip</td>
<td>E17</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Poly I:C, 5 mg/kg, ip</td>
<td>E12</td>
</tr>
<tr>
<td>Impaired male sexual behavior</td>
<td>Rat</td>
<td>LPS, 250 µg/kg, ip</td>
<td>E21</td>
</tr>
</tbody>
</table>

CRF: corticotropin-releasing factor; FGF: fibroblast growth factor; LI: latent inhibition; MBP: myelin basic protein; mPFC: medial prefrontal cortex; Nacc: nucleus accumbens; Shh: sonic hedgehog; SN: substantia nigra; TH: tyrosine hydroxylase.

\(^1\) LI and USPEE represents learning paradigm in which the associative history of a stimulus influences the stimulus’ associability in normal animals.
Chapter II: Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring
1. Preface

The rationale behind my thesis draws from observations in epidemiological research indicating that maternal infection during pregnancy increases the risk for psychiatric illnesses of neurodevelopmental origin such as schizophrenia (SCZ) and autism (Brown 2006). Infection caused by different types of pathogens have been implicated in these studies, suggesting that a common element would be inducing some changes in the developmental trajectory of the fetus. It has been hypothesized that this common element would be part of the maternal immune response to pathogens. Therefore, we wanted to know if maternal immune activation (MIA) by a molecular immunogen could alter neural development in rodents. More specifically, we wanted to determine if MIA could affect offspring behavior.

Though maternal infection is considered a risk factor for SCZ, most pregnancies complicated by infections do not result in neurological or psychiatric adverse effects. We hypothesized that an additional environmental challenge may interact with MIA to result in altered neurodevelopment. In epidemiological studies, obstetric complications are often cited as environmental risk factors for SCZ, in particular those associated with neonatal hypoxia (Cannon et al. 2002). Thus we set up an animal model to investigate whether MIA was sufficient to induce behavioral alterations in offspring by itself, and whether a subsequent period of anoxia would interact with the first insult to result in neurodevelopmental effects.
2. Manuscript

Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring

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Keywords: Behavior; Dopamine; Endotoxin; Perinatal; Hypoxia; Lipopolysaccharide; Maternal infection; Schizophrenia; Startle
Abstract

An increased incidence of schizophrenia has been associated with several perinatal insults, most notably maternal infection during pregnancy and perinatal hypoxia. This study used a rat model to directly test if maternal exposure to bacterial endotoxin (lipopolysaccharide, LPS) during pregnancy alters behaviors relevant to schizophrenia, in offspring at adulthood. The study also tested if postnatal anoxia interacted with gestational LPS exposure to affect behavior. At adulthood, offspring from dams administered LPS on days 18 and 19 of pregnancy showed significantly increased amphetamine-induced locomotion, compared to offspring from saline-treated dams. A period of anoxia on postnatal day 7 had no effect on amphetamine-induced locomotion and there was no interaction between effects of gestational LPS and postnatal anoxia on this behavior. Offspring from LPS-treated dams also showed enhanced acoustic startle responses as adults, compared to offspring from saline-treated dams. In offspring tested for pre-pulse inhibition (PPI) of acoustic startle and for apomorphine modulation of PPI, no effects of either gestational LPS or of postnatal anoxia and no interactions between LPS and anoxia were observed. It is concluded that maternal LPS exposure during pregnancy in the rat may be a useful model to study mechanisms responsible for effects of maternal infection on behaviors relevant to schizophrenia, in offspring.

1. Introduction

Substantial epidemiologic evidence points to in utero and perinatal insults as etiological factors in schizophrenia. In particular, increased incidence of schizophrenia has been observed following maternal infections with either viral or bacterial pathogens during pregnancy (e.g., Watson et al., 1984; Barr et al., 1990; O’Callaghan et al., 1994; Suvisaari et al., 1999; Brown et al., 2000). This suggests that infection during pregnancy might contribute to the pathophysiology of schizophrenia through adverse effects on brain development. In this context,
animal models may prove useful to directly test if infection during gestation produces long term changes in CNS functions relevant to schizophrenia.

Several groups have administered viruses to neonatal rodents shortly after birth to model effects of perinatal infection on later behavior (Solbrig et al., 1998; Rothschild et al., 1999; Engel et al., 2000; Pletnikov et al., 2002), while one recent study has administered human influenza virus to mice during gestation (on embryonic day 9.5, E9.5) resulting in offspring with deficits in social interaction and other behaviors relevant to schizophrenia (Shi et al., 2003). Modeling of bacterial infections during pregnancy has recently been approached by using maternal administration of lipopolysaccharide (LPS), a component of the cell wall of gram negative bacteria (Borrell et al., 2002). Systemic administration of LPS is a widely accepted model of infection, resulting in reproducible production of fever and induction of pro-inflammatory cytokines, the main chemical mediators of the host defense response to infection (Luheshi and Rothwell, 1996; Larson and Dunn, 2001). Compared to live pathogens, use of LPS confers control over time course and dose of endotoxin exposure, thus reducing potential variability and allowing identification of windows of vulnerability and dose related effects.

Therefore, the first aim of this study was to test if LPS administration to rat dams during pregnancy produces long term changes, in offspring, in behaviors relevant to schizophrenia. The behaviors tested were amphetamine (AMPH)-induced locomotion, prepulse inhibition (PPI) of acoustic startle and apomorphine disruption of PPI. In rodents, locomotion in response to low dose AMPH is routinely used as a behavioral indicator of activity in the mesolimbic dopamine (DA) pathway (Castall et al., 1977; Porrino et al., 1984), and there is now compelling in vivo imaging evidence for a dysregulation of presynaptic striatal dopaminergic function in the genesis of positive symptoms of schizophrenia (Laruelle et al., 1996, 1999; Breier et al, 1997; Abi- Dargham et al., 1998; Laruelle and Abi-Dargham, 1999). PPI is a form of sensorimotor gating that is conserved across species and is defined as an inhibition of the startle response
when a low intensity stimulus, the prepulse, precedes the startling stimulus. Deficits in PPI in schizophrenic compared to control subjects have been reproducibly observed in numerous studies (Braff et al., 2001). Strong evidence indicates that PPI is regulated by nucleus accumbens dopaminergic mechanisms, and disruption of PPI by DA agonists like apomorphine is a frequently used animal model of PPI deficits in schizophrenic subjects (Geyer et al., 2001).

In addition to maternal infection during pregnancy, obstetric complications, particularly those involving neonatal hypoxia at the time of labor and delivery, are also associated with an increased incidence of schizophrenia (McNeil et al., 2000). This raises the question as to whether there might be interactive or additive effects of maternal infection and perinatal hypoxia on CNS function. Support for such an interaction has been provided in animal models of brain injury, where prior exposure to LPS has been reported to alter the degree of damage incurred by a later hypoxic-ischemic insult, under some conditions. For example, in adult rats, LPS priming 3-4 days before middle cerebral artery occlusion provides protection against hypoxia-ischemia induced CNS injury, through a mechanism mediated by the cytokine, tumor necrosis factor-α (TNF-α) (Tasaki et al., 1997; Dawson et al., 1999). In contrast, LPS administered to immature rats 1–4 h before hypoxic-ischemic insult actually enhances neuronal cell damage (Eklind et al., 2001; Coumans et al., 2003). It is not known if LPS pre-exposure might interact with milder global hypoxic insult in immature brain to affect more subtle CNS functions, and whether this interaction may occur over a longer time interval of several days between insults. Thus, the second aim of this study was to test if gestational LPS and later global anoxic insult, at postnatal day 7, interact to affect behavior in adult offspring.

The experimental design of the current study consisted of administering either LPS or saline to rat dams on days 18 and 19 of pregnancy (E18, E19). On postnatal day 7 (P7), male offspring from these dams were placed in a chamber containing either 100% N₂ (anoxia) or air (air control) for 7 min. At adulthood,
The offspring were behaviorally tested for AMPH-induced locomotion, acoustic startle responses, PPI of startle and apomorphine disruption of PPI.

The epidemiology of schizophrenia suggests that the late 2nd trimester of human pregnancy may be a period of increased fetal vulnerability to maternal infection (Barr et al., 1990; O’Callaghan et al., 1994; Suvisaari et al., 1999; Brown et al., 2000). Rat CNS is developmentally less mature than is human, with rat brain at approximately postnatal day 7–14 estimated to be developmentally equivalent to newborn human brain (Romijn et al., 1991; Avishai-Eliner et al., 2002). Thus LPS should be administered near term in the pregnant rat (gestation length=22 days) to mimic late 2nd trimester infection in humans. However rats (and other species) have high mortality rates very near term (E20–E22) in response to doses of LPS that produce fever and no mortality in non-pregnant rats and in pregnant rats before E20 (Martin et al., 1995). Thus, in the current experiments LPS was administered on E18 and E19, to mimic an acute maternal infection during pregnancy. Anoxia was delivered on P7 since rat brain at this age is nearing the developmental stage of the human brain at birth, a time when humans experience labor and delivery complications involving hypoxia that are associated with increased schizophrenia. In addition, rat brain shows enhanced vulnerability to hypoxic damage at this early postnatal time (Slotkin et al., 1986; Jensen et al., 1991; Yager et al., 1996).

2. Materials and methods

2.1. Gestational LPS and postnatal anoxia treatment; experimental design

All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee. For gestational LPS treatment, timed pregnant Sprague–Dawley rats (Charles River, Quebec, Canada) were injected on day 18 and 19 of pregnancy as follows: 13 pregnant rats were injected
intraperitoneally with 50 µg/kg LPS (Sigma L2755 from Escherichia coli, serotype 0128:B12) and 10 were injected with saline (control), once daily. Offspring in groups receiving gestational LPS were thus derived from 13 separate dams, while those in groups receiving gestational saline were derived from 10 separate dams. 50 µg/kg LPS was chosen as a dose producing significant fever and induction of serum cytokines (interleukin-6, IL-6) in dose–response studies in non-pregnant rats (data not shown), and no reduction in survival when administered to pregnant rats on E18 and E19.

On the day of birth, a small quantity of indelible ink was injected into one of the paws of each pup to identify animals from different birth groups. Pups were cross-fostered with surrogate dams in mixed litters. Only male pups were retained for the study. On P7, half of the pups born from both LPS-treated mothers and saline-treated mothers were subjected to anoxia. For this, each pup was placed for 7 min in a chamber containing 100% N$_2$ (flow rate=9 liters per min) at 34ºC. Pups were given a 10 min recovery period before being returned to their foster dam. The other half of the pups (from both LPS-treated and saline-treated mothers) received control treatment on P7; this consisted of placing the pup for 7 min in a chamber with ambient air at 34ºC, instead of N$_2$. Pups were weaned at 21 days of age and grown to adulthood. Animals were group housed (two animals/cage) in random treatment combinations and maintained on a 12 h:12 h light:dark cycle (lights on at 8:00 h) with free access to food and water. Behavioral testing of these offspring began at 70 days of age. For behavioural testing, all animals were first tested for baseline PPI of acoustic startle. One week later the animals were tested for AMPH-induced locomotion. The next week the animals were tested for PPI after receiving a saline injection, and one week later they were tested for PPI after apomorphine administration.

2.2. Average startle response (ASR) and prepulse inhibition (PPI) of acoustic startle
Startle reactivity was measured using two SR-LAB startle apparatuses (San Diego Instruments, San Diego, USA). Each sound attenuated startle chamber contained a clear Plexiglas cylinder resting on a piezoelectric transducer that detected the vibrations caused by the movements of the animals. A computer control unit stored startle responses and controlled the timing and presentation of acoustic stimuli. A SR-LAB calibration unit was used to produce consistent response sensitivity across chambers and trials. Testing took place between 9:00 and 17:00 h. Each test session began with a 5 min acclimatization period in the presence of 70 dB white noise, which continued throughout the session. One orienting pulse alone trial (120 dB for 30 ms) was then presented, which was excluded from the data analysis. Next, 11 pulse alone trials (120 dB for 30 ms), five null trials with no stimulus, and five prepulse+pulse trials at each of five different prepulse intensities were presented, in a pseudo-random order and with an average inter-trial interval of 17 s (range: 9–29 s). The prepulse+pulse trials consisted of a 30 ms prepulse (at 73, 76, 79, 82 or 85 dB), followed by a 70 ms delay, then a startle pulse (120 dB, 30 ms). The testing session lasted 15 min. When assessing effects of saline injection or of apomorphine on PPI, saline or apomorphine (0.18 mg/kg) was injected 10 min before the animal was placed in the startle apparatus and tested for PPI using the protocol described above. For each animal, a background startle value (average of the 5 null trials) was subtracted from the startle amplitude of the pulse alone and prepulse+pulse trials before further calculations were performed. Prepulse inhibition is expressed as %PPI, defined as (1-[startle amplitude on prepulse+pulse trial/mean startle amplitude on pulse alone trials])x100.

2.3. Amphetamine (AMPH)-induced locomotion

Locomotor activity was monitored using 11 activity chambers (30x40x40 cm), each equipped with two parallel infrared light beams aligned with photoelectric switches. The consecutive interruption of the two beams was recorded as one locomotor act. Each test session began with 1h of habituation,
followed by saline injection and another hour of recording. D-amphetamine sulphate (0.5 mg/kg) was then injected subcutaneously and locomotor activity was recorded for an additional 2h. Data are presented as locomotor acts (photocell counts) summed for each successive 10 min interval.

2.4. Data analysis

Data analyses for baseline ASR and for effects of apomorphine on ASR were performed using two-way analysis of variance (ANOVA) with gestational LPS treatment and postnatal anoxia as between-subject factors. Data for baseline PPI and PPI modulated by apomorphine were analyzed using three-way ANOVA, with LPS treatment and anoxia as between-subject factors, and prepulse intensity as a within-subject repeated measure. Similarly, data analyses for locomotor activity were performed using three-way ANOVA, with LPS treatment and anoxia as between-subject factors, and time as a within-subject repeated measure. Separate analyses were performed on data for baseline locomotion during habituation, for locomotion following saline and for locomotion after AMPH administration. Post-hoc Newman–Keuls tests were performed, where indicated, to assess differences between treatments at one specific time-point or prepulse intensity.

2.5. Plasma Interleukin-6 (IL-6) levels

The cytokine, IL-6, is an important mediator of fever and readily detectible in the circulation of febrile animals in response to systemic administration of LPS (Cartmell et al., 2000). In the present study, plasma IL-6 levels were measured to confirm that the dose of LPS administered to pregnant rat dams effectively elicited a cytokine response. Pregnant rat dams received injections of LPS (50 µg/kg, n=7) or saline (n=5) on E18 and on E19. Three hours after the last LPS or saline injection, animals were rapidly decapitated and a sample of trunk blood was collected into sterile tubes containing pyrogen-free heparin (10 U/ml). Samples
were centrifuged (5300 g, 4°C, 10 min) and plasma stored at -70°C until assayed. Plasma was analyzed for IL-6 using a rat specific ELISA (kindly provided by Dr. Stephen Poole, National Institute for Biological Standards and Control, NIBSC, UK) as described previously (Rees et al., 1999; Cartmell et al., 2000). Intra- and interassay coefficients of variability for the IL-6 assay were <5% and the detection limit was 20 pg/ml.

3. Results

The design of this study generated 4 experimental groups of offspring, i.e., those receiving gestational LPS+postnatal anoxia (LPS+anoxia group, n=14), gestational LPS+postnatal air (LPS group, n=18); gestational saline+postnatal anoxia (Anoxia group, n=9) or gestational saline+postnatal air (Control group, n=13). At adulthood, these offspring were behaviorally tested for amphetamine-induced locomotion, acoustic startle responses, prepulse inhibition (PPI) of startle and apomorphine disruption of PPI.

3.1. Survival, plasma interleukin-6, gestation length, and weight

Plasma IL-6 levels were measured to confirm that LPS elicited a cytokine response in pregnant rats. Table 1 shows that dams injected with LPS on E18 and E19 had significantly higher levels of plasma IL-6 on E19, in comparison to saline-injected dams.

Dams injected with LPS on E18 and E19 exhibited clear signs of sickness-type behaviors including decreased locomotor activity, piloerection and general malaise for up to 12h post injection. These are typical responses to endotoxin exposure in rats. Survival of dams treated with either LPS or saline during gestation was 100%. Gestation length was similar for LPS- and saline treated dams (Table 1), indicating that this dose of LPS does not induce premature labor. However the percentage of stillborn pups was somewhat greater from LPS-treated
(6/142=4.2%) compared to saline treated dams (1/124=0.8%). In preliminary experiments, we determined that 7 min of anoxia (100% N\textsubscript{2}) on P7 was the length of anoxia at which considerable acute mortality occurred. Exposure of pups to 7 min of anoxia on P7 resulted in the death of 17.9% (5/28) of the pups on the day of treatment, while no air-treated pups (0/31) died on P7. For animals who lived until P8, long term survival until adulthood was 100% in all four experimental groups. There were no significant group differences in body weight of offspring from birth through to adulthood (Table 1b).

3.2. Amphetamine-induced locomotion in adult offspring

On the day of locomotor testing, the four experimental groups were tested for baseline locomotion (1 h), followed by locomotor responses to saline (1 h) and to low dose (0.5 mg/kg) AMPH (2 h). Three way ANOVA of baseline locomotion revealed a significant LPS x time interaction (F\textsubscript{5,245} = 2.476, P=0.033), but no significant effect of anoxia (F\textsubscript{1,49} = 0.863, P=0.357) and no significant interactions of anoxia with LPS or time (Anoxia x LPS: F\textsubscript{1,49} = 1.489, P=0.228; Anoxia x time: F\textsubscript{5,245} = 0.130, P=0.985; Anoxia x LPS x time: F\textsubscript{5,245} = 0.456, P=0.809). Three way ANOVA of saline-induced locomotion showed no significant effects of LPS (F\textsubscript{1,49} = 2.278, P=0.138) or of anoxia (F\textsubscript{1,49} = 0.093, P=0.761) and no interactions (LPS x time: F\textsubscript{5,245} = 0.314, P=0.904; Anoxia x time: F\textsubscript{5,245} = 0.818, P=0.538; Anoxia x LPS: F\textsubscript{1,49} = 0.964, P=0.331; Anoxia x LPS x time: F\textsubscript{5,245} = 1.057, P=0.385). Three way ANOVA of locomotion in response to low dose (0.5 mg/kg) AMPH also revealed a significant LPS x time interaction (F\textsubscript{11,495} = 2.441, P=0.006), but no significant effect of anoxia (F\textsubscript{1,45} = 0.014, P=0.908) and no interactions of anoxia with LPS or time (Anoxia x LPS: F\textsubscript{1,45} = 0.076, P=0.783; Anoxia x time: F\textsubscript{11,495} = 0.558, P=0.862; Anoxia x LPS x time: F\textsubscript{11,495} = 1.285, P=0.229).

Since there were no significant interactions between gestational LPS and postnatal anoxia, effects of these two treatments are shown separately in Figs.1
and 2. Fig.1 shows the effects of gestational LPS on locomotion in the subsample of offspring that were treated with air (control treatment) on P7 (omitting those treated with anoxia on P7). Offspring from LPS-treated dams showed significantly greater stimulation of locomotor activity at 30, 40 and 50 min after AMPH administration (t=150, 160 and 170 min in Fig.1), compared to offspring from saline-treated dams. When data from the total sample of offspring (i.e. those treated with both anoxia and air at P7) were included in the analysis of effects of gestational LPS, a similar result was obtained, i.e. offspring from LPS-treated dams again showed significantly greater locomotion at 30 (P<0.01), 40 (P<0.01) and 50 (P<0.05) min after AMPH administration, compared to offspring from saline-treated dams (data not shown).

Fig.2 shows that anoxia on P7 had no significant effect on locomotor activity at adulthood. The figure shows effects of postnatal anoxia in the subsample of offspring treated with saline (control treatment) during gestation (omitting those treated with LPS during gestation). For baseline, saline-induced and AMPH-induced locomotor activity, there were no significant differences between anoxia- and air-treated animals. Similarly no effects of postnatal anoxia on locomotion were found, when the analysis included the total sample of offspring treated with both LPS and saline during gestation (data not shown).

Three way ANOVA of data for baseline acoustic startle responses (ASR) indicated a significant effect of trial ($F_{10,490}=24.045$, $P<0.0001$) across all experimental groups. Post hoc analysis of the trial effect revealed that ASRs during trials 1, 2 and 3 were significantly different from ASRs during trials 4–11 (all Ps<0.0001), and that there were no significant differences in ASR within trials 4–11 (all Ps>0.579) (Fig.3a). Thus it appeared as if all experimental animals required an habituation of three startle trials in order to attain stable and reproducible ASR across the next eight trials (trials 4–11). Therefore in subsequent analyses of startle response, we discarded data from the first three startle trials, and performed analysis on average ASR from trials 4–11 (similarly
for analyses of PPI data in Section 3.4, we discarded data from prepulse+pulse trials that were intercalated between the first three startle trials. This resulted in the elimination of one trial at each of the five prepulse intensities, and average results from the four remaining trials at each prepulse intensity were included in the analyses).

Using average ASR from the last eight startle trials (trials 4–11) as the measure of ASR for each animal, two way ANOVA on baseline ASR data showed a significant effect of LPS (F1,49=4.622, P=0.037), but no effect of anoxia (F1,49=0.025, P=0.876) and no LPS x anoxia interaction (F1,49=0.011, P=0.917). All offspring receiving gestational LPS showed significantly increased baseline ASRs, compared to those receiving saline injections during gestation (Fig.3a and b). Anoxia on P7 had no effect on ASR at adulthood.

Apomorphine significantly (P<0.01) enhanced ASR in all experimental groups of animals. For ASR after apomorphine administration, two way ANOVA showed no significant effect of LPS (F1,53=1.361, P=0.249) or of anoxia (F1,53=0.053, P=0.818) and no LPS x anoxia interaction (F1,53=0.181, P=0.672). Thus gestational LPS and postnatal anoxia had no effects on apomorphine-induced ASR in adult offspring (Fig.3c).

3.4. PPI of acoustic startle in adult offspring

Three way ANOVA of data for baseline PPI of acoustic startle showed no significant effects of LPS (F1,49=0.004, P=0.950) or of anoxia (F1,49=2.249, P=0.140) and no interactions (LPS x prepulse intensity: F4,196=1.188, P=0.317; Anoxia x prepulse intensity: F4,196=0.894, P=0.468; Anoxia x LPS: F1,49=0.019, P=0.891; Anoxia x LPS x prepulse intensity: F4,196=0.564, P=0.689). Thus both gestational LPS and postnatal anoxia were without effect on PPI in adult offspring, under conditions of the present experiment, and the two insults did not interact to affect PPI (Table 2a).
Three way ANOVA of data for PPI after apomorphine showed no significant effects of LPS (F\(_{1,51}=1.077, P=0.304\)) or of anoxia (F\(_{1,51}=0.968, P=0.330\)) and no interactions (LPS×prepulse intensity: F\(_{4,204}=0.456, P=0.768\); Anoxia × prepulse intensity: F\(_{4,204}=0.874, P=0.480\); Anoxia × LPS: F\(_{1,51}=1.513, P=0.224\); Anoxia × LPS × prepulse intensity: F\(_{4,204}=1.053, P=0.381\)). The low dose of apomorphine used significantly (P<0.05) inhibited PPI only at the 81 dB prepulse intensity, for all experimental groups. Thus, both gestational LPS and postnatal anoxia were without effect on apomorphine modulation of PPI in adult offspring (Table 2b).

4. Discussion

The main finding of this study is that administration of bacterial endotoxin to rat dams on days 18 and 19 of pregnancy produces long-term enhancement of AMPH-induced locomotion and of acoustic startle responses in the resulting offspring. Pharmacologic lesioning and imaging studies indicate that locomotion in response to low dose AMPH in the rat largely reflects activity in the midbrain-ventral striatal (nucleus accumbens) DA pathway (Castall et al., 1977; Porrino et al., 1984). There is substantial evidence that dysregulation of subcortical DA function plays a critical role in the pathophysiology of schizophrenia. For example, drugs like AMPH, which enhance DA transmission, can exacerbate psychotic symptoms in schizophrenic subjects and induce a schizophrenia-like syndrome with chronic use in normals (reviewed by Yui et al., 1999). More recently, in vivo imaging studies have provided more direct evidence that enhanced subcortical DA activity may contribute to positive symptoms of schizophrenia. These studies demonstrated that, compared to controls, untreated schizophrenic subjects show enhanced striatal DA release in response to AMPH, that correlates with worsening of psychotic symptoms (Laruelle et al., 1996, 1999; Breier et al., 1997; Abi-Dargham et al., 1998; Laruelle and Abi-Dargham, 1999). Our findings with gestational LPS in the rat model support the idea that maternal...
infection during pregnancy could contribute to the genesis of such subcortical dopaminergic overactivity in schizophrenia.

While gestational LPS increased acoustic startle responses in adult offspring, this insult was without effect on PPI of startle or on apomorphine modulation of PPI. Enhanced ASR (i.e. deficits in habituation of ASR) in schizophrenic subjects compared to controls has been reported in several studies, particularly in paradigms utilizing a series of pulse alone trials (with no pre-pulse trials) to determine habituation (Geyer and Braff, 1982; Bolino et al., 1992; Braff et al., 1992; Parwani et al., 2000). The finding of deficient habituation of ASR appears, however, to be less robust than is the finding of PPI deficits in schizophrenia (Braff et al., 2001).

In experiments in which LPS (1 mg/kg) was administered to pregnant rats every second day throughout pregnancy, Borrell et al. (2002) have recently reported disrupted PPI of startle using either an acoustic prepulse of 55dB or a photic prepulse, in adult offspring. Differences in timing or dosage of LPS exposure may account for the differing behavioral alterations in their study versus ours (i.e., reduced PPI versus enhanced AMPH-induced locomotion and ASR). LPS administered on E18 and E19 may more closely mimic an acute second trimester infection in humans. The model of Borrell et al. may approximate a chronic infection during the first and second trimester of pregnancy, although tolerance to repeated LPS is known to develop (West and Heagy, 2002). Enhanced AMPH-induced release of striatal DA appears to be associated with exacerbations of psychotic symptomatology (Laruelle and Abi-Dargham, 1999), while PPI deficits correlate with cognitive symptoms such as thought disorder and distractibility (Karper et al., 1996; Perry et al., 1999; Braff et al., 2001). Thus, comparison of our findings to those of Borrell et al. suggest that it may be possible to model differing aspects of the schizophrenic phenotype by administering LPS during different times of gestation in the rat. In addition, the current study employed a paradigm of cross-fostering of pups with untreated
surrogate dams, while animals in the study by Borrell et al. appear to have been raised postnatally by their original LPS- or saline-treated mothers. Differential postnatal maternal care may have contributed to behavioral findings in the latter study, since maternal deprivation has been reported to have long term effects on both PPI and AMPH-induced locomotion in offspring (Zimmerberg and Shartrand, 1992; Matthews et al., 1996; Ellenbroek and Cools, 2002; Meaney et al., 2002).

LPS administration induces production of pro-inflammatory cytokines, most prominently IL-1β, IL-6 and TNF-α, which collectively mediate defence responses to systemic infection including fever, hypophagia, enhancement of slow wave sleep, sickness behavior and glucocorticoid secretion, primarily via direct actions in the CNS (Luheshi and Rothwell, 1996; Larson and Dunn, 2001). In addition to affecting adult CNS function, cytokines have multiple effects on developing neurons, making them prime candidates as mediators of effects of maternal infection on fetal brain development. For example, LPS, IL-1, IL-6 and/or TNF-α influence conversion of mesencephalic progenitor cells into differentiated DA neurons and survival of embryonic dopaminergic and serotonergic neurons in vitro (von Coelln et al., 1995; Jarskog et al., 1997; Ling et al., 1998). Cultured DA neurons are selectively injured by low doses of LPS that have no effect on developing hippocampal or cortical neurons (Kim et al., 2000). IL-1β has also been reported to regulate expression of neurotrophic molecules, such as brain-derived neurotrophic factor, a potent regulator of the survival and differentiation of DA neurons, as well as nerve growth factor (Lapchak et al., 1993; Heese et al., 1998). In our study, pregnant rat dams administered LPS on E18/19 had increased levels of plasma IL-6, confirming that the dose of LPS used elicited a cytokine response in these animals. LPS administered to rat dams on E18 has also been shown to increase IL-1 β and TNF-α mRNA in fetal brain (Cai et al., 2000), while increased IL-1 β, IL-6 and TNF-α in the placenta and a small decrease in fetal brain TNF-α have been reported following LPS on E16 (Urakubo et al., 2001). Independent of other effects of
cytokines, LPS-induced fever might also affect brain development. For example, heat stress early in gestation has been reported to produce gross CNS anomalies in developing rodents (Mottola et al., 1993; Yitzhakie et al., 1999).

In addition to affecting development of dopaminergic systems, prenatal LPS may lead to lasting dysregulation of cytokine function in offspring as adults, and resulting dysregulation of cytokine-DA interactions in adult brain. In this context, Borell et al. (2002) reported that LPS administration throughout pregnancy results in offspring with increased serum IL-6 as adults. Elevated IL-6 levels may alter DA function since systemic IL-6 administration in adult rats has been reported to alter central DA turnover and enhance AMPH-induced locomotor activity (Zalcman et al., 1994, 1999; Song et al., 1999). Another possible mechanisms by which maternal LPS might increase AMPH-induced locomotion in offspring is by decreasing AMPH metabolism due to LPS effects on liver drug metabolizing enzymes or other processes in offspring, although this mechanism would not account for effects of prenatal LPS on ASR.

In the current study, 7 min of anoxia on P7 had no effect on baseline or AMPH-induced locomotion and no effect on PPI or apomorphine modulation of PPI, at adulthood. This finding is in agreement with previous reports on behavioral effects of postnatal anoxia in the rat. Postnatal anoxia administered on P1 (Speiser et al., 1983), P2 (Iuvone et al., 1996), P4 (Shimomura and Ohta, 1988), P5 or P10 (Jensen et al., 1992) has been reported to produce transient baseline hyperactivity in rats as adolescents but no lasting effects on locomotion in adulthood. With repeated anoxic insults (at P2 and P6) or chronic mild (P2–P6) or episodic (P7–P11) hypoxia, hypoactivity or hyperactivity have been observed at adulthood (Lun et al., 1990; Nyakas et al., 1991; Decker et al., 2003). None of these previous studies have reported on AMPH-induced locomotion or PPI, although spatial and working memory deficits at adulthood have been observed in some models of postnatal anoxia (Iuvone et al., 1996; Decker et al., 2003). In contrast to the lack of effect of acute postnatal anoxia, we have
previously reported that the subtle insult of Caesarean section (C-section) birth is sufficient to produce lasting increases in AMPH-induced locomotion in rats and guinea pigs (El-Khodor and Boksa, 1998; Vaillancourt and Boksa, 1998, 2000). Addition of 15 min of anoxia to the C-section procedure did not exacerbate or ameliorate the C-section-induced effect on AMPH-induced locomotion in the rat. However in the guinea pig, a model with greater CNS maturity than the rat, C-section with 1–2 min of additional birth anoxia resulted in further changes in AMPH-induced locomotion, compared to C-section alone. Thus it appears as if dopaminergic systems might exhibit greater susceptibility to long term dysregulation by subtle insults during pregnancy and at birth (gestational LPS, C-section birth, birth anoxia), compared to relative resistance to acute postnatal anoxia.

We observed no interaction between effects of gestational LPS and postnatal anoxia on behavior at adulthood. Thus pre-exposure to LPS during gestation did not sensitize the animals to effects of postnatal anoxia at P7, and effects of gestational LPS were neither exacerbated nor ameliorated by later exposure to postnatal anoxia. LPS pre-exposure 1–4 h before hypoxic ischemic or excitotoxic insult has been reported to exacerbate neuronal injury in immature rats (Lee et al., 2000; Eklind et al., 2001; Coumans et al., 2003). Together with our findings, this suggests that the sensitizing effects of LPS exposure may be only short-lived, and that LPS and anoxia might have interactive effects on immature brain only when administered in close temporal proximity.

In conclusion, maternal exposure to bacterial endotoxin during pregnancy resulted in long term changes in behaviors relevant to schizophrenia, while postnatal anoxia on P7 was without effect and the two insults did not interact. Animal modeling of obstetric insults may contribute to an understanding of the types of complications that have the most pronounced impact on specific CNS systems and their mechanisms of action.
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Figures and legends

![Graph showing effects of LPS during gestation on AMPH-induced locomotion](image)

**Fig. 1. Effects of LPS during gestation on AMPH-induced locomotion in adult offspring.** Rat dams were administered LPS (50 µg/kg) or saline on days 18 and 19 of pregnancy. On postnatal day 7 (P7), their offspring were exposed to either 100% N₂ (anoxia) or air (air control) for 7 min. At adulthood, offspring were tested for baseline locomotion, followed by locomotor responses to saline and to low dose (0.5 mg/kg) AMPH. Since no significant interactions between LPS+anoxia were observed, the figure shows the effects of gestational LPS on locomotion in the subsample of offspring treated with air (control treatment) at P7. Asterisks denote values significantly different from saline during gestation at P<0.05 (*) and P<0.01 (**). LPS during gestation significantly enhanced AMPH-induced locomotion in adult offspring.
Fig. 2. Effects of postnatal anoxia on AMPH-induced locomotion in adult offspring. Rats were administered LPS or saline during gestation, followed by anoxia or air on postnatal day 7. At adulthood, they were tested for locomotor responses at baseline and in response to saline and to low dose (0.5 mg/kg) AMPH. Since no significant interactions between LPS+anoxia were observed, the figure shows the effects of postnatal anoxia on locomotion in the subsample of offspring treated with saline (control treatment) during gestation. Postnatal anoxia had no significant effect on AMPH-induced locomotion in adult offspring.
Fig. 3. Effects of LPS during gestation and of postnatal anoxia on baseline and apomorphine-induced acoustic startle responses (ASR) in adult offspring. Rats were administered LPS or saline during gestation, followed by anoxia or air on postnatal day 7, and were tested for acoustic startle responses at adulthood. (a) shows baseline ASRs across 11 successive trials in the four experimental groups, i.e., animals receiving gestational LPS+postnatal anoxia (LPS+Anoxia), gestational LPS+postnatal air (LPS); gestational saline+postnatal anoxia (Anoxia) or gestational saline+ postnatal air (Control). For all experimental groups, ASRs during trials 1, 2 and 3 were significantly different.
from ASRs during trials 4–11 (Ps<0.0001). (b) shows average baseline ASR (from trials 4–11) for each of the four experimental groups. *=significantly different at P<0.05. LPS during gestation significantly increased ASR at adulthood. Postnatal anoxia had no effect on ASR and there was no interaction between LPS+anoxia.(c) shows average apomorphine-induced ASR (from trials 4–11) for each of the four experimental groups. LPS during gestation and postnatal anoxia had no significant effects on apomorphine-induced ASR.

Tables

Table 1. (a) Maternal plasma interleukin-6 levels, length of gestation and (b) offspring weights after treatment with gestational lipopolysaccharide (LPS) and/or postnatal anoxia

(a) Gestational LPS Gestational saline

| Interleukin-6 levels in maternal plasma (pg/ml ±SEM) | 1846±217 (n=7)* | 136±41 (n=5) |
| Gestation length (mean # of days ±SEM) | 22.6±0.1 (n=13) | 22.1±0.1 (n=10) |

(b) Weight (g) ±SEM of offspring on postnatal day:

<table>
<thead>
<tr>
<th></th>
<th>P1</th>
<th>P7</th>
<th>P10</th>
<th>P70</th>
<th>P100</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS+Anoxia</td>
<td>6.5±0.2</td>
<td>16.0±0.4</td>
<td>20.4±0.4</td>
<td>434.6±12.2</td>
<td>520.0±12.0</td>
</tr>
<tr>
<td>LPS</td>
<td>6.3±0.3</td>
<td>16.1±0.5</td>
<td>20.9±0.4</td>
<td>428.0±7.1</td>
<td>519.4±9.1</td>
</tr>
<tr>
<td>Anoxia</td>
<td>6.3±0.3</td>
<td>15.8±1.0</td>
<td>20.1±0.9</td>
<td>426.7±12.4</td>
<td>528.3±15.8</td>
</tr>
<tr>
<td>Control</td>
<td>6.7±0.3</td>
<td>15.6±1.1</td>
<td>20.6±0.7</td>
<td>425.7±8.0</td>
<td>512.9±8.6</td>
</tr>
</tbody>
</table>

Rat dams were administered LPS (50 µg/kg) or saline on days 18 and 19 of pregnancy. On postnatal day 7 (P7), their offspring were exposed to either 100% N₂ (anoxia) or air (air control) for 7 min. Offspring were then grown to adulthood. Using a separate cohort of dams, IL-6 was measured in maternal blood samples taken 3 h after the last LPS or saline injection.

* Significantly different from values for saline-treated dams at P<0.001.
Table 2. Effects of LPS during gestation and of postnatal anoxia on baseline pre-pulse inhibition (PPI) of acoustic startle (a) and on PPI after apomorphine (b), in adult offspring

(a) Baseline PPI

<table>
<thead>
<tr>
<th>% PPI at pre-pulse intensity of</th>
<th>73dB</th>
<th>76dB</th>
<th>79dB</th>
<th>82dB</th>
<th>85dB</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS+Anoxia</td>
<td>29.1±7.1</td>
<td>42.0±5.2</td>
<td>60.4±5.1</td>
<td>69.3±4.1</td>
<td>77.0±3.9</td>
</tr>
<tr>
<td>LPS</td>
<td>15.9±8.2</td>
<td>41.1±6.1</td>
<td>55.8±5.6</td>
<td>60.5±7.1</td>
<td>72.4±7.8</td>
</tr>
<tr>
<td>Anoxia</td>
<td>45.1±8.0</td>
<td>56.7±7.4</td>
<td>70.7±9.1</td>
<td>71.4±7.8</td>
<td>78.4±8.6</td>
</tr>
<tr>
<td>Control</td>
<td>15.4±7.2</td>
<td>48.1±5.9</td>
<td>64.2±5.5</td>
<td>70.5±3.0</td>
<td>72.0±4.2</td>
</tr>
</tbody>
</table>

(b) PPI after apomorphine

<table>
<thead>
<tr>
<th>% PPI at pre-pulse intensity of</th>
<th>73dB</th>
<th>76dB</th>
<th>79dB</th>
<th>82dB</th>
<th>85dB</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS+Anoxia</td>
<td>37.4±10.3</td>
<td>23.1±10.6</td>
<td>57.8±7.1</td>
<td>72.7±6.2</td>
<td>84.9±4.7</td>
</tr>
<tr>
<td>LPS</td>
<td>36.5±8.4</td>
<td>23.5±7.6</td>
<td>61.2±4.0</td>
<td>73.5±4.6</td>
<td>84.0±2.3</td>
</tr>
<tr>
<td>Anoxia</td>
<td>44.3±10.1</td>
<td>39.9±7.9</td>
<td>52.1±9.6</td>
<td>71.1±5.8</td>
<td>80.2±4.4</td>
</tr>
<tr>
<td>Control</td>
<td>37.4±9.8</td>
<td>4.1±10.7</td>
<td>48.0±7.6</td>
<td>59.7±9.2</td>
<td>76.5±3.8</td>
</tr>
</tbody>
</table>

Rats were administered LPS or saline during gestation, followed by anoxia or air on postnatal day 7. They were tested for baseline PPI and apomorphine (0.18 mg/kg) modulation of PPI at adulthood. Gestational LPS and postnatal anoxia alone or in combination had no significant effects on either baseline PPI or PPI after apomorphine.
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Iuvone L, Geloso MC, Dell’Anna E. Changes in open field behavior, spatial memory, and hippocampal parvalbumin immunoreactivity following enrichment in rats exposed to neonatal anoxia. Experimental Neurology 1996;139:25–33.


3. Conclusion

This first experiment revealed that LPS exposure during gestation by itself was sufficient to alter offspring behavior, a fact that had not yet been well established at the time this study was completed. Contrary to our expectations, exposure to anoxia on postnatal day 1 or 7 had no additive or interactive effects with gestational LPS on the behaviors tested. As mentioned in the discussion, closer proximity in time seems to be necessary to observe an interaction between the two insults. In most cases, LPS pre-exposure seems to increase the damages induced by hypoxia (Wang et al. 2009), although at low doses, it may be protective (Lin et al. 2009). Regardless, as we did not observe any effects of anoxia in our experiment, we decided not to investigate the effects of anoxia further.

A limitation of this experiment was the fact that all the pups received an anoxia or air control treatment, which introduced neonatal handling as a confounding variable. This may partly explain why, contrary to many other studies (Shi et al. 2003; Ozawa et al. 2006; Smith et al. 2007; Wolff and Bilkey 2008; Makinodan et al. 2008; Meyer et al. 2008c), we did not observe a significant PPI disruption, although there was a tendency for decreased PPI at 76, 79 and 82dB. Accordingly, in chapter IV, we observed a significant PPI disruption in MIA-exposed offspring following a similar protocol, but without the confounding effects of the anoxia/air treatment.
Chapter III: Model of maternal immune activation using chronic LPS exposure via osmotic pump
1.1 Introduction

The overall aim of this project was to develop an animal model allowing us to study the effects of the maternal response to an immunogen during pregnancy on offspring nervous function, in particular their behavior. The experiment detailed in the preceding chapter indicated that maternal immune activation (MIA) by lipopolysaccaride (LPS) administration alone can alter adult offspring behavior, namely amphetamine (AMPH)-induced locomotion. We were then interested to know if some aspects of the maternal immune challenge, such as the type of immunogen, duration and gestational timing of exposure, may have a critical impact on offspring behavioral changes. The first aspect we explored was the effect of the duration of exposure to the maternal immune response. We examined if a maternal immune response of longer duration resulted in more severe or simply different behavioral alterations in the offspring. To elicit this longer immune response, we decided to deliver LPS via a subcutaneously implanted osmotic minipump.

When using molecular immunogens in physiological research, it is important to take into account the development of tolerance to their immune effects. Immune tolerance is characterized by a transient state of low responsiveness to pyrogen bioactivity, i.e. a decreased production of pro-inflammatory cytokines and subsequent attenuated fever in response to repeated exposure to LPS or other exogenous pyrogen. Tolerance to repeated injection of LPS has been documented in many species such as rabbits, guinea pigs and rats (Yamashiro et al. 1993; Zeisberger and Roth 1998). However, tolerance to chronic LPS exposure in rodents, such as delivery by minipump, had not been studied. Consequently, before using minipump delivery in our model of MIA, we wanted to evaluate the duration of the immune response to LPS administered via this method. In the first experiment of this chapter, we measured the duration of the immune response to LPS delivered via minipumps. We also compared it to the duration of the immune response elicited by repeated LPS injections at 24h intervals, such as used in chapter II. In the second experiment of the chapter, we
investigated the behavioral changes in offspring induced by chronic prenatal LPS exposure via osmotic minipump.

**EXPERIMENT I: DEVELOPMENT OF TOLERANCE TO THE PYROGENIC EFFECTS OF LPS DELIVERED BY OSMOTIC MINIPUMP OR BY REPEATED INJECTION.**

**1.2 Methods**

Adult male Sprague-Dawley rats (275-300g) were used in this experiment. Core body temperature was monitored in freely moving animals by remote biotelemetry using small battery-operated, temperature-sensitive radio transmitters (DataScience, St. Paul, MN), which were previously implanted in the abdominal cavity under anesthesia (50 mg/kg ketamine, 5 mg/kg xylazine, and 0.5 mg/kg acetopromazine). Animals were allowed a minimum of 7 days postsurgery for recovery. Transmitter output frequency (Hz) was monitored at 10-min intervals by antennas mounted in receiver boards situated beneath each animal’s cage. This information was relayed to a computer where frequency measurements were converted to degrees Celsius using Dataquest software (DataScience). A first group of animals (n=5) received either repeated daily injections of LPS (from *E. coli* serotype 0111:B4, L-2630, Sigma; 50 µg/kg i.p.) for 4 days, or a corresponding volume of saline (n=5). Injections were administered between 10:00 and 12:00, during the light phase of the normal light-dark cycle. A second group of rats were subcutaneously implanted with osmotic pumps (Alzet, CA) containing either LPS solubilized in saline released at a continuous rate of 6.25 µg/hour (n=5), or a control saline solution (n=4). For pump implantation, animals were temporarily anaesthetized by gas inhalation with isoflurane, and a small (1.5 cm) incision was made in the skin between the shoulder blades, into which an osmotic pump was placed. The incision was immediately closed with suture clips and the animal was left to recover under red light for 45 min before being returned to its home cage. The minipump released LPS or sterile saline during 5 days, after which animals were euthanized.
Temperature measurements were averaged over 2h and analyzed using two-way ANOVA, with treatment as the between-subjects factor and time as a within-subjects repeated measure. Significant interactions were analyzed using simple main effect tests.
Figure 1. Changes in body temperature in response to LPS administration via different routes. (A) Repeated LPS injection (50 µg/kg i.p., n=5; saline, n=5). (B) Continuous LPS delivery by osmotic minipump (6.25 µg/h s.c., n=5; saline, n=4). Both LPS-treated groups responded with fever, but the minipump delivery group displayed fever for a longer period. Black boxes indicate hours of darkness. *p<0.05, **p<0.01

1.3 Results

Fig. 1 shows the body temperature of the animals following LPS or saline treatment. Each data point indicates the body temperature averaged over the last two hours, such that in Fig. 1A, body temperature at time of injection (t=0, 24, 48, 72h) actually represents the average body temperature of the two hours preceding the injection. LPS induced a significant rise in average body temperature compared to the saline treated controls, when administered either by injection (Fig. 1A) or by osmotic pump (Fig. 1B). In the group receiving repeated daily LPS injections, temperature was significantly elevated at 6h (p<0.02) and 8h (p<0.01) following the 1st injection, and 4h (p<0.0001) and 6h (p<0.04) after the 2nd injection. There was a tendency for elevated body temperature 4h after the first LPS injection (p=0.07). No deviation from control values was observed after the 3rd and 4th injections of LPS.

Continuous LPS infusion resulted in a more sustained increase of core body temperature (Fig. 3.1b). Results of the ANOVA indicated a significant time x treatment interaction (p<0.01). A simple main effect test was used to decompose the interaction, and revealed that the effect of LPS treatment was significant (p<.05) from 2h to 10h, from 16h to 24h and at 32h. The body temperature of the LPS treated animals returned to normal around 36h after pump implantation, and thereafter resembled the circadian variation in temperature exhibited by the saline treated controls.
1.4 Conclusion

Our results supported previous observations indicating that there is tolerance to the pyrogenic effects of repeated LPS injections, and demonstrated that continuous LPS delivery through minipumps also eventually leads to tolerance. However under our experimental conditions, LPS administration by osmotic pump induced fever for a longer time period than did i.p. injection of LPS. In the infection model using repeated injections, rats displayed an elevated temperature for 4h after the first injection and for 4h after the second, yielding a total of 8h of fever. By the 3rd injection, rats had become tolerant to the pyrogenic effects of LPS, which corroborates observations previously made in rabbits, rats and guinea pigs (Yamashiro et al. 1993; Zeisberger and Roth 1998). This indicates that in the case of acute LPS treatment, two injections 24h apart are sufficient to induce a fever of optimal duration. This corresponds to the treatment regimen used in chapter II, V and VI. In the case of minipump delivery, animals exhibited an overall elevated body temperature from 2h to 24h, which was statistically significant for a total of 20h. Hence, total duration of the febrile response to LPS was markedly longer when LPS was administered via minipump compared to repeated i.p. injections. This prolonged fever implies that there was also enhanced release of cytokines (IL-1β, IL-6, TNF-α) responsible for mediating the LPS–induced fever. Thus, in order to determine if a more prolonged prenatal LPS-induced inflammatory/febrile response is associated with behavioral alterations in offspring, we proceeded to investigate the effects of gestational LPS treatment by osmotic minipump on adult offspring behavior.
EXPERIMENT II: EFFECTS OF PRENATAL CHRONIC LPS EXPOSURE IN LATE GESTATION ON DOPAMINE MEDIATED BEHAVIOR IN THE OFFSPRING

2.1 Introduction

After having demonstrated that LPS administered via osmotic minipumps induced a longer febrile response (20h) compared to repeated injections at 24h intervals (8h of fever), we went on to investigate the effects of chronic gestational LPS exposure on offspring behavior. In this experiment, we used s.c. minipumps to deliver LPS to pregnant dams from E18 to birth, and investigated the resulting behavioral changes in the offspring. As previously, we measured two dopamine-mediated behaviors relevant to SCZ: prepulse inhibition of acoustic startle (PPI) and amphetamine (AMPH)-induced locomotion. A third behavioral measure was added, behavioral sensitization, in which repeated administration of AMPH or other psychostimulants in a rodent leads to an augmentation of behavioral responses to subsequent challenge with the drug. The amphetamine-sensitized state is believed to model some of the positive symptoms and cognitive deficits observed in patients with SCZ (Featherstone et al. 2007).

2.2 Methods

For gestational LPS treatment via osmotic pump, E18 timed pregnant Sprague–Dawley rats (Charles River, Quebec, Canada) were temporarily anaesthetized by gas inhalation with isoflurane, and a small (1.5 cm) incision was made between the shoulder blades into which an osmotic pump (Alzet, CA) was implanted. The incision was immediately closed with suture clips and the animal recovered for 45 min under red light before being returned to its home cage. The minipump released LPS (from *E. coli* serotype 0111:B4, L-2630, Sigma, Canada) solubilized in saline at a continuous rate of 6.25 µg/hour, or control saline solution, for 5 days (i.e. until delivery). Rats were observed daily but left undisturbed for the rest of the pregnancy. This procedure did not cause mortality or fetal loss in the pregnant dams.

On the day of birth, a small quantity of indelible ink was injected into one of the paws of each pup to identify animals from different treatment groups and
litters. Pups were cross-fostered with untreated surrogate dams in mixed litters, with pups from the same birth litter always cross-fostered to at least two different surrogate dams. Pups were weaned on postnatal day 21 (P21) and group housed. PPI of acoustic startle was measured in these offspring at adulthood (P70) as described previously (chapter II). There were 16 rats per group, and no more than 3 pups per group came from the same dam. One week and two weeks after measurement of PPI, about half of these offspring (n=9) were tested on AMPH-induced locomotion in response to different doses of AMPH. Locomotor activity was monitored in clear Plexiglass chambers (42cm x 42cm x 30cm) equipped with a grid of infrared light beams (VERSAMAX animal activity monitoring system, AccuScan Instruments, OH). Each test session began with 30 min of habituation, followed by saline injection and 40 min of recording. D-amphetamine sulphate (0.5 mg/kg sc on week 1 and 2.0 mg/kg sc on week 2) was then injected subcutaneously and locomotor activity was monitored for an additional 100 min. Another group of adult offspring was used to evaluate behavioral sensitization to the effects of AMPH. Animals from each of the two birth groups were assigned to one of two pre-treatment conditions: sensitized and non-sensitized, for a total of 4 experimental groups. There were 12 rats per group, and no more than 3 pups per group came from the same dam. At the beginning of each of five consecutive daily sessions, animals were injected with 2.0 mg/kg AMPH (sensitized) or an equivalent volume of saline (non-sensitized), and were placed inside a locomotion chamber for 30 min. One week after the fifth pre-treatment session, all animals were challenged with 0.5 mg/kg AMPH and their locomotor activity was recorded for 100 min. The 50 min of locomotor activity following AMPH injection were analyzed using three-way ANOVA with prenatal LPS exposure and AMPH pre-treatment as between-subject factors, and time as a within-subject factor.
2.3 Results

![Graph showing effects of gestational treatment with lipopolysaccharide (LPS) via osmotic pump on prepulse inhibition of startle (PPI) in adult male offspring. Data show the mean percentage PPI (+SEM) at different prepulse intensities. Gravid females were implanted sc with an osmotic pump delivering LPS (6.25 µg/h for 5 days) or saline on E18. Offspring prenatally treated with LPS displayed significantly lower PPI compared to control offspring (n=16). *significant effect of treatment, p<.05 versus saline.](image)

Figure 2  Effects of gestational treatment with lipopolysaccharide (LPS) via osmotic pump on prepulse inhibition of startle (PPI) in adult male offspring. Data show the mean percentage PPI (+SEM) at different prepulse intensities. Gravid females were implanted sc with an osmotic pump delivering LPS (6.25 µg/h for 5 days) or saline on E18. Offspring prenatally treated with LPS displayed significantly lower PPI compared to control offspring (n=16). *significant effect of treatment, p<.05 versus saline.

Results indicated no significant differences in baseline acoustic startle response between rats gestational exposed to LPS and controls (data not shown). However, chronic exposure to LPS during late pregnancy significantly decreased PPI in rat offspring (Fig. 2). Two-way ANOVA revealed a significant effect of prenatal treatment (F$_{1,75}$=6.24, $p=.01$), a significant effect of prepulse intensity (F$_{4,75}$=30.14, $p<.01$), and no significant treatment x prepulse intensity interaction.
Figure 3  Effects of gestational treatment with LPS via osmotic pump on amphetamine (AMPH)-induced locomotion in adult male offspring. Chronic gestational exposure to LPS (6.25 µg/h sc, E18 to birth) had no significant effects on the locomotor response to AMPH in adult male offspring. Data
show the mean distance traveled (± SEM) in response to (A) low dose (0.5 mg/kg) and (B) high dose (2.0 mg/kg) AMPH (n=9).

Statistical analysis indicated that chronic exposure to LPS via osmotic pump during late gestation had no significant effect on AMPH-induced locomotion in adult male offspring (Fig. 3). Two-way ANOVA of the response to low dose AMPH injection (0.5 mg/kg) revealed no significant effect of prenatal treatment, a significant effect of time (F\(_{9,144}=28.93, p<.01\)) and no significant time x treatment interaction. The same analysis performed on the response to high dose AMPH injection (2.0 mg/kg) indicated no significant effect of prenatal treatment, a significant effect of time (F\(_{9,144}=2.19, p=.03\)) and no significant time x treatment interaction.

![Figure 4](image_url)

**Figure 4** Effects of gestational LPS exposure via osmotic pump on behavioral sensitization to AMPH in adult male offspring. Adult rats gestationally exposed to LPS (6.25 µg/h sc, E18 to birth) and saline-exposed
controls were injected with AMPH (sensitized) or saline (non-sensitized) for one week prior to the AMPH challenge. The graph shows locomotion in response to the AMPH (0.5 mg/kg) challenge. Following AMPH challenge, animals pre-treated with AMPH exhibited significantly higher locomotor activity than animals pre-treated with saline. However, locomotor activity was not significantly different between sensitized (i.e. AMPH pre-treated) animals prenatally treated with LPS and prenatal saline controls (n=12, *p<.05 versus non-sensitized.)

Next, we examined the sensitizing effect of pre-exposure to AMPH on locomotion induced by a subsequent AMPH challenge. Statistical analysis of the 50 min of locomotor activity following the AMPH challenge (t=90 to 130 min) revealed no significant prenatal LPS treatment x AMPH pre-treatment x time interaction. There was a significant AMPH pre-treatment x time interaction (F_{4,41}=7.57, p<.00), indicating that the effects of AMPH pre-exposure on locomotion were significantly more pronounced at certain timepoints than others (Fig. 4). Decomposing the interaction with simple main effect tests indicated that AMPH pre-exposure significantly increased locomotion between 90 and 130 min, compared to saline pre-treatment, which is expected under the sensitization paradigm. However, there was no significant prenatal LPS treatment x AMPH pre-treatment interaction (F_{1,44}=2.68, p=.11), which indicated that AMPH pre-treatment had the same effect in both prenatal LPS-treated and prenatal saline-treated animals. Prenatal LPS treatment by itself had no significant effect on this test (F_{1,44}=0.24, p=.63). Thus overall, we concluded that there was no effect of prenatal LPS treatment on AMPH-induced sensitization in adult male offspring.

2.4 Discussion
Chronic gestational exposure to LPS via subcutaneously-implanted osmotic minipump lead to some degree of behavioral changes in adult offspring, namely a significant decrease in PPI at all prepulse intensities. However, we did
not find a significant effect of chronic gestational LPS exposure on AMPH-induced locomotion or on sensitization to the effects of AMPH. This contrasts with the results obtained with acute gestational LPS exposure presented in chapter II. In that experiment, LPS was injected twice, once on E18 and once E19, and neonates were subject to an anoxic or air treatment. Under these conditions, prenatal LPS significantly increased AMPH-induced locomotion at adulthood, but had no significant effect on PPI. Some differences between the protocols of the two studies may explain the discrepant results. First, whereas acute LPS injections were given on E18 and 19, minipumps released LPS from E18 until birth (i.e. E21/22). We showed that this longer exposure resulted in a febrile response of longer duration, and thus indicated a prolonged cytokine release. PPI disruption may thus require an immune challenge that is more severe in that respect. Alternatively, since prenatally LPS-exposed animals displayed a tendency for an average lower PPI at 76, 79 and 82 dB in the repeated injection model (chapter II, Table 2), it is possible that using larger groups would have revealed a significant prenatal treatment effect. Another crucial difference between the two study designs is the anoxia/air treatment. In the repeated injection study, all of the pups were subjected to anoxia (100% N₂) or air (control) treatment for 7 min, then allowed 10 min of recovery on a heated platform. Although this period of handling and maternal separation was brief, it may have caused an additional stress that, together with LPS exposure, was sufficient to induce changes in the neurocircuitry subserving AMPH-induced locomotion. However, this remains speculative.

In conclusion, we demonstrated that MIA induced by chronic LPS administration via osmotic pump significantly reduced offspring PPI. In contrast, it appeared to have no effect on dopamine-mediated behaviors such as AMPH-induced locomotion and sensitization.
Chapter IV: The viral mimic, polyinosinic:polycytidylic acid, induces fever in rats via an interleukin-1-dependent mechanism
1. Preface

In the preceding chapters, we investigated the behavioral changes elicited by exposure to maternal immune activation (MIA) induced by bacterial endotoxin, a membrane component of gram(-) bacterial cell walls. However, a significant proportion of studies linking maternal infection to increased risk for schizophrenia were based on infections of viral origin, most notably influenza. Thus, we wanted to determine if MIA elicited by a viral-like immunogen, such as polyinosinic:polycytidylic (poly I:C), would similarly affect offspring behavior. Poly I:C is a viral-like synthetic double stranded RNA known to cause fever and trigger components of the innate immune response in a variety of species (Kimura et al. 1994b; Toth 1996). However, basic understanding of this viral infection model was limited, including knowledge of the cytokine cascade underlying the immune response. In fact, even the optimal dose required to induce a pyrogenic response in rats was unknown. Hence, before using poly I:C in our model of developmental alteration by MIA, we wanted to learn more about the basic characteristics of the immune response this molecule induces. We were particularly interested in studying the febrile response, altered food intake and cytokine cascade that were elicited by poly I:C, all of which could potentially affect fetal development.
2. Manuscript

Title: The viral mimic, polyinosinic:polycytidylic acid, induces fever in rats via an interleukin-1-dependent mechanism

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Keywords: cytokines; fever; food intake
ABSTRACT

Polinosinic:polycytidylic acid (poly I:C) is a synthetic double-stranded RNA that is used experimentally to model viral infections in vivo. Previous studies investigating the inflammatory properties of this agent in rodents demonstrated that it is a potent pyrogen. However, the mechanisms underlying this response have not been fully elucidated. In the current study, we examined the effects of peripheral administration of poly I:C on body temperature and cytokine production. Male rats were implanted with biotelemetry devices and randomly assigned to one of the following three groups: poly I:C + saline, poly I:C + interleukin-1 receptor antagonist (IL-1ra), or saline + saline. Maximal fever of 1.6°C above baseline was observed 3 h after an intraperitoneal injection of poly I:C (750 µg/kg). Pretreatment with IL-1ra diminished this response by >50% (maximum body temperature = 0.6°C above baseline). Plasma IL-6 concentration increased fivefold 2 h post-poly I:C compared with saline-injected rats; levels returned to baseline 4 h postinjection. Pretreatment with IL-1ra prevented this rise in IL-6. Plasma tumor necrosis factor (TNF)-α was also increased more than fourfold 2 h postinjection but remained unaffected by IL-1ra treatment. IL-1β and cyclooxygenase-2 mRNA were significantly upregulated in the hypothalamus of poly I:C-treated animals. Finally, poly I:C decreased food intake by 30%, but this response was not altered by pretreatment with IL-1ra. These results suggest that poly I:C induces fever, but not anorexia, through an IL-1 and prostaglandin-dependent mechanism.

INTRODUCTION

Administration of bacterial endotoxin [lipopolysaccharide (LPS)] to laboratory animals is a widely used model of infection and inflammation. Similar to infection with live pathogens, LPS induces fever and a variety of sickness behaviors, including decreased food intake, weight loss, and increased sleep. The mechanisms underlying LPS-induced fever and sickness behaviors have been well characterized and shown to be mediated by proinflammatory cytokines, the most important being interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α.
Unlike bacterial infection, little is known about sickness responses to viral infection. Because most viruses produce double-stranded RNA (dsRNA) at some point during their replication (17), a synthetic viral-like dsRNA that stimulates antiviral activities of the innate immune system, polyinosinic: polycytidylic acid (poly I:C), has been used to mimic viral infections. Advantages of using poly I:C over live viruses include safety, convenience, but more importantly reproducibility and control over dose and time of administration of the immunological challenge. Similar to LPS, central or systemic administration of poly I:C results in the induction of the acute-phase reaction, fever, and sickness behaviors in a variety of species, including mice, rabbits, guinea pigs, and rhesus monkeys (11, 16, 19, 20, 48). Most studies examining the pyrogenic properties of poly I:C have been carried out in rabbits injected intravenously with low doses (2.5–50 µg/kg) of poly I:C, which resulted in fevers of ~1°C in magnitude (20, 22, 46). Studies using rats or mice reported comparable increases in body temperature in response to higher doses of poly I:C (20–600 µg/animal; see Refs. 10, 15, 32, 48). However, none of these studies examined the poly I:C-induced febrile response in relation to cytokine expression. Thus, in contrast with infection models using LPS where the cytokine cascade leading to fever and sickness behavior has been extensively studied (13, 29), the mechanisms underlying the pyrogenic response to poly I:C have not been fully elucidated.

Poly I:C is best known as a potent inducer of interferon (IFN)-α and -β in vitro and in vivo (32, 34). It has also been reported to induce IL-6, IL-12, and TNF-α in vitro in human and mouse leukocyte cultures (2, 34). Proinflammatory cytokine mRNAs were also detected in the lungs of mice inoculated intratracheally with poly I:C (49). In contrast, very little is known about the poly I:C-induced plasma profiles of the three principal proinflammatory cytokines (IL-1β, IL-6, and TNF-α), and no studies have explored the role of these cytokines in the pyrogenic response to poly I:C treatment. Thus the aim of the present study was to examine the effects of peripheral administration of poly I:C on body temperature, food intake, and cytokine production and to test if the febrile effects of poly I:C, like those induced by LPS, are IL-1 dependent.
MATERIALS AND METHODS

Adult male Sprague-Dawley rats (250–300 g body wt, Charles River, Quebec, Canada) were used in all experiments. The animals were housed individually in a controlled environment at an ambient temperature of 21 ± 2°C and a 12:12-h light-dark cycle (lights on from 08:00 to 20:00). Food and water were provided ad libitum. In one study, powdered food (powdered 5012; Ralston Purina, Ottawa, Canada) was used to monitor changes in food intake. All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee.

Core body temperature was monitored in freely moving animals by remote biotelemetry using small battery-operated, temperature-sensitive radio transmitters (DataScience, St. Paul, MN), which were previously implanted in the abdominal cavity under anesthesia (50 mg/kg ketamine, 5 mg/kg xylazine, and 0.5 mg/kg acetopromazine). Animals were allowed a minimum of 7 days postsurgery for recovery. Transmitter output frequency (Hz) was monitored at 10-min intervals by antennas mounted in receiver boards situated beneath each animal’s cage. This information was relayed to a personal computer where frequency measurements were converted to degrees Celsius using Dataquest software (DataScience).

Pyrogenic and anorexic responses to poly I:C. To investigate the effects of poly I:C on body temperature, rats were injected intraperitoneally with either 500, 750, or 1,000 µg/kg poly I:C (n = 6/dose; Sigma) or a corresponding volume of saline (n = 7) 2 h after lights on.

Once the optimal dose of poly I:C was established, a second experiment was conducted to determine the role of IL-1 in poly I:C-induced fever and anorexia. To prepare animals for testing the effect of poly I:C on food intake and weight loss, animals were habituated to powdered food (powdered 5012; Ralston Purina) placed in special dispensers designed to avoid spillage and handled for 2 days before the experiment. On the day of the experiment, animals were given a single injection of poly I:C (750 µg/kg ip) or saline (1 ml/kg ip) at 0 h (2 h after
light onset), together with either recombinant human IL-1 receptor antagonist (IL-1ra; a kind gift from Dr. Robert Thompson, Synergen) or saline (at 0 and again at 1 h, n = 5–7 for various treatment groups). The dose of IL-1ra used (1 mg/kg ip) was based on our earlier studies (35) where it was used to inhibit LPS induced fever. No IL-1ra alone-treated group was included in these studies as we, and others, have previously shown it not to have any effects on any of the parameters investigated in our experiments (27, 30, 45). Core body temperature was monitored at 10-min intervals starting at 24 h before and up to 48 h after treatment with poly I:C. Changes in body weight were monitored by weighing the animals 24 h before the start of the experiment, at the time of poly I:C injection, and at 24 and 48 h post-treatment. Food intake was assessed at the same time points by weighing the special dispensers (PFF16D jar) containing the powdered food. The amount of food consumed by individual animals was calculated by subtracting the weight of the dispensers after treatment from the weight at the start of the experiment.

Cytokine measurements. To assess changes in circulating cytokine levels in response to poly I:C, rats were injected with poly I:C (750 µg/kg) 2 h after light onset and killed with an overdose of anesthetic at 0, 1, 2, 4, 6, and 8 h after injection (n = 3 at 0, 2, and 4 h; n = 4 at 1, 6, and 8 h). Blood was collected by cardiac puncture using sterile heparinized syringes and placed in sterile tubes containing pyrogenfree heparin (10 U/ml). The samples were then centrifuged (5,300 g, 10 min at 4°C), and plasma was collected and stored at -80°C until assays were performed for IL-1β, IL-6, and TNF-α. In a separate experiment, animals were given a single injection of poly I:C (750 µg/kg ip) together with either recombinant human IL-1ra (1 mg/kg ip) or saline at -1 and 0 h (n = 6). Unlike the studies on fever and food intake described above, IL-1ra was administered at these time points to ensure its presence at the site of inflammation, given that local IL-1β is induced by LPS within 1 h (36). Control animals received the same number of injections of either saline or IL-1ra alone (n = 5/group). Rats were killed at 2 h, and blood samples were collected and processed as described above and then assayed for IL-6 and TNF-α. Cytokine
levels were measured using a two-site, rat-specific ELISA (NIBSC, Potters Bar, UK), as previously described (41). All samples were assayed in duplicate. The ELISA reagents used in this study were specific to the target protein and were tested for cross reactivity with other cytokines.

RT-PCR. To investigate brain mechanisms involved in poly I:C induced fever, we examined two markers of inflammation, namely IL-1β and cyclooxygenase (COX)-2. We measured mRNA rather than protein since both markers occur in relatively low levels in the hypothalamus after systemic injections of inflammatory stimuli. Two hours after injection with either saline (n = 5) or poly I:C (750 µg/kg ip, n = 6), animals were perfused intracardially with diethyl pyrocarbonate saline. Brains were removed, frozen on dry ice, and stored at -80°C. The hypothalami were then dissected before use and homogenized in TRIzol Reagent (Invitrogen), and total RNA was isolated following the manufacturer’s instructions. The air-dried RNA pellet was dissolved in 100 µl autoclaved water, and cDNA was synthesized using murine myeloleukemia virus RT (MMLV; Invitrogen). RNA (1 µg) was incubated with random primers (5 µM; Applied Biosystems) for 10 min at 65°C, and cDNA synthesis was performed by adding dithiothreitol (10 µM; Invitrogen), MMLV RT (200 units), dNTPs (1 mM; Sigma-Aldrich), and first-strand buffer (Invitrogen) and incubating for 1 h at 37°C, followed by 5 min at 90°C to inactivate the enzyme. PCR amplification of cDNA was performed using ReadyMix RedTaq PCR Reaction Mix with 1.5 mM MgCl₂ (Sigma-Aldrich) and 6 pmol primers for β-actin (forward: 5’-GCCGTCTTCCCCCTCCATCGTG-3’; reverse: 5’-TACGACCAGAGGCATACAGG-GA-CAAC-3’), IL-1 β (forward: 5’-CCCAAGCACCCTTTTCTTCTTCATCTTT-3’; reverse: 5’-CAGGGGTGGG-TGTGCCCGTCTTTC-3’), or COX-2 (forward: 5’-GCTGTACAGCAGTAGTGCTTGTTGTA-3’), using a Gene Amp PCR system 9700 Thermocycler (Applied Biosystems). The linear phase of PCR amplification was determined by performing RT-PCR on a sample from each treatment group for an increasing number of cycles (20–50). The
optical density (absorbance) of each PCR product was quantified by densitometry using Northern Eclipse version 6.0 (Empix Imaging) and plotted against the number of cycles. A cycle number within the exponential phase of the reaction was then selected and used for all subsequent PCRs. The following cycling parameters were used: 1) 5 min at 94°C; 2) 30 s at 94°C, 30 s at 60°C, followed by 45 s at 72°C (µ-actin and IL-1µ) or 2 min at 72°C (COX-2), for 26, 32, and 46 cycles, respectively; and 3) 72°C for 10 min. The absorbance of the PCR products was quantified and expressed as a percentage of actin absorbance [relative absorbance (gene X/actin mRNA X 100)].

Statistical analysis. Temperature measurements were averaged over 30 min and then analyzed using two-way ANOVA, with treatment as the between-subjects factor and time as a within-subjects repeated measure. Significant interactions between time and treatment were decomposed using simple main effects tests, and post hoc pairwise comparisons were performed to assess differences between specific treatments at a given time point. Food intake and body weight data were analyzed in the same fashion. ELISA results were analyzed with one-way ANOVA and RT-PCR data with Student’s t-tests. Post hoc Newman-Keul’s tests were performed where indicated. Statistical analyses resulting in probabilities of $P = 0.05$ were considered significant.

RESULTS

Poly I:C induced fever and decrease in food intake. Intraperitoneal injection of 750 or 1,000 µg/kg poly I:C induced a significant rise in body temperature compared with saline treated controls, starting at 120 and 150 min after injection, respectively, and remaining significant up to 420 min (Fig. 1; all $P < 0.05$). The fever appeared monophasic in both cases, although all groups of animals exhibited a transient hyperthermia at 30 min, most likely because of handling stress during the injection. Interestingly, the largest increases in body temperature were observed after the administration of the medial dose of poly I:C (750 µg/kg), with a maximal amplitude of 1.6°C at 3 h. The largest dose of poly I:C, 1,000 µg/kg, induced a rise in body temperature peaking 4.5 h after injection at 1°C above baseline. The difference between these two doses was significant early in
the course of the febrile response \( [P < 0.05 \text{ at time } (t) = 120 \text{ and } 180 \text{ min}] \). The lowest dose of poly I:C (500 µg/kg) did not significantly alter body temperature compared with the saline-treated controls, except for two time points \( (t = 240 \text{ and } 300 \text{ min}) \).

In a second experiment, injection of poly I:C (750 µg/kg) again resulted in a significant fever 120–420 min after injection (Fig. 2, all \( P < 0.01 \)), which reached a peak amplitude of 1.1°C at \( t = 150 \text{ min} \). Administration of IL-1ra at 0 and 1 h after poly I:C injection significantly attenuated the febrile response (Fig. 2, \( P =<0.05 \text{ at } t = 120–330 \text{ min} \) for poly I:C + saline vs. poly I:C + IL-1ra). However, the poly I:C + IL-1ra-treated group still displayed a significantly higher body temperature than the saline controls \( (P < 0.05 \text{ at } t = 210–330 \text{ and } 390 \text{ min}) \), with a peak difference of 0.6°C at \( t = 240 \text{ min} \). The fevers lasted for 7 h (Fig. 2), after which body temperature returned to baseline (8–48 h, data not shown).

In the same experiment, poly I:C alone reduced food intake by 31% over the 24-h period after treatment (Fig. 3A, \( P < 0.01 \)), with the animals eating, on average, 8.3 g less food than was consumed over the same period before treatment (Fig. 3A, \( P < 0.01 \)). Poly I:C-treated animals lost an average of 3.3 ± 1.9 g body wt in the 24 h after treatment, whereas their controls gained an average of 5.0 ± 1.0 g/day in each of the 2 days after saline injection (Fig. 3B, \( P < 0.05 \)). These anorexic effects of poly I:C lasted for 24 h, after which food intake returned to baseline levels. To study the role of IL-1 in poly I:C-induced anorexia, a third group of rats was injected one time with poly I:C (750 µg/kg) and with IL-1ra (1 mg/kg) at 0 and again at 1 h. In contrast to its effects on body temperature, IL-1ra had no effect on the decrease in food intake or weight loss induced by poly I:C. Animals treated with poly I:C + IL-1ra consumed significantly less food (8.8 g or 33%) over the 24-h period after the injection compared with the same period before treatment (Fig. 3A, \( P < 0.01 \)). They also exhibited a 5.2 ± 3.5 g decrease in body weight in the 24 h after treatment (Fig. 3B, \( P < 0.01 \)), which was similar to the poly I:C alone-treated group. All groups of animals returned to baseline food consumption levels 48 h after treatment (day 2; Fig. 3A). Poly I:C alone and poly I:C + IL-1ra animals gained more weight 48 h after treatment (day 2) than on the
day preceding the treatment (day 0; P < 0.05). The saline group did not show this rebound effect.

**Inflammatory cytokine response to poly I:C.** The decrease in fever resulting from the concurrent administration of IL-1ra and poly I:C suggests that IL-1 plays an integral role in the pyrogenic response to poly I:C. To further investigate the role of this cytokine, as well as that of IL-6 and TNF-α, we measured the plasma concentration of these mediators in a separate study. These experiments revealed that plasma IL-1β concentrations were close to the detection limit of the assay and did not deviate significantly from baseline levels (t = 0) at any of the time points tested (Fig. 4A). In contrast, poly I:C administration resulted in a fivefold increase in plasma IL-6 concentrations, which peaked at 358 ± 43 pg/ml 2 h after the injection (Fig. 4B, P < 0.01 compared with baseline). The increase in plasma IL-6 was transient, with levels returning to baseline 4 h after the injection, where they remained until the end of the study (8 h). A similar trend was seen with TNF-α, where a fourfold elevation in plasma TNF-α was observed 2 h postinjection (267 ± 60 pg/ml; Fig. 4C, P < 0.01), whereas no significant changes from baseline levels were observed at the other time points tested.

To determine whether the poly I:C-induced increase in plasma IL-6 was IL-1 dependent, poly I:C-injected rats were pretreated with IL-1ra in a separate experiment. After the poly I:C injection (2 h), poly I:C alone induced a significant rise in plasma IL-6 (134 ± 48 pg/ml; Fig. 5, P < 0.05) compared with saline or IL-1ra controls, although values for the latter two groups did not deviate from baseline. The poly I:C-induced rise in plasma IL-6 concentration was attenuated significantly in the presence of IL-1ra (value for poly I:C + IL-1ra = 49 ± 19 pg/ml). In the same study, the poly I:C-induced increase in plasma TNF-α concentration was not affected by IL-1ra treatment (data not shown).

In a separate study designed to examine the brain mechanisms underlying poly I:C-induced fever, the hypothalami of saline- and poly I:C-treated rats were microdissected, and COX-2 and IL-1 β mRNA, two well-established inflammatory mediators in the central nervous system, were analyzed using semiquantitative PCR. Poly I:C induced an approximately twofold increase in IL-
1 β mRNA compared with saline-treated controls (Fig. 6A, P < 0.01). Similarly, hypothalamic COX-2 mRNA, which appears downstream of IL-1 β during fever and is an important indicator of pyrogenic activity, was significantly higher in poly I:C-treated animals relative to saline controls (Fig. 6B, P < 0.01). Analysis of the linear relationship between IL-1β and COX-2 mRNA in the poly I:C- and saline-treated animals revealed that the slopes and intercepts of both regression lines were not significantly different, making the analyses of the linear regression of the combined data possible. Analysis of pooled data from poly I:C- and saline treated animals revealed a significant positive correlation between hypothalamic IL-1β and COX-2 mRNA levels (Fig. 6C; y = 1.09x + 9.42; Pearson correlation coefficient r = 0.88; P < 0.01), which suggests central COX-2 is induced by IL-1β after poly I:C injection.

DISCUSSION

In the present study, we demonstrate that poly I:C induces a significant long-lasting fever in rats that is comparable in magnitude and time course to that observed after treatment with LPS. A dose-response study using this synthetic viral product showed that the animals responded maximally to a dose of 750 µg/kg ip (Fig. 1). Although the doses in our study are relatively high compared with those used in studies with rabbits [e.g., 5 and 33 µg/kg iv (22)], they are consistent with those used in other rodent species [e.g., guinea pig: 800 µg/kg im (11); mouse: 600 µg/animal ip (48)]. Whereas LPS-induced fever in rats is generally biphasic (42), the febrile response to poly I:C in the present study was monophasic, starting at 2 h, peaking at 3 h, and returning to basal temperatures at 8 h. Interestingly, the highest poly I:C dose induced a fever response that was significantly lower than that resulting from the medial dose. This suggests that the dose response has a “bellshaped” pattern, an observation made previously by others in mice injected with poly I:C (49).

The most significant observation in this study is that IL-1ra significantly attenuated the febrile response to poly I:C, thus demonstrating for the first time that the pyrogenic action of poly I:C is IL-1 dependent. This is similar to our
previous findings and those of others using LPS in the presence and absence of IL-1ra (27, 45), suggesting a common axis between the inflammatory responses caused by the two stimuli. Curiously, we failed to detect any changes in the levels of circulating IL-1β at any time point examined; however, this was also observed in our earlier studies using LPS (27). We have previously shown that the contribution of endogenous IL-1β to the febrile response to exogenous inflammatory stimuli is most likely at the level of the local site of inflammation (9, 35). Locally induced IL-1β will in turn result in the induction of circulating IL-6, which acts as a pyrogenic signal to the brain (9, 35). This hypothesis is consistent with the results of the present study, since the poly I:C-induced rise in plasma IL-6 was inhibited by IL-1ra. However, in contrast to LPS, which resulted in a significant increase in this cytokine over an extended period (1–6 h after LPS; see Ref. 27), the effect of poly I:C was relatively transient. A significant increase in circulating IL-6 levels was only detected 2 h after the injection, during the rising phase of the fever. This suggests that IL-6 may only act as a trigger for poly I:C-induced fever and that other pathway(s) are involved in maintaining the pyrogenic response. Although the absolute plasma IL-6 concentrations 2 h after treatment with poly I:C were different between the time course study (358 pg/ml; Fig. 4B) and that involving IL-1ra pretreatment (134 pg/ml; Fig. 5), analysis of the data indicated that the quality controls performed on each ELISA plate used in our experiments fell within a 10% range of the expected values. Furthermore, an interassay coefficient of variation of 1.05% indicated that our assays are reliable. Variability in plasma IL-6 observed between the two experiments may be because of the fact that the two studies were performed at different times, using different animals. This could have been a significant factor since subtle variations in the way animals are raised can affect several parameters in the adult animal, including their response to stressors (3). Regardless of these differences, it is important to note that, in both experiments, the increase in IL-6 concentrations relative to control (i.e., 5-fold) was the same.

In contrast to IL-6, IL-1ra failed to attenuate the poly I:C-induced increases in plasma TNF-α concentrations, suggesting that the upregulation of this
cytokine in the circulation is not IL-1 dependent. A difference was also observed between the time course of the plasma TNF-α response to poly I:C and to LPS from earlier studies. Similar to IL-6, significant increases in circulating TNF-α were only observed 2 h after the poly I:C injection. In contrast, TNF-α is the first cytokine normally detected at the site of inflammation and in the plasma after treatment with LPS, where it reaches maximal levels as early as 1 h after the injection (26), with IL-6 generally peaking 2–4 h after treatment (26, 28). This suggests that poly I:C might trigger a different peripheral cytokine cascade than the one induced by LPS. The difference in the time profiles between IL-6 and TNF-α does not preclude the latter from involvement in mediating the fever response to poly I:C, especially since IL-1ra failed to completely abolish the increase in body temperature. We have previously demonstrated that TNF-α is involved in mediating fever after localized inflammation in rats (31), and others have shown that it induces COX-2 in the brain (8). The role of TNF in fever, however, remains controversial, with some suggesting that it acts as an endogenous cryogen (26). The involvement of this cytokine in poly I:C-induced fever will need to be explored further in future studies.

At the level of the brain, our results suggest that poly I:C induces fever through similar central mechanisms to those triggered by LPS. Similar to LPS-induced fever (5, 37), hypothalamic IL-1β and COX-2 mRNA were both significantly upregulated by poly I:C in the present study. COX-2, an enzyme responsible for prostaglandin synthesis, is essential for the generation of the febrile response to LPS or pyrogenic cytokines such as IL-1 (7, 25). COX activity appears to be equally important for the mediation of poly I:C-induced fever, where inhibition of this enzyme was demonstrated to significantly attenuate the febrile response in rabbits (1, 43, 44). In the current study, the linear relationship between levels of IL-1β and COX-2 mRNA after poly I:C treatment also suggests that COX-2 is induced by IL-1β in the course of poly I:C-induced fever, since it is in the course of fever induced by other inflammatory agents, such as LPS (6, 24). These observations suggest that the mechanisms involved in triggering the febrile response to poly I:C include the induction of all three proinflammatory cytokines
tested, with IL-6 probably acting as the circulating signal to the brain. In addition, we provide some evidence to suggest that, at the level of the hypothalamus, both IL-1β and subsequently COX-2 are involved in activating the febrile response to systemic viral stimulation.

In contrast to poly I:C-induced fever, which was largely mediated by IL-1, decreases in body weight and food intake appeared totally insensitive to the administration of IL-1ra. This contrasts with LPS-induced anorexia, which is inhibited by IL-1ra (14, 23). It is unlikely that the lack of effect of IL-1ra on the poly I:C-induced reduction in food intake is because of loss of activity over time, since we have previously demonstrated that a similar dose of IL-1ra inhibited the effect of the appetite suppressant leptin over a 22-h period (30). The observations made in the present study exclude IL-1 and, by extension, IL-6 as mediators for poly I:C-induced anorexia but do not, however, exclude TNF-α, which increases significantly after poly I:C treatment and is not inhibited by IL-1ra. This cytokine has previously been implicated in mediating a decrease in food intake after infection and inflammation (38, 47) and provides a viable alternative to IL-1 and IL-6 for mediating the anorexic effects of poly I:C. However, this would need to be confirmed by neutralization studies in vivo. Other cytokines may also be involved. For example, IFN-α, which is produced in significantly high concentrations in response to poly I:C (39), has been shown to decrease food intake and activity in humans and mice (12, 40), and is suggested to play a role in the poly I:C-induced decrease in physical activity (18).

In addition to humoral signals, other pathways have been described that can relay the peripheral inflammatory signal to the brain, namely afferent fibers of the vagus nerve. Vagal afferents have been shown to mediate some of the LPS-induced behavioral effects (e.g., decreased social interaction, decreased food intake, and reduction in food-motivated behavior; see Refs. 4, 21, and 33). It is therefore possible that vagal afferents play a role in poly I:C-induced anorexia, using central IL-1-independent mechanisms, although no direct evidence exists to implicate this route of propagating the peripheral poly I:C-induced inflammatory signal to the brain.
In conclusion, the current study clearly demonstrates that effects of poly I:C on fever and on food intake are mediated via differing pathways, with effects on fever being IL-1 dependent and those on food intake IL-1 independent. Further studies are needed to explore these pathways in greater detail.

FIGURES

Fig. 1. Fever in response to increasing doses of polyinosinic:polycytidylic acid (poly I:C). Rats were injected ip with 500, 750, or 1,000 µg/kg poly I:C (n = 6) or saline (n = 7) at time (t) = 0 min. Only the 2 higher doses induced a significant rise in body temperature, with 750 µg/kg inducing the highest (750 µg/kg vs. saline: P < 0.01 at t = 120–420 min; 1,000 µg/kg vs. saline: P < 0.01 at t = 150–360 min, P < 0.05 at t = 420 min). Arrow depicts the time of injection.
Fig. 2. Role of interleukin (IL)-1 in poly I:C-induced fever. Pretreatment with IL-1 receptor antagonist (ra; 1 mg/kg at $t = 0$ and 60 min, $n = 6$) attenuated poly I:C (750 µg/kg ip, $n = 7$)-induced fever but did not completely abolish it (saline, $n = 5$). Poly I:C + saline vs. saline: $P < 0.01$ at $t = 120–420$ min after injection; poly I:C + saline vs. poly I:C + IL-1ra: $P < 0.05$ at $t = 120–330$ min; poly I:C + IL-1ra vs. saline: $P < 0.05$ at $t = 210–330$ min and at 390 min. Arrows depict the time of injection.
Injection of poly I:C \((t = 0, 750 \, \mu g/kg \, ip, \, n = 7)\) significantly reduced food intake \((A)\) and body weight \((B)\) compared with saline controls \((n = 5)\). IL-1ra treatment \((1 \, mg/kg \, at \, t = 0 \, and \, 60 \, min, \, n = 6)\) did not inhibit poly I:C-induced anorexia and weight loss. *\(P < 0.05\) and **\(P < 0.01\) vs. saline.
Fig. 4. Time course of plasma IL-1β (A), IL-6 (B), and tumor necrosis factor (TNF)-α (C) concentrations in response to poly I:C. Poly I:C administration (750 µg/kg ip, n = 3 at 0, 2, and 4 h; n = 4 at 1, 6, and 8 h) resulted in a 5-fold increase in plasma IL-6 concentration 2 h after injection (**P < 0.01 vs. 0 h). Poly I:C also induced a 4-fold elevation in plasma TNF-α 2 h after injection (**P < 0.01 vs. 0 h). Plasma concentrations of both cytokines returned to baseline levels at 4 h, where they remained until the end of the study. There was no detectable change in plasma IL-1β. Dotted lines represent the detection limits of the assays.
Fig. 5. Plasma IL-6 induction by poly I:C in the presence or absence of IL-1ra. IL-1ra treatment (1 mg/kg, 1 and 0 h) significantly attenuated the poly I:C (750 µg/kg ip)-induced rise in plasma IL-6 (poly I:C + saline and poly I:C + IL-1ra, n = 6; IL-1ra + saline and saline + saline, n = 5). P < 0.05, poly I:C + IL-ra vs. poly I:C (#) and poly I:C + saline vs. IL-1ra + saline and saline + saline (*). Dotted line represent the detection limit of the assay.
Fig. 6. Effect of poly I:C on brain IL-1β and cyclooxygenase (COX)-2 mRNA. IL-1β (A) and COX-2 (B) mRNA were upregulated significantly in the rat hypothalamus in response to peripheral poly I:C injection (750 µg/kg ip, n = 6) compared with saline treatment (n = 5). **P < 0.01. C: in addition, analysis of combined data from saline- and poly I:C-treated animals revealed that hypothalamic levels of mRNA for IL-1β and for COX-2 were significantly correlated (r = 0.88, P = 0.01).
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3. Conclusion

At the time this study was conducted, administration of poly I:C was known to cause fever and trigger components of the innate immune response in a variety of species (Kimura et al. 1994b; Toth 1996), but basic understanding of the immune reaction in rats was extremely limited, including knowledge of the cytokine cascade underlying the immune response. The first step was to identify the lowest dose at which a maximal febrile response was induced in adult rats. This would constitute a physiologically relevant dose to use in our model of MIA. Next, we had to determine if this poly I:C dose induced circulating pro-inflammatory cytokines, as they are candidate mediators for the effects of MIA. We indeed confirmed the induction of plasma interleukin-6 and tumor necrosis factor-α. Finally, we measured a significant decrease in body weight and food intake, which would constitute another potential mediator of the disruptive effects of MIA. With this information, we were ready to include poly I:C in our model of MIA and study its effects on offspring behavior.
Chapter V: Effects of prenatal infection on prepulse inhibition in the rat depend on the nature of the infectious agent and the stage of pregnancy
1. Preface

Chapter V describes experiments in which we compare effects of three different types of immunogen [bacterial endotoxin (lipopolysaccharide, LPS), polyinosinic: polycytidylic acid (poly I:C), and turpentine] on prepulse inhibition (PPI) of acoustic startle. We have shown in chapters II and III that maternal immune activation (MIA) during gestation induced by bacterial endotoxin (LPS) elicited behavioral alterations in the offspring, such as changes in amphetamine (AMPH)-induced locomotion (chapter II) and prepulse inhibition of acoustic startle (PPI, chapter III). Compared to bacterial infections, however, a greater number of studies linking maternal infection to increased risk of schizophrenia (SCZ) were based on viral infections, most notably influenza (Brown 2006; Patterson 2009). Thus, we wanted to know if MIA triggered by a product of viral origin, poly I:C, would similarly affect offspring behavior. After describing the characteristics of the immune response to poly I:C in adult rats in Chapter IV, we were ready to test effects of poly I:C, as an inducer of MIA, on offspring behavior.

In our model of MIA, it is unlikely that LPS would act directly on the fetus. However, we cannot discount the possibility that LPS and poly I:C could bind to Toll-like receptors situated on the placenta and activate the immune response locally (Beijar et al. 2006; Abrahams et al. 2006), in which case the mother’s own immune reaction may have little effect on the developmental outcome. Use of local inflammation in a model of MIA could help ascertain the role of the maternal immune system in offspring neurodevelopmental alterations. Intramuscular injection of turpentine, which causes a sterile abscess inducing a systemic immune response, is a well-characterized model of local inflammation (Luheshi et al. 1997; Leon 2002). Unlike LPS and poly I:C, this substance does not enter the systemic circulation. Thus, observing neurodevelopmental alterations elicited by turpentine-induced MIA would substantiate the hypothesis that the deleterious consequences of maternal infection are mediated via fever or
circulating cytokines rather than the immunogen acting directly on the fetus or the placenta.

To compare the effects of MIA induced by different agents, we selected PPI as an outcome measure. PPI is a robust phenomenon that occurs in almost all mammals, and can be investigated in different species using similar methods (Swerdlow et al. 2002). PPI is particularly relevant to schizophrenia research as it reveals deficits in sensory information gating believed to be an important feature of the illness in humans. PPI deficits in schizophrenic patients are well documented (Kumari et al. 2004).

In addition to comparing the effects of MIA induced by different agents, gestational windows of vulnerability to MIA were also investigated. Epidemiological studies indicate that increased SCZ risk is more strongly associated with prenatal infection occurring during specific periods of pregnancy (see Chapter I for a discussion). In experimental study of MIA, gestational windows of susceptibility have been observed for the disruption of certain behaviors, such as explorative and perseverative behaviors (Meyer et al. 2006b). Therefore, in the following experiment, we compared the effects of MIA induced by three different agents (LPS, poly I:C or turpentine) administered at three different periods during gestation (embryonic day (E)10–11, E15–16 and E18–19).
2. Manuscript

Effects of prenatal infection on prepulse inhibition in the rat depend on the nature of the infectious agent and the stage of pregnancy

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Abstract

Maternal infection during pregnancy is a risk factor for some psychiatric illnesses of neurodevelopmental origin such as schizophrenia and autism. In experimental animals, behavioral and neuropathological outcomes relevant to schizophrenia have been observed in offspring of infected dams. However, the type of infectious agent used and gestational age at time of administration have varied. The objective of the present study was to compare the effects of prenatal challenge with different immune agents given at different time windows during gestation on behavioural outcomes in offspring. For this, pregnant rats were administered bacterial endotoxin (lipopolysaccharide, LPS), the viral mimic polyninosinic: polyribidylic acid (poly I:C), or turpentine, an inducer of local inflammation, at doses known to produce fever, at three different stages in pregnancy: embryonic day (E)10-11, E15-16 and E18-19. Prepulse inhibition of acoustic startle (PPI) was later measured in male adult offspring. PPI was significantly decreased in offspring after prenatal LPS treatment at E15-16 and E18-19. Intramuscular injection of pregnant dams with turpentine at E15-16 also decreased PPI in adult offspring. Maternal poly I:C administration had no significant effect on PPI in offspring. In contrast to prenatal LPS exposure, acute LPS administration to naive adult males had no effect on PPI. Thus, prenatal exposure both to a systemic immunogen and to local inflammation at brief periods during later pregnancy produced lasting deficits in PPI in rat offspring. These findings support the idea that maternal infection during critical windows of pregnancy could contribute to sensorimotor gating deficits in schizophrenia.
1. Introduction

It is generally accepted that schizophrenia is a disorder of neurodevelopmental origin [31], caused by both genetic and environmental factors [57]. Among environmental risk factors, an increased incidence of schizophrenia has been documented following prenatal infections with viruses such as influenza, measles, rubella, varicella-zoster and polio [10,11,40,54,56], and with the protozoa causing toxoplasmosis [12]. Increased risk for schizophrenia has also been associated with maternal pneumonia during pregnancy [45], which is most commonly bacterial in origin [25]. The wide variety of infectious agents associated with increased risk for schizophrenia suggests that mechanisms common to various prenatal infections may affect fetal development.

To model maternal infection during pregnancy, live viruses as well as molecular immunogens like the bacterial endotoxin, lipopolysaccharide (LPS), or the viral mimic polyinosinic: polycytidylic acid (poly I:C) have been administered to pregnant rodents. Following gestational immune activation, changes relevant to the pathophysiology of schizophrenia have been observed in the offspring, including deficits in prepulse inhibition of acoustic startle (PPI) [41,46], latent inhibition [62], object recognition [46], social interaction [51], increases in amphetamine-induced locomotion [19,61], other alterations in dopaminergic function [4,32], and hippocampal abnormalities [24]. However, there is a high degree of variability among these studies with respect to the nature of the immune challenge used and the gestational stage at which it is administered.

We and others [2,7,42,46] have suggested that the deleterious consequences of maternal infection on fetal neurodevelopment may be mediated via circulating cytokines induced by the immunogens, rather than the immune agent acting directly on the fetuses. Intramuscular (im) turpentine injection, a well-documented model of local inflammation [16,30], may be used to investigate this question, since unlike LPS and poly I:C, it does not enter the systemic circulation. Though these three immune models (LPS, poly I:C, turpentine) all induce sickness behaviors and febrile responses through circulating cytokines
[15,20,37], the exact timing and quantity of cytokine released vary for each immunogen. Hence, their effect on fetal neurodevelopment may not be equivalent. We were therefore interested to compare the effects of these three different maternal immune treatments on offspring behavior.

Among behavioral outcomes investigated after prenatal immune treatment, PPI is particularly relevant to schizophrenia as it reveals deficits in sensory information gating believed to be an important feature of the illness. PPI is a well-established sensorimotor gating paradigm defined as “a profound decrease in startle magnitude when the startling pulse is preceded by a weak prepulse” [55]. PPI deficits in schizophrenic patients are well documented [9,28,34]. In mice, administration of influenza virus or poly I:C on embryonic day (E)9.5 or poly I:C on E12-17 decreases PPI in offspring [46,51]. Similarly, in rats, administration of LPS on alternate days throughout the entire pregnancy leads to a marked PPI disruption [8]. However, no study has compared the effects of maternal immune treatment at different gestational periods on PPI. A recent study by Meyer et al. [43] indicated that maternal infection affected offspring explorative and perseverative behavior differentially depending on the gestational stage at which the immune agent was administered. This supports the idea that a window of vulnerability to infection during gestation may also exist for the production of other behavioural deficits, such as PPI disruption.

Thus, our aim was to compare the effects of maternal infection induced by physiological doses of different immune agents administered at different periods during gestation on PPI in the adult offspring. In order to do so, we administered pyrogenic doses of LPS, poly I:C, or turpentine to pregnant rats at one of three time points during gestation: E10-11, E15-16, and E18-19. These time points are representative of what has been used in animal models of maternal infection, and more importantly, coincide with different neurodevelopmental events, in particular with regard to the dopaminergic (DA) system [5,47], known to be involved in the regulation of PPI [23] and possibly in the pathophysiology of schizophrenia [29].
Finally, to investigate whether the disruptive effects of LPS on PPI are strictly developmental, we also examined the effects of acute LPS administration on PPI in normal adult rats. A large body of literature indicates that LPS decreases performance in a number of cognitive tasks, including different types of conditioning [48,52], and spatial learning [50]. This suggested that acute LPS could also disrupt the pre-cognitive processes involved in PPI.

2. Materials and methods

2.1. Subjects and treatments

All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee. For gestational LPS and poly I:C treatment, timed pregnant Sprague–Dawley rats (Charles River, Quebec, Canada) were injected intraperitoneally (ip) with LPS (from *E. coli* serotype 0111:B4, L-2630, Sigma, Canada) or poly I:C (Sigma, Canada) at the doses indicated, once daily, on two consecutive days, at one of the following gestational times: E10-11, E15-16, or E18-19. Turpentine was injected intramuscularly (im) only once at each time point (E10, E15, E18), as the abscess it induces lasts at least 18h [16]. Injection of a corresponding volume of sterile saline (ip or im) was used as a control. Although all three substances induce an innate immune reaction, their administration does not necessarily result in identical cytokine profile, HPA activation and other physiological effects. In addition, fever and cytokine responses are reported to differ in magnitude when the same immunogen is administered to pregnant dams at different gestational times [14,18,39]. Taking this into consideration, our strategy for selecting the doses of each of the immunogens to be used was to choose doses that gave physiologically relevant, optimal febrile responses in male rats in our lab [20,36], and that were generally tolerated by pregnant females (i.e. 100 µg/kg LPS 0111:B4 ip, 750-1000 µg/kg poly I:C ip, 10µl turpentine im). Despite these precautions, initial administration of 100 µg/kg LPS at specific gestational time points (E18-19 and E10-11) led to
appreciable maternal and fetal mortality (see Table 1), so lower LPS doses (50 and 25 µg/kg) had to be used for these time points.

On the day of birth, a small quantity of indelible ink was injected into one of the paws of each pup to identify animals from different treatment groups and litter. Pups were cross fostered with surrogate dams in mixed litters, with pups from the same birth litter always cross-fostered to at least two different surrogate dams. Pups were weaned on postnatal day 21 (P21), group housed (two animals from the same treatment/cage) and maintained on a 12 h:12 h light:dark cycle (lights on at 8:00 h) with free access to food and water. Two pups per treated dam were used in each treatment group, except for poly I:C and turpentine on E15, where three pups from one litter were used. Only male pups were retained for the study. This was done to avoid confounding effects of hormonal variation in females, since it has been shown that PPI is influenced by estrogen [59] and varies across the female rat estrous cycle [27]. Behavioral testing of these offspring began at adulthood (P70). To study the effects of acute LPS on PPI in adults, naive male rats (350 g) were injected with LPS (250 µg/kg), and were tested for PPI 3h later.

2.2. PPI

Average startle response (ASR) and PPI of acoustic startle were measured using four SR-LAB startle apparatuses (San Diego Instruments, San Diego, USA). Each sound attenuated startle chamber contained a clear Plexiglas cylinder resting on a piezoelectric transducer that detected the vibrations caused by the movements of the animals. A computer stored startle responses and controlled the timing and presentation of acoustic stimuli. A SR-LAB calibration unit was used to produce consistent response sensitivity across chambers and trials. Testing took place between 10:00 and 18:00 h. Each test session began with a 7 min acclimatization period in the presence of 65 dB white noise, which continued throughout the session. Two orienting pulse alone trials (120 dB for 30 ms) were then presented, followed by 10 pulse alone trials (120 dB for 30 ms), five null trials with no stimulus, and five prepulse+pulse trials at each of three different
prepulse intensities, in a pseudo-random order and with an average intertrial interval of 17 s (range: 9–29 s). The prepulse+pulse trials consisted of a 30 ms prepulse (at 69, 73 and 77 dB), followed by a 70 ms delay, then a startle pulse (120 dB, 30 ms). The testing session lasted 15 min. For each animal, a background startle value (average of the 5 null trials) was subtracted from the startle amplitude of the pulse alone and prepulse+pulse trials before further calculations were performed. Prepulse inhibition is expressed as % PPI, defined as (1-[startle amplitude on prepulse+pulse trial/mean startle amplitude on pulse alone trials])x100.

2.3. Statistical analyses

To analyze the effects of treatment and gestational time across prepulse intensities, separate two-way ANOVAs were conducted at each gestational time, with treatment as between-subject factor and prepulse intensity as a within-subject measure. Post-hoc Tukey’s Honestly Significant Difference tests were performed where indicated, to assess differences between treatments at specific prepulse intensities. \( P < 0.05 \) was considered significant.

To evaluate the overall effect of each treatment across gestational periods, separate two-way ANOVAs were conducted. To this end, each animal’s PPI results were averaged over all three prepulse intensities, and ANOVAs were conducted with treatment and gestational time as between-subject factors. To facilitate this analysis, each immunogen was considered as a single treatment, even though it was sometimes necessary to use different doses at different gestational times in order to avoid maternal or fetal mortality. The dose of 50 \( \mu g/kg \) LPS at E10-11 was selected for this analysis, as it is the same as the one used on E18-19.

3. Results

3.1. Pregnancy outcome following gestational immune treatment

In order to model a physiological maternal infection, we aimed to administer to pregnant dams doses of the three immunogens which have been
shown to produce optimal fever and cytokine induction in the rat, while having limited impact on maternal and pup survival (i.e. 50-100 µg/kg of LPS; 750-1000 µg/kg of poly I:C; 10 µl turpentine) [20,36,37]. Table 1 shows that contrary to systemic administration of poly I:C and local inflammation by turpentine, systemic administration of LPS to pregnant rats may have a negative impact on gestation outcome, depending on the timing of administration. Administration of 100 µg/kg LPS on E10-11 led to a 100% rate of fetal demise, although all dams survived the treatment. Consequently, lower doses had to be used (50 and 25 µg/kg) at this gestational time. On the other hand, 100 µg/kg of LPS was well tolerated by pregnant animals when given on E15-16. Since preliminary work suggested late gestation was a particularly sensitive time, lower doses of systemic immunogens (50 µg/kg LPS and 750 µg/kg poly I:C) were used at E18-19 to maximize dam survival. Yet, only 62.5% of dams survived administration of 50 µg/kg LPS on E18-19.

3.2 Effects of systemic LPS or poly I:C administration during gestation on PPI in adult offspring

Adult male offspring from dams who received LPS (50 µg/kg), poly I:C (750 µg/kg) or saline on E18 and 19 were tested for PPI at three prepulse intensities (Fig. 1a). The results indicated that exposure to LPS, but not poly I:C, on E18-19 significantly decreased PPI in rat offspring. Two-way ANOVA revealed a significant effect of prenatal treatment (F_{2,29}=4.66, p=.02), a significant effect of prepulse intensity (F_{2,58}=27.53, p<.01), and no significant treatment x prepulse intensity interaction. Post-hoc analysis of the treatment effect indicated that prenatal LPS-treated animals had significantly lower PPI compared to saline (p<.05) or poly I:C (p<.01) treated animals. PPI in poly I:C-treated individuals did not differ significantly from that measured in saline controls.

To investigate the effects of maternal infection at earlier stages of pregnancy, rat dams were administered LPS, poly I:C or saline on either E15 and 16 or on E10 and 11. Fig. 1b shows PPI data for adult offspring from dams treated on E15-16. Prenatal treatment with LPS (100 µg/kg), but not poly I:C (1
mg/kg), significantly \((p<.05)\) reduced PPI in these offspring. Two-way ANOVA of the LPS and saline data indicated a significant effect of treatment \((F_{1,16}=5.90; p=.03)\) and prepulse intensity \((F_{2,32}=8.23; p<.01)\), and no significant interaction.

On E10-11, administration of 100 µg/kg LPS to dams led to a 100% fetal death rate (Table 1). Consequently, lower doses (25 µg/kg and 50 µg/kg) of LPS, well tolerated by pregnant dams, were used. Prenatal treatment with these two lower doses of LPS had no significant effect on offspring PPI (Fig. 1c). Statistical analysis indicated no significant effect of treatment \((F_{2,26}=0.89; p=.42)\), a significant effect of prepulse intensity \((F_{2,52}=25.22; p<.01)\), and no significant interaction \((F_{4,52}=0.67; p=.61)\). Administration of poly I:C (1 mg/kg) on E10-11 was well tolerated by the pregnant animals, but was also without significant effect on PPI in adult male offspring (Fig. 1d). Analysis of the data indicated no significant effect of treatment, a significant effect of prepulse intensity \((F_{2,40}=15.17; p<.01)\), and no significant interaction.

Finally, to evaluate the global effect of prenatal LPS treatment across the three gestational time points, offspring PPI values were averaged across prepulse intensities for each animal, and a two-way ANOVA was performed, with treatment (LPS) and gestational time (E10-11, E15-16, E18-19) as between-subject factors. (As discussed in Methods, this analysis was performed on data using the maximal tolerated dose of LPS at each gestational time point, i.e. 50, 100 and 50 µg/kg LPS at E18-19, E15-16 and E10-11, respectively). This analysis indicated a significant effect of LPS treatment \((F_{1,53}=7.64, p<.01)\), a significant effect of gestational time of administration \((F_{2,53}=13.76, p<.01)\), and no significant treatment x gestational time interaction. Although this combined analysis suggests that maternal LPS treatment at all three gestational time points inhibited offspring PPI, inspection of the data indicates that the effect is much less pronounced on E10-11 than on E18-19 and E15-16. Similar analysis of the poly I:C data indicated no significant effect of treatment or gestational time and no interaction.

3.3 Effects of local inflammation during gestation on PPI in adult offspring
To investigate effects of local inflammation during gestation, 10 µl turpentine was injected intramuscularly into the hind leg of pregnant females at E18, E15 or E10 (i.e., at similar gestational ages as in experiments with LPS and poly I:C). Fig 2a shows PPI in adult male offspring born to mothers injected with saline or turpentine on E18. Animals born to mothers treated with turpentine on E18 did not differ significantly from controls. Two-way ANOVA indicated no significant effect of treatment, a significant effect of prepulse intensity ($F_{2,36}=19.34$, $p<.01$), and no significant interaction. However, turpentine injected slightly earlier in gestation (E15) significantly decreased PPI in adult male offspring (Fig. 2b). ANOVA revealed a nearly significant treatment effect ($F_{1,14}=3.91$; $p<.07$), a significant effect of prepulse intensity ($F_{2,28}=4.99$; $p=.01$) and a significant treatment x prepulse intensity interaction ($F_{2,28}=3.27$; $p=.05$). Post-hoc analysis indicated that turpentine-treated animals displayed significantly lower PPI at the 8dB prepulse intensity ($F_{1,42}=8.81$; $p<.01$). In contrast, turpentine injected around mid-gestation (E10) had no significant effect on PPI in adult male offspring (Fig. 2c). ANOVA indicated no significant effect of treatment, a significant effect of prepulse intensity ($F_{2,40}=30.52$, $p<.01$), and no significant interaction. Finally, ANOVA to evaluate the global effect of prenatal turpentine across the three gestational time points (with offspring PPI values averaged across prepulse intensities) revealed no significant effect of treatment or time of administration, but showed a tendency towards a treatment x gestational time interaction ($F_{2,52}=2.56$, $p=.09$).

3.4. Effects of acute LPS administration on PPI in normal adult rats

In order to compare long-term effects of prenatal LPS exposure, observed above, to acute effects of LPS on PPI, we measured PPI in normal adult male rats 3h after injection of LPS (250 µg/kg, ip). Despite observable sickness behavior due to LPS, acute LPS administration had no significant effect on PPI (Fig. 3). Data analysis clearly indicated no significant effect of treatment, a significant effect of prepulse intensity ($F_{2,44}=35.52$, $p<.01$), and no significant interaction.
3.5 Baseline startle

Table 2 shows the average baseline acoustic startle responses obtained in the different experiments described above. Baseline startle did not differ significantly between treated animals and controls.

4. Discussion

In this study, we provide evidence that both systemic and local inflammation during specific gestational periods can alter PPI in the resulting adult offspring. Maternal infection with LPS on E15-16 or E18-19 significantly decreased PPI in adult male offspring, while LPS exposure on E10-11 had only marginal effects. Local inflammation by turpentine on E15, but not on E18 or E10, reduced PPI in adult male offspring. In contrast, a pyrogenic dose of poly I:C administered to pregnant rats on E18-19, E15-16 or E10-11 had no significant effect on PPI in adult male offspring. In addition, LPS given acutely to naïve adult male rats showed no effect on PPI. Another major effect of LPS that differed across gestational ages was the effect on dam and fetal mortality. A dose of LPS that was well tolerated at E15-16 was lethal to dams at E18-19, whereas earlier in gestation (E10-11), it induced fetal death but all dams survived.

To allow comparison between different immunogens, our strategy was to select, for each of them, a dose that gave an optimal febrile response (i.e. 100 µg/kg LPS 0111:B4 ip, 750-1000 µg/kg poly I:C ip, 10µl turpentine im). These doses, frequently used in experimental animal models studying the physiology of the immune response, have been shown to induce a clear cytokine response and expression of sickness behavior in adult male rats [20,35-37], while having minimal impact on fetal survival when administered to pregnant animals at some gestational time points. These doses would thus appear to be physiologically relevant to what occurs during maternal infections in humans. It is known that pregnant dams display different responses to immunogens at different stages of gestation with respect to fever, cytokine response, HPA activation and survival [14,18,39]. These variations are an inherent part of studies examining gestational windows of vulnerability to infection, and contribute to their interest. A caveat to
this approach, however, is that due to this varying immune response, in particular with regards to fetal survival, it was not always possible to use identical doses of immunogens across all stages of gestation. In the cases where maternal or fetal survival were compromised, doses as close as possible to those optimal for fever were used. Appreciably higher doses of LPS or poly I:C have been used in some previous studies on effects of prenatal infection in rodents. However, it is notably difficult to compare LPS doses between studies, as the bioactivity of the substance varies depending on the lot and serotype of the *E. coli* strain from which the LPS is extracted.

In the current study, the effects of prenatal infection on PPI depended on the type of immunogen (LPS, poly I:C or turpentine) administered. This may not be all that surprising since the exact peripheral cytokine cascade triggered by each immunogen differs in some respects, although all three immune treatments induce fevers mediated by cytokines. For instance, after LPS administration in rats, TNF-α is the first cytokine normally detected at the site of inflammation and in the plasma, reaching maximal levels as early as 1h after the injection [33], while IL-6 generally peaks 2–4h after treatment [33,36]. However, following poly I:C administration in rats, IL-6 and TNF-α both peaked together 2h after injection [20]. Furthermore, in contrast to LPS, poly I:C is a potent inducer of interferon (IFN)–α and –β [1,38]. While moderate doses of poly I:C and LPS induce fevers lasting approximately 8h [20,35], local injection of turpentine induces much longer lasting fever (greater than 18h), during which IL-6 remains elevated in the plasma [16]. However, no elevation of TNF-α is detected in the circulation during turpentine-induced inflammation [16,37]. In addition to cytokine induction, further consequences of maternal infection, such as fever and enhanced glucocorticoid secretion [6,58] could also contribute to eventual PPI deficits in offspring. The fact that poly I:C, a weak corticoid stimulator [22] relative to LPS, was without effect on offspring PPI in this study, may hint towards a role for stress hormones in the fetal effects of maternal infection, although this hypothesis warrants more detailed investigation.
As demonstrated in this study, maternal administration of 50 µg/kg LPS on E18-19 or 100 µg/kg on E15-16 decreases PPI in adult male offspring. This corroborates the findings of Borrell et al. [8], who observed a decrease in rat offspring PPI following administration of 1 mg/kg LPS on alternate days throughout the whole pregnancy. However, our study indicates that an immune challenge of much shorter duration within a critical window is sufficient to disrupt sensorimotor gating in adult offspring. Additionally, in a previous study [19], we observed a tendency for reduced PPI in offspring following gestational LPS treatment on E18-19, that may have been obscured by early handling to which those pups were subjected. In contrast, gestational administration of LPS (25 and 50 µg/kg) on E10-11 did not significantly affect offspring PPI. This might be due to the lower doses used. However, at this time during gestation, a higher dose of LPS (100 µg/kg) led to fetal demise in all dams injected.

Gestational administration of poly I:C had no significant effect on PPI in rat offspring, using physiological doses of poly I:C previously shown to produce fever and cytokine induction in male rats [20]. In contrast to our results with rats, studies in mice have shown that poly I:C can alter offspring PPI when administered on E9 [41,51] or E12 through E17 [46]. However, in these studies with mice, the doses of poly I:C used were relatively large (20mg/kg in [51]; 5-10 mg/kg in [41,46]) or the magnitude of the difference was limited [46]. In addition, Meyer’s group [41] administered poly I:C via an intravenous route, which induces a stronger immune response than the ip route [60], used in the other studies.

Although turpentine injection has been routinely used to study local inflammation, this is the first study to use it to investigate the consequences of maternal immune activation. Maternal turpentine injection on E15 significantly reduced offspring PPI. Turpentine causes inflammation through tissue injury, and as such does not enter the circulation. Therefore our findings with turpentine support the idea that maternal infection may affect fetal neurodevelopment through actions of circulating cytokines and/or fever, rather than via direct effects of the infectious agent on the fetus.
Additionally, our results suggest there is a gestational window of vulnerability for PPI disruption by immune activation towards the middle to end of gestation, i.e. around E15-E19. Gestational days 15-16 appear to be a particularly sensitive period, as two out of the three immune challenges affected PPI when administered at this time. Our finding parallels that of Meyer et al. [43], who observed a deficit in reversal learning in offspring following a prenatal immunogen administration on E17, but not on E9. It is unknown at this point whether this gestational window of vulnerability arises from the variation in the maternal immune response throughout gestation, the neurodevelopmental stage of the fetus, or a conjunction of both. With regard to the development of the dopaminergic (DA) system, known to be involved in the regulation of PPI [23], on E10-11 in the rat, neuroblasts are being induced to differentiate into future mesencephalic DA neurons. On E15-16, mesencephalic DA neurons are at a maturational stage where they start sending out projections while at a later stage (E18-19), their morphology more closely resembles adult brain DA neurons [47,53]. The period from E12 to E22 in the rat represents a general equivalent of the 2nd human trimester, based on criteria such as development of CNS electrical activity, neurotransmitter expression and synaptogenesis [49]. Interestingly, numerous epidemiological studies have identified the 2nd trimester as a critical period when exposure to influenza virus [3] and other infections [13,45,54] leads to an increased risk for schizophrenia.

Although it is well known that postnatal maternal care has an important impact on offspring behaviour in rodents, ours is the only group who have used cross-fostering to control for this potential confounding variable in studies on effects of prenatal immune challenge on PPI. In a recent experiment designed specifically to investigate this possible confound in studies of maternal infection, Meyer et al. [44] observed deficits in conditioning in prenatal control mice that had been adopted by surrogate mothers treated with poly I:C during gestation, thus indicating that postnatal care by a gestationally-treated mother can affect offspring behavior by itself. Our study demonstrates that behavioral effects of prenatal immune challenge (LPS, turpentine) are present even when maternal care
is standardized through cross-fostering. In addition, it is possible that some prenatal immunogens (e.g. poly I:C) do not disrupt PPI in rats by themselves, but only do so in conjunction with another mild challenge, such as poorer maternal care.

As opposed to the long term behavioural consequences of LPS administration, the short term effects of LPS injection are well known. Behavioural responses to acute LPS administration, collectively known as sickness behaviour [26], include increased sleep, anorexia, adipsia, and a decreased interest in the surroundings. A decreased performance in a number of cognitive tasks has also been observed, including deficits in aversive conditioning [48,52], and spatial learning in the water maze [50]. However, there is still considerable debate over whether the altered performance in cognitive tasks truly reflect specific deficits, or whether they arise from a decrease in motivation induced by LPS administration, as suggested by some studies [21]. PPI, being an automatic pre-cognitive cognitive task without conscious attentional processing [17], may avoid the latter confound. Our results, showing no acute effect of LPS on PPI, indicate that the CNS pathways involved in this information processing task remain functional in the presence of the inflammatory response to LPS. However, it should be noted that PPI does not include any learning or memory component, and in this, and other respects, is quite different from the other tasks previously investigated.

In summary, in this study we observed deficits in PPI in adult rat offspring following prenatal systemic immune challenge with bacterial endotoxin or local inflammation with turpentine, but not following systemic poly I:C. These effects occurred using physiologically relevant doses of the immunogens, known to produce fever in the rat, and were independent of effects of maternal infection on maternal care of the offspring. These PPI deficits were observed after immune challenge during brief time windows during later stages of pregnancy (E15-19), with little or no effect at an earlier stage (E10-11). However fetal survival appeared to be more sensitive to the toxic effects of bacterial endotoxin at the earlier stage of pregnancy. These findings support the premise that maternal
infection during critical periods during pregnancy could contribute to sensorimotor gating deficits observed in schizophrenia.

Acknowledgements

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Figures

Fig. 1. Effects of systemic treatment with lipopolysaccharide (LPS) and polyinosinic: polycytidylic acid (poly I:C) at different gestational periods on prepulse inhibition of startle (PPI) in adult male offspring. Data show the mean percentage PPI (+ SEM) at different prepulse intensities (dB above background). (A) Offspring prenatally treated on E18-19 with LPS (50 µg/kg) displayed significantly lower PPI compared to saline- (treatment main effect, \( p < .05 \)) and poly I:C (750 µg/kg)- \( (p < .01) \) treated offspring \( (n=10-12) \). (B) Offspring prenatally treated on E15-16 with LPS (100 µg/kg) displayed significantly lower PPI compared to saline-treated offspring (treatment main effect, \( p < .05 \), \( n=9 \)). (C) Prenatal treatment with LPS (25 or 50 µg/kg) on E10-11 did not significantly alter PPI in adult offspring \( (n=9-11) \). Treatment with a higher dose (100 µg/kg) led to fetal resorption. (D) Prenatal treatment with poly I:C (1 mg/kg) on E10-11 did not significantly affect PPI in adult offspring \( (n=9-11) \). *=significant effect of treatment, \( p < .05 \) versus saline.
Fig. 2. Effects of local inflammation induced by turpentine at different times during gestation on PPI in adult male offspring. Data show the mean percentage PPI (+ SEM) at different prepulse intensities (dB above background) for male offspring (n=9-11) born to dams treated with intramuscular turpentine (10 µl) on (A) E18, (B) E15 or (C) E10. Maternal turpentine administration on E15 significantly decreased PPI in adult offspring at the 8dB prepulse intensity. * = p<.01.
Fig. 3. PPI in normal adult rats after acute systemic LPS administration. Data show the mean percentage PPI (+ SEM) at different prepulse intensities (dB above background) for male rats (n=11-12) tested 3h after systemic treatment with LPS (250 µg/kg) or saline.

Tables

**Table 1.** Effects of various inflammatory treatments on survival of pregnant dams and litters

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<td>Saline</td>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>E18</td>
<td>Turpentine (10 µl)</td>
<td>5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>E15</td>
<td>Saline</td>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>E15</td>
<td>Turpentine (10 µl)</td>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>E10</td>
<td>Saline</td>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>E10</td>
<td>Turpentine (10 µl)</td>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
E18-19, E15-16, E10-11 = gestation days 18-19, 15-16, 10-11, respectively. “Dam Survival” shows the % of dams surviving to term following injection with the indicated immunogen. “Dams Delivering Live Litters” shows the % of dams that gave birth to live litters at term following injection with the indicated immunogen.

**Table 2.** Baseline acoustic startle responses (a) in adult rats exposed prenatally to immunogens and (b) in normal adult rats following acute LPS treatment

<table>
<thead>
<tr>
<th>Time of Treatment</th>
<th>Treatment and Dose</th>
<th>Startle Response (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E18-19</td>
<td>Saline</td>
<td>187.8±25.6</td>
</tr>
<tr>
<td>E18-19</td>
<td>LPS (50 µg/kg)</td>
<td>187.8±18.4</td>
</tr>
<tr>
<td>E18-19</td>
<td>Poly I:C (750 µg/kg)</td>
<td>192.4±34.5</td>
</tr>
<tr>
<td>E15-16</td>
<td>Saline</td>
<td>377.7±82.9</td>
</tr>
<tr>
<td>E15-16</td>
<td>LPS (100 µg/kg)</td>
<td>232.5±23.7</td>
</tr>
<tr>
<td>E15-16</td>
<td>Poly I:C (1 mg/kg)</td>
<td>258.4±49.7</td>
</tr>
<tr>
<td>E10-11</td>
<td>Saline</td>
<td>241.8±33.5</td>
</tr>
<tr>
<td>E10-11</td>
<td>LPS (25 µg/kg)</td>
<td>204.5±40.3</td>
</tr>
<tr>
<td>E10-11</td>
<td>LPS (50 µg/kg)</td>
<td>203.1±45.6</td>
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<tr>
<td>E10-11</td>
<td>Saline</td>
<td>205.9±28.7</td>
</tr>
<tr>
<td>E10-11</td>
<td>Poly I:C (1 mg/kg)</td>
<td>246.6±42.6</td>
</tr>
<tr>
<td>E18</td>
<td>Saline</td>
<td>180.9±24.7</td>
</tr>
<tr>
<td>E18</td>
<td>Turpentine (10 µl)</td>
<td>128.6±20.5</td>
</tr>
<tr>
<td>E15</td>
<td>Saline</td>
<td>225.6±52.4</td>
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<tr>
<td>E15</td>
<td>Turpentine (10 µl)</td>
<td>186.6±29.2</td>
</tr>
<tr>
<td>E10</td>
<td>Saline</td>
<td>260.2±58.9</td>
</tr>
<tr>
<td>E10</td>
<td>Turpentine (10 µl)</td>
<td>178.6±35.0</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>Saline</td>
<td>146.2±29.2</td>
</tr>
<tr>
<td>Adult</td>
<td>LPS (250 µg/kg)</td>
<td>118.7±13.3</td>
</tr>
</tbody>
</table>
References


3. Conclusion

Our results, together with those of others, demonstrated that gestational timing of the immune challenge is a critical factor when examining the effects of maternal immune activation (MIA) on behavior. A number of behavioral alterations have now been shown to be influenced by gestational timing of administration, such as changes in exploratory behavior (Meyer et al. 2006b), latent inhibition (Meyer et al. 2006a), perseverative behavior and working memory deficits (Meyer et al. 2006a; Meyer et al. 2006b). The timing of gestational windows of vulnerability are likely determined in part by the maturational stage of the fetus’ CNS at the time of administration. However, it may also be critically influenced by maternal and placental physiology, which changes throughout gestation, including in their immune response.

We also showed that maternal immune activation induced by turpentine, a model of local inflammation, could affect offspring behavior. This suggests an important role for the maternal immune response in the effects exposure to maternal infection. The mechanisms that would underlie these alterations would be common to both systemic and local inflammation. Pro-inflammatory cytokines have been suggested, but other intermediates such as HPA-axis activation and reduced food intake must not be discounted. These possible mechanisms are discussed in more detail in the general discussion section.
Chapter VI: Immunological response in adult animals that had been exposed to maternal immune activation during gestation
1.1 Introduction

The neurological consequences of various early adverse events, such as stress exposure, malnutrition and infection have been widely studied. However, their consequences on other systems such as the immune system have received less attention. Starting in 2004, Pittman and colleagues conducted a series of experiments to investigate the effects of neonatal immunogen exposure on adult immune function. In their first experiment, fourteen-day-old (P14) rats were submitted to a dose of LPS that elicited an immune reaction. When given a second LPS injection during adulthood, they responded with an attenuated fever compared with that of rats which had not been exposed to LPS as neonates (Boisse et al. 2004). At the same time, exposure to LPS exclusively at maturity did not cause fever attenuation when subjects were challenged again two months later. A follow-up study demonstrated that this attenuated fever was caused by an enhanced hypothalamic-pituitary-adrenal axis (HPA)-axis activation and corticosterone secretion in response to the adult LPS challenge (Ellis et al. 2005). Excess corticosterone in turn limited the rise of circulating pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α (Ellis et al. 2005), both critical mediators of the febrile response.

In addition to these observations, some immunological changes have been found in animals exposed to maternal immune activation (MIA) in utero. For instance, Borrell and colleagues observed that rats exposed to MIA throughout gestation displayed increased baseline levels of circulating IL-2 and IL-6 on P100 and P170, as well as increased circulating TNF-α throughout their lifetime (P21, 39, 70, 170 and 400) (Borrell et al. 2002; Romero et al. 2008). They also exhibited morphological signs of astroglial and microglial reactivity, indicative of immune activation in the brain, which were very mild on P100 but more apparent on P300 (Borrell et al. 2002). Results from another group showed increased TNF-α levels in the adult brain of MIA-exposed rodents (Ling et al. 2004). In
another model, animals born from mothers subjected to repeated stress during gestation have been known to display long-term behavioral and physiological alterations, including a depressed immune function (Kay et al. 1998).

A permanent dysregulation of the immune systems may affect brain function. Altered immune function has been observed in certain human mental illnesses such as depression (Zunszain et al. 2010; Piser 2010) and schizophrenia (SCZ) (Potvin et al. 2008). For instance, major depression has been associated with elevated plasma IL-6 levels (Alesci et al. 2005). IL-6 is a major inducer of sickness behavior, an organized behavioral response to infection consisting in anorexia, increased sleep, reduced physical activity and social withdrawal (Kelley et al. 2003). Circulating cytokines have also been shown to affect rodent behavior and brain neurochemistry. Increase in circulating IL-6 have been shown to alter brain DA turnover and sensitize AMPH-induced locomotor activity, indicating modulation of brain DA systems by circulating IL-6 (Zalcman et al. 1994; Zalcman et al. 1999; Song et al. 1999).

On the basis of these reports, we hypothesized the following: animals exposed to MIA in utero would exhibit an attenuated immune response in reaction to an immune challenge administered during adulthood. To investigate these questions, we first examined the pyrogenic response to LPS or poly I:C in adult rats that had been exposed to LPS in utero. In the second experiment, we measured circulating pro-inflammatory cytokine changes following LPS injection in adult animals exposed to LPS in utero.

1.2 Materials and methods

Prenatal treatment. Timed pregnant Sprague–Dawley rats were injected intraperitoneally (ip) with 50μg/kg E. coli LPS or 750μg/kg poly I:C, once daily on gestational days (E) 18 and 19. Injection of an equivalent volume of sterile saline (ip) was used as a control. Pups were cross-fostered with surrogate dams in mixed litters and weaned on postnatal day 21 (P21). For each experiment, pups came from a minimum of four different mothers per group. Animals were housed
two subjects per cage under standardized conditions, and maintained on a 12 h:12 h light:dark cycle with free access to food and water, until they reached adulthood.

Experiment 1: Febrile response to adult immune challenge in MIA-exposed animals. At 3 months of age, a small battery-operated, temperature-sensitive radio transmitter was implanted in the animals’ abdominal cavity under anesthesia (50 mg/kg ketamine, 5 mg/kg xylazine, and 0.5 mg/kg acetopromazine). Antennas mounted in receiver boards situated beneath each animal’s cage monitored the transmitter output frequency (Hz) at 10-min intervals. This information was relayed to a personal computer where frequency measurements were converted to degrees Celsius using Dataquest software (DataScience). Animals were allowed a minimum of 7 days for postsurgery recovery. On the day of the experiment, after 7h of baseline recording, injections were administered between 10:00 and 12:00, during the light phase of the normal light-dark cycle, and changes in core body temperature were monitored continuously for 15h after treatment. Subjects were treated as follows: animals prenatally exposed to LPS either received an LPS injection (100 µg/kg ip, prenatal LPS/LPS, n=6) or an equivalent volume of saline (prenatal LPS/saline n=4); animals prenatally exposed to poly I:C received poly I:C (750 µg/kg ip, prenatal poly I:C/poly I:C n=5); animals prenatally exposed to saline as control were either given LPS (100 µg/kg ip, prenatal saline/LPS, n=6) or poly I:C (750 µg/kg ip, prenatal saline/ poly I:C, n=5). As the number of receiver platforms available was limited, there was no group treated with saline both prenatally and as adults (prenatal saline/saline) and no group exposed to poly I:C prenatally and then to saline as adults (prenatal poly I:C/saline). However, since the question under investigation was whether MIA-exposed animals had an altered immune response compared with non-exposed animals, these two groups were judged to be the least informative for this question and were thus omitted.

Experiment 2: Cytokine response to adult immune challenge. In this second experiment, LPS was the sole molecular immunogen used, as the cytokine cascade triggered by this molecule had already been well characterized (Conti et al. 2004). Rats were prenatally exposed to LPS-induced MIA (50µg/kg) or saline
as described above. Since no effect of prenatal LPS at a dose of 50µg/kg was observed on fever responses in experiment 1, a group of rats was also exposed to a higher dose of LPS (100µg/kg) using the same protocol. We had previously observed that administering this higher dose of LPS to E18 dams may sometimes lead to mortality (around 40% in Chapter IV). However, in this experiment, no dam death was observed, and all pregnant animals gave birth to live pups, who were then cross-fostered and weaned as explained above. When the animals reached adulthood, (i.e. at P83-86), they were challenged with either LPS (100µg/kg ip) or saline and sacrificed with an overdose of anesthetic, 2h after LPS/saline injection. Blood was collected in heparinized tubes by cardiac puncture and centrifuged, after which the plasma was stored at -80°C until assayed. IL-1β and TNF-α levels were measured using a two-site, rat-specific ELISA, as previously described (Rees et al. 1999). It is important to note that, in order to maximize information and minimize use of experimental animals, one month before sacrifice three behavioral tests were conducted on this cohort of animals, each one separated by 7 days: prepulse inhibition (PPI) of acoustic startle, light PPI and social recognition. The animals were challenged with LPS and sacrificed as described above, after one week of rest following the last behavioural test. These behavioural tests are non-invasive, brief and do not involve any drug injections.

Data analysis. Temperature measurements were averaged over 1h periods and analyzed using two-way mixed model ANOVA, with the treatment group as a between-subject factor and time as a within-subject factor. Significant interactions were decomposed using simple main effects tests, and post-hoc Tukey’s tests were performed when appropriate. ELISA results were analyzed using two-way independent measures ANOVA and post-hoc Tukey’s tests. Statistical analysis resulting in probabilities of $p<.05$ were considered significant.
1.3 Results

Experiment 1. Rats were exposed to MIA induced by LPS or poly I:C injection on E18 and E19, or to maternal saline injection as control. At adulthood, they were challenged with LPS, poly I:C or saline injection and their body temperature was monitored (Fig. 1). Baseline body temperature did not differ among treatment groups. Two-way ANOVA of body temperature measurements for the 10h following LPS or saline injection (Fig 1A) indicated a significant treatment x time interaction ($F_{20,120}=5.04$, $p<.01$). Decomposing this interaction with simple effects tests allowed us to look at the effect of treatment at each time point revealing a significant effect of treatment between 4 to 8h after injection (all $p<.01$), and a significant effect of time on all treatments (all $p<.01$). Most, importantly, post-hoc pairwise comparisons indicated that there were no significant differences in body temperature between the prenatal LPS/LPS group and the prenatal saline/LPS group at any time point, indicating that prenatal LPS exposure had no effect on the subsequent febrile response to LPS at adulthood. As expected, body temperature of LPS/LPS group differed significantly from the LPS/Saline group from 4h to 7h after injection (Fig.1A, all $p<.01$), indicating that LPS induces a febrile response in adult rats that had been exposed prenatally to LPS. Body temperature of the Saline/LPS group was also significantly different from the LPS/Saline group, in this case from 5h to 7h post-injection (all $p<.05$). A separate analysis was performed to examine the effects of adult poly I:C challenge in rats prenatally exposed to poly I:C or saline (Fig. 1B). Two-way ANOVA revealed no significant treatment x time interaction ($F_{10,70}=0.89$, $p=.55$), a significant effect of time ($F_{10,70}=2.82$, $p<.01$) and no significant effect of treatment ($F_{10,70}=0.14$, $p=.72$). Thus prenatal poly I:C exposure had no effect on the response of body temperature to poly I:C challenge at adulthood.
Fig. 1. Febrile response to immune challenge in adult rats exposed to maternal immune activation (MIA) *in utero*. Pregnant animals were injected ip with lipopolysaccharide (LPS, 50µg/kg), polyinosinic: polycytidylic acid (poly I:C, 750µg/kg) or saline on days 18 and 19 of gestation. When the offspring reached adulthood, they were treated again with an immune agent (LPS 100µg/kg, poly I:C 750µg/kg) or with saline as control. Data show the average body temperature (±SEM) between 7h before and 15h after injection, depicted by the arrows. (A) Rats prenatally exposed to LPS or saline both displayed a significant increase in body temperature in response
to LPS injection at adulthood \((p<.01\) at \(t=4-7h\) and \(p<.05\) at \(t=5-7h\), respectively) compared with rats prenatally exposed to LPS that received saline injection at adulthood as a control. Their average febrile response was not significantly different from one another (B) Rats prenatally exposed to poly I:C or saline did not significantly differ from one another in their body temperature response to poly I:C challenge at adulthood. \(*p<.05\ **p<.01\) vs prenatal LPS/Saline.

**Experiment 2.** Animals were exposed to LPS induced MIA on E18 and E19. At adulthood they were challenged with LPS or saline, and their plasma cytokine response was measured 2h after challenge (Fig. 2). Two-way ANOVA of plasma IL-1\(\beta\) levels indicated no significant interaction between prenatal and adult treatment (\(F_{2,25}=0.96, p=.40\)), a significant effect of adult immune challenge (\(F_{1,25}=89.3, p<.001\)) and no effect of prenatal treatment (\(F_{2,25}=0.90, p=.42\); Fig. 2A). All groups, regardless of prenatal treatment, showed an increase in plasma IL-1\(\beta\) levels in response to LPS challenge at adulthood, and this LPS-induced IL-1\(\beta\) response did not differ in magnitude between prenatal treatment groups. This increase in IL-1\(\beta\), which is not affected by prenatal treatment, is consistent with a normal LPS response. Similarly, analysis of plasma TNF-\(\alpha\) levels revealed no significant interaction between prenatal and adult treatment (\(F_{2,25}=1.10, p=.35\)), a significant effect of adult LPS challenge (\(F_{1,25}=62.3, p<.001\)) and no effect of prenatal treatment (\(F_{2,25}=1.08, p=.35\); Fig. 2B). Thus, MIA-exposed exposed animals did not significantly differ from the non-exposed animals in their cytokine response to LPS challenge.
**Fig. 2.** Effect of MIA exposure on LPS-induced cytokine response. Rats exposed to LPS (50 or 100µg/kg) or saline on day 18 and 19 of gestation were challenged again with LPS (100µg/kg) or saline during adulthood and sacrificed 2h after injection. Figure shows plasma levels (±SEM) of interleukin (IL)-1β (*A*), and tumor necrosis factor (TNF)-α (*B*). LPS injection at adulthood resulted in a significant rise in IL-1β and TNF-α. However, there was no effect of prenatal treatment the cytokine response. (Prenatal saline-LPS: n=3; prenatal LPS 100µg/kg-LPS: n=4; all other groups: n=6) ***p<.001 vs adult saline injection
1.4 Discussion

Based on studies of neonatal exposure to immune challenge, we initially hypothesized that prenatal exposure to MIA may alter the immune response in the adult rat. However, our study demonstrated that animals exposed to MIA on E18 and 19 did not exhibit an altered immune response. Firstly, animals whose mother were exposed to LPS or poly I:C during gestation did not show an altered febrile response when exposed to the same immunogen at maturity. Secondly, animals whose mothers were exposed to LPS during the same gestational period did not show an altered cytokine response to a LPS challenge at adulthood.

Contrary to our results with gestational treatment, Pittman et al showed that animals exposed neonatally (P10) to an immunogen displayed a reduced febrile and cytokine response, which was caused by an increased corticosterone response to the immune challenge (Boisse et al. 2004; Ellis et al. 2005). One possible reason for the discrepancy between prenatal and post-natal treatment is that direct contact between the fetus and the immunogen would be required for the programming of the immune system to occur. This hypothesis is supported by further studies by Pittman and colleagues who observed that these changes were immunogen-specific, i.e. that rats exposed to LPS on P10 did not show an altered immune reaction to poly I:C at adulthood and vice versa. LPS and poly I:C signalling cascades are very similar, the main difference being their specific receptors, located on monocytes; LPS signalling occurs through toll-like receptor-4 (TLR4) (Lien et al. 2000), while poly I:C signals through TLR3 (Alexopoulou et al. 2001). It was therefore concluded that, for this immunogen-specificity to occur, the immune changes in the exposed offspring needed to occur at the level of TLR signalling (Ellis et al. 2006). However, molecules like LPS or poly I:C may not come into direct contact with the fetus, when injected into the maternal compartment. For instance, our laboratory has showed that radiolabeled LPS injected intraperitoneally to a pregnant rat did not penetrate the fetal cavity (Ashdown et al. 2006). At the present, it is not clear whether common live
viruses such as influenza, much less individual viral-like like particles poly I:C, can cross the placenta, (Aronsson et al. 2002; Shi et al. 2005).

According to a study by Spencer et al. (2006), programming of the immune system by neonatal LPS exposure in the rat occurred only when the immunogen was administered during a critical period, corresponding roughly to the 2nd post-natal week. Thus administering LPS on E18-19 may expose the dam/fetus to immunogen outside a critical period required for long term effects on immune function in offspring. At the present, whether some changes in the immune response would occur if the immune agent was administered earlier in pregnancy is unknown. Studies examining the long term immune effects of earlier MIA exposure observed fluctuations in baseline cytokine levels, such as increased circulating IL-2, IL-6 and TNF-α (Borrell et al. 2002; Romero et al. 2008). However, these studies did not investigate if such alterations extended to the response to an immune challenge at adulthood. In the present study, we did not observe an effect of prenatal treatment on baseline IL-1β or TNF-α. These studies also used a more extensive immune treatment (LPS injected either daily or every other day throughout pregnancy), which may bring about more extensive alterations.

In conclusion, the current study clearly demonstrates that exposure to MIA on E18 and 19 in the rat does not alter fever and cytokine responses to immunogen administration at adulthood. Further studies are needed to determine if these observations extend to exposure during an earlier gestational period.
Chapter VII: General discussion
The rationale of our study is based on observations from epidemiological research indicating that maternal infection during pregnancy increases the risk for the offspring of developing schizophrenia (SCZ) later in life (Brown 2006; Patterson 2009). Infections from different types of viruses and bacteria have been implicated in these studies, suggesting that a common element would be inducing some changes in the developmental trajectory of the fetus. It has been hypothesized that this common element would be part of the maternal immune response to pathogens. As the diagnosis of SCZ is based on behavioral symptoms, we wanted to examine the possibility that activation of the maternal immune response by itself could affect offspring behavior in adulthood. To investigate this question, we decided to develop an animal model of maternal immune activation (MIA) and observe if it induced behavioral changes later in life. We were also interested in investigating what aspects of the immune challenge had an impact on the developmental outcome. When this project started in 2001, very few experimental studies had been conducted on the impacts of maternal infection and MIA on offspring neurodevelopment, especially in relation to their behavior. In recent years, however, the number of publications on the subject has grown tremendously, and MIA is now routinely used to try and understand the processes linking maternal infection to altered neurodevelopment, and how these processes could be involved in the etiology of human mental illnesses.

Briefly, we injected pregnant rats with a molecular immunogen and grew their offspring to adulthood. The offspring then underwent various behavioral tests, such as amphetamine (AMPH)-induced locomotion and prepulse inhibition of acoustic startle (PPI), to evaluate the effect of MIA on offspring behavior. We then examined to what extent different parameters of the MIA challenge, such as the duration of the maternal immune response, the type of immunogen used, or the gestational timing of exposure, influenced the offspring behavioral outcome.

Though maternal infection is considered a risk factor for SCZ, most pregnancies complicated by influenza infections do not result in neurological or
psychiatric adverse effects in the offspring. Consequently, we first investigated whether MIA needed to occur in conjunction with another type of challenge to have an effect on development, or whether MIA alone was sufficient to alter offspring behavior into adulthood. In epidemiological studies of SCZ, obstetric complications are often cited as environmental risk factors, in particular those associated with neonatal hypoxia (Cannon et al. 2002). Hypoxia may be linked to an inflammatory state in the brain (Dammann and Leviton 2004), and pro-inflammatory cytokines, which are also suspected to play a role in the negative consequences of maternal infection, have been shown to alter neuronal cell fate and survival (Deverman and Patterson 2009). Thus we were interested to find out whether MIA by itself was sufficient to induce behavioral alteration in an animal model, and whether a subsequent period of anoxia would interact with the first insult to alter offspring neurodevelopment. Experimental results presented in chapter II revealed that LPS administration on gestational day (E)18 and 19 was sufficient to induce behavioral changes in offspring, comprising increased AMPH-induced locomotion and baseline acoustic startle. The addition of a period of anoxia on the day of birth or 7 days later (which would roughly correspond to the brain maturity of a human newborn (Romijn et al. 1991; Avishai-Eliner et al. 2002)) did not worsen or diminish the alterations. In later chapters, MIA-induced alterations were observed without additional early-life challenges, and we can thus conclude that a period of anoxia or another environmental challenge is not necessary to observe behavioral effects of MIA.

Next, we investigated the effect of the duration of the MIA exposure on offspring behavioral alterations. In chapter II, MIA was induced using two LPS injections given at a 24h interval. In chapter III, we wanted to know if an LPS challenge of longer duration would result in more extensive or simply different behavioral alterations. Subcutaneous (s.c.) osmotic minipumps were used to deliver a constant flow of LPS solution. To compare the immune response induced by intraperitoneal (i.p.) LPS injection at 24h intervals with one caused by continuous LPS infusion via minipumps, fever induced by both methods was measured using remote biotelemetry in adult male rats. In the infection model
using repeated injections, rats displayed an elevated temperature for 4h after the first injection and for 4h after the second. By the 3rd injection, rats had become tolerant to the pyrogenic effects of LPS, which corroborates observations previously made in rabbits, rats and guinea pigs (Yamashiro et al. 1993; Zeisberger and Roth 1998). In the case of minipump delivery, the animals exhibited a significantly elevated body temperature for a total of 18h. This prolonged fever implies an enhanced release of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) responsible for mediating the LPS-induced fever. Next, to investigate if a prolonged MIA exposure was associated with different behavioral alterations in offspring, pregnant rats were implanted with minipumps that continuously delivered LPS from E18 to delivery. This type of MIA challenge resulted in a significant decrease in PPI at all prepulse intensities but, as opposed to repeated LPS injection, did not increase AMPH-induced locomotion. Besides the duration of the maternal immune response, other factors could contribute to the difference between the results obtained with the two methods, notably the route of administration (i.p. versus s.c.), and the fact that that all the pups in the first experiment received an anoxia or air treatment, which introduced neonatal handling as a confounding variable. PPI disruption by MIA in rodents has since become a well established finding, with MIA often induced by only one or two injections of molecular immunogen (Shi et al. 2003; Ozawa et al. 2006; Smith et al. 2007; Fortier et al. 2007; Wolff and Bilkey 2008; Makinodan et al. 2008; Meyer et al. 2008c).

The second factor investigated was whether MIA-induced behavioral alterations are stimulus specific. In chapters II and III, we characterized changes following MIA induced by LPS, which is of bacterial origin. However, most studies linking maternal infection to increased risk of SCZ were based on infections of viral origin, most notably influenza. Thus, we wanted to know if MIA triggered by a product of viral origin would similarly affect offspring behavior. Polyinosinic: polycytidylic (poly I:C), a viral-like synthetic double stranded RNA, was selected to induce MIA. At the time this study was conducted, administration of poly I:C was known to cause fever and trigger
components of the innate immune response in a variety of species (Kimura et al. 1994b; Toth 1996), but basic understanding of the model was limited, including knowledge of the cytokine cascade underlying the immune response. Consequently, before using poly I:C in a model of developmental alteration by MIA, we wanted to know more about the basic characteristics of the immune response this molecule induces. We first identified the lowest dose at which a maximal febrile response was induced in adult rats. Following poly I:C injection, a significant rise in the pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis (TNF)-α was detected in the plasma. Co-injection of poly I:C and IL-1 receptor antagonist (IL-1ra) revealed that the febrile response and the release of IL-6, but not of TNF-α, was IL-1 dependent. This dependency on IL-1, and the hypothalamic rise in IL-1β and cyclooxygenase-2 mRNA that we detected, parallel what has been observed following LPS injection, and suggest a common axis between the inflammatory responses caused by LPS and poly I:C. However, some differences between the two responses were also detected, notably in the temporal profiles of circulating IL-6 and TNF-α release, and in the mechanisms underlying inflammatory-induced anorexia. Since poly I:C was shown to reliably induce fever and circulating cytokines, we decided to use it alongside LPS in our model of MIA-induced developmental alterations.

Contrary to LPS, which has been shown to be unable to cross the placenta (Goto et al. 1994; Ashdown et al. 2006a), whether poly I:C can cross the placental barrier remains undetermined. Conflicting evidence has been reported as to whether the influenza virus can cross the placenta and enter the fetal cavity (Aronsson et al. 2002; Shi et al. 2005), and to the author’s knowledge, no study directly addressed this question regarding poly I:C itself. Nevertheless, Toll-like receptors (TLR)-3 and -4 are present in the placenta, and through their binding, circulating poly I:C and LPS could potentially activate the innate immune response locally (Beijar et al. 2006; Abrahams et al. 2006) and affect placental functions. We were therefore interested to know whether MIA induced by an agent that would not have access to the fetal or placental unit could also induce behavioral alterations in the offspring. This would clearly indicate that the
maternal immune response, even in the absence of a pathogen, can alter offspring neurodevelopment. Intramuscular injection of turpentine, an organic solvent causing tissue necrosis at the site of injection (Wusteman et al. 1990), is a well-characterized model of local inflammation. It causes a sterile abscess, with local pro-inflammatory cytokine production and subsequent cytokine release into the circulation, which triggers a systemic immune response (Luheshi et al. 1997; Leon 2002). We decided to integrate turpentine into our study of the developmental effects of MIA, and compare its effects to those of LPS- and poly I:C-induced MIA.

In addition to comparing the effects of MIA induced by different agents, another factor was investigated: windows of vulnerability to MIA during gestation. For many developmental insults, it has been established that “susceptibility to teratogenesis varies with the developmental stage at the time of exposure” (Wilson 1973). Furthermore, epidemiological studies have suggested that increased SCZ risk is associated with prenatal infection occurring during specific trimesters (see Chapter I for a discussion). In the experiment presented in Chapter V, pregnant rats were injected with either LPS, poly I:C or turpentine at one of three periods during gestation: embryonic day (E)10–11, E15–16 and E18–19. PPI was then measured in the offspring. The results demonstrated that the gestational timing of the immune challenge is a critical factor, drastically influencing the effects of MIA. For instance, LPS-induced MIA significantly decreased offspring PPI when the immune challenge occurred on E15–16 and E18–19, but not on E10-11. Similarly, turpentine-induced MIA also disrupted PPI when turpentine challenge occurred on E15–16, but not on E10-11 or E18-19. We did not detect an effect of poly I:C-induced MIA at the dose administered. These results indicate that both parameters studied, i.e. gestational period and nature of immune agent, can affect the consequences of MIA. The fact that turpentine-induced MIA can affect behavior is particularly interesting, in that it indicates that neither direct contact between the immunogen and the fetus, or between the immunogen and placenta, are required for MIA to exert an influence on offspring development. This strongly supports the hypothesis that the maternal
immune response plays an active role in the developmental alterations following maternal infection.

Other groups also observed the critical influence of the gestational timing of MIA on behavioral outcomes. In a series of experiments, Meyer and colleagues compared offspring behavioral changes following gestational administration of poly I:C on E9 to those obtained by treating mice on E17. Alterations in exploratory behavior (Meyer et al. 2006b), latent inhibition (Meyer et al. 2006a) and prepulse inhibition (Meyer et al. 2008c) were induced only when poly I:C was administered on E9. Perseverative behavior and specific working memory deficits, however, emerged only when MIA was administered at E17 (Meyer et al. 2006a; Meyer et al. 2006b). Other behavioral changes, such as increased AMPH-induced locomotion (Meyer et al. 2006a) and loss of the unconditioned stimulus pre-exposure effect (Meyer et al. 2008c), were present regardless of the gestational timing of MIA.

These observations, together with ours, highlight the importance of critical windows of sensitivity. When thinking about critical windows of susceptibility, the focus is logically on the maturational stage of the fetus itself. Indeed, proliferating cells are often sensitive to developmental cues (e.g. growth factors, cytokines) during specific developmental periods. However, in the case of MIA, the precise timing of gestational windows of vulnerability could be critically influenced by two additional factors: the maternal immune system and the placenta. Maternal physiology undergoes many changes during pregnancy, including its reaction to pathogens. It has been observed that in many species, including rats, the LPS-induced febrile response gradually dampens towards the end of the pregnancy, leading to hypothermia and even death when close to parturition (Martin et al. 1995; Fofie and Fewell 2003). These changes are accompanied by an increase in LPS-induced IL-1ra and decrease in IL-1β and IL-6 compared to non-pregnant females’ responses (Fofie et al. 2005; Ashdown et al. 2006b). Towards the end of pregnancy, hypothalamic–pituitary–adrenal (HPA)-axis reactivity also decreases, as LPS fails to increase ACTH and corticosterone plasma concentrations in near-term rats (Russell et al., 2005). These gradual
changes in the gestating animal’s physiology could influence the alterations induced by a maternal immune challenge.

The placenta forms an active barrier that prevents most common pathogens and toxins from coming into direct contact with fetal tissue, while still allowing specific immune signals to cross. However, placental physiology also goes through changes throughout gestation. For instance, in rats, IL-6 crosses the placenta in far greater amounts in mid-gestation (E11-13) compared to late gestation (E17-19) (Dahlgren et al. 2006). Interestingly, Meyer and colleagues reported that the MIA-induced alterations in their model appeared generally more severe when poly I:C was administered earlier (E9) compared to later (E17) in gestation (Meyer et al. 2007). Yet, the maternal circulating IL-6 response did not differ between the two time points (Meyer et al. 2006b). In summary, the periods of sensitivity we actually observe are likely the result of combined influences in the maternal, placental and fetal compartment.

It might be observed that, contrary to us, Meyer’s group and others found a decreased PPI after gestational administration of poly I:C. However, they used a dose 4 to 60 times greater than ours, and, in some studies, a different route of administration (i.v. vs i.p.) (Borrell et al. 2002; Meyer et al. 2005; Ozawa et al. 2006; Romero et al. 2007; Romero et al. 2008), while we chose to use a dose that gave a moderate fever in adults, a challenge that can be expected to occur in a natural setting. This exemplifies the many methodological differences that exist between laboratories, i.e. in choice and dose of immune agent, gestational time and route of administration, outcome measure and age of testing. All these render comparison between studies extremely difficult. Nevertheless, the fact that MIA alone can cause behavioral, morphological and biochemical alterations is now well established. The next step is to look for the mechanisms underlying these changes.
Importance of cytokines as part of the underlying mechanism mediating MIA-induced behavioural alterations.

Meyers’ group demonstrated that overexpression of IL-10, a potent anti-inflammatory cytokine, by macrophages in the fetus prevented or attenuated some MIA-induced behavioral alterations, including PPI disruption, decreased exploration, latent inhibition deficiency and increased locomotor activity by MK-801 (Meyer et al. 2008b). However, some behavioral changes remained present in MIA-exposed transgenic mice, such as enhanced AMPH-induced locomotion. Smith et al. showed that a single maternal injection of the pro-inflammatory cytokine IL-6 caused behavioral changes in offspring strikingly similar to changes induced by MIA, such as PPI and latent inhibition (LI) deficits (Smith and Kluger 1992). Furthermore, co-administration of poly I:C with anti-IL-6 antibodies to pregnant rodents prevented the PPI, LI, and exploration deficits otherwise associated with poly I:C-induced MIA. Taken together these results suggest that some of the behavioural alterations produced by MIA may be mediated via IL-6 and/or modulated by IL-10. However, studies measuring pro-inflammatory cytokine levels in fetal and neonatal brains following MIA have yielded inconsistent results, suggesting that direct effects of cytokines on fetal brain may not be responsible for altered neurodevelopment in offspring. Besides being able to directly influence neuronal cells on their own, cytokines also regulate systemic aspects of the immune response in the pregnant mother, such as fever, decreased food intake, and HPA-axis activation. These systemic changes remain as important candidate mechanisms that may mediate effects of MIA on fetal neurodevelopment. A recent study by Cui et al. (2009) showed that co-administration of a cyclo-oxygenase inhibitor, ibuprofen, with LPS to pregnant rat dams inhibited LPS-induced fever but was unable to reverse prenatal LPS-induced deficits in hippocampal neurogenesis in offspring. This study thus ruled out fever as a mediator of prenatal LPS-induced alterations in hippocampal neurogenesis. The possible contributions of fever, altered food intake and/or
HPA-axis activity on this and other MIA-induced alterations in CNS function in offspring warrant further investigation.

In conclusion, the work in this thesis and investigations carried out by other laboratories in the last decade using rodent models have provided clear evidence that maternal immune activation during pregnancy can cause lasting alterations in CNS function and behaviour in resulting offspring. This lends strong support to the idea that the association between prenatal infection and increased schizophrenia in human epidemiological studies actually reflects a causal relationship, i.e., prenatal infection causes changes in neurodevelopment contributing to symptoms of the disorder. Animal models should continue to provide useful tools for unravelling the precise mechanisms by which maternal infection during pregnancy alters brain function in offspring both acutely and in the long-term.
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