PRENATAL INFLAMMATION AND NEURODEVELOPMENTAL DISORDERS: THE ROLE OF CYTOKINES

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Abstract

Prenatal infection has been linked to the development of schizophrenia in the offspring, most likely through the maternal inflammatory response triggered by infection. A number of studies in animal models demonstrated that acute inflammatory episodes were sufficient to trigger brain alterations in the adult offspring, especially in the mesolimbic dopamine (DA) system, involved in the pathophysiology of schizophrenia. The aim of this research program was to identify which elements of the maternal inflammatory response are implicated in the alteration of the mesolimbic DA neurotransmission of the offspring. The pro-inflammatory interleukin (IL)-6, the anti-inflammatory IL-1 receptor antagonist (ra) and the immune modulator leptin were of particular interest. To investigate this, a model of aseptic clinical trauma was used, which consisted of a localized intramuscular injection of turpentine oil (TURP). This approach allows for the study of the endogenous mediators whilst avoiding the direct targeting of the foetal compartment, since the exogenous immunogen remains at the site of injection. In the work presented here it was demonstrated that maternal inflammatory response to TURP varied with the gestational day (GD), which was associated with the magnitude of the behavioural effects in the offspring. Early TURP treatment, at GD 15, induced significant impairments in pre-pulse inhibition and the cued-task of the Morris-water maze, whereas it enhanced cued-fear conditioning and locomotor response to AMPH; these effects were largely absent when TURP was given to the dam at GD 18. Neutralization of IL-6 and IL-1ra, the main circulating mediators induced by TURP, during the maternal acute inflammatory response at GD 15 prevented the effects of prenatal TURP on enhanced locomotor response to AMPH and increased synthesis of DA in the nucleus accumbens of the offspring. Leptin, a cytokine-like hormone implicated in inflammation, appeared to be involved in the induction of enhanced behavioural plasticity following repeated AMPH administration. Finally, it was demonstrated that inflammation induced an IL-6-mediated hypoferremia that was involved in the induction of enhanced sensitization to AMPH and tyrosine

hydroxylase in the nucleus accumbens. Collectively, the work presented in this thesis provides extensive and novel evidence showing that several immune mediators affect neurodevelopment of the mesolimbic DA system directly or via secondary mechanisms, and may be involved in the increased risk for schizophrenia in the human population.

Résumé

L'infection chez la mère a été liée au développement de la schizophrénie chez l'enfant, probablement due à la réponse inflammatoire déclenchée par l'infection. De nombreuses études utilisant des modèles animaux ont démontré que l'inflammation est suffisante pour déclencher des altérations dans le cerveau chez la progéniture adulte, en particulier dans le système dopaminergique mésolimbique (DA), impliqué dans la physiopathologie de la schizophrénie. L'objectif de ce programme de recherche était d'identifier quels éléments de la réponse inflammatoire sont impliqués dans les changements observés dans la neurotransmission DA de la progéniture. La cytokine pro-inflammatoire interleukine (IL)-6, l'antagoniste du récepteur de l'IL-1 (IL-1ra) et la leptine ont été d'un intérêt particulier. Pour induire une réponse inflammatoire, un modèle de traumatisme clinique aseptique a été utilisé. Il s'agissait d'une injection intramusculaire de l'essence de térébenthine (TURP), qui évite de cibler directement le compartiment fœtal par l'immunogène. Dans le travail présenté ici, il a été démontré que la réponse inflammatoire maternelle induite par la TURP varie selon le jour de gestation (JG), où celle-ci a été associée à l'ampleur des effets sur le comportement de la progéniture. Lorsque administré au JG 15, la TURP a induite des déficiences importantes dans l'inhibition de pré-pulse et dans le labyrinthe aquatique de Morris, alors qu'elle a accentué la peur conditionnée et la réponse locomotrice à l'AMPH. Ces effets ont été largement absents lorsque la TURP a été donnée au JG 18. La neutralisation de l'IL-6 et de l'IL-1ra lors de la réponse inflammatoire de la mère au JG 15 a empêché les effets de la TURP sur la réponse locomotrice à l'AMPH et l'augmentation de la synthèse de DA dans le noyau accumbens de la progéniture. La leptine quand à elle, semble être impliquée dans l'induction de la plasticité comportementale après des administrations répétées d'AMPH. Finallement, il a été démontré que l'inflammation déclenche l'hypoferremie, une réduction de fer dans la circulation et le placenta, qui a été impliqué dans l'induction de la sensibilisation à l'AMPH et à l'hydroxylase de tyrosine dans le noyau accumbens. Collectivement, le travail présenté dans cette thèse fournit de nombreux et nouveaux éléments montrant que

plusieurs médiateurs immunitaires affectent le développement neurologique du système DA et peuvent être impliqués dans le risque accru de schizophrénie dans la population.

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Introduction

A number of psychiatric disorders have been hypothesised to have a neurodevelopmental origin (Ehninger et al., 2008; Levitt, 2005; Rapoport et al., 2005) arising from a combination of genetic and environmental risk factors that lead to disrupted brain development (Levitt, 2005; Tsuang et al., 2001). Based on epidemiological studies, maternal infection has been linked to some of these psychiatric conditions, including cerebral palsy, autism spectrum disorders, schizophrenia (SCZ) and other forms of psychosis (Brown and Derkits, 2010a; Deverman and Patterson, 2009; Patterson, 2009). Evidence for the link between this prenatal infection and SCZ is abundant, and it has been estimated that about 33 % of SCZ cases involve events of maternal infection [reviewed in (Brown and Derkits, 2010a)].

To investigate the mechanistic link between maternal infection and the development of SCZ, a number of experimental approaches using rodents have been developed and are now widely used in this area of research (Boksa, 2010a; Meyer and Feldon, 2010). The most frequently used models involve the systemic injection of immunogenic agents such as bacterial lipopolysaccharide (LPS) (Fortier et al., 2004) or the viral mimic polyinosinic:polycytidylic acid (poly I:C) (Meyer et al., 2005; Shi et al., 2003; Zuckerman et al., 2003) to pregnant rats or mice. In the animal models, the consequences of these prenatal insults were remarkably similar to several aspects of SCZ and other psychiatric disorders [reviewed in (Boksa, 2010a; Meyer and Feldon, 2010)]. These include alterations in cognition, such as deficits in working memory and sensorimotor gating, impairments in social interaction and enhancement of mesolimbic dopaminergic (DAergic) neurotransmission (Boksa, 2010a; Meyer and Feldon, 2010).

The body of evidence from experimental animal models has provided considerable support to the tenet that maternal infection is sufficient to trigger many of the neuropathophysiological features of SCZ and other psychiatric disorders (Meyer and Feldon, 2010). What remain to be established are the mechanisms that lead to these effects. It has been proposed that the inflammatory

response to infection, triggered during viral, bacterial and other types of infection, may underlie this association (Patterson, 2009). The inflammatory response to infection is orchestrated by a family of endogenous mediators, known as cytokines (Konsman, 2002), which are released into the systemic circulation following treatment with pathogen-associated molecular patterns (PAMPs) such as LPS or poly I:C (Medzhitov et al., 1997) and the subsequent activation of the toll-like family of receptors (Alexopoulou et al., 2001; Poltorak et al., 1998; Qureshi et al., 1999). Cytokines target the brain, where they ultimately lead to the initiation of fever, anorexia, lethargy and stress response, among other inflammatory responses (also know as sickness behaviours) (Luheshi, 1998; Luheshi et al., 1996b). In addition, cytokines induce hypoferremia and hypozincemia, defined as the decrease in the levels of circulating non-haeme iron and zinc, respectively, mainly through their targeting of the liver (Grieger and Kluger, 1978; Hentze et al., 2004; Liuzzi et al., 2005; Morimoto et al., 1989). Most of these responses are potentially damaging for the developing foetus, in particular cytokines themselves (Ashdown et al., 2006a; Dahlgren et al., 2006; Girard et al., 2010) and hypoferremia (Beard and Connor, 2003) which can alter many neurodevelopmental processes (Beard, 2003; Deverman and Patterson, 2009). It is therefore of critical importance to determine whether these specific mediators are involved in the alterations of the normal course of neurodevelopment leading to the alterations in brain neurochemistry, morphology and behaviour induced by prenatal infection.

The research project presented in this thesis is based on the hypothesis that the maternal inflammatory response, triggered during infection, is involved in the effects induced on the foetal brain. In order to test this, and rather than using a systemically administered immunogen, we opted to use an animal model of clinical trauma and inflammation induced by turpentine oil (TURP). This aseptic inflammatory agent is administered locally [intramuscular (i.m.)] and remains secluded in the site of injection, where it induces local tissue damage (Wusteman et al., 1990) and activation of inflammatory cells (Sheikh et al., 2006). Subsequently, the production of a number of pro-inflammatory cytokines such as

tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β) and IL-6 is triggered, of which only IL-6 enters the circulation following this treatment (Aguilar-Valles et al., 2007; Luheshi et al., 1997; Tron et al., 2005; Turnbull et al., 2003).

This model of inflammation has several advantages over the more commonly used systemic agents, LPS or poly I:C. For example, administration of LPS or poly I:C to pregnant dams has been shown to induce foetal and maternal mortality, and pre-term delivery (Fortier et al., 2007; Girard et al., 2010; Koga et al., 2009). These effects are due to their direct targeting of the placenta and the systemic induction of IL-1β and TNFα (Girard et al., 2010; Haider and Knofler, 2009; Kakinuma et al., 1997), which are not significantly released into the systemic circulation following TURP administration (Cooper et al., 1994; Luheshi et al., 1997; Turnbull et al., 2003). In addition, LPS and poly I:C can be readily detected in the circulation following administration and can, and indeed do, interact directly with the foetal compartment (Ashdown et al., 2006a). This makes it difficult to determine whether the effects seen in the offspring are due to the direct actions of the inflammatory agent or the secondary, endogenous maternal mediators (i.e. cytokines, hypoferremia, etc.). Therefore, whilst avoiding the toxic, potentially confounding, effects of systemic LPS or poly I:C treatment, TURP still activates the systemic acute phase response common to all types of infection (Gershenwald et al., 1990; Kozak et al., 1998; Leon, 2002; Tron et al., 2005; Turnbull et al., 1998).

In Chapter II and part of Chapter III of this thesis, two studies are presented demonstrating the effects that the stage of pregnancy has on the inflammatory response to TURP administration. In particular, we explored the possibility of alteration in the levels of circulating mediators like IL-6 and IL-1 receptor antagonist (IL-1ra), which are the main circulating pro- and anti-inflammatory cytokines, respectively, released following TURP administration (Horai et al., 1998; Kozak et al., 1998; Leon, 2002). The rationale behind these studies are the well established observations that the maternal immune system and inflammatory response undergo dramatic alterations throughout gestation, which are proposed to allow foetal development to progress without recognition of foetal antigens as

alien to the dam's immune system (Luppi, 2003; Sacks et al., 1999). In addition, it has also been observed that the mother's ability to release cytokines from peripheral circulating cells, under basal and stimulated conditions, is dynamically altered throughout gestation (Amoudruz et al., 2006; Pillay et al., 1993; Shimaoka et al., 2000). In animal models, it has also been observed that fever, a hallmark inflammatory response to infection, is attenuated in a number of species near term and during lactation in response to LPS and other immunogens (Cooper et al., 1988; Eliason and Fewell, 1997; Kasting et al., 1978; Martin et al., 1995; Simrose and Fewell, 1995; Stobie-Hayes and Fewell, 1996; Zeisberger et al., 1981). Therefore, we aimed to establish whether this was also the case for TURP-induced inflammation, which had not been used in pregnant rodents prior to the studies presented in this thesis.

Chapter III consists of a study aimed to ascertain whether the differential inflammatory response to early vs. later administration of TURP, has diverse consequences on the behaviour of the offspring. The behavioural tasks used in the adult offspring were chosen in order to measure different aspects of the animal's cognitive ability, including sensorimotor gating, spatial and associative learning and memory. In addition, we sought to determine the activity of the mesolimbic DA system by determining the locomotor response elicited by the DA indirect agonist amphetamine (AMPH). This neurotransmitter is fundamentally involved in the pathophysiology of SCZ and other forms of psychoses and may contribute to the alterations in the cognitive and positive symptom domains of SCZ (Davis et al., 1991).

Having determined, the elements of the maternal inflammatory response stimulated by TURP (Chapter II) and the behavioural consequences in the offspring (Chapter III), the studies in Chapters IV and V were designed to investigate whether the maternal circulating cytokines IL-6, IL-1ra and leptin (Chapter IV), or inflammation-induced hypoferremia (Chapter V), were involved in the effects of prenatal TURP on the DA neurons of the adult offspring. In Chapter IV, maternal cytokines were targeted using neutralizing antisera for each individual mediator, whereas in Chapter V, hypoferremia was counterbalanced by

supplementing the mothers through parenteral iron administration, without affecting maternal cytokine levels (specifically IL-6), which allowed us to discern whether the effect of IL-6 could be prevented by attenuating the hypoferremic response, at least in regard to the offspring's DA function. Throughout these studies we sought to establish the contribution of these elements of the inflammatory response on the neurodevelopment of the offspring, resulting in altered brain function as adults.

Chapter I: Comprehensive review of the literature

I.1. Neurodevelopmental hypothesis of psychiatric disorders

The term 'neurodevelopmental psychiatric disorders' refers to a large group of diseases whose clinical onset occurs during periods of maturation and development (Ehninger et al., 2008; Levitt, 2005). These disorders are associated, in general, with complex neuropsychiatric features including intellectual and specific learning disabilities, attention and social interaction deficits, epilepsy and/or psychosis, amongst others (Ehninger et al., 2008). They are thought to be caused by a variety of genetic and environmental factors (Sawa et al., 2004) that induce alterations in processes such as neurogenesis, cell migration, and establishment of neuronal connectivity (Ehninger et al., 2008; Sawa et al., 2004). In this regard, the brain is considered to be especially vulnerable during development, since insults involving a minor impact in adults can result in significant pathologies when occurring prior to maturation (Ehninger et al., 2008). One of these disorders, SCZ, has been hypothesized to have a neurodevelopmental origin (Weinberger, 1987): a behavioural outcome of an aberration in developmental processes within the brain, which begins long before the onset of the clinical symptoms (Fatemi and Folsom, 2009; Rapoport et al., 2005). There are numerous independent lines of evidence supporting this hypothesis (Fig. I-1). For example, there is a conspicuous absence of gross physical damage or signs of progressive neurodegeneration and ongoing brain inflammatory processes (i.e. gliosis) for SCZ patients (Rapoport et al., 2005; Weinberger, 1987). In general, for many psychiatric disorders the underlying pathophysiology is rather complex and involves microscopic, rather than macroscopic, alterations in brain development (DiCicco-Bloom et al., 2006; Levitt, 2005), which is suggestive of early-life insults, rather than brain tissue degeneration and concomitant inflammation (Rapoport et al., 2005). In addition, pre-schizophrenic children present behavioural, physical and brain morphological alterations, before the clinical onset of psychosis (Danielyan and Nasrallah, 2009; Fatemi and Folsom, 2009; Rapoport et al., 2005; Ross et al., 2006). These alterations include delays in motor and speech development; 'soft' neurological

symptoms; poor peer relationships (with boys being more disruptive and girls more withdrawn), social isolation, social anxiety, lower cognitive abilities (assessed by formal cognitive tests or by educational achievement) and subtle alterations in brain morphology (Danielyan and Nasrallah, 2009; Fatemi and Folsom, 2009; Rapoport et al., 2005; Ross et al., 2006). These alterations are present prior to the manifestation of psychotic illness and before treatment with neuroleptic agents, meaning that they are not the result of progressive degenerative processes, or side-effects of the psychotropic medication (Danielyan and Nasrallah, 2009).

Finally, individuals who develop SCZ are more likely to have experienced pre- or peri-natal adverse events (Gilmore et al., 1998; Weinberger, 1987), and/or late adolescent disturbances in brain development, compared to healthy, control individuals (Fatemi and Folsom, 2009; Feinberg, 1982; Mathalon et al., 2003; Rapoport et al., 2005) (Fig. I-1). The discovery of subtle abnormalities in neuronal migration and organization, and reduced neuronal size and arborisation in post-mortem brains from SCZ patients that can be the consequence of adverse events during perinatal development, supports the hypothesis of a neurodevelopmental origin for this disease (Mathalon et al., 2003).

Figure I-1. Developmental cascade towards schizophrenia

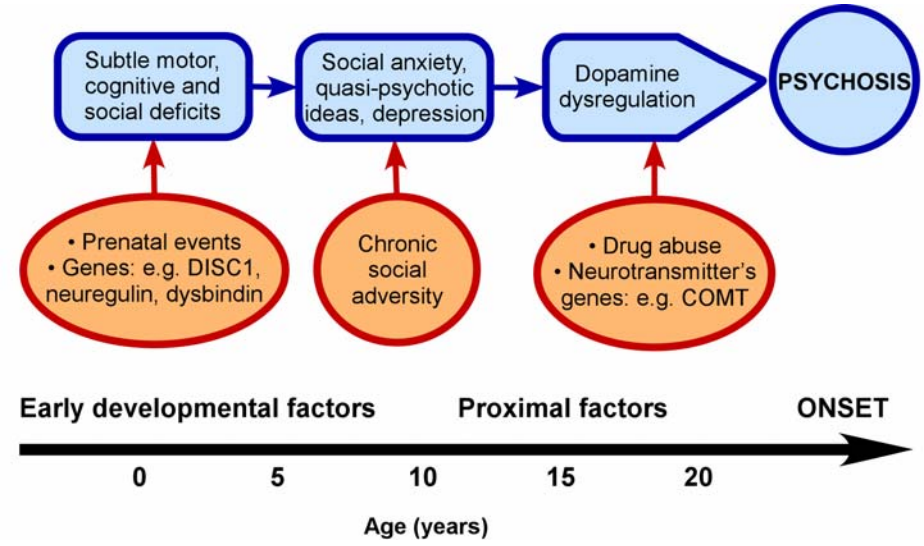

Figure I-1. Schizophrenia is hypothesised to develop from a combination of genetic and environmental factors that affect early brain development. These

effects express themselves as subtle alterations in neurological, cognitive, motor and social functions. Further adverse events later in life (drug abuse, stress) along with other genetic risk factors trigger the onset of psychosis during early adulthood. Modified from (Murray et al., 2008).

I.2. The aetiology of neurodevelopmental psychiatric disorders: interaction of genetic and environmental risk factors

A large body of evidence indicates that genetic factors play an important role in the aetiology of neurodevelopmental disorders (1988; 2008; Arnold et al., 2005; Ehninger et al., 2008; Miller et al., 2005; Sawa et al., 2004). For example, concordance data for monozygotic twins for mood disorders (i.e. major depression and bipolar disorder) is ~40-50 % (Sawa et al., 2004). The genetic component of SCZ is well established and illustrated by varying degrees of lifetime prevalence. For the general population the risk is of ~1 %, whereas it increases in the relatives of SCZ patients, depending on the degree of biological kinship to the patient. For example, monozygotic twins, sharing 100 % of the genome with the SCZ patient, have a risk of 40-50 % (Sawa et al., 2004; Tsuang et al., 2001), whereas siblings or dizygotic twins (i.e. first-degree relatives) that share about 50 % of their genes have a risk of 9 % (Tsuang et al., 2001). Adoption studies have confirmed the concordance data derived from twin studies for SCZ (Tsuang et al., 2001). In addition, many of the pathological features of SCZ are observed in first-degree relatives of clinically diagnosed patients suggesting a role for genetic factors in the aetiology of this disorder (Danielyan and Nasrallah, 2009).

Heritability data indicates that the contribution of genetic variation for SCZ is between 60 and 81 % (Sullivan et al., 2003), whereas for autism-spectrum disorders this value is >90 % (DiCicco-Bloom et al., 2006). Despite the seemingly large contribution of genetic factors to these psychiatric disorders, their genetic

constituent is considered to be composed of multiple genes with relatively small individual risk factors. There are, for example, over 280 genes identified and designated as causing mental retardation (Inlow and Restifo, 2004), with 15 or more genes linked to autism-spectrum disorders (Veenstra-VanderWeele et al., 2004). Up to 50 genes may be involved in the aetiology of SCZ (Lewis et al., 2003; Shirts and Nimgaonkar, 2004).

Classic linkage studies combined with analysis of SCZ-associated single nucleotide polymorphisms within linkage areas revealed several candidate genes for SCZ, including *dysbindin*, *neuregulin-1* (*NRG1*), *G72*, *the regulator of G-protein signalling-4* (*RGS4*) and *catechol-O-methyltransferase* (*COMT*) (reviewed in (Sawa et al., 2004)). In addition, *disrupted-in-schizophrenia-1* (*DISC1*) was identified, by a cytogenetic approach, in a Scottish family with a chromosome translocation and disruption of *DISC1* inside its open reading frame (Millar et al., 2000a; Millar et al., 2000b). This translocation segregated with several major psychiatric illnesses, including SCZ and mood disorders (Sawa et al., 2004). Intriguingly, most of these genes have been identified to have roles in brain development (Sawa and Kamiya, 2003; Sawa and Snyder, 2002), which strongly supports the role of neurodevelopmental alterations in the aetiology of these psychiatric conditions. In addition, recent studies have identified a number of rare structural chromosomal variants linked to SCZ. These alterations included micro-deletions and micro-duplications of genomic segments, many of which affect genes involved in brain development, synaptic transmission, neuregulin signalling, axonal guidance and integrin signalling (Walsh et al., 2008), as well as alterations in copy number of genes located on chromosomes 1, 15 and 22 (2008).

Although the genetic contribution to neurodevelopmental psychiatric disorders is well-established, the available evidence implicates gene-environment interactions as fundamental factors in their aetiology (Ehninger et al., 2008; Levitt, 2005). In this regard, the monozygotic twin concordance rate for SCZ and mood disorders is less than perfect (~50 % or less), clearly indicating the existence of non-genetic contributing causes (Caspi and Moffitt, 2006; Tsuang et al., 2001).

Indeed, environmental risk factors have been documented for substance abuse (Heath and Nelson, 2002), depression (Kendler et al., 2002), mental retardation (Levitt, 2005) and SCZ (Tsuang et al., 2001), whereas evidence is more limited for disorders such as autism (Arndt et al., 2005; Trottier et al., 1999) and ADHD (Caspi and Moffitt, 2006).

The environmental factors identified include prenatal events [i.e. maternal stress, famine, substance abuse, infection or exposure to neurotoxins (thalidomide or anticonvulsants such as valproic acid) during pregnancy], low birth weight and birth complications and postnatal events (i.e. early postnatal trauma, deprivation of normal parental care during infancy, childhood physical maltreatment and neglect, premature parental loss, family conflict and violence, substance abuse during adolescence, and head injury) (Arndt et al., 2005; Caspi and Moffitt, 2006; Levitt, 2005; Trottier et al., 1999) (Figure I-1).

Meta-analysis of population-based data for perinatal events associated with the development of SCZ revealed significant estimates for three main categories of obstetric complications, with a pooled odds ratio of their effect on the development of SCZ of 2.0 (Cannon et al., 2002; Geddes and Lawrie, 1995). These include complications during pregnancy (i.e. bleeding, pre-eclampsia, diabetes and rhesus incompatibility), abnormal foetal growth and development (low birth weight, congenital malformations and small head circumference) and complications of delivery (including asphyxia, uterine atony and emergency Caesarean section) (Cannon et al., 2002; Geddes and Lawrie, 1995). Some of these factors are believed to have a direct causative effect, while others may simply be markers for other underlying casual processes. For example, low birth weight and small head circumference reflect foetal growth retardation, but themselves can be caused by a multitude of factors, such as maternal malnutrition or placental damage leading to foetal growth restriction (Rapoport et al., 2005), any of which may in turn be responsible of the increased risk for SCZ. Finally, most of these environmental risk factors constitute a nonspecific risk for many disorders, for example, complications at birth have been found to be linked not only to SCZ, but also to ADHD and mental retardation/autism spectrum disorders

(Caspi and Moffitt, 2006; Levitt, 2005). As a result, they cannot be considered predictive of a particular disorder.

Another important consideration is that although environmental risk factors independently account for a few clinical cases, they are regarded as to be mostly contributory, since exposure to them does not always generate the disorder (Caspi and Moffitt, 2006; DiCicco-Bloom et al., 2006; Rapoport et al., 2005) (Fig. I-1). In this regard, heterogeneity of response characterizes all known environmental risk factors for psychopathology, including the most overwhelming of traumas (Caspi and Moffitt, 2006). Such response heterogeneity is associated with pre-existing differences known to be under genetic influence (Caspi and Moffitt, 2006). This hypothesis implies that in any given population, individual genetic predisposition is directly responsible for the vulnerability to the environmental causes of a number of psychiatric conditions (Caspi and Moffitt, 2006), including SCZ (Dean et al., 2009; Rapoport et al., 2005) (Fig. I-1).

One of the environmental factors identified as a risk for developing psychiatric disorders is maternal infection during pregnancy. Since its identification in 1988 (Mednick et al., 1988), the link between maternal infection and SCZ has been found in several independent studies (reviewed in (Brown and Derkits, 2010a)), leading to the suggestion that this environmental factor may be a major risk factor in the aetiology of SCZ.

I.3. Maternal infection as a risk factor for neurodevelopmental psychiatric disorders

Maternal infection has been identified as a risk factor for several neurodevelopmental disorders such as periventricular leukomalacia (the leading cause of cerebral palsy), mental retardation, autism and SCZ (Brown and Derkits, 2010a; Deverman and Patterson, 2009). However, the epidemiological evidence establishing the link between prenatal infection and postnatal development of SCZ is better described than for any other psychiatric condition. Ecological studies, including those based on the subjective report of illness, suggest that SCZ is more prevalent in the offspring of populations of women that were pregnant

during periods of influenza epidemics (Machon et al., 1983; Mednick et al., 1988), as well as other types of viral infections, including diphtheria, pneumonia, measles, varicella zoster, mumps and poliovirus (O'Callaghan et al., 1994; Suvisaari et al., 1999; Torrey, 1988; Watson et al., 1984). Overall, some of these studies have been suggested the existence of a significant association between exposure to the influenza epidemics and other viral infections during the second trimester of gestation and SCZ (Adams et al., 1993; Barr et al., 1990; Kendell and Kemp, 1989; Limosin et al., 2003; McGrath and Castle, 1995; Mednick et al., 1994; Mednick et al., 1988; Selten et al., 1999; Sham et al., 1992; Takei and Murray, 1993).

Further evidence has indirectly suggested a relationship between prenatal infection and SCZ. For instance, this disorder is more prevalent among individuals born to pregnancies that occur during winter (Hare et al., 1972; Machon et al., 1983). There are nearly 50 studies indicating that there is a 5-15 % excess of SCZ cases among individuals born in the northern hemisphere during the months of January and March (reviewed in (Fatemi and Folsom, 2009). This finding is subject to several interpretations, including that winter is a season associated with an increased frequency of respiratory infections (Fatemi and Folsom, 2009). Alternatively, low vitamin D levels during winter, another risk factor for SCZ (McGrath et al., 2003), may be a contributing underlying factor.

Even though the link between prenatal influenza and adult SCZ has been supported by 12 independent ecological studies [see for instance (Izumoto et al., 1999; Limosin et al., 2003)]; 9 separate studies, published about the same time, failed to confirm this connection [as in (Mino et al., 2000; Westergaard et al., 1999); reviewed in (Brown and Derkits, 2010a)]. The main limitation of these is that ‘exposure to infection’ was defined solely by the fact that the individual was pregnant during the time of the epidemic (i.e. based on the date of birth of the offspring). Therefore, the studies may have included a significant number of non-exposed cases, which would clearly mask any true, underlying association between infection during gestation and the development of SCZ. Using individual biomarkers of illness, including analysis of maternal serum or clinical diagnoses

(Brown and Derkits, 2010a), as the parameters to define exposure to infection, it was shown that SCZ in the offspring was significantly associated with clinically diagnosed maternal respiratory infections (Brown et al., 2000b), as well as maternal serologically documented influenza (Brown et al., 2004a; Susser et al., 2000), rubella (Brown et al., 2000a; Brown et al., 2001), *Toxoplasma gondii* (Brown et al., 2005; Mortensen et al., 2007), herpes simplex virus-2 (Buka et al., 2008; Buka et al., 2001a), maternal genital or reproductive infections (Babulas et al., 2006), and maternal bacterial infections (Sorensen et al., 2009). The interpretation of these results is, however, hampered by the fact that some were obtained using a broad definition of psychosis, where both non-affective (e.g. SCZ) and affective psychoses (e.g. major depression or bipolar disorders with psychotic features) were included (Brown et al., 2001; Buka et al., 2001a; Buka et al., 2001b). This however suggests that maternal infection may be involved in the development of psychotic features that may not be necessarily restricted to those that characterise SCZ, but several other disorders as well.

The attributable proportion of maternal infections for SCZ is the proportion of case subjects in the population who would not have developed SCZ if maternal infections (particularly influenza, *T. gondii* and genital/reproductive infections) were removed (Brown and Derkits, 2010a). This has been estimated to be 33 % and is based on the calculated odds ratio for each of these infections individually (3.0, 2.6 and 5 for influenza, *T. gondii* and genital/reproductive infections respectively) (Brown and Derkits, 2010a).

The wide variety of infections associated with SCZ suggests that there may be a common factor underlying increased susceptibility (Gilmore and Jarskog, 1997). Therefore, it has been hypothesized that maternal immune activation, and the inflammatory response that follows all types of infection (Brown and Derkits, 2010a), may be fundamentally involved. Epidemiological studies have provided some evidence supporting this hypothesis. Increased levels of circulating pro-inflammatory cytokines, specifically interleukin (IL)-8 during the second and early third trimester (Brown et al., 2004b) and tumour necrosis

factor (TNF)α at birth (Buka et al., 2001b), are associated with a higher risk of psychosis or SCZ in the offspring.

What remains unclear from all of these studies is the critical stage(s) of gestation during which the developing brain may be more vulnerable to this prenatal insult. A number of the studies mentioned did not have the statistical power to define this feature. Those that did suggested variable stages, frequently including the first or second trimesters (Brown et al., 2004a; Brown et al., 2000b; Sorensen et al., 2009), but also the third trimester (Brown et al., 2004b; Buka et al., 2001b). Despite these inconsistencies, data indicates that maternal infection is indeed linked to the development of SCZ in the offspring (Brown and Derkits, 2010a).

I.4. Schizophrenia: generalities and symptoms

SCZ is a complex and heterogeneous psychiatric disorder. It has been referred to as the "worst disease affecting mankind" (1988) and, in economic terms, the seventh most costly medical illness to modern society (Freedman, 2003). SCZ is characterised by: psychotic symptoms such as delusions and hallucinations (also known as the positive symptom dimension); alterations in drive and volition, including lack of motivation, blunted affect, social withdrawal and reduction in spontaneous speech (the negative symptom dimension) and alterations in neurocognition, including difficulties in memory, attention and executive functioning (the cognitive symptom dimension) (Ross et al., 2006; van Os and Kapur, 2009; Walker et al., 2004).

The major areas of neurocognitive deficits in SCZ are verbal and spatial memory, learning, abstraction, selective attention and language disabilities. Of these, executive function and memory, which are associated with the frontal and medial temporal regions of the brain, are the most severely affected (Danielyan and Nasrallah, 2009). Pre-pulse inhibition (PPI), an operational measure of sensorimotor gating (Swerdlow et al., 2001), is a feature of pre-attentive cognitive processing consistently reported to be impaired in SCZ patients (Li et al., 2009a). PPI is the normal reduction of the amplitude of the startle reflex in response to an

intense startling stimulus (the pulse) when this is shortly preceded by a weaker, non-startling sensory stimulus (the pre-pulse) (Li et al., 2009a). Its impairments are considered to be an indication of a failure of inhibitory filtering mechanisms that can lead to sensory overload and consequent cognitive fragmentation (Geyer, 2006). The most robust correlates of the deficits in PPI are abnormalities in distractibility and thought disorder (Geyer, 2006).The negative symptom dimension is linked to the neurocognitive deficits (van Os and Kapur, 2009), whereas there appears to be no relationship between the negative and positive dimension symptoms over time (Eaton et al., 1995). There is, as yet, no single theory that fully explains the complex pattern of psychosis, negative symptoms, cognitive impairment and associated psychopathology in SCZ (Danielyan and Nasrallah, 2009).

Additional features in the diagnosis of SCZ, as opposed to other forms of psychosis, are the long duration of the symptoms, the bizarreness of the delusions, the presence of the negative dimension symptoms and absence of affective symptoms (i.e. depressive or manic symptoms) (van Os and Kapur, 2009; Walker et al., 2004). The onset of SCZ most commonly occurs during the second or third decade of life, ranging from childhood to old age (Ross et al., 2006) and there is an average age of diagnosis in males four years earlier than in females (Riecher-Rossler and Hafner, 2000).

Most of the cognitive and neurological deficits exhibited by SCZ patients can also be found in many other neurological and non-neurological disease processes (Danielyan and Nasrallah, 2009). Similarly, the psychotic dimension symptoms of SCZ overlap with several other psychiatric disorders (Table I-1). Intriguingly, it has been observed that some schizophrenia-like psychopathological abnormalities (i.e. paranoid delusional thinking and auditory hallucinations) are expressed in an attenuated form in 5-8 % of healthy population, especially in individuals with schizotypal or schizoid personality traits (van Os and Kapur, 2009). This extensive overlapping of symptoms with other psychiatric and even neurological conditions is suggestive of a common underlying neuropathophysiology for these disorders, which, rather than discrete

diagnoses, may perhaps represents a continuum that extends to the general population (van Os and Kapur, 2009).

Table I-1. DMS-IV categories of psychiatric disorders that share the presence of psychotic dimension symptoms

Non affective psychotic disorders:	**Affective psychoses:**	**Substance-induced psychotic disorders:**	**Psychotic disorder due to a general medical condition**
• Schizophrenia • Schizoaffective disorder • Schizophreniform disorder • Delusional disorder • Brief psychotic disorder • Psychotic disorder not otherwise specified	• Bipolar disorder with psychotic features • Major depressive disorder with psychotic features	• Alcohol-induced • Other substance-induced	

Adapted from (American Psychiatric Association., 2000).

I.5. Neuropathology and pathophysiology of SCZ

Over the last hundred years, since the modern descriptions of SCZ (Mc, 1949; Varga and Kroll, 1977), there have been significant efforts to elucidate the brain abnormalities that underlie this disorder. These efforts have been largely frustrating, as no common gross structural and histological abnormalities have been identified to date and only a few of the reported abnormalities have been seen across studies.

However, one consistent observation has been that unlike neurodegenerative diseases, SCZ brains do not present features such as inclusion bodies, dystrophic neuritis or reactive gliosis, the hallmarks of neurodegeneration (Arnold et al., 1998; Ross et al., 2006). There have been reports suggesting subtle defects, including cytoarchitectural abnormalities in the entorhinal grey matter and other corticolimbic regions (Arnold et al., 1997); higher frequency of neurons in the white matter underlying the prefrontal cortex (PFC), and the temporal and parahippocampal regions (Arnold et al., 2005); reduction in the volume of cortical neuropil, without comparable neuronal loss (Selemon and Goldman-Rakic, 1999; Selemon et al., 1995); and deficits in synaptic connectivity (Ross et al., 2006). As with the behavioural symptoms, all of these histological abnormalities, including a number mentioned in the subsequent sections, are nonspecific to this disorder and have a high-frequency prevalence in the general population, making them poor predictors of SCZ (Rapoport et al., 2005).

5.1. Structural alterations in the SCZ brain

The most consistent structural alterations described in SCZ are increased ventricular volume (particularly in the lateral and third ventricle) and decreased whole brain volume, with larger effects in the prefrontal and temporal cortical areas, including the hippocampus (Danielyan and Nasrallah, 2009; Steen et al., 2006). In addition, more localized reductions in grey matter volume and density appear in the frontal eye fields, supplementary motor, sensorimotor, parietal and temporal cortical areas, cerebellum, the cingulates, insula, thalamus, caudate, the

parahippocampal region, hippocampus and amygdala. While the functional consequences of these volumetric alterations in SCZ are not known, they have been associated with deficits in the cognitive dimension (i.e. deficits in attention, executive functioning and pre-morbid cognitive functioning in patients) (Danielyan and Nasrallah, 2009), but not with symptom severity (reviewed in (Danielyan and Nasrallah, 2009; Rapoport et al., 2005)).

Supporting the neurodevelopmental hypothesis of SCZ, several of these volumetric changes in the SCZ brain have been found to be present at the first sign of illness (Danielyan and Nasrallah, 2009). However, some controversy exists, as a few longitudinal studies, following SCZ patients before and after the onset of psychosis, have reported evidence of progressive changes in grey matter after clinical onset. Some studies have also shown a progressive enlargement of the lateral ventricles in patients, followed for up to four years after their first psychotic episode (DeLisi et al., 1997; Gur et al., 1998; Mathalon et al., 2001; Nair et al., 1997; Pantelis et al., 2003; Rapoport et al., 2005). This evidence has been used to suggest that, to some extent, the pathophysiology of SCZ involves neurodegeneration. However, as brain structural alterations can also be found in patients with first-episode of psychoses and in the non-schizophrenic relatives of the patients, and since no comparable neuronal loss has been found to explain the changes in grey matter volume, the evidence, if not perfectly, better supports a neurodevelopmental hypothesis.

5.2. Gene expression and myelination in SCZ

Gene expression microarray studies in brain tissue from SCZ patients have revealed altered expression of genes involved in synaptic transmission and energy metabolism, amongst others (Arion et al., 2007; Dean et al., 2009; Dean et al., 2007; Ross et al., 2006). Focus has been centered on the dorso-lateral PFC, since functional imaging studies have suggested that metabolic brain activity is decreased in this brain region in patients with the disorder (Danielyan and Nasrallah, 2009). Recent microarray analyses of the PFC transcriptome revealed changes in expression of genes related to synaptic function, myelination, signal

transduction, as well as up-regulation of gene expression of chaperones of heat shock protein family and immune markers (Arion et al., 2007). The increased expression of immune mediators seems to be dissociated from acute, active inflammatory processes. In fact, this has been interpreted as residual markers of earlier life stressors (Arion et al., 2007), among which maternal infection may be a preeminent event (Borrell et al., 2002).

Several lines of evidence have suggested that myelination may be altered, most likely decreased, in the brains of SCZ individuals, and this is now considered a major component in the development and pathophysiology of this disorder (Danielyan and Nasrallah, 2009). Gene expression analyses, using either high throughput microarrays or selected gene expression by qPCR, suggest that several mRNAs whose proteins are involved in myelination are decreased, including *myelin oligodendrocyte glycoprotein* (*MOG*) in the PFC and *myelin-associated protein* (*MAG*), *transferrin* (*Tf*), and other genes in the anterior cingulated cortex (Arion et al., 2007; Dean et al., 2007; Hakak et al., 2001; Haroutunian et al., 2007; McCullumsmith et al., 2007). White matter impairments have also been suggested by reduced fractional anisotropy in frontal and temporal brain regions, and in the genu and splenium of the corpus callosum [reviewed in (Danielyan and Nasrallah, 2009)]. This has been suggested to result in disturbed cerebral connectivity (Danielyan and Nasrallah, 2009).

5.3. Alterations in neurotransmission in SCZ

In addition to the gene expression and brain imaging evidence suggesting altered brain connectivity and synaptic connectivity, alterations in several neurotransmitter systems have been implicated in the pathophysiology of SCZ. There are consistent pharmacological data for the involvement of glutamate (Glu), and dopamine (DA). Neuropathological studies also suggest a role for γ-amminobutyric acid (GABA) neurotransmission (Dean et al., 2009; Ross et al., 2006).

5.3.1. Glutamate and γ-amminobutyric acid

The role of Glu in SCZ started with the observation that the N-methyl-D-aspartic acid (NMDA) receptor antagonists ketamine and phencyclidine cause psychotic and cognitive abnormalities that resemble SCZ symptoms (Coyle, 2006). In addition, SCZ patients show greater sensitivity to the psychomimetic effects of these drugs than non-SCZ individuals (Coyle, 2006). Supporting the role of Glu in SCZ pathophysiology is the evidence suggesting that this disorder, especially its negative symptoms, can be treated with NMDA receptor modulators, such as D-serine, glycine and sarcosine, which increase the receptor's function (Ross et al., 2006). Similarly, neuropathological evidence indicates reduced expression of the GABA transporter in a specific subtype of GABAergic interneurons in cortical areas, the chandelier neurons, suggestive of reduced GABAergic neurotransmission (Hashimoto et al., 2008; Lewis et al., 2005). The expression of other genes involved in this neurotransmitter system has also been found to be decreased, including *glutamate decarboxylase 1* (*GAD1*) or GAD67 which is responsible for GABA synthesis, and several $GABA_A$ receptor subunits (Hashimoto et al., 2008). These findings have been linked to a decrease in brain-derived neurotrophic factor (BDNF) signalling and to a decreased in NMDA receptor function (Lewis et al., 2005; Ross et al., 2006). In addition, there are reports of alterations in specific epigenetic tags, such as DNA methylation, in the proximal promotor region of the *GAD1* gene in the PFC of SCZ patients (Huang and Akbarian, 2007). These alterations were accompanied with decreased *GAD1* mRNA levels (Huang and Akbarian, 2007). Similarly, expression of DNA methyltransferase 1 is found to be reduced in the GABAergic interneurons of the PFC of SCZ patients (Ruzicka et al., 2007). Changes in epigenetic regulation of GABAergic function could be interpreted as the result of the hypothesised environmental factors involved in triggering this disorder. Although it is still unclear, alteration in GABA neurotransmission have been proposed to be involved in the manifestation of the negative and cognitive dimension symptoms of SCZ. The role of Glu may be more widespread, however, influencing the positive dimension of symptoms as well (Ross et al., 2006).

5.3.2. Dopamine

The "classical" DAergic hypothesis of SCZ (Carlsson and Lindqvist, 1963) is that hyperactivity of the DA system is responsible for the symptoms of the disorder. More recently, this hypothesis was expanded to include the proposal that the hyperactivity of the mesolimbic DA system [whose neuronal cell bodies are located in the ventral tegmental area (VTA) and terminals innervate the nucleus accumbens (NAcc) in the striatum (STR), Fig. I-2] contributes to positive symptoms in SCZ; whereas impaired function of the DA system in the PFC (released by neurons whose cell bodies also lie in the VTA) contributes to the cognitive symptom dimension [Fig. I-2, (Davis et al., 1991; Weinberger, 1987)].

The DA hypothesis of SCZ derives, in part, from the identification of the mechanisms of action of antipsychotics, many of which act as DA receptor 2 (D2 receptor) blockers (Ross et al., 2006). In fact, it was determined that there is a correlation between effectiveness of classical neuroleptics and their D2 receptor blocking potency (Creese et al., 1976; Seeman and Lee, 1975). Furthermore, pharmacological studies showed that a single exposure to amphetamine (AMPH), a stimulant drug that increases extracellular levels of DA in striatal and cortical regions via release and reverse transport (Erreger et al., 2008; Sulzer et al., 1995), evokes or exacerbates positive symptoms in SCZ patients at doses which do not induce psychosis in healthy subjects (Lieberman et al., 1987; Lieberman et al., 1997; Yui et al., 1999). This is also true for other DA enhancing drugs such as methylphenidate and L-3,4-dihydroxyphenylalanine (L-DOPA) (Lieberman et al., 1987). Results from imaging studies demonstrated that a significant number of non-medicated schizophrenic patients show marked elevation of AMPH-induced striatal dopamine release in comparison to healthy volunteers (Abi-Dargham et al., 1998; Abi-Dargham et al., 2009; Laruelle et al., 1996). This response correlated significantly with the emergence or worsening of positive symptoms (Abi-Dargham et al., 1998; Abi-Dargham et al., 2000; Breier et al., 1997; Laruelle et al., 1999; Meyer-Lindenberg et al., 2002). Baseline occupancy of DA receptors in the striatum is also increased in SCZ (Abi-Dargham, 2004). In addition, post-mortem studies showed that striatal density of D2 receptors in SCZ patients is

increased (Lee et al., 1978; Seeman et al., 1987); as neuroleptic administration has been shown to up-regulate D2 receptors (Soares and Innis, 1999), it was proposed that this finding was a consequence of the antipsychotic medication. However, increased D2 levels are also found in non-mediated SCZ patients (Crow et al., 1978; Taeuber et al., 1979; Toru et al., 1988).

Sensitized mesolimbic DA function observed in SCZ patients is tightly associated with hypo-functioning of DA neurotransmission in the PFC. Indeed, recent studies using imaging techniques and cognitive tasks associated with PFC function have shown that the degree of abnormal PFC activation in schizophrenic patients predicts the magnitude of the exaggerated subcortical DA function (Bertolino et al., 1999; Meyer-Lindenberg et al., 2002). Additionally, SCZ patients exhibit deficits in cognitive tasks that are dependent on PFC function [for review (Bunney and Bunney, 2000)]. This may be modulated by mesocortical DA neurotransmission, as PFC DA has been shown to be involved in executive function and working memory (Phillips et al., 2004; Sawaguchi and Goldman-Rakic, 1994; Williams and Goldman-Rakic, 1995) and abnormalities in PFC DA, including decreased DA innervation to this region, are observed in SCZ (Abi-Dargham et al., 2002; Akil et al., 1999; Daniel et al., 1991; Weinberger et al., 1988).

The simultaneous presence of multiple behavioural abnormalities, along with underlying alterations in brain morphology, connectivity and neurotransmission, seem to collectively define SCZ. However none of these features, individually, identifies this disorder, as they can also be found in individuals with other psychiatric diagnoses, and also among the general population. In order to fully understand the aetiology of SCZ, it is necessary to test the hypothesis that individual risk factors identified in epidemiological studies, have a causal relationship with this disorder or some aspects of it.

Figure I-2. The mesocorticolimbic dopamine system

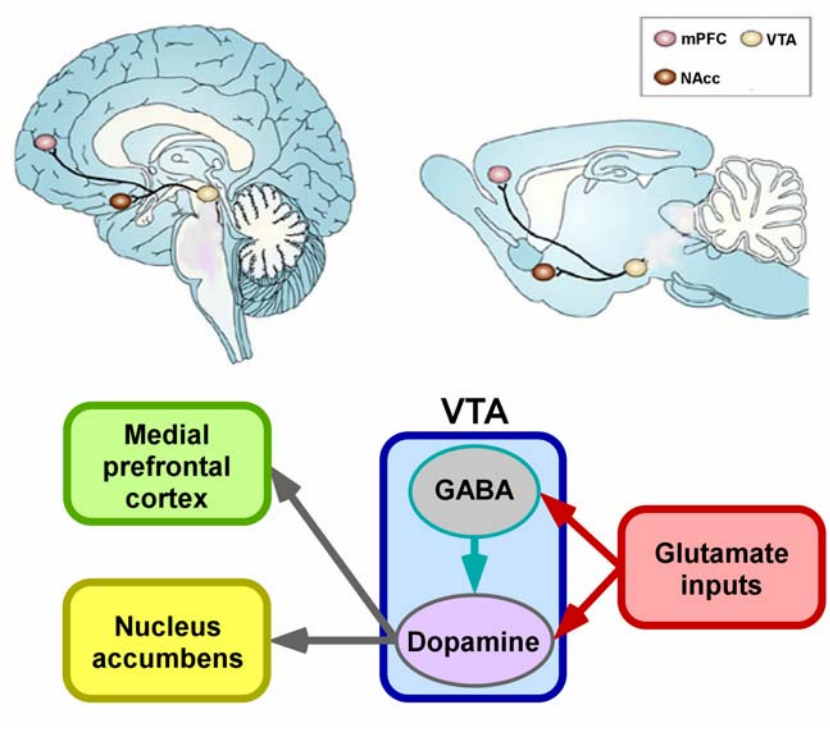

Figure I-2. Human (top left) and rat (top right) brains, showing the mesolimbic and mesocortical dopamine (DA) pathways, which originate in the ventral tegmental area (VTA) and send ascending projections to the nucleus accumbens (NAcc) and medial prefrontal cortex (mPFC), respectively. Schematic showing the DA and GABA neuronal populations within the VTA (lower panel). GABA neurons provide inhibitory input to DA neurons. In addition, both neuronal populations receive excitatory glutamatergic inputs, which can regulate the relative activity of DA and GABA activity in the VTA. Modified from (Laviolette and van der Kooy, 2004).

I.6. Animal models for studying the effects of maternal infection on the neurodevelopment of the offspring

It is not possible to asses the relative magnitude of the inherited and environmental contributors in the aetiology of psychiatric disorders of neurodevelopmental origin without the use of animal models (Boksa, 2004; Nawa

and Takei, 2006). This is especially true when examining the involvement of environmental risk factors, since their effects on brain development are poorly understood. A number of research laboratories have investigated the consequences of maternal exposure to several immunogenic agents on the neurodevelopment, neurochemistry, brain structure and behaviour of the offspring.

6.1. Behavioural effects of prenatal infection

Initial approaches used prenatal infection with influenza virus [at gestational day (GD) 9 in mice], followed by the application of a battery of behavioural tests, relevant to SCZ, in the adult offspring (Shi et al., 2003). These studies showed that the adult offspring of infected mothers presented, compared to the offspring of control dams, decreased social interaction, reduced exploration in the open field, a measure of anxiety, impaired performance in the novel object test, indicative of impaired working memory as observed in SCZ and diminished PPI of acoustic startle (Shi et al., 2003). These behavioural alterations are similar to aspects of SCZ (see section I.4).

Further studies investigated the consequences of maternal immune activation using molecular immunogens. The viral mimic polyinosinic:polycytidylic acid (poly I:C) has been used to stimulate the maternal immune system (with one or multiple injections), at several stages of pregnancy in mice or rats, ranging from GD 9 until GD 17 [reviewed in (Boksa, 2010a; Meyer and Feldon, 2010)]. Overall, prenatal stimulation with poly I:C induced deficits in PPI and in latent inhibition (LI) of conditioned learning, a task to measure associative learning and selective attention. These offspring showed in addition decreased social interaction, novel object recognition and impairments in spatial working memory (Fortier et al., 2007; Meyer et al., 2008a; Meyer et al., 2008b; Meyer et al., 2006a; Meyer et al., 2008c; Meyer et al., 2006b; Meyer et al., 2008e; Ozawa et al., 2006; Shi et al., 2003; Smith et al., 2007; Vuillermot et al., 2010; Wolff and Bilkey, 2008; Zuckerman et al., 2003; Zuckerman and Weiner, 2003,

2005). These behavioural alterations mimic some negative and cognitive dimension symptoms of SCZ (Section I.4).

In addition, there were described impairments in exploration of the open field, and increased sensitivity to the locomotor activating effects of the NMDA non-competitive blocker, MK801, AMPH and methamphetamine, whose locomotor effects depend on the mesolimbic DA system. Similar findings have been related to positive symptoms of SCZ (Meyer et al., 2008a; Meyer et al., 2008b; Meyer et al., 2006a; Meyer et al., 2008c; Meyer et al., 2006b; Meyer et al., 2008e; Ozawa et al., 2006; Smith et al., 2007; Vuillermot et al., 2010; Zuckerman et al., 2003; Zuckerman and Weiner, 2005).

The role of bacterial infection has also been investigated by using the Gram-negative bacterial cell wall component, lipopolysaccharide (LPS). In rats, injections at several stages of gestation, raging from GD 9 until birth, induced, in the offspring, impairments in PPI, decreases in latency to fall from the roto-rod, spatial learning in the Morris-water maze and the radial arm maze, associative learning in the cued version of the Morris-water maze and in passive avoidance. Conversely, these treatments resulted in increases in anxiety, ethanol intake and preference, and sensitivity to the locomotor effects of AMPH (Fortier et al., 2004; Fortier et al., 2007; Girard et al., 2009; Girard et al., 2010; Golan et al., 2006; Golan et al., 2005; Hava et al., 2006; Lante et al., 2008; Lante et al., 2007; Liu et al., 2004; Wang et al., 2010). LPS has also been administered either in alternate days (Borrell et al., 2002) or daily throughout pregnancy (Romero et al., 2006; Romero et al., 2008). Similarly to acute LPS administration, these chronic prenatal treatments also induced impairments in PPI (Borrell et al., 2002; Romero et al., 2006; Romero et al., 2008).

Both bacterial and viral mimics have been proven to be able to induce several behavioural alterations in the offspring when administered during gestation in animal models. Many of these behavioural effects can be related to some of the behavioural alterations observed in patients with SCZ. Further analysis of the brain morphology, histo- and neurochemistry have revealed

striking parallels between the alterations induced by maternal infection in the offspring's brain in animal models and those found in the SCZ population.

Importantly, some of the alterations in behaviour have been shown to appear in the adult but not in the juvenile offspring (Ozawa et al., 2006; Romero et al., 2008; Vuillermot et al., 2010; Zuckerman and Weiner, 2003), as occurs in SCZ patients. Also significant is the observation that a number of behavioural alterations were shown to be reversed by either acute or chronic treatment with several antipsychotic drugs (i.e. haloperidol, chlorpromazine or clozapine) in the adult or adolescent animals (Meyer et al., 2008e; Ozawa et al., 2006; Romero et al., 2006; Shi et al., 2003; Zuckerman et al., 2003), which constitute the primary pharmacological treatment for psychotic illness (Seeman and Kapur, 2000).

6.2. Alterations in DA neurotransmission induced by prenatal infection in animal models

Given the central role of DA neurotransmission in SCZ (see 5.3.2), several studies investigated the effects of prenatal immune activation on this neurotransmitter system. One often used approach is the measurement of tissue DA content and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). DOPAC and HVA are the products of the enzymatic degradation of DA by the monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). A large amount of DA is degraded intracellularly, following its reuptake by the DA transporter (DAT) in the STR or the noradrenaline (NA) transporter (NAT) in the PFC. In addition, HVA/DA or DOPAC/DA ratios are often used as a measure of the turnover of this neurotransmitter.

Prenatal poly I:C treatment at GD 15 [intravenous (i.v.), 4 mg/kg] induced enhanced release of DA from striatal explants in the adult offspring [postnatal day (P) 100] (Zuckerman et al., 2003). In addition, poly I:C treatment at GD 9 (i.v., 5 mg/kg) has been reported to result in increased DA and DOPAC levels in the PFC and the globus pallidus (GP) and HVA in the NAcc and GP of adult mice (Winter et al., 2009). Similarly, several injections of poly I:C (i.p., 5 mg/kg, GD 12-17) resulted in greater levels of DOPAC and HVA in the adult STR (Ozawa et al.,

2006). Prenatal LPS treatment has been shown to have rather variable effects on DA, depending on the age, brain region, and dose regime of LPS treatment during gestation. For example, daily administration of LPS throughout the entire pregnancy [subcutaneous (s.c.) injection, 2 mg/kg) resulted in increased DA levels in the NAcc of adult animals (P 120, 170 or 400), and lower levels in younger animals (P 39) (Romero et al., 2006; Romero et al., 2008). Additionally one of these reports also showed increased DOPAC levels in the dorsal STR (dSTR) (Romero et al., 2006). Interestingly, a single LPS administration at GD 10 (i.p. 1 mg/kg), resulted in a decrease of DA in the dSTR, but an increase in the HVA/DA ratio in this region in weanling animals (P 21) and adults (P 84, 120, 420 or 510) (Ling et al., 2004a; Ling et al., 2002; Ling et al., 2006; Ling et al., 2009; Ling et al., 2004b; Wang et al., 2009). Decreased DA was also found in the NAcc, frontal cortex, amygdala, hippocampus and hypothalamus, accompanied by decreased levels of HVA in the NAcc and amygdala, and increased HVA/DA ratio in the frontal cortex, amygdala and hypothalamus (P 120) (Wang et al., 2009). Similarly, decreased DA levels in the NAcc at P 83 were found when escalating doses of LPS were administered daily from GD 15 until 19 (from 20 to 80 μg/kg) (Bakos et al., 2004).

Immunoreactivity of tyrosine hydroxylase (TH), the rate limiting enzyme for synthesis of DA and NA, is a commonly used marker of catecholaminergic neurons. This and other elements involved in DA neurotransmission have also been studied. Prenatal poly I:C treatment (i.v., GD 9) results in increased TH immunoreactivity in the mesencephalon of embryonic mice at GD 13 and 17 (Meyer et al., 2008a), as well as in the NAcc and SN of adult mice (P 120) (Vuillermot et al., 2010). In the NAcc, TH immunoreactivity was decreased at P 35 (Vuillermot et al., 2010) but increased at P 70 (Meyer et al., 2008c; Vuillermot et al., 2010), in both cases compared to age matched controls. Accompanying these effects, DAT immunoreactivity was also found to be increased in the foetal mesencephalon (GD 17) (Meyer et al., 2008a), but decreased in the dSTR at P 35, as well as in the NAcc at GD 19 and P 35 (Vuillermot et al., 2010). Immunoreactivity of DA receptors, D1 and D2, was reduced in the adult mice's

PFC (Meyer et al., 2008c; Meyer et al., 2008d) and increased in the NAcc (for both D1 and D2) and dSTR (only D1) (Vuillermot et al., 2010). In contrast, Ozawa *et al* reported that DA D2 receptor's binding was reduced in the striatum of adult mice (Ozawa et al., 2006).

The effects of prenatal LPS administration on these markers of DA neurotransmission are also rather conflicting. Borrell *et al* (Borrell et al., 2002) found increased TH immunoreactivity in the NAcc and bed nucleus of the stria terminalis in adult rats whose mothers were treated with LPS (s.c., 1 mg/kg) on alternate days during the entire pregnancy. In contrast, Ling *et al* reported in a number of studies that a single dose of LPS at GD 10 (i.p., 1 mg/kg) leads to a significant decrease in TH immunoreactivity, which was significant in the SN, at several postnatal ages (P 21, 120, 210, 420, 510) as well as in the VTA of post-weanling rats (Ling et al., 2004a; Ling et al., 2002; Ling et al., 2006; Ling et al., 2009; Ling et al., 2004b; Wang et al., 2009). Interestingly, prenatal LPS-induced reduction in TH immunoreactivity is accompanied with greater susceptibility to DAergic neuronal death by subsequent postnatal insults in adult rats [i.e. a secondary LPS injection, rotenone or 6-hydroxydopamine (6-OHDA) administration] (Ling et al., 2004a; Ling et al., 2006; Ling et al., 2004b; Zhu et al., 2007). TH immunoreactivity levels were not assessed in the terminal regions in these studies.

The reason for these seemingly divergent scenarios has yet to be investigated. However, they may stem from the type and/or dose of immunogens and/or the stage of pregnancy at which this is administered. Some of the effects of prenatal inflammatory insults have been observed to vary with the stage of pregnancy during which the immune challenge was induced (Cui et al., 2009; Fortier et al., 2007; Li et al., 2009b; Meyer et al., 2006a; Meyer et al., 2007a; Zuckerman and Weiner, 2005) and with the type of immunogen used (Fortier et al., 2007).

6.3. Additional alterations in animals models of maternal infection

A multitude of additional behavioural, structural and neurochemical alterations have been reported in the offspring of rats or mice treated with either poly I:C or LPS (see (Boksa, 2010a; Meyer and Feldon, 2010) for detailed reviews).

Relevant findings parallel the pathophysiological features of SCZ (section 5.2). These include the finding of enlarged lateral ventricles (Li et al., 2009b), alterations in the white matter microstructure (Li et al., 2010) and reduced myelination (Cai et al., 2000; Rousset et al., 2006). Given the recent emphasis on hippocampal abnormalities in SCZ (Freedman and Goldowitz, 2010; Schmajuk, 2001), morphological alterations in this region have been studied. For example, there are reports of decreased hippocampal neurogenesis (Cui et al., 2009; Meyer et al., 2006a); as well as decreased dendritic arborisation, especially in the CA1 field (Baharnoori et al., 2009); changes in width and length of several hippocampal subdivisions (Golan et al., 2005) and increased cell density (Fatemi et al., 2002; Fatemi et al., 1999; Golan et al., 2005) among others. There are also reports of alterations in serotonin, GABA and glutamate neurotransmitter systems in the hippocampal formation (Fatemi et al., 2008; Lante et al., 2007; Lowe et al., 2008; Meyer et al., 2006a; Nyffeler et al., 2006; Roumier et al., 2008; Winter et al., 2009).

Collectively, the effects of prenatal inflammatory insults in the offspring are consistent with the epidemiological link between such events in the population of pregnant women and increased risk of SCZ and other forms of psychosis in the offspring. More specifically, they have demonstrated that immune activation during gestation can indeed induce changes in neurodevelopment that result in alteration of brain function in the offspring. What remain to be determined are the mediators and mechanisms through which maternal infection impairs neurodevelopment.

I.7. Possible inflammatory mediators involved in the neurodevelopmental effects of prenatal infection

Inflammatory agents such as poly I:C or LPS initiate the cascade of inflammatory events by binding to a specific class of receptors that recognize pathogen-associated molecular patterns (PAMPs), i.e. the toll-like receptors (TLR). LPS is recognized by TLR4 (Poltorak et al., 1998; Qureshi et al., 1999), whereas double stranded RNA, like poly I:C, is recognized by TLR3 (Alexopoulou et al., 2001). Following TLR receptor binding, PAMPs activate several intracellular signalling pathways that ultimately lead to the nuclear localization of the transcription factor, NFκB (Anderson, 2000; Takeda et al., 2003), which triggers the transcription of several genes, including cytokines (Medzhitov et al., 1997) (Fig I-3A).

Cytokines are small, mostly secreted proteins originally characterized as immune modulators. They have since been found to mediate a number of functions in several tissues, including the central nervous system (Deverman and Patterson, 2009). Cytokines act, in the context of innate immune response to infection and injury, as pro-inflammatory/pyrogenic [IL-1β, IL-6, IL-8, TNFα, and interferon (IFN)γ] or anti-inflammatory/cryogenic intermediaries [IL-1 receptor antagonist (IL-1ra) and IL-10 amongst others] (Kluger, 1991; Leon, 2002; Roth, 2006); their expression is dramatically increased following inflammatory events. The increase in the levels of these mediators is usually short-lived, with the duration depending on the type and dose of immunogen administered as well as the stage of gestation (Ashdown et al., 2007; Fofie and Fewell, 2003; Fofie et al., 2005; Meyer et al., 2006a).

These mediators are involved in the downstream activation of several host responses to infection, collectively called inflammation, and whose initial component is known as the acute phase response. This response includes fever, hypothalamus-pituitary-adrenal (HPA) axis activation, anorexia and hypoferremia (Baumann and Gauldie, 1994). IL-6 has been proposed as a major peripheral mediator in the induction of several of these responses, including fever (Rummel

et al., 2006), HPA axis activation (Turnbull et al., 2003) and hypoferremia (Nemeth et al., 2004a) (Fig I-3B).

Several of these maternal responses have been proposed to be involved, either collectively or individually, in the induction of the alterations in the neurodevelopment of the offspring (Boksa, 2010a; Brown and Derkits, 2010a; Patterson, 2009). Central to these responses is the involvement of cytokines and the possible influence they may exert on the normal foetal development during pregnancy.

Figure I-3. Induction of inflammation by pathogen-associated molecular patterns

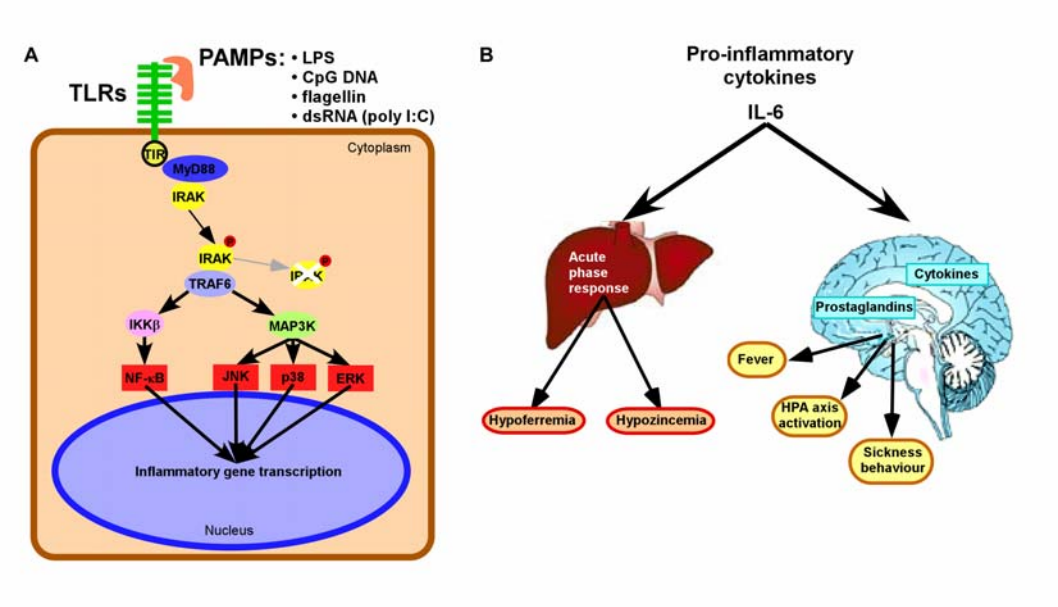

Figure I-3. (A) Toll-like receptors (TLRs) expressed on professional immune cells (monocytes, macrophages, dendritic cells and microglia) recognize and respond to microbial infection. TLRs are activated by products containing pathogen-associated molecular patterns (PAMPs), derived from bacteria [lipopolysaccharide (LPS), DNA with non-methylated cytosine-guanine motifs (CpG DNA), flagellin] or from viruses [double-stranded RNA (dsRNA)]. All TLR family members have a unique intracellular TIR (Toll/IL-1 receptor) signaling domain. In response to activation by the corresponding ligands, TIR domains interact with the TIR domain of the signaling adaptor MyD88 (myeloid differentiation factor 88), which transduces the signal to a family of IL-1 receptor-associated kinases (IRAKs). Phosphorylation of IRAK, a serine-

threonine kinase, by other IRAK family members induces cascades of signaling through TRAF6 (tumor necrosis factor receptor-associated factor 6). TRAF6 transduces the signal to IKKbeta (IkappaB kinaseβ) and to MAP3K (mitogen-activated protein kinase kinase kinase). This signaling results in transcriptional responses, mediated primarily by nuclear factor-κB (NF-κB), extracellular-signal regulated kinase (ERK) and stress-activated protein kinases, such as c-Jun N-terminal kinase (JNK) and p38, leads to expression of pro-inflammatory cytokines. Modified from (Kariko et al., 2004). (B) Pro-inflammatory cytokines released to the systemic circulation, especially interleukin-6 (IL-6), trigger the systemic inflammatory response by targeting several organs. Actions on the brain induce the expression of secondary mediators (i.e. cytokines, prostaglandins) that in turn induce fever, activation of the hypothalamus-pituitary-adrenal (HPA) axis and other sickness behaviours. Activation of the liver, on the other hand, leads to the release of several acute phase response proteins, and to the initiation of responses such as the decrease in circulating iron (hypoferremia) and zinc (hypozincemia).

7.1. Cytokines and brain development

The involvement of pro-inflammatory cytokines in the aetiology of neurodevelopmental psychiatric disorders is suggested by the finding of increased levels of pro-inflammatory cytokines (IL-8 or TNFα) in serum samples from pregnant women that give rise to individuals that later develop psychosis (Brown et al., 2004b; Buka et al., 2001b). Intriguingly, other serum maternal pro-inflammatory cytokines were analysed in these studies (IL-1β, IL-2 and IL-6) and no definite association between these and SCZ in the offspring was found (Brown et al., 2004b; Buka et al., 2001b).

A more causal role for elevated maternal cytokines in SCZ-related alterations has been established through the study of their effects in animal models. Administration of exogenous cytokines to pregnant rats or mice has been shown to be sufficient to induce several molecular and behavioural effects in the offspring. For example, prenatal administration of IL-6 in mice (at GD 9, 5 μg,

i.p.) resulted in impairments in PPI and latent inhibition in the adult offspring, whereas a similar treatment with IFNγ had no effect (Smith et al., 2007). In addition, IL-6 administration for three days (either at GD 8, 10 and 12 or GD 16, 18 and 20; 9 μg/kg each day, i.p.) resulted in impairments in spatial learning in the Morris-water maze (Samuelsson et al., 2006b) and HPA-axis hyperactivity (Samuelsson et al., 2004). It was also described a reduction in the number of neurons, an increase in astrogliosis, as well as increased expression of apoptosis markers in the hippocampus (Samuelsson et al., 2006b). These results, along with those from epidemiological studies in humans (Brown et al., 2004b; Buka et al., 2001b), seem to suggest a negative outcome from exposure to pro-inflammatory cytokines. However, further evidence suggests a more complex scenario. In this regard, the offspring of transgenic female mice engineered for macrophage-specific overexpression of the anti-inflammatory cytokine IL-10 [IL-10 transgenic mice (IL-10tg)], presented some behavioural alterations similar to those seen after administration of poly I:C at GD 9 (2 mg/kg, i.v.), including decreased open-field exploration and impaired latent inhibition (Meyer et al., 2008b). This finding led to the proposal that rather than a net increase in cytokine concentration, it is an imbalance between pro- and anti-inflammatory cytokines that may underlie the association between maternal infection and SCZ (Meyer et al., 2008b).

In addition to the demonstration that prenatal increases in cytokine levels is sufficient to induce neurodevelopmental alterations in the offspring, a complementary series of studies demonstrated that IL-6 is necessary for the neurodevelopmental effects of prenatal infection. In this case, functional inhibition of poly I:C-induced IL-6 in pregnant mice prevented several of the behavioural effects of prenatal poly I:C in the offspring, including impaired PPI and latent inhibition, decreased locomotion and exploration of the centre of the open field, and decreased social interaction (Smith et al., 2007). In addition, the offspring of IL-6 “knock-out” mothers treated with poly I:C, do not present these alterations (Smith et al., 2007). Furthermore, administration of poly I:C to IL-10tg mothers failed to induce the reduction in open field exploration, impaired PPI and latent inhibition, and hyperlocomotion induced by the NMDA non-competitive

antagonist, MK-801 (Meyer et al., 2008b). IL-10tg mothers presented a mildly attenuated induction of IL-6 and TNFα by poly I:C in the circulation, which could be one the mechanisms through which poly I:C-induced alterations in the offspring are prevented (Meyer et al., 2008b). Intriguingly however, IL-10 overexpression did not prevent the increase in acoustic startle response or the enhanced AMPH-induced locomotion induced by the prenatal treatment with poly I:C (Meyer et al., 2008b), suggesting that the anti-inflammatory effect of IL-10 was not sufficient to avert all the effects of prenatal inflammation.

Other than the role of individual mediators, what still remains unknown is the relevant site of action of maternal cytokines. The general consensus has been that circulating cytokines act at the level of the foetal brain and/or the placenta. Another possibility is that secondary induction of cytokines in the foetal compartment may be playing a significant role. Analyses of foetal cytokine expression following maternal treatment with LPS or poly I:C has, however, shown large inconsistencies between studies. These could simply be ascribed to the heterogeneity in doses and/or immunogenicity of the different lots of immunogens, stages of gestation and the time points analyzed (see (Boksa, 2010a) for a detailed review). For example, foetal brain cytokine levels and expression following LPS administration have both been shown to be induced, reduced or unaffected by the maternal insults (Ashdown et al., 2006a; Cai et al., 2000; Paintlia et al., 2004; Paintlia et al., 2008; Urakubo et al., 2001). An effect of similar variability has been observed following maternal treatment with poly I:C (Gilmore et al., 2005; Meyer et al., 2008b; Meyer et al., 2006a).

Immune activation of the placenta by the maternally administered LPS (Ashdown et al., 2006a; Cai et al., 2000; Gayle et al., 2004; Girard et al., 2010; Urakubo et al., 2001)or poly I:C (Koga et al., 2009) has also been shown. For example, induction of expression of IL-1β, IL-6, TNFα and INFγ has been demonstrated. These findings raise the possibility that cytokines from placental origin may either interfere with the normal function of the placenta or contribute to the cytokine cascade that targets the foetal brain.

7.1.1. IL-1 and TNFα

Regardless of their tissue source, cytokines may directly target the foetal brain. Their effects on the development of this organ can be varied (Deverman and Patterson, 2009; Huleihel et al., 2004). IL-1β, for example, is expressed in the embryonic spinal cord neuroepithelium in rats where it has a role in cell proliferation (de la Mano et al., 2007). This cytokine also acts as a mitogen for astrocytes (Giulian et al., 1988). TNFα regulates developmental apoptosis (Barker et al., 2001; Sedel et al., 2004) and synaptogenesis (Deverman and Patterson, 2009) and is involved in the induction of compensatory changes in synaptic strength in response to prolonged changes in synaptic activity (Stellwagen and Malenka, 2006). This represents a form of homeostatic plasticity known as synaptic scaling, which is thought to provide stability to neuronal networks (Turrigiano and Nelson, 2004) and may have functional roles during developmental synaptic refinement (Kaneko et al., 2008). Both IL-1β (Zeise et al., 1992) and TNFα (Stellwagen et al., 2005) have been shown to regulate inhibitory synaptic transmission and, in addition, IL-1β can inhibit hippocampal long-term potentiation (LTP) (Cunningham et al., 1996; Katsuki et al., 1990). LTP is a form of synaptic plasticity, thought to represent a cellular mechanism of learning and memory (Kotaleski and Blackwell, 2010).

Another important component of the IL-1 signalling is the endogenous antagonist IL-1ra, which normally limits the effects of inflammation by blocking the activity of IL-1 on its receptor (Dinarello, 1991; Spulber et al., 2009). In the context of acute inflammation, IL-1β is not always released into the systemic circulation, whereas large increases in IL-1ra are commonly found in the circulation following LPS (Pohl et al., 2009b) and turpentine oil (TURP) injection (Aguilar-Valles et al., 2007). In these circumstances, IL-1ra may act to inhibit locally expressed IL-1β in the brain and other organs in order to exert its inhibitory function. Some have reported that this cytokine can play a protective role for the foetuses during events of maternal infection (Girard et al., 2010), however the precise role of this endogenously expressed mediator on the effects of inflammation on neurodevelopment remains largely unknown.

7.1.2. Leptin

Leptin was originally identified as the product of *ob* gene (Zhang et al., 1994) and was characterized as the hormone that regulates food intake and energy expenditure (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Leptin is primarily produced by adipose tissue and secreted into the circulation, where levels correlate positively with body fat mass (Frederich et al., 1995; Maffei et al., 1995). Unlike classic cytokines (i.e. IL-1β and IL-6) leptin is constitutively produced by adipose tissue and is present in nanogram concentrations in the general circulation (Friedman and Halaas, 1998).

Since its identification, it has become clear however, that this hormone has a multitude of physiological roles, such as control of neuroendocrine function (Ahima et al., 1996; Coleman, 1978; Dallman et al., 1999; Korbonits et al., 1997), regulation of sexual maturity during puberty (Ahima et al., 1997; Barash et al., 1996; Chehab et al., 1996), angiogenesis (Park et al., 2001; Sierra-Honigmann et al., 1998), glucose metabolism (Burcelin et al., 1999; Rossetti et al., 1997; Sivitz et al., 1997), lipogenesis (Chen et al., 1996), hematopoiesis (Bennett et al., 1996) and immune cell activation (Lord et al., 1998) both *in vitro* and *in vivo*.

Regarding immunity, rising levels of leptin have direct stimulatory effects on both innate (Gainsford et al., 1996; Loffreda et al., 1998) and adaptive immune functions (Lord et al., 1998; Martin-Romero et al., 2000), as well as the acute and chronic inflammatory processes (Faggioni et al., 2000a; Faggioni et al., 2000b; Ikejima et al., 2005; Mancuso et al., 2002; Matarese et al., 2002; Takahashi et al., 1999). For example, leptin treatment induces production of classical pro-inflammatory cytokines including TNFα, IL-1β, IL-6, and IFN-γ (Zarkesh-Esfahani et al., 2001) (Dixit et al., 2004; Maedler et al., 2004) and regulates the release of pro- and anti-inflammatory cytokines in response to LPS (Faggioni et al., 1999; Loffreda et al., 1998; Santos-Alvarez et al., 1999), *in vitro* and *in vivo*. The converse is also true, as inflammatory stimuli increase leptin synthesis above baseline levels. In animal models this effect has been demonstrated for TNFα, IL-1β (Grunfeld et al., 1996b; Sarraf et al., 1997), LPS, TURP and carrageenan (Faggioni et al., 1998; Gualillo et al., 2000a; Mastronardi et al., 2001). Similarly

to IL-6 induction after LPS or TURP, elevation of leptin following either of these treatments depends on IL-1β (Faggioni et al., 1998). During the acute inflammatory response, leptin is involved in the induction of several sickness-type responses, such as anorexia (Harden et al., 2006; Sachot et al., 2004). It appears that leptin controls this response through the induction of brain synthesis of cytokines, such as IL-1β (Luheshi et al., 1999; Wisse et al., 2004). In addition, leptin is involved in the induction of the fever response to LPS (Harden et al., 2006; Luheshi et al., 1999; Phillips et al., 1996; Sachot et al., 2004; Turek et al., 2004) through the induction of brain IL-1β (Hosoi et al., 2002; Sachot et al., 2004; Wisse et al., 2004) and COX-2 (Inoue et al., 2006).

Despite its clear involvement in several aspects of the inflammatory response to infection, the role of leptin in foetal development has not yet been extensively studied. However it is conceivable that elevated leptin levels during pregnancy may affect several aspects of gestation, as leptin has been shown to regulate early embryonic development (Kawamura et al., 2003), implantation (Ramos et al., 2005) and placental endocrine function (Islami et al., 2003a, b; Lappas et al., 2005). Another intriguing aspect of leptin is that, similarly to IL-6, upon binding to its receptor, leptin activates the intracellular signalling molecule gp130 (Tartaglia, 1997; Tartaglia et al., 1995), which results in the activation of the JAK2/STAT3 signalling pathway. Therefore, it may affect the expression of similar target genes to those activated by IL-6 (Kishimoto et al., 1994) (see section 7.1.3).

7.1.3. IL-6

IL-6, can affect development of neurons and glia by affecting processes such as proliferation, survival, death, neurite outgrowth and gene expression (Patterson, 2002). This cytokine and its receptor (IL-6R) mRNAs are co-expressed in neurons and are developmentally regulated in the rat brain, with the highest levels of expression in the adult hippocampus, striatum and neocortex (Gadient and Otten, 1994). High doses of IL-6 increase the susceptibility of cerebellar neurons in culture to toxic insults and results in cell damage and death (Conroy et al., 2004). In contrast, lower doses of this cytokine have

neuroprotective effects (Conroy et al., 2004). Similar dual effects have been observed in embryonic or postnatal midbrain catecholaminergic and spinal cholinergic neurons in culture (Kushima et al., 1992; Kushima and Hatanaka, 1992). The nature of the effects of IL-6 seems to depend on the dose and duration of the stimulus and the cell type under analysis. IL-6 also has a role in neurotransmission, especially in the regulation of hippocampal synaptic plasticity. IL-6 levels are dramatically upregulated by LTP induction *in vivo* (Balschun et al., 2004), and application of an anti-IL-6 antibody 90 min after tetanus prolonged LTP and improved long-term memory (Balschun et al., 2004). In contrast, IL-6 administration was demonstrated to inhibit LTP (Bellinger et al., 1995; Li et al., 1997; Tancredi et al., 2000).

Furthermore, activation of gp130, the molecule through which IL-6R signals, by a recombinant fusion protein of IL-6 and its soluble receptor, increased the myelinating capacity of embryonic stem cells derived from oligodendrocyte precursors (Zhang et al., 2006). IL-6 and other members of the gp130 family of cytokines [including ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and IL-11] are also capable of enhancing oligodendrocyte survival *in vitro* (Barres et al., 1993). They can also regulate self-renewal of neuroepithelial/radial glial cells, which function as the scaffolds for radially migrating neurons and as precursors for neurons, astrocytes, oligodendrocytes and adult neural progenitors (Deverman and Patterson, 2009).

IL-6 has been suggested as a major mediator of the effects of maternal infection in neurodevelopment of the offspring. This hypothesis is based on the fact that this cytokine plays a major role as a systemic mediator of inflammation, the varied effects that it has on normal brain development and, more importantly, on the finding that its administration to pregnant dams induces some of the same effects as prenatal poly I:C, and that its neutralization at the time of poly I:C treatment prevents the effects of the latter in the offspring. IL-6, however, can act in more than one way to affect neurodevelopment. Its effects may be by the direct activation of IL-6R in the foetal brain or, alternatively, it may alter neurodevelopment through one of the several secondary inflammatory responses

that it triggers in the mother during the acute phase response. These include hypoferremia that characterises the acute phase response and is triggered by all types of infection (Grieger and Kluger, 1978; Kluger and Rothenburg, 1979). Appropriate iron homeostasis is fundamental for normal brain development, especially for the DAergic neurons (Beard and Connor, 2003).

7.2. Hypoferremia

Hypoferremia is a decrease in circulating non-haeme iron, a consequence of its cellular retention, which occurs during infection and inflammation. This response plays a beneficial role by withholding an essential nutrient from invading pathogens (Grieger and Kluger, 1978; Kluger and Rothenburg, 1979; Nemeth and Ganz, 2006). During inflammatory hypoferremia, iron accumulates in macrophages and intestinal iron absorption is interrupted (Hentze et al., 2004). The mechanisms leading to hypoferremia have been recently characterized at a molecular level. IL-6 has been identified as the most important cytokine in the induction of this process, during inflammation, following diverse inflammatory challenges, such as TURP (Merle et al., 2007; Nicolas et al., 2002b; Pigeon et al., 2001), LPS (Kemna et al., 2005; Pigeon et al., 2001; Yeh et al., 2004) and Freund's adjuvant (Anderson et al., 2002). Circulating IL-6 targets hepatocytes, where it induces the expression and release of hepcidin-1 (HAMP) (Nemeth et al., 2004a; Nemeth et al., 2003).

HAMP is a small protein that acts by binding to ferroportin-1 (FPT1), the only iron exporter identified to date, inducing its internalization and degradation. As a consequence, HAMP-induced internalization of FPT1 blocks iron flow into the blood and other extracellular fluids from cells that store (i.e. macrophages and hepatocytes) or acquire this nutrient (i.e. duodenal enterocytes) (Donovan et al., 2000; Donovan et al., 2005; Gruper et al., 2005; Hentze et al., 2004; Knutson et al., 2005; Nemeth and Ganz, 2006; Nemeth et al., 2004b; Nicolas et al., 2002a). HAMP is considered the systemic regulator of iron homeostasis (Pigeon et al., 2001) and is responsible for inflammatory hypoferremia. HAMP-deficient mice fail to develop hypoferremia after an inflammatory challenge with TURP (Nicolas

et al., 2002b). The continuous use of plasma iron for erythrocyte production rapidly depletes the non-haeme iron from blood. When this is prolonged, haemoglobin synthesis is affected, leading to anaemia of inflammation, also known as anaemia of chronic disease (Nemeth and Ganz, 2006; Weinstein et al., 2002).

Hypoferremia of inflammation during pregnancy may represent a major challenge to the foetus, since IL-6-induced interruption of iron flow, from both the maternal diet and depots, may compromise the supply of this micronutrient. During pregnancy, intestinal absorption of iron plays a major role in the supply of this nutrient to both the mother and the foetus (Millard et al., 2004). In fact, the rate of maternal intestinal absorption increases with the increasing size of the foetus and is maximal just prior to parturition (Gruper et al., 2005; McArdle et al., 2003): this is also when materno-foetal transfer of iron is maximal (Millard et al., 2004). Therefore, by limiting the external input of iron (Nemeth and Ganz, 2006) to the pregnant dam, together with the obstruction of iron flow from hepatic and plasmatic depots and, likely, the passing via the placenta, IL-6-driven hypoferremia may significantly interrupt the supply of iron to the developing foetus. The consequences of this during development could be numerous and serious since iron is involved in basic cell metabolism, in the development of the hippocampus, myelination, and, most importantly, in the development and function of DAergic neurons. For instance, iron is abundant within all the DA-rich regions of the brain, such as the pallidum, caudate-putamen, NAcc, SN and VTA (Hill and Switzer, 1984; Youdim et al., 1984), and is a cofactor of TH (Beard and Connor, 2003), which suggests a functional role in the mesolimbic and mesocortical DA systems. In addition, numerous studies have reported long-term effects of developmental iron deficiency, which include alterations in DA neurotransmission.

7.2.1. Developmental iron deficiency and adult DA function

In vivo microdialysis studies in rats exposed to a dietary iron deficiency from weaning (P 21) onwards showed that, as adults, these animals presented elevated levels of extracellular DA and metabolites in the striatum (Beard et al.,

1994; Lozoff et al., 2006; Nelson et al., 1997). Additionally, there was an elevation in the tissue content of DA in the NAcc (Nelson et al., 1997) and an increased sensitivity to the locomotor activating effect of AMPH (Youdim and Yehuda, 1985). Moreover, post-weanling iron deficiency has been shown consistently to reduce the number of DA transporter (DAT) binding sites in the NAcc, as well as other striatal nuclei (Beard et al., 2003; Beard et al., 2002; Burhans et al., 2006; Erikson et al., 2000; Felt et al., 2006; Wiesinger et al., 2007). As previously described, DAT represents an important clearance mechanism for extracellular DA and, in addition, NAcc DAT is the molecular target of the psychomotor stimulant cocaine, which can act as DAT inhibitor. Cocaine increases the extracellular levels of DA, thereby inducing its locomotor activating effects. Accordingly, iron-deficient animals show impaired cocaine-induced increases in extracellular DA (Nelson et al., 1997) and diminished locomotor activating effects of this psychostimulant (Erikson et al., 2000). This suggests that decreased DAT expression may contribute to the increase in extracellular levels of striatal DA and to the increased sensitivity to AMPH induced by dietary iron deficiency.

Apart from its role in DA function during post-weanling stages, deficits in iron supply during gestation have been shown to result in long lasting alterations in DA metabolism and function, presumably due to alterations in neurodevelopment. Iron deficiency throughout gestation and lactation has been shown to increase tissue-content of striatal DA and metabolites, in the post-weanling and adult offspring (Beard et al., 2006; Kwik-Uribe et al., 2000), which is similar to the effects of postnatal iron deficiency. Interestingly, when iron deficiency was restricted to a period spanning from GD 15 until P 4, it also induced increases in tissue content of DA and metabolites in the medial PFC (mPFC) and STR, accompanied with increased striatal TH expression and phosphorylation in the offspring (Unger et al., 2007). TH phosphorylation results in increased enzymatic activity, which could contribute to the increased DA levels induced by developmental iron deficiency.

Collectively, the alterations induced by iron deficiency during development are suggestive of increased DA synthesis and release from the striatal DAergic terminals, accompanied by deficient reuptake. These effects bear a striking resemblance to a number of the aforementioned effects of prenatal infection on the DA neurons in rodent models, as well as to key pathophysiological aspects of SCZ in humans. Interestingly, micronutrient deficiency during gestation is associated with increased risk of developing SCZ (Brown and Susser, 2008; Zammit et al., 2007) and, specifically, low maternal haemoglobin during gestation, which is highly correlated with maternal iron levels, is associated with an increased risk of SCZ in adult offspring (Brown and Susser, 2008). Additionally, some polymorphisms in the *transferrin* (*Tf*) gene, which encodes for a protein involved in non-haeme iron transport and storage, have been associated with SCZ (Qu et al., 2007) and *Tf* mRNA expression has been found to be decreased in the dorsolateral PFC of SCZ patients (Arion et al., 2007; Hakak et al., 2001). These observations strongly suggest that altered iron homeostasis during gestation could be involved in increasing the risk of developing SCZ, thus, this represents a plausible mechanism through which maternal infection may affect the neurodevelopment of the offspring.

I.8. Turpentine as a model of maternal inflammation

TURP is an inflammatory agent whose injection [usually intramuscular (i.m.)] produces localised necrotic damage (Wusteman et al., 1990) and the sequential induction of TNFα and IL-1β at the site of injury (Luheshi et al., 1997; Zheng et al., 1995). These cytokines in particular IL-1β, in turn trigger IL-6 synthesis and release into the circulation, where it acts as the major pro-inflammatory circulating mediator [Figure I-4, (Luheshi et al., 1997)]. TURP-induced inflammation is an established model for studying the role of IL-6 in the induction of the acute phase responses, including fever and hypoferremia (Aguilar-Valles et al., 2007; Kozak et al., 1998; Kozak et al., 1997; Nicolas et al., 2002b; Turnbull et al., 2003). This model has the additional advantage that it strongly activates the immune response without the confounding presence of the

injected exogenous immunogen in the circulation (which could be toxic for foetuses, independently of its immune activation abilities). For instance, injection of LPS and poly I:C in pregnant animals is associated with high maternal and foetal mortality rates (Martin et al., 1995; Meyer et al., 2005). These effects make it difficult to ascertain if the immunogenic agent is directly responsible for the effect on the foetal compartment or if maternal inflammatory mediators or responses are sufficient to trigger the developmental alterations leading to increased vulnerability to psychiatric disorders in the offspring.

The use of TURP has an additional advantage over direct injection of exogenous IL-6 in that the endogenously released cytokine produces biological responses at plasma concentrations ranging from pico to nanogram / mL concentrations (Cartmell et al., 2000), whereas pharmacological amounts of recombinant IL-6 (in the order of μg/mL) would have to be injected to achieve any significant effects (Blatteis et al., 1990; Cartmell et al., 2000; Harre et al., 2002; Roth, 2004).

Based on these properties and the capabilities that this model provides to study the role of circulating inflammatory mediators in isolation, all the work presented in this thesis was conducted using localized injury with TURP.

Figure I-4. Turpentine-induced inflammation

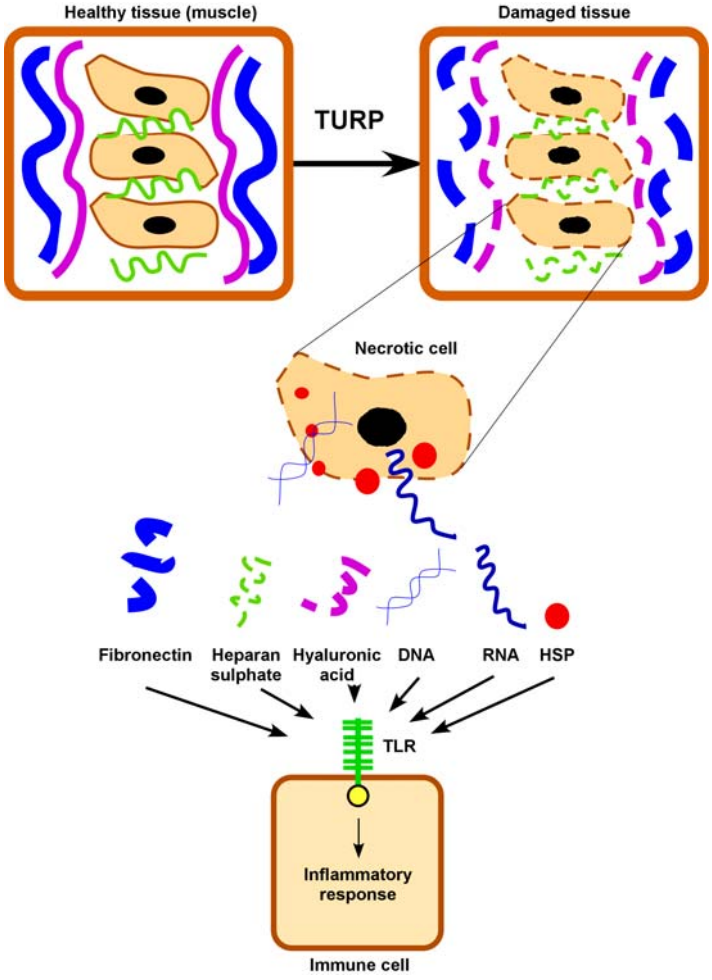

Figure I-4. Molecules derived from injured tissue, blood vessels, and necrotic cells activate Toll-like receptors and induce inflammation. Following tissue and cell injury by agents like turpentine oil (TURP), endogenous ligands of Toll-like receptors (TLRs) are generated and/or released. Fragments of extra-cellular matrices (hyaluronic acid, fibronectin, heparan sulfate) and heat shock proteins (HSP) released from damaged tissue, blood vessels, and necrotic cells, respectively, activate TLR4, the LPS receptor. RNA and chromatin-associated DNA released from necrotic cells activate TLR3 and TLR9, respectively. When endogenous ligands activate TLRs the resulting immune response is similar to that induced by microbial products. Modified from (Kariko et al., 2004).

I.9. Hypothesis

Collectively, evidence derived from epidemiological studies in humans and experiments conducted in animal models suggest that the inflammatory response elicited by a wide variety of infections in pregnancy affects the neurodevelopment of the foetuses resulting in alterations in brain morphology, neurochemistry and behaviour in the adult offspring. In particular, pro- and anti-inflammatory cytokines may play a significant role in the induction of these alterations. Some of these cytokines, however, may exert their effect through the induction of a secondary inflammatory response. Based on this evidence we propose that maternal circulating cytokines released during inflammation, particularly IL-6, IL-1ra and leptin, are responsible for the effects of maternal infection in the neurodevelopment of the offspring. In particular, IL-6, the main circulating pro-inflammatory mediator, will be fundamentally involved. In addition, we propose that at least some of the effects on neurodevelopment are induced by IL-6-activated hypoferremia, which may specifically target the development of the DA neurons. A hypothetical scheme describing this is presented in Fig. I-5.

Figure I-5. Hypothetical model of the mechanisms through which maternal inflammatory insults lead to altered neurodevelopment of the offspring

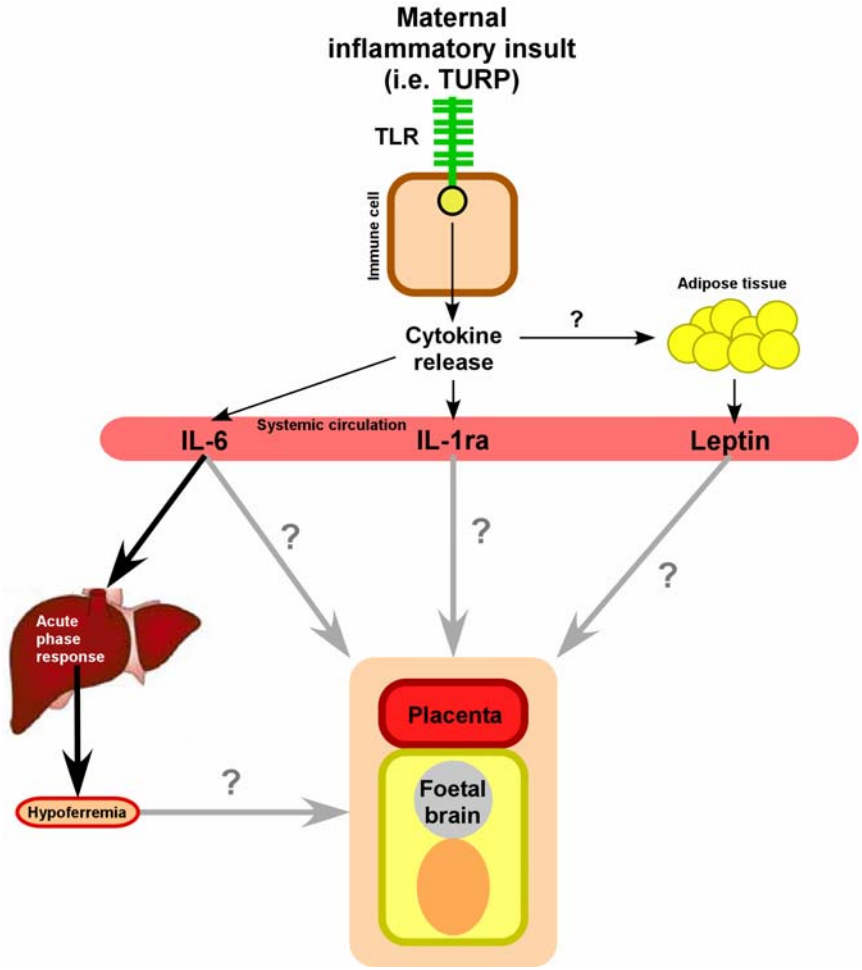

Figure I-5. A maternal inflammatory insult [i.e. locally administered turpentine oil (TURP) or systemic injection of LPS or poly I:C] leads to activation of immune cells, which release cytokines into the systemic circulation. The cytokines that reach the circulation are, mainly, the pro-inflammatory interleukin (IL)-6 and the anti-inflammatory IL-1 receptor antagonist (IL-1ra). In parallel, leptin release is stimulated from the adipose tissue. Each of these cytokines may in turn target the foetal compartment (placenta and/or the foetal brain directly) resulting in aberrant neurodevelopment. IL-6 activates acute phase responses such as hypoferremia, through its targeting of the maternal liver, which may also impact negatively on the normal development of the foetal brain.

I.10. Objectives

The aims of the work presented in this thesis are:

1. ***To determine the effect of gestational age on the inflammatory response to TURP administration***

This will be conducted by comparing the inflammatory response to TURP in normal cycling females and pregnant dams at different stages of gestation (e.g. GD 18 vs. GD 15).

2. ***To establish whether the effect of prenatal TURP on the cognitive and DA function of the offspring depends on the stage of gestation at which the insult occurs***

These studies will involve the assessment of behavioural performance of offspring born to mothers treated with TURP at either GD 15 or GD 18, focusing on behavioural tasks that gauge sensorimotor gating, spatial and associative learning and memory, as well as the function of the mesolimbic DA system.

3. ***To analyse the effects of the maternal cytokines induced by TURP in a pregnant rat on the DA neurotransmission of the adult offspring***

The approach here will involve the neutralization of the biological function of targeted endogenous cytokines identified as key components in the developmental cascade induced by the inflammatory stimulus in the pregnant animals. The outcomes will be assessed in the offspring by measuring behavioural and biochemical parameters of DA neurotransmission.

4. ***To examine the role of prenatal hypoferremia on the effects of prenatal inflammation on the DA neurotransmission of the adult offspring***

This study will involve the analysis of iron levels in both the mother and the foetal compartment following treatment with TURP. The aim will be to investigate whether the inflammation-induced hypoferremia will alter the normal development of the foetal brains by, again, assessing DA function in the adult offspring. The involvement of hypoferremia will be tested by assessing if

maternal iron replenishment prevents the effects of prenatal inflammation in the offspring.

Chapter II: Attenuated fever in rats during late pregnancy is linked to suppressed interleukin-6 production after localized inflammation with turpentine

II.1. Preface

The maternal inflammatory response, which is thought to be fundamentally involved in the effects of prenatal infection on neurodevelopment, endures profound changes throughout gestation in humans and other mammalian species (Luppi, 2003; Spencer et al., 2008). Fever, a hallmark of the acute phase response to infection, has been shown to be strongly attenuated in near term rodents and other mammals, challenged with a variety of immunogenic agents (Cooper et al., 1988; Eliason and Fewell, 1997; Kasting et al., 1978; Martin et al., 1995; Simrose and Fewell, 1995; Stobie-Hayes and Fewell, 1996; Zeisberger et al., 1981). The data from humans is less conclusive in this regard (Spencer et al., 2008); however, analysis of the mechanisms behind this attenuated form of the acute phase response, using animal models, may be important in revealing some of the alterations in the function of the immune system during pregnancy, which may be relevant for the human population as well.

Several attempts have been made to define the mechanisms that lead to reduced fever to LPS in late gestation. Although some studies suggested an alteration in peripheral cytokine production (Fofie and Fewell, 2003; Fofie et al., 2005), others failed to replicate this finding (Harre et al., 2006; Mouihate et al., 2005b), and instead reported an attenuated production of central prostaglandins (PG) (Mouihate et al., 2002), especially the pyrogenic PGE_2 (Imai-Matsumura et al., 2002), and in addition a decreased sensitivity to exogenous PGE_2 administration (Eliason and Fewell, 1997; Stobie-Hayes and Fewell, 1996). Others have suggested that at least some of these alterations may be due to increased expression of the nitric oxide synthase (NOS), and production of NO in the central nervous system of near term pregnant rats (Begg et al., 2007; Begg et al., 2008). It however remains to be determined if peripheral mechanisms may be playing a role in the gestational attenuation of fever, especially as greater production of IL-1ra was described for pregnant rats treated with LPS at GD 18 when compared to non-pregnant females (Ashdown et al., 2007). It is also important to define if these alterations extend to other types of immunogens, i.e. TURP, since the mechanisms through which this pyrogenic agent differ from

those activated by systemic LPS administration. The response to localized tissue damage is mediated solely by the maternal cytokines entering the circulation from the site of injection, whereas LPS can directly target the brain for fever induction (Cartmell et al., 2001; Givalois et al., 1994; Turrin et al., 2001). This fundamental difference may reveal different alterations in the inflammatory response through which fever is decreased during gestation. We therefore undertook this study to determine, for the first time, the characteristics of maternal fever to TURP during late gestation (i.e. GD 18), in comparison to non-pregnant females and to elucidate the potential mechanisms underlying this difference.

II.2. Manuscript

Title: Attenuated fever in rats during late pregnancy is linked to suppressed interleukin-6 production after localized inflammation with turpentine

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Abstract

An attenuated fever response to pathogens during late pregnancy is a phenomenon that has been described in several mammalian species, and although mechanisms are not completely understood, decreased prostaglandin E_2 (PGE_2) synthesis has been implicated. Upstream of PGE_2, there is evidence to suggest that anti-inflammatory cytokines such as interleukin-1 receptor antagonist (IL-1ra) could play a significant role. In the present study we addressed the role of pro-inflammatory cytokines during late pregnancy, specifically interleukin-6 (IL-6), an important circulating mediator in fever. Turpentine oil (TURP), a very potent pyrogen and activator of IL-6, was injected into the hind-limb muscle of rats at 18^{th} day of pregnancy (GD 18) or in non-pregnant (NP) age-matched female controls. As expected, TURP injection induced a highly significant fever in the NP animals, which peaked 11 h post-injection and lasted for over 24 h. This was accompanied by a significant rise in circulating IL-6 levels, which correlated with changes in PGE_2 synthesizing enzymes expression in the hypothalamus. In complete contrast, TURP-induced fever was totally absent in GD 18 animals whose body temperature did not deviate from basal values. The lack of response was additionally reflected by the absence of change in IL-6 concentration and by the significant attenuation of PGE_2 synthesizing enzymes expression, which correlated with the suppressed expression of SOCS3, a hypothalamic marker of IL-6 activity. Contrary to the changes in circulating IL-6 levels at GD 18, IL-1ra was induced to levels comparable to those of NP females, suggesting that the influence of this anti-inflammatory cytokine on the fever response to TURP is at best minimal. These data further confirm the importance of IL-6 in fever generation and provide evidence that it may be a key component of the attenuated fever response in late pregnancy.

Introduction

Fever is a hallmark response to injury or infection with a well-established adaptive value (Kluger, 1991; Kluger and Rothenburg, 1979; Roth, 2006); its induction, duration, magnitude and relapse are tightly regulated by the balance between the members of a family of endogenous mediators known as cytokines that can act as pro-inflammatory/pyrogenic [interleukin (IL)-1β; tumour necrosis factor α (TNFα) and IL-6] or anti-inflammatory/cryogenic intermediaries [the endogenous IL-1 receptor antagonist (IL-1ra) and IL-10 among others] (Kluger, 1991; Leon, 2002; Roth, 2006). In the central nervous system, pyrogenic cytokines induce prostaglandin E_2 (PGE_2) synthesis, the ultimate step in fever induction, (Ivanov et al., 2002; Ivanov and Romanovsky, 2004; Rummel et al., 2006) by the transcriptional induction of cyclooxigenase-2 (COX-2) and microsomal PGE synthase-1 (mPGES-1), the rate limiting enzymes of this pathway (Ivanov and Romanovsky, 2004; Lacroix and Rivest, 1998; Matsumura and Kobayashi, 2004). Disruption of this tightly controlled process could result in an abnormal fever response, such as that observed during late pregnancy where an attenuated response to exogenous pathogens has been documented in several mammalian species. In these studies experimental animals including mice, rats, sheep and guinea pigs (Kasting et al., 1978; Martin et al., 1995), exhibited a reduction in the fever response to infusion of either the viral mimic polyinosinic: polycytidylic acid [poly I:C (Cooper et al., 1988)], endotoxin lipopolysaccharide (LPS) (Kasting et al., 1978; Martin et al., 1995; Zeisberger et al., 1981) or endogenous pyrogens such as IL-1β (Simrose and Fewell, 1995) and PGEs (Eliason and Fewell, 1997; Stobie-Hayes and Fewell, 1996). Several studies have linked this to a reduction in central PGE_2 synthesis (Imai-Matsumura et al., 2002) probably as a result of blunted hypothalamic COX-2 induction (Imai-Matsumura et al., 2002; Mouihate et al., 2002). Recently, the involvement of central nitric oxide synthase suppressing LPS-induced fever at late pregnancy has been suggested (Begg et al., 2007). What remains uncertain is the nature of the peripheral signals responsible for the reduced COX-2 expression and ultimately,

the fever response. We have recently reported (Ashdown et al., 2006b) that higher than normal circulating levels of IL-1ra in LPS-treated pregnant rats at the later stages of pregnancy, could be partly responsible for these effects. In support of these findings we further demonstrated a causal link between increased levels of circulating IL-1ra and hypothalamic COX-2 induction. Unlike IL-1ra however, no consistent differences were reported on the ability of animals at late stages of pregnancy to synthesize pro-inflammatory/pyrogenic cytokines in response to LPS. In these animals the LPS-induced production of IL-1β and IL-6 were reported to be reduced when compared to virgin females (Fofie et al., 2005), but not when compared to earlier stages of pregnancy or in lactating dams, whose febrile response is higher in magnitude than dams in late pregnancy (Mouihate et al., 2005a).

It has been also suggested that near-term pregnancy suppression of fever may stem from the inability to mount an appropriate/full response to circulating pro-inflammatory cytokines (Chen et al., 1999). This, however, has not been supported by the finding that intracellular signalling pathways triggered by LPS/IL-1β or LPS-induced IL-6 to induce COX-2 remained intact with no differences reported between pregnant dams at early and late stages of pregnancy (Harre et al., 2006; Mouihate et al., 2005a). Particularly surprising about these findings is in regard to IL-6, which we and others have demonstrated to be a critical component of the fever response (Cartmell et al., 2000; Kozak et al., 1997; Kozak et al., 2006; Rummel et al., 2006). The circulating levels of IL-6 increase dramatically following a systemic inflammatory challenge and correlate significantly with the fever response. During late pregnancy the levels of IL-6 appear to remain unchanged when compared to GD 15 controls (Harre et al., 2006). This observation argues against the importance of this cytokine in the regulation of the fever response. One possibility for this anomaly may lie in the type of stimulus being used. For instance the majority of studies investigating this aspect (Cooper et al., 1988; Fofie and Fewell, 2003; Fofie et al., 2005; Harre et al., 2006; Mouihate et al., 2005a), including ours (Ashdown et al., 2006b), have used a generalized systemic challenge namely LPS or poly I:C. These

immunogens tend to act by triggering a robust immune response, which involves the targeting of multiple organs/systems to induce cytokines' release (Cartmell et al., 2001; Givalois et al., 1994; Turrin et al., 2001). Such an overwhelming response may be a factor masking any intricate changes that may occur in the levels of circulating pyrogens such as IL-6. We have previously described a model of localised inflammation, namely the intramuscular (i.m.) injection of turpentine oil (TURP), that induces fever by the activation of febrile effectors in a more defined serial fashion (Leon, 2002; Luheshi et al., 1997). The mode of action of this potent pyrogen involves the production of localised necrotic damage (Wusteman et al., 1990), which results in the sequential induction of TNFα and IL-1β in the site of injury (Luheshi et al., 1997; Zheng et al., 1995). The locally increased cytokines, particularly IL-1β, induce IL-6 synthesis and release into the circulation (Luheshi et al., 1997; Turnbull et al., 2003). A particular advantage of this approach is that it facilitates the study of IL-6 without the influence of other circulating cytokines. Given this property, we sought to characterise the particular role of IL-6 in fever generation in late-pregnant rats [gestational day 18 (GD 18)] by measuring the changes in its levels in the circulation and how they correlate with the fever response, and by investigating changes in brain mechanisms regulating the fever response to this important circulating pyrogen.

Methods

Adult female Sprague-Dawley rats (Charles River, Saint Constant, Quebec, Canada) were used in all experiments; two kinds of animals were employed: randomly cycling non-pregnant (NP) females (250-300 g) and primiparous pregnant females at gestational day 18 (GD 18; 250-340 g). All the individual experimental procedures for this study were approved by the Animal Care Committee of McGill University. Care throughout the duration of the experiment was provided according to the Canadian Council of Animal Care guidelines. Animals were housed individually in a controlled environment at an ambient temperature of 21 ± 2 °C; they had free access to food and water and were handled for at least 7 days prior to experimentation. Observations were carried out in a 12 h:12 h light–dark cycle (lights on from 08:00 to 20:00 h).

In all studies, animals received a single intramuscular (i.m.) injection of 100 μL of purified turpentine oil (TURP) (Riedel-deHaën, Sleeze, Germany) or 100 μL of sterile physiological saline (SAL) into the gastrocnemius muscle of the left hind limb. Animals were killed (see details below) 11 h after either treatment.

Measurement of body temperature

Changes in core body temperature were measured using remote radio-biotelemetry (Data Sciences, St Paul, MN, USA) as previously described (Sachot et al., 2004). Briefly, anaesthetized animals (i.m.; 50 mg kg^{-1} ketamine hydrochloride, 5 mg kg^{-1} xylazine hydrochloride, 0.5 mg kg^{-1} acepromazine maleate; 1 μL [g body weight]$^{-1}$) were implanted intraperitoneally (i.p.) with precalibrated temperature-sensitive radio transmitters (TA10TA-F40, Data Sciences). The level of anaesthesia was assessed by the withdrawal reflex to a toe pinch. Pregnant females were implanted on GD 7 and allowed to recover until GD 18. Transmitter output frequency (Hz) was monitored, at 10 min intervals, by an antenna mounted in a receiver board, situated beneath the cage of each animal. The output data from each transmitter were transformed into °C by Dataquest software (Data Sciences). On the day of the experiment, NP and GD 18 females

were separated into SAL or TURP groups (n=5-7 per group). Injections were administered between 08:00 and 9:00 h and the changes in core body temperatures monitored up to 11 h post-treatment. Body temperature data were analyzed by calculating the area under the temperature-*versus*-time curve (AUC or fever index) for each animal; mean AUC per group was used for statistical analysis.

Plasma cytokine measurements

Plasma concentrations of IL-6, IL-1β and IL-1ra were determined from animals sacrificed at the peak of the febrile response, 11 h after TURP injection, in order to measure the maximum humoral responses to the TURP challenge. Blood was collected from animals deeply anaesthetized with a lethal dose of pentobarbital sodium (i.p.; 60 mg k [g body weight]$^{-1}$) via cardiac puncture (n = 5-7 per group) using sterile heparinised syringes and placed into sterile tubes. Samples were then centrifuged (5300 g, 15 min at 4 °C), aliquoted and stored at – 80 °C until assays were performed. Sandwich enzyme-linked immunosorbent assays (ELISA) for IL-6, IL-1β and IL-1ra (NIBSC, Potters Bar, UK) were performed as previously described (Rees et al., 1999), except that plasma samples and secondary antibodies (NIBSC) were diluted in a buffer containing 0.5 M NaCl, 2.5 mM NaH_2PO_4, 7.5 mM Na_2HPO_4, 0.1 % Tween 20, 1 % ovalbumin and 1:100 normal sheep serum (Sigma-Aldrich, St. Louis, MO, USA). Intra-assay and inter-assay variations were below 10 %. The detection limit for IL-6 and IL-1β was 40 pg mL^{-1}, and 78 pg mL^{-1} for IL-1ra. All samples were assayed in duplicate.

Hypothalamus dissection

Killed animals designated for plasma collection were perfused with sterile physiological saline (prepared in DEPC-treated H_2O); brains were dissected, frozen on dry ice and stored at -80 °C until used. Hypothalami were microdissected from frozen tissue and divided into right and left hemispheric

portions; the left hemisphere was used for RNA extraction and the right for protein extraction.

RNA extraction and RT-PCR in the hypothalamus

In order to asses the changes in transcription of SOCS3, IL-1β, COX-2 and mPGES-1 genes in the hypothalamus, reverse transcription (RT), followed by PCR were performed as previously reported (Rummel et al., 2006). Briefly, total RNA was extracted by disaggregating tissues in 1 mL of TRIzol (Invitrogen, Burlington, ON, Canada). Total RNA was isolated according to the manufacturer's protocol and the air-dried RNA pellet dissolved in 50 μL of DEPC-treated water. One microgram of total RNA was transcribed into DNA. PCR reactions were performed for COX-2, mPGES-1, SOCS3, IL-1β and β-actin using 1.8 μL of cDNA and 6 pmol of specific primers (Alpha DNA, Montreal, QC, Canada) for COX-2 (forward: 5'-TGATAGGAGAGACGATCAAGA-3'; reverse: 5'-ATGGTAGAGGGCTTTCAACT-3'), mPGES-1 (forward: 5'-TTTCTGCTCTGCAGCACACT-3''; reverse: 5'-CATGGAGAAACAGGTGAACT-3'), SOCS3 (forward: 5'-CCAGCGCCACTTCTTCAC-3'; reverse: 5'-GTGGAGCATCATACTGGTCC-3'), IL-1β (forward: 5'-CCCAAGCACCTTCTTTTCCTTCATCTT-3', reverse: 5'-CAGGGTGGGTGTGCCGTCTTTC-3') and β-actin (forward: 5'-GCCGTCTTCCCCTCCATCGTG-3'; reverse: 5'-TACGACCAGAGGCATACAGGGACAAC-3') using a Gene Amp PCR system 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA). The following parameters were used: (1) 5 min at 94 °C (all primer pairs); (2) 30 s at 94 °C, 30 s at 60 °C (for SOCS3, IL-1β and β-actin) or 57 °C (for mPGES-1, COX-2), followed by 45 s at 72 °C for 20, 28, 34, 36 or 40 cycles (for β-actin, COX-2, mPGES-1, IL-1β and SOCS3 respectively); and (3) 72 °C for 10 min (for all primer pairs). PCR products were separated by gel electrophoresis (1.5 % agarose) and band densities were obtained using GeneTool image analysis software (Syngen, Frederick, MD, USA). Each cDNA data were expressed as a ratio of β-actin optical density and then relative to measurements obtained from

the SAL group of NP females [(gene X/β-actin mRNA)/(mean of gene X/β-actin from SAL-NP group)] in order to pool data from different gels. Initial PCR experiments using total RNA were performed for each pair of primers to ensure that products did not result from genomic DNA amplification. Additionally, the linear phase of PCR amplification of cDNA was determined by performing RT-PCR on a sample from each treatment group for an increasing number of cycles (20-50 cycles).

Protein extraction and hypothalamic COX-2 Western blot analysis

The right side of the hypothalamus was disaggregated in lysis buffer (50 mM Tris-HCl, 2 mM EDTA and 1 % Nonidet) that included a protease inhibitor cocktail for general use (Sigma-Aldrich). Protein content was quantified using Bradford's reagent (Sigma-Aldrich) following manufacturer's instructions, aliquoted and frozen at -80 °C until used.

On the day of the analytical procedure the protein was mixed (1:1) with Laemmli running buffer (Bio-Rad Laboratories, Mississauga, ON, Canada) mixed with β-mercaptoethanol (50 μL of β-mercaptoethanol per mL of Laemmli buffer; Sigma-Aldrich) and incubated at 95 °C for 5 min. This (50 μg) was then loaded into pre-cast acrylamide gels (4-20 % Tris-glycine gel, Invitrogen) and electrophoresed for 2 hours at 125 V. The protein was then transferred (overnight at 15 V) to nitrocellulose membranes (Hybond ECL, Amersham Biosciences Corp, Piscataway, NJ, USA) using XCell II Blot Module (Invitrogen), following the manufacturer's instructions for liquid transference.

For immunodetection, membranes were blocked with 1 X Tris-Buffer-Saline (TBS), 10 % non-fat dry milk (Bio-Rad Laboratories) and 0.1 % Tween 20 for 2 hours at room temperature. Membranes were then incubated overnight (at 4 °C) with a mixture of antibodies raised against murine COX-2 (1:1000; Cayman Chemical, Hornby, ON, Canada) and actin (1:10 000, Sigma-Aldrich) diluted in 1 X TBS, 5 % milk and 0.1 % Tween 20. After, antibody excess was washed (1 X for 15 min and 4 X 5 min) with 1 X TBS, 0.1 % Tween 20, membranes were incubated with a detection antibody (1:2000; donkey anti-rabbit IgG-HRP, Santa

Cruz Biotechnology, Santa Cruz, CA, USA; diluted in 1 X TBS, 1 % milk and 0.1 % Tween 20) for 1 hour at ambient temperature. After a similar series of washing steps, the membranes were incubated with ECL western blotting detection reagents (0.125 mL cm^{-2}; Amersham Biosciences Corp) for 1 minute and then exposed to a chemoluminescence sensitive film (Hyperfilm ECL, Invitrogen) for 1-5 minutes. Films were digitized and optical density was determined using GeneTool image analysis software (Syngen, Frederick, MD, USA). Two bands were detected, one at approximately 42 kDa corresponding to actin and another at ~72 kDa corresponding to COX-2. Levels of COX-2 expression were expressed as a ratio of the β-actin signal and then, as for PCR data, as relative amounts of the measurements obtained for the SAL-NP females in order to pool data from different membranes.

Data analysis

All data are presented as mean values ± S.E.M. and were analysed using StatView software (version 4.57, Abacus Concepts Inc, Berkeley, CA, USA). One-way ANOVA was used to analyse the data from the temperature, ELISA, PCR and Western blot studies. In cases where comparisons using ANOVA were significant, Newman-Keuls multiple comparisons test was performed. Correlations between parameters were analysed and expressed as fitness to linear curve (r^2); statistical significance was determined by ANOVA. In all cases, *P* values less than 0.05 were deemed statistically significant.

Results

TURP-induced fever is abolished in late pregnancy

Basal core body temperature (T_c) was generally lower in pregnant animals compared to NP females (Fig. II-1). This finding is consistent with previous reports that show reduced light-phase basal T_c during late pregnancy (Fewell et al., 2002; Fofie and Fewell, 2003). Similar to previous reports (Cooper and Rothwell, 1991; Luheshi et al., 1997), i.m. injection of 100 μL of TURP into the left rear-limb of NP females (n=7), induced a significant increase in T_c that started 2.5 hours after injection and peaked between 10-11 h post-injection (~39.5 °C), with a maximum increase in temperature of 2.4 °C (Fig. II-1). Fever persisted during the light phase of the second day but was no longer evident during that day's dark phase (data not shown). In contrast, and somewhat surprising given the potency of the TURP stimulus, rats at a late pregnancy stage (GD 18) exhibited no change in body temperature, which did not deviate from basal over the time course of the study (n=6; Fig. II-1). As would be expected given the lack of response in GD 18 animals, the comparison of the fever index showed that NP females injected with TURP had significantly higher fever index than NP-SAL (n=5) and GD 18-TURP groups (one-way ANOVA $F_{(3,22)}=8.72$, $P=0.0008$; Newman-Keuls test *versus* SAL- injected NP females: $P<0.001$ and *versus* TURP-injected GD 18 females: $P<0.01$; inset Fig. II-1), while that of GD 18-TURP did not differ from the one of GD 18-SAL (n=5; Newman-Keuls test: $P>0.05$). Interestingly, and despite the absence of the fever response in the pregnant females, TURP-induced oedema of the hind-limb (a hallmark sign of localized inflammation) was present in GD 18 animals, and was similar in severity to that exhibited by the counterpart cycling females. None of these animals showed signs of extreme discomfort that necessitated the premature termination of the study.

Circulating levels of cytokines: dampened IL-6 induction is associated with the absence of fever response

At the peak of the fever response, 11 h after TURP injection, NP females showed a 5.4-fold increase in the concentration of IL-6, from 27.4 ± 9.2 pg mL^{-1} in the SAL group (n=5) up to 146 ± 29 pg mL^{-1} in the TURP-injected animals (n=7; one way ANOVA: $F_{(3,19)}$=6.42, P<0.01; Newman-Keuls test *versus* SAL-injected NP females: P=0.0012; Fig. II-2A). IL-6 levels in the SAL-injected GD 18 group (n=5; 38.6 ± 2.2 pg mL^{-1}) did not differ from those of the SAL-injected NP females (Newman-Keuls test: P=0.74). Late-pregnant rats did not show a significant induction of IL-6 after TURP injection (n=6; 60.5 ± 21.9 pg mL^{-1}; Newman-Keuls test *versus* SAL-injected GD 18 dams: P=0.5), and compared to TURP-injected NP-females, these levels were significantly lower (Newman-Keuls test: P=0.0094; Fig. II-2A).

NP-females showed a significant correlation between plasma IL-6 concentration and T_c (r^2=0.74, P=0.0003; top panel Fig. II-2B) which is consistent with the proposed role of this cytokine in driving the fever response after localised infection or inflammation (Cartmell et al., 2000; Rummel et al., 2006). In contrast, no such correlation was observed in the GD 18 pregnant dams (bottom panel Fig. II-2B) in concordance with their suppressed febrile response.

Circulating IL-1β and IL-1ra levels were also assayed. IL-1β levels were found to be below detection limits of the assay in all animals tested (NP and GD 18 females; data not shown). TURP-injected groups had a similar rise in IL-1ra concentrations (one way ANOVA: $F_{(3,17)}$=9.86, P=0.0005; Newman-Keuls test: P>0.05), from 406 ± 49 pg mL^{-1} and 500± 55 pg mL^{-1} under the SAL treatment up to 3151 ± 668 pg mL^{-1} and 2067 ± 386 pg mL^{-1} 11 h after TURP injection in NP and GD 18 animals respectively (Newman-Keuls test SAL- *versus* TURP-injected NP females: P<0.01; and SAL- *versus* TURP-injected GD 18 dams: P<0.05; Fig. II-2C).

Changes in circulating IL-6 levels correlate with hypothalamic SOCS3 mRNA expression

To further confirm that circulating IL-6 was acting on the hypothalamus, SOCS3 mRNA was measured in the same animals used for cytokine determination. SOCS3, which is induced by the IL-6-activated intracellular signalling pathway (Lebel et al., 2000b), is part of a negative feedback mechanism that limits the pro-inflammatory actions of this cytokine (Croker et al., 2003).

In NP females, 11 h after TURP injection, SOCS3 mRNA was induced ~1.8 fold compared to SAL-injected females (one way ANOVA: $F_{(3,19)}$=8.79, p=0.0007; Newman-Keuls test *versus* SAL-injected NP group: P<0.001; Fig. II-3A); these changes correlated significantly with circulating IL-6 levels and T_c (r^2=0.53, P=0.0074 for IL-6; r^2=0.55, P=0.0059 for T_c; top panel of Figs. II-3B and C). In contrast, pregnant females did not show any significant increase in SOCS3 mRNA (Newman-Keuls test *versus* SAL-injected GD 18 dams: P>0.05; Fig. II-3A) nor a correlation between this mRNA and T_c or IL-6 levels (bottom panels Figs. II-3B and C). Similar to plasma IL-6, SOCS3 mRNA levels in TURP-injected GD 18 dams were significantly lower compared to those of TURP-injected NP-females (Newman-Keuls test: P=0.019; Fig. II-3A).

Fever pathways in the hypothalamus: evidence of blunted prostaglandin synthesis and reduced induction of IL-1β

Expression of COX-2 was determined by RT-PCR; 11 h after TURP injection hypothalamic COX-2 mRNA was significantly induced (1.9 fold) in NP-females compared to SAL-injected ones (one way ANOVA: $F_{(3,19)}$=11.39, P=0.0002; Newman-Keuls test: P<0.001; Fig. II-4A). In GD 18 females TURP injection also caused a significant induction of COX-2 mRNA (1.4 fold, Newman-Keuls test *versus* SAL-injected GD 18 dams: P<0.05), although this was significantly lower compared to the induction in NP females (Newman-Keuls test: P<0.05; Fig. II-4A).

In order to confirm the RT-PCR data, COX-2 expression was assayed by Western Blot. NP females had 3-fold more COX-2 levels after TURP injection

(one way ANOVA: $F_{(3,18)}$=4.6, *P*=0.015; Newman-Keuls test *versus* SAL-injected NP group: *P*<0.01; Fig. II-4B). In contrast, pregnant females injected with TURP did not show such an increase compared to their own control group (Newman-Keuls test: *P*>0.05; Fig. II-4B). Although induced levels of COX-2 were greater in NP-females than in GD 18 dams (3.0 ± 0.77 a.b. *versus* 2.1 ± 0.29 a.b. respectively) this difference was not statistically significant (Newman-Keuls test: *P*=0.15).

Similar to the changes in COX-2, mPGES-1 mRNA levels were significantly up-regulated in NP (2.15 fold; one way ANOVA: $F_{(3,19)}$=11.8, *P*=0.0001; Newman-Keuls test *versus* SAL-NP females: *P*<0.0001) and GD 18 females (1.7 fold; Newman-Keuls test *versus* SAL-GD 18 dams: *P*<0.05; Fig. II-5). mPGES-1 mRNA levels differed significantly between the two TURP-injected groups (Newman-Keuls test: *P*<0.05; Fig. II-5).

In NP females, expression levels of COX-2 (measured as mRNA or as protein) and mPGES-1 correlated significantly with circulating IL-6 levels (Table II-1). SOCS3 mRNA levels correlated with the expression levels of both enzymes (Table II-1). In late-pregnant rats no correlation was found between IL-6 and COX-2 or mPGES-1 (Table II-1). However, similar to cycling females, SOCS3 mRNA levels correlated with those of COX-2 and mPGES-1 (Table II-1).

Although IL-1β was not detected in the circulation, it has been proposed that centrally it plays a significant role in TURP-induced fever (Luheshi et al., 1997). Therefore we decided to measure the expression of its mRNA levels within the hypothalamus. In TURP-injected NP-females IL-1β mRNA was significantly induced (2.4 fold, one way ANOVA: $F_{(3,19)}$=13.26, *P*=0.0001; Newman-Keuls test *versus* SAL-injected NP group: *P*<0.0001; Fig. II-6A) and correlated with T_c (r^2=0.77, *P*=0.0002; top panel Fig. II-6C), IL-6 concentration in plasma (r^2=0.45, *P*=0.017; top panel Fig. II-6B) and SOCS3 mRNA (r^2=0.84, *P*<0.0001; figure not shown). Similarly, GD 18 females showed a significant induction of hypothalamic IL-1β (1.7 fold, Newman-Keuls test *versus* SAL-injected GD 18 females: *P*<0.05), although these values were significantly lower than those in TURP-injected NP-females (Newman-Keuls test: *P*<0.05; Fig. II-6A) and did not

correlated with T_c (r^2=0.16, *P*=0.22; bottom panel Fig. II-6C) or with SOCS3 mRNA (r^2=0.18, *P*=0.19; figure not shown). However, hypothalamic IL-1β mRNA showed a low but significant correlation with circulating IL-6 levels (r^2=0.34, *P*=0.049; bottom panel Fig. II-4B). T_c and IL-1β mRNA only correlated with expression levels of COX-2 and mPGE-1 in NP females, and not in GD 18 dams (Table II-1).

Discussion

In the present study we demonstrated that a TURP challenge, which would normally induce a highly significant and prolonged fever response in non-pregnant rats, was almost totally ineffective during late pregnancy (Fig. II-1). The absence of this response appeared to be the result of an attenuated IL-6 release into the circulation (Fig. II-2A), reflected in a diminished hypothalamic SOCS3 mRNA induction (Fig. II-3A) and a reduction in COX-2 and mPGES-1 synthesis in this brain structure (Figs. II-4 and II-5). A functional link between fever, peripheral IL-6 and hypothalamic expression of SOCS3, COX-2, mPGES-1, was confirmed in the cycling females treated with TURP by the induction of these markers and more crucially by the significant correlation between them and the fever response (Figs. II-2B, II-3B, II-3C and Table II-1). Importantly, this relationship was absent in the GD 18 females, where these molecules were scantily induced by TURP. The levels of the anti-inflammatory cytokine IL-1ra were comparable in both NP and GD 18 females injected with TURP (Fig. II-2C). This contrasts with our earlier observations made in pregnant females injected with LPS (Ashdown et al., 2006b) where it was evident that rather than IL-6, the main factor regulating the attenuated fever response to LPS was in fact a higher than normal production of IL-1ra in the pregnant females. To understand this discrepancy the differences between systemic challenges like i.p. LPS injection and localised activation of the immune system, as occurs with TURP, must be considered. LPS could target directly several tissues that produce IL-1ra (Turrin et al., 2001), such as white adipose tissue (WAT), the amount of which increases during pregnancy (Dayer et al., 2006), most likely resulting in the over-production of this cytokine during late pregnancy. In contrast, TURP effects are confined to the local site of inflammation thus diminishing the possibility of stimulating fat deposits which are more accessible to a circulating stimulus.

Our current observations demonstrating drastically reduced levels of IL-6 (and consequently hypothalamic SOCS3 mRNA) and COX-2 after TURP injection during late pregnancy, provide a possible mechanism underlying the

reported reduction in PGE_2 levels in the brain, the ultimate step in fever generation (Fewell et al., 2002; Imai-Matsumura et al., 2002; Mouihate et al., 2002). These findings are similar to those reported previously in IL-6 "knock out" mice which were shown to be resistant to TURP-induced fever (Kozak et al., 1997), and exhibit significantly attenuated COX-2 induction (Turnbull et al., 2003). These results coupled with ones made in the present study strongly support our recent observations demonstrating a direct link between circulating IL-6 and brain COX-2 expression (Rummel et al., 2006). In those experiments we clearly demonstrated that IL-6 injection induces COX-2 expression in endothelial cells lining the microvasculature of the brain (Rummel et al., 2006) and that neutralisation of LPS-induced circulating IL-6 almost totally abolishes the expression of this enzyme in the same structures.

In addition to the changes in the levels of COX-2 in the current study, we have observed a significant reduction in the levels of mPGES-1 expression after TURP in late pregnant animals. This enzyme is involved in the last step of PGE_2 synthesis, downstream of COX-2, and is fundamental in TURP-induced fever (Saha et al., 2005). In the central nervous system, regulation of mPGES-1 expression has been shown to be linked with that of COX-2 after LPS challenge (Ivanov et al., 2002; Ivanov and Romanovsky, 2004; Yamagata et al., 2001). Our findings support the hypothesis of overlapping mechanisms of transcriptional regulation for both enzymes (Ivanov et al., 2002; Ivanov and Romanovsky, 2004; Yamagata et al., 2001), which is likely controlled by IL-6 in the case of TURP-induced inflammation.

Traditionally, a central role in the regulation of COX-2 and mPGES-1 expression has been ascribed to IL-1β and other molecules classically involved in NF-□B activation (Kojima et al., 2004; Laflamme et al., 1999; Nadjar et al., 2005; Sooranna et al., 2006). The observations made in the current study do not negate this hypothesis since we have demonstrated that hypothalamic IL-1β is induced in both groups of TURP treated animals but are reduced significantly in pregnant females 11 h after TURP injection (Fig. II-6). However, at earlier time points, 4 and 6 h after TURP injection, when fever and increased COX-2 and

mPGES-1 expression were already evident (data not shown), hypothalamic IL-1β mRNA was not induced, while circulating IL-6 and hypothalamic SOCS3 mRNA levels were already significantly up-regulated (data not shown). At the later time point tested (11 h), however, there was a high degree of correlation between COX-2 or mPGES-1 expression levels and those of IL-1β mRNA in the hypothalamus (Table II-1) similar to the one found for IL-6 or SOCS3 mRNA (Table II-1). Given the established role of IL-1β in COX-2 and mPGES-1 regulation, it is tempting to suggest that its central induction may contribute to regulation of PGE_2-synthesising enzymes expression in a sustained febrile response as occurs after TURP injection. The anomaly of this hypothesis is that there is no data suggesting a direct link between peripheral IL-6 and brain IL-1β, and our preliminary observations (data not shown) do not support the existence of such an association.

The observation that pregnant animals responded to the TURP challenge with a significant induction of both hypothalamic COX-2 and mPGES-1 mRNAs (Figs. II-5A and II-6), although this was not reflected in changes in core temperature (Fig. II-1) is paradoxical. There are two possible explanations for this finding: i) The existence of an additional regulatory step that uncouples transcription from translation of COX-2, by which COX-2 protein levels are not increased even with augmented transcription of the COX-2 gene; accordingly our results showed that hypothalamic COX-2 protein levels were not significantly elevated in late-pregnant dams (Fig. II-5B). ii) The sensitivity to the pyrogenic effect of central PGE_2 could be attenuated in near-tem pregnant rats. This, in fact, has been previously demonstrated by infusing PGE_2 directly into the lateral ventricles of near-tem pregnant rats, which resulted in an attenuated pyrogenic effect (Chen et al., 1999; Eliason and Fewell, 1998, 1999). The mechanisms underlying the attenuated febrile response to PGE_2 are not completely clear, although they may involve alteration in PGE receptor expression (Mouihate et al., 2002), changes in brain prostaglandin clearance and/or catabolism (Ivanov and Romanovsky, 2003), or increased expression of the antipyretic arginine-vasopressin (Chen et al., 1999; Eliason and Fewell, 1998, 1999).

In previous reports, the inability to find altered molecular fever pathways in the central nervous system after LPS during late pregnancy, upstream of PGE_2 synthesizing enzymes (Harre et al., 2006; Mouihate et al., 2005a), could stem from the nature of the given stimulus. As mentioned earlier, LPS is capable of stimulating several organs to produce fever mediators that act in a highly redundant fashion, as is further evidenced by the lack of effect of targeted mutations ("knock out") of cytokines on LPS-febrile response (Kopf et al., 1994; Kozak et al., 1997; Leon, 2002; Zheng et al., 1995). Several of these mediators could activate similar intracellular signalling pathways, responsible for COX-2 induction, which could have masked our ability to detect alterations in such pathways. In contrast, in TURP-induced fever, the respective situation is such that elimination of any of the identified components in the cytokine pathway (TNFα, IL-1β and IL-6) abolishes this response (Kozak et al., 1997; Leon, 2002; Luheshi et al., 1997; Zheng et al., 1995) and this is readily reflected in the activation state of downstream mediators, as we report in the present study. Additionally, the use of different controls groups (NP females in the current study and GD 15 and lactating females in Mouihate *et al*., 2005 and Harre *et al*., 2006) could explain some of these inconsistencies.

The mechanisms underlying the blunted IL-6 production during pregnancy were not directly addressed in our study; however, several interesting possibilities arise. TURP-induced fever depends largely on IL-6 induction and release into the circulation, as a direct result of localised release of other pyrogenic cytokines such as TNFα and IL-1β (Luheshi et al., 1997; Turnbull et al., 2003). Our previous demonstration that IL-1ra induction is enhanced after LPS injection in late-pregnant rats (Ashdown et al., 2006b), does not extend to all inflammation models such as the one used in the current study; however, it is still feasible that IL-1ra induction in the local site of inflammation (where subcutaneous adipose tissue may be activated) may be enhanced, thus indirectly preventing IL-6 synthesis by inhibiting the action of IL-1β at the level of the necrotic tissue. Other plausible candidates for the reduced IL-6 production during near-term pregnancy are progesterone and oestrogen, that act as generalised modulators of both, innate and

adaptive immunity (Beagley and Gockel, 2003; Doria et al., 2006). Oestrogen levels rise towards the end of pregnancy (from GD 17 onwards), while progesterone levels, that are maintained at a very high level throughout most of the duration of pregnancy, diminish around GD 19 (Mann and Bridges, 2001). Oestrogen has been shown to decrease the inflammatory effects of carrageenan (Cuzzocrea et al., 2001), IL-1β (Ospina et al., 2004) and LPS (Mouihate and Pittman, 2003; Vegeto et al., 2001). Its anti-inflammatory effect seems to stem from its ability to arrest NF-κB-mediated transcription (Ghisletti et al., 2005) of genomic targets that include IL-6 (Galien and Garcia, 1997) and COX-2 genes (Mouihate and Pittman, 2003; Ospina et al., 2004). This could account for the abolished effects of TURP in dams at GD 18 described in our study. Mouihate & Pittman (2003) showed that oestrogen and progesterone replacement reduced circulating IL-6 induction by IL-1β, but not after LPS injection, although fever and COX-2 expression were modulated in the opposite direction. However, the effect of different doses of oestrogen, in the presence of low levels of progesterone, has not been determined.

The physiological significance of reduced fever or immune activation during pregnancy is generally regarded as a protective mechanism against the detrimental effects of an abnormal rise in core body temperature or cytokines on foetal development. This is based on a growing body of evidence suggesting that immune activation during mid- or late-pregnancy is a risk factor strongly associated with increased incidence of psychiatric disorders such as cerebral palsy, autism and schizophrenia (Ashdown et al., 2006a; Boksa, 2004; Fortier et al., 2004; Meyer et al., 2005; Meyer et al., 2006a). Accordingly, prenatal exposure to IL-6 was shown to induce abnormalities in hippocampal structure, deficits in spatial working memory, hypertension and alterations in stress response in the adult offspring (Samuelsson et al., 2006b; Samuelsson et al., 2004). Based on these observations and the results from the current study, it would appear that this cytokine, which has been shown to cross the blood/placental barrier (Dahlgren et al., 2006), plays a significant role in the reported neurodevelopmental defects

associated with maternal infection, stress or injury during gestation and could provide a promising pharmacological target to help meliorate these effects.

Acknowledgements

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Figures and Tables

Figure II-1. TURP-induced fever is attenuated in late pregnancy

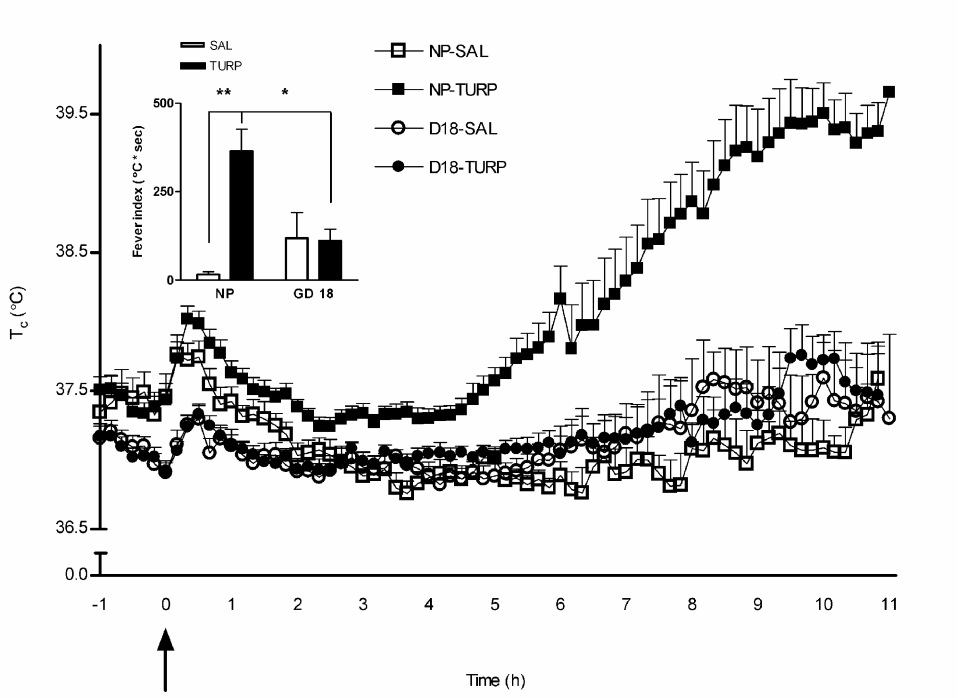

*Figure II-1. Injection of TURP (100 µL; i.m.) induced a significant rise in core body temperature in NP-females (Tc, °C). Animals in the 18th day of pregnancy (GD 18) were completely resistant to the febrile effects of TURP. The arrow indicates the time of injection (animals were injected between 8:00 and 9:00 h). Insert, Fever indexes (or area under the curve, AUC) were calculated for each group. AUC in the NP females injected with TURP was significantly greater than both SAL-injected NP females and TURP-injected GD 18 dams. NP females versus SAL-injected NP females: ** P<0.01 and versus TURP-injected GD 18 females: * P<0.05.*

Figure II-2. Circulating IL-6 and IL-1ra levels after TURP injection are differentially modulated in late pregnant dams

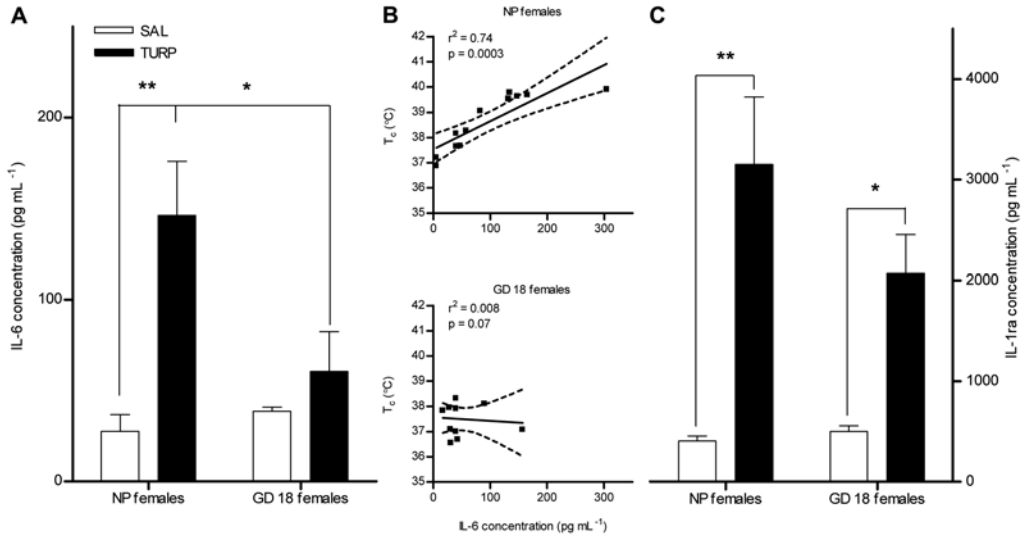

*Figure II-2. (A) 11 h after the injection of TURP (100 μL; i.m.), IL-6 concentration was significantly increased in NP-females; this increase was not evident in late pregnant dams (GD 18), whose levels did not differed from those found in the corresponding SAL-injected controls. TURP-injected NP females versus SAL-injected NP females: ** P<0.01 and versus TURP-injected GD 18 females: * P<0.05. (B) Top panel, correlation analysis between plasma IL-6 levels (X axis) and temperature 11 h after injection (Y axis) from NP-females revealed a significant relationship between these parameters (n=12 including SAL and TURP injected animals; r^2=0.74, P=0.0003). Bottom panel, the same analysis showed that in GD 18 dams this correlation was not evident (n=11; r^2=0.08, P=0.07). (C) IL-1ra concentration was significantly increased in both NP and GD 18 females after TURP injection; there was no significant difference in the induced levels of the anti-inflammatory cytokine between these two groups. TURP-injected versus SAL-injected NP females: ** P<0.01; TURP-injected versus SAL-injected GD 18 females: * P<0.05.*

Figure II-3. Hypothalamic SOCS3 mRNA, a marker of IL-6 activity, is not induced after TURP challenge during late pregnancy

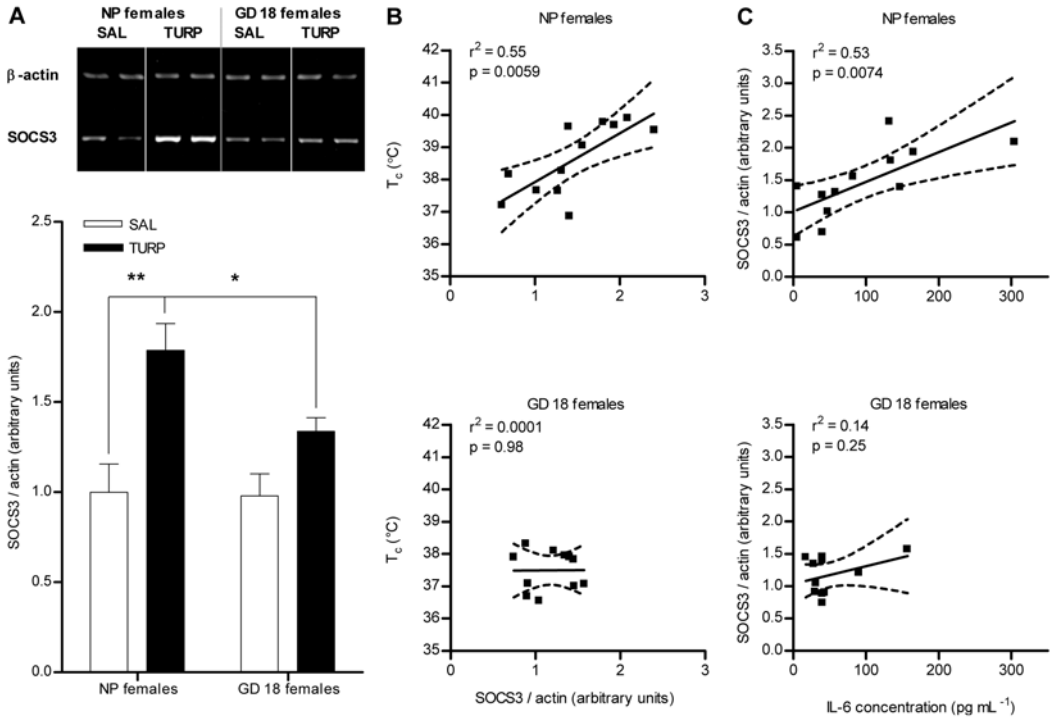

*Figure II-3. (A) In NP-females, SOCS3 mRNA expression in the hypothalamus was significantly induced 11 h after TURP injection (100 µL; i.m.), in contrast this increase was blunted in GD 18 dams. TURP-injected NP females versus SAL-injected NP females: ** P<0.01 and versus TURP-injected GD 18 females: * P<0.05. Each band in the gel represents one animal; two animals per group are shown. (B) Top panel, correlation analysis between hypothalamic SOCS3 mRNA levels (X axis) and temperature 11 h after injection (Y axis) from NP-females revealed a significant relationship between these parameters (n = 12 including SAL and TURP injected animals; r^2=0.55, P=0.0059). Bottom panel, the same analysis showed that in GD 18 dams this correlation was not evident (n=11; r^2=0.0001, P=0.89). (C) Top panel, in NP-females correlation analysis as in B for plasma IL-6 levels (X axis) and hypothalamic SOCS3 mRNA levels (Y axis) revealed a supports the significant link between these parameters (n=12 including SAL and TURP injected animals; r^2=0.53, P=0.0074). Bottom panel, in contrast in GD 18 dams this correlation (IL-6 versus hypothalamic SOCS3 mRNA) was not evident (n=11; r^2=0.14, P=0.25).*

Figure II-4. COX-2 expression, at the level of mRNA and protein, is differentially regulated in NP- and GD 18 females after TURP injection

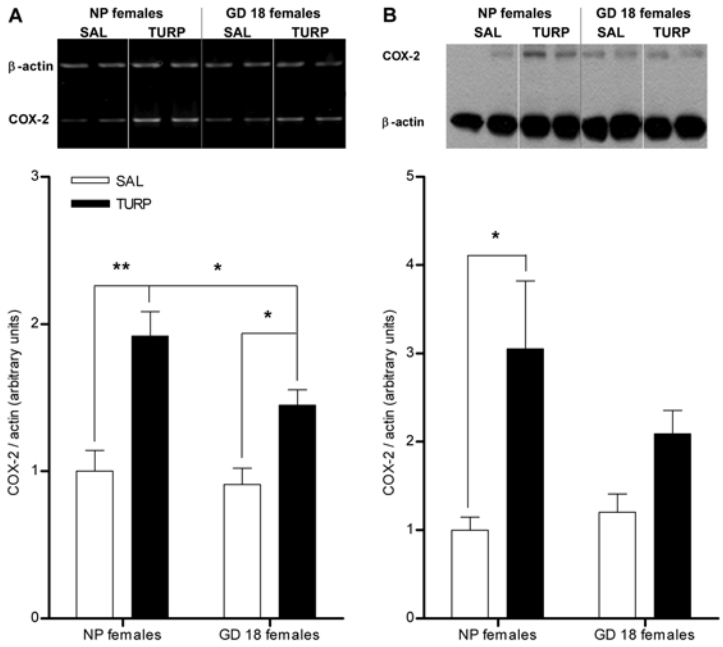

*Figure II-4. (A) TURP injection (100 μL; i.m.) induced a significant increase of COX-2 mRNA expression in the hypothalamus; this induction was reduced, but still present, in GD 18 dams. TURP-injected NP females versus SAL-injected NP females: ** P<0.01; TURP-injected GD 18 females versus SAL-injected GD 18 females and versus TURP-injected NP females: * P<0.05. Each band in the gel represents one animal; two animals per group are being shown. (B) COX-2 protein levels (measured by Western Blot) in the hypothalamus of NP females were increased 11 h after TURP injection (100 μL; i.m.); this increase was not present in GD 18 dams. TURP-injected NP females versus SAL-injected NP females: * P<0.05. Each band in the gel represents one animal; two animals per group are shown.*

Figure II-5. Attenuated mPGES-1 mRNA expression in the hypothalamus of GD 18 females

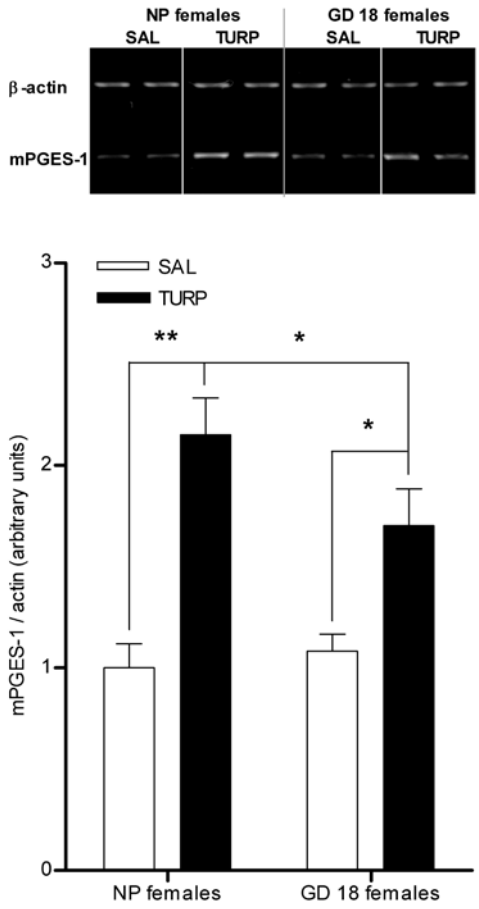

*Figure II-5. RT-PCR analysis of mPGES-1 mRNA levels in the hypothalamus revealed a significant induction in NP-females after TURP injection (100 μL; i.m.), in comparison, levels of this mRNA were increased to a lesser extent in GD 18 dams. TURP-injected NP females versus SAL-injected NP females: ** P<0.01; TURP-injected GD 18 females versus SAL-injected GD 18 females and versus TURP-injected NP females: * P<0.05. Each band in the gel represents one animal; two animals per group are being shown.*

Figure II-6. Hypothalamic IL-1β mRNA induction after TURP is attenuated GD 18-females

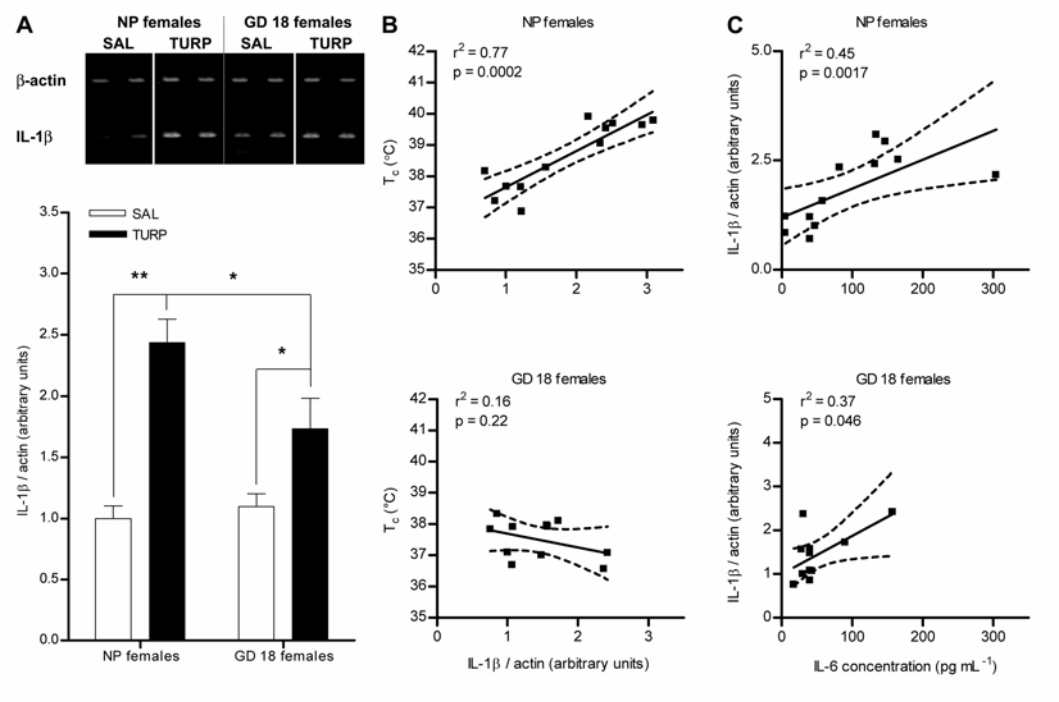

*Figure II-6. (A) Similar to other central markers of inflammation, IL-1β mRNA expression increased in the hypothalamus of NP-females, 11 h after TURP injection (100 μL; i.m.). In TURP-injected GD 18 dams the levels of this mRNA were lower, but still significantly induced. TURP-injected NP females versus SAL-injected NP females: ** P<0.01; TURP-injected GD 18 females versus SAL-injected GD 18 females and versus TURP-injected NP females: * P<0.05. Each band in the gel represents one animal; two animals per group are being shown. (B) Top panel, hypothalamic IL-1β mRNA levels (X axis) correlated significantly with temperature 11 h after TURP injection (Y axis) in NP-females (n=12 including SAL and TURP injected animals; r^2=0.77, P=0.0002). Bottom panel, the same analysis showed that no such correlation existed in GD 18 dams (n=11; r^2=0.16, P=0.22). (C) Top panel, as in B, in NP-females hypothalamic IL-1β mRNA levels (Y axis) showed a low, but significant correlation with plasma IL-6 levels (X axis) (n=12 including SAL and TURP injected animals; r^2=0.45, P=0.017). Bottom panel, in GD 18 dams this correlation was still present (n=11;*

r^2=0.46, P=0.046). The expression of microsomal PGE synthase-1 is attenuated in GD 18 dams after TURP injection.

Table II-1. Correlation of hypothalamic COX-2 and mPGES-1 expression levels with Tc, plasma IL-6, hypothalamic IL-1β and SOCS3 mRNAs

	Core temperature (T_c)		Plasma IL-6		Hypothalamic IL-1β mRNA		Hypothalamic SOCS3 mRNA	
	NP	**D18**	**NP**	**D18**	**NP**	**D18**	**NP**	**D18**
COX-2 mRNA	r^2 = 0.68**	r^2 = 0.01	r^2 = 0.47**	r^2 = 0.09	r^2 = 0.79***	r^2 = 0.09	r^2 = 0.70**	r^2 = 0.72**
COX-2 protein	r^2 = 0.39*	r^2 = 0.02	r^2 = 0.33*	r^2 = 0.12	r^2 = 0.26	r^2 = 0.26	r^2 = 0.70**	r^2 = 0.33
mPGES-1 mRNA	r^2 = 0.69**	r^2 = 0.005	r^2 = 0.5 **	r^2 = 0.05	r^2 = 0.84***	r^2 = 0.07	r^2 = 0.64**	r^2 = 0.41*

*r^2 denotes goodness of fit to a linear curve and significance was evaluated by ANOVA. n = 12 for NP group and 11 for D18 group. * = p < 0.05; ** = p < 0.01; *** = p < 0.0001.*

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II.3. Supplementary results and discussion

Circulating IL-6 and IL-1ra at the time of fever induction by TURP

The present study clearly demonstrated that during late gestation (GD 18), induction of IL-6 by TURP was blunted, at the peak of the inflammatory response, and that the expression of downstream signalling molecules (SOCS3, IL-1β) and enzymes involved in PGE_2 synthesis (COX-2 and mPGES1) was also attenuated, suggesting that diminished IL-6 underlies the suppressed/attenuated febrile response to TURP in late pregnancy. Importantly, we observed equivalent induction of IL-1ra following TURP injections to NP-females and GD 18 dams, which suggests that the role of this cytokine is less prominent in the attenuation of fever in this model of inflammation, compared to LPS, where induction of IL-1ra is greater in late gestation (Ashdown et al., 2007). We observed in addition that the febrile response to TURP at GD 18 was suppressed throughout the time course of the study (Fig. II-1). Therefore, we sought to determine whether at earlier phases of the febrile response, more specifically during induction, the mechanisms of fever attenuation were similar to those found later on, at the peak of the response. For this purpose, we analysed circulating cytokine levels 6 h after TURP injection, which is the time point when it is possible to detect IL-6 in the serum of NP-females for the first time (data not shown). Circulating IL-6 levels were significantly induced at this time point by TURP in NP-females (n=5/group; one way ANOVA: $F_{(3,19)}$=3.64, P=0.032; Newman-Keuls test *versus* SAL-NP females: P<0.05; Fig. II-7A), in contrast and similar to what we observed at the 11 h time point, this effect was completely absent in TURP-treated GD 18 dams compared to SAL-treated counterparts (n=6/group; Newman-Keuls test *versus* SAL-GD 18 females: P>0.05; Fig. II-7A). Intriguingly, circulating IL-6 levels in the SAL-treated GD 18 dams tended to be greater than those in NP-females; however this effect did not reach statistical significance (Fig. II-7A). Similar observations were made for circulating IL-1ra levels (Fig. II-7B), whereby TURP's ability to induce this cytokine were suppressed in the GD 18 group compared to the NP-females (one way ANOVA: $F_{(3,14)}$=6.19, P=0.0067;

Newman-Keuls test TURP *versus* SAL-NP females: $P<0.01$; TURP *versus* SAL-GD 18 females: $P>0.05$; Fig. II-7B). Unlike the 11 h time point, IL-1ra levels were not induced by TURP at GD 18, however, similar to IL-6, basal levels of this cytokine (i.e. in the SAL-treated groups) were greater for the GD 18 group compared to the NP-females at the 6 h time point (Newman-Keuls test SAL-NP females *versus* SAL-GD 18 females: $P<0.05$). These findings bear a striking resemblance to what is observed in humans, where the ability of peripheral immune cells to produce IL-6 and IL-1ra in basal/non-stimulated conditions is increased (Amoudruz et al., 2006; Pillay et al., 1993), whereas following immune stimulation with PAMPs, these cells' ability to release pro-inflammatory cytokines is blunted (Amoudruz et al., 2006; Luppi et al., 2002). Collectively, these data support the hypothesis that towards the end of gestation, there is a dampening in the capacity of maternal peripheral immune system to produce some cytokines, particularly IL-6, which may underlie the attenuated fever. In addition, it is intriguing that basal IL-1ra levels were significantly greater in the GD 18 group, which suggests that greater basal levels of this cytokine may participate in the blockade of fever induction, as previously reported (Fofie and Fewell, 2003). However, this difference may be dependent on the time of the day at which measures are taken, since this effect was not present at the 11 h time point (Fig. II-2), which were sacrificed at the end of the light phase (at around 20 h), whereas that for the 6 h time point, animals were sacrificed at around 15 h. Circadian variation in circulating cytokine levels has been shown to occur in humans (Haus and Smolensky, 1999; Miles et al., 2008) and alterations in these circadian rhythms may be involved in the mechanisms resulting in attenuated fever during late pregnancy. It has been previously shown that the time of the day will affect the inflammatory response to infection (Marpegan et al., 2009; Ucar et al., 1983).

Figure II-7. IL-6 and IL-1ra levels are differentially modulated by gestation and TURP treatment

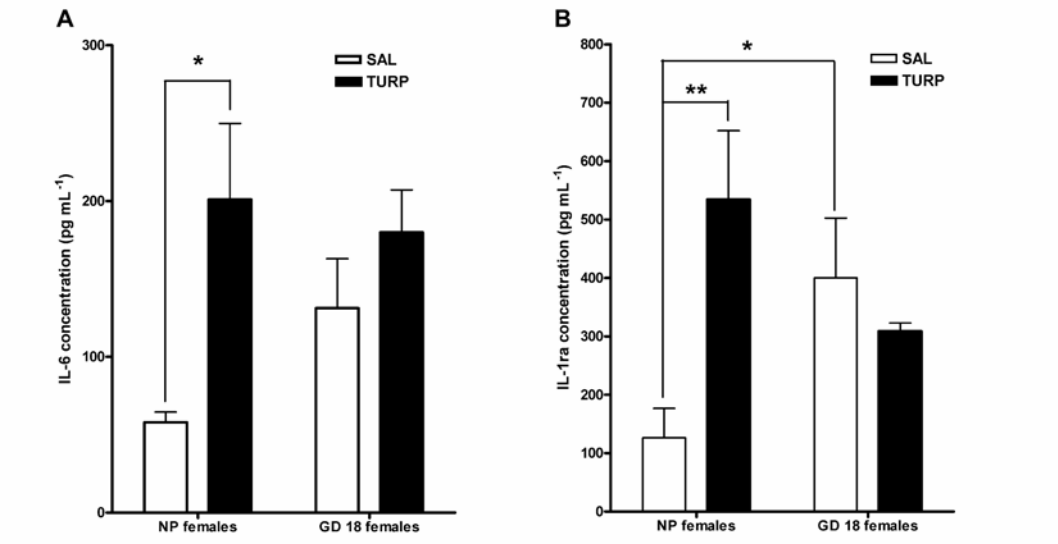

*Figure II-7. (A) 6 h after TURP (100 μL; i.m.), IL-6 circulating levels were significantly induced among NP females. This effect was completely blocked in the pregnant females at GD 18. This group, however, showed a non-significant increase in the basal levels (i.e. in the SAL treated group) of this cytokine. TURP-NP females versus SAL-NP females: * P<0.05. (B) IL-1ra levels were also significantly induced at this time point in the TURP-treated NP-females and not affected by the inflammatory treatment at GD 18. Similar to IL-6, IL-1ra levels were significantly greater in the SAL-GD 18 group compared to their NP counterparts. TURP-NP females versus SAL-NP females: ** P<0.01; SAL-treated NP versus GD 18 females: * P<0.05 .*

Hypothalamic expression of inflammatory signals at the time of fever induction by TURP

To complete our analysis of the pyrogenic signalling molecules at the induction phase of fever, mRNA expression of SOCS3, COX-2 and mPGES-1, as well as COX-2 protein levels, were analysed in the hypothalamus of GD 18 and NP-females at the 6 h time point (Fig. II-8). TURP treatment of NP-females significantly induced the expression of SOCS3 (one way ANOVA: $F_{(3,19)}$=3.99,

P=0.023; Newman-Keuls test TURP *versus* SAL-NP females: *P*<0.05; Fig. II-8A), mPGES-1 (one way ANOVA: $F_{(3,18)}$=5.67, *P*=0.0065; Newman-Keuls test TURP-NP females *versus* SAL-NP females or *versus* TURP-GD 18 females: *Ps*<0.05; Fig. II-8B) and COX-2 (one way ANOVA: $F_{(3,18)}$=5.90, *P*=0.0055; Newman-Keuls test TURP *versus* SAL-NP females: *P*<0.01; Fig. II-8C) mRNAs and COX-2 protein levels (one way ANOVA: $F_{(3,14)}$=5.08, *P*=0.014; Newman-Keuls test TURP *versus* SAL-NP females: *P*<0.05; Fig. II-8D). According to the observations made for circulating IL-6 levels, neither of the molecules measured were significantly induced by TURP in the GD 18 group compared to their SAL counterparts (Newman-Keuls tests TURP *versus* SAL-GD 18 females: *Ps*>0.05). SOCS3 (Fig. II-8A) and COX-2 (Fig. II-8C) mRNA levels in the SAL-treated GD 18 dams tended to be greater than those in the SAL-treated NP-females, similar to what is observed for IL-6 and IL-1ra circulating levels (Figs. II-7A and B), however these effects did not reach statistical significance.

Finally, IL-1β mRNA levels were also determined in the hypothalami of the groups described above. At this time point, neither of the TURP-treated groups showed a significant change in the mRNA levels of this molecule, compared to their respective controls (Fig. II-9). These data suggests that IL-1β expression in the brain is not necessary for fever induction, at least in this model of inflammation. Therefore, its role maybe restricted to either later phases of the fever response (i.e. maintenance) or in the induction of additional sickness-type responses such as anorexia, reduced social interaction, etc (Konsman et al., 2002; Laye et al., 2000).

Figure II-8. Hypothalamic expression of SOCS3, mPGES-1 and COX-2 mRNAs and COX-2 protein levels are not induced by TURP during late pregnancy

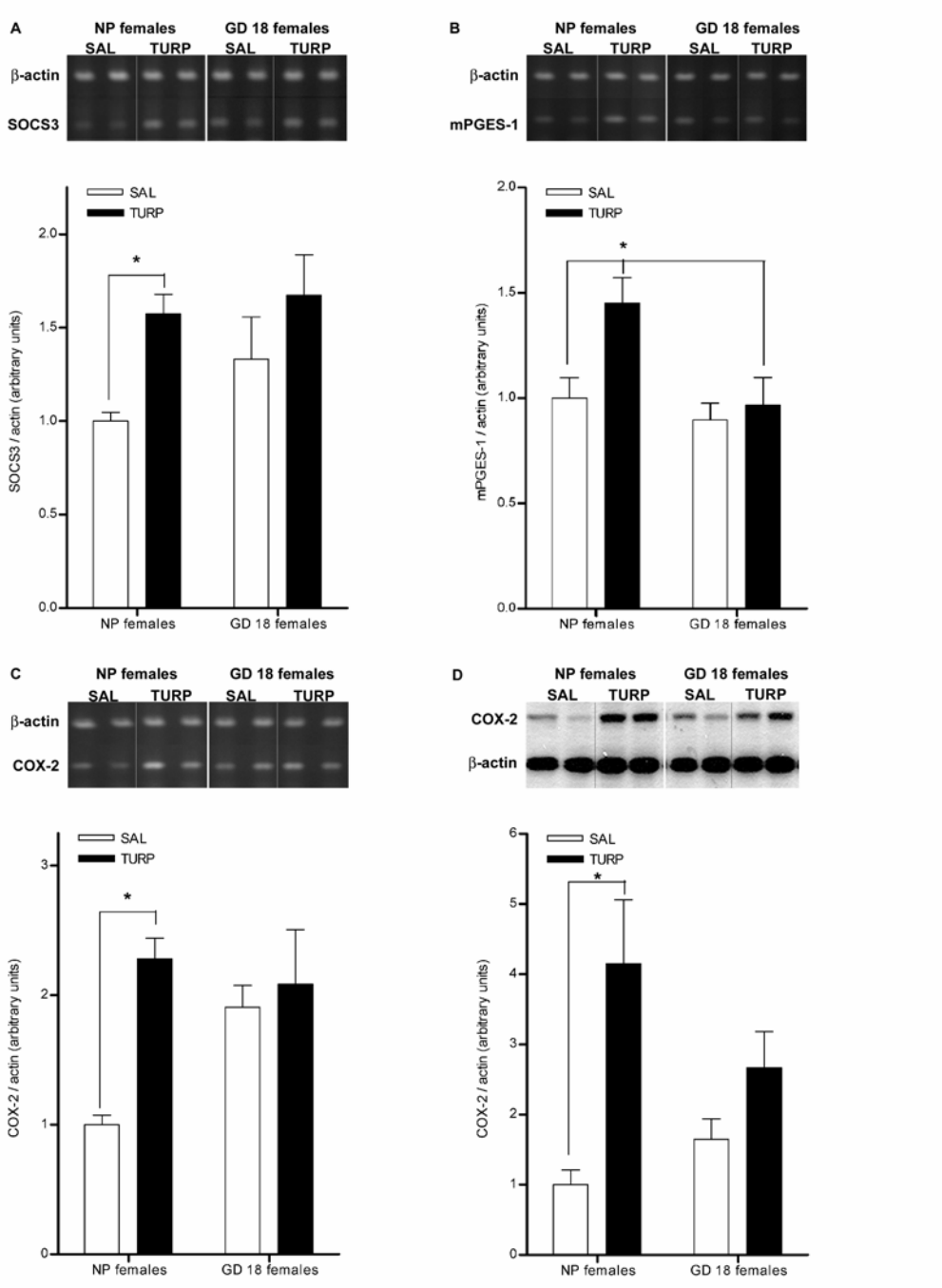

*Figure II-8. (A) Hypothalamic expression of SOCS3 was elevated following TURP treatment at the time of fever induction (6h after treatment), but only in the NP females group. TURP-NP females versus SAL-NP females: * P<0.05. (B) A similar effect was observed for hypothalamic mPGES-1 mRNA*

*expression. In this case, the effect of TURP on the expression of this gene in the NP females resulted in significantly greater mRNA levels than both their SAL counterparts and the TURP-treated GD 18 females. TURP-NP females versus SAL-NP or versus TURP-GD 18 females: * P<0.05. (C) COX-2 mRNA levels and (D) COX-2 protein levels were also significantly increased in the TURP-NP females group, whereas the inflammatory treatment was not effective in the late pregnant group. TURP-NP females versus SAL-NP females: * P<0.05.*

Figure II-9. Hypothalamic IL-1β mRNA levels are not induced by TURP treatment in neither NP nor GD 18 females

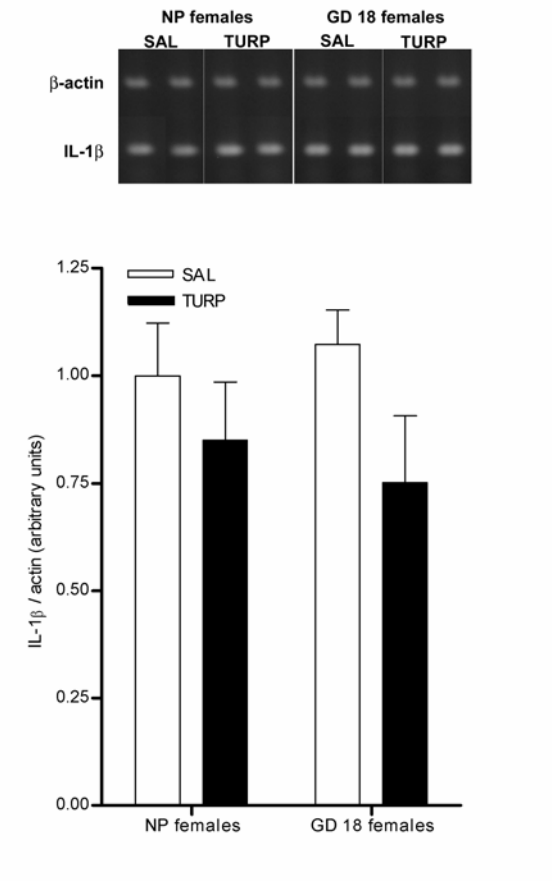

Figure II-9. IL-1β mRNA levels remained unchanged following TURP treatment to either NP or GD 18 females at the 6 h time point.

Altogether these data support the hypothesis that attenuated induction of pro-inflammatory cytokines, along with normal induction of anti-inflammatory mediators, underlies the attenuation in the febrile response in late pregnancy, and possibly several other acute phase responses, following a localized inflammatory challenge, like TURP administration. Given the adaptive value of fever (Kluger, 1978; Kluger et al., 1975) and the inflammatory response in general (Baumann and Gauldie, 1994; Kushner, 1988), this attenuation in near term dams may be reflective of the potential damage that inflammatory mediators represent for the developing foetus. Given this possibility, we sought to determine whether the response to TURP treatment differed between stages of pregnancy, and whether this insult administered at different stages of gestation had differential consequences in the adult offspring.

Chapter III: Alterations in cognitive function and behavioural response to amphetamine induced by prenatal inflammation are dependent on the stage of pregnancy

III.1. Preface

From the previous study (Chapter II) we determined that the levels of IL-6, the main pro-inflammatory cytokine released to the circulation during TURP-induced inflammation, were attenuated at GD 18 compared to NP-females. We sought to determine whether the difference between these physiological states was also evident when comparing GD 18 to an earlier gestational stage, namely GD 15. At this earlier stage of gestation, the mechanisms that lead to attenuated fever have not as yet been engaged (Begg et al., 2007; Begg et al., 2008; Harre et al., 2006), suggesting that these animals will still have an intact inflammatory response similar to that exhibited by NP-females. In addition, we sought to investigate whether this potentially different inflammatory response correlated with behavioural effects in the offspring of mothers treated at the two gestational ages.

In studying the behavioural consequences of prenatal inflammation in the offspring, we focused on several aspects that have previously been shown to be altered in SCZ. In particular, we determined the offspring's responses in the pre-pulse inhibition (PPI) of acoustic startle, an operational measure of sensorimotor gating; learning and memory of spatial information in the Morris-water maze and of an associative emotional task in the form of fear conditioning; and finally, the locomotor response to the dopamine (DA) indirect agonist, AMPH, which depends on the function of the mesolimbic DA neurons.

III.2. Manuscript

Title: Alterations in cognitive function and behavioural response to amphetamine induced by prenatal inflammation are dependent on the stage of pregnancy

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Abstract

Maternal infection during human pregnancy has been associated with the development of schizophrenia in the adult offspring. The stage of development and the maternal inflammatory response to infection, which undergoes quantitative and qualitative changes throughout gestation, are thought to determine critical windows of vulnerability for the developing brain. In order to investigate how these two factors may contribute to the outcome in the offspring, we studied the inflammatory response to turpentine (TURP) injection (100 μl/dam) and its consequences in the adult offspring, in pregnant rats at gestational day (GD) 15 or 18, which correspond to late first and early second trimester of human pregnancy, respectively. Maternal inflammatory response to TURP was different between the two GDs, with fever and circulating levels of the pro-inflammatory interleukin (IL)-6 significantly attenuated at GD 18, compared to GD 15. In the adult offspring, TURP challenge at GD 15 induced a significant decrease in pre-pulse inhibition (PPI) of acoustic startle, increased latency in the cued task of the Morris-water maze, prolonged conditioned fear response and enhanced locomotor effect of amphetamine. In contrast, the same immune challenge at GD 18 induced only a prolonged conditioned fear response. These results suggest a window of vulnerability at GD 15, at which TURP seems to affect several behaviors that are strongly modulated by dopamine. This was supported by increased tyrosine hydroxylase expression in the nucleus accumbens of the adult offspring of mothers treated at GD 15.

Introduction

Maternal infection has been linked to the development of schizophrenia in the adult offspring. It is estimated, based on the magnitude of the effects reported for maternal influenza, toxoplasmosis and genital/reproductive infections, that prevention of these infections during pregnancy could theoretically avoid up to a third of all schizophrenia cases in the adult progeny (Brown and Derkits, 2010b). Infectious agents as diverse as bacteria, viruses and protozoa have been shown to be involved in the etiology of this psychiatric disorder, leading to the hypothesis that, although infection-specific mechanisms may be involved, an inflammatory response common to all types of infection would have a major effect on neurodevelopment (Brown and Derkits, 2010b; Gilmore and Jarskog, 1997).

At least two factors are deemed critical to determining windows of vulnerability for maternal infection to affect fetal development. First, the stage of neurodevelopment at which the inflammatory insult occurs could determine which ongoing developmental processes could be affected (Boksa, 2010b; Brown and Derkits, 2010b; Fortier et al., 2007; Meyer et al., 2006a; Meyer et al., 2008d; Meyer et al., 2007b). However, the critical stage of gestation at which vulnerability of the fetus may be greater to develop schizophrenia following maternal infection remains poorly defined (Brown and Derkits, 2010b). Studies have shown significant associations during the first (Brown et al., 2004a; Sorensen et al., 2009), second (Brown et al., 2004b; Brown et al., 2000b; Sorensen et al., 2009) or third (Brown et al., 2004b; Buka et al., 2001b) trimester of gestation.

Secondly, it is now well documented that the maternal immune system and inflammatory response undergo profound changes throughout pregnancy (Luppi, 2003; Sacks et al., 1999). For example, whereas basal cytokine production by peripheral blood cells shows a progressive increase during pregnancy, immunogen-stimulated release of the pro-inflammatory cytokine interleukin (IL)-6 is significantly attenuated (Amoudruz et al., 2006; Luppi et al., 2002) whilst that of the anti-inflammatory cytokine IL-1 receptor antagonist (ra) is significantly

enhanced (Pillay et al., 1993). Interestingly, these changes have also been reported in animal models of maternal infection particularly in rodents. For example, in near-term rats, intraperitoneal (IP) lipopolysaccharide (LPS) injection resulted in increased induction of IL-1ra (Ashdown et al., 2007; Fofie and Fewell, 2003), whereas IL-6 induction was blunted following intramuscular (IM) injection of turpentine oil (TURP) (Aguilar-Valles et al., 2007). These alterations in circulating cytokine levels may underlie the well characterized observation of attenuated/suppressed febrile response to LPS (Martin et al., 1995; Zeisberger et al., 1981), polyinosinic: polycytidylic acid (poly I:C, a viral mimic) (Cooper et al., 1988) and TURP (Aguilar-Valles et al., 2007).

Studies of this type have contributed to our understanding of the link between the infection-induced immune response in the mother with pathological outcomes in the offspring that are relevant for schizophrenia and other psychiatric disorders (Boksa, 2010b; Meyer and Feldon, 2010; Meyer et al., 2009a; Patterson, 2009). They also represent an extremely useful tool for unraveling the complex mechanisms underlying these pathologies.

In the present study we sought to determine whether the offspring's susceptibility to develop behavioral alterations as adults may depend on the age of gestation at which the inflammatory insult occurred and at the same time, how this related to the prenatal inflammatory response. We focus our studies on the effects of maternal inflammation on the offspring's cognitive function (Elvevag and Goldberg, 2000) and the mesolimbic dopamine (DA) function (Laruelle et al., 1999), which are recognized as central in the pathophysiology of schizophrenia. In addition, alterations in cognitive function prior to the onset of schizophrenia (Ellman et al., 2009) as well as following its clinical onset (Brown et al., 2009) and psychotic symptoms (Zammit et al., 2009), are significantly associated with prenatal infection.

For this purpose, we used a model of clinical trauma, involving aseptic localized injury and inflammation, induced by IM injection of TURP (Wusteman et al., 1990). Following its administration, TURP remains secluded in the site of injection (Wusteman et al., 1990), where it induces tissue damage, activation and

recruitment of immune cells to the site of injury (Sheikh et al., 2006), and local release of tumor necrosis factor (TNF)α, IL-1β and IL-6, of which only IL-6 enters the circulation (Aguilar-Valles et al., 2007; Luheshi et al., 1997; Tron et al., 2005; Turnbull et al., 2003). This approach allows for the study of the endogenous inflammatory circulating mediators in isolation (Gershenwald et al., 1990; Kozak et al., 1998; Leon, 2002; Tron et al., 2005; Turnbull et al., 1998) whilst avoiding the confounding factor of having the systemically injected immunogen (i.e. LPS or poly I:C) directly targeting the placenta (Ashdown et al., 2006a) and the fetal compartment, which often results in fatalities (Fortier et al., 2007; Girard et al., 2010). Importantly, we have previously demonstrated that prenatal TURP treatment induces significant behavioral alterations in the offspring, congruent with neurodevelopmental psychiatric disorders (Aguilar-Valles et al., 2010; Fortier et al., 2007). In addition, others have implicated IL-6 (the only pro-inflammatory mediator that appears in the circulation after TURP treatment) as an important component involved in these neurodevelopmental changes (Smith et al., 2007).

Methods

Animals and treatment

Time pregnant primiparous Sprague-Dawley rats (Charles Rivers, QC, Canada) were used in all experiments. Dams were received on gestational day (GD) 7, when they were individually housed in a controlled environment at an ambient temperature of 21±1 ºC on 12:12 h light-dark cycle (lights on at 0800 h) with free access to food and water. Pregnant dams were handled daily from GD 11 or GD 14 onwards, depending on the stage at which the mothers would be treated, and habituated to a rectal probe to determine core temperature (Physitemp Instruments, NJ, U.S.A.). On the day of the experiment core body temperature was measured at baseline (0), and animals were injected intramuscularly with 100 □L of purified TURP (Riedel-deHaën, Sleeze, Germany), at either GD 15 (TURP-15) or 18 (TURP-18); control dams received an equivalent volume of saline (SAL) at the same GDs (SAL-15 or SAL-18). All injections were administered between 0900 and 1000 h. Core temperature was additionally recorded 8, 10 and 24 h after the IM treatment. Only the male offspring were used for the behavioral and tyrosine hydroxylase (TH) expression studies; these were housed under the same environmental conditions, except that they were pair-housed with animals from the same experimental group.

GD 15 and 18 were chosen since they roughly correspond to late first or early second trimester of human pregnancy, respectively, in terms of markers of brain development (Clancy et al., 2001). Specifically, the early stage corresponds to a period , in the development of the DA neurons at which axons are moving towards their targets, whereas at GD 18 innervation of the stratum is occurring (Van den Heuvel and Pasterkamp, 2008). These two stages of rodent gestation may in addition represent two functionally distinct periods in the time course of the changes in immunity that occur during a normal pregnancy, as cytokine production [both from lymphocyte T helper (Th)1 and Th2] by peripheral blood cells decreases in the second but not during the first trimester of human pregnancy (Shimaoka et al., 2000).

All experimental procedures were approved by McGill University's Animal Care Committee, pursuant of the Canadian Council of Animal Care. All efforts were made to minimize the number of animals used.

Experiment 1: Analysis of the maternal inflammatory response at GD 15 and 18

This experiment was performed to determine the effect of gestational stage on the maternal inflammatory response to TURP. Mothers treated at GD 15 or 18 (n=6/group) were sacrificed 10-11 h after IM treatment with a lethal dose of pentobarbital sodium (IP, 60 mg/kg) and blood was collected via cardiac puncture. Placentas (3/mother) were also collected. This time point represents the peak of fever and cytokine responses to TURP (Aguilar-Valles et al., 2007). Serum cytokines were determined, as well as placenta mRNA levels of inflammatory mediators.

Plasma cytokine determination by ELISA

Blood samples were collected using sterile heparinized syringes and placed into sterile tubes. Samples were then centrifuged (5300 g, 15 min at 4 °C), aliquoted and stored at –80 °C until assays were performed. Sandwich enzyme-linked immunosorbent assays (ELISA) for IL-6, IL-1ra and leptin (NIBSC, Potters Bar, UK) were performed as previously described (Inoue et al., 2008; Rees et al., 1999) using species-specific ELISA (NIBSC, Potters Bar, UK). Intra-assay and inter-assay variations were below 10%. The sensitivities of the assays were 3.9 pg/ml for IL-1ra, 7.8 pg/ml for IL-6 and leptin.

Real time RT-PCR and Western blotting in placenta

Total RNA was extracted and reverse transcribed as described previously (Pohl et al., 2009a) from placenta. Real-time PCR was performed in duplicate using pre-optimized primer/probe mixture (TaqMan Gene Expression Assays, Applied Biosystems, ON, Canada) and TaqMan universal PCR master mix (Applied Biosystems). The housekeeping gene 18S was used to normalize levels

of cDNA expression for each sample. Levels of gene expression were calculated as the X-fold difference from the control groups at each GD. The mRNA levels of the following genes were assessed: IL-6, IL-1β, IL-1ra, TNFα and SOCS3, used as a marker of activity of the JAK/STAT3 signaling pathway (Lebel et al., 2000a), which is activated by cytokines that target the gp130 signaling molecule, including IL-6 and leptin. The assay ID for each gene is as follows: IL-6: Rn99999011-m1; IL-1β: Rn00580432-m1; TNFα: Rn99999017-m1; IL-1ra: Rn00573488-m1; SOCS3: Rn00585674-s1 and 18S: EUK-18S-rRNA4352930.

For western blot studies, placentas were frozen and protein extracted and the analysis conducted as previously described (Aguilar-Valles et al., 2007). Blocked nitrocellulose membranes were incubated with antibodies against phosphorylated STAT3 (pSTAT3, 1:1000, Cell Signaling Technology, Sanvers, MA, U.S.A.), STAT3 (1:8000, Santa Cruz Biotechnology) and actin (HRP-coupled, 1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Data was expressed as either pSTAT3/STAT3 or STAT3/actin ratios.

Experiment 2: Behavioral consequences of TURP treatment at either GD 15 or 18 in the adult offspring

These experiments were designed to test the differential effects of TURP treatment at GD 15 or 18 on the behavior of the adult offspring [SAL (pooling GD 15 and 18): n=16, TURP-15: n=9, TURP-18: n=9, three independent experiments were performed). Mothers treated as described above were allowed to give birth and their male offspring were marked, weighed and cross-fostered to surrogate dams in mixed litters (Fortier et al., 2007). Offspring were weaned from their foster mothers at postnatal day (P) 21 and used at P 60-62 for behavioral testing, which was limited to two tasks/adult animal. This included pre-pulse inhibition (PPI) of acoustic startle (SAL: n=30, TURP-15: n=27, TURP-18: n=18), amphetamine (AMPH)-induced locomotion (SAL: n=30, TURP-15: n=24, TURP-18: n=21), Morris-water maze (SAL: n=24, TURP-15: n=27, TURP-18: n=12) or fear conditioning (SAL: n=16, TURP-15: n=9, TURP-18: n=9). An equal number of pups per dam was used for each behavioral task in order to counterbalance any

possible litter effect (3/dam for PPI, AMPH-induced locomotion and Morris-water maze and 1/dam for fear conditioning). Each pup only performed 2 tasks, at least 7 days of rest were allowed in between. All tests were performed between 1000 and 1600 h.

PPI of acoustic startle

PPI was measured as previously described (Fortier et al., 2007). Briefly, basal startle response and PPI of acoustic startle were measured using six SR-LAB startle apparatuses (San Diego Instruments, San Diego, USA). Each test session began with a 5 min acclimatization period in the presence of 70 dB white noise, which continued all throughout. 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 pre-pulse + pulse trials at each of three different pre-pulse intensities, in a pseudo-random order and with an average inter-trial interval of 17 s (range: 9–29 s). The pre-pulse + pulse trials consisted of a 30 ms pre-pulse (at 76, 82 or 85 dB), followed by a 70 ms delay, then a startle pulse (120 dB, 30 ms). The testing session lasted 15 min. PPI is expressed as % PPI, defined as (1−[startle amplitude on pre-pulse + pulse trial/mean startle amplitude on pulse alone trials]) × 100. Basal startle response was also analyzed.

Morris-water maze

Animals were trained in the spatial version of the Morris-water maze, as previously described (Brouillette and Quirion, 2008). The apparatus consisted of a white circular pool (140 cm in diameter) filled with opaque water (by the addition of skim milk powder, 24 °C) to a 60 cm height and surrounded by visuo-spatial cues posted on the walls of the room (Morris, 1984). Animals were tracked using a video recording system (HVS, Buckingham, UK). Animals were trained to find a hidden platform (14 cm diameter) located 1 cm below the water level. Training consisted of three trials per day, with inter-trial interval of 45 min, for 4 consecutive days. If an animal did not find the hidden platform within 90 s, it was

guided to it. Animals were pseudo-randomly started from a different position for each trial. Latency to reach the platform was recorded and averaged per day. On day 5, a probe task to test spatial memory was performed by removing the platform and recording the time swam on each quadrant (expressed as percentage of the total swimming time, 60 s = 100 %). After a 1-h resting period, the cued task was performed by placing the visible platform into the maze. Latency to reach the visible platform and swim speed were recorded. After each trial, animals were placed under heat lamps to dry and prevent hypothermia.

Fear conditioning

Conditioning was performed using a Fear Conditioning System (Kinder Scientific, Poway, CA, U.S.A.). Each conditioning chamber (24.13 cm wide x 60.33 cm long) was equipped with a high density cage rack with 7 x 15 infrared photobeams for movement quantification. Data was acquired and analyzed using Motor Monitor software (Kinder Scientific). The chamber's grid floor was connected to a programmable shocker unit for foot shock delivery. Freezing time was automatically recorded and defined as the time spent in immobility (i.e. no break of an adjacent infrared beam for 5 milliseconds) and expressed as % of the time bin. One day after a 30-min acclimation session, animals were conditioned by pairing (10 X, 2-min inter trial period) a 30-s, 85 dB tone, produced by a 2.9 kHz tone module, to a 1-s, 0.5 mV foot shock, delivered in the last second of the tone. Freezing time was recorded during each 30-s tone presentation. The next day, animals returned to the conditioning chamber and freezing time in the chamber was recorded for 5 minutes. This was used as a measure of background contextual fear conditioning and compared to the freezing time in the minute prior to conditioning. Animals remained in the chamber for another 25 min to extinguish the contextual fear memory. The last day, animals were introduced to dissimilar chambers (with different wall color, floor and chamber's aroma) for 10 min and then exposed to the conditioned stimulus for 5 min and freezing time recorded.

Basal and AMPH-induced locomotion

Locomotor response to d-AMPH sulphate salt (Sigma-Aldrich, Dorset, UK) was measured using a well-established protocol (Fortier et al., 2004; Yetnikoff et al., 2007). Briefly, locomotor activity was quantified (as distance traveled in cm) with an infrared activity-monitoring apparatus for rats (AccuScan Instruments, Columbus, OH, U.S.A.). Rats were habituated to the boxes for 30 minutes (basal locomotor activity) and placed back in the boxes for 30 minutes the next day. Total locomotion was compared between the two 30-min periods to obtain a general measure of habituation. Immediately after, the animals were injected with AMPH (2 mg/kg, IP) and locomotor activity was monitored for an additional 90 minutes.

Experiment 3: Effect of prenatal TURP treatment on brain tyrosine hydroxylase (TH) expression

This experiment was designed to measure the expression of TH, the rate limiting enzyme in the synthesis of DA, in selected brain areas of the offspring at several postnatal ages (P) to monitor the time course in alterations of expression. Based on the results from experiments 1 and 2 (see results section below), only mothers treated at GD 15 were used (n=6 mothers/group). Cross-fostered male offspring were sacrificed by live decapitation at post-weaning (P 22), peri-pubertal (P 35) and adult (P 60) ages. Collected brains (n=5-7/group/age) were immersed in 2-methylbutane (Fisher Scientific, Hampton, NH, U.S.A.) and chilled with dry ice. Western blot analysis was performed from bilateral punches from the ventral tegmental area (VTA), substantia nigra (SN), nucleus accumbens (NAcc, including core and shell), dorsal striatum (dSTR) and medial prefrontal cortex (mPFC, including cingulated areas 1 and 2). Brain regions were excised from 300 □m thick cryostat sections, using previously described procedures (Yetnikoff et al., 2007). Western blot membranes were incubated with antibodies against TH (1:5000, Chemicon, Temicula, CA, U.S.A.) and actin. Data are expressed as a ratio of TH over actin optical densities.

Statistical analyses

All data are presented as mean values ± s.e.m.; p values < 0.05 values were deemed significant. All data presented characteristics of a normal distribution; therefore only parametric analyses were used.

Body temperature was analyzed using three-way ANOVA with gestational age (GD 15 vs. 18) and treatment (SAL vs. TURP) as between-groups factors and time after treatment as the within-group factor. Two- and three-way interactions were further analyzed using simple effect ANOVAs and Tukey's HSD post hoc tests. Despite differences in the time course of basal body temperature, we chose to asses the febrile response to TURP using the absolute temperature values, rather than using the change in body temperature from the pre-injection value. This was done since it has been demonstrated that absolute temperature provides a better representation of the physiological characteristics of fever (Feng et al., 1989; Inoue et al., 2008).

Circulating cytokines, placental real time PCR and Western blot data and some behavioral measures (basal startle response during PPI test; latency in the cued task, swim speed and number of passes through the platform location on probe task of the Morris-water maze; basal freezing response to the tone in fear conditioning) were analyzed using one-way ANOVAs and Tukey's HSD post hoc test.

The remaining of the behavioral data (PPI; training and the probe task in the Morris-water maze; conditioning, context and cued test in the fear conditioning task; and basal and AMPH-induced locomotion) were analyzed using two-way ANOVAs, with prenatal treatment (SAL vs. TURP-15 vs. TURP-18) as the between-groups factor and a within-group factor which was specific to the data from each behavioral task (i.e. pre-pulse intensity for PPI, day of training or quadrant for Morris-water maze, trial or time bin for fear conditioning and time bin or day for basal locomotion and time bin for AMPH-induced locomotion). TH expression was also analyzed using two-way ANOVA, for each brain region, with age (P 22 vs. 35 vs. 60) and prenatal treatment (SAL-15 vs. TURP-15) used as between-groups factors. If a main effect of prenatal treatment was found, analysis

was continued with Tukey's HSD post hoc test, and if a significant interaction between main effects was found, analysis was continued with simple effect ANOVA and then Tukey's HSD post hoc tests.

Results

Experiment 1: Maternal inflammatory response to TURP depends on the gestational stage of treatment

The febrile response is differentially induced at GD 15 and 18

Body temperature was significantly lower in the GD 18 SAL-treated dams throughout the experiment, compared to similarly treated animals at GD 15 (gestational age x treatment interaction $F_{(1,45)}$=6.94, p=0.0115; simple effect of gestational age for the SAL-treated dams $F_{(1,45)}$=5.16, p=0.0279, Fig. III-1A).

Treatment with TURP induced a significant increase in core body temperature at both gestational stages (simple effect of treatment for the GD 15 groups $F_{(1,45)}$=66.52, p<0.0001; simple effect of treatment for the GD 18 groups $F_{(1,45)}$=19.63, p<0.0001, Fig. III-1A). TURP-induced increase in temperature was significant at the 8 h time point (treatment x time interaction $F_{(3,135)}$=22.53, p<0.0001; simple effect of treatment at 8 h $F_{(1,180)}$=58.18, p<0.0001, Fig. III-1A), reached a peak at 10 h for both groups (simple effect of treatment at 10 h $F_{(1,180)}$=102.98, p<0.0001, Fig. III-1A) and receded 24 h after treatment at both gestational stages. Importantly however, the febrile response was significantly greater for GD 15 than for GD 18 dams (simple effect of gestational age for the TURP-treated groups $F_{(1,45)}$=35.96, p<0.0001, Fig. III-1A).

Circulating IL-6 correlates with the variations in maternal febrile response

IL-6 and IL-1ra, the two main pro- and anti-inflammatory circulating cytokines induced by TURP (Cartmell et al., 2001; Cartmell et al., 2000; Luheshi et al., 1997), were measured. IL-6 was significantly induced by TURP at GD 15 (effect of treatment $F_{(3,21)}$=51.67, p<0.0001, SAL-15 vs. TURP-15 p<0.0001, Fig. III-1B). At GD 18, IL-6 induction by TURP was also significant (SAL-18 vs. TURP-18 p=0039), however it was greatly diminished in comparison to that at GD 15 (TURP-18 vs. TURP-15 p<0.0001, Fig. III-1B). Interestingly, circulating IL-1ra was induced by TURP at both gestational stages (effect of treatment $F_{(3,22)}$=9.03, p=0.0006; SAL-15 vs. TURP-15 p=0.007; SAL-18 vs. TURP-18

p=0.0125, Fig. III-1C) to comparable levels between the two immune stimulated groups (TURP-15 vs. TURP-18 p=0.135, Fig. III-1C).

Placenta inflammatory response to maternal TURP is modulated independently of the stage of pregnancy

We determined the effect of the maternal localized inflammatory insult with TURP on the expression of inflammatory mediators in the placenta, as inflammation in this organ is thought to be significant in the pathological processes that affect fetal development (Ashdown et al., 2006a; Boksa, 2010b). Since IL-6 is the predominant pro-inflammatory mediator in the maternal circulation following TURP treatment, we first determined the activation of the JAK/STAT signaling pathway. STAT3 phosphorylation was significantly induced by TURP treatment in placentas of dams at GD 15 (1.55-fold change; effect of treatment $F_{(3,22)}$=11.69, p=0.0001, SAL-15 vs. TURP-15 p=0.0015, Fig. III-2A), as well as at GD 18 (1.75-fold change, SAL-18 vs. TURP-18 p=0.0003, Fig. III-2A) in contrast to the marked differences in IL-6 induction in the maternal circulation, which suggests the involvement of additional mediators. STAT3 total protein levels (data not shown; effect of treatment $F_{(3,22)}$=1.69, p=0.20) and SOCS3 mRNA levels (effect of treatment $F_{(3,23)}$=0.035, p=0.79, Fig. III-2B) were not affected by the TURP treatment at any gestational stage.

We then analyzed the mRNA levels of IL-6, IL-1β, IL-1ra and TNFα (Fig. III-3). IL-6 mRNA expression was significantly induced by TURP injection at both gestational stages, with a 1.8-fold change compared to control values at GD 15 (effect of treatment $F_{(3,22)}$=14.79, p<0.0001, SAL-15 vs. TURP-15 p=0.0006, Fig. III-3A) and a 2.2-fold change from control values at GD 18 (SAL-18 vs. TURP-18 p<0.0001, Fig. III-3A). The increase in IL-6 mRNA levels did not differ between the GD 15 and 18 groups (TURP-15 vs. TURP-18 p=0.102). In contrast, TURP led to a significant 2.85-fold decrease at GD 15 in IL-1β mRNA levels (effect of treatment $F_{(3,22)}$=8.86, p=0.0007, SAL-15 vs. TURP-15 p=0.002, Fig. III-3B) and a statistically comparable 1.77-fold reduction at GD 18 (SAL-18 vs. TURP-18 p=0.0259, Fig. III-3B). mRNA expression levels of IL-1ra (effect of

treatment $F_{(3,22)}$=2.39, p=0.10) and TNFα (effect of treatment $F_{(3,22)}$=1.13, p>0.36, Fig. 3D) were not significantly affected by TURP at any GD, although there was a trend for a reduction in IL-1ra at GD 15 (1.57-fold reduction, Fig. III-3C).

One possible mediator that activates JAK/STAT signaling and the release of IL-6 from human placenta is leptin (Lappas et al., 2005). We indeed observed that circulating leptin levels were significantly induced after treatment with TURP at both gestational stages (main effect of prenatal treatment $F_{(1,18)}$=5.77, p=0.027, Fig. III-10), therefore supporting the likelihood of its involvement in immune activation of the placenta.

Experiment 2: Behavioral alterations in the offspring differ according to the stage of gestation at which the immune challenge occurred

To determine the consequences of maternal inflammation at either GD 15 or GD 18 on the offspring's cognitive performance and behavioral effect of AMPH, treated mothers were allowed to give birth. TURP did not induce any significant effect on the total time of gestation, body weight of the offspring at birth, litter size or maternal survival at either gestational stage of treatment (data not shown).

PPI is impaired in the offspring of mothers treated with TURP at GD 15, but not at GD 18

PPI of acoustic startle was used as an operational measure of sensorimotor gating (Swerdlow et al., 2001), which has been reported to be impaired in schizophrenic patients (Geyer, 2006). Maternal TURP treatment induced a significant impairment of PPI in the offspring treated at GD 15 (significant effect of prenatal treatment $F_{(2,146)}$=87.71, p<0.0001, SAL vs. TURP-15 p=0.0003, Fig III-4A). There was a marked trend for reduction of PPI in the offspring of mothers treated at GD 18, which did not reach statistical significance (SAL vs. TURP-18 p=0.079, Fig. III-4A). Since no pre-pulse alone trials were included, we cannot entirely rule out that the PPI deficit may be due to altered reactivity to the pre-pulse. However, the magnitude of the startle response, determined in the startle-alone trials of the task, was not significantly altered by any prenatal treatment

(effect of treatment $F_{(2,74)}$=0.03, p=0.966, Fig. III-4B), suggesting that no effects on general reactivity or the sensitivity to the sound can account for the PPI deficits.

Prenatal TURP at GD 15 does not affect spatial learning but alters performance in the cued task

Hippocampal abnormalities are commonly found in schizophrenia (Heckers and Konradi, 2002). In addition, there are functional alterations of hippocampal physiology and structure in the offspring of immune challenged mothers (Baharnoori et al., 2009; Cui et al., 2009; Ito et al., 2010; Lowe et al., 2008; Makinodan et al., 2008; Meyer et al., 2006a), similar to those that have been linked to PPI deficits (Bast and Feldon, 2003; Swerdlow et al., 2001). Therefore, we evaluated the behavioral performance in a spatial learning and memory task in the Morris-water maze, which is highly reliant on the functional integrity of the hippocampus (D'Hooge and De Deyn, 2001).

All animals showed a significant decrease in the daily average escape latency to find the platform, from day 1 throughout day 4 (significant effect of time $F_{(3,180)}$=70.13, p<0.0001, Fig. III-5A), without effect of prenatal treatment ($F_{(2,60)}$=0.43, p=0.65, Fig. III-5A). On day 5, all animals spent more time than by chance (i.e. 25%) on Q1, where the platform was located during training (95% confidence interval of the % average time in Q1: SAL=30.9 to 37.8 %, TURP-15=31.1 to 38.4 % and TURP-18=26.6 to 39.8 %, Fig. III-5B). Prenatal TURP did not affect the time spent in each quadrant (effect of prenatal treatment $F_{(2,59)}$=0.06, p=0.943). The equal capacity for spatial learning and memory between the different groups was also observed in the amount of crosses on the previous location of the platform (effect of prenatal treatment $F_{(2,56)}$=2.1, p=0.132, Table III-1). All groups showed identical swim speed (effect of prenatal training $F_{(2,61)}$=0.9, p=0.41, Table III-1) suggesting similar locomotor activity. Finally, the latency to reach the visible platform in a new location was recorded as a measure of the motivational state of the animal. The offspring of dams treated with TURP at GD 15 presented a significantly greater latency to reach the platform under the

new conditions (effect of prenatal training $F_{(2,58)}$=3.72, p=0.030, SAL vs. TURP-15 p=0.0084), whereas the offspring of dams treated with TURP at GD 18 did not show this effect (SAL vs. TURP-18 p=0.28, Fig. III-5C).

Prenatal TURP at GD 15 and GD 18 induces longer lasting conditioned freezing response

We aimed to asses whether an additional form of learning and memory could be affected by prenatal inflammation. Therefore, we performed fear conditioning to determine the ability of the offspring to learn the associative memory task. Baseline freezing response to the tone was not different between groups (26.6 ± 3, 29.8 ± 4 and 32.0 ± 7 % for SAL, TURP-15 and TURP-18 groups respectively, effect of prenatal treatment $F_{(2,37)}$=0.42, p=0.66, Fig III-6A). All groups showed a significant increase in freezing behavior after the first tone-shock pairing (effect of conditioning $F_{(9,333)}$=29.1, p<0.0001, 1st vs. 2nd trial p<0.0001), which reached a plateau after the 3rd trial and was not affected by prenatal treatment (effect of prenatal treatment $F_{(2,37)}$=0.04, p=0.96). Accordingly, freezing time to the context was greater 24 h after than prior to conditioning (effect of conditioning $F_{(5,185)}$=5.19, p=0.0002, Fig. III-6B) and was not altered either by the prenatal treatments (effect of prenatal treatment $F_{(2,37)}$= 0.49, p=0.62; interaction prenatal treatment x conditioning $F_{(10,185)}$=1.19, p=0.30, Fig. III-6B). Prior to the cued test, freezing time to the unfamiliar chamber did not differ between groups (significant time x prenatal treatment interaction $F_{(10,180)}$=3.97, p<0.0001, simple effect of prenatal treatment before tone onset $F_{(2,216)}$=2.21, p=0.12, Fig. III-6C). Finally, after tone onset, all animals substantially increased their freezing time (effect of tone presence $F_{(5,180)}$=52.0, p<0.0001, Fig. III-6C), which was not affected by of prenatal treatment for the first 4 minutes (simple effects of treatment at minutes 1-4 after tone onset, $F_{(2,216)}$<3.1, p>0.05). However, during the last minute of exposure (i.e. minute 5), control animals decreased the freezing time, whereas the TURP groups remained freezing significantly more time (simple effect of prenatal treatment at minute 5 after tone onset $F_{(2,216)}$=3.76, p=0.0268, SAL vs. TURP-15 p=0.011 and SAL vs. TURP-18 p=0.018, Fig. III-

6C). These data suggest that learning and memory of a conditioned fear response were not affected by prenatal TURP treatment, but there is evidence of a prolonged conditioned response to the tone in both prenatal TURP-treated groups.

AMPH-induced locomotion is only affected by prenatal inflammation at GD 15

We tested the mesolimbic dopamine function by measuring the locomotor response to AMPH given the relevance of this neurotransmitter system for schizophrenia (Davis et al., 1991; Weinberger, 1987). In addition, impaired responses in PPI and the cued task of the Morris-water maze, as well as increased freezing after fear conditioning may be associated with enhanced midbrain dopamine function (Morice et al., 2007; Pezze and Feldon, 2004; Swerdlow et al., 2001; Weiss et al., 2007).

Locomotor activity decreased as a function of time during the first acclimation session (significant effect of time $F_{(2,74)}$=156.1, p<0.0001, Fig. III-7A) for all groups, with no effect of prenatal treatment (effect of prenatal treatment $F_{(2,37)}$=0.07, p=0.94, Fig III-7A). Likewise, total locomotor activity was significantly reduced from the first to the second acclimation session (effect of time $F_{(1,37)}$=9.99, p=0.0031, Fig. III-7B) and this measure of habituation was not altered by prenatal treatments (effect of prenatal treatment $F_{(2,37)}$=0.01, p=0.99, Fig. III-7B). Locomotor activity was greatly increased following AMPH injection in all groups (Fig. III-7C). Importantly, the locomotor response to AMPH was greater in the offspring of TURP-treated mothers at GD 15 than in the SAL and TURP-18 groups (significant main effect of prenatal treatment $F_{(2,71)}$=5.51, p=0.006, SAL vs. TURP-15 p=0.002; TURP-15 vs. TURP-18 p=0.014, Fig. III-7C), whereas such response in the TURP-18 group was identical to control (SAL vs. TURP-18 p=0.63, Fig. III-7C).

Experiment 3: Longitudinal analysis of TH expression in mesocorticolimbic and nigrostriatal circuits in the offspring of TURP-treated mothers at GD 15

The results from experiments 1 and 2 suggest that a significantly greater inflammatory response at GD 15 than at GD 18 may be inducing alterations in neurodevelopment of the offspring, leading to: enhanced locomotor response to AMPH, impaired PPI and performance in the cued-task of the Morris-water maze, and prolonged conditioned fear response. In contrast, the offspring treated at GD 18 presented attenuated/absent behavioral alterations induced by TURP, notably the enhancement of locomotor response to AMPH was completely absent. Some behavioral effects of TURP treatment at GD 15 suggested alterations in DA neurotransmission; we therefore decided to explore this possibility by analyzing TH expression in several DA-rich brain regions in these offspring. These analyses were performed at several postnatal ages (22, 35 and 60) in order to explore whether the effects of the prenatal immune insult were age dependent or present throughout the animal's postnatal development.

In the VTA, TH expression was not significantly altered by prenatal TURP at any age (Fig. III-8A). Interestingly, in the NAcc, there was a marked effect of prenatal TURP at P 60, when TH levels were significantly greater than the control offspring (significant main effect prenatal treatment $F_{(1,28)}$=6.48, SAL-P 60 vs. TURP-P 60 p=0.005, Fig. III-8B). There was no effect of prenatal TURP on TH levels at early ages in the NAcc (Fig. III-8B) or in the mPFC at any age (Fig. III-8C). Finally, TH was not significantly affected by prenatal TURP in the nigrostriatal DA system at any age (SN, Fig. III-9A; dSTR, Fig. III-9B).

Discussion

In this report, we established that maternal pro-inflammatory response (i.e. release of IL-6 and fever) to locally administered TURP is attenuated at GD 18, compared to GD 15. In contrast, there were comparable levels of the anti-inflammatory cytokine IL-1ra between the two gestational stages following TURP treatment. These results resemble those described when comparing non-pregnant females to GD 18 dams after TURP treatment (Aguilar-Valles et al., 2007). Changes in peripheral pro-inflammatory cytokine production have not always been detected in models of systemic inflammation (Harre et al., 2006; Mouihate et al., 2005a). Nevertheless, our results firmly support the hypothesis that reduced ability to induce IL-6 upon a localized inflammatory stimulus underlies the attenuated febrile response towards the end of pregnancy (Aguilar-Valles et al., 2007) and recapitulate reports of reduced induction of IL-6 by immunogenic agents in pregnant women near term (Amoudruz et al., 2006; Luppi et al., 2002).

Other pro-inflammatory cytokines, such as IL-1β and TNFα, are clearly engaged in TURP-induced inflammation (Cooper et al., 1994; Luheshi et al., 1997). However, their involvement is confined to either the initial cascade of inflammatory mediators within the site of injection, where they may have a paracrine effect, or centrally for development of sickness-type behaviors (Aguilar-Valles et al., 2007; Cooper et al., 1994; Luheshi et al., 1997). Therefore, their role as endocrine mediators may be minor or even absent in this model of inflammation (Luheshi et al., 1997; Rivier, 2002).

Although the pro-inflammatory component of the systemic maternal response to TURP was attenuated by the stage of pregnancy, placental immune activation was rather similar between the two stages of gestation. More specifically, placental STAT3 activation and IL-6 mRNA were similarly stimulated, whereas IL-1β mRNA expression was suppressed at both GDs. This is the first demonstration that placental inflammatory mediators can be altered by a localized immune challenge administered to the mother. Since TURP remains localized in the site of injection (Wusteman et al., 1990) the most likely

instigators at the level of the materno-fetal interface are endogenous maternal inflammatory mediators. TURP-induced IL-6 was dramatically attenuated at GD 18, therefore additional systemic mediators may be involved in altering placental JAK/STAT signaling and cytokine mRNA expression (Jinbo et al., 2002; Sheikh et al., 2006; Venihaki et al., 2001). One feasible candidate is leptin, a pleiotropic hormone that activates the JAK/STAT signaling pathway, is involved in the acute inflammatory response (Luheshi et al., 1999; Rummel et al., 2010; Rummel et al., 2008; Sachot et al., 2004) and is able to release IL-6 from human placenta (Lappas et al., 2005). Leptin levels were induced by TURP, regardless of the gestational stage, suggesting a possible involvement for this mediator.

Downstream transcriptional targets of leptin, such as SOCS3 and IL-1β, were not induced in the placenta and our finding demonstrating suppressed IL-1β mRNA expression in placental tissue was especially intriguing. One possible explanation for this rather surprising effect is that at the expression of these two molecules peaked earlier in the inflammatory process, as occurs for the expression of other acute phase proteins (Sheikh et al., 2007; Sheikh et al., 2006; Tron et al., 2005). Further time course studies would be required to clarify this finding.

Animal models of maternal systemic inflammation, such as IP LPS injection, have been associated with extensive placental damage, involving profound reduction in perfusion, cell death and macrophage invasion (Girard et al., 2010). This can be ascribed to a direct effect of LPS on the placental cells, as we have previously demonstrated (Ashdown et al., 2006a). Accompanying these effects, there are significant increases in placental pro- and anti-inflammatory cytokine expression (Ashdown et al., 2006a; Girard et al., 2010; Urakubo et al., 2001), which are also observed after systemic poly I:C treatment (Gilmore et al., 2005; Koga et al., 2009). These effects are linked to an increased rate of fetal and maternal mortality (Fortier et al., 2007; Girard et al., 2010) and/or pre-term delivery (Koga et al., 2009) which involve IL-1 and TNFα induction (Girard et al., 2010; Haider and Knofler, 2009; Kakinuma et al., 1997). In contrast, maternal treatment with TURP does not stimulate the release of these two cytokines into the maternal circulation (Cooper et al., 1994; Luheshi et al., 1997; Turnbull et al.,

2003), and we observed in the present study that their placental expression was either unaffected (TNFα) or decreased (IL-1β). This alone suggests that toxicity to the placenta mediated by these cytokines may be absent in this model of maternal inflammation. In addition, prenatal treatment with TURP has consistently resulted in unaffected litter sizes, body weight of the offspring at birth and lack of maternal mortality [(Fortier et al., 2007) and present study] which were all normal and altogether may suggest attenuation of cytokine-induced damage to the placental.

The behavioral effects of prenatal TURP in the adult offspring were markedly different between the groups treated at the two gestational ages. The earlier immune challenge (i.e. at GD 15) produced a clear impairment in PPI, an increased latency in the cued task of the Morris-water maze, prolonged conditioned freezing response in fear conditioning and augmented locomotor response to the dopamine indirect agonist AMPH. In contrast, all of these changes were absent in the offspring born to mothers treated with TURP at GD 18, with the exception of the effect on the freezing response to the tone in the cued fear-conditioning test. Differential effects by maternal infection on the offspring, depending on the gestational ages, have also been shown for the poly I:C and LPS models (Cui et al., 2009; Fortier et al., 2007; Li et al., 2009b; Meyer et al., 2006a; Meyer et al., 2008d; Zuckerman and Weiner, 2005). One proposal derived from these reports is that immune insults earlier in pregnancy produce more marked effects on the offspring (Meyer et al., 2007b), which our results strongly support.

A significant deficit in PPI was found in the offspring of TURP-treated mothers at GD 15, and trend for the same effect was found in the offspring of mothers treated at GD 18. These results confirm our previous findings (Fortier et al., 2007), and strengthen the proposal that a specific window of vulnerability exists for PPI at GD 15 for it to be affected by maternal immune stimulation. In humans, impaired PPI is interpreted as an indication of a failure of inhibitory filtering mechanisms that can lead to sensory overload and consequent cognitive fragmentation (i.e. distractibility and thought disorder) in schizophrenia (Geyer, 2006). Hippocampal dysfunction could underlie PPI deficits, as well as alterations

on locomotor activity, learning and memory (Bast and Feldon, 2003), however we found that spatial learning and memory, and basal locomotor activity were not affected by prenatal TURP treatment, at either gestational stage. This suggests that hippocampal processes underlying locomotion and spatial processing were spared by the prenatal immune insult. These findings corroborate previous reports where normal spatial learning was found in the offspring of mice treated with LPS at GD 17 (Golan et al., 2005) or poly I:C at GD 12 (Ito et al., 2010). Others however, have shown decreased spatial learning when LPS was administered to rats at GD 19 (Lante et al., 2008; Lante et al., 2007). The difference between the latter studies and those of Golan et al (Golan et al., 2005) and ours may reflect either a late-pregnancy window of vulnerability for the hippocampus, or differences due to the dose and/or type of immunogen. Our data suggests that the observed deficit in sensorimotor gating did not concur with deficits in spatial learning or locomotion, and may therefore arise from developmental alteration of other neuronal systems. However, we cannot completely rule out functional deficit of the hippocampus in the offspring of TURP-treated mothers, as we have demonstrated that prenatal LPS treatment results in significant alterations in adult neurogenesis (Cui et al., 2009) and neuronal excitability (Lowe et al., 2008).

Animals born to mothers treated at GD 15 did, however, show increased latency to reach the platform in the cued version of the maze. Similar results have been reported for mice born to mothers treated at GD 17 with LPS (Golan et al., 2005). These workers (Golan et al., 2005) interpreted their findings as a deficit in associative learning. Our results in the present study indicate that animals born to TURP-treated dams at both GDs exhibited normal learning and memory for conditioned fear (both contextual and cued), which represents a form of associative learning. In fact, these same animals showed a prolonged conditioned freezing response during the cued memory test. This finding, along with the deficit in the cued task of the Morris-water maze, may be indicative of impairment in the animal's ability to adapt its behavioral responses to changes in the predictive value of a given cue (i.e. location of platform or tone). Importantly

these effects seemed to be more prominent in the offspring of mothers treated at GD 15 than at GD 18.

Increased latency to reach a visible platform in the Morris-water maze is also found in "hyper-DAergic" mice, generated by genetic inactivation of the DA transporter (DAT) (Morice et al., 2007; Weiss et al., 2007). These mice have constitutively elevated extracellular levels of DA (Dumartin et al., 2000; Fumagalli et al., 1998) and, similarly to what we found in our present studies, display intact spatial memory (Weiss et al., 2007). DAT knock-out mice however show strong deficits in reversal learning, suggesting that the differences observed in the cued Morris-water maze could be attributed to increased preservative behavior (Morice et al., 2007) as a consequence of enhanced DA neurotransmission (Morice et al., 2007). It is therefore tempting to suggest that the behavioral similarities between the "hyper-DAergic" mice and the rats born to TURP-treated mothers in our present study may stem from alterations in the DA neurotransmission. In this regard, enhanced mesolimbic DA neurotransmission has been reported to also impair PPI (Swerdlow et al., 2001) and to increase conditioned freezing response (Pezze and Feldon, 2004; Pezze et al., 2002; Pezze et al., 2001). In the present study we showed that animals born to TURP-treated mothers at GD 15, but not at GD 18, present greater locomotor response to the DA indirect agonist, AMPH.

The altered response to AMPH in the TURP-15 offspring prompted us to analyze TH expression in the brains of these animals. Our data suggested that one of the underlying alterations in these offspring occurs in the mesolimbic DA neurons, which modulate the behavioral response to AMPH, as TH was found increased in the NAcc of adult animals treated with prenatal TURP at GD 15. Intriguingly, as we have reported recently (Aguilar-Valles et al., 2010), the levels of this enzyme in the other DA-rich brain areas analyzed were not affected by the prenatal insult. In addition, effects on the mesolimbic, but not on the mesocortical or nigrostraiatal DA systems have been observed in the adult offspring of mothers treated with poly I:C (Vuillermot et al., 2010) or LPS (Romero et al., 2010). Since we did not assess the level of expression of TH in the offspring of TURP-treated

mothers at GD 18, we cannot completely rule out the involvement of DA in the alterations induced in these offspring. It is possible however that given the two different stages of development of the mesencephalic TH positive neurons that project to the STR (both dorsal and NAcc) and the mPFC, the effects of inflammation may vary. DA axonal projections from mesencephalic neurons will have only reached the ganglionic eminence and the NAcc striatum at GD 15 (Van den Heuvel and Pasterkamp, 2008). At GD 18 the axonal projection will have reached the striatum and some will have, by this time, started to advance towards the mPFC (Van den Heuvel and Pasterkamp, 2008). Based on this developmental timeline, it is likely that an inflammatory insult at GD 15 may affect the innervation of the NAcc, whereas at GD 18, these processes may be less vulnerable to the maternal/placental mediators.

The effects on TH are not only spatially restricted, but also appear to be temporally constrained, as they were not apparent at earlier postnatal ages. The mechanisms underlying the post-pubertal appearance of TH alterations in the NAcc are currently unknown and deserve further investigation. These data supports the hypothesis that a common underlying mechanism may be involved in the induction of neurodevelopmental alterations by diverse types of inflammatory/infectious agents (Meyer and Feldon, 2009).

Collectively, the data presented in this report suggest a significant link between the magnitude of the inflammatory response, governed by the time of pregnancy, during gestation and the consequences in the adult offspring. In this regard, TURP-induced maternal IL-6 may be playing a central role in the long term effects on the offspring, as others have shown in the prenatal poly I:C model (Smith et al., 2007). The effect of this cytokine may be direct on the developing fetus, as it has been shown that IL-6 from the maternal side can be transported to the fetal compartment, with greater efficiency at mid- (GD 11-13) than late-pregnancy (GD 17-19) (Dahlgren et al., 2006). The relative contribution of placental vs. maternal inflammatory mediators in the effects of prenatal infection in the offspring's development is currently unknown. Locally expressed cytokines in the placenta could have a paracrine or autocrine effect on placental cells,

altering functions such as the materno-fetal exchange of nutrients and signaling molecules that support and control fetal development (Fowden et al., 2008; Malassine et al., 2003). Placental cytokines could in addition directly target the fetus, along with maternally derived cytokines.

Another intriguing possibility is that IL-1ra, which was elevated following TURP treatment, may be playing a role. The main function of IL-1ra is to limit the inflammatory response by targeting IL-1 activity at the level of its receptor (Cartmell et al., 2001; Dinarello, 1991; Luheshi et al., 1996a; Miller et al., 1997). Despite the potentially beneficial effect of IL-1ra as an anti-inflammatory agent (Girard et al., 2010), abnormally high IL-1ra levels may also directly target the fetal compartment (Pillay et al., 1993), directly blocking neurodevelopmental processes in which IL-1 may be normally involved (Cunningham et al., 1996; Schneider et al., 1998; Spulber et al., 2009). Supporting this rationale is the direct evidence of detrimental effects described for the anti-inflammatory cytokine IL-10, whose overexpression during gestation can induce some of the same effects demonstrated with prenatal poly I:C treatment (Meyer et al., 2008b).

By using this model of localized inflammation (TURP), we demonstrated that the systemic induction of maternal cytokines at GD 15 is associated with a greater amount of behavioral alterations than those caused when the same immune challenge is administered at GD 18.

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Conflict of interest

All authors declare that they have no conflicts of interest that are relevant to this manuscript.

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Figures and Tables

Figure III-1. Maternal inflammatory response to turpentine (TURP) was significantly affected by the gestational day (GD) of treatment

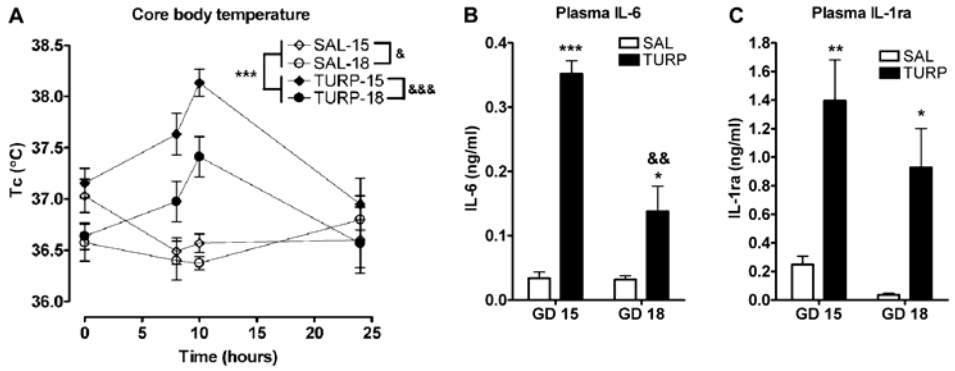

*Figure III-1. Maternal febrile response was determined by measuring rectal temperature at baseline (0), 8, 10 and 24 h after saline (SAL) or TURP injections (i.m., 100 µl/dam) at GD 15 or 18 (A). Circulating interleukin (IL)-6 (B) and IL-1 receptor antagonist (IL-ra) (C) were determined from blood samples collected 10-11 h after SAL or TURP treatment of a subset of dams used for fever determination (A). * P<0.05, ** P<0.01 and *** P<0.0001 vs. corresponding SAL treated group. & P<0.05, && P<0.01 and &&& P<0.0001 vs. same treatment group at one of the GDs.*

Figure III-2. TURP-induced activation of placental JAK/STAT signaling pathway occurred independently of the GD

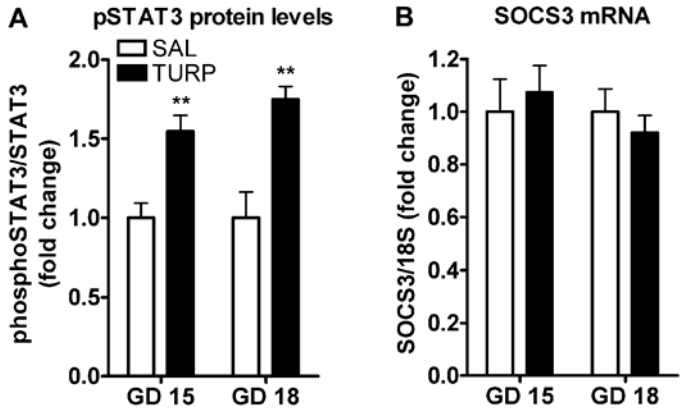

*Figure III-2. Phosphorylated STAT3 protein levels were determined 10-11 h after SAL or TURP injection (as in Fig. III-1) (A). SOCS3 mRNA levels were also measured from the same animals (B). ** P<0.01 vs. corresponding SAL treated group.*

Figure III-3. Maternal treatment with TURP altered placental cytokine mRNA expression independently of GD

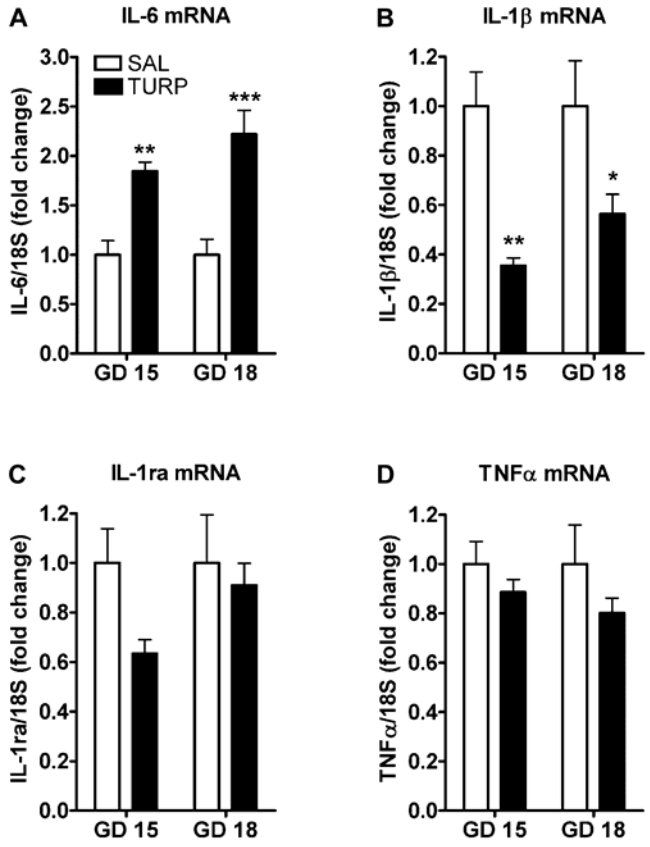

*Figure III-3. IL-6 (A), IL-1β (B), IL-1ra (C) and tumour necrosis factor (TNF)α (D) mRNA levels were determined from the same samples as in Fig. 2B. * P<0.05, ** P<0.01 and *** P<0.0001 vs. corresponding SAL treated group.*

Figure III-4. Pre-pulse inhibition (PPI) of acoustic startle was significantly attenuated by prenatal treatment with TURP at GD 15, but not at GD 18

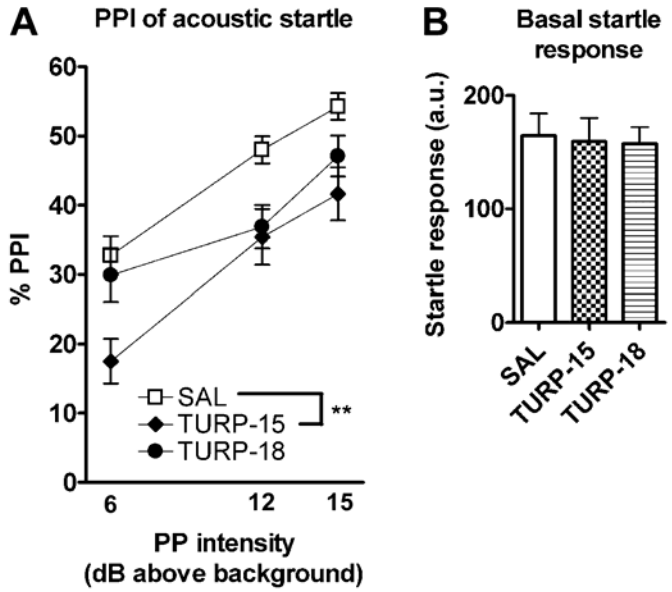

*Figure III-4. PPI was determined in the adult offspring of mother treated with SAL or TURP at GD 15 or GD 18 (A). Pre-pulse intensities assayed were 6, 12 and 15 dB above background sound levels (70 dB). Throughout the PPI experiment, the startle response to a 120 dB tone was also determined (B). ** P<0.01 vs. SAL treated group.*

Figure III-5. Prenatal TURP at GD 15 increased latency in cued task of Morris-water maze, but did not affect spatial learning and memory

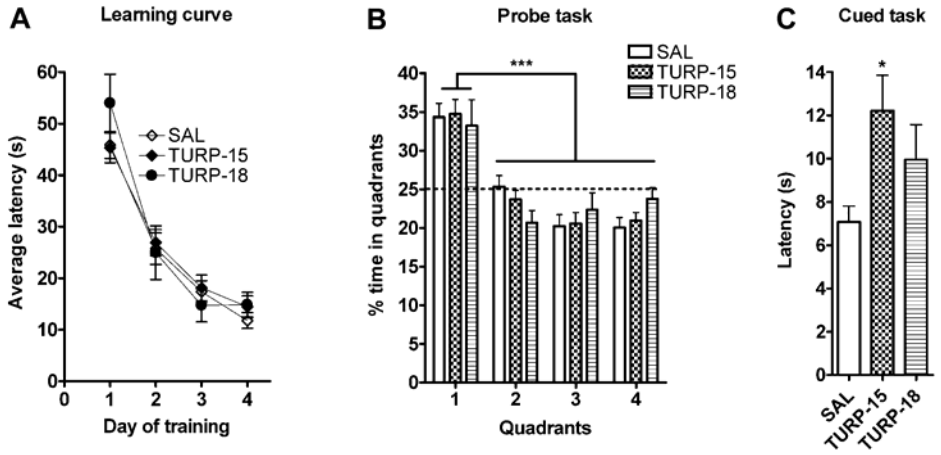

*Figure III-5. Training (A) in the Morris-water maze consisted of 3 trials per day for 4 consecutive days, during which latency (s) to reach the hidden platform was recorded. On day 5, the probe task was performed (B) to measure spatial memory. In this task the time spent swimming on the quadrant where the platform was located during training (Q1) was determined. After completion of the probe task, the cued task was performed (C), where latency to reach the visible platform was measured. *** P<0.0001 vs. any other quadrant. * P<0.05 vs. SAL treated group.*

Figure III-6. Prenatal TURP prolonged the conditioned fear response at either GD

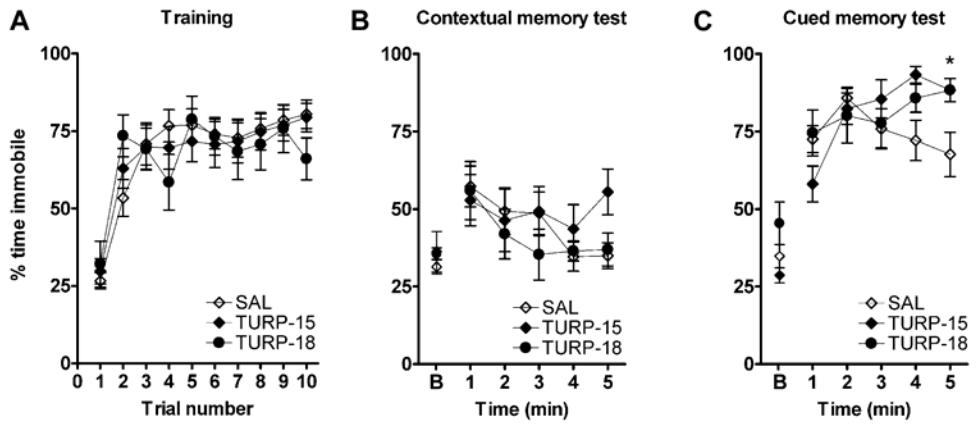

*Figure III-6. Animals were trained to associate a 30-s, 85 dB tone to a 1-s, 0.5 mV foot shock (10 trials), (A). Time spent in freezing behavior (i.e. immobility time) during each tone presentation was determined. The next day, animals returned to the same context where conditioning took place and freezing response (immobility time) determined (5 minutes) as a measure of background contextual memory of conditioned fear (B). This response was compared to the immobility time before conditioning (basal [B] on X-axis). Subsequently, 24 h later, animals were brought to a different context and their basal (B on X-axis) and tone-induced freezing response were determined as a measure of the cued conditioned fear memory (C). * P<0.05 vs. SAL treated group.*

Figure III-7. Amphetamine (AMPH)-induced locomotion was significantly increased by prenatal TURP at GD 15, but not at GD 18

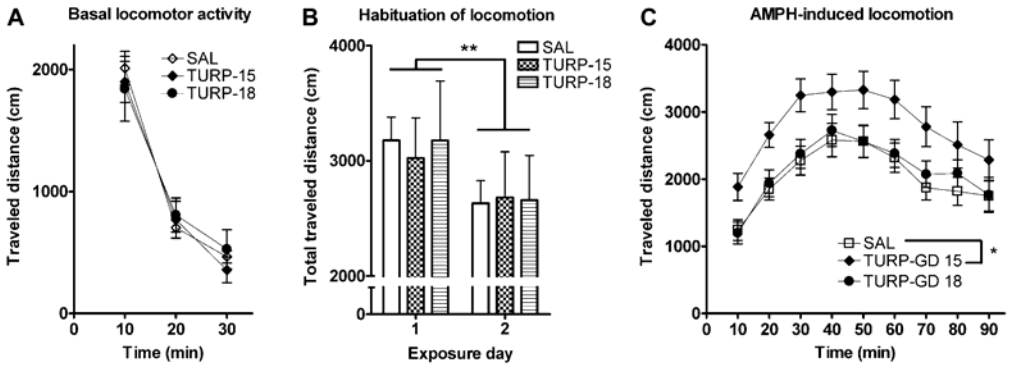

*Figure III-7. Locomotor activity (distance traveled) was measured for 30 min (presented in 10 –min bins) under basal conditions (A). The following day, animals were reintroduced to the same chambers and locomotion was measured for another 30 minutes and compared to the total activity of the previous day, in order to assess habituation (B). Following this second basal activity period, animals were treated with AMPH (i.p., 2 mg/kg of body weight) and their locomotor response determined for 90 min (presented in 10-min bins), (C). ** P<0.01 1st vs. 2nd day in the activity chambers. * P<0.05 vs. SAL treated group.*

Figure III-8. Tyrosine hydroxylase (TH) levels in the nucleus accumbens (NAcc) were increased in the adult offspring of mothers treated at GD 15

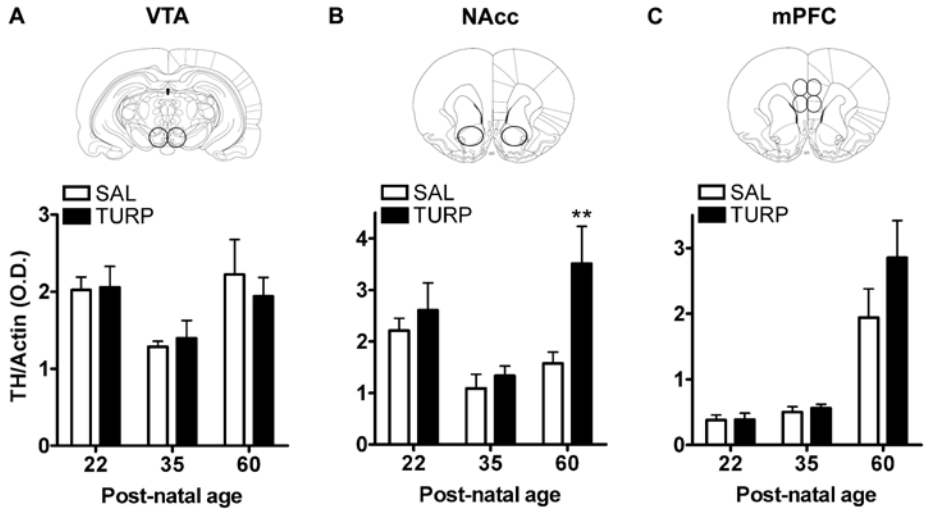

*Figure III-8. TH, the rate limiting enzyme in the synthesis of dopamine, was determined from protein extracts in the mesocorticolimbic DA regions: the ventral tegmental area (VTA) (A), NAcc (B) and the medial prefrontal cortex (mPFC) (C). Brains were collected at several postnatal days (P) representing post-weaning (P 22), peri-pubertal (P 35) and adult (P 60) ages. Upper panels in each graph depict a representative coronal view of the brain at which the corresponding brain regions were excised (Paxinos, 1998). ** P<0.01 vs. SAL treated group.*

Figure III-9. TH expression in nigrostriatal DA regions was not affected by prenatal TURP treatment at GD 15

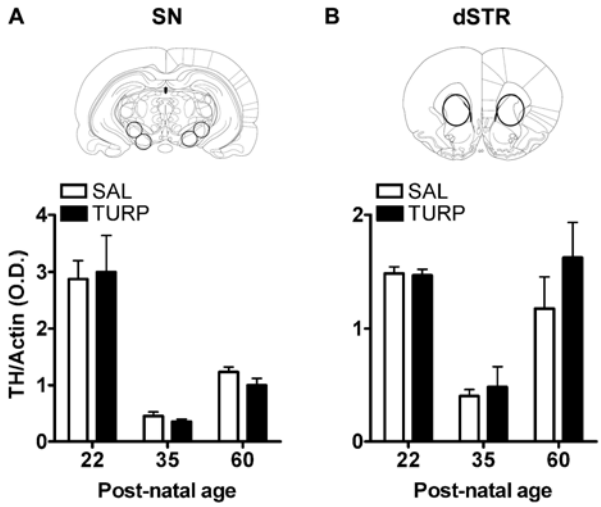

Figure III-9. TH was determined from the substantia nigra (A) and the dorsal striatum (B) from the same animals used in Fig 8. Upper panels in each graph depict a representative brain section from where the corresponding brain regions were excised (Paxinos, 1998).

Figure III-10. Circulating leptin levels

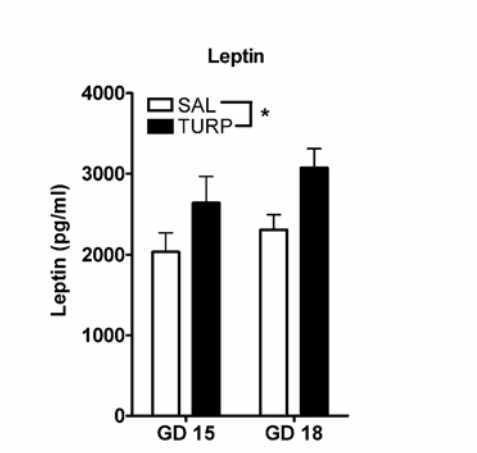

*Figure III-10. Circulating leptin levels were determined 10-11 h after saline (SAL) or turpentine (TURP) injection (i.m. 100 µl/dam), using same samples as in Fig. 1B and C. * P<0.05 vs. SAL treated groups.*

Table III-1. Mnemonic and locomotor parameters of the Morris-water maze

Prenatal treatment	Platform crossings	Swim speed
SAL	6.0 ± 0.4	24.8 ± 0.7
TURP-15	6.0 ± 0.5	24.1 ± 0.6
TURP-18	4.4 ± 0.7	25.8 ± 1.5

Number of crosses on the previous location of the platform during the probe task and swim speed (cm/s) in the Morris-water maze. Saline (SAL), turpentine at gestational day (GD) 15 (TURP-15) or at GD 18 (TURP-18).

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Chapter IV: Maternal cytokines are involved in the effects of prenatal infection on dopamine function of the offspring

IV.1. Preface

Our previous study established that GD 15 is a stage of the rat's pregnancy at which maternal immune response is still intact, and importantly leads to several behavioural changes in the offspring, including impaired PPI and enhanced response to AMPH. These alterations were accompanied by an increased expression of TH in the NAcc for the adult, but not peri-pubertal or weanling offspring. Maternal cytokines, the mediators of the systemic inflammatory response may be involved in the induction of these alterations in the offspring. Others have shown that by altering the levels or bio-activity of cytokines in the context of a model of maternal viral infection (Meyer et al., 2008b; Smith et al., 2007) the effects on the offspring can be prevented. From these studies, it has been suggested that an imbalance between pro- and anti-inflammatory cytokines caused by maternal infection may result in the effects of the offspring. We sought to further explore the possibility that maternal circulating cytokines affect neurodevelopment. Therefore, we undertook a study to determine the contribution of each circulating cytokine in the maternal circulation to the effects of maternal inflammation in the offspring, by using specific neutralizing antisera to each individually in separate studies. We aimed to target the main circulating pro-inflammatory (IL-6) and anti-inflammatory (IL-1ra) mediators, as well as the pleiotropic hormone, leptin, which has been described to be stimulated by PAMPs (Faggioni et al., 1998; Mastronardi et al., 2001) and other inflammatory stimuli, such as TURP (Faggioni et al., 1998), carageenan (Gualillo et al., 2000b), and pro-inflammatory cytokines (Grunfeld et al., 1996a; Sarraf et al., 1997) and to have a fundamental role in several acute phase responses such as fever and anorexia (Gardner et al., 1998; Grunfeld et al., 1996a; Luheshi et al., 1999; Rummel et al., 2009; Rummel et al., 2008; Sachot et al., 2004).

IV.2. Manuscript

Title: Maternal cytokines are involved in the effects of prenatal infection on dopamine function of the offspring

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In preparation

Keywords: maternal infection, IL-6, IL-1ra, leptin, dopamine, tyrosine hydroxylase, amphetamine, sensitization

Abstract

Maternal inflammatory response to infection is thought to underlie the link between infection during gestation and mental disorders, like schizophrenia, in the offspring. It has been suggested that maternal cytokines are the inflammatory agents that could affect neurodevelopment. We sought to test the contribution of the pro-inflammatory cytokine, interleukin (IL)-6, as well as the anti-inflammatory IL-1 receptor antagonist (IL-1ra) and the immune modulator, leptin, to the neurodevelopmental alterations following prenatal inflammation. We assessed the roles of these mediators by neutralization studies using species specific antibodies in several behavioural and neurochemical markers of mesolimbic dopamine (DA) neurotransmission in the offspring of mothers treated with turpentine oil (TURP). Previously, we have shown that this model of aseptic clinical trauma, while activating the systemic endogenous inflammatory responses, avoids the toxic side effects of systemic administration of immunogens. We also demonstrated that administration of TURP at gestational day (GD) 15 induced enhanced locomotor response to the DA indirect agonist, amphetamine (AMPH) and increases the expression of the DA synthetic enzyme tyrosine hydroxylase, as well as DA and its metabolites in the nucleus accumbens (NAcc) of the adult offspring. In the present study we demonstrated that these effects were mediated by the induction of maternal IL-6 and, quite unexpectedly, by IL-1ra as their neutralization resulted in the prevention of all of these alterations. Interestingly, maternal increases in leptin appeared to be involved in the enhancement of the mechanisms that lead to behavioural sensitization following repeated administration of AMPH, as well as it contributes to the increased synthesis of DA in the NAcc, but it does not affect the elevated levels of NAcc DA metabolites. Our results provide new evidence to suggest that both pro and anti-inflammatory maternal mediators contribute equally to neurodevelopmental alterations in the offspring that lead to a sensitized DA function in the adult offspring. These results are relevant for schizophrenia and other forms of psychosis.

Introduction

Maternal infection during the first or second trimester of human pregnancy has been identified as a risk factor for schizophrenia (Brown and Derkits, 2010a), and other psychiatric disorders (Deverman and Patterson, 2009). Among the infection agents that have been significantly associated with schizophrenia in the offspring are influenza and other respiratory infections (Brown et al., 2004a; Brown et al., 2000b; Susser et al., 2000), rubella (Brown et al., 2000a; Brown et al., 2001), *Toxoplasma gondii* (Brown et al., 2005; Mortensen et al., 2007), herpes simplex virus-2 (Buka et al., 2008; Buka et al., 2001a), maternal genital or reproductive infections (Babulas et al., 2006) and bacterial infections (Sorensen et al., 2009). The leading hypothesis on the mechanisms through which maternal infection increases the risk for developmental psychiatric disorders posits that an imbalance of pro- and anti-inflammatory cytokines during the acute phase response affects neurodevelopment (Ashdown et al., 2006a; Brown and Derkits, 2010a; Meyer et al., 2008b). Interestingly, increased levels of circulating pro-inflammatory cytokines have been found associated with higher risk of psychosis or schizophrenia (Brown et al., 2004b; Buka et al., 2001b).

It has recently become possible to test this hypothesis by means of using animal models of prenatal infection (Boksa, 2010a; Meyer and Feldon, 2010; Patterson, 2009). In this regard, maternal immune stimulation with Gram-negative bacterial cell wall component, lipopolysaccharide (LPS), the viral mimic, double stranded RNA, polyinosinic:polycytidylic acid (poly I:C), or an aseptic inflammatory agent, turpentine oil (TURP), induce several behavioural, brain morphological and neurochemical alterations in the adult offspring, which resemble some of those found in schizophrenia and other psychiatric disorders (Aguilar-Valles et al., 2010; Boksa, 2010a; Fortier et al., 2007; Meyer and Feldon, 2010; Patterson, 2009). Furthermore, administration of IL-6 to pregnant mothers is sufficient to trigger several alterations in the offspring (Samuelsson et al., 2006a; Samuelsson et al., 2006b; Samuelsson et al., 2004; Smith et al., 2007) and this cytokine has been shown to be necessary for the effects of prenatal poly I:C in

the offspring (Smith et al., 2007). Intriguingly, overexpression of the anti-inflammatory cytokine IL-10, although preventing some of the effects of prenatal poly I:C treatment, also mimicked some behavioral effects of poly I:C (Meyer et al., 2008b).

In the present study, we sought to determine the contribution of circulating pro- and anti-inflammatory cytokines released during inflammation, to the effects of prenatal immune challenge on the DA neurotransmission in the adult offspring. In order to achieve this objective, we utilized an animal model of aseptic inflammation which involves intramuscular (i.m.) administration of TURP. Unlike other models of prenatal inflammation, such as LPS or poly I:C that are administered systemically, TURP does not enter the maternal circulation (Wusteman et al., 1990), thus allowing for the study of maternal endogenous mediators in the absence of another direct influence exerted by the administered immunogen on the foetal compartment. We have previously demonstrated that maternal treatment with TURP induces several behavioral and neurochemical alterations in the offspring (Aguilar-Valles et al., 2010; Fortier et al., 2007), like those induced by other immunogenic agents, and that resemble pathophysiological aspects of schizophrenia.

In this study, we focused on the contribution of the main circulating pro-inflammatory cytokine, IL-6, and two other important mediators, IL-1 receptor antagonist (IL-1ra) and leptin. IL-1ra, a major anti-inflammatory mediator, regulates the magnitude and duration of the maternal inflammatory response (Ashdown et al., 2007; Horai et al., 1998; Luheshi et al., 1996a) by inhibiting the effects of locally expressed IL-1β (Luheshi et al., 1996a; Miller et al., 1997). The role of endogenously released IL-1ra during maternal inflammation for the foetus development remains unexplored. However, administration of IL-1ra prevents some of the damaging effect of prenatal LPS administration, especially at the level of the placenta (Girard et al., 2010). Leptin, the product of the *obese* gene (Zhang et al., 1994), apart from its prominent role in food intake and energy expenditure (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995), contributes significantly to the acute responses to infection, such as fever,

anorexia, cachexia and other brain controlled sickness behaviours (Fantuzzi and Faggioni, 2000; Luheshi et al., 1999; Rummel et al., 2009; Sachot et al., 2004). In addition, it has been shown to regulate several aspects of gestation, including implantation (Ramos et al., 2005), placental endocrine function (Islami et al., 2003a; Lappas et al., 2005) and foetal development (Kawamura et al., 2003).

Methods

Animals

Primiparous time-pregnant Sprague-Dawley rats (Charles Rivers, QC, Canada) were used. On GD 7 animals were individually housed in a controlled environment at an ambient temperature of 21±1 ºC on 12:12 h light-dark cycle (lights on at 0800 hours) with free access to food and water. Only male offspring were used, which were housed under the same environmental conditions. Mothers were treated with the inflammatory stimulus at GD 15. This day of pregnancy was used since it has been proposed to be roughly equivalent to the late 1st trimester of human pregnancy (Clancy et al., 2001), which has been significantly linked with the increased risk of developing schizophrenia in the offspring of infected mothers (Brown and Derkits, 2010a). Experimental procedures were approved by the Animal Care Committee from the Douglas Mental Health University Institute and McGill University pursuant of the Canadian Council of Animal Care. All efforts were made to minimize the number of animals used.

Treatment and experimental protocols

Pregnant dams were handled daily from GD 11 onwards and habituated to a rectal probe to determine core temperature (Physitemp Instruments, NJ, U.S.A.). At GD 15 basal core temperature was recorded and a maternal inflammatory response was elicited by injecting intramuscularly with 100 μL of purified TURP (Riedel-deHaën, Sleeze, Germany); control dams received an equivalent volume of saline (SAL). SAL- (n=9) and some of the TURP-treated (n=10) dams were injected with normal sheep serum (NSS, i.p., 3.5 ml/kg; Sigma Aldrich, St. Louis MO, U.S.A.), whereas other TURP-treated dams were co-administered with anti-IL-6 (n=6) (Rummel et al., 2006), anti-Leptin (n=6) (Rummel et al., 2009) or anti-IL-1ra (n=6) (Cartmell et al., 2001) crude antiserum raised in sheep (i.p. 3.5 ml/kg). Each of these three antisera has been proven to block the biological function and appearance of its targeted cytokine in the circulation of treated

animals, minimally affecting the release of non-target cytokines (Cartmell et al., 2001; Rummel et al., 2009; Rummel et al., 2006). All injections were administered between 0900 and 1000 hours. Core body temperature was measured in addition at 8, 10 and 24 h after i.m. treatment and supplementary doses of NSS or the different antisera were administered 10 and 24 h after TURP injection.

Food intake and body weigh of the mothers were also recorded at baseline, and then 6, 10, 24, 48, 72 and 96 h after i.m. injections, and used as a markers of the biological activity of the neutralizing antisera (Sachot et al., 2004). Food intake data was expressed as cumulative food intake (in grams from baseline) and body weight data expressed as cumulative change in body weight (expressed as percentage from baseline measure) (Sachot et al., 2004).

Mothers were allowed to give birth and the newborn offspring were tattooed and cross fostered to naïve mothers (Aguilar-Valles et al., 2010; Fortier et al., 2004). Offspring were weaned at postnatal day (P) 22 and allowed to grow until adulthood (P 60), when some animals from each experimental group were sacrificed by decapitation for brain collection, whereas the rest was used for behavioral experiments.

An additional group of pregnant dams, treated as described above, except that they were only injected with SAL or TURP (n=5-7/group), were used to determine the time course of cytokine induction by TURP (Aguilar-Valles et al., 2010). Blood was collected by tail nick just before i.m. injection (0 time point), then 6, 10, 24 and 48 h after treatment for determinations of IL-6, IL-1ra and leptin.

ELISA

Blood samples were allowed to cloth at room temperature for 1 hour, spun at 4000 rpm for 20 min at 4 ºC to obtain serum and stored at -80 ºC until used. Maternal serum samples were analyzed for IL-6 and IL-1ra (the main cytokines released into the circulation by TURP treatment (Aguilar-Valles et al., 2007) using species specific ELISA (NIBSC, Potters Bar, UK) as described previously (Inoue et al., 2008; Rees et al., 1999). Leptin was determined using a rat leptin

ELISA kit (Millipore Corp., Billerica, MA, U.S.A.) and following the manufacturer's instructions.

Behavioral procedures

We determined the locomotor response of the offspring following a single AMPH administration, as well as after repeated AMPH injection to induce behavioral plasticity, as we have previously reported (Aguilar-Valles et al., 2010; Yetnikoff et al., 2007). Briefly, animals were allowed to habituate to the apparatus for 30 minutes (AccuScan Instruments, Columbus, OH, U.S.A. n=23-50 per group). The next 5 days, animals received either a saline injection (saline pre-treatment group, 1 μl/g, i.p., n=11-26 per group) or d-AMPH sulphate salt (AMPH pre-treatment group, 2 mg/kg, i.p., n=11-26 per group) and were placed back in the boxes for 90 minutes. Seven days after termination of saline or AMPH pre-treatment, a test for behavioral sensitization was conducted, where all animals, regardless of the pre-treatment condition, received a single injection of AMPH (1 mg/kg, i.p.). Locomotor activity was monitored for 90 minutes. This lower dose of AMPH for the sensitization test was chosen to avoid stereotypy. All behavioral measurements were performed between 0900 and 1600 hours.

HPLC and western blotting

Collected brains were immersed in 2-methylbutane (Fisher Scientific, Hampton, NH, U.S.A.) and chilled with dry ice as previously reported (Aguilar-Valles et al., 2010). 300 μm thick cryostat sections were obtained and bilateral punches from the Nucleus accumbens (NAcc) were excised using our previously described procedures (Aguilar-Valles et al., 2010; Yetnikoff et al., 2007).

HPLC analysis was conducted as we recently reported (Aguilar-Valles et al., 2010; Grant et al., 2009). Briefly, tissue punches were re-suspended in 150 or 200 μL 0.1 m phosphate buffer, pH 7.0, and filtered using 0.45-μm syringe filters. A 3-5 μL volume of this filtrate was loaded onto a 15-cm C-18 reverse-phase column via manual injection ports (20-μL loop). Dual-channel coulometric III detectors (model 5100A; ESA, Inc., Bedford, MA, U.S.A.) were used to measure

the reduction and oxidation currents for dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC). EZChrom Data Chromatography Data System (Scientific Software, Inc., San Ramon, CA, U.S.A) was used to analyzed and quantify DA and DOPAC concentrations.

Western blots were conducted as previously described (Aguilar-Valles et al., 2007; Yetnikoff et al., 2007). Membranes were incubated with antibodies against tyrosine hydroxylase (TH, 1:5000, Chemicon, Temicula, CA, U.S.A.) and actin (HRP-coupled, 1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Data are expressed as a ratio of TH over actin optical densities.

Data analysis

All data were expressed as mean values ± S.E.M.; p values lower than 0.05 were deemed significant. Two-way ANOVA was used to analyze maternal serum cytokine levels, fever, food intake and body weight data, as well as basal and AMPH induced locomotion of the offspring with experimental group used as between-groups variable (SAL vs. TURP for serum cytokines and SAL-NSS, TURP-NSS, TURP-anti-IL-6, TURP-anti-Leptin or TURP-anti-IL-1ra for the other data sets) and time point as a within-group variable. Locomotion data during the sensitization test were also analyzed using a two-way ANOVA, with prenatal treatment (SAL-NSS, TURP-NSS, TURP-anti-IL-6, TURP-anti-Leptin or TURP-anti-IL-1ra) and pre-treatment (saline vs. AMPH) as between-groups variables. Levels of TH, DA and DOPAC in the NAcc were analysed using one-way ANOVA, with experimental group as the between-groups variable. When main effects or interactions were significant, analyses were followed up by simple effect ANOVA and Fisher's LSD posthoc tests.

Results

Maternal inflammatory insult with TURP induced the simultaneous release of IL-6, IL-1ra and leptin

Treatment with TURP to pregnant dams at GD 15 resulted in a significant increase in the circulating levels of IL-6 (Fig. IV-1A), IL-1ra (Fig. IV-1B) and leptin (Fig. IV-1C). The time courses of IL-6 and IL-1ra induction were very similar; the levels of these mediators deviated significantly from controls after the 6 h time point, peaked at 10 h (IL-6: treatment x time point interaction $F_{(5,60)}$=3.98, p=0.0035; simple effect of treatment at the 10 h time point $F_{(1,69.28)}$=17.5, p<0.0001. IL-1ra: treatment x time point interaction $F_{(5,72)}$=11.04, p<0.0001; simple effect of treatment at the 10 h time point $F_{(1,71.39)}$=52.2, p<0.0001) and receded by 24 h post-injection.

Unlike "classical" cytokines, like IL-6 and IL-1ra, the basal levels of leptin in the serum are readily detectable in the circulation, in the order of ng/ml (Friedman and Halaas, 1998; Maffei et al., 1995) and they further increase during pregnancy (Gavrilova et al., 1997). Under our experimental conditions, basal circulating leptin levels at GD 15 were 7.98 ± 0.72 ng/mL (Fig. IV-1C). In the SAL-treated group, these levels tended to decrease towards the end of the lights-on phase, whereas in those treated with TURP, levels remained more or less stable, with no decrease at the 10 h time point compared to the basal levels, resulting in significantly greater levels in the TURP-treated group (effect of treatment $F_{(1,10)}$=9.02, p= 0.013). This effect was only observed when data was expressed as percentage change from baseline (Fig. IV-1C), but not in the absolute values of this hormone in the maternal circulation (inset Fig. IV-1C). This may be due to the variability in the levels of adiposity among the pregnant rats (Inoue et al., 2008; Maffei et al., 1995), which makes the change from baseline a more adequate/accurate method to determine the effect of inflammation on leptin levels.

Neutralization of IL-6 reversed TURP-induced fever and decreased food intake, whereas leptin is involved in the TURP-induced anorexia and cachexia

Having determined the time course of circulating cytokine induction in pregnant rats at GD 15, we proceeded to neutralize each of these cytokines by means of using specific anti-serum. In order to monitor the effectiveness of the neutralization by the anti-serum treatments, we measured fever and changes in food intake and body weight as biological markers of IL-6, IL-1ra and leptin activity during inflammation. TURP induced a significant increase in core body temperature [effect of treatment at the 6 ($F_{(4,33)}$=6.57, p=0.0005) and 10 h ($F_{(4,33)}$=8.73, p=0.0005) time points, SAL-NSS vs. TURP-NSS Fisher's LSDs, p<0.05; Fig. IV-2A] and decreases in food intake [effect of treatment at the 48 ($F_{(4,198)}$=4.12, p=0.0041), 72 ($F_{(4,198)}$=13.2, p<0.0001) and 96 h ($F_{(4,198)}$=20.12, p<0.0001) time points, SAL-NSS vs. TURP-NSS Fisher's LSDs, p<0.05, Fig. IV-2B] and body weight gain [effect of treatment at the 72 ($F_{(4,156)}$=7.23, p<0.0001) and 96 h ($F_{(4,156)}$=4.17, p=0.0041) time points, SAL-NSS vs. TURP-NSS Fisher's LSDs, p<0.05; Fig. IV-2C]. Co-treatment of TURP and the IL-6 antiserum resulted in a complete attenuation of the febrile response (6 and 10 h time points: TURP-NSS vs. TURP-anti-IL-6 Fisher's LSDs, p<0.05; SAL-NSS vs. TURP-anti-IL-6 Fisher's LSDs, p>0.05; Fig. IV-2A) and a reversal in the decreased food intake (48 h time point: SAL-NSS vs. TURP-anti-IL-6 Fisher's LSDs, p>0.05; 72 h time point: TURP-NSS vs. TURP-anti-IL-6 Fisher's LSDs, p<0.05 and SAL-NSS vs. TURP-anti-IL-6 Fisher's LSDs, p>0.05; 96 h time point: TURP-anti-IL-6 vs. SAL-NSS or TURP-NSS Fisher's LSDs, p<0.05; Fig. IV-2B), whereas it left intact the effect of TURP on body weight (effect of treatment at 48 h($F_{(4,156)}$=4.1, p=0.0045), TURP-NSS vs. TURP-anti-IL-6 Fisher's LSDs, p>0.05; at 72 and 96 h time points: TURP-NSS vs. TURP-anti-IL-6 Fisher's LSDs, p>0.05 and SAL-NSS vs. TURP-anti-IL-6 Fisher's LSDs, p<0.05; Fig. IV-2C). Leptin antiserum did not affect TURP-induced fever (6 h time point: TURP-NSS vs. TURP-anti-leptin Fisher's LSDs, p>0.05; at 10 h time point: TURP-NSS vs. TURP-anti-leptin Fisher's LSDs, p>0.05 and SAL-NSS vs. TURP-anti-leptin Fisher's LSDs, p<0.05; Fig. IV-2A), however it completely reversed TURP-induced anorexia (at

48, 72 and 96 h time points: TURP-NSS vs. TURP-anti-leptin Fisher's LSDs, $p<0.05$; Fig. IV-2B) and the decrease in body weight gain (at 48, 72 and 96 h time points: TURP-NSS vs. TURP-anti-leptin Fisher's LSDs, $p<0.05$; Fig. IV-2C). The anti-leptin antiserum treatment to TURP-injected dams resulted in a greater-than control cumulative food intake (at 72 and 96 h time points: SAL-NSS vs. TURP-anti-leptin Fisher's LSDs, $p<0.05$) and gain in body weight (at 48 and 72 h time points: SAL-NSS vs. TURP-anti-leptin Fisher's LSDs, $p<0.05$). Finally, and quite unexpectedly, IL-1ra antiserum did not change any of the physiological responses measured (Fig. IV-2A, B and C).

None of the treatments (either TURP or the co-administration of any of the antisera) induced any significant change in the duration of gestation, litter size, body weight of the offspring at birth or throughout the postnatal development (data not shown).

Prenatal TURP enhanced the acute response to AMPH through IL-6 and IL-1ra, whereas its effects on behavioural sensitization seem to depend on leptin

Prenatal TURP-NSS treatment at GD 15 induced a significant increase in the locomotor activating effects of AMPH in the adult offspring (effect of prenatal treatment $F_{(1,31)}=4.35$, $p=0.045$; Fig. IV-3B), without affecting the basal locomotor response of these animals (Fig. IV-3A) and similar to what we have previously demonstrated (Aguilar-Valles et al., 2010). Co-administration of anti-IL-6 antiserum resulted in a reversal of this phenotype (effect of prenatal treatment $F_{(4,69)}=2.87$, $p=0.029$, TURP-NSS vs. TURP-anti-IL-6 Fisher's LSD $p<0.05$) as the offspring of TURP-anti-IL-6 treated dams presented a locomotor response statistically identical to that of the SAL-NSS offspring (SAL-NSS vs. TURP-anti-IL-6 Fisher's LSD $p>0.05$; Fig. IV-3C). Intriguingly, neutralization of IL-1ra during prenatal inflammation also resulted in the prevention of the sensitized locomotor response to AMPH induced by TURP (SAL-NSS vs. TURP-anti-IL-1ra Fisher's LSD $p>0.05$; Fig. IV-3C), suggesting a causative role for this mediator in the effects of prenatal inflammation in the neurodevelopment of the DA function of the offspring. Finally, prenatal neutralization of leptin during

TURP-induced inflammation did not prevent the enhancement of the locomotor response to AMPH induced by maternal inflammation (SAL-NSS vs. TURP-anti-leptin Fisher's LSD $p<0.05$ and TURP-NSS vs. TURP-anti-leptin Fisher's LSD $p>0.05$; Fig. IV-3C).

Administration of AMPH for five consecutive days, in order to produce behavioural plasticity, induced a gradual increase in the locomotor response to AMPH in all offspring groups (effect of administration day $F_{(4,292)}=30.64$, $p<0.0001$; Fig. IV-4A). This effect was significantly greater in the adult offspring of TURP-NSS treated dams, compared to the offspring of control, SAL-NSS-treated dams (SAL-NSS vs. TURP-NSS Fisher's LSD $p<0.05$; Fig IV-4A). The administration of anti-IL-6 antiserum to TURP-treated dams effectively prevented this effect in the adult offspring, confirming the role of this pro-inflammatory cytokine in the effects of prenatal inflammation on the behavioural responses to AMPH in the adult offspring (SAL-NSS vs. TURP-anti-IL-6 Fisher's LSD $p>0.05$; Fig IV-4A). Importantly, as we had observed for the acute response to AMPH, neutralization of IL-1ra in TURP treated dams also resulted in the reversal of the effect of prenatal inflammation on AMPH-induced locomotion (SAL-NSS vs. TURP-anti-IL-1ra Fisher's LSD $p>0.05$; Fig IV-4A). Compared to the other two mediators, the effect of leptin on these behavioural responses was rather unique. Prenatal administration of anti-leptin antiserum to TURP-treated mothers did not alter the enhanced locomotor response to AMPH, induced by TURP, for the first two days of exposure to the psychomotor stimulant (Fig IV-4A). However, on the third day of the AMPH pre-treatment phase, the locomotor response in this group dropped to levels comparable with those in the SAL-NSS offspring, and did not differ from this groups for the rest of the treatment days (SAL-NSS vs. TURP-anti-leptin Fisher's LSD $p>0.05$; Fig IV-4A).

On the day of the sensitization test, the AMPH-pre-treated offspring from all of the experimental groups showed a greater locomotor effect to the lower dose of AMPH compared to their saline-pre-treated counterparts (effect of pre-treatment $F_{(1,137)}=90.52$, $P<0.0001$; Fig. IV-4B). Importantly, this sensitized locomotor response was significantly greater in the TURP-NSS offspring,

compared to the SAL-NSS counterparts (simple effect of prenatal treatment in the AMPH-pre-treated animals $F_{(4,137)}$=2.98, p=0.02; SAL-NSS vs. TURP-NSS Fisher's LSD p<0.05; Fig IV-4B). Similarly to what we observed during the pre-treatment phase, neutralization of either IL-6 or IL-1ra during the prenatal inflammatory challenge resulted in the prevention of the effects of TURP on this behavioural response (SAL-NSS vs. TURP-anti-IL-6 or TURP-anti-IL-1ra Fisher's LSDs p>0.05 and TURP-NSS vs. TURP-anti-IL-6 or TURP-anti-IL-1ra Fisher's LSDs p<0.05; Fig IV-4B). These results strongly suggest that these two cytokines, despite having opposite roles during inflammation, have a similar contribution to the effects of prenatal inflammation on neurodevelopment, which lead to an enhanced locomotor response to AMPH treatment in the adult offspring.

Administration of anti-leptin antiserum to TURP treated mothers also prevented the effect of TURP on the sensitized response to AMPH (SAL-NSS vs. TURP-anti-leptin Fisher's LSD p>0.05 and TURP-NSS vs. TURP-anti-leptin Fisher's LSD p<0.05; Fig. IV-4B). These results suggest that prenatal TURP-induced leptin may be involved in the alterations of brain mechanisms that give rise to enhanced behavioural plasticity following repeated AMPH administration. In contrast to IL-6 and IL-1ra, leptin's contribution to the induction of a baseline sensitized state of the DA system, which modulates the initial responses to AMPH in the adult offspring, may be negligible. These data also suggests that alteration of these two phenomena by prenatal inflammation (i.e. acute vs. sensitized locomotor response to AMPH) may be due to effects on segregated brain mechanisms.

Prenatal TURP increases baseline expression of tyrosine hydroxylase and DA levels in the nucleus accumbens through IL-6, IL-1ra and leptin

We have demonstrated that prenatal TURP-treatment results in the increase of TH, DA and DOPAC in the NAcc of the adult offspring (Aguilar-Valles et al., 2010). This effect seems to be highly restricted to this brain region, as no changes are detected in other DA terminal or somatodendritic regions (Aguilar-Valles et al., 2010). We therefore started by analysing the expression of TH in the NAcc of

the adult offspring, in order to determine the relative contribution of TURP-induced IL-6, IL-1ra or leptin on the increased expression of this marker. TH expression levels were increased by prenatal TURP-NSS treatment at GD 15 compared to the control counterparts (effect of prenatal treatment $F_{(4,24)}$=2.94, p=0.04, SAL-NSS vs. TURP-NSS Fisher's LSD p<0.05; Fig. IV-5A). Co-administration of anti-IL-6 anti-serum, as well as anti-IL-1ra or anti-leptin antiserum, totally abolished this increase with the TH levels in the NAcc being comparable to those found in the SAL-NSS control group (SAL-NSS vs. TURP-anti-IL-6 or TURP-anti-leptin or TURP-anti-IL-1ra Fisher's LSDs p>0.05 and TURP-NSS vs. TURP-anti-IL-6 or TURP-anti-IL-1ra Fisher's LSDs p<0.05; Fig. IV-5A). All of the three mediators tested seem to contribute in a similar fashion to the increased expression of NAcc TH in the adult offspring.

We then determined the contribution of each cytokine to the effects of prenatal TURP on NAcc DA and DOPAC levels. DA levels were significantly increased by the prenatal TURP-NSS treatment (effect of prenatal treatment $F_{(4,28)}$=3.47, p=0.02, SAL-NSS vs. TURP-NSS Fisher's LSD p<0.05; Fig. IV-5B). This effect was prevented by maternal co-administration of anti-IL-6 antiserum (SAL-NSS vs. TURP-anti-IL-6 Fisher's LSD p>0.05 and TURP-NSS vs. TURP-anti-IL-6 Fisher's LSD p<0.05; Fig. IV-5B), as well as by prenatal administration of either anti-IL-1ra or anti-leptin antiserum (SAL-NSS vs. TURP-anti-IL-1ra or TURP-anti-leptin Fisher's LSD p>0.05 and TURP-NSS vs. TURP-anti-IL-1ra or TURP-anti-leptin Fisher's LSD p<0.05; Fig. IV-5B). These data correspond with the effects of all of the tested mediators on TH expression, and suggests that IL-6 as well as IL-1ra and leptin contribute to the increased synthetic ability of DA in the NAcc in the offspring of TURP-treated mothers.

Finally, we measured NAcc DOPAC and found that the offspring of TURP-NSS-treated mothers presented a marked trend for increased levels of this metabolite (effect of prenatal treatment $F_{(4,26)}$=3.85, p=0.0138, SAL-NSS vs. TURP-NSS Fisher's LSD p=0.09; Fig. IV-5C). Importantly, maternal co-administration of anti-IL-6 or anti-IL-1ra antiserum prevented the appearance of this effect (SAL-NSS vs. TURP-anti-IL-6 or TURP-anti-IL-1ra Fisher's LSDs

$p>0.05$ and TURP-NSS vs. TURP-anti-IL-6 or TURP-anti-IL-1ra Fisher's LSDs $p<0.05$), whereas that of anti-leptin antiserum was ineffective in reversing it (SAL-NSS vs. TURP-anti-leptin Fisher's LSDs $p<0.05$ and TURP-NSS vs. TURP-anti-leptin $p>0.05$; Fig. IV-5C). These results, along with our behavioural data, suggest that prenatal increases in IL-6 and IL-1ra are involved in the induction of very similar alterations in the DA neurotransmission of the adult offspring, suggestive of effects on neurodevelopment through a common mechanism. In contrast, prenatal increases in leptin make distinct contribution to the affected DA neurotransmission, affecting mainly the mechanisms of behavioural sensitization by repeated AMPH administration.

Discussion

In the present study, we determined that maternal cytokines, including the pro-inflammatory IL-6, the anti-inflammatory IL-1ra and the immune modulator leptin, contribute to the effects of prenatal inflammation on the DA neurotransmission of the adult offspring. IL-6 and IL-1ra, despite having been described to play opposite roles in inflammation (Bluthé et al., 2000; Cartmell et al., 2001), appear to have a similar contribution to the induction of alterations in the adult offspring. Neutralization of either IL-6 or IL-1ra during prenatal inflammation prevented the appearance of several TURP-induced phenotypes in the adult offspring, including the enhanced response to acute and repeated AMPH administration, and increased sensitised locomotor response to AMPH. These two cytokines were in addition involved in the elevated TH, DA and DOPAC levels in the NAcc of the adult offspring. Elevation of leptin levels during inflammation is involved instead in more specific effects, for example in the alteration of the mechanisms that lead to enhanced sensitization following repeated AMPH administration in the adult offspring and in the induction of elevated TH and DA in the NAcc. This suggests that whereas IL-6 and IL-1ra may be acting through a common downstream mediator/mechanism to affect foetal neurodevelopment, leptin may have a different target.

Similar to prenatal TURP administration, other inflammatory agents, namely LPS and poly I:C, induce an enhancement of the locomotor response to acute AMPH injection and alter the expression of several markers of DA neurotransmission in the mesolimbic system (Fortier et al., 2004; Meyer et al., 2008a; Meyer et al., 2008b; Meyer et al., 2006a; Meyer et al., 2008c; Meyer et al., 2006b; Meyer et al., 2008e; Ozawa et al., 2006; Smith et al., 2007; Vuillermot et al., 2010; Zuckerman et al., 2003; Zuckerman and Weiner, 2005). The elucidation of the inflammatory mechanisms leading to this phenotype is fundamental, given the preeminent role of enhanced mesolimbic DA function in the pathophysiology of psychosis, including schizophrenia (Davis et al., 1991; Weinberger, 1987).

Previous studies indicated a central role for the pro-inflammatory cytokine IL-6 for the behavioural effects of prenatal poly I:C administration (Smith et al., 2007), however the impact of this mediator on markers of DA neurotransmission was not reported. In addition, others showed that overexpression of the anti-inflammatory IL-10, while preventing several behavioural effects of poly I:C in the adult offspring, did not prevent the enhanced locomotor response to AMPH (Meyer et al., 2008b). We previously reported that hypoferremia of inflammation, a response triggered by IL-6 during induced inflammation (Nemeth et al., 2004a), is indeed involved in triggering the enhanced behavioural response to AMPH in the adult offspring and in some of the biochemical markers of DA neurotransmission, especially TH expression in the NAcc (Aguilar-Valles et al., 2010). It is therefore feasible that IL-6 may be affecting neurodevelopment through its effects on iron metabolism; alternatively this cytokine may exert direct effects on the foetal compartment (Dahlgren et al., 2006). These two scenarios are not mutually exclusive, and the data presented in the current study suggests that this may be the case, as all the effects of prenatal TURP administration on the markers of DA neurotransmission were prevented by neutralizing IL-6 during the maternal inflammatory response.

Neutralization of IL-1ra during maternal inflammation resulted in a similar phenotype to that obtained when neutralizing IL-6, suggesting that these mediators may be acting through the same downstream mechanism. This was rather unexpected, since it has been recently shown that administration of IL-1ra to pregnant dams prevented the damaging effects of LPS administration in the placenta and the foetal brain (Girard et al., 2010). It is important to note, however, that when administered from an exogenous source, IL-1ra may have different effects compared to the role of the endogenously expressed mediator. This is especially true considering that following LPS administration to late pregnant females, there is a greater that normal induction of circulating IL-1ra (Ashdown et al., 2007). We and others have observed that addition of human recombinant IL-1ra (hrIL-1ra), in the context of acute inflammation, can attenuate the release of pro-inflammatory cytokines and several sickness behaviours (Ashdown et al.,

2007; Bluthé et al., 1992; Girard et al., 2010; Luheshi et al., 1996a; Luheshi et al., 1997; Miller et al., 1997). In the case of the present study, however, the neutralization of IL-1ra did not alter any of the sickness responses measured, including fever, decreased food intake and body weight gain. Although one possible explanation for this effect is simply the lack of effectiveness of the anti-serum, the fact that it prevented all of the markers of DA neurotransmission and sensitization of the AMPH locomotor responses in the offspring, suggests that the intervention was indeed effective.

In male rats, neutralization of IL-1ra prolonged the intra-pouch LPS-induced fever (another model of localized inflammatory challenge), without affecting the peak of fever curve, suggesting a role of this mediator in deffervescence or termination of fever (Cartmell et al., 2001). However, co-administration of hrIL-1ra with TURP (Luheshi et al., 1997) or LPS (Luheshi et al., 1996a; Miller et al., 1997) results in an overall blunted febrile response. The different results between these two approaches suggests that some of the observed effects of administration of hrIL-1ra may reflect the roles of IL-1β or a pharmacological effect, rather than the physiological role of the endogenous mediator. Neutralizing the biological activity of the endogenous mediator appears as a more adequate strategy to elucidate the role of inflammation-induced IL-1ra. Finally, the discrepancy between the study of the role of endogenous IL-1ra in deffervescence (Cartmell et al., 2001) and ours, showing a lack of effect, may also be explained by the hypothesis that IL-1ra may not be playing a central role in the control of fever and other inflammatory responses during pregnancy, at least for models of localised inflammation (Aguilar-Valles et al., 2007).

Although we cannot rule out the possibility that IL-1ra may be affecting neurodevelopment through an indirect mechanism, as IL-6 may do (Aguilar-Valles et al., 2010), these two cytokines could directly target the foetal brain. In fact, it has been shown that in rats IL-6 can trespass into the foetal circulation (Dahlgren et al., 2006); although similar data for IL-1ra is not yet available. Our results suggest that these two mediators could trigger similar alterations in the foetal brain. The main biological function of IL-1ra is as a competitive antagonist

of the activity of the IL-1 receptor (IL-1RI) (Dinarello, 1991). It is therefore conceivable that increased levels of this cytokine during inflammation may affect the foetal development through the blockade of IL-1. For example, the absence of a functional IL-1RI in mice throughout development induces an increase in the size of the terminal tree of DA neurons innervating the striatum (Parish et al., 2002). Intriguingly this same phenotype was observed in the IL-6 knock out mice (Parish et al., 2002). These effects are rather similar to the phenotype of rats born to TURP treated mothers, which present increased expression of TH in the NAcc, but not in the VTA, where the cell bodies of the neurons that innervate the NAcc are located, suggesting increased innervation of this region probably as a result of the prenatal insult [present study and (Aguilar-Valles et al., 2010)].

The effects of cytokines on developing DA neurones may however depend on the dose and/or the length of the stimulus, as it has been demonstrated for IL-1β, IL-6 and IL-1ra. For example, chronic IL-1β overexpression in the SN causes a progressive degeneration and death of DA neurons of this region (Ferrari et al., 2006; Koprich et al., 2008; Viviani et al., 2004). While these effects are reversed by IL-1ra administration (Koprich et al., 2008), overexpression of IL-1ra itself induced a decrease in the concentrations of DA and its metabolites in striatum and other brain regions (Oprica et al., 2005). The congenital absence of IL-6 during development (i.e. IL-6 "knock-out" mice), results in a smaller number of mesencephalic TH positive neurons, suggesting a role for this cytokine in survival and/or differentiation of DA neurons (Parish et al., 2002). Indeed, *in vitro*, IL-6 induces survival and differentiation of neuronal cells (Gadient and Otten, 1997; Satoh et al., 1988), especially mesencephalic TH positive neurons (Hama et al., 1991). Finally, IL-6 released from astrocytes following a mild inflammatory insult enhances the survival of DA neurons, whereas a greater inflammatory stimulation leads to death of the same cells (Li et al., 2009c).

As IL-1 can stimulate the synthesis of IL-6 in both neurons and astrocytes (Benveniste et al., 1990; Gadient and Otten, 1997; Horie et al., 1997; Norris et al., 1994), one possible mechanism through which IL-1ra and IL-6 may act to affect neurodevelopment of DA cells could be that IL-1ra through inhibiting the

induction of foetal brain IL-6, will promote sprouting of DA terminals, as occurs with the congenital absence of either IL-1RI or IL-6 (Parish et al., 2002).

The results from the present study suggest that leptin has a slightly different role in the effects of prenatal inflammation on the dopamine function of the adult offspring, affecting mainly mechanisms that lead to sensitization following repeated AMPH administration. As the actions of AMPH in the NAcc are associated with its ability to enhance locomotor activity (Heusner et al., 2003; Hoebel et al., 1983), whereas its actions in the VTA are associated with the induction of behavioural sensitization (Vezina, 2004), it could be expected that the effect of elevated levels of leptin during gestation may be more related with sensitization.

It is also possible that the observed effects of this cytokine may be linked to its role as a regulator of energy balance, which is shifted towards a reduction in food intake and subsequent weight loss in the pregnant mothers, following inflammation. Leptin, however, may also control the expression of cytokines in the placenta (Lappas et al., 2005), which in turn may be more centrally involved in the described effects. Finally, increased levels of this cytokine may be reaching the foetal compartment, leading to altered neurodevelopment of the DA systems. In the case of the latter scenario, leptin receptors are known to be expressed in the mesencephalon as early as GD 14 (Matsuda et al., 1999; Udagawa et al., 2000). Leptin is able to stimulate signalling (STAT3 phosphorylation) in the DA and GABA neurons of the VTA that project to the NAcc (Fulton et al., 2006; Hommel et al., 2006). This cytokine-like hormone has also been shown to increase AMPH-induced locomotion (Fulton et al., 2006; Hao et al., 2004) and to modulate brain reward circuitry by altering performance for rewarding brain stimulation (Figlewicz et al., 2006; Fulton et al., 2004; Fulton et al., 2000; Shalev et al., 2001). More specifically, genetic deficiency of leptin (i.e. the o*b/ob* mice) induces diminished locomotor response to AMPH, sensitization after repeated AMPH administration, reduced release of DA in the NAcc and decreased expression of TH in the NAcc and VTA (Fulton et al., 2006; Leinninger et al., 2009). All these alterations are reversed by chronic leptin administration (Fulton et al., 2006). In

addition, acute leptin administration increases AMPH-induced release of DA in the NAcc, by modulating the activity of TH and DAT, without affecting their expression (Figlewicz et al., 1998; Perry et al., 2010). It is considered that leptin plays an important role in the reorganization of the brain circuits that underlies the sensitization process (Fulton et al., 2006) by regulating somatodendritic DA release in the VTA (Roseberry et al., 2007).

Our data suggests that inflammation-induced increases in IL-6 and IL-1ra are involved in the induction of an enhanced basal DA neurotransmission in the offspring, whereas leptin would be more specifically involved in affecting those mechanisms involved in the development of sensitization. What we observed in the offspring of mothers treated with any of the antisera was a prevention of the effects of prenatal TURP and their return to levels found in the control animals. This suggests that the neutralization of inflammatory mediators reflected their role in neurodevelopment during episodes of prenatal inflammation and not a side effect of the treatment.

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Figures

Figure IV-1. Maternal treatment with turpentine oil induces the release of interleukin (IL)-6, IL-1 receptor antagonist (ra) and leptin

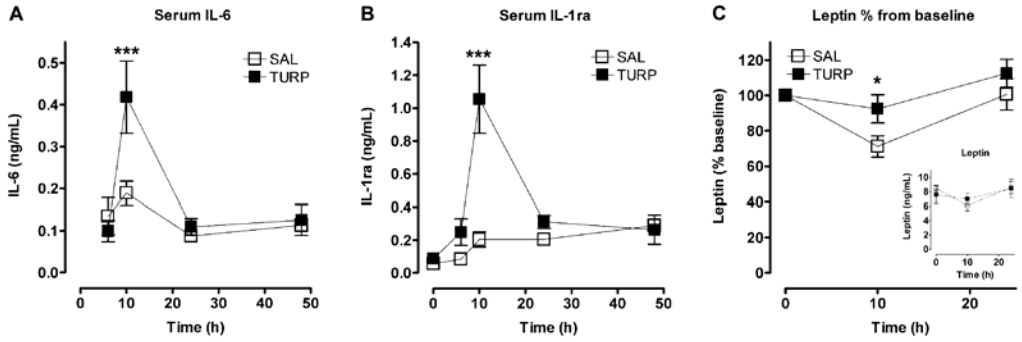

*Figure IV-1. (A) Maternal administration of turpentine oil (TURP, i.m., 100 μL/rat) induced a significant increase in circulating IL-6, 10 h after treatment (n=5-7/group). (B) A similar kinetics was observed for the TURP-induced IL-1ra. (C) Leptin levels were significantly greater at the 10 h time point, when expressed as change from baseline (0 h) values. The actual levels of leptin (inset) were not significantly altered, however, due to individual variations in adiposity, which affect leptin levels in the circulation, a more adequate analysis of the effects of inflammation would be to compare changes in concentration from the baseline levels. *** p<0.0001 and * p<0.05 compared to SAL-treated dams at the same time point.*

Figure IV-2. Neutralization of maternal cytokines during TURP-induced inflammation differentially modulates fever, anorexia and maternal body weight

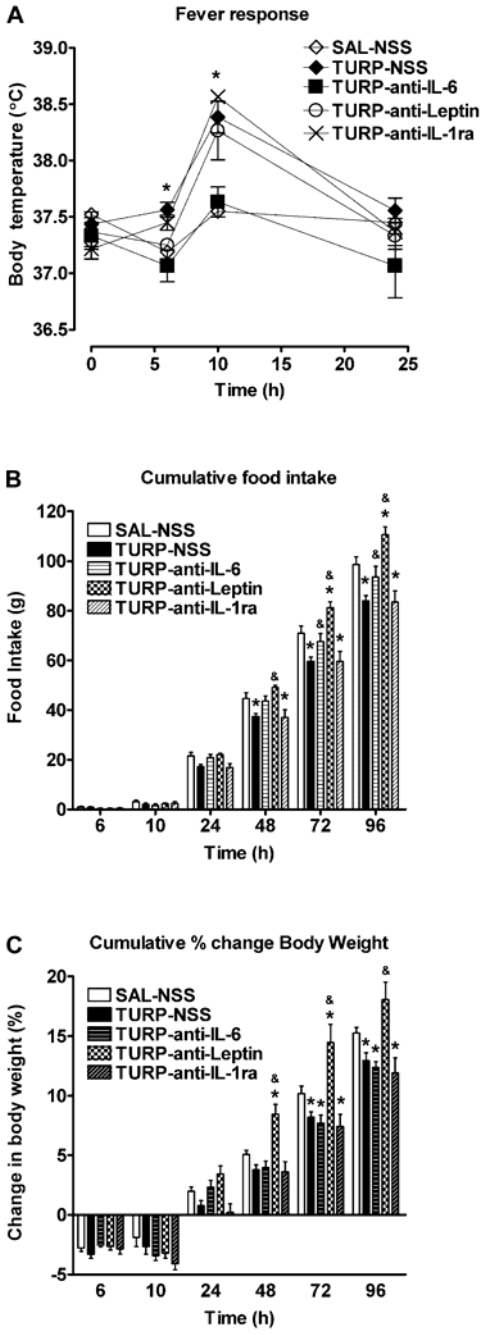

Figure IV-2. (A) Maternal treatment with TURP (as in Fig. IV-1) induced a significant increase in core body temperature 6 and 10 h after injection (n=10).

*This effect was completely prevented by co-treatment with anti-IL-6 antiserum (n=6), whereas neutralization of wither IL-1ra (n=6) or leptin (n=6) did not change the maternal febrile response. (B) TURP induced as well a reduced in maternal cumulative food intake. This effect was significant 48, 72 and 96 hours after the inflammatory treatment. Neutralization of either IL-6 or leptin reversed this effect at all time points, however anti-leptin antiserum administration resulted in food intake levels greater than that of SAL-NSS (n=9) treated dams. Anti-IL-1ra antiserum did not affect this response to inflammation. (C) TURP-induced reductions in food intake were followed by a decrease in the amount of body weight gained. This decrease rate in body weight gain was prevented only by anti-leptin antiserum co-administration, whereas IL-6 and IL-1ra did not appear to be involved. * p<0.05 vs. SAL-NSS and & p<0.05 vs. TURP-NSS at the corresponding time point.*

Figure IV-3. Maternal neutralization of IL-6 or IL-1ra prevents enhancement of locomotor response to AMPH induced by prenatal TURP

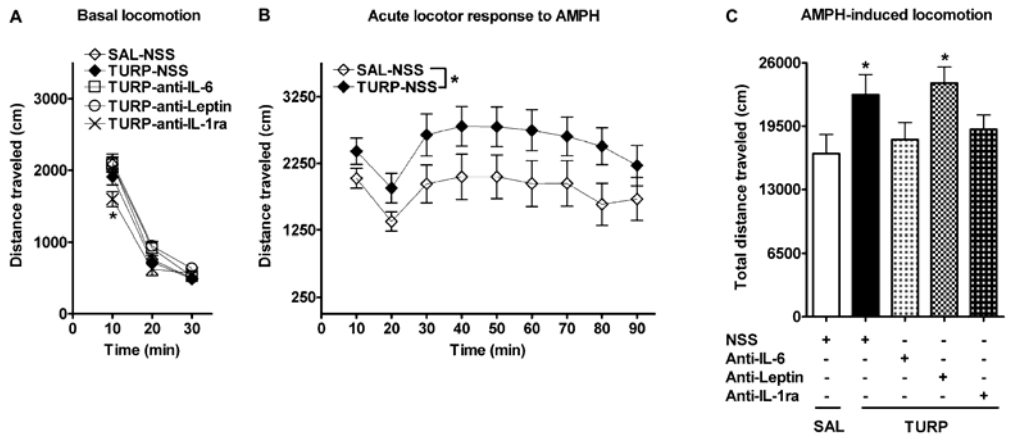

Figure IV-3. (A) Basal locomotor activity was not affected in the offspring by prenatal TURP administration or co-treatment with anti-IL-6, anti-IL-1ra or anti-leptin antiserum (n=23-50 per group). Following AMPH administration (i.p. 2 mg/kg), locomotion increased in the offspring of SAL-NSS or TURP-NSS treated dams. This effect was, however, significantly greater in the TURP-NSS offspring.

*(C) Total AMPH-induced locomotor activity was significantly induced by prenatal TURP-NSS treatment, and reversed by either anti-IL-6 or anti-IL-1ra antiserum co-administration. In contrast, prenatal leptin neutralization did not alter the effect of prenatal TURP in this response of the adult offspring. * p<0.05 vs. SAL-NSS group.*

Figure IV-4. Maternal IL-6, IL-1ra and leptin contribute enhanced behavioural sensitization to repeated AMPH administration induced by prenatal TURP

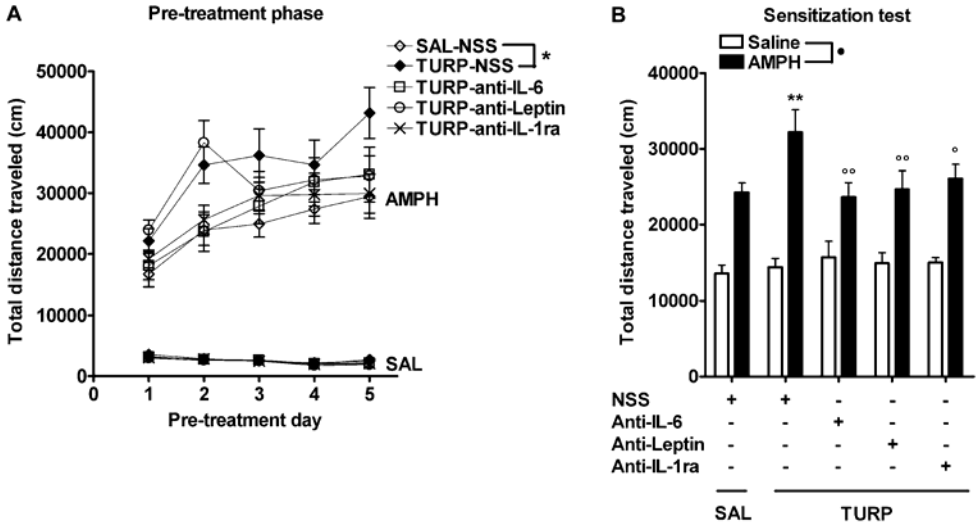

Figure IV-4. (A) Offspring were treated with AMPH (as in Fig. IV-3B) or saline for 5 consecutive days and locomotor response recorded on each day. Prenatal TURP-NSS treatment induced an overall significant increase in the response to AMPH. Neutralization of either IL-6 or IL-1ra, as during the first day of AMPH administration, prevented this effect. Anti-leptin antiserum co-administration had a different effect: while sparing the effect of prenatal TRUP for the first two days of AMPH injection, it prevented the enhancement induced by prenatal inflammation from the third day of AMPH administration onwards. (B) Seven days after the pre-treatment phase, saline and AMPH pre-treated animals were given a dose of AMPH (1 mg/kg) and locomotor response compared. All AMPH-pre-treated animals presented greater locomotor response than their saline-pre-

*treated counterparts. This sensitization effect was significantly greater in the TURP-NSS offspring, which was prevented by prenatal neutralization of either IL-6, IL-1ra or leptin. * p<0.05 TURP-NSS vs. SAL-NSS offspring; ● p<0.05 saline vs. AMPH pre-treatment; ** p<0.01 vs. AMPH pre-treated SAL-NSS offspring; ○ p<0.05 and ○○ P<0.01 vs. AMPH pre-treated TURP-NSS offspring.*

Figure IV-5. Neurochemical markers of DA neurotransmission in the nucleus accumbens are increased by prenatal TURP through the differential effect of IL-6, IL-1ra and leptin

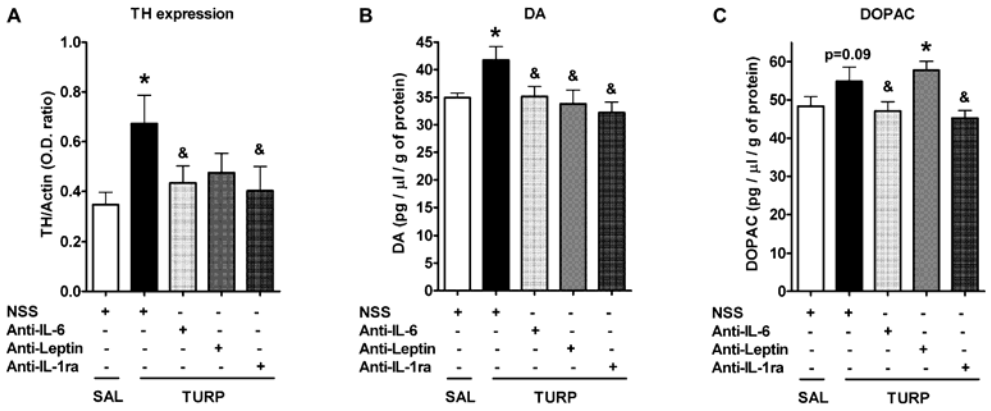

*Figure IV-5. (A) Prenatal TURP-NSS treatment resulted in increased levels of tyrosine hydroxylase (TH) in the nucleus accumbens (NAcc) of the adult offspring. This effect was prevented by the co-administration of either anti-Il6, anti-IL-1ra or anti-leptin antiserum. (B) Accompanying increased TH expression, NAcc dopamine (DA) levels were also significantly greater in the TURP-NSS offspring compared to their control counterparts. As for the effects on TH expression, all the tested cytokines contribute to this increase in DA, as their neutralization prevented the effect of prenatal TURP. (C) DOPAC was also increased in the NAcc of the offspring of TURP-NSS treated mothers. Increased DOPAC was also found in the offspring whose mothers received anti-leptin antiserum as well, whereas it was prevented by either anti-IL-6 or anti-IL-1ra antiserum. * P<0.05 vs. SAL-NSS and & p<0.05 vs. TURP-NSS.*

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Chapter V: Prenatal Inflammation-Induced Hypoferremia Alters Dopamine Function in the Adult Offspring in Rat: Relevance for Schizophrenia

V.1. Preface

Prenatal inflammatory stimulation with TURP leads to a significant alteration of the mesolimbic DA neurotransmission (Chapters III and IV). Maternal cytokines released into the circulation, especially IL-6 and IL-1ra, are involved in alterations that lead to a sensitized state of the accumbal DA neurotransmission, which underlie behavioural alterations like increased AMPH-induced locomotion (Chapter IV) and possibly, impaired PPI (Chapter III). Circulating IL-6, which others showed to be involved in the effects of prenatal poly I:C treatment (Smith et al., 2007), has been reported to cross the rat's placenta and reach the foetal compartment (Dahlgren et al., 2006), which may be one of the mechanisms through which it affects brain development. However, IL-6 induces a number of potentially damaging responses in the mother during inflammation, such as fever, stress response and hypoferremia. Although we cannot rule out a role for fever and stress hormones in the effects of infection on the developing brain, hypoferremia of inflammation is a feasible mechanism through which maternal infection may affect the development of DA neurons. Hypoferremia of inflammation is a conserved acute phase response that consists in the reduction of circulating non-haeme iron levels (see section 7.2 of Chapter I) that may lead to restriction of foetal iron supply. We observed that hypoferremia occurs during gestation, if the inflammatory insult occurs at GD 15, but not at GD 18 (Chapter III). Restricted iron supply to the developing rat has been extensively demonstrated to cause long-term effects in the DA neurotransmission (Beard and Connor, 2003), in particular increased release of DA (Unger et al., 2007) and increase behavioural response to AMPH (Youdim and Yehuda, 1985). We sought to test the contribution of inflammatory hypoferremia in the alterations induced by prenatal treatment with TURP. The strategy for this study was to supplement iron, via intraperitoneal injections of the nutrient, to a group of females that will be treated with either SAL or TURP at GD 15. The aim of parenteral iron supplementation was to bypass the regulated intestinal absorption (Nemeth and Ganz, 2006), which may be blocked during hypoferremia, and to inhibit the

induction of hepatic HAMP expression, which has been observed in instances of iron overloading (Nemeth et al., 2004a).

V.2. Manuscript

Title: Prenatal Inflammation-Induced Hypoferremia Alters Dopamine Function in the Adult Offspring in Rat: Relevance for Schizophrenia

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Abstract

Maternal infection during pregnancy has been associated with increased incidence of schizophrenia in the adult offspring. Mechanistically, this has been partially attributed to neurodevelopmental disruption of the dopamine neurons, as a consequence of exacerbated maternal immunity. In the present study we sought to target hypoferremia, a cytokine-induced reduction of serum non-haeme iron, which is common to all types of infections. Adequate iron supply to the foetus is fundamental for the development of the mesencephalic dopamine neurons and disruption of this following maternal infection can affect the offspring's dopamine function. Using a rat model of localized injury induced by turpentine, which triggers the innate immune response and inflammation, we investigated the effects of maternal iron supplementation on the offspring's dopamine function by assessing behavioral responses to acute and repeated administration of the dopamine indirect agonist, amphetamine. In addition we measured protein levels of tyrosine hydroxylase, and tissue levels of dopamine and its metabolites, in ventral tegmental area, susbtantia nigra, nucleus accumbens, dorsal striatum and medial prefrontal cortex. Offspring of turpentine-treated mothers exhibited greater responses to a single amphetamine injection and enhanced behavioral sensitization following repeated exposure to this drug, when compared to control offspring. These behavioral changes were accompanied by increased baseline levels of tyrosine hydroxylase, dopamine and its metabolites, selectively in the nucleus accumbens. Both, the behavioral and neurochemical changes were prevented by maternal iron supplementation. Localized prenatal inflammation induced a deregulation in iron homeostasis, which resulted in fundamental alterations in dopamine function and behavioral alterations in the adult offspring. These changes are characteristic of schizophrenia symptoms in humans.

Introduction

Environmental factors, combined with genetic predisposition, are now recognized as key events underlying a number of psychiatric disorders of neurodevelopmental origin, including schizophrenia. Of the environmental events, maternal infection during critical stages of human gestation has been associated with increased incidence of schizophrenia in the adult progeny (Babulas et al., 2006; Brown, 2006; Brown and Derkits; Sorensen et al., 2009). Studies in animal models of maternal infection have demonstrated a plethora of behavioral, molecular and structural alterations relevant to schizophrenia in the adult offspring (Baharnoori et al., 2009; Borrell et al., 2002; Cui et al., 2009; Fortier et al., 2004; Fortier et al., 2007; Lowe et al., 2008; Meyer et al., 2008a; Meyer et al., 2005; Ozawa et al., 2006; Romero et al., 2006; Shi et al., 2003; Zuckerman and Weiner, 2005). The mechanisms responsible for these alterations are unknown. However, because a wide variety of viral and bacterial pathogens are implicated (Brown, 2006; Ellman and Susser, 2009), it is thought that a response common to all forms of infection is involved in the aetiology of the disorder (Brown and Derkits; Patterson, 2009). One such response is hypoferremia, a cytokine-mediated reduction of circulating non-haeme iron (Lee et al., 2005; Nemeth et al., 2004a; Nemeth et al., 2003; Nicolas et al., 2002b). In normal individuals, this response is triggered to limit the availability of this essential nutrient to the invading pathogens and is thus considered an inherent protective mechanism (Grieger and Kluger, 1978; Kluger and Rothenburg, 1979; Nemeth and Ganz, 2006). Hypoferremia results from the hepcidin (HAMP)-mediated interruption of iron trafficking into the blood from body stores such as macrophages, hepatocytes and from duodenal enterocytes, which mediate dietary absorption of iron (Hentze et al., 2004; Nemeth and Ganz, 2006). Hypoferremia during pregnancy may have serious repercussions for the developing foetus. Sufficient iron supply is necessary for neurodevelopmental processes; in fact, reduction in iron supply at several stages of development results in enduring changes in dopamine (DA) neurotransmission (Beard and Connor, 2003; Beard et al., 2006; Kwik-Uribe et al.,

2000; Unger et al., 2007) that outlast the iron deficient periods (Felt et al., 2006; Kwik-Uribe et al., 2000).

We hypothesized that inflammation-induced hypoferremia causes a disruption of foetal brain development, which leads to functional defects in adulthood, synonymous with psychiatric disorders such as schizophrenia. The goal of this study was, therefore, to investigate whether changes in iron traffic induced by maternal inflammation would have an impact on DA function and DA-related behaviours in the adult offspring. To this end, we conducted studies using a rat model of localized injury and inflammation induced by an intramuscular (i.m.) injection of turpentine (TURP). In contrast to the more commonly used models of systemic bacterial [lipopolysaccharide (LPS)] or viral (poly I:C) infection, TURP remains localized at the injection site (Wusteman et al., 1990). This feature allows us to study the role of endogenous inflammatory mediators on foetal development, in the absence of possible confounding factors triggered by systemically injected immunogens, which could act directly on the foetal compartment (Ashdown et al., 2006a; Cai et al., 2000; Gayle et al., 2004; Liverman et al., 2006; Meyer et al., 2006a) and often lead to high maternal mortality (Fortier et al., 2007). In recent studies, we demonstrated that treatment of pregnant rats with TURP induces reproducible behavioral changes in the adult offspring, similar to those induced by maternal LPS or poly I:C treatments (Fortier et al., 2007). The aim of the present study was to investigate whether these effects are due to alteration in DA function resulting from reduced iron levels during a critical neurodevelopmental period (i.e. gestational day [GD] 15). Our results strongly support a major role for inflammation-induced hypoferremia in the development of enhanced DA function in the offspring.

Methods

Animals

Time pregnant primiparous Sprague-Dawley rats (Charles Rivers, QC, Canada) were used in all experiments. On GD 7 or 8 animals were individually housed in a controlled environment at an ambient temperature of 21±1 ºC on 12:12 h light-dark cycle (lights on at 0800 hours) with free access to food and water. Only male offspring were used, which were housed under the same environmental conditions. Mothers were treated with the inflammatory stimulus at GD 15. This day of pregnancy was used since it has been proposed to be roughly equivalent to the late 1st trimester of human pregnancy (Clancy et al., 2001), which has been significantly linked with the increased risk of developing schizophrenia in the offspring of infected mothers (Brown and Derkits, 2010a). Importantly, our previous studies in pregnant rats treated with TURP at GD 10, 15 or 18, suggested a window of vulnerability for the adult offspring treated at GD 15 (Fortier et al., 2007).

Ethics statement

Experimental procedures were approved by the Animal Care Committee from the Douglas Mental Health University Institute and McGill University pursuant of the Canadian Council of Animal Care (Animal Use Protocol # 4306). All efforts were made to minimize the number of animals used.

Treatments and experimental protocols

Pregnant dams were handled daily from GD 11 onwards and habituated to a rectal probe to determine core temperature (Physitemp Instruments, NJ, U.S.A.). Some animals (13 dams) received an intraperitoneal (i.p.) injection of 10 mg/kg of the aqueous complex of poly-nuclear iron (III)-hydroxide in sucrose (Venofer, American Reagent, NY, U.S.A.) or an equivalent volume of vehicle (either saline [7 dams] or 30% sucrose [7 dams]) at GD 13 and 14. Iron was administered to the dams from GD 15 until GD 18 at larger doses (i.p., 20 mg/kg). At GD 15 basal core temperature was recorded and a small sample of tail blood was collected to

determine basal levels of cytokines and serum iron. Subsequently, a maternal inflammatory response was elicited by injecting intramuscularly with 100 μL of purified TURP (Riedel-deHaën, Sleeze, Germany); control dams received an equivalent volume of saline (SAL). All injections were administered between 0900 and 1000 hours. Using a rectal probe core body temperature was measured at 8, 10, 24, 48 and 72 hours after i.m. treatment and tail blood collected at each time point. The animals were then sacrificed at GD 19 (96 hours after the i.m. treatment) with a lethal dose of pentobarbital sodium (i.p., 60 mg/kg) and a final blood sample was collected via cardiac puncture. Maternal liver, placentas, foetal livers and brains were excised and immersed in 2-methylbutane (Fisher Scientific, Hampton, NH, U.S.A.) and chilled with dry ice. Serum cytokine, iron and Tf saturation levels were determined, as well as maternal liver, placenta, foetal liver and brain tissue iron levels.

In a separate experiment, SAL and TURP treated dams (i.m., 7 rats per group) were sacrificed 10-11 hours after i.m. treatment, which represents the peak of fever and cytokine responses (Aguilar-Valles et al., 2007), and livers collected to measure mRNA expression of molecules involved in iron homeostasis.

To determine the effects of prenatal TURP and iron supplementation on DA function and related behaviours in the adult offspring, pregnant dams (6-12 per group) were treated as described above, with the exception that tail blood collection was not performed. The male offspring from each mother were marked, weighed and cross-fostered with surrogate dams in mixed litters (Fortier et al., 2004; Fortier et al., 2007). Offspring were weaned from their foster mothers at postnatal day (P) 22 and used at P 60-62, for either behavioral testing or biochemical analyses. Behavioral sensitization to AMPH was used to gauge midbrain DA function and plasticity in the adult offspring. In addition, protein expression levels of TH were determined by Western blotting from extracts obtained from the VTA, SN, NAcc, dSTR and mPFC. Tissue levels of DA and metabolites (DOPAC and HVA) were determined by High Performance Liquid Chromatography (HPLC) from NAcc, dSTR and mPFC.

Behavioral testing

We measured locomotor responses to either single or repeated injections of d-amphetamine sulphate salt (AMPH, Sigma-Aldrich, Dorset, UK) in adult male offspring, using our well-established protocol (Yetnikoff et al., 2007). Briefly, locomotor activity was quantified with an infrared activity-monitoring apparatus for rats (AccuScan Instruments, Columbus, OH, U.S.A.). On day 1 all rats (n=23-50 per group) were habituated to the boxes for 30 minutes (basal locomotor activity). On day 2 all animals received a saline injection (1 μl/g, i.p.) and were placed back in the boxes for 30 minutes. Immediately after, one half of the animals (saline pre-treatment group, 11-26 per group) received another saline injection, and the other half was injected with AMPH (2 mg/kg, i.p.); locomotor activity was monitored for an additional 90 minutes. On days 3, 4, 5 and 6, animals received an injection of either saline (saline pre-treatment group) or AMPH (2 mg/kg, AMPH pre-treatment group) and their locomotor activity was measured for 90 minutes. Finally, on day 14, a week after termination of saline or AMPH pre-treatment, a test for behavioral sensitization was conducted, where all animals, regardless of the pre-treatment condition, received a single injection of AMPH (1 mg/kg, i.p.). Locomotor activity was monitored for 90 minutes. This lower dose of AMPH for the sensitization test was chosen to avoid stereotypy. All behavioral measurements were performed between 0900 and 1600 hours.

Serum cytokine and iron determination

All blood samples were allowed to cloth at room temperature for 1 hour, spun at 4000 rpm for 20 min at 4 ºC to obtain serum and stored at -80 ºC until used. Maternal serum samples were analyzed for IL-6 and IL-1ra (the main cytokines released into the circulation by TURP treatment (Aguilar-Valles et al., 2007)) using species specific ELISA (NIBSC, Potters Bar, UK) as described previously (Rees et al., 1999). Serum iron (SI) was measured using an iron kit (RANDOX, Mississauga, ON, Canada). In parallel, a direct measure of total iron binding capacity (TIBC) of the serum was determined using a TIBC kit (RANDOX). For both procedures the manufacturer's protocols were followed.

Once both measurements were obtained (expressed as μg/dL of serum), serum T) saturation was calculated by expressing the serum iron content as percentage of TIBC (Tf saturation = SI/TIBC x 100).

Three foetuses per dam were collected, and the results from their individual measurements averaged to obtain one value per mother. For tissue iron content, maternal liver, placenta, foetal liver and brain were homogenized in 1:10 (w/v) high-purity water. One volume of protein-precipitation solution (1 N HCl and 10 % (v/v) trichloroacetic acid in high purity water) was then added to the samples, blank and iron standards and incubated for 1 hour at 95 ºC. The samples were then allowed to cool at room temperature for 2 min, vortex mixed and centrifuged at 10 000 x g for 10 min at room temperature (Hofer et al., 2008; Rebouche et al., 2004) and assayed for iron content.

Quantitative RT-PCR

Total RNA was extracted and reverse transcribed from maternal livers, placenta, foetal liver and brain as described previously (Pohl et al., 2009b). Real-time PCR was performed in duplicate using pre-optimized primer/probe mixture (TaqMan Gene Expression Assays, Applied Biosystems, ON, Canada) and TaqMan universal PCR master mix (Applied Biosystems). The housekeeping gene 18S was used to normalize levels of cDNA expression for each sample. Levels of gene expression were calculated as the X-fold difference from the control groups (SAL-VEH group). The mRNA levels of the following genes was assessed: suppressor of cytokine signalling 3 (SOCS3), used as a marker of activity of the JAK/STAT3 signalling pathway activated by IL-6 (Lebel et al., 2000a); HAMP and zip14, a dual iron/zinc importer that has been shown to be induced by TURP and may be involved in the cellular sequestration of iron (Liuzzi et al., 2006; Liuzzi et al., 2005). The assay ID for each gene is as follows: SOCS3: Rn00585674-s1, HAMP: Rn00584987-m1, zip14 (slc39a14): Rn01468335-g1 and 18S: EUK-18S-rRNA4352930.

Western blotting and HPLC

We explored biochemical markers of DA neurons, including protein expression of TH and tissue levels of DA and its metabolites, DOPAC and HVA. Brains collected from adult male offspring (P 60-62) by decapitation were immersed in 2-methylbutane (Fisher Scientific, Hampton, NH, U.S.A.) and chilled with dry ice. 300 μm thick cryostat sections were obtained. There were no behavioral differences between the animals born to mothers supplemented with either SAL or sucrose. Thus, to minimize the number of animals used, only SAL-SAL and TURP-SAL groups were included. Bilateral punches from the VTA, SN, NAcc (including core and shell), dSTR and mPFC (including cingulated areas 1 and 2) were excised using our previously described procedures (Yetnikoff et al., 2007) using the Paxinos and Watson rat brain atlas (Paxinos, 1998).

Western blots were conducted as previously described (Aguilar-Valles et al., 2007; Yetnikoff et al., 2007). Membranes were incubated with antibodies against TH (1:5000, Chemicon, Temicula, CA, U.S.A.) and actin (HRP-coupled, 1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Data are expressed as a ratio of TH over actin optical densities.

HPLC analysis was conducted as we recently reported (Grant et al., 2009). Briefly, tissue punches were re-suspended in 100 μL 0.1 m phosphate buffer, pH 7.0, and filtered using 0.45-μm syringe filters. A 10-μL volume of this filtrate was loaded onto a 15-cm C-18 reverse-phase column via manual injection ports (20-μL loop). Dual-channel coulometric III detectors (model 5100A; ESA, Inc., Bedford, MA, U.S.A.) were used to measure the reduction and oxidation currents for DA and DA metabolites (one channel was used for DA and the other for DA metabolites). EZChrom Data Chromatography Data System (Scientific Software, Inc., San Ramon, CA, U.S.A) was used to analyzed and quantify DA, DOPAC and HVA concentrations.

Data analyses

All data were analyzed as raw values, except for HPLC results, where the data were analyzed as percentage of the average value for the SAL-VEH group,

due to large inter-assay variability between two different batches of samples assayed. All data are presented as mean values ± s.e.m. p values < 0.05 values were deemed significant.

Maternal fever, serum cytokines and iron: data were analyzed using a repeated measures 2-way ANOVA, with time as a within-group variable and pre-natal treatment (SAL vs. TURP) as a between-groups variable.

Maternal liver PCR data: data from the 10-11 hours experiment were analyzed using two-tailed Student's t-tests to compare data from the SAL-VEH and TURP-VEH groups.

Maternal serum Tf saturation: data were analyzed using a 3-way ANOVA with prenatal treatment (SAL vs. TURP) and maternal supplementation (VEH vs. IRON) used as between-groups variables in all cases and time point used as a within-group variable. When the interactions of main effects were significant, analysis was followed by performing simple-effects ANOVA and Fisher's LSD post hoc tests.

Tissue iron content, HAMP mRNA levels (96 h time point), western blotting and HPLC: data were analyzed using 1-way ANOVA, with maternal treatment (SAL-VEH vs. TURP-VEH vs. SAL-IRON vs. TURP-IRON) as between-groups variable and furthered with Fisher's LSD post hoc tests when ANOVA was significant.

Offspring's basal and AMPH-induced locomotion: data were analyzed using 3-way ANOVAs, with prenatal treatment (SAL vs. TURP) and maternal supplementation (VEH vs. IRON) as between-groups variables and time as a within-group variable. When interactions were significant, analyses were followed by simple-effects ANOVA and Fisher's LSD post hoc tests.

AMPH sensitization test: data were analyzed using a 3-way ANOVA, with prenatal treatment (SAL vs. TURP), maternal supplementation (VEH vs. IRON) and pre-treatment (saline vs. AMPH) used as a between-groups variables, followed by simple-effects ANOVA and Fisher's LSD post hoc tests when interactions were significant.

Results

TURP induces an acute-phase response and hypoferremia in GD 15 pregnant dams

In order to characterize the maternal inflammatory response to TURP and the alterations that this may induce in systemic iron trafficking, we determined the kinetics of several inflammatory markers and maternal serum iron levels at GD 15. TURP induced a significant increase in core body temperature at 10 hours, with a 1.9 ºC difference in comparison to the SAL group and returned to basal 24 h after injection (Fig. V-1A; treatment by time interaction, $F_{(4,48)}$=7.74, p<0.001, simple effect of treatment at 10 h: $F_{(1,53.84)}$=37.82, p<0.0001). Serum pro- [interleukin (IL)-6)] and anti- [IL-1 receptor antagonist (IL-1ra)] inflammatory cytokines were significantly induced at the same time point (10 h) after TURP injection and no longer detectable by 24 h (GD 16; Fig. V-1B and C, respectively; IL-6: treatment by time interaction, $F_{(5,60)}$=3.98, p=0.004, simple effect of treatment at 10 h $F_{(1,69.28)}$=17.5, p<0.0001; IL-1ra: treatment by time interaction $F_{(6,62)}$=11.04, p<0.0001, simple effect of treatment at 10 h $F_{(1,71.39)}$=52.2, p<0.0001).

TURP treatment dramatically reduced serum non-haeme iron levels, 10 and 24 h after injection (Fig. V-1D; treatment by time interaction, $F_{(6,72)}$=14.10, p<0.0001; simple effect of treatment at 10 h, $F_{(1,78.88)}$=12.34, p=0.0007; at 24 h, $F_{(1,78.88)}$=69.26, p<0.0001), returning to control levels at 48 h (GD 17) after treatment. No further differences in non-haeme iron levels were observed between TURP and control groups. However, there was a reduction in the serum levels of iron with the progress of pregnancy towards term, with non-haeme iron levels at GD 19 becoming significantly lower than those at GD 15, 16, 17 and 18 (Fig. V-1D; main effect of time, $F_{(6, 72)}$=16.36, p<0.0001, 96 h vs. 0, 24, 48 or 72 h Fisher's LSDs p< 0.05). This decrease in maternal serum non-haeme iron towards term is in fact normally observed, as iron is used to sustain increased requirement for this nutrient due to expansion of the maternal erythrocyte mass and the high demand of the growing foetus (Millard et al., 2004; Murray and Stein, 1971).

TURP's effect on circulating iron levels was accompanied by an induction of hepatic expression levels of HAMP mRNA by 25-fold (Fig. V-1E; $t_{(12)}$=5.27, p=0.0002) and zip14 mRNA by 5.5-fold (Fig. V-1E; $t_{(12)}$=4.48, p=0.0007). Zip14 is a dual iron/zinc importer involved in the cellular uptake of iron (Liuzzi et al., 2006). In addition, we observed an 11.6-fold increase in the expression levels of SOCS3 mRNA after TURP treatment (Fig. V-1E; $t_{(12)}$=7.6, p<0.0001), indicating the activation of the JAK/STAT signaling pathway, most likely by IL-6 (Lebel et al., 2000b), which is involved in the induction of HAMP and zip14 mRNA expression.

Maternal iron supplementation reverses hypoferremia, but has no effect on other inflammatory responses

We determined if parenteral iron supplementation had any side effect on the inflammatory responses induced by TURP, namely cytokine production or fever. Maternal febrile response, as well as induction of IL-6 and IL-1ra by TURP remained intact following the iron supplementation schedule, with no significant effects of iron supplementation detectable on all three parameters (Fig. V-8). Maternal serum non-haeme iron levels, analyzed as transferrin (Tf) saturation, were differentially affected by TURP, depending on the maternal supplementation (Fig. V-2A). Tf saturation was strongly diminished by TURP treatment in vehicle (VEH) co-treated animals (Fig. V-2A; prenatal treatment by maternal supplementation interaction, $F_{(1,23)}$=7.85, p=0.01; simple effect of maternal treatment for VEH supplemented animals, $F_{(1,23)}$=24.40, p<0.0001), supporting the effect seen by analyzing raw serum non-haeme iron levels (Fig. V-1D). Importantly, TURP treatment did not have any effect on serum Tf saturation in the animals supplemented with iron (Fig. V-2A; simple effect of maternal treatment for iron supplemented animals, $F_{(1,23)}$=0.95, p=0.33), suggesting a blockade of the TURP effect on serum iron levels when mothers were over-loaded with this micronutrient. Basal Tf saturation among the iron supplemented animals was significantly lower than VEH mothers (supplementation by time interaction, $F_{(5,115)}$=4.91, p=0.0004, simple effect of supplementation at 0 h, $F_{(1,135.6)}$=10.8,

p=0.0013), which may be due to the induction of hepatic HAMP mRNA expression, as observed in both SAL-IRON and TURP-IRON groups at GD 19 (96 h after i.m. injections; Fig. V-2C; effect of treatment, $F_{(3,21)}$=5.89, p=0.044; SAL-IRON or TURP-IRON vs. SAL-VEH or TURP VEH Fisher's LSDs, p<0.05). Accordingly, there was an accumulation of non-haeme iron in the maternal liver of iron-supplemented dams at this same time point (Fig. V-2B; effect of treatment, $F_{(3,22)}$=23.18, p<0.0001; SAL-IRON or TURP-IRON vs. SAL-VEH or TURP VEH Fisher's LSDs, p<0.0001), in the absence of all the other TURP-induced effects, which returned to basal/control levels [maternal serum non-haeme iron (Fig. V-1D); hepatic non-haeme iron content (Fig. V-2B) and maternal hepatic HAMP mRNA expression (Fig. V-2C)].

TURP induces long-term effects on foetal iron content, which are prevented by maternal iron supplementation

We performed foetal iron content and HAMP mRNA expression measurements 96 h after TURP treatment. This time point was chosen since the acute inflammatory responses to TURP (i.e. maternal fever, cytokine induction and decreased serum non-haeme iron) had returned to basal/control levels, enabling us to study the extended effects of the inflammatory challenge on foetal tissue in the absence of the maternal immune responses. In the placenta, non-haeme iron content was significantly reduced in the TURP-VEH treated group (effect of treatment, $F_{(3,71)}$=4.36, p=0.007; SAL-VEH vs. TURP-VEH Fisher's LSD, p<0.05), and this effect was reversed by iron supplementation in TURP-IRON treated dams (Fig. V-3A; TURP-VEH vs. TURP-IRON Fisher's LSD, p<0.05). HAMP mRNA expression levels in this organ paralleled the effects seen in iron content, with a reduction induced by TURP treatment (effect of treatment, $F_{(3,22)}$=6.37, p=0.0029; non significant SAL-VEH vs. TURP-VEH Fisher's LSD, p=0.07) and a recovery of HAMP mRNA expression in the TURP-IRON group, to levels significantly greater than those of SAL-VEH (Fig. V-3D; TURP-IRON vs. SAL-VEH or TURP VEH or SAL-IRON Fisher's LSDs, p<0.05).

There were no effects on foetal liver iron content (Fig. V-3B; effect of treatment, $F_{(3,68)}$=0.71, p=0.55). However, foetal hepatic HAMP mRNA levels were significantly increased in the foetuses from TURP- and SAL-IRON treated mothers (Fig. V-3E; effect of treatment, $F_{(3,21)}$=4.17, p=0.018; SAL-VEH vs. TURP-VEH or SAL-IRON Fisher's LSD, p<0.05), whereas in foetal livers from TURP-IRON treated mothers, HAMP mRNA levels were back to control levels (Fig. V-3E; TURP-IRON vs. SAL-IRON or TURP-VEH Fisher's LSDs, p<0.05).

In the foetal brain, non-haeme iron content was significantly reduced in the foetuses of TURP-IRON treated dams, compared to the foetal brains from SAL-VEH and TURP-VEH treated mothers (Fig. V-3C; effect of treatment, $F_{(3,73)}$=2.96, p=0.038; TURP-IRON vs. SAL-VEH or TURP-VEH Fisher's LSDs, p<0.05). However, there were no effects on HAMP mRNA expression (Fig. V-3F; effect of treatment, $F_{(3,23)}$=0.46, p=0.71).

Maternal inflammation and iron supplementation differentially alter sensitivity to the effects of AMPH on locomotor activity in the adult offspring

We first assessed the effects on litter size and body weight at birth, and found that none of the prenatal treatments had a significant effect on these variables (data not shown), suggesting that neither TURP and/or iron supplementation induced foetal mortality nor gross physical changes at birth.

In the adult offspring, locomotion in basal conditions or in response to amphetamine (AMPH, 2 mg/kg of body weight) or saline injection was determined. AMPH or saline were administered to animals of each experimental group for 5 consecutive days. No effects were observed in basal locomotion (Fig. V-4A) or in response to injection of saline throughout the 5 days of pre-treatment (Fig. V-4B) for individuals born to TURP and SAL-treated mothers, supplemented with either VEH or iron. However, when treated with AMPH, animals born to TURP-VEH mothers showed significantly greater locomotion throughout the 5 pre-treatment days, in comparison to their SAL-VEH counterparts (Fig. V-4B; prenatal treatment by supplementation interaction, $F_{(1,62)}$=4.84, p=0.032; simple effect of treatment in the VEH condition, $F_{(1,62)}$=6.96,

p=0.01). In contrast, there was no difference in AMPH-induced locomotion between the offspring of TURP and SAL-treated, iron-supplemented mothers (simple effect of treatment in the iron condition, $F_{(1,62)}$=0.22, p=0.64), which exhibited locomotor responses to AMPH statistically similar to those of the SAL-VEH group (Fig. V-4B; simple effect of supplementation in the SAL condition, $F_{(1,62)}$=2.32, p=0.13). During days 1 and 2, iron supplemented animals presented a trend for enhanced response to AMPH in comparison to SAL-VEH, which disappeared from day 3 onwards; this effect did not reach statistical significance.

In the test for behavioral sensitization (7 days after last pre-treatment injection), all animals were challenged with a single injection of AMPH (1 mg/kg). As expected, all AMPH pre-treated rats exhibited sensitized response to the AMPH challenge in comparison to those pre-treated with saline (Fig. V-4C; main effect of pre-treatment, $F_{(1,129)}$=63.9, p<0.0001). However, among the AMPH pre-treated animals, offspring of dams exposed to TURP-VEH showed a significantly greater sensitized response than the offspring of the SAL-VEH group (Fig. V-4C; three-way prenatal treatment by maternal supplementation by pre-treatment interaction, $F_{(1,129)}$=7.83, p=0.006; simple effect of prenatal treatment for the AMPH pre-treated VEH animals, $F_{(1,129)}$=10.39, p=0.0016). In contrast, offspring of TURP-IRON treated dams were sensitized to AMPH similar to the SAL-VEH offspring (Fig. V-4C; simple effect of maternal supplementation for the AMPH pre-treated TURP animals, $F_{(1,129)}$=12.17, p=0.0007). Intriguingly, iron supplementation alone during pregnancy, also resulted in enhanced behavioral sensitization in the adult offspring (Fig. V-4C; simple effect of maternal supplementation for the AMPH pre-treated SAL animals, $F_{(1,129)}$=9.94, p=0.02; simple effect of treatment for the AMPH pre-treated IRON animals, $F_{(1,129)}$=8.77, p=0.0037).

Maternal inflammation leads to biochemical alterations in the adult DA system, which are partly reversed by maternal iron supplementation

We analyzed baseline tyrosine hydroxylase (TH) expression in DA rich regions by Western blotting (Fig. V-5). Consistent with our behavioral results,

nucleus accumbens (NAcc) TH expression was significantly increased in the adult offspring of the TURP-VEH group compared to the saline controls (Fig. V-5A). This effect was not observed in the offspring of iron supplemented mothers (TURP-IRON and SAL-IRON groups), whose levels of TH were comparable to those of the control group (Fig. V-5A; effect of treatment, $F_{(3,23)}$=3.63, p=0.028; TURP-VEH vs. SAL-VEH or TURP-IRON Fisher's LSDs, p<0.05). These changes were region specific, as levels of TH expression in the dorsal striatum (dSTR; Fig. V-5B; effect of treatment, $F_{(3,22)}$=0.62, p=0.60), medial prefrontal cortex (mPFC; Fig. V-5C; effect of treatment, $F_{(3,21)}$=0.88, p=0.46), ventral tegmental area (VTA; Fig. V-6A; effect of treatment, $F_{(3,23)}$=0.58, p=0.63) and substantia nigra (SN; Fig. V-6B; effect of treatment, $F_{(3,23)}$=0.04, p=0.96) were not significantly altered by any prenatal treatment.

We also measured tissue content of DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in DA terminal regions (Fig.V-7). Consistent with effects seen in TH expression, NAcc levels of DA in the TURP-VEH group were elevated (Fig. V-7A; effect of treatment, $F_{(3,18)}$=3.83, p=0.028; SAL-VEH vs. TURP-VEH Fisher's LSD, p<0.05), as well as those of DOPAC (effect of treatment, $F_{(3,18)}$=18.27, p<0.0001; SAL-VEH vs. TURP-VEH Fisher's LSDs, p<0.05) and HVA (effect of treatment, $F_{(3,18)}$=3.83, p=0.041, SAL-VEH vs. TURP-VEH Fisher's LSDs, p<0.05). Intriguingly, DA levels in the SAL-IRON and TURP-IRON groups were also increased (Fig. V-7A; SAL-VEH vs. SAL-IRON or TURP-IRON Fisher's LSDs, p<0.05); the same was true for NAcc HVA and DOPAC (SAL-VEH vs. SAL-IRON or TURP-IRON Fisher's LSDs, p<0.05), with DOPAC levels in TURP-IRON animals being even greater than the TURP-VEH or SAL-IRON groups (Fig. V-7A; TURP-IRON vs. SAL-IRON and TURP-VEH Fisher's LSDs, p<0.05). As for TH, effects of prenatal treatments appeared to be specific to the NAcc since neither dSTR (Fig. V-7B) nor mPFC (Fig. V-7C) DA and HVA were altered and dSTR DOPAC was significantly increased only in the offspring of SAL-IRON treated dams (Fig. V-7B; effect of treatment, $F_{(3,20)}$=3.26, p=0.043; SAL-VEH vs. SAL-IRON Fisher's LSD, p<0.05).

Discussion

In the current study we provided the first evidence, to our knowledge, that localized maternal inflammation during gestation renders adult offspring significantly more sensitive to the locomotor activating effects of a single AMPH injection, and to the behavioral plasticity following repeated exposure to this drug. In addition, we demonstrated increased baseline expression of TH and tissue levels of DA, DOPAC and HVA, which were specific to the NAcc. The fact that TURP does not enter the circulation suggests that the effects observed in the offspring in the current study, are most likely due to secondary downstream mediators and not to direct effects on the foetal compartment, as reported for poly I:C (Meyer et al., 2008b; Meyer et al., 2007b; Ozawa et al., 2006; Vuillermot et al.; Zuckerman et al., 2003) and LPS (Ashdown et al., 2006a; Borrell et al., 2002; Feleder et al.; Fortier et al., 2004; Romero et al., 2006; Romero et al., 2008).

We demonstrated that maternal iron supplementation, which counteracts inflammation-induced hypoferremia, effectively prevented the prenatal TURP-induced increased sensitivity to the behavioral effects of single and repeated AMPH administration in the adult offspring. These results suggest that maternal hypoferremia, induced by the TURP injection, causes long-lasting behavioral alterations in the offspring. Remarkably, prenatal TURP-induced increase in NAcc TH expression was also completely abrogated by maternal iron supplementation.

Increased levels of TH and DA, restricted to the NAcc, have also been reported by others in the offspring of mothers treated with poly I:C (Vuillermot et al., 2010) and LPS (Romero et al., 2008). As with the effect of TURP, there is no clear explanation on how these prenatal inflammatory insults, leads to changes in TH, DA and metabolites that are restricted to this brain region. One possibility may be linked to the differential distribution of iron in the brain. Iron is particularly abundant within DA-rich regions of the brain, where it has been shown to have a role in DA metabolism and function (Beard and Connor, 2003; Kwik-Uribe et al., 2000; Lozoff et al., 2006). Of these regions, the NAcc and the

dSTR are among the most enriched areas of the brain (Beard and Connor, 2003), and they are sensitive to systemic variations in iron (i.e. dietary restriction or supplementation), which in turn affect DA neurotransmission. Likewise, iron deficiency during gestation has been shown to affect DA neurotransmission in the whole striatum, including the NAcc (Beard, 2003; Beard and Connor, 2003; Burhans et al., 2005; Burhans et al., 2006; Lozoff et al., 2006). Interestingly, although less studied, mPFC DA neurotransmission seems largely unaffected by early manipulations in iron (Kwik-Uribe et al., 2000). This is supported by our findings which show that the effects of TURP on DA was restricted to the NAcc, and that iron supplementation alone affected DA metabolites only in the NAcc and the dSTR, whereas neither treatment affected the mPFC TH, DA or HVA levels. These results suggest that the anatomical localization of iron may underlie the effects of maternal TURP and/or iron supplementation to modulate striatal DA transmission, as opposed to cortical DA function, although it does not fully explain the differential effect between NAcc and dSTR.

Increased NAcc TH, which is consistent with augmented basal NAcc DA concentration, may result from either a change in expression level per neuronal terminal, or from structural reorganization within the mesolimbic circuitry, the latter resulting in axonal sprouting of NAcc DA terminals. Prenatal inflammation may therefore lead to profound presynaptic alterations in NAcc DA synthesis. However, additional effects on DA receptors, transporter (Vuillermot et al.) and/or catabolic enzymes cannot be excluded, as baseline levels of DOPAC and HVA were also increased by prenatal TURP. Our findings of increased DA function in the NAcc at baseline are likely to contribute to increased behavioral response to acute AMPH injection and perhaps, to the enhanced AMPH-induced behavioral plasticity following repeated treatment (Featherstone et al., 2007; Vezina, 2004). Intriguingly, altered levels of basal NAcc DA, DOPAC and HVA remained increased in the offspring of TURP-IRON treated mothers, despite normalization of TH expression. This suggests additional alterations in the synthetic and/or catabolic pathways of DA that may account for the observed alterations. It is important to note however, that prenatal TURP treatment caused

two mechanistically differentiable effects in the offspring. One was the increased response to a single AMPH injection, which may stem from changes in the release of DA in the NAcc (Sulzer et al., 1995), therefore is more directly related to increased basal expression of TH and to the increased basal content of DA in the NAcc. In contrast, behavioral plasticity after repeated AMPH administration is a phenomenon that requires long-term neural adaptations that occur in several brain structures, notably the VTA, where the cell bodies of the mesolimbic DA neurons lie (Flores et al., 2005; Flores et al., 2000; Yetnikoff et al., 2007), therefore TURP may affect additional cellular and molecular targets. In this regard, iron supplementation to TURP-treated mothers seems to have prevented only some of the effects of TURP that impact the response to acute AMPH injection (i.e. increased NAcc TH expression, but no greater DA and metabolites) as well as those mechanisms underlying the sensitized response after repeated AMPH administration.

Interestingly, iron supplementation alone (i.e. SAL-IRON group) induced several behavioral and biochemical changes in the offspring, which resembled the effects of prenatal inflammation (i.e. enhanced sensitivity to acute and repeated AMPH injection and increased basal levels of DA, DOPAC and HVA in the NAcc). SAL-IRON group also exhibited a greater AMPH-induced stereotypy during the pre-treatment phase (data not shown) and increased basal levels of DOPAC in the dSTR. This surprising finding is most likely due to iron overload in iron sufficient animals (Archer and Fredriksson, 2007), although the doses of iron used in our study were chosen to be below those that cause toxic side effects (Jiang et al., 2007; Legssyer et al., 2003). This is further exacerbated by the fact that parenteral administration of iron bypasses the regulatory mechanisms that control its dietary absorption (Hentze et al., 2004), therefore iron administered through dietary supplementation, which normally does not cause overload (Casanueva and Viteri, 2003), may spare the offspring from the undesired effects described in the present study. To the best of our knowledge, there are no studies on the effects of maternal-iron supplementation on the DA function of the adult offspring, however it is normally considered that excess of iron results in toxic

effects due to oxidative stress (Hentze et al., 2004). These findings, although unexpected, nevertheless emphasize the critical importance of this micronutrient to normal development of mesolimbic DA neurons.

Given that maternal circulation constitutes the only source of iron to the developing foetus (Millard et al., 2004), and that HAMP down-regulates FPN1 (Nemeth et al., 2004b), which is essential for the transport of iron through the materno-foetal interface (Donovan et al., 2000; Donovan et al., 2005; Gruper et al., 2005; Mok et al., 2004a; Mok et al., 2004b), we rationalized that the inflammation-induced reduction in maternal iron will restrict the amounts being transported through the materno-foetal interface. To confirm this, we measured iron levels in the placenta, foetal liver and brain collected from all the treatment groups. As expected, placental iron levels were dramatically reduced, paralleling the maternal supply of this nutrient and completely recovered by iron replenishment in TURP treated dams. The TURP-induced reduction in placental iron content may reflect either the reduced availability of maternal serum iron and/or the demand to sustain the foetal development during the hypoferremia (Gambling et al., 2009).

The changes in iron levels in the placenta were very closely paralleled by the levels of HAMP expression, which may be merely responding to the tissue's fluctuation in iron concentration (Pigeon et al., 2001) or conversely, responding to a signal from outside the placenta, and in turn modulating iron export rates from this tissue to the foetal compartment (Gambling et al., 2009). Interestingly, regulation of HAMP mRNA expression in foetal liver was the reverse of that seen in the placenta. In this tissue, TURP-treatment resulted in induced expression of HAMP mRNA at a time point when TURP-induced maternal circulating cytokines are no longer elevated, suggesting that mediators other than cytokines may be involved in this effect. One likely possibility is that iron flow from the placental stores into the foetus resulted in higher than normal iron levels in the foetal circulation, thus triggering foetal liver HAMP mRNA expression (Gambling et al., 2009). In fact, foetuses of iron supplemented (SAL-IRON) dams also showed increased foetal liver HAMP mRNA expression, which is

presumably due to a direct effect of the supplemented micronutrient (Gambling et al., 2009), as no inflammatory pathways were activated in this condition.

Iron levels in the foetal brains taken from all four treatment groups did not show any alteration, other than a small but significant reduction in TURP-IRON group. This latter observation is rather puzzling and it possibly reflects a lower than normal supply of iron in the TURP-IRON group. The latter effect can be mediated by the increased placental HAMP in the TURP-IRON group, which would lead to iron trapping within this organ, at the expense of the foetal brain, similar to the effects of maternal hepatic HAMP on maternal liver iron content (De Domenico et al., 2007). However, it is important to note that in the present study we measured whole brain iron levels, and that marked regional differences in iron acquisition capabilities of the brain are possible and have indeed been reported (Beard and Connor, 2003; Connor et al., 2001; Moos et al., 2007). In fact, brain DA regions are known to be enriched in iron (Hill and Switzer, 1984; Youdim et al., 1984) and susceptible to systemic variations of this nutrient (Connor et al., 2009), suggesting that specific effects of inflammation and iron supplementation may be found in the mesolimbic DA neurons, which would then be involved in the induction of the marked effects on DA function observed in the adult offspring. Altogether our data are consistent with a scenario where inflammation-induced maternal hypoferremia leads to placental deficiency in this nutrient. This could be due, at least partly, to a putative increase in iron transport to the foetal compartment that keeps whole-tissue levels of iron normal. In addition, placental iron deficiency may in turn result in altered function of this tissue. For example, dietary iron deficiency leads to increased cytokine expression in the placenta (Gambling et al., 2002) and altered transport of amino acids towards the foetal compartment (McArdle et al., 2006; McArdle et al., 2003). This may, along with direct restriction of iron supply in specific brain areas, impact aspects of neuronal development, resulting in the effects observed in the adult offspring.

Hypoferremia has long been recognized as one of the main components of the host's response to infection/inflammation, and the mechanisms involved in the

induction of this process are fairly well understood. The strongest evidence to date suggests that the pro-inflammatory cytokine IL-6, which is readily detectable in the circulation of infected individuals regardless of the pathogenic trigger, is the main circulating mediator of hepatic HAMP mRNA expression (Lee et al., 2005; Nemeth et al., 2004a; Nemeth et al., 2003; Nicolas et al., 2002b). IL-6 has been suggested to be strongly involved in the aetiology of the offspring alterations induced by maternal inflammation (Smith et al., 2007). We cannot rule out the direct involvement of either other cytokines (Meyer et al., 2008b) or other mediators, such as inflammation-induced alteration in zinc homeostasis (hypozincemia) (Coyle et al., 2009). However, our results strongly indicate that during prenatal inflammation, hypoferremia plays a fundamental role in the developmental effects of this maternal insult on the mesolimbic DA function, and that the effect of IL-6 on the developing foetus may be mediated trough this mechanism.

Our results clearly demonstrate that a localized inflammatory insult during gestation has profound affects on DA function, which may be relevant for schizophrenia, where increased striatal DA is proposed to underlie the so-called positive symptoms of the disorder (Abi-Dargham et al., 1998; Abi-Dargham et al., 2000; Breier et al., 1997; Laruelle et al., 1999). In addition, we showed for the first time that this risk factor for schizophrenia also increased the animal's vulnerability to develop sensitized behavioral response to an AMPH challenge given one week after repeated exposure. Repeated administration of drugs of abuse, including AMPH, has been shown to also result in sensitization to the rewarding effects of these drugs (Vezina, 2004). Thus, our findings further strengthen the link between these two psychiatric disorders, whose co-morbidity has been proposed to stem from DA dysfunction (Chambers et al., 2001). Finally, we provided new evidence for the involvement of foetal/maternal iron homeostasis in the developmental processes that render the offspring more susceptible to enhanced DA function.

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Figures and Tables

Figure V-1. TURP induced a maternal inflammatory response and hypoferremia in pregnant rats at GD 15

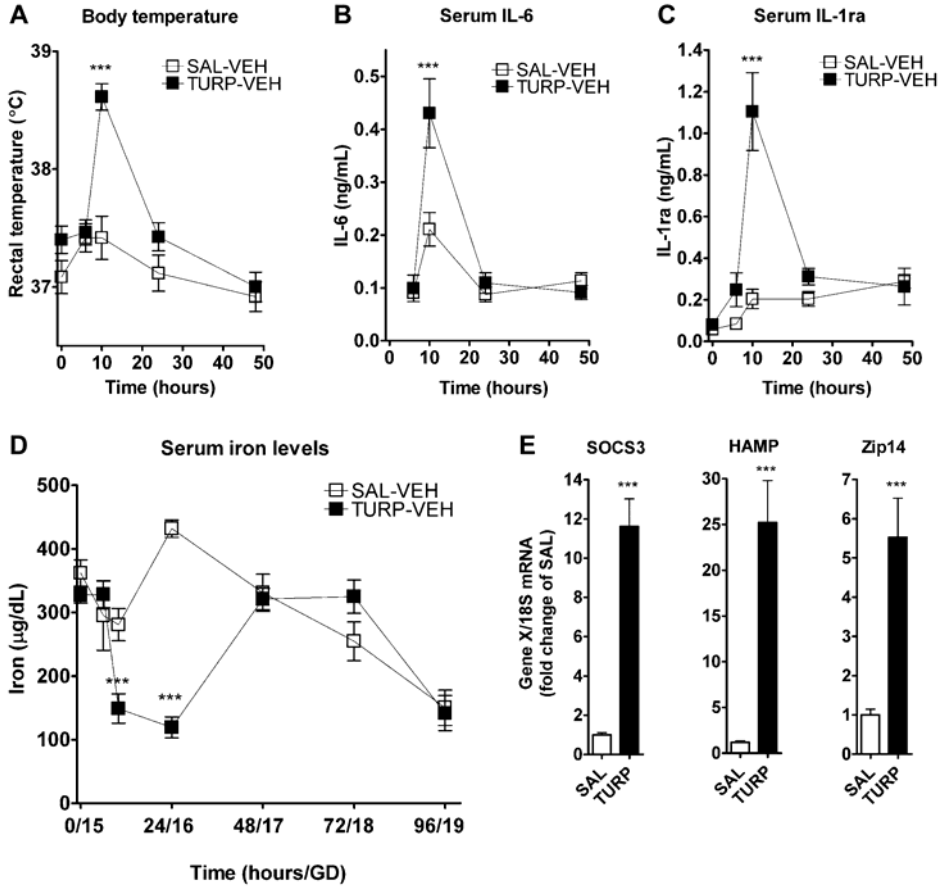

*Figure V-1. (A) Sterile saline (n=6) or TURP (n=8) was injected (i.m., 100 µl/rat) and core body temperature determined immediately before (0 time point) and 8, 10, 24 and 48 h after. TURP induced a significant increase in core body temperature 10 h after TURP injection, which receded 24 h after injection. p<0.0001: *** SAL vs. TURP. (B and C) Serum IL-6 and IL-1ra levels were determined by ELISA from samples collected 6, 10, 24 and 48 h after SAL or TURP injections in the same rats as in (A). TURP induced a significant increased in the levels of both cytokines 10 h after injection and returned to baseline by 24 h. p<0.0001: *** SAL vs. TURP. (D) Non-haeme iron levels were also measured in the serum samples from the same animals used in (A-C). TURP induced*

*significant reduction in non-haeme levels 10 and 24 hours after TURP injection, returning to control levels by 48 hours. Non-haeme iron levels decreased as pregnancy progressed, independently of treatment. p<0.0001: *** SAL vs. TURP. (E) Hepatic gene expression in dams sacrificed at the peak of fever and cytokine induction, 10-11 hours after i.m. injection. SOCS3 (left panel), HAMP (middle panel) and zip14 (right panel) mRNAs were significantly induced in the TURP treated dams (n=7 per group). p<0.0001: *** SAL vs. TURP.*

Figure V-2. TURP effect on maternal serum Tf saturation was prevented by parenteral iron supplementation

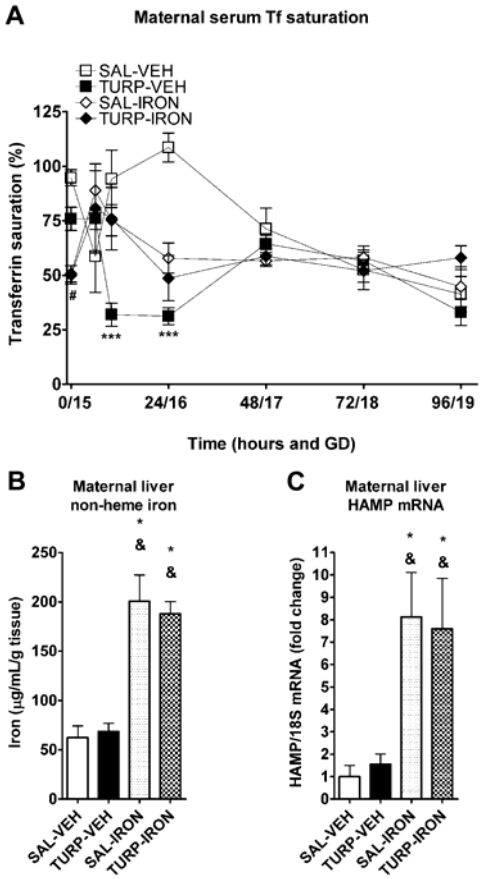

Figure V-2. (A) Serum Tf saturation levels were significantly reduced 10 and 24 h after TURP injection, this effect was absent in TURP-IRON treated dams. Baseline Tf saturation levels in iron supplemented animals was significantly

*reduced. (B and C) Hepatic non-haeme iron levels, 96 h after i.m. injection, were unaffected by TURP treatment, but dramatically increased in iron supplemented dams. In parallel, iron supplementation led to a strong induction maternal hepatic HAMP mRNA expression. p<0.05 * vs. SAL-VEH, & vs. TURP-VEH.*

Figure V-3. Foetal non-haeme iron and HAMP mRNA levels are modulated by maternal TURP treatment and iron supplementation

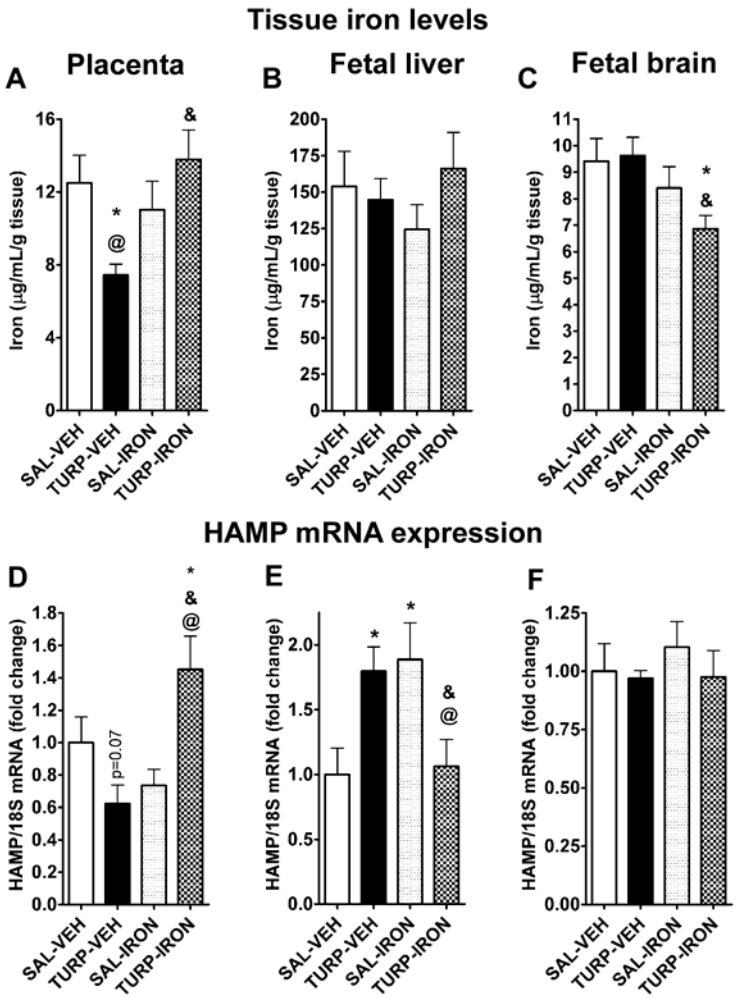

*Figure V-3. (A) Non-haeme iron levels in the placenta were lower in the TURP-treated dams, and this effect was reversed in the TURP-IRON treated dams. p<0.05: * vs. SAL-VEH, & vs. TURP-VEH, @ vs. SAL-IRON. SAL-VEH n=6, TURP-VEH n=8, SAL-IRON n=7 and TURP-IRON n =6. (B) Foetal liver non-haeme iron content was unaffected by maternal treatments. (C) In the foetal brain,*

*the only significant effect was a significant decrease in the non-haeme iron content of TURP-IRON treated mothers. p<0.05: * vs. SAL-VEH, & vs. TURP-VEH. (D) Placental HAMP mRNA expression was decreased in TURP-VEH mothers, whereas this effect was significantly reversed in the TURP-IRON treated dams. p<0.05: * vs. SAL-VEH, & vs. TURP-VEH, @ vs. SAL-IRON. (E) In the foetal liver, HAMP mRNA expression was significantly increased by TURP and by iron supplementation alone, whereas when both treatments were given together these effects is blocked. p<0.05: * vs. SAL-VEH, & vs. TURP-VEH, @ vs. SAL-IRON. (F) HAMP mRNA expression was not significantly altered by any treatment in the foetal brain.*

Figure V-4. Prenatal TURP treatment increased acute and sensitized AMPH-induced locomotion, which are prevented by maternal iron co-administration

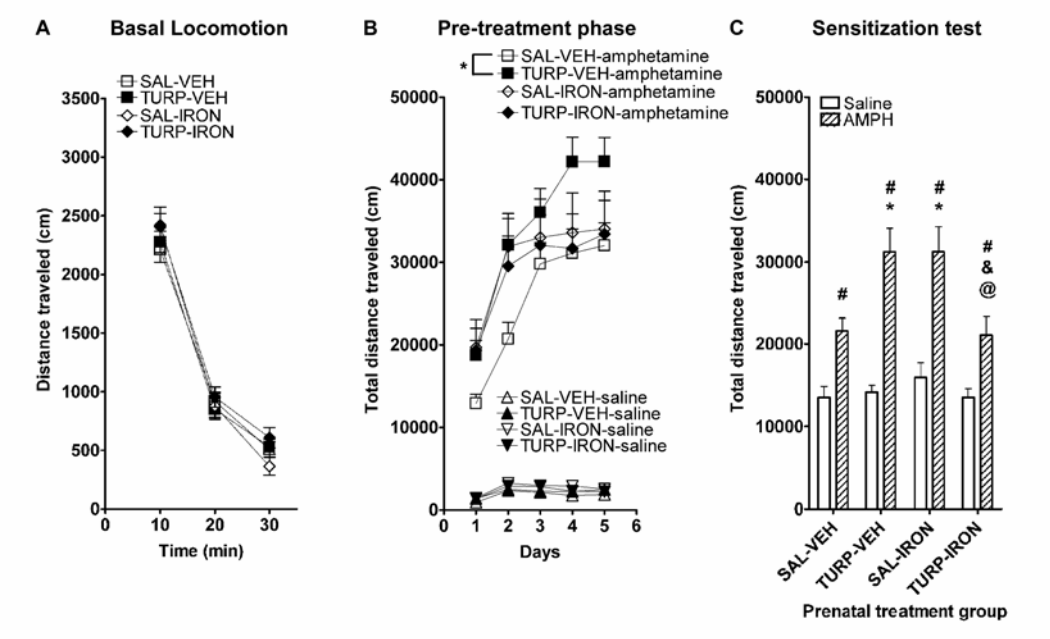

Figure V-4. (A) Basal locomotion in the adult offspring of SAL-VEH (n=45), TURP-VEH (n=50), SAL-IRON (n=23) and TURP-IRON (n=26) treated dams was not affected by any treatment when animals were introduced to the behavioral chambers for the first time. (B) AMPH administration (i.p. 2 mg/kg) for 5 consecutive days induced a progressive enhancement of the locomotor

*activating effects, which was overall grater among the offspring of TURP-VEH treated dams compare to their SAL-VEH counterparts. This effect was completely prevented by iron co-treated, with the offspring of the TURP-IRON and SAL-IRON groups showing identical levels of locomotor behavior, which were also indistinguishable from those of the SAL-VEH group. Saline pre-treated: SAL-VEH n=24, TURP-VEH n=26, SAL-IRON n=12 and TURP-IRON n=12. AMPH pre-treated: SAL-VEH n=21, TURP-VEH n=24, SAL-IRON n=11 and TURP-IRON n=14. (C) Seven days after the last saline or AMPH injection, all animals were tested for sensitization, with a lower dose of AMPH (i.p. 1 mg/kg). All AMPH pre-treated animals showed significantly greater response to this dose of AMPH (dashed bars) than the saline-pre-treated animals (white bars). Among the AMPH pre-treated animals, those born to TURP-VEH and SAL-IRON presented significantly greater sensitization response, whereas in the offspring of TURP-IRON dams, sensitization response was comparable to control animals. $p<0.01$: # vs. saline pre-treated counterpart, * vs. AMPH pre-treated SAL-VEH, & vs. AMPH pre-treated TURP-VEH, @ vs. AMPH pre-treated SAL-IRON.*

Figure V-5. Iron supplementation prevented increased NAcc TH levels induced by prenatal TURP treatment

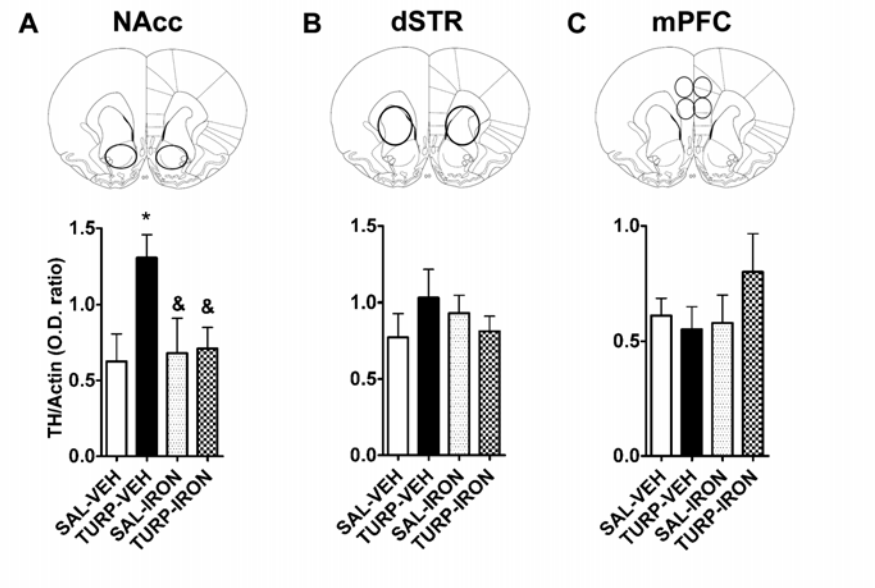

*Figure V-5. (A) Basal TH levels in the NAcc were measured by western blotting from adult animals' protein extracts. These were significantly greater in the offspring of TURP-treated dams, which was prevented by maternal iron supplementation. SAL-VEH n=5, TURP-VEH n=7, SAL-IRON n=5 and TURP-IRON n=6. $p<0.05$ * vs. SAL-VEH, & vs. TURP-VEH. (B and C) TH protein levels from dSTR (B) and mPFC (C) were not significantly affected by any prenatal treatment.*

Figure V-6. TH expression in the somatodendritic DA regions is not affected by maternal treatments

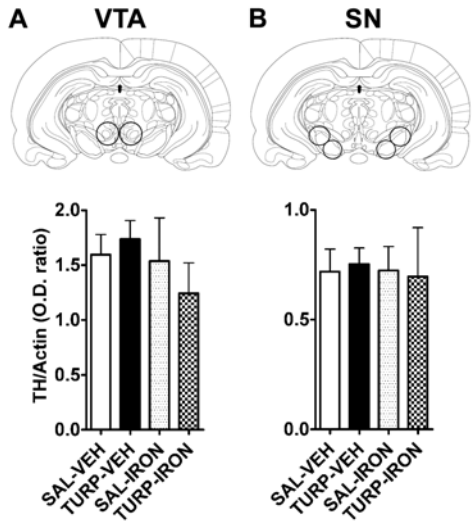

Figure V-6. (A and B) TH protein levels were not significantly altered by maternal manipulations in VTA (A) and SN (B).

Figure V-7. Prenatal inflammation and iron supplementation increase NAcc levels of DA, DOPAC and HVA

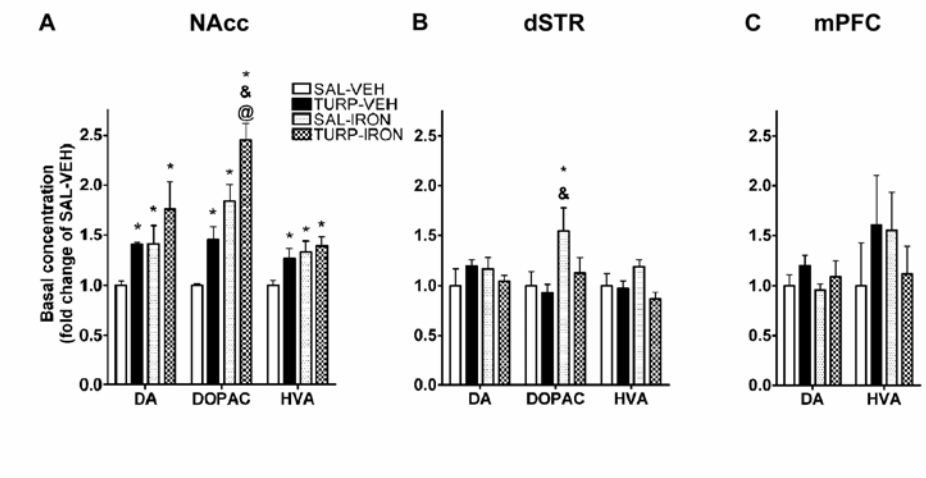

Figure V-7. (A) In the NAcc prenatal TURP induced a significant induction in the content of DA, DOPAC and HVA. These effects were also present in the offspring of SAL-IRON-treated dams and in the TURP-IRON offspring, who also showed

*DOPAC levels greater that all of the other groups. SAL-VEH n=5, TURP-VEH n=7, SAL-IRON n=5 and TURP-IRON n=6. p<0.05 * vs. SAL-VEH, & vs. TURP-VEH, @ vs. SAL-IRON. (B) In the dSTR only DOPAC content was significantly greater in the offspring of SAL-IRON dams, whereas no other significant effect was found on the metabolite or in DA and HVA levels. (C) In contrast, in the mPFC no changes were detected in DA and HVA (DOPAC was under detection limits of the assay for this region).*

Figure V-8. Inflammatory response remained intact in iron supplemented mothers

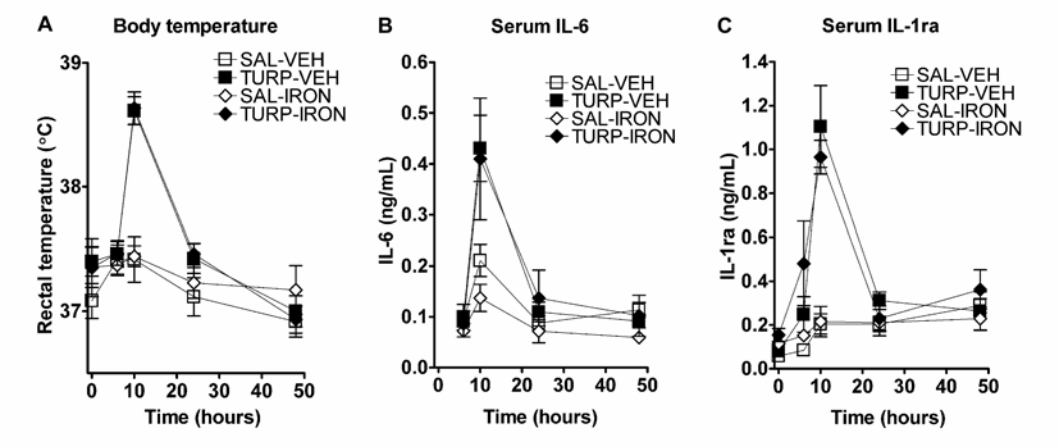

Figure V-8. (A) Febrile response followed the same kinetics in vehicle and iron supplemented mothers, peaking at 10 h after TURP injection and returning to baseline 24 h later. Basal temperature was not affected by iron supplementation. (B and C) Serum IL-6 and IL-1ra levels followed the same kinetics in the TURP-IRON group compared to the TURP-VEH group.

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V.3. Supplementary results and discussion

Hypoferremia of inflammation is differentially stimulated by TURP at GD 15 and GD 18

We compared the induction of hypoferremia at the two stages of pregnancy used in Chapter III, at the peak of the inflammatory response (Fig. V-9). TURP treatment at GD 15 induced a significant reduction in serum iron levels (effect of treatment $F_{(3,16)}$=13.15, p<0.0001, SAL-15 vs. TURP-15 p<0.001, Fig. V-9A) and transferrin (Tf) saturation (effect of treatment $F_{(3,16)}$=9.22, p<0.001, SAL-15 vs. TURP-15 p<0.01, Fig V-9B). In contrast, at GD 18, TURP did not affect maternal circulating iron levels or Tf saturation (SAL-18 vs. TURP-18 ps>0.05, Figs. V-9A and B). Importantly, basal iron levels and Tf saturation at GD 18 (i.e. in the SAL-treated group) were significantly lower than those at GD 15 (SAL-15 vs. SAL-18 ps<0.01). This reduction is basal iron levels has been observed to occur during gestation, as iron requirements of the foetus increase towards the end of this period, and as a consequence, maternal iron is diverted towards them (Millard et al., 2004).

Figure V-9. Maternal circulating non-haeme iron levels are modulated by inflammation and gestation

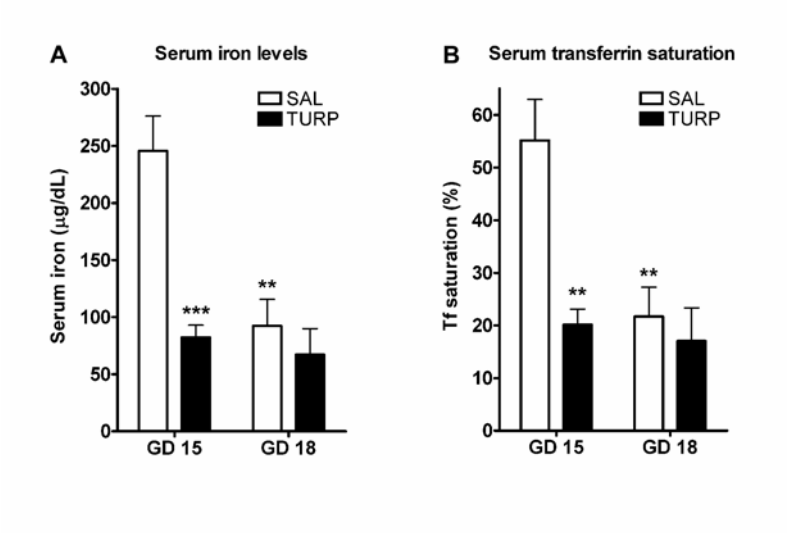

*Figure V-9. Serum iron levels were determined 10-11 h after SAL or TURP injection (as in Fig. III-1) (A). Transferrin (Tf) saturation was also measured from the same animals (B). *** P<0.0001 and ** P<0.01 vs. SAL-15 group.*

The results confirmed that during gestation the maternal response to a localised inflammatory challenge undergoes a profound change, whereby it is almost completely attenuated towards the end of gestation, in all aspects of the acute phase response that were analysed (i.e. circulating IL-6, fever, hypoferremia). This differential activation of the immune response, in addition, resulted in greatly different pattern of behavioural alterations in the adult offspring of mothers treated with TURP at two different stages of gestation (Chapter III).

Maternal haematological parameters and serum iron are not affected by inflammation at birth

In the present study, it was demonstrated that maternal hypoferremia plays a significant role in the induction of functional alterations of the mesolimbic DA

system induced by prenatal inflammation. During gestation, TURP-induced inflammatory challenge resulted in a significant reduction of non-haeme iron level in the circulation and the placenta. The latter was evident 96 h after the TURP challenge, when circulating levels of iron have returned to control values, suggesting prolonged effects of acute inflammation on iron homeostasis during gestation. In addition, prolonged reduction of circulating non-haeme iron can lead to the development of anaemia of inflammation or anaemia of chronic disease (Nemeth and Ganz, 2006; Weinstein et al., 2002). The effect of TURP on the maternal circulating iron levels receded 48 hours after TURP treatment, however we wanted to asses whether this could have had a long-term effect on haematological markers of the mother, indicative of anaemia. Therefore, maternal haematological markers were measured from blood samples obtained from mothers at P0, after they had given birth and when offspring had been cross fostered to naïve mothers. Measurements included haemoglobin levels, hematocrit, red blood cell (RBC) count, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) (Table V-1). Neither of these parameters was significantly altered by maternal treatment with TURP or iron supplementation (Table V-1). The lack of effect of the inflammatory stimulus on the haematological parameters confirms the acute nature of the inflammatory stimulus. In addition, since iron supplementation did not result in changes in the haematological parameters, this suggests that the mothers' diet is enough to sustain the demand for this nutrient during gestation, therefore any form of iron supplementation, especially parenteral administration as in the case of the present study, accumulated in the store tissues and resulted in overloading of this nutrient.

Table V-1. Haematological parameters after delivery of dams with or without iron-sucrose supplementation and i.m. injection of saline or turpentine at GD 15

	Treatment	HMG	HMT	RBC	MCV	MCH	MCHC
VEH	**SAL**	126.3 ± 4.96	0.345 ± 0.015	5.89 ± 0.27	58.08 ± 0.39	21.54 ± 0.21	370.3 ± 1.99
	TURP	136.1 ± 9.22	0.372 ± 0.026	6.35 ± 0.46	58.45 ± 0.47	21.49 ± 0.23	367.4 ± 2.5
IRON	**SAL**	147.4 ± 15.89	0.402 ± 0.046	6.90 ± 0.83	58.20 ± 0.73	21.40 ± 0.37	367.6 ± 2.9
	TURP	136.5 ± 14.24	0.376 ± 0.039	6.47 ± 0.75	58.75 ±0.92	21.24 ± 0.36	363.9 ± 2.77

Mothers were administered with vehicle (VEH, saline or sucrose) or iron for 7 days (GD 13-18) and treated with either saline (SAL) or turpentine (TURP) at GD 15. Haematological parameters were determined after delivery. Haemoglobin (HMG, g/L); haematocrit (HMT, L/L); red blood cell counts (RBC, $x10^{12}$/L); mean corpuscular volume (MCV, fL); mean corpuscular haemoglobin (MCH, pg/cell); mean corpuscular haemoglobin concentration (MCHC, g/L).

Serum non-haeme iron levels and Tf saturation were also determined from the same groups of mothers after delivery (Fig. V-10). Serum iron levels were significantly lower in the iron supplemented groups compared to vehicle supplemented animals, regardless of the inflammatory treatment (effect of supplementation, $F_{(1,29)}$=14.19, p=0.0008; Fig. V-10A). A similar effect was observed for Tf saturation data, whereby maternal iron supplementation resulted in lower proportion of saturated Tf (effect of supplementation, $F_{(1,26)}$=8.82, p=0.0063; Fig. V-10B). This effect is similar to what is observed at the time of TURP administration at GD 15 (i.e. 0 h time point in Fig. V-2A), and most likely reflects the accumulation of this nutrient in maternal tissues like the liver (Fig. V-2B), as a result of iron-induced hepatic HAMP expression (Fig. V-2C). Interestingly, Tf saturation values in all of the groups of dams after delivery were greater than those measured at GD 19 (Fig. V-2A) suggesting a recovery of maternal iron levels shortly after delivery.

These results suggest that the inflammatory challenge with TURP to the mothers is enough to alter the iron homeostasis in the placenta and possibly the

foetus, however it is not strong enough to induce maternal anaemia, excluding the possibility that the effect of prenatal acute inflammation may be complicated by the development of anaemia of inflammation.

Figure V-10. Maternal circulating iron levels at birth are not affected by inflammation, but reduced by parenteral supplementation

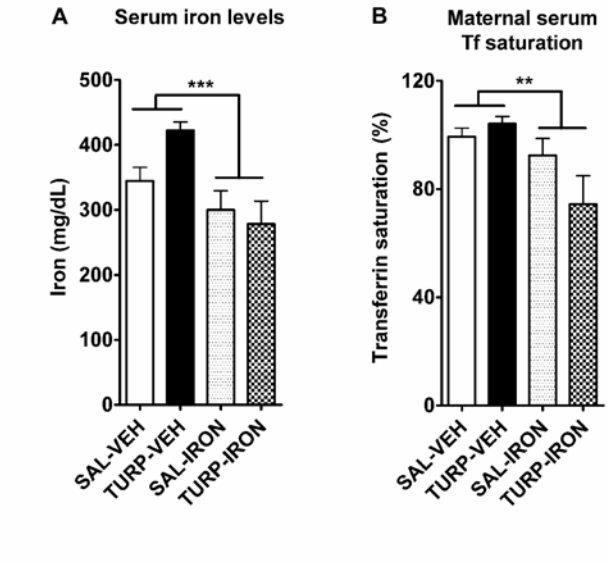

*Figure V-10. (A) Serum iron levels were significantly decreased at birth, in the maternal circulation by parenteral iron supplementation. A similar effect of iron supplementation was observed in the maternal serum Tf saturation (B). p<0.01 ** or p<0.0001 *** IRON vs. VEH supplemented animals.*

Stereotypy induced by AMPH is increased by prenatal inflammation, which is prevented by maternal iron supplementation

Behavioural analysis in the adult offspring in response to AMPH administration showed that prenatal TURP resulted in greater locomotor response to daily administration of the DA indirect agonist, which was evident throughout the whole pre-treatment phase of the testing (days 1-5, Fig. V-4B) and during the sensitization test (Fig. V-4C). These effects were prevented by maternal iron supplementation to TURP-treated dams, however, some were mimicked by

maternal iron supplementation alone. In order to determine if the dual effect of iron supplementation on locomotion could have not been confounded by an effect on the offspring's stereotypical behaviour induced by AMPH, we determined this behavioural response at the same time as locomotion (Fig. V-11). During the pre-treatment phase (Fig. V-11A) all AMPH-treated groups presented greater stereotypy counts than the saline-treated counterparts, and this effect increased every day of the pre-treatment phase (effect of time, $F_{(4,63)}$=53.32, p<0.0001). Throughout this phase however, SAL-IRON offspring presented significantly more stereotypy counts compared to their SAL-VEH counterparts (prenatal treatment by supplementation interaction, $F_{(1,63)}$=11.71, p=0.001; simple effect of supplementation for the offspring of SAL treated mothers, $F_{(1,63)}$=9.77, p=0.003) and compared to the TURP-IRON offspring (simple effect of prenatal treatment for the offspring of IRON supplemented dams, $F_{(1,63)}$=9.73, p=0.003). These results suggest that although the AMPH-induced locomotion in the SAL-IRON group seemed comparable to that of the SAL-VEH offspring (Fig. V-4B), the SAL-IRON animals were indeed more sensitive to the effects of AMPH, as they present significantly greater levels of stereotypy counts than the control animals. This effect was not observed in the TURP-IRON offspring, suggesting that iron supplementation is beneficial for the offspring of mothers that undergo inflammatory insults.

Finally, on the day of the sensitization test (Fig. V-11B), all AMPH-pre-treated groups presented a greater stereotypy counts than their saline-pre-treated counterparts (effect of pre-treatment, $F_{(1,127)}$=84.73, p<0.0001). Among the AMPH-pre-treated groups, SAL-IRON and TURP-VEH presented significantly greater stereotypy counts than the SAL-VEH animals (prenatal treatment by supplementation by pre-treatment threeway interaction, $F_{(1,127)}$=11.54, p=0.0009; simple effect of maternal supplementation for the AMPH-pre-treated offspring of SAL-treated mothers, $F_{(1,127)}$=20.94, p<0.0001; simple effect of prenatal treatment for the AMPH-pre-treated offspring of VEH-supplemented mothers, $F_{(1,127)}$=7.38, p=0.0075). The stereotypy counts of the TURP-IRON group did not differ from those in the SAL-VEH controls and were significantly lower than that in both

TURP-VEH and SAL-IRON groups (simple effect of maternal supplementation for the AMPH-pre-treated offspring of TURP-treated mothers, $F_{(1,127)}=11.88$, $p=0.0008$; simple effect of prenatal treatment for the AMPH-pre-treated offspring of IRON-supplemented mothers, $F_{(1,127)}=28.16$, $p<0.0001$). These observations confirm our finding that in the SAL-IRON group there is a greater sensitivity for the induction of stereotypy by AMPH administration. More importantly, they support our proposal that imbalance of foetal iron homeostasis is involved in the effects of prenatal inflammation in the adult offspring's DA function.

Figure V-11. AMPH-induced stereotypy counts are increased by prenatal TURP treatment and IRON supplementation administered separately, but not when given together

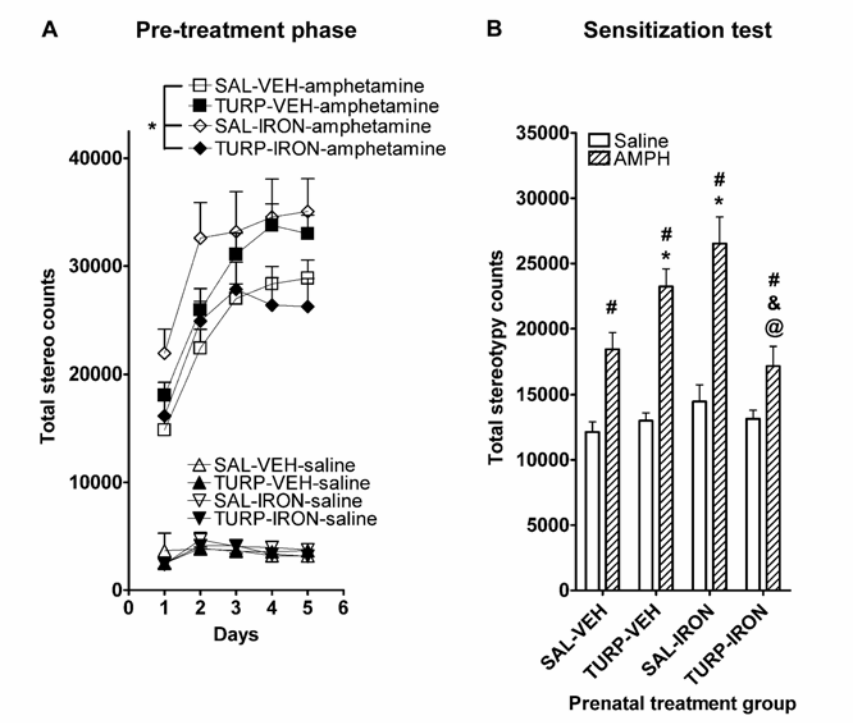

*Figure V-11. (A) AMPH administration (i.p. 2 mg/kg) for 5 consecutive days induced a progressive enhancement of the induction of stereotypy, which tended to be overall greater among the offspring of TURP-VEH treated dams compared to their SAL-VEH counterparts. This effect was even greater for the SAL-IRON group and completely prevented in the offspring of TURP-IRON dams, whose behaviour was indistinguishable from those of the SAL-VEH group. * vs. AMPH pre-treated SAL-VEH or TURP-IRON. (B) Seven days after the pre-treatment phase all animals were tested for sensitization, with a lower dose of AMPH (i.p. 1 mg/kg). All AMPH pre-treated animals (dashed bars) showed significantly greater stereotypy than the saline-pre-treated animals (white bars). Among the AMPH pre-treated animals, those born to TURP-VEH and SAL-IRON presented significantly greater stereotypy, whereas in the offspring of TURP-IRON dams, sensitization response was comparable to control animals. $p<0.01$: # vs. saline pre-treated counterpart, * vs. AMPH pre-treated SAL-VEH, & vs. AMPH pre-treated TURP-VEH, @ vs. AMPH pre-treated SAL-IRON.*

General discussion and conclusion

In this thesis work it was firmly established that a localized inflammatory challenge with TURP (i.m.) to a pregnant rat results in significant long-term alterations in behaviour and brain neurochemistry of the adult offspring. These changes were shown to be linked to increased maternal cytokines either directly or through secondary mechanisms such hypoferremia. The gathered evidence indicates that during gestation, the inflammatory response of the mother is dynamically modulated (Chapters II and III). A baseline increase in the levels of the main pro-inflammatory cytokine, IL-6 (Chapter II), was described. This effect is consistent with the changes in the basal levels of circulating inflammatory mediators that occurs in pregnant women (Amoudruz et al., 2006; Luppi et al., 2002; Pillay et al., 1993). In addition, inflammation-induced levels of this cytokine were strongly attenuated at GD 18, compared to either GD 15 (Chapter III) or non-pregnant females (Chapter II). This blunted IL-6 response may underlie the attenuated febrile response found in the late-pregnancy group of dams (Aguilar-Valles et al., 2007), but not earlier in gestation. IL-6 is involved in the induction of COX-2 and mPGES-1 (Rummel et al., 2006), which are involved in the production of the pyrogenic prostaglandin PGE_2. We observed attenuation in the induction of the expression of these synthetic enzymes in response to TURP at GD 18 (Chapter II).

What remains to be determined is the mechanism through which IL-6 release is attenuated near term. We speculated that the increasing levels of oestrogen during gestation, towards GD 17 (Mann and Bridges, 2001), may be involved in this response (Cuzzocrea et al., 2001; Ghisletti et al., 2005; Mouihate and Pittman, 2003; Ospina et al., 2004; Vegeto et al., 2001). This hypothesis deserves further investigation.

Intriguingly, although attenuated fever is commonly found in several mammalian species and using several inflammatory agents (Cooper et al., 1988; Eliason and Fewell, 1997; Kasting et al., 1978; Martin et al., 1995; Simrose and Fewell, 1995; Stobie-Hayes and Fewell, 1996; Zeisberger et al., 1981), not all

models of inflammation have been found to result in similar alterations in the profile of released cytokines (Harre et al., 2006; Mouihate et al., 2005b). Notably, extensive research has been made using the systemic injection of LPS, which has produced different results. In this case, some have reported normal levels of IL-6 (Harre et al., 2006), but increase IL-1ra induction (Ashdown et al., 2007); in addition, central anti-pyretic mechanisms have also been involved (Begg et al., 2007; Begg et al., 2008; Chen et al., 1999; Imai-Matsumura et al., 2002; Mouihate et al., 2002). For example, it has been shown that there is a decrease in the febrigenic response to the injection of prostaglandins (Chen et al., 1999) and increase expression of nitric oxide synthase (NOS), which may exert an anti-pyretic effect in the hypothalamus (Begg et al., 2007; Begg et al., 2008). These data, along with our own studies, suggest a rather complex network of mechanism that converge to reduce the febrile response in late pregnancy. On one hand, the response to a spatially confined inflammatory insult can be attenuated by means of peripheral mechanisms that reduce the ability of the organism to release IL-6, the main circulating pro-inflammatory mediator (Figure discussion-1). Alternatively, a more overwhelming stimulus, such as the systemic injection of LPS, and perhaps poly I:C, would overcome the peripheral mechanisms that limit the release of IL-6 from immune cells by targeting a greater number of cell types, whose response may not necessarily be affected by the systemic increases in oestrogen. Then, the central anti-pyretic mechanisms (hyporesponsiveness to prostaglandins, increased production of NO) may come to play a significant role to attenuate the febrile response. In addition, by directly targeting the adipose tissue, an important source of inflammatory mediators in the context of systemic inflammation (Axelsson et al., 2005; Dayer et al., 2006; Zoccali et al., 2005), LPS may induce the release of greater amounts of the anti-inflammatory IL-1ra (Ashdown et al., 2006b; Dayer et al., 2006). In humans, data on the febrile response towards the end of gestation are not yet available (Spencer et al., 2008). What has, however, been shown is the alteration in basal and immunogen-induced levels of cytokines from peripheral immune cells (Amoudruz et al., 2006; Luppi

et al., 2002; Pillay et al., 1993), which are comparable to those described in animal models, especially TURP described in this thesis.

The consequences of having an intact febrile response towards the end of gestation are unknown, although prolonged hyperthermia has teratogenic effects in the developing foetus (Chambers et al., 1998; Edwards, 2006). Given the adaptive value that fever and other host inflammatory/sickness responses have (Baumann and Gauldie, 1994; Kluger et al., 1998; Kushner, 1988; Murray and Murray, 1979), it has been speculated that their suppression occurs as a consequence of the potential damage that they may pose for the foetus (Ashdown et al., 2006a; Boksa, 2010a; Meyer et al., 2009b; Mouihate et al., 2008). The observations that suggest the existence of multiple mechanisms contributing to attenuate inflammation during late gestation support the hypothesis that these represent protective mechanisms.

Somewhat in line with this point of view, the consequences of prenatal infection for the offspring seem to be more marked when the insult occurs early in gestation, when inflammatory responses are still intact, compared to late-pregnancy [Chapter II and (Cui et al., 2009; Fortier et al., 2007; Li et al., 2009b; Meyer et al., 2006a; Meyer et al., 2007a; Zuckerman and Weiner, 2005)]. However, if the mechanisms that prevent a normal inflammatory response in late pregnancy were to protect the normal foetal development, then one may expect for them to appear earlier in gestation. More evidence is required to better understand what would happen if febrile response in late gestation and lactation were to be restored to levels observed in non-pregnant females or in early/mid-pregnancy.

Figure Dicsussion-1. Mechanisms that lead to attenuated inflammation in late-pregnancy

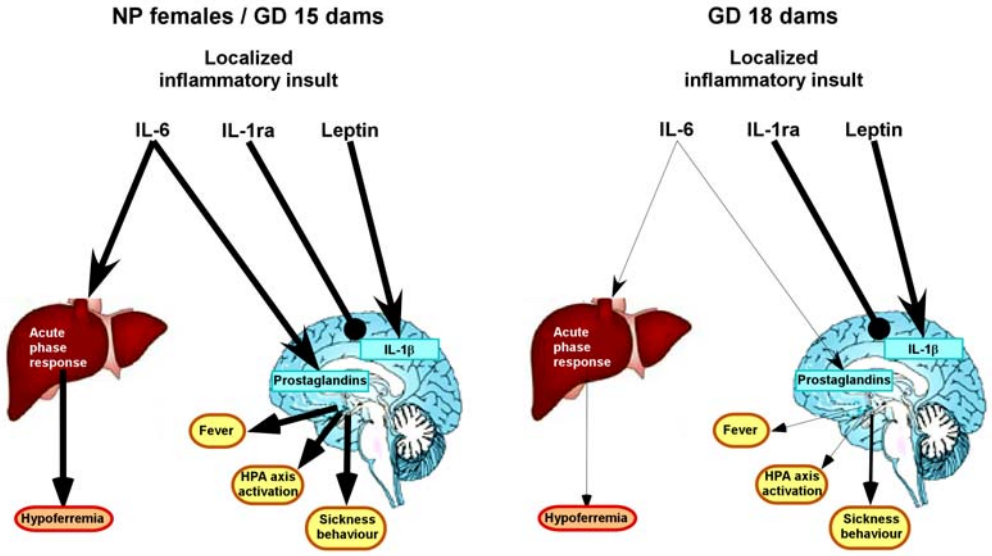

Figure Discussion-1. Inflammatory response to a localised insult is modulated by pregnancy. In dams at GD 15 or in NP females (left), TURP elicits a significant increase in the circulating levels of the pro-inflammatory cytokine IL-6, the anti-inflammatory IL-1ra and leptin. IL-6 targets the liver to induce HAMP expression, which in turn leads to the induction of hypoferremia. In the brain, IL-6 is involved in the induction of synthesis of prostaglandins, which initiate the febrile response, the activation of the HPA axis and other sickness behaviours. Leptin is proposed to induce the expression of brain cytokines, such as IL-1β, as well as to control the induction of decreases in food intake and body weight. IL-1ra inhibits the effects of locally expressed IL-1β, thereby controlling the duration of several sickness responses. In late gestation, i.e. GD 18, the release of IL-6 into the circulation is inhibited, therefore all downstream responses are attenuated. In contrast, IL-1ra and leptin are induced at comparable levels. These changes may reflect a shift in the balance of anti-and pro-inflammatory mediators, resulting in the attenuation of many inflammatory responses, whereas some, mediated by leptin, may remain intact.

The main objective of this research program was to identify the inflammatory mechanisms involved in the effects of prenatal infection on the neurodevelopment of the offspring, especially regarding the mesolimbic DA system. In this regard, IL-6, the main circulating pro-inflammatory mediator in acute inflammation (Harden et al., 2006; Liuzzi et al., 2005; Nemeth et al., 2004a; Roth, 2004; Rummel et al., 2008; Rummel et al., 2006; Turnbull et al., 2003), had been recognized in animal models of maternal infection, to be centrally involved in the induction of behavioural and neurochemical alterations in the offspring (Smith et al., 2007). It is important to bear in mind that the interest in IL-6 was a central component of the rational for choosing TURP as a model of inflammation. As discussed above and in section I.8, the systemic effects of TURP depend on the elevation of circulating IL-6 levels, whereas models of systemic inflammation (LPS or poly I:C) trigger a more complex profile of cytokine release (Givalois et al., 1994; Turrin et al., 2001).

Administration of TURP at GD 15, but not at GD 18, induced the release of maternal IL-6 and this correlated with induction of enhanced AMPH-induced locomotion (among other behavioural alterations) and increased expression of TH in the NAcc of the adult, but not the weanling or peri-pubertal offspring (Chapter II). This was the first piece of evidence, obtained in the present studies, implicating IL-6 in the effects of prenatal TURP on the DA neurotransmission of the offspring. In addition, direct confirmation of this proposal was obtained from the studies in Chapter III. In these, it was observed that neutralization of IL-6 during the inflammatory challenge to the mother prevented several TURP-induced alterations in the offspring, including: enhanced locomotor response to acute and repeated AMPH-administration, enhanced behavioural sensitization following repeated AMPH treatment, the increased expression of TH and, importantly, the elevated levels of DA and DOPAC in the NAcc. Collectively these data provided more definitive support for the involvement of IL-6 in the neurodevelopmental defects that lead to sensitized DA neurotransmission.

Circulating IL-6 could target the placenta to exert its detrimental effects on the offspring (Fitzgerald et al., 2008). We determined the activation of the

JAK/STAT3 signaling pathway in this tissue, following TURP administration to the mother at GD 15 or 18 (Chapter III), and found that it occurred irrespectively of the presence of significant levels of IL-6 in the maternal circulation. This data implies the involvement of additional mediators capable of the activation of this pathway, such as leptin (Tartaglia, 1997; Tartaglia et al., 1995).

Another possibility was then explored. IL-6 is involved in the induction of hypoferremia during inflammation (Nemeth et al., 2004a). This response is potentially quite detrimental for the developing brain, as iron is involved in a number of basic metabolic function of all cell types (Beard and Connor, 2003; Beard et al., 2006). In addition, fluctuation in iron levels can impact the function and development of DA neurons (Beard and Connor, 2003; Beard et al., 2006; Burhans et al., 2005; Erikson et al., 2001; Unger et al., 2007). The studies described in Chapter V indicated that TURP administration to an iron-replenished dam almost completely prevented the induction of a sensitized mesolimbic DA system. Iron replenishment was used to block the induction of HAMP, the systemic controller of iron homeostasis, during TURP-induced inflammation. This approach indeed prevented the drop in maternal circulating and placental levels of non-haeme iron during gestation (Fig. Discussion-2). However, it was also observed that this form of iron supplementation has a negative outcome for the offspring when given to an iron-sufficient mother.

Despite the clear cut evidence implicating the alterations in maternal iron homeostasis during inflammation on the development of alterations in DA function in the offspring, the mechanisms of action in the foetal brain remained elusive. There were no whole tissue alterations in foetal brain iron content that could relate to the alterations in DA neurotransmission observed in the adult offspring, although regionally restricted changes may occur. As mentioned, TURP induced decrease in iron content in the placenta and this was prevented in the iron-supplemented mothers. This could suggest that IL-6-induced hypoferremia may result in altering the physiology of this organ, which may in turn affect neurodevelopment of the foetus. However, it is important to note that compromised placental physiology, as induced by prenatal LPS (Girard et al.,

2010), leads to drastic decreases in blood flow, induction of reactive oxygen species and cell death in this tissue. These effects negatively impact foetal development, inducing growth restriction, major damage to the foetal brain and even foetal death (Girard et al., 2010). In the present studies it was clearly demonstrated that prenatal TURP alters the expression of inflammatory molecules (Chapter III) and iron content (Chapter V) in the placenta. However, this was not associated with changes in body weight of the newborns or litter size, indicatives of drastic placental damage.

Another possibility is that HAMP, whose expression was induced in the foetal liver by either maternal TURP treatment or maternal iron supplementation alone, has recently been described to activate the JAK/STAT3 signalling pathway (De Domenico et al., 2010). This effect, mediated by the binding of HAMP to FPT, alters the expression levels of many genes, including cytokines (De Domenico et al., 2010). It is tempting to suggest that foetal HAMP may be targeting neurons in the developing brain, which express FPT (Moos and Rosengren Nielsen, 2006; Moos et al., 2007), thereby inducing alteration in the development of the mesolimbic DA, among other systems (Fig. Discussion-2).

Along with the induction of hypoferremia, which in turn affects development of DA neurones, IL-6 may cross directly into the foetal compartment at this stage of gestation (Dahlgren et al., 2006), resulting in additional alterations in brain physiology (Samuelsson et al., 2006a; Samuelsson et al., 2006b; Samuelsson et al., 2004; Smith et al., 2007).

Figure Dicsussion-2. Inflammatory mechanisms involved in the effects of prenatal infection on the neurodevelopment of the offspring

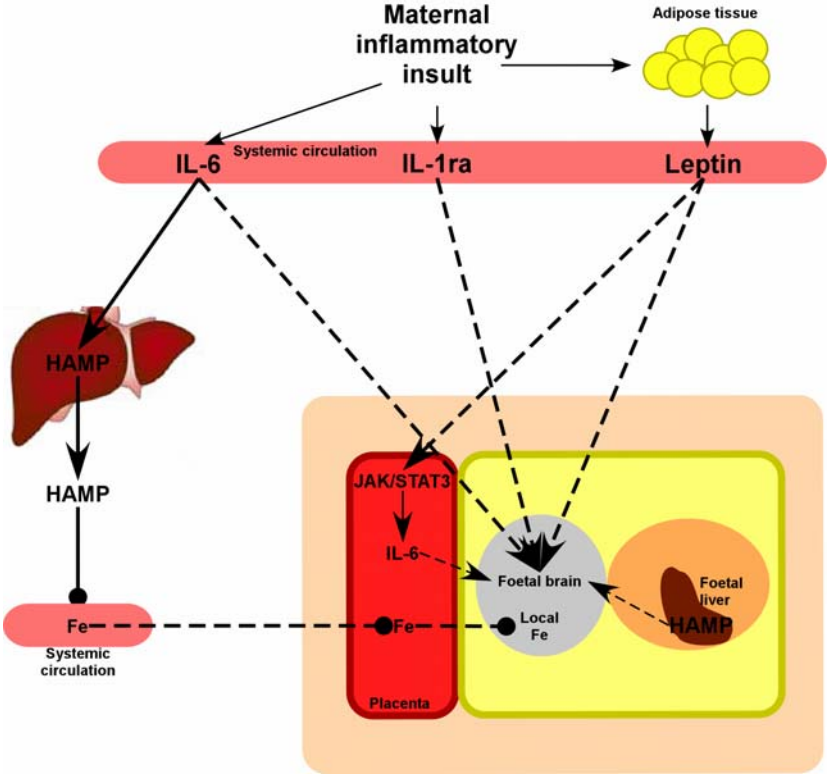

Figure Discussion-2. Maternal infection leads to the release of multiple inflammatory mediators, many of which are involved in affecting neurodevelopment of the offspring, especially that of the mesolimbic DA system. IL-6 is fundamentally involved in the induction of neurodevelopmental alterations that lead to a sensitized DA neurotransmission in the adult offspring. IL-6 may exert its effect directly on the foetal brain, as it can cross the placental-blood barrier. Alternatively or in parallel, IL-6-induced hypoferremia disrupts iron homeostasis, leading to a decrease of non-haeme iron levels in the maternal circulation and the placenta. The latter effect may influence neurodevelopment through malfunction of this organ or may restrict the supply of this nutrient into specific foetal brain regions. Leptin seems to be responsible for the activation of the JAK/STAT3 signalling pathway in the placenta, which may lead to the increased expression of IL-6. Leptin and IL-1ra are also involved in the effects of prenatal inflammation on the DA function of the offspring. Finally, maternal

TURP results in the induction of HAMP in the foetal liver, which may alter gene expression in the foetal brain.

Studies in Chapter IV involved two other inflammatory mediators, IL-1ra and leptin, in the induction of alterations in DA neurotransmission in the offspring of TURP-treated mothers (Fig. Discussion-2). IL-1ra is an anti-inflammatory cytokine (Dinarello, 1991), whose administration to pregnant dams prevents the toxic effects of LPS in the foetuses (Girard et al., 2010). Therefore it was initially hypothesized that this mediator would act as a protective agent and its neutralization would lead to a worsening of the effects of prenatal inflammation on the neurodevelopment of the offspring. Surprisingly, this intervention showed that IL-1ra was involved in induction of several alterations of prenatal TURP in DA neurotransmission, as evidenced by the prevention of the enhancement of the locomotor responses to AMPH injection and the increased levels of TH, DA and DOPAC in the NAcc. These effects completely overlapped with those triggered by IL-6, suggesting the existence of a common mechanism for these two cytokines with seemingly antagonistic effects. Data from this study however, indicated that Leptin, is only involved in the induction of neurodevelopmental alterations that lead to enhanced behavioural sensitization following repeated AMPH administration, as well as the induction of basal increases in TH and DA. It has not yet been defined how TURP-induced elevations in maternal IL-1ra and leptin may affect brain development. Several studies indicate that these two cytokines can affect the function and development of the DA neurons. For example, genetically ablated expression of IL-1RI, the receptor where IL-1ra exerts its antagonistic effect, leads to increased sprouting of the terminal regions of mesencephalic DA neurons (Parish et al., 2002), possibly a similar effect of TURP-induced IL-1ra. A similar model for leptin (i.e. the *ob/ob* mice) indicates that this hormone can induce activation of the mesolimbic DA neurons and control of remodelling of this circuitry during sensitization (Figlewicz et al., 2006; Fulton et al., 2004; Fulton et al., 2000; Shalev et al., 2001).

Finally, by means of using TURP as a model of inflammation, it was possible to establish that a wide range of behavioural and neurochemical alterations in the offspring are induced in the absence of systemic exogenous immunogens, like LPS or poly I:C. This distinction is fundamental, as it provides pivotal evidence that the maternal endogenously expressed mediators and inflammatory mechanisms are, without doubt, responsible for the effects in the foetal compartment by underlying the link between maternal infection and mental disorders in the offspring.

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