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Late Prenatal Infection and Neurodevelopmental Disorders:

Characterization of an Immune-Mediated Mouse Model

Settore Scientifico-Disciplinare BIO/14

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1. INTRODUCTION

Compelling evidence suggests that the aetiology of multifactorial and multisymptomatic psychiatric diseases include exposures to adverse events during prenatal and early postnatal life, which may disrupt the correct maturation of neural system and lead to long-lasting changes in brain function. Prenatal insults, and in particular prenatal infection, could thus act as a "primer" for the neuropsychiatric route, even if the specificity of the illness that develops is strongly influenced by the genetic and environmental context in which the process occurs (Meyer, 2013b). A similar concept of "early-life programming of adult disease" has been put forward by the seminal work of David Barker conducted in the context of cardiovascular disease (Dover, 2009), suggesting that specific environmental factors acting during sensitive prenatal or early postnatal developmental periods can induce persistent changes in physiological, emotional and behavioural functions throughout life (Bale *et al*, 2010). Against this background, the aim of my PhD thesis was to investigate and further characterize an established murine model of prenatal infection that is based on maternal administration of the viral mimic polyriboinosinic-polyribocytidilic acid [Poly(I:C)], focusing on different aspects of the relationship between altered neurodevelopment and psychiatric disease. First, I will give an overview of the state of the art regarding the association between prenatal infection and different neurodevelopmental illnesses as identified by human epidemiological studies, and then I will present our preclinical results obtained in an experimental model system of prenatal immune activation.

1.1 Neurodevelopment and Mental Illness

In the past decades, research has suggested a pivotal role of gestational and early life stressors in susceptibility to mental disorders, and this role has been increasingly investigated by multiple epidemiological and birth cohort studies. Early life is, in fact, a period of high vulnerability to a wide range of exogenous adverse events, which can alter the course of normal brain development and compromise the integrity and function of various brain systems with permanent consequences. In particular, fetal brain development is a complex and delicate process that takes place in a protected environment inside the mother's body. It is thus conceivable that a variety of exogenous maternal factors could alter the course of fetal brain development and subsequent maturation predisposing the individual to the development of multiple diseases later in life. On this basis, it is plausible that specific environmental factors acting during sensitive prenatal or early postnatal developmental periods could 'program' adult disease by inducing persistent changes in physiological, emotional and behavioural functions throughout life (Bale *et al*, 2010). As normal brain development follows a regulated and programmed pathway that gives rise to distinct and specific regional structures and networks (Bayer *et al*, 1993) (Herschkowitz *et al*, 1997), numerous exogenous agents that interfere with this process could alter the course of normal brain development, compromising the integrity of structure and function of the central nervous system. In recent years, the early life environment has garnered increasing attention with respect to its role in elevating the susceptibility to psychiatric disorders that are characterized by neurodevelopmental components, including schizophrenia, autism, and bipolar disorder. This has been supported by different lines of research. In particular, different birth cohort studies with large databases on prenatal and perinatal risk factors have come to the age when various psychiatric disorders typically emerge, and data collection methods have been improved, in order to systematically identify different risk factors during prenatal and postnatal periods (Brown, 2011). Moreover, researchers have implemented many translational models that incorporate measures of the environment into animal models. The latter provide an indispensable tool to study causal relationships between early-life exposures to specific environmental adversities and abnormal brain development.

These studies have greatly increased our knowledge regarding the interaction between early life environment and neurodevelopment, especially in light of its impact on later development of mental illnesses. It is now widely recognised that factors such as maternal stress, prenatal infection, undernutrition, obstetric complications, childhood trauma and abuse all confer increased risk for the development of psychiatric disorders later in life (Brown, 2011). For example, different studies demonstrated an increased risk of schizophrenia and autism among individuals born and raised in an urban area compared to a rural area (Lauritsen *et al*, 2014; March *et al*, 2008; Mortensen *et al*). *Per se*, this coincidence does not represent an early life adversity, but it could determine fetal exposure to environmental stressors like pollution and infectious microbes. Prenatal exposure to infectious agents has, in fact, been identified as a noticeable risk factor for many mental disorders, which will be discussed in depth in the following sections of this thesis.

Maternal nutrition has also been identified as a possible factor involved in the fetal programming of adult disease, as demonstrated by epidemiological studies from the Dutch Hunger Winter and the Chinese Famines (Brown and Susser, 2008; Brown *et al*, 2000b; Xu *et al*, 2009) that found a statistically significant increase in the risk of developing schizophrenia and major affective disorder as a consequence of prenatal malnutrition. Moreover, prenatal deficiency of specific micronutrients, as for example folate, iron and vitamin D, has also been associated with adult mental disease (Brown *et al*, 2007; Eyles *et al*, 2013; McGrath, 2011). Apart from these environmental factors, maternal psychological stress during pregnancy also plays a critical detrimental role in fetal development. Exposure to bereavement stress, natural disaster, war, serious injury or death of a close relative have all been associated with a higher risk of psychiatric disease, as for example schizophrenia (Herman *et al*, 2006; Khashan *et al*, 2008; van Os and Selten, 1998), affective disorders (Kleinhaus *et al*, 2013), and autism (Class *et al*, 2014). Maternal stress, and in particular elevated levels of fetal glucocorticoids, detrimentally reprograms the fetal HPA axis, and this in turn could increase long-term mental disease risk (Harris and Seckl, 2011). Since higher basal HPA axis activity coupled with greater stress reactivity is associated with the development of depressive and anxiety-related disorders, it has been proposed that children born to stressed mothers during pregnancy could have a higher risk of developing affective and emotional disorders such as major depression and anxiety (Koenig, 2006; Weinstock,

2005, 2008). Lastly, obstetric complications during delivery, as for example asphyxia, uterine atony, hypoxia and emergency C-section, are among the first epidemiological findings shown to contribute to susceptibility for schizophrenia (for detailed reviews see (Cannon *et al*, 2002; Matheson *et al*, 2011)), and also seem to be associated with an increased risk for autism (Kolevzon *et al*, 2007) and cerebral palsy (Rennie *et al*, 2007).

As the human brain continues to mature during adolescence, the postnatal environment also has a critical influence on programming disease risk. Increasing evidence unequivocally reveal that adults exposed to child abuse, neglect, trauma, and infection are at greater risk of developing psychiatric disorders. For example, results from the Adverse Childhood Exposure study demonstrate that childhood exposure to a variety of adverse experiences like psychological maltreatment, physical abuse, sexual abuse, child neglect, caregivers's substance/alcohol use, caregiver's depressive symptoms, caregiver's being treated violently, and criminal behaviour in the household, is associated with an increased risk of general health complaint and, in particular, with the development of affective disorders (Chapman *et al*, 2004; Felitti, 2009; Felitti *et al*, 1998). Being bullied in school also increases susceptibility to depression and difficult peer relationships in adulthood (Lund *et al*, 2009; Sourander *et al*, 2009), as well as divorce of parents and inter-parental conflict, even if the overall increase of risk is somewhat low (Fryers and Brugha, 2013). Childhood abuse, and in particular sexual abuse, stands out as one of the main postnatal risk factors for developing adult mental disorders and can lead to various pathological outcomes. Increased risk following sexual and physical abuse has in fact been demonstrated for depression, anxiety, personality disorders, and to a lesser extent schizophrenia (Fryers *et al*, 2013).

Against this background, researchers have sought to shed light on the mechanisms underlying the association between these pre- and postnatal environmental insults and the increased vulnerability to psychological illness. Clearly, the common target of these early life stressors is, among others, fetal and early brain development, even if this process can be impacted by different factors in different ways. In particular, many researchers hypothesize that environmental insults converge on different pathogenetic processes that act to disrupt correct neurodevelopment, including oxidative stress, apoptosis, inflammatory processes and HPA-mediated mechanisms (Meyer and Feldon, 2010a; Steullet *et al*, 2014).

Increased oxidative stress plays an important role in schizophrenia (Wood *et al*, 2009) as it could affect neurogenesis, neuronal proliferation, migration and differentiation, as observed for example in autistic individuals (Kern and Jones, 2006; Miller *et al*, 2005). Moreover, Do et al. argue that the detrimental effects of many environmental insults are mediated by oxidative and nitrosative stress, which in turn could impact myelination and NMDA receptor functioning (Do *et al*, 2009). In particular, recent evidence leads to the hypothesis that the redox, neuroimmune and glutamatergic systems form a central 'hub', and that a dysregulation of any of these systems could lead to molecular abnormalities typical, for example, of schizophrenia, such as abnormalities in parvalbumin interneurons and white matter (for a detailed review see (Steullet *et al*, 2014), ultimately leading to the social, cognitive and affective abnormalities found in this disease.

Apoptosis, or programmed cell death, is an ubiquitous process involved in many biological processes and pivotal for normal brain development, as it is critical for turnover of cells and brain shaping by ensuring that only growing, migrating and synapse forming neurons survive (Lockshin and Zakeri, 2002; Penaloza *et al*, 2006). As such, this process has been repeatedly associated with ethiopathological processes involved in schizophrenia and other psychiatric diseases (Jarskog, 2006). Interestingly, apoptosis is affected by many environmental stimuli, such as oxidative stress, hypoxia, ischemia and proinflammatory cytokines, rendering it a preferential mechanism through which differential stressors could enact their detrimental effects on brain development.

Cytokines are small pleiotrophic soluble proteins that have multiple roles, such as coordinating the hosts' response to infection as well as mediating physiological signalling between cells of non-immune tissues, including the CNS. They can act both locally or have endocrine effects. These soluble mediators have important roles in neurodevelopment and function, as they contribute to many different processes such as temporal regulation of neurogenesis and gliogenesis, progenitor migration, proliferation and axon path-finding, neuronal survival and synapse modulation and elimination (Boulanger, 2009; Deverman and Patterson, 2009; Suvisaari and Mantere, 2013). Against this background, different (but not mutually exclusive) hypotheses that could explain why abnormal prenatal exposure to these agents increases the risk of developing psychiatric disorders have been developed. The prenatal cytokine hypothesis, first proposed by Gilmore and Jarskog, postulates that exaggerated levels of

cytokines produced by the mother could disturb normal fetal brain development, increasing the risk of developmental psychiatric diseases such as schizophrenia (Gilmore and Jarskog, 1997). This theory has been extensively supported by many epidemiological and preclinical studies, and has further been broadened to include the concept that infection-induced developmental neuroinflammation could also be relevant beyond early life (Meyer, 2013a). In particular, exposure to prenatal immune challenge could prime early alterations in peripheral and central inflammatory response systems that could in turn disrupt processes that are pivotal for normal brain development, such as myelination, synaptic pruning and neuronal remodelling, which occur mainly during postnatal brain maturation (Meyer, 2013a). This topic will be discussed in detail in subsequent sections of my thesis.

Activation of the HPA axis is a common end point of many environmental insults, as for example stress, malnutrition, hypoxia and infection. Moreover, psychosocial factors such as migration, low socioeconomic status and living in a hostile or dangerous area can similarly induce HPA hyperactivity. Ultimately, activation of the HPA axis results in the release of glucocorticoids, which are detrimental for normal brain development (Painter *et al*, 2012). In particular, glucocorticoids exert their biological functions through two different types of receptors (the glucocorticoid and the mineralocorticoid receptor) that are expressed at relatively high levels during fetal development (Welberg and Seckl, 2001). Thus, altered levels of glucocorticoids in the maternal host and fetal environment could readily affect programming of physiological, endocrinological and behavioural functions (Seckl, 2004; Welberg *et al*, 2001) (Owen *et al*, 2005).

1.2 Prenatal Infection and Psychiatric Disorders

In recent years, a plethora of epidemiologic, clinical and preclinical studies have provided evidence in support of the notion that prenatal infection represents a risk factor for various psychiatric disorders, in particular those characterized by a neurodevelopmental component such as schizophrenia and autism. Indeed, early-life exposures to microbial pathogens have been clearly documented to determine congenital brain abnormalities and a variety of learning and behavioural abnormalities in adulthood (Remington et al., 2006). Thus, investigators began to examine whether in

utero exposure to infectious agents was related to an increased incidence of various psychiatric conditions. In the following sections I will summarize the most relevant epidemiological, clinical and preclinical studies that provide support for this association.

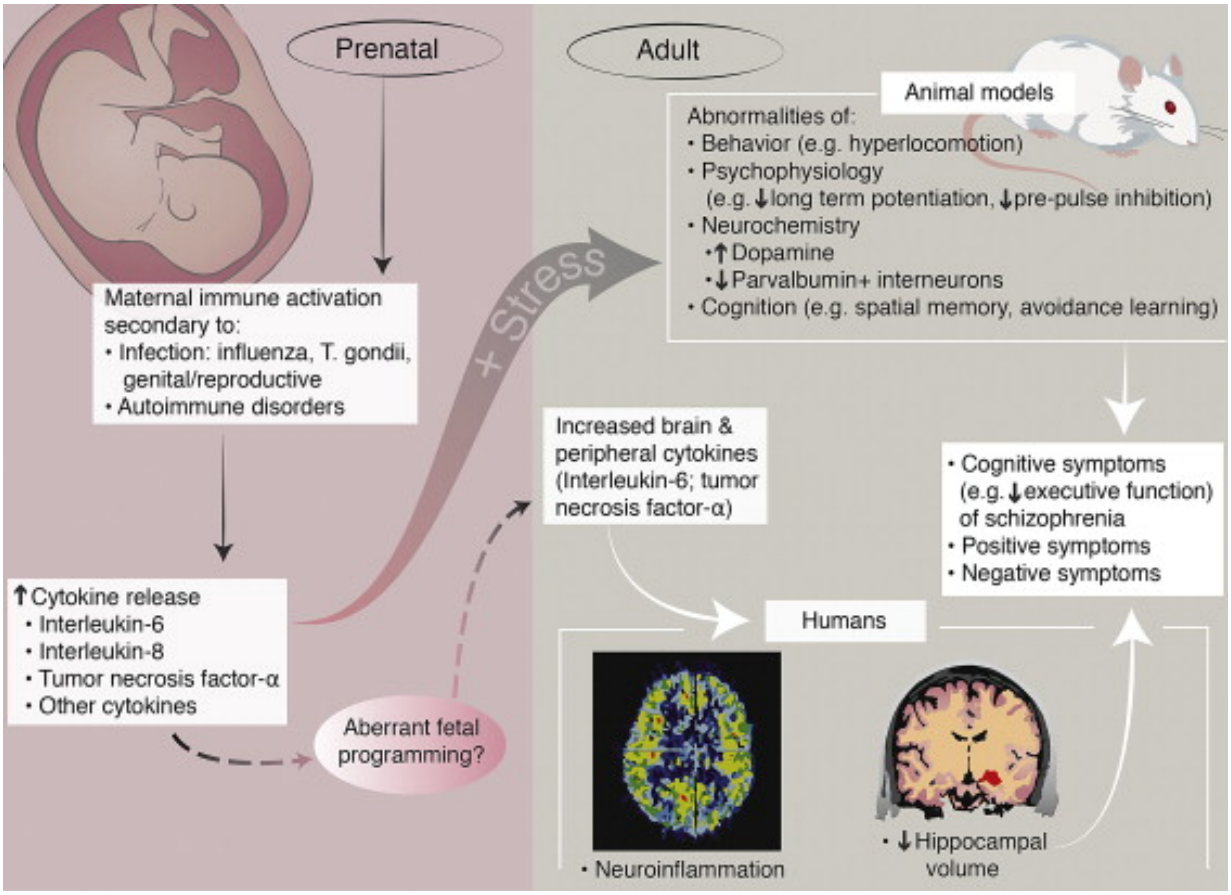

Figure1. The cytokine model of schizophrenia. From Ragy et al., 2014.

1.2.1 Epidemiological Studies

The earliest studies regarding the association between psychiatric disorders and prenatal infection were derived from ecological data. In these studies, the exposure status is based, for example, on the presence of influenza pandemics in a certain population at a given time. Thus, exposure is examined in relation to the date at which individuals, who later developed psychiatric conditions, were in utero. Even considering the limitations of such approach, many studies found a significant association between exposure to prenatal infection and the subsequent development of psychiatric disorders, in particular schizophrenia. The first line of evidence supporting such neuroimmune mechanisms dates back more than a 100 years, when Karl A. Menninger observed a link

between influenza exposure and psychotic disease in patients who were admitted to the Boston Psychiatric Hospital after the influenza pandemic of 1918 (Menninger, 1919). This hypothesis was fuelled in the 1970s by Torrey et al., who suggested that latent viruses could be involved in the development of schizophrenia (Torrey and Peterson, 1973). Since then, this field of investigation has greatly expanded, and many other infectious agents are now considered to play a role in the development of different psychiatric conditions. In the 1980s, Mednick et al. reported an increased risk of schizophrenia after prenatal exposure to the influenza pandemic in greater Helsinki (Mednick *et al*, 1988) in the second trimester of pregnancy, and his findings were replicated by subsequent studies evaluating other influenza epidemics (Barr *et al*, 1990; Kendell and Kemp, 1989; Kunugi *et al*, 1995; McGrath and Castle, 1995; O'Callaghan *et al*, 1991). The association between prenatal infection and schizophrenia was revealed also by studies on other infections, including respiratory viral infections (O'Callaghan *et al*, 1994; Watson *et al*, 1984), measles (Torrey *et al*, 1988), varicella-zoster (O'Callaghan *et al*, 1994) (Torrey *et al*, 1988), and polio (Suvisaari *et al*, 1999). There have been, however, numerous studies that failed to replicate these associations (Erlenmeyer-Kimling *et al*, 1994; Selten *et al*, 2010; Susser *et al*, 1994; Westergaard *et al*, 1999), probably because of the methodological limitations of using ecological data, such as misclassification of exposure and assumption of full term pregnancy. For this purpose, investigators started to conduct birth cohort studies, which sought to document infections by measuring biomarkers in individual pregnancies and to relate confirmed exposure to the development of schizophrenia among individuals followed up into the age risk of the disorder. These studies provided serologic evidence for various pathogens implicated in the prenatal infectious aetiology of schizophrenia, such as rubella (Brown *et al*, 2001; Brown *et al*, 2000a), influenza (Brown *et al*, 2004), *Toxoplasma Gondii* (Brown *et al*, 2005; Mortensen *et al*, 2007), *Herpes simplex virus type 2* (Buka *et al*, 2008; Buka *et al*, 2001) and other prenatal infections such as genital/reproductive infections (Babulas *et al*, 2006), bacterial infection (Sorensen *et al*, 2009) and pyelonephritis (Clarke *et al*, 2009). A similar link, even if less established, has also been demonstrated for other psychiatric diseases. In a study based on the Danish Medical Birth register, Atladottir et al. observed an increased risk for developing autism after both viral and bacterial infections (Atladottir *et al*, 2010). In subsequent studies, different groups demonstrated an increased risk for autism following increased levels of

proinflammatory citokines in the maternal compartment and amniotic fluid (Abdallah *et al*, 2012; Goines *et al*, 2011). Recently, Parboosing and colleagues demonstrated a 4-fold increase in bipolar disorder risk after exposure to maternal influenza in a serologically documented prospective epidemiological setting (Parboosing *et al*, 2013).

1.2.2 Animal Models of Prenatal Infection

Based on the accumulating epidemiological evidence that links prenatal exposure to maternal infection with the development of psychiatric disorders later in life, investigators have developed a number of in-vivo models of prenatal immune activation in both rodents and monkeys in order to test the hypothesis of causality that arises from human studies. The use of animal models, in fact, allows studying the neurobiological basis of brain and behavioural disturbances relative to various psychiatric disorders, and also to implement and evaluate novel pharmacological treatments. In particular, animal research provides the opportunity to overcome the ethical and technical reasons that limit the capacity of unravelling the causal relationship that exists between prenatal infection and psychiatric disorders in humans, seeing as the investigation of the precise cellular and molecular mechanisms that affect normal brain development is impossible in human subjects. Moreover, the use of animal models allows a stringent experimental control of subjects in genetically homogeneous populations and facilitates the recognition and evaluation of diverse neurobiological factors that contribute to different aspects of brain and behavioural alterations relevant to psychiatric disorders.

On this basis, since 2000 there has been a consistent increase of studies using in-vivo animal models to characterize the effects of systemic and local inflammation on brain development and behaviour, in particular with respect to psychiatric disorders. It must be noted, however, that the attempt to model any psychiatric condition in animals is often met with some degree of scepticism, as the clinical manifestations of most of these diseases are typically 'human', including delusions, hallucinations, mood and thought disorders, which are quite difficult to ascertain without structured interviews. The main approach is thus to focus on individual physiological, neuroanatomical or behavioural phenotypes of the disorder, rather than to model the entire syndrome (Kellendonk *et al*, 2009; Lipska and Weinberger, 2000). In order to do so, various cross species translational paradigms have been developed for the identification and characterization of neuropsychological, psychopharmacological, cognitive and

behavioural core dysfunctions implicated in psychotic disorders (Meyer *et al*, 2010a) and the most commonly used are summarized in the following paragraph.

Prepulse inhibition (PPI) of the acoustic startle reflex is an operational measure of sensorimotor gating that reflects the ability to filter or gate intrusive sensorimotor information. This parameter can be operationally measured in animals and humans and is impaired in schizophrenic individuals. Latent inhibition (LI) is a form of associative learning considered to represent and organism's capacity to ignore irrelevant stimuli, and again it is impaired in schizophrenic patients with acute positive symptoms or abnormally enhanced in patients with marked negative symptoms. Working memory, on the other hand, is a special short-term memory buffer with a limited temporal capacity that is used to hold relevant information active for guiding on-going behaviour such as reasoning, decision-making and problem-solving, and can be readily tested in numerous experimental paradigms. Social behaviour is referred to as behaviour that takes place in a social context and results from the interaction between and among individuals, and since commonly used experimental animals are highly social animals, social interaction and recognition tests can be efficiently used to study social behaviour in different experimental conditions. Other cross-species experimental paradigms that are typically used to assess functional brain abnormalities related to schizophrenia and other neurodevelopmental disorders include stereotypy, sustained attention and vigilance, executive functions and dopamine and glutamate associated neurotransmission (behavioural sensitivity to dopamine receptor agonists or NMDA-receptor antagonists).

Importantly, the degree to which it is possible to extrapolate information from animal models to humans, and thus the value of the information obtained from animal models, largely depends on how the model in question responds to three main validity criteria. In particular, *face validity* refers to phenomenological and symptomatological similarities between the model and the clinical condition, and reflects the descriptive capacity of the model to mimic the behavioural abnormalities seen in the human psychological condition. *Construct validity* refers to the degree of similarity between mechanisms underlying the pathological phenotype in the animal model and in the human condition, and accounts for mechanistic similarities between the two conditions. Lastly, *predictive validity* implies that the model allows extrapolation of the effects of a particular manipulation from one species to another and from one condition to the other. In a narrower context, such as pharmacological treatment, this criteria implies

that treatments known to influence a clinical state in humans should have similar effects in the animal model. Of note, validity criteria are usually restricted to the purpose of the model, no animal model will likely fulfil all validity criteria at the same time, and there is no general indication on how to way the different validity criteria in the model validation process (van der Staay *et al*, 2009).

Animal models that attempt to assess the effects of maternal immune activation on central nervous system development and association with psychopathology in the offspring have been largely established in mice and rats, and more recently in monkeys (Short *et al*, 2010). In these attempts, both bacterial and viral infections have been modelled, using different main immunogenic approaches, such as administration of influenza virus, lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (PolyI:C) and individual pro-inflammatory cytokines. One aspect of these models is that the specific gestational timing of exposure can be varied across different studies of prenatal infection, ranging from daily administration throughout pregnancy to a single administration during early or late gestation. Of note, mapping the developmental stage of human perinatal brain on that of rodents is a complex issue which is still a subject of ongoing research, even if the rat brain at roughly postnatal day 7-13 is generally considered equivalent to the developmental stage of the human brain at term (Avishai-Eliner *et al*, 2002; Romijn *et al*, 1991). Thus, the first and second halves of rat (and mouse) pregnancy are usually considered to approximate the first and second trimester of pregnancy in humans, even if this could result in an oversimplification (Clancy *et al*, 2007). This issue is of particular importance when considering that different stages of pregnancy correspond to different stages of brain maturation, and thus insults acting at different time points could likely impact different systems and lead to very different behavioural and pathological outcomes.

Fatemi et al. were the first to implement an experimental mouse model of prenatal exposure to human influenza virus, in which pregnant mice receive, with an intranasal infusion, a sublethal dose of a mouse-adapted human influenza strain on gestation day 9. The long term behavioural and neurochemical effects are then compared between control and influenza-exposed offspring. Fatemi and colleagues demonstrated that this prenatal immunological insult leads to a multitude of postnatal neuropathological and behavioural manifestations in the offspring that are relevant to different core aspects of schizophrenia and autism, such as impaired prepulse inhibition,

reduced social interaction and exploratory behaviour, increased sensitivity to NMDA receptor antagonists, reduced cortico- and neurogenesis in the hippocampus and prefrontal cortex, reduced purkinje cells in the cerebellum, increased gliolisis and reduced expression of GABAergic markers such as reelin in the frontal cortex and hippocampus (Fatemi *et al*, 2004; Fatemi *et al*, 2000; Fatemi *et al*, 2002; Fatemi *et al*, 2008; Fatemi *et al*, 1998a; Fatemi *et al*, 1998b; Shi *et al*, 2003). In addition to these behavioural and morphological alterations, prenatal exposure to influenza virus leads to persistent changes in gene expression levels in the offspring's CNS (Fatemi *et al*, 2005; Fatemi *et al*, 2008), and long-term deficiency in serotonin production has also been noted in offspring born to influenza-infected mice (Winter *et al*, 2008). Interestingly, this model has also been used to investigate the impact of the precise prenatal timing of the insult, by exposing pregnant dams to the immunological insult at different times during pregnancy (Kneeland and Fatemi, 2013). In light of these findings, it is clear that the prenatal influenza model is highly suitable for the investigation of the relationship between exposure to viral infection during pregnancy and increased risk of psychopathology later in life. Moreover, the model is characterized by a high level of construct and face validity, and accounts for one of the well-known environmental factors implicated in the aetiology of schizophrenia and autism. However, when compared to other models of prenatal immune activation that do not use live pathogens, this model allows a lower degree of control over the time course and dose of immunogen exposure, while on the other hand it more closely mimics the entire course of a propagating viral infection compared to other models that use immune-activating agents instead of live pathogens. The model has been recently expanded to experimental investigations in rhesus monkeys, demonstrating the emergence of reduced grey and white matter in different cortical and parieto-cortical brain regions (Short *et al*, 2010). This finding is particularly relevant in light of the fact that corticogenesis is much more complex in monkeys than in rodents, and therefore primate models help to verify the relevance of the findings in rodent models to human conditions. Lastly, significant alterations in postnatal brain structure and function have been observed in rodents born to mothers exposed to other viral and bacterial infections, such as *Campylobacter Rectus* (Offenbacher *et al*, 2005; Yeo *et al*, 2005) and *Escherichia Coli* (Pang *et al*, 2005; Rodts-Palenik *et al*, 2004), even if the long-term neurobehavioural consequences have only been marginally investigated.

Models based on the use of live viral or bacterial pathogens do not provide a satisfactory answer to the question as to whether the behavioural and neurological abnormalities that arise in the offspring of exposed mothers are due to specific direct effects of the pathogen or to the indirect activation of the maternal immune system. In order to answer this issue, investigators developed prenatal infection models based on the use of immune activating agents that evoke cytokine-associated immune responses without using live viral or bacterial pathogens. In this way, it is possible to directly investigate the cytokine hypothesis put forth by Gilmore and Jarskog which postulates that cytokines play a key role in mediating the association between prenatal infection and abnormal brain development in the offspring (Gilmore *et al*, 1997). Two of the best established models are based on maternal exposure to the bacterial endotoxin lipopolysaccharide (LPS) or the synthetic analogue of double stranded RNA polyinosinic:polycytidylic acid (PolyI:C). The latter will be discussed in detail in the next section of the thesis, so for now I will focus only on the LPS model.

LPS is an inherent cell wall component of gram-negative bacteria recognized mainly by the pathogen recognition receptor transmembrane protein toll-like receptor 4 (Triantafilou *et al*, 2002a; Triantafilou and Triantafilou, 2002b). Upon binding to TLR4, LPS stimulates the production of and release of different pro-inflammatory cytokines (Fortier *et al*, 2004a; Kimura *et al*, 1994), leading to an innate immune response typically seen after infection with gram-negative bacteria (Triantafilou *et al*, 2002a). In particular, administration of LPS leads to cytokine induction, fever, inflammation, complement cascade activation and sickness behaviour (Aderem and Ulevitch, 2000). Upon binding to TLR4 on macrophages and immune cells, LPS triggers a signalling cascade involving the activation of transcription factors such as nuclear factor kappa B (NfkB), that in turn promote the transcription of genes encoding for pro- and anti-inflammatory cytokines, as well as other mediators of inflammation (Aderem *et al*, 2000). In particular, LPS elicits the production of interleukin-1 (IL-1), tumour necrosis factor alpha (TNF-α) and IL-6. The use of LPS presents various advantages when compared to the use of live pathogens, for example that it can be handled without stringent biosafety precautions and that the experimenter can readily control the intensity and duration of the cytokine response. The immunological challenge induced by LPS is, in fact, time-limited, ranging from 24 to 48h depending on the dose used (Meyer *et al*, 2005) (Cunningham *et al*, 2007). However, each batch of LPS typically

contains different pyrogenic and cytokinogenic activity, since it is purified from bacteria (Ray *et al*, 1991), and it also exhibits tolerance, which could be important in paradigms characterized by daily administration of LPS (Chen *et al*, 2005).

Numerous studies in both rats and mice have proven that LPS precipitates a number of behavioural and neurochemical abnormalities that are relevant to schizophrenia and other psychotic disorders. Indeed, prenatal exposure to LPS has been shown to induce impaired sensorimotor gating in the offspring (Borrell *et al*, 2002; Fortier *et al*, 2004a; Fortier *et al*, 2007; Romero *et al*, 2007; Romero *et al*, 2010), increased sensitivity to psychotomimetic drugs (Fortier *et al*, 2004a; Fortier *et al*, 2007), impaired working memory and spatial learning (Coyle *et al*, 2009; Hao *et al*, 2010; Lante *et al*, 2008) and impaired social interaction (Golan *et al*, 2005; Kirsten *et al*, 2012). Of note, the precise functional abnormalities that emerge after prenatal exposure to LPS are critically influenced by the timing of the prenatal manipulation (Meyer and Feldon, 2009a; Meyer *et al*, 2007). Moreover, prenatal immune challenge with LPS induces also many morphological and neurochemical abnormalities, such as reduced hippocampal neurogenesis (Cui *et al*, 2009; Lin and Wang, 2014), decreased dendritic length, arborisation and spine density in the hippocampus and medial prefrontal cortex (Baharnoori *et al*, 2009) and white matter abnormalities (Kumral *et al*, 2007; Paintlia *et al*, 2008). Investigator also report reduced tyrosine hydroxylase immunoreactive neurons ((Ling *et al*, 2002; Ling *et al*, 2006; Ling *et al*, 2009; Ling *et al*, 2004), decreased dopamine levels or innervation in the nucleus accumbens or striatum (Bakos *et al*, 2004; Ling *et al*, 2009; Romero *et al*, 2010; Snyder-Keller and Stark, 2008), and decreased levels of serotonin in various brain areas (Wang *et al*, 2009). Interestingly, some of the behavioural and cognitive abnormalities can be rescued by acute or chronic antipsychotic treatment (Borrell *et al*, 2002; Romero *et al*, 2007). All in all, these findings suggest that the prenatal infection models that are based on LPS enjoy a high level of face and predictive validity, in particular with regard to schizophrenia-like psychopathology. This is true also when considering the post-pubertal onset of many of the behavioural alterations, which is consistent with the post-pubertal onset of full blown psychotic behaviour in schizophrenia and related disorders (Fatemi and Folsom, 2009a).

Another valuable approach to investigate the impact of increased cytokine levels on brain development and on consequent brain psychopathology is to treat animals with

specific individual cytokines during pregnancy, including IL-1β, IL-6, TNF-α and IFN-γ. This approach was pioneered by Samuelsson et al. (2006) in rats and by Smith et al. (2007) in mice, and both groups demonstrated that administration of exogenous IL-6 to pregnant mothers is sufficient to evoke various long-lasting structural and functional abnormalities in the offspring, such as impaired working memory, impaired sensorimotor gating and impaired latent inhibition (Samuelsson *et al*, 2006; Smith *et al*, 2007). Moreover, when IL-6 is eliminated from the maternal immune response by genetic interventions or by IL-6 blocking antibodies, maternal immune challenge by poly(I:C) is no longer capable of in inducing behavioural abnormalities in the offspring, suggesting that IL-6 may play a key role in mediating the effects of maternal immune activation on brain development. On the other hand, administration of other pro-inflammatory cytokines alone, such as IL-1β, TNF-α and IFN-γ is insufficient in precipitating similar behavioural deficits, and co-administration of IL-1β and IFN-γ receptor antagonists does not prevent the effects of prenatal exposure to poly(I:C) (Smith *et al*, 2007).

Lastly, there have been recent attempts to explore whether local maternal inflammation during pregnancy could be sufficient to determine long-term behavioural and functional alterations relevant to psychopathology in the offspring. In particular, different studies analysed the effects of intramuscular injection with turpentine oil in pregnant dams at different times during pregnancy (Aguilar-Valles *et al*, 2010; Aguilar-Valles *et al*, 2012; Aguilar-Valles and Luheshi, 2011; Fortier *et al*, 2007). Turpentine oil remains confined at the site of the injection, where it causes tissue damage, inflammation, recruitment and activation of immune cells and secretion of pro-inflammatory cytokines (Aguilar-Valles *et al*, 2010; Aguilar-Valles *et al*, 2012; Aguilar-Valles *et al*, 2011). In this way, investigators can study the effects of circulating inflammatory mediators that are produced exclusively by the maternal immune system, with no contribution of placental-derived inflammatory factors. Aguilar-Valles et al. successfully demonstrated that turpentine oil injection is capable of inducing long-term behavioural and pharmacological alterations in the offspring, such as impaired sensorimotor gating, increased sensitivity to psychotomimetic drugs and cognitive deficits. Moreover, these behavioural alterations are paralleled by neurochemical abnormalities such as increased expression of tyrosine hydroxylase and increased dopamine and its metabolites in the nucleus accumbens (Aguilar-Valles *et al*, 2010;

Aguilar-Valles *et al*, 2012; Aguilar-Valles *et al*, 2011). These findings provide further support to the hypothesis that induction of the maternal immune response during pregnancy could be a key factor in mediating the association between prenatal infection and increased risk of developing psychiatric diseases later in life. Moreover, they could encourage human epidemiological studies focusing on potential associations between maternal physical trauma and increased risk of neurodevelopmental disorders in the offspring, seeing that turpentine injection causes local tissue damage.

1.3 The PolyI:C Model

As discussed previously, in order to answer the question as to whether the deleterious effects of prenatal infection are mediated by specific characteristics of the individual pathogens or by the activation of the maternal immune system, investigators have developed different models based on the use of immune activating agents that do not contain live pathogens. One such model that has gained great recognition in the last decade, and on which I have focused my PhD thesis, is based on the use of the inflammatory agent polyriboinosinic:polyribocytidilic acid (poly(I:C)), a synthetic analogue of a double-stranded RNA. In this model, pregnant dams are exposed to the immunological challenge at a specific gestational age and the neurobiological and behavioural effects are then compared in the resulting offspring relative to offspring born to vehicle treated mothers. Poly(I:C) is commercially available, and it mimics the double stranded RNA that is generated during viral infection as a replication intermediate for single-stranded RNA or as a by-product of the transcription in DNA viruses (Takeuchi and Akira, 2007). Once in contact with the host, it is recognized as foreign by the transmembrane protein toll-like receptor 3 (TLR3), a class of pathogen recognition receptors that recognize constitutive structures present or associated with viral or bacterial pathogens (Alexopoulou *et al*, 2001). Upon binding to TLR3, poly(I:C) elicits the production and release of many pro inflammatory cytokines, including IL-1β, IL-6 and TNF-α, thanks to the translocation of NFkB to the nucleus where it explicates its role of transcription factor (Doughty *et al*, 2006; Fortier *et al*, 2004b; Koga *et al*, 2009; Meyer *et al*, 2006a). Moreover, poly(I:C) is a potent inducer of type I interferons IFN-α and IFN-β (Fortier *et al*, 2004b), thus efficiently mimicking the acute phase response to viral infection (Kimura *et al*, 1994; Traynor *et al*, 2004). There are several advantages of

using viral-like immune activating agents, such as poly(I:C), compared to live viral agents: first of all, they can be easily handled without stringent biosafety precautions; secondly, the intensity of the cytokine associated immune response can be readily controlled by appropriate dose-response studies (Meyer *et al*, 2005; Shi *et al*, 2003); thirdly, the acute poly(I:C)-induced immunological challenges are time limited, ranging from 24 to 48h depending on the dose used (Cunningham *et al*, 2007; Meyer *et al*, 2005); finally, prenatal poly(I:C) exposure is capable of altering cytokine levels in three compartments of the maternal-foetal interface, namely the placenta, the amniotic fluid and the foetus (Graciarena *et al*, 2010; Meyer *et al*, 2006a; Meyer *et al*, 2008d; Urakubo *et al*, 2001). These aspects are particularly important with respect to investigating the contribution of different factors in the aetiopathogenesis of psychiatric disorders.

Control over the intensity of the response is a relevant factor for experimental attempts to investigate gene-environment and environment-environment interactions in the pathogenesis of neurodevelopmental disorders such as schizophrenia. Indeed, induction of a blunted phenotype by prenatal immune activation with poly(I:C) at low dosage allows the identification of synergistic gene-environment or environment-environment interactions in the development of schizophrenia-relevant behavioural abnormalities. For example, the effects of poly(I:C) are prevented in transgenic animals overexpressing IL-10 or with the deletion of IL-6 (Meyer *et al*, 2008d; Smith *et al*, 2007), while recent studies have demonstrated synergistic effects between poly(I:C) and aberrant expression of genetic susceptibility factors of schizophrenia, such as DISC1 (Abazyan *et al*, 2010; Ibi *et al*, 2010), Nurr1 (Vuillermot *et al*, 2012) and Neuregulin-1 (O'Leary *et al*, 2014). Moreover, low doses of poly(I:C) have also been demonstrated to have additive effects with an environmental risk factor for schizophrenia, namely pubertal stress exposure (Giovanoli *et al*, 2013).

The fact that the immune response induced by poly(I:C) is restricted in time is particularly relevant when prenatal immune activation models are designed to explore the impact of precise timing in the relationship between prenatal infection and postnatal brain and behavioural alterations. Epidemiological studies conducted in humans, in fact, suggest that the association between prenatal infection and enhanced risk of psychiatric disorders is influenced by the precise prenatal timing of exposure. Indeed, initial studies found an association between prenatal infection and schizophrenia when the infection occurred during the second trimester of pregnancy (Mednick *et al*, 1994) reviewed in

(Brown and Derkits, 2010), while recent studies suggest a critical impact of infections occurring in early pregnancy ((Babulas *et al*, 2006; Brown *et al*, 2004; Sorensen *et al*, 2009). On these basis, it is clear that animal models, such as the poly(I:C) model, represent valuable tools to examine the 'critical window' hypothesis of this association, and this can be achieved by comparing the effects of prenatal immune challenge at distinct gestational ages. Meyer et al. designed a series of experiments that directly explored this issue, and confirmed that the precise timing of the prenatal immunological activation is indeed a critical determinant of the specificity of both the brain and behavioural abnormalities that develop in the offspring (Meyer *et al*, 2006a; Meyer *et al*, 2006b; Meyer *et al*, 2008d; Meyer *et al*, 2006c). In particular, the gestational stage of the manipulation determines specific psychopathological and neuropathological symptom clusters in the offspring. More specifically, prenatal immune activation on gestation day 9 (GD9) (poly(I:C) 5mg/kg) leads to a pathological profile characterized by reduced exploratory behaviour, alterations in selective associative learning in the form of LI disruption, impairments in sensorimotor gating, enhanced sensitivity to psychotomimetic drugs and deficiency in spatial working memory when the demand on temporal retention is high (Meyer *et al*, 2006b; Meyer *et al*, 2008d). On the other hand, an identical manipulation on GD17 leads to perseverative behaviour, deficits in spatial working memory and recognition memory even when the demand on temporal retention is low, potentiated response to amphetamine and the non-competitive NMDA antagonist dizolcipine, abolition of the US-pre-exposure effect and social interaction deficits (Bitanihirwe *et al*, 2010a; Bitanihirwe *et al*, 2010b; Meyer *et al*, 2006a; Meyer *et al*, 2006b; Meyer *et al*, 2008d; Richetto *et al*, 2013a). It is clear that some pathological traits are specific to different symptom clusters arising from different stages of prenatal manipulations, while others overlap, suggesting that the vulnerability of specific forms of post-natal brain dysfunctions to prenatal infection varies across gestational stages. Moreover, the differences between the long-term functional effects of prenatal immune challenge with poly(I:C) on GD9 and GD17 may be related to the different symptom clusters of schizophrenia, which appear to follow separate developmental courses (Gross, 1997; Murray *et al*, 1992; Sporn *et al*, 2004), respond to different treatment strategies (Maguire, 2002), and are suggested to be associated with dysfunctions in specific brain regions. One hypothesis that emerges from these considerations is that early *vs* late prenatal immune challenge could capture the dichotomy between the

positive and negative symptoms of schizophrenia, with early/middle viral-like immune activation leading to a variety of abnormalities associated with the positive symptoms of schizophrenia, and late infection leading to a cluster of behavioural and cognitive abnormalities relevant (but not limited) to the cognitive/negative symptoms of schizophrenia. This hypothesis is also supported by neurochemical data: early/middle prenatal infection leads to a hyperdopaminergic state in mesocorticolimbic areas (Winter *et al*, 2008), while late prenatal infection induces a marked hypodopaminergic and hypoglutamatergic state in the prefrontal cortex and hippocampus of the offspring (Bitanihirwe *et al*, 2010a). These different effects capture the bidirectional changes in dopamine function and impaired glutamatergic signalling that are suggested to mediate the different symptom clusters of schizophrenia (Guillin *et al*, 2007; Howes and Kapur, 2009; Knable and Weinberger, 1997). Thus, the poly(I:C) model represents a powerful tool to investigate distinct neuropathological signs relevant to positive and negative/cognitive symptoms of schizophrenia, respectively, and it could be used to link specific neuronal and neurochemical dysfunctions with distinct forms of schizophrenia-related behaviours. As discussed in the following chapters, my PhD aimed at evaluating such associations, focusing entirely on the consequences of late prenatal immune activation. Our interest in choosing this precise stage of gestation was given by the fact that still little is know regarding the molecular mechanisms underlying the negative and cognitive symptoms of schizophrenia, and seeing as these symptoms still remain the most difficult to treat pharmacologically (Wallace *et al*, 2011), new insights on the molecular players that underlie these manifestations could hopefully serve the development of new treatment strategies.

The poly(I:C) model also includes various aspects of maternal/foetal inflammation by altering the levels of pro- and anti-inflammatory cytokines in different compartments of the maternal-foetal interface, thus modelling one of the main mechanisms that is thought to mediate the long-term effects of prenatal immune activation. A summary of the acute changes induced by prenatal poly(I:C) in the foetal compartments is summarized in **Table 1**. Recently, an elegant study by Garay et al. investigated changes in fetal brain cytokines during development in different brain regions, demonstrating that maternal immune activation with poly(I:C) induces a complex profile of cytokine alteration that is both age and region specific (Garay *et al*, 2013).

Alterations	Immunogen	Time	Species	Reference
↓TNF-α (24h fetal brain) ↓TNF-α (Liver, Spleen) ↑ TNF-α (placenta)	Poly(I:C) 10-20mg/kg ip	GD16	Rat	Gilmore et al. (2005)
↓IL-1β, IL-10 ↑ IL-6 (3h fetal brain) ↑ IL-6, IL-1β, TNF-α (6h fetal brain)	Poly(I:C) 2-5mg/kg iv	GD9	Mouse	Meyer et al. (2006, 2008)
↑ IL-6, IL-1β, TNF-α, IFN-γ (2-4h placenta)	Poly(I:C) 4.5mg/kg ip	GD16.5	Mouse	Koga et al. (2009)
↑ IL-10, IL-1β (3h fetal brain) ↑ IL-6 (6h fetal brain)	Poly(I:C) 5mg/kg iv	GD17	Mouse	Meyer et al. (2006)
↓TNF-α (2-24h fetal brain)	Poly(I:C) 5mg/kg sc	GD20	Spiny Mouse	Ratnayake et al. (2014)
↑ IL-7, IL-1β, IL-13, MCP-1, MIP1- α, MIG, VEGF (6h fetal brain) ↑ IL-1β (24h fetal brain)	Poly(I:C) 20mg/kg ip	GD16	Mouse	Arrode-Bruses et al. (2012)

Table 1. Significant acute changes in immune mediators in the fetal compartment after prenatal exposure to poly(I:C).

Having highlighted the importance of the maternal immune system in mediating the long-term effects of prenatal infection, it seems clear that the individual maternal immune response to the immune activating agent could be an important determinant of the behavioural and pathological outcomes in the offspring,(Bronson *et al*, 2011). In particular, individual differences in maternal cytokine response could account for differential behavioural neuropathological manifestations in the offspring. This hypothesis has recently been investigated in the poly(I:C) model by various studies, based initially on the observation that differential maternal body weight response to the immune challenge correlates with differential behavioural outcome in the offspring (Bronson *et al*, 2011; Vorhees *et al*, 2012). Interestingly, Missault et al. have demonstrated that maternal weight response to maternal immune activation reflects differences in cytokine response, and that this correlates with the pathological manifestations in the offspring. In particular, pregnant dams that lost weight after the prenatal immune activation showed increased levels of TNF-α compared to controls, unlike animals that increased weight. Moreover, offspring of dams that lost weight showed more severe behavioural deficits (Missault *et al*, 2014). This issue is particularly

interesting and warrant further examination, especially with respect to the human population where individual responses to infection might account for the wide variety of effects that have been observed after prenatal exposure to infection.

Of note, environmental insults targeting the pregnant maternal host may affect brain and behavioural development differently in male and female offspring, and numerous studies investigating the effects of stress on brain development have highlighted critical sex differences in the development of long-term structural and brain abnormalities (Mueller and Bale, 2008; Weinstock, 2007). The poly(I:C) model offers the opportunity to investigate this issue also after prenatal exposure to infection, even if this aspect has so far received less attention because many existing studies focus only on male offspring in order to avoid the possible confounds and interpretative problems arising from hormonal fluctuations in female subjects (Fortier *et al*, 2007; Romero *et al*, 2007; Wolff and Bilkey, 2008). Of the studies that have considered this issue, the majority did not reveal marked sex differences in brain and behavioural alterations (Meyer *et al*, 2005; Meyer *et al*, 2006b; Ozawa *et al*, 2006; Shi *et al*, 2003; Zuckerman *et al*, 2003a; Zuckerman and Weiner, 2003b), as opposed to a few studies that observed clear sex-dependent alterations in specific pathological domains (Meyer *et al*, 2008a; Schwendener *et al*, 2009; Zhang *et al*, 2012).

Lastly, it must be noted that the poly(I:C) model, when compared to infection models that are based on the use of live pathogens, falls short in mimicking the precise immunological insult that occurs in the human environment, as it does not reproduce the full spectrum of immune responses normally induced by viral exposure (Meyer *et al*, 2009b). Actually, it mimics especially the viral-like acute phase responses in the maternal host, and this needs to be taken into account when investigating the precise cellular and molecular mechanisms that mediate the deleterious effects of prenatal viral infection. However, this limitation does not undermine the relevance of the model, as it does not influence its capacity in reproducing many behavioural neurochemical and anatomical features of schizophrenia related disorders.

1.3.1 Behavioural Phenotype

As previously discussed, the poly(I:C) model is capable of inducing numerous behavioural and cognitive abnormalities that are relevant to schizophrenia and other psychiatric disorders (Boksa, 2010; Meyer *et al*, 2010a). A summary of these alterations

is provided in **Table 2**. Interestingly, most of the schizophrenia-related behavioural effects induced by poly(I:C) exposure are consistent with the post-pubertal onset of full-blown psychotic behaviour in schizophrenia, and emerge only at post-pubertal stages of life (Meyer *et al*, 2006b; Meyer *et al*, 2008d; Ozawa *et al*, 2006; Richetto *et al*, 2013a; Richetto *et al*, 2013b; Vuillermot *et al*, 2010; Zuckerman *et al*, 2003a), thus efficiently modelling the time profile of schizophrenic pathology. The prevalent hypotheses behind the delayed onset of schizophrenia symptomatology are related to the functional maturation of intracortical connectivity (Weinberger and Lipska, 1995), hormonal refinement during peri-adolescence (Halbreich and Kahn, 2003) and exposure to stressful life events (and associated changes in the stress response system) such as trauma and drug abuse (Corcoran *et al*, 2003; Phillips *et al*, 2006). Thus, the poly(I:C) model represents a valuable tool to investigate the developmental character of progressive brain changes relevant to schizophrenia and other psychotic disorders, and allows the evaluation of possible additive/precipitating factors postnatal factors. Interestingly, consistent with the endogenous sensitization hypothesis of schizophrenia, which postulates that dopaminergic transmission may function in a sensitized state even before the onset of full blown psychotic behaviour (Fusar-Poli *et al*, 2011; Laruelle, 2000), early prenatal infection leads to heightened sensitivity to amphetamine even in pre-pubertal stages of life (Meyer *et al*, 2008a; Vuillermot *et al*, 2010). Against this background, in my PhD thesis I evaluated whether different aspects of cognitive function could also emerge before the onset of the full-blown spectrum of abnormalities induced by prenatal immune activation, in line with the observation that, in the case of schizophrenia, cognitive symptoms appear to be present before the onset of the illness (Bellack *et al*, 2007; Reichenberg, 2010; Reichenberg *et al*, 2010).

The prenatal poly(I:C) model has also been recently expanded to experimental investigations in rhesus monkeys by Bauman et al. In their study, the authors demonstrate that maternal immune activation with poly(I:C) late in the first trimester and late in the second trimester determines altered affiliative vocalizations and increased repetitive behaviours. Moreover, first trimester offspring displayed abnormal social behaviour (Bauman *et al*, 2014). These results extend the findings in rodent models to more human-like behaviours and strengthens the relevance of the polyI:C models for modelling pathological abnormalities common to autism and schizophrenia.

All in all, the wide spectrum of behavioural and cognitive abnormalities that ensue after prenatal treatment with poly(I:C) highlights the models' excellent degree of face validity for the pathological manifestations of schizophrenia, psychosis and, to some extent, autism.

Species	Gestational Period	Behavioural Alterations	References
Mouse	Early/Middle	↓PPI, LI, Social Behaviour, Exploratory Behaviour, Working Memory ↑ Sensitivity to DA-R agonists and NMDA-R Agonists	Shi et al. (2003); Meyer et al. (2005, 2006a,b,c, 2008b,c,d, 2010b); Makinodan et al. (2008); Li et al. (2009); Vuillermot et al. (2010, 2011); Li et al. (2014)
Mouse	Middle or Middle/Late	↓PPI, Exploratory Behaviour, Working Memory ↑ Sensitivity to DA-R agonists, Repetitive Behaviours	Ozawa et al. (2008); Cardon et al. (2010); De Miranda et al. (2010); Wolff et al. (2011); Connor et al. (2012); Xuan et al. (2014)
Mouse	Late	↓ Social Behaviour, Working Memory, Cognitive Flexibility, Ultrasound Vocalizations ↑ LI, Repetitive Behaviours**,** Sensitivity to DA-R agonists and NMDA-R Agonists	Meyer et al. (2006b,c, 2008c, 2010b); Li et al. (2009); Bitanihirwe et al. (2010a,b); Richetto et al. (2013); Malkova et al. (2012)
Rat	Middle/Late	↓PPI, LI, Working Memory ↑/↓ Cognitive Flexibility, Sensitivity to DA-R agonists and NMDA-R Agonists	Zuckerman et al. (2003); Zuckerman and Weiner (2003, 2005); Wolff and Bilkey (2008); Piontkewitz et al. (2009, 2011a,b) Dickerson et al., (2010) Bronson et al. (2010); Han et al. (2011); Zhang et al. (2012) Richtand et al. (2011); Wolff et al. (2011); Savanthrapadian et al. (2013)

Table 2. Significant behavioural alterations of offspring exposed to prenatal poly(I:C). PPI: Prepulse Inhibition, LI: Latent Inhibition.

1.3.2 Morphological, Neurochemical and Molecular Characteristics

Along with the behavioural alterations, prenatal immune activation with poly(I:C) is also capable of inducing a variety of morphological and neurochemical abnormalities that are relevant for various psychiatric conditions. Morphological changes have so far been investigated in detail in the mouse, with few studies addressing this issue in the rat, and some of the most replicated findings seem to be cell death in white matter, reduced neurogenesis and reduced myelin basic protein immune staining ((Liu *et al*, 2013; Makinodan *et al*, 2008; Meyer *et al*, 2006b). At the neurochemical level, prenatal immune activation studies focused primarily on dopamine (DA), serotonin (5-HT), and glutamate. With respect to dopamine, there are disparate results that vary depending of the timing of the prenatal manipulation, with a hyperdopaminergic state occurring after early prenatal poly(I:C) exposure, and a hypodopaminergic state occurring after late prenatal manipulation (Bitanihirwe *et al*, 2010a; Winter *et al*, 2009). Moreover, the results vary depending on the brain region considered. Studies examining serotoninergic parameters point to a decrease in serotoninergic measures (Ohkawara *et al*, 2014; Winter *et al*, 2008), as those studying glutamate function (Bitanihirwe *et al*, 2010a; Meyer *et al*, 2008a). A summary of the most relevant neurochemical and morphological alterations is provided in **Table 3**.

The molecular characterization of the model, on the other hand, has been less pursued, especially with respect to the molecular alterations that characterize adult offspring and the time profile with which these alterations develop. Initial studies point to alterations in myelin basic protein (Makinodan *et al*, 2008), changes in reelin and parvalbumin (Meyer *et al*, 2006b; Meyer *et al*, 2008d), alterations in gene expression patterns in the brains of fetal offspring (Garbett *et al*, 2012), and altered GSK3β signalling (Willi *et al*, 2013). The limited information regarding the molecular profile of the alterations induced by prenatal poly(I:C) stimulated many aspects of my PhD project, also in light of the fact that identifying the molecular signatures which underlie specific behavioural phenotypes could eventually lead to the development of new pharmacological strategies to treat various aspect of psychiatric diseases.

Species	Gestational Period	Morphological Alterations	References
Mouse	Early/Middle	↓Cortico-/neurogenesis in the hippocampus ↓axonal size, myelin thickness in HPC, MBP immunostaining ↓Cerebellar Purkinje Cells ↑ lateral and 4th ventricle volumes by MRI	Meyer et al. (2006a); Makinodan et al. (2008); Li et al. (2009); Shi et al. (2009); Liu et al. (2013)
Mouse	Late	↓Cortico-/neurogenesis in the HPC ↑ lateral and 4th ventricle volumes by MRI	Meyer et al. (2006a); Li et al. (2009);
Rat	Middle/Late	Pyknotic pyramidal cells	Zuckerman et al. (2003); Piontkewitz et al. (2010)
Species	**Gestational Period**	**Neurochemical Alterations**	**References**
Mouse	Early/Middle	↓ D1 in mPFC ↓ 5-HT, 5-HTIAA in NAc, GP, HPC, taurine in HPC ↓ GluR1 subunit in NAc ↓ $GABA_{A\alpha2}$ subunit in Amy ↑ DA, DOPAC in PFC and GP ↑ THir, DATir in mesencephalon ↑ TH immunoreactivity in NAc and ST ↑ TH positive cells in SN and VTA	Meyer et al. (2008a,c,e); Nyffeler et al. (2006); Winter et al. (2008a); Vuillermot et al. (2010)
Mouse	Late	↓DA, Glutamate in PFC and HPC ↓GABA in HPC ↓NR1 subunit immunoreactivity in ST ↓D2 receptor binding in ST ↑ DOPAC, HVA in ST	Ozawa et al. (2006); Meyer et al. (2008); Bitanihirwe et al. (2010)
Rat	Late	↑KCl-induced DA release from striatal slices	Zuckerman et al. (2003)

Table 3. Significant morphological and neurochemical alterations of offspring exposed to prenatal poly(I:C). HPC: Hippocampus; PFC: Prefrontal Cortex; Amy: Amygdala; GP: Globus Pallidus**;** NAc: Nucleus Accumbens; ST: Striatum; VTA: Ventral Tegmental Area; SN: Substantia Nigra.

1.3.3 Pharmacological Interventions

Lastly, an important feature of the poly(I:C) model is its predictive validity, in the sense that at least some of the behavioural and cognitive abnormalities induced by the prenatal immunological manipulation can be normalized by acute/chronic antipsychotic treatment. Initially, Zuckerman et al. demonstrated that prenatal infection-induced alterations in latent inhibition were normalized by acute treatment with both haloperidol and the atypical antipsychotic clozapine (Zuckerman *et al*, 2003a). In line with these initial finding, Ozawa et al. reported that chronic treatment with clozapine normalized the prenatal poly(I:C) induced impairments in the novel object recognition test (Ozawa *et al*, 2006), while Meyer et al. demonstrated that the same drug was capable of improving spatial working memory deficits induced by prenatal poly(I:C) exposure (Meyer *et al*, 2010b). In addition, acute treatment with preferential dopamine D1 or D2 receptor antagonists was sufficient to normalize poly(I:C)-induced PPI deficits, demonstrating a critical role of the dopaminergic system abnormalities in the development of sensorimotor gating deficits induced by prenatal immune challenge (Vuillermot *et al*, 2010). Interestingly, recent findings demonstrate that treatment with clozapine or the atypical antipsychotic risperidone during an asymptomatic period of adolescence prevents the emergence of schizophrenia-like behavioural and structural brain abnormalities in adult offspring exposed to gestational poly(I:C) (Piontkewitz *et al*, 2011b; Piontkewitz *et al*, 2009). Clozapine has also been shown to revert iNOS increase and microglial activation (Ribeiro *et al*, 2013) and ameliorate neuronal synchronization in rats exposed to prenatal poly(I:C) (Dickerson *et al*, 2012).

In light of these findings, and considering its robust construct and face validity, it appears clear that the poly(I:C) model may be used as a pharmacological screening test against different aspects of schizophrenia related psychopathology. Indeed, the model represents a valid tool for the establishment and characterization of novel antipsychotic drug treatments, as it accounts for the developmental nature of different psychiatric diseases and incorporates the aetiological significance of these disorders. Moreover, given that the precise timing of the prenatal poly(I:C) exposure gives rise to different clusters of behavioural alterations that in some way mimic the symptom clusters of schizophrenia, this offers the possibility to evaluate a compound's antipsychotic efficacy against specific symptom profiles. On these bases, we used this model to evaluate possible beneficial therapeutic potential of a selective class of positive allosteric

modulators of the $GABA_A$ receptor against the cognitive alterations induced by prenatal poly(I:C).

Of note, in vivo rodent models of prenatal immune activation also provide a unique opportunity to establish and study early and preventive interventions in order to reduce the risk of developing brain disorders associated with in-utero exposure to infection. In particular, these models offer the opportunity to implement interventions during the preconceptional period (vaccinations), during the acute phase of the maternal immune response (acute anti-inflammatory treatments) and during the early phases of the offspring's development (antipsychotic or antidepressant treatment during the prodromal phase) (reviewed in (Meyer *et al*, 2009b)).

2. AIM OF THE PROJECT

Compelling evidence suggests that prenatal infection confers increased risk for the development of psychiatric disease later in life. In particular, environmental insults could disrupt the normal course of neurodevelopment leading to long-lasting changes in brain function. Prenatal infection could thus act as a 'primer' for the neuropsychiatric route, even if the specificity of the illness that develops is strongly influenced by the genetic and environmental context in which the process occurs. Moreover, prenatal infection-induced neuroinflammation could prime alterations in central and peripheral inflammatory systems and impair correct development of neural systems from juvenile to adult stages of life, extending its pathological relevance beyond prenatal periods. In this context, the aim of my thesis was to further characterize a mouse model of prenatal infection based on the use of the viral mimetic poly(I:C) administered to pregnant dams in a late phase of gestation (GD17). The choice of this stage was based on previous findings showing that GD17 poly(I:C) treatment is capable of inducing working memory deficiency in adulthood (Bitanihirwe *et al*, 2010a) and to alter postpartum maternal behaviour (Schwendener *et al*, 2009). Hence, as discussed in more detail in subsequent sections, this gestational window could be particularly significant for the development of behavioural phenotypes relevant to the cognitive and affect-related symptoms of neurodevelopmental psychiatric diseases.

During my three years of thesis, I focused on analysing five different aspects of this animal model, developing my work into five main different projects in order to address some of the unresolved questions regarding the association between prenatal infection and mental illness. The results of these studies are presented in the form in which they have been published, except for the genome-wide study that is still ongoing, and thus presented as unpublished material.

In particular, I first investigated whether prenatal immune activation could induce long-term maturational alterations in GABAergic gene transcription (GABA transcriptome project). Neuronal dysfunctions in the cortical GABAergic system have been widely documented in neuropsychiatric disorders with prenatal infectious aetiologies (Gonzalez-Burgos and Lewis, 2012; Lewis *et al*, 2012; Stan and Lewis, 2012),

even if the extent to which prenatal immune activation can cause long-lasting changes in GABAergic gene expression remains essentially unexplored.

Second, in the neonatal cross-fostering project, I went on to dissect the relative contribution of prenatal and postnatal maternal effects on the development of cognitive impairments and alterations in the GABAergic system in the offspring. While it has been acknowledged that the long-term behavioural and molecular alterations manifest in prenatally immune-challenged offspring may stem from alterations in early prenatal brain development (Garbett *et al*, 2012; Meyer *et al*, 2008a; Stolp *et al*, 2011; Vuillermot *et al*, 2010), the role of the postpartum milieu, in which offspring of infected mothers are raised, has not been thoroughly investigated. As recent studies suggest that disruption of the intricate mother-infant relationship resulting from immune activation in late pregnancy could confer additional risk for the offspring to develop brain pathology in later life (Schwendener *et al*, 2009), we were motivated to address this issue in further detail by implementing an additional neonatal cross-fostering design.

Third, in keeping with our findings showing cognitive impairments and GABAergic abnormalities following prenatal exposure to Poly(I:C), I explored possible beneficial effects of a pharmacological intervention targeted at the GABAergic system. Numerous evidence suggest, in fact, that impaired GABA signalling may contribute to the emergence of cognitive deficits associated with psychiatric disorders (Gonzalez-Burgos *et al*, 2011; Lewis *et al*, 2012), and it has been proposed that pharmacological interventions targeting GABA abnormalities may prove useful in correcting both cognitive impairments and dopaminergic dysfunctions present in patients with schizophrenia (Guidotti *et al*, 2005; Stan *et al*, 2012). We thus directly explored this hypothesis in the GABA modulator project, and we specifically investigated the ability of the benzodiazepine positive allosteric modulator SH-053-2'F-S-CH_3 to modulate the behavioural defects of adult mice that were exposed to Poly(I:C) during late gestation.

Fourth, we investigated the effects of prenatal immune activation on the behavioural and cognitive functions during the process of aging. It has been suggested that early-life environmental insults such as prenatal infection could negatively influence brain aging (Krstic *et al*, 2012). This hypothesis has been mainly driven by findings demonstrating progressive neuronal dysfunctions associated with prenatal exposure to immune challenge (Piontkewitz *et al*, 2011a; Richetto *et al*, 2014; Vuillermot *et al*, 2010). Hence, the progression of neuronal abnormalities might be pathologically

relevant beyond the adult period and could thus further affect cognitive functions during aging.

Last, I implemented an unbiased genome-wide approach in order to find new potential mechanisms underlying the association between prenatal infection and psychiatric disorders and broaden the list of putative targets for future therapeutic intervention during adult life or for disease prevention in adolescence. Using established microarray techniques, I analysed the gene expression profile of two brain areas that are relevant for psychiatric disease, namely the prefrontal cortex and the nucleus accumbens, in order to uncover long-term transcriptional signatures of prenatal infection.

3. MATERIALS AND METHODS

3.1 Animals

C57BL/6 mice were used throughout the whole study. Female and male breeders were obtained from the in-house specific pathogen free colony of the Physiology and Behaviour Laboratory (Swiss Federal Institute of Technology (ETH) Zurich) at the age of 12–14 weeks. Breeding always began after two weeks of acclimatization to the new animal holding room, which was a temperature and humidity controlled (21±11C, 55±5%) holding facility under a reversed light–dark cycle (lights off: 08:00–20:00 hours). All animals were kept in Macrolon type II cages (365 × 207 × 140 mm, length × width × height), and they had *ad libitum* access to standard laboratory chow food (Kliba 3430, Klibamühlen, Kaiseraugst, Switzerland) and water unless specified otherwise. All procedures described in the present study had been previously approved by the Cantonal Veterinarian's Office of Zurich and are in agreement with the principles of laboratory animal care in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 86-23, revised 1985).

3.1.1 Maternal Immune Activation during Pregnancy

For the purpose of the maternal immunological manipulation during pregnancy, female mice were subjected to a timed mating procedure in which groups of 2–3 females were moved to a partitioned cage with one male, thus allowing olfactory but not physical contact between male and female animals. On the third day of partitioning, the females were brought together with one male and allowed to mate. Successful mating was verified next morning by the presence of a vaginal plug, and the day was referred to as gestational day (GD) 0. Pregnant dams on gestation day 17 (GD 17) received either a single injection of poly(I:C) (potassium salt; Sigma- Aldrich) at a dose of 5 mg/kg or vehicle (sterile pyrogen-free 0.9% NaCl) according to protocols established before (Meyer *et al*, 2005; Meyer *et al*, 2008b; Meyer *et al*, 2006b; Meyer *et al*, 2008d). The late gestational stage (i.e., GD 17) was selected because of our previous findings showing that GD17 poly(I:C) treatment is capable of inducing working memory deficiency in

adulthood (Bitanihirwe *et al*, 2010a) and to alter postpartum maternal behaviour (Schwendener *et al*, 2009). Poly(I:C) was dissolved in sterile pyrogen-free 0.9% NaCl (= vehicle) solution to yield a final concentration of 1.0 mg/ml and was administered via the intravenous (i.v) route at the tail vein under mild physical constraint. All solutions were freshly prepared at the day of administration and injected with a volume of 5 ml/kg.

3.1.2 Allocation of Offspring

Five independent cohorts of animals were prepared for the experiments included in this project and were allocated as follows.

3.1.2.1 Neonatal cross-fostering project

The implementation of the neonatal cross-fostering procedure closely followed the protocols established by Schwendener et al. (2009): On the day of birth (postnatal day [PND] 1), offspring born to poly(I:C)- and vehicle-treated dams were culled to litters of six to eight animals each (with three to four animals per sex) and cross-fostered to surrogate rearing mothers. Male and female pups were removed from the original mother and gently placed in a cage containing sawdust bedding for a maximum of 10 min. Half of a given litter was then placed with a poly(I:C)-treated surrogate rearing mother and the other half with a vehicle-treated rearing mother. Each surrogate mother thus simultaneously fostered pups originating from both prenatal treatment conditions, but not its own offspring. PolyI:C and vehicle offspring were marked on the left and right hind paw, respectively. For the behavioural experiments, a total of 24 litters [12 poly(I:C) and 12 vehicle] were cross-fostered to 24 rearing mothers, half of which had been subjected to polyI:C exposure during pregnancy and the other half to vehicle treatment only. Another 12 litters [6 poly(I:C) and 6 vehicle] were used for the generation of the offspring assigned to the molecular investigations. The cross-fostering thus resulted in four experimental treatment groups: (1) offspring subjected to prenatal vehicle exposure and raised by a vehicle-treated surrogate mother (CON-CON), (2) offspring subjected to prenatal vehicle exposure and raised by a poly(I:C)-treated surrogate mother (CON-POL), (3) offspring subjected to prenatal poly(I:C) exposure and raised by a vehicle-treated surrogate mother (POL-CON), and (4) offspring subjected to

prenatal poly(I:C) exposure and raised by a poly(I:C)-treated surrogate mother (POL-POL).

The offspring were weaned and sexed on PND 21. Littermates of the same sex were caged separately and maintained in groups of 2-3 animals per cage. The animals were left undisturbed until commencement of the behavioural and molecular investigations, which were conducted in pubescence and adulthood so as to assess possible maturation-dependent effects of the prenatal and postnatal manipulations. Hence, one group of animals was behaviourally tested in pubescence (PND 30 to 45) and one in adulthood (PND 70 onwards). Likewise, the molecular investigations were performed once the offspring reached PND 45 (pubescence) and 90 (adulthood), respectively. The pubescent and adult stages were defined based on the gradual attainment of sexual maturity and age-specific behavioural discontinuities from younger to older animals (Spear, 2000). These two developmental stages roughly correspond to a period between 11–14 years and 20 years onward, respectively, in humans. For pubescent and adult behavioural testing, two separate cohorts of fostered offspring were used in order to avoid potential confounds arising from repeating testing across peri-pubertal development. In addition, separate cohorts of behaviourally naïve offspring were used for the molecular analyses in pubescence and adulthood so as to avoid possible confounding effects on gene transcription arising from prior stress exposure due to behavioural testing.

3.1.2.2 Prefrontal GABAergic transcriptome project

All offspring weaned and sexed at postnatal day (PND) 21. Littermates of the same sex were caged separately and maintained in groups of 2-4 animals per cage as described above. Only male subjects were included in all behavioural and cognitive tests to circumvent avoid bias arising from sexual dimorphism. They stemmed from multiple independent litters (N = 14 for poly(I:C) and N = 15 for vehicle control litters). Gene expression analyses were conducted when the offspring reached either the peri-pubertal (postnatal day [PND] 35) or adult (PND 100) stage of development. Cognitive testing was similarly performed both in peri-puberty (PND 28-40) and adulthood (PND 95-106). Peri-pubertal and adult stages were defined as explained previously. Separate cohorts of behaviourally naïve animals were used for all gene expression analyses and

cognitive testing in order to avoid possible confounds and carry-over effects arising from prior testing.

3.1.2.3 GABA modulator project

All offspring were weaned and sexed at postnatal day (PND) 21. Littermates of the same sex were caged separately and maintained in groups of 2-4 animals per cage as described above. Only male mice were included in all experiments to circumvent bias arising from sexual dimorphism. A first cohort of behaviourally naïve male animals comprising of 11 control (CON) and 9 poly(I:C) (POL) offspring was allocated to gene expression analyses of GABAA receptor subunits in prefrontal and hippocampal tissues (see below). A second cohort of male animals comprising of 34 control (CON) and 34 poly(I:C) (POL) offspring was assigned to the behavioural and cognitive testing, which included (1) spatial working memory in the Y-maze, (2) social approach and cognition, and (3) AMPH sensitivity tests. To minimize the number of animals required to complete all tests of interest, mice were repeatedly tested starting with the spatial working memory test and completing with the AMPH sensitivity test. A resting period of 1 week was inserted between each of these tests so as to allow sufficient drug wash-out from one test to another. Previous drug histories (i.e., SH-053-2'F-S-CH3 or vehicle treatment, see below) were counterbalanced across the prenatal treatment conditions from one test to another in order to further minimize possible confounds from drug carry-over effects. CON and POL offspring in both cohorts used in this study stemmed from multiple independent litters (16 CON litters and 14 POL litters) in order to avoid litter effects. Hence, 1-2 offspring per litter were used either for the gene expression analyses or the behavioural and cognitive testing.

3.1.2.4 Aging project

All offspring were weaned and sexed on postnatal day (PND) 21. Littermates of the same sex were caged separately and maintained in groups of 3-5 animals per cage. Only male mice were included in all experiments to circumvent bias arising from sexual dimorphism. For each age of testing, the offspring stemmed from multiple independent litters (N = 8-12 for each prenatal treatment conditions) to avoid possible confounds arising from litter effects; and 1-2 male offspring per litter were randomly allocated to the behavioural/cognitive, immunohistochemical, gene expression, or serological tests

at each testing age. All experiments were conducted when the offspring reached the pubescent (4-7 weeks of age), adult (4-5 months of age), or aged (22-24 months of age) stages of life. Pubescent and adult stages were defined as explained previously,0 and the aged stage was defined based on previous normative aging studies in mice (Flurkey et al., 2007). Separate cohorts of pubescent, adult, and aged poly(I:C)- and control offspring were used for the behavioural and cognitive tests at each age in order to avoid possible confounds and carry-over effects arising from experimental testing at prior ages. In a first series of experiments, offspring in every age cohort were repeatedly subjected to cognitive testing assessing (1) short-term spatial recognition memory, (2) acquisition and expression of contextual fear memories, and (3) spatial reference learning and memory. These cognitive domains were studied using tests that are commonly used to evaluate hippocampus-regulated cognitive functions in rodent models (Bannerman *et al*, 1999; Deacon *et al*, 2002a; Maren and Fanselow, 1997; Morris *et al*, 1982; Moser *et al*, 1993; Otto and Poon, 2006). In a second series of experiments, independent cohorts of pubescent, adult, and aged offspring were subjected to an open field test assessing basal locomotor activity and innate anxiety-like behaviour, followed by a test of food hoarding behaviour. The open field test served to ascertain whether prenatal immune activation would lead to altered innate anxiety-like behaviour and/or locomotor activity, both of which could affect the animals' performance in the cognitive tests of primary interests. The food hoarding test was served to extend the cognitive phenotyping to a test that measures species-typical behaviour known to be sensitive to hippocampal damage (Deacon *et al*, 2002a).

In subsequent experimental series, additional separate cohorts of behaviourally naïve offspring were used for the immunohistochemical, gene expression and serological analyses to circumvent possible confounding effects of stress by prior behavioural testing on glial and cytokine markers (Giovanoli *et al*, 2013).

3.1.2.5 Microarray analyses

All offspring of the 18 independent litters were weaned and sexed at postnatal day (PND) 21. Littermates of the same sex were caged separately and maintained in groups of 2-4 animals per cage. For the purpose of this study, only male subjects were used in order to circumvent bias arising from sexual dimorphism. The animals were left undisturbed until commencement of behavioural testing in adulthood, specifically from

PND92 to PND106. The animals underwent a Y-maze test, and a social interaction and recognition test. After nearly two weeks of washout from the behavioural testing, animals were sacrificed at PND114.

3.2 Behavioural Testing

3.2.1 Open Field

The open field exploration test is a widely used behavioural assay to evaluate innate anxiety-like behaviour and locomotor responses to novel environments in rodents (Belzung and Griebel, 2001). The test was conducted in 4 identical square arenas (40 × 40 cm) surrounded by walls (35 cm high). The apparatus was made of grey Plexiglas and was located in a testing room under diffused lighting (25 lux as measured in the centre of the arenas). A digital camera was mounted directly above the four arenas. Images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Noldus Information Technology, The Netherlands) tracking system. The animals were gently placed in the centre of the arena and allowed to explore for 1 h. For the purpose of data collection, the arena was conceptually partitioned into two zones: a centre zone (measuring 13.5×13.5 cm) in the middle of the arena and a peripheral zone occupying the remaining area. The dependent measures were (a) total distance moved in the entire arena, (b) total time spent in the centre zone, and (c) total distance travelled in the centre zone.

3.2.2 Spatial Working Memory in the Y-Maze

The spatial novelty preference test in the Y-maze assesses spatial working memory and uses the natural tendency of rodents to explore novel over familiar spatial environments (Dellu et al., 1992). The apparatus was made of transparent Plexiglass and consisted of three identical arms (50 × 9 cm; length × width) surrounded by 10-cm high transparent Plexiglass walls. The three arms radiated from a central triangle (8 cm on each side) and were spaced 120° from each other. Access to each arm from the central area could be blocked by a removable opaque barrier wall. The floor of the maze was covered with sawdust bedding, which was changed between both the testing phases and the trials. The maze was elevated 90 cm above the floor and was positioned in a dedicated testing

room enriched with distal spatial cues. A digital camera was mounted above the Y-maze apparatus. Images were captured at a rate of 5 Hz and transmitted to a PC running the EthoVision tracking system (Noldus Information Technology), which calculated the time spent and distance moved in the three arms and centre zone of the Y-maze. The working memory test in the Y-maze consisted of two phases, called the sample and choice phases.

• Sample phase: The animals were allowed to explore two arms (referred to as 'start arm' and 'familiar arm'). Access to the remaining arm ('novel arm') was blocked by a barrier wall door. To begin a trial, the animal was introduced at the end of the start arm and was allowed to freely explore both the start and the familiar arms for 5 min. Test timing was initiated once the animal entered the start arm, as detected by the EthoVision tracking system. The animal was then removed and kept in a holding cage prior to commencement of the choice phase. The barrier door was removed and the sawdust flooring changed to avoid olfactory cues.

• Choice phase: The animal was introduced to the maze following a retention interval of 1 min. During the choice phase, the barrier wall was removed so that the animals could freely explore all arms of the maze for 2 min. The animal was then removed from the maze and returned to the home cage. The sawdust flooring was changed in preparation for the next trial. On each trial, the time spent in each of the three arms was recorded. The relative time spent in the novel arm during the choice phase of the test was calculated by the formula (time spent in the novel arm/time spent in all arms) × 100 and used as the index for working memory performance. In addition, total distance moved on the entire maze was recorded and analysed in order to assess general locomotor activity.

3.2.3 Working Memory in a Matching-to-Position Dry Maze Paradigm

All animals were first tested in matching-to-position dry maze paradigm using protocols established before (Bitanihirwe *et al*, 2010b). It was positioned in a well-lit room enriched with distal spatial cues. A digital camera was mounted above the dry maze. Images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Noldus Information Technology, Wageningen, The Netherlands) tracking system, which calculated the distance moved to reach the reward on each trial.

Before testing, all animals were progressively food deprived during the initial habituation phase (days 1-5) until a minimal 90% free-feeding weight was reached.

During habituation, animals were placed on the dry maze for 2 daily trials of 2 min each. To begin a trial, the animals were placed gently in the centre of the dry maze. The inter-trial interval (ITI) was approximately 1 min. On day 6 and 7, the animals were then pre-trained in the dry maze using the visual cue for 2 consecutive trials. One random hole was cued with the flag and rewarded with the milk solution (75 µl of freshly prepared condensed milk [Alicommerce SAS] used in a 1:4 [milk/water] dilution.). For both trials, each animal was left on the maze for 2 min. If it failed to reach the rewarded hole during this time, it was gently guided to the rewarded hole by the experimenter. All animals consumed the reward within the allowed maximum time of 15 sec.

Following the habituation (days 1-5) and pre-testing (days 6 and 7) phases, working memory testing was started and lasted for 3 testing days (days 8-10). The working memory task was based on the matching-to-position paradigm, in which the animals were required to learn the novel position of a rewarded hole revealed to them on trial 1 of each day in order to navigate effectively to the same location (i.e., matching) on the subsequent trial on the same day. Hence, each test day included 2 trials. For both trials, each animal was left on the maze for 2 min. If it failed to reach the rewarded hole during this time, it was gently guided to the rewarded hole by the experimenter as described above. The reward remained in the same position across trials on a given test day, but took a new position on each test day. To begin a trial, the animals were placed gently in the centre of the dry maze as described before. The allocation of the rewarded holes to a specific spatial location was counterbalanced across the 4 experimental conditions. An ITI of 1 min was used at each of the 3 test days, corresponding to the minimal amount of time needed to clean the dry maze surface with water and dry it from the first to the second trial. Working memory was indexed by the reduction in distance moved and time needed to find the location of the reward in trial 2 relative to trial 1. All animals were returned to *ad libitum* food upon completion of the matching-to-position dry maze test.

3.2.4 Spatial Reference Learning and Memory in the Morris Water Maze

To study spatial reference learning and memory, we used a standard Morris water maze task, in which the animals are required to find an invisible platform with the aid of distal spatial cues. The apparatus consisted of a circular tank made of white fiberglass measuring 1 m in diameter. It was positioned in the middle of a well-lit room enriched

with distal spatial cues. The water maze was filled daily with fresh tap water to a depth of 19 cm, with the temperature of the water maintained at approximately 22±1 °C. A solid piece of cylindrical clear Plexiglas, 7 cm in diameter and 18.5 cm high, was used as an escape platform. When positioned in the water maze, the platform surface was 0.5 cm below the water surface and remained invisible to the animals. The location of the platform could be made visible by a cue, in the form of a black miniature flag mounted onto the platform. A digital camera was mounted above the water maze tank. Images were captured at a rate of 5 Hz, and transmitted to a PC running the Ethovision tracking system (Noldus Information Technology, The Netherlands), which calculated the escape latency and distance swum on each trial. Four points, equally spaced along the circumference of the water maze, were arbitrarily assigned as north (N), east (E), south (S) and west (W), which also defined four quadrants (NE, SE, NW, and SW) of equal size. For each trial (including visible platform training), the animals were allowed to spend 5 s on the platform before being placed into a waiting box containing sawdust embedding. For each animal, the inter-trial interval was approximately 5 minutes on each day of testing. When an animal failed to locate the platform within the 60 s limit, an escape latency of 60 s was assigned and the animal was guided to the platform by the experimenter.

On the first day, the animals were pretrained in the water maze using the visible cued platform (positioned in the centre of the water maze) across 3 consecutive trials. This served to familiarize the animals with water exposure stress and to assess general sensorimotor functions. To begin a trial, the animal was released into the WM against the maze wall at a randomly chosen start position (N, E, S or W).

On the second day, acquisition of spatial reference memory started. This lasted for 5 days, during which each mouse underwent 3 trials. Hence, a total of 15 acquisition trials were given to each mouse. During this period, the platform was not equipped with the visual cue and was therefore invisible to the mouse. For each mouse, the platform was placed in the same position on every day and every trial, but the platform position was randomized between mice. A different starting position was used on each of the daily trials, and different sequences of starting positions were used between days, as well as between animals within a day. Acquisition of reference memory was indicated by reduction of escape latency and distance swum to find the invisible platform over training trials.

One day after acquisition training ended, a probe test was performed, in which the platform was removed and the animals were allowed to search for it for 60 s. This served as a test of spatial memory retention. The percent time spent in the target quadrant, i.e. the quadrant in which the platform was located during the preceding acquisition phase, was taken to analyse retention of spatial reference memory.

3.2.5 Contextual Fear Conditioning

We used a standard contextual fear conditioning test to assess associative fear learning and memory. The apparatus comprised 4 Coulbourn Instruments (Allentown, PA, USA) operant chambers (Model E10-10), each installed in a ventilated, sound-insulated chest. The chambers measured 30 cm (wide) × 25 cm (long) × 29 cm (high), but the animal was confined to an area of 17 cm (wide) × 13 cm (long) in the centre of the chamber by a transparent Plexiglas enclosure. Illumination inside the chamber was provided by a house light (2.8 W) positioned on the panel wall, 21 cm above the grid floor. The grid floor was made of stainless steel rods (4 mm in diameter) spaced at regular intervals of 10 mm centre to centre, through which scrambled electric foot shock of 1 s duration and 0.3 mA intensity (the unconditioned stimulus, US), generated by a shock scrambler (Model E13-14) could be delivered. A miniature digital camera was mounted 30 cm directly above the centre of the area of interest. The output of the camera was fed to a multiplexer (YSQ-430, Sony, Japan) before being transmitted to a PC running the NIH Image software (version 1.61) for real-time analysis. The algorithm of the freezing response detection procedure has been validated before and is fully described elsewhere (Richmond *et al*, 1998). Briefly, successive digitized images (192 × 144 = 27,648 pixels, at 8-bit grey scale) obtained at a rate of 1 Hz were compared. The number of pixels differing between adjacent frames was then computed. If this was less than 0.05% of the total number of pixels in a frame, the animal was considered to be freezing in that 1-s interval.

The contextual fear test was adapted from protocols established by (Deacon *et al*, 2002a) and consisted of two phases, separated by 24 h:

On the first day, the animals were placed into the designated chambers and received 3 electric foot shocks (1-s, 0.3mA), which were separated by a 30-s interval. The first shock was delivered after an initial 3-min habituation period, during which no stimulus other than the house light was presented. The animals were removed from the

conditioning chambers 30 s after they received the last foot shock, and they were brought back to their home cages. During the conditioning day, the amount of freezing during each 30-s post-shock period provided a measure for the evaluation of the acquisition of conditioning.

On the second day, the animals were placed back to the same conditioning chambers for a period of 3 min, during which their freezing behaviour was recorded. This served as a test of measuring conditioned fear towards the context. The expression of context freezing during the 3-min test period was indexed as percent time freezing and expressed as a function of 30-s bins. After completion of the contextual fear test, the animals were brought back to their home cages.

3.2.6 Food Hoarding

The apparatus used to assess food hoarding consisted of modified home cages (40 cm long, 25 cm wide, and 15 cm high) that were kept in a temperature- and humidity-controlled (21 ± 1°C, 55 ± 5%) holding facility under a reversed light–dark cycle (lights off: 8:00 A.M. to 8:00 P.M.) as described above. Each home cage consisted of standard sawdust embedding and a Plexiglas house (Tecniplast) in the form of a triangle (150 mm wide, 110 mm wide, and 77 mm high). The cages contained stainless steel grid tops, through which drinking bottles could be inserted to allow *ad libitum* water access throughout the entire testing period. Each cage was individually connected to a wire mesh tube (32 cm long, 6 cm in diameter). The mesh consisted of 1 cm squares, and was double-rolled with the meshes misaligned to prevent food pellets dropping through. Each tube was filled with 100 g food pellets (Kliba 3430, Provimi Kliba, Switzerland), which were placed at the distal end of the hoarding tube by pouring them in at the other end with the tube held vertically. The proximal end of the wire mesh tube was sealed with a removable wooden plug.

To habituate the animals to the modified home cages, they were placed individually into the cages 12 h before the food hoarding test commenced. During this habituation period time, access to the hoarding tube was prevented with a wooden bung, so that the animals were food deprived overnight for 12 h (i.e. from 8:00 P.M. to 8:00 A.M.). The overnight food deprivation served to induce a mild state of hunger before the commencement of testing (Deacon, 2012). On the next morning, the wooden bung was removed just prior to the onset of the dark phase (lights off at 8:00 A.M.) to

allow access to the hoarding tube. Each animal had free access to the hoarding tube for a total of 24 h, during which they were left undisturbed. After this period, all food pellets that had been hoarded into each home base box were collected and weighed. This provided a measure of food hoarding behaviour. The pellets that remained in the tube were also weighed to assess the amount of food intake during the 24-h test period. The amount of food eaten was calculated by the formula: Total amount (in g) of food pellets placed into the hoarding tube – (food pellets displaced into the home cage + food pellets remaining in the tube). All measures were taken and analysed by an experimenter who was blind to the experimental conditions.

3.2.7 Social Interaction and Recognition

The apparatus was the same used for the Y-maze test, except that two of the three arms contained rectangular wire grid cages (13 × 8 × 10 cm, length × width × height; bars horizontally and vertically spaced 9 mm apart). The third arm did not contain a metal wire cage and served as the start zone (see below). The apparatus was located in an experimental testing room under dim diffused lighting (~35 lux as measured in the individual arms).

The test of social interaction consisted of two phases, namely the 'dummy phase', which is an index of 'social approach', and the 'novelty phase', which is an index of social recognition.

• *Dummy phase:* The animals were allowed to explore the three arms (referred to as 'start arm', 'dummy arm' and 'live arm'). During this phase, one metal wire cage contained an unfamiliar C57BL6 mouse ('live mouse'), and the other wire cage contained an inanimate object ('dummy mouse'), which was made of black LEGO™ bricks and took the shape of a mouse. To begin a trial, the animal was introduced at the end of the start arm and was allowed to freely explore all three arms for 5 min. Behavioural observations were made by an experimenter blind to the experimental conditions, and social interaction was defined as nose detection within a 2-cm interaction zone. The percent time spent with the live mouse was calculated by the formula (time spent with the live mouse/(time spent with the live mouse + time spent with the dummy object)) × 100 and used to assess relative exploration time between a congenic mouse and an inanimate dummy object. On completion of the 'dummy phase',

the animal was removed and kept in a holding cage, during which the sawdust flooring was changed to avoid olfactory cues.

- *Novelty phase:* Another unfamiliar C57BL6 mouse, which is referred to as the 'novel mouse' during the test phase, now replaced the inanimate dummy mouse. The other cage contained the 'familiar mouse' previously used in the 'dummy phase'. The allocation of the 'novel mouse' and 'familiar mouse' to the two wire cages was counterbalanced across experimental groups. To start the 'novelty phase', the animal was introduced into the maze again and was allowed to freely explore all three arms for 5 min. Behavioural observations for social interaction and locomotor activity were scored as described before. The percent time spent with the novel mouse was calculated by the formula (time spent with the novel mouse/(time spent with the novel mouse + time spent with the familiar mouse)) × 100 and used to assess relative exploration time between the familiar and unfamiliar congenic mouse.

3.2.8 Amphetamine Sensitivity Test

The amphetamine sensitivity test was conducted in 4 identical open-field arenas (40 × 40 × 35-cm high) made of wood and painted grey. They were located in a testing room under dim diffused lighting (approximately 35 lux as measured in the centre of the arenas). A digital camera was mounted directly above the 4 arenas. Images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Noldus, Wageningen, The Netherlands) tracking system to record locomotor activity indexed by the distance moved in the entire open field arena.

To acclimatize the animals to the open field, they were placed in the centre of the arena and allowed to explore freely for 30 min. At the end of this time period, the animals were removed from the apparatus and injected intraperitoneally (i.p.) with saline (isotonic 0.9% NaCl) solution to ascertain possible group differences to acute stress exposure induced by the injection procedure. Following saline injection, the animals were immediately returned to the same arenas and allowed to explore for another 30 min. Subsequently, the animals were briefly removed from the apparatus once more, administered with AMPH, and returned to the same arenas again. The locomotor responses to the acute drug challenge were then monitored for a period of 90 min. *D*-amphetamine sulfate (Sigma-Aldrich) was dissolved in isotonic 0.9% NaCl solution to achieve the desired concentration for injection. AMPH was administered i.p.

at a dose of 2.5 based on previous findings (Meyer et al., 2008c). The volume of injection was 5 ml/kg for all solutions. All solutions were freshly prepared on the day of testing.

3.3 Molecular Analyses

3.3.1 Collection of Brain Samples

Behaviourally naïve offspring of the different projects were decapitated on PND 45 (pubescence) or after PND 90 (adulthood) for the purpose of measuring gene and protein expression levels in prefrontal and hippocampal tissues. Following decapitation of the animals, the brains were immediately extracted from the skull and placed dorsal side up on an ice-chilled plate. This was directly followed by preparing 1-mm coronal brain sections using razorblade cuts and subsequent micro-dissection of the brain areas of interest. In particular, for the cross-fostering project we dissected the medial prefrontal cortex (mPFC; bregma: +2.3 to +1.3 mm) and dorsal hippocampus (dHPC; bregma -1.3 to -2.3 mm); for the GABAergic transcriptome project we dissected the medial prefrontal cortex (mPFC; bregma: +2.3 to +1.3 mm); for the GABA-modulator project we dissected the medial prefrontal cortex (mPFC; including anterior cingulate, prelimbic, and infralimbic subregions, bregma: +2.3 to +1.3 mm), striatum (Str; including dorsomedial and -lateral caudate putamen, bregma +1.5 to +0.5 mm), dorsal hippocampus (dHPC; bregma −1.5 to −2.5 mm), and ventral hippocampus (vHPC; bregma −2.5 to −3.5 mm); for the aging project we dissected the dorsal hippocampus (dHPC; bregma −1.5 to −2.5 mm); for the microarray study we dissected the medial prefrontal cortex (mPFC; bregma: +2.3 to +1.3 mm) and the nucleus accumbens (bregma +1.5 to +0.5 mm). Brain specimens were collected in 96-well microtiter plates kept on dry ice and allowed to freeze before storage at −80°C until further use.

3.3.2 RNA Preparation and Quantitative Real-Time PCR Analyses

In the neonatal cross-fostering project, the GABAergic project and the GABA modulator project, total RNA was isolated by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy), according with the manufacturer's instructions and quantified by spectrophotometric analysis. On the other hand, for the aging project and the microarray analysis, total RNA was isolated

using the Qiagen RNeasy Mini kit (Qiagen Italia) according to the manufacturer's instructions, and quantified by spectrophotometric analysis.

An aliquot of each sample was then treated with DNase to avoid DNA contamination. RNA was analysed by TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iScript one-step RT-PCR kit for probes (Bio-Rad Laboratories). The samples were run in 384-well formats in triplicates as multiplexed reactions with a normalizing internal control (36B4). We choose 36B4 as internal standard for gene expression analyses since its expression was not affected by prenatal treatment and further manipulations. In some cases, the results were validated also with β-actin and Rn18s as internal standards.

Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reaction. Relative target gene expression was calculated according to the 2(-Delta Delta C(T)) method. The probe and primers sequences used are summarized in **Table 4.**

Gene	Forward Primer	Reverse Primer	Probe
gad65	5'-ACCGAGAAGGCTATGAAATGG-3'	5'-TCTGGCTTTAATCACTGGCG-3'	5'-AGGCGGCTCATTCTCTCTTCATTGTC-3'
gad67	5'-GCAGATCCTGGTTGACTGTAG-3'	5'-GAACATATTGGTATTGGCAGTCG-3'	5'-TGGTTTAGCTGGTGAATGGCTGACA-3'
vgat	5'-ACGACAAACCCAAGATCACG-3'	5'-AAGATGATGAGGAACAACCCC-3'	5'-CAGCACGAACATGCCCTGAATGG-3'
alpha1	5'-TGAGAGCTGAATGCCCAATG-3'	5'-TCTGCTACAACCACTGAACG-3'	5'-CCTGCCCACTAAAATTCGGAAGCTATGC-3
alpha 2	5'-CCATGAGGCTTACAGTCCAAG-3'	5'-ACGGAGTCAGAAGCATTGTAAG-3'	5'-CGTAGCTTCCAAATTTCAGTGGGCA-3'
alpha 3	5'-ACAATATGACCACACCCAACA-3'	5'-AGCTTCCAAACTTCAGTGGG-3'	5'-CAATACACGCTGAATGCCCCATGC-3'
alpha 4	5'-GCCTGCCCTTTGAAATTTGG-3'	5'-GATACAGTCTGCCCAATGAGG-3'	5'-ATCTACACCTGGACCAAAGGCCC-3'
alpha 5	5'-GGGAATGGACAATGGAATGC-3'	5'-TGTCATTGGTCTCGTCTTGTAC-3'	5'-CATTTGCGAAAAGCCAAAGTGACTGGA-3'
nkcc1	5'-GCATTCAATCCGTCTTTCTGG-3'	5'-GGCCACAGATCATTAAACCAAC-3'	5'-AGTAAAGCAGGCCGTGAGTTTGGA-3'
kcc2	5'-TCAAACAGATGCACCTCACC-3'	5'-CCTCTGGCTTCTCCTCATTG-3'	5'-CTCCCGTTCCCGCTCGTTCTT-3'
bdnf	5'-AAGTCTGCATTACATTCCTCGA-3'	5'-GTTTTCTGAAAGAGGGACAGTTTAT-3'	5'-TGTGGTTTGTTGCCGTTGCCAAG-3'
ngf	5'-CCCAATAAAGGTTTTGCCAAGG-3'	5'-TTGCTATCTGTGTACGGTTCTG-3'	5'-TGGACATTACGCTATGCACCTCACTG-3'
ntf3	5'-CAGAACATAAGAGTCACCGAGG-3'	5'-GTTTCACAGGAGAGTTACCGG-3'	5'-TGGTGTCCCCGAATGTCAATGGC-3'
mog	5'-CTCCATCGGACTTTTGATCC-3'	5'-AGCAGATGATCAAGGCAACC-3'	5'-ATTGTGCCTGTTCTTGGACC-3'
mag	5'-CCTTCAACCTGTCTGTGGAGTT-3'	5'-CGGGTTGGATTTTACCACAC-3'	5'-CCCATAATCCTTCTGGAGTCAC-3'
mobp	5'-TTCTTCGAGGATGGGTGCAT-3'	5'-AGCAGCTCACACGTACAAGA-3'	5'-CACCATTTCTTCCTCCTGTTCC-3'
mal	5'-CGTGGTCCATGCTGTGTTTT-3'	5'-TTTCTCCACCATCCAGTCTGTG-3'	5'-GCCCATCTTCCCCATTAACTTC-3'
36B4	5'-AGATGCAGCAGATCCGCAT-3'	5'-GTTCTTGCCCATCAGCACC-3'	5'-CGCTCCGAGGGAAGGCCG-3'
β-actin	5'-ACCTTCTACAATGAGCTGCG-3'	5'-CTGGATGGCTACGTACATGG-3'	5'-TCTGGGTCATCTTTTCACGGTTGGC-3'
Rn18s	5'-GTAACCCGTTGAACCCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'	5'-TGCAATTATTCCCCATGAACGAGG-3'

Table 4: Sequences of forward and reverse primers and probes used in qRT-PCR analysis and purchased from Eurofins MWG Operon (Germany).

3.3.3 Protein Extraction and Western Blot Analysis

Western blot analysis was used to investigate $GAD_{65/67}$ and VGAT protein levels in the total homogenate of mPFC tissue. Brain samples were manually homogenized using a glass-glass potter in a pH 7.4 cold buffer (containing 0.32 M sucrose, 0.1 mM EGTA, 1mM HEPES solution in presence of a complete set of protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors) and then sonicated for 10s at a maximum power of 10-15% (Bandelin Sonoplus). Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin as calibration standard.

Equal amounts of protein were run under reducing conditions on Any Kd Criterion TGX precast gels (Bio-rad Laboratories) and then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The blots were blocked with 10% non-fat dry milk and then incubated with the primary antibodies. Because of the limited availability of mPFC tissue in small animals such as mice, we were constrained to focus our Western blot analyses on two analyses only. Therefore, we used an anti-$GAD_{65/67}$ polyclonal antibody (1:1000, 4°C overnight; Millipore) that is able to simultaneously recognize both GAD isoforms (65 and 67 kDa) and a primary anti-VGAT polyclonal antibody (1:1000, 4°C overnight, Millipore). Membranes were then incubated for 1 h at room temperature with a peroxidase-conjugated anti-rabbit IgG (1:1000 for $GAD_{65/67}$, 1:2000 for VGAT) and immunocomplexes were visualized by chemiluminescence using the Chemidoc MP imaging system (Bio-Rad Laboratories). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa after probing the membranes with a polyclonal antibody (1:10'000, Sigma–Aldrich) followed by a 1:10'000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma–Aldrich). Protein levels of $GAD_{65/67}$ (measured together without discriminating between bands) and VGAT were calculated using an up-to-date Image Lab software (Bio-Rad Laboratories).

3.3.4 Immunohistochemistry

In the aging project, we conducted immunohistochemical analyses of synaptic proteins and glial cells in pubescent (4 weeks old), adult (4 months old) and aged (= 22 months old) male offspring. Animals were deeply anesthetized with an overdose of Nembutal (Abbott Laboratories) and perfused transcardially with 0.9% NaCl, followed by 4%

phosphate-buffered paraformaldehyde solution containing 15% picric acid. The dissected brains were postfixed in the same fixative for 6 h and processed for antigen retrieval involving overnight incubation in citric acid buffer (pH = 4.5) followed by a 90 s microwave treatment at 480 W according to protocols established and validated before (Giovanoli et al., 2013). The brains were then cryoprotected using 30% sucrose in phosphate-buffered saline (PBS), frozen with powdered dry ice, and stored at −80 °C until further processing.

Perfused brain samples were cut coronally at 30 μm thickness from frozen blocks with a sliding microtome. Eight serial sections were prepared for each animal. The sections were rinsed in PBS, and stored at −20 °C in antifreeze solution until further processing. For immunohistochemical staining, the slices were rinsed three times for 10 min in PBS, and blocking was done in PBS, 0.3% Triton X-100, 10% normal serum for 1 h at room temperature. Primary rabbit anti-synaptophysin (SYN) (Sigma, CatNo# SAB4502906; diluted 1:3000) and mouse monoclonal anti-postsynaptic density protein 95 (PSD95) (Pierce Antibody Products, CatNo# ABR MA1-045; diluted 1:800) were used to assess presynaptic and postsynaptic proteins, respectively. These synaptic proteins were selected because they are commonly used to assess synaptic integrity in human and rodent studies of brain aging (Benice *et al*, 2006; Berchtold *et al*, 2013; Morrison and Baxter, 2012; Nyffeler *et al*, 2007). Primary rat anti-cluster of differentiation 68 (CD68) (AbD Serotec, CatNo# MCA1957; diluted 1:5000) was used to visualize microglia cells, and rabbit anti-glial fibrillary acidic protein (GFAP) (DAKO, CatNo# Z0334; diluted 1:5000) to visualize astrocytes (Giovanoli *et al*, 2013). All antibodies were diluted in PBS containing 0.3% Triton X-100 and 2% normal serum, and the sections were incubated free-floating overnight at room temperature. After three washes with PBS (10 min each), the sections were incubated for 1 h with the biotinylated secondary antibodies diluted 1:500 in PBS containing 2% NGS and 0.3% Triton X-100. Sections were washed again three times for 10 min in PBS and incubated with Vectastain kit (Vector Laboratories) diluted in PBS for 1 h. After three rinses in 0.1 M Tris-HCl, pH 7.4, the sections were stained with 1.25% 3,3-diaminobenzidine and 0.08% H_2O_2 for 10–15 min, rinsed again four times in PBS, dehydrated, and coverslipped with Eukitt (Kindler).

3.3.5 Optical Densitometry

We also performed optical densitometry analyses in the aging project. Quantification of SYN and PSD95 immunoreactivities was achieved by means of optical densitometry using ImageJ software (NIH). Optical densitometry was chosen because SYN and PSD95 are highly enriched at pre- and postsynaptic sites, respectively, in the hippocampal areas of interest (see below). Digital images were acquired at a magnification of 40× (NA 0.075) using a digital camera (Axiocam MRc5, Zeiss) mounted on a Zeiss Axioplan microscope. Exposure times were set so that pixel brightness was never saturated. Pixel brightness was measured in the hippocampal areas of interest of one randomly selected brain hemisphere. In addition, pixel brightness was measured in the corpus callosum as background area. The background-corrected optical densities were averaged per brain region and animal. Three to 4 coronal brain sections per animal were analysed by an experimenter who was blind to the experimental conditions.

SYN and PSD95 optical densities were measured in the pyramidal cell layer of cornu ammonis areas 1 and 3 (CA1 and CA3, respectively) and in the granule cell layer of the dentate gyrus (DG) on hippocampal sections ranging from Bregma -1.5 to -3.0 mm. These areas were delineated according to "*The Mouse Brain in Stereotaxic Coordinates*" by Franklin and Paxinos (2008). Schematic illustrations of the hippocampal cell layers, in which the relative optical density of SYN and PSD95 were measured, are provided in corresponding figure legends.

3.3.6 Stereological Investigations

Lastly, we performed stereological analyses of microglia and astrocytes. The numbers of CD68- and GFAP-immunoreactive cells were determined by unbiased stereological estimations using the optical fractionator method (Gundersen *et al*, 1988). With the aid of the image analysis computer software Stereo Investigator (version 6.50.1; MicroBrightField), every section of a one-in-eight series was measured, resulting in an average of 4-5 sections per brain sample. The following sampling parameters were used: (1) a fixed counting frame with a width of 60 µm and a length of 60 µm; and (2) a sampling grid size of 200 × 150 µm. The counting frames were placed randomly at the intersections of the grid within the outlined structure of interest by the software. The cells were counted following the unbiased sampling rule using the 40× oil lens

[numerical aperture (NA), 1.3] and included in the measurement when they came into focus within the optical dissector (Howard and Reed, 2005).

CD68- and GFAP-immunoreactive cell numbers were quantified in the CA1-CA3 and DG regions of the hippocampal formation ranging from bregma -1.5 to -3.0 mm. These areas were delineated according to "*The Mouse Brain in Stereotaxic Coordinates*" by Franklin and Paxinos (2008). Initial analyses showed that there were no subregion-specific differences between control and poly(I:C)-exposed offspring in any of the glial markers studied, and therefore, the individual CA areas were collapsed to a single hippocampal CA region for the final analysis and presentation of data. All stereological estimates were acquired by an experimenter who was blind to the animals' treatment conditions

3.3.7 Serum Cytokine Measurements

Pubescent (4 weeks old), adult (4 months old), and aged (= 22 months old) male offspring were used for the determination of serum cytokine levels. All animals were killed by decapitation, and trunk blood was collected into Eppendorf tubes. The collected blood was allowed to clot at room temperature for 1 hour before centrifugation at 4°C according to protocols established before (Meyer *et al*, 2006b). The resulting serum from each animal was subdivided to permit storage at −80°C until the cytokine assay was performed. Serum cytokine levels were measured using a multiplexed particle-based flow cytometric cytokine assay as described previously (Meyer *et al*, 2006b). Fluorokine MAP (Multi Analyte Profiling) mouse kits for interleukin (IL)-1β, IL-4, IL-6, IL-10, interferon-γ (IFN-γ), and tumour necrosis factor-α (TNF-α) were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). These cytokines were selected so as to include key members of the pro-inflammatory (IL-1β, IL-6, IFN-γ, and TNF-α) and anti-inflammatory (IL-4 and IL-10) family (Curfs *et al*, 1997). All procedures followed the manufacturer's instructions. The analysis was conducted using a conventional flow cytometer (LSR II; BD Biosciences). The detection limits (in pg/ml) were 1.0 for IL-1β, 2.0 for IL-4, 0.5 for IL-6, 0.4 for IL-10, 3.0 for IFN-γ and 0.3 for TNF-α.

3.3.8 Microarray Analyses

Gene expression microarray assays were performed using Mouse Gene 1.0 ST Array Strips on GeneAtlas platform (Affymetrix), following the 3'IVT one cycle labelling and amplification protocol described in the Affymetrix GeneChip Expression Analysis Technical Manuals and in the GeneAtlas™ WT Expression Kit User Manual. Mouse Gene 1.0 ST Array Strips are comprised of more than 530,000 probes covering more than 36,000 transcripts and variants, which represent more than 20,000 genes mapped through UniGene or via RefSeq annotation.

To synthesize First-Strand cDNA, 250ng RNA were reverse-transcribed with the Gene Atlas 3'IVT Express Kit or WT Expression Kit (Affymetrix, Santa Clara, CA, USA) using T7 oligo(dT) primer. Second-Strand cDNA synthesis was carried out using DNA polymerase and RNase H to simultaneously degrade RNA and synthesize second-strand cDNA. This step was followed by the in vitro transcription using IVT Labelling Master Mix to generate multiple copies of biotin-modified antisense-RNA (aRNA) from the double-stranded cDNA templates. Subsequently strand DNA was purified to remove unincorporated NTPs, salts, enzymes and inorganic phosphate. Labelled cDNA (10ug) was then fragmented and 7.5µg were hybridized onto Mouse Gene 1.0 ST Array Strips. The reactions of hybridation, fluidics and imaging were performed on the Affymetrix Gene Atlas instrument according to the manufacturer's protocol.

3.4 Drug Administration

In the GABA modulator project, we treated animals acutely with the benzodiazepine positive allosteric modulator SH-053-2'F-S-CH3. A detailed description of SH-053-2'F-S-CH3 synthesis has been described previously (Cook et al., 2009). Synthesis of (S)-ethyl 8-ethynyl-6-(2-fluorophenyl)-4-methyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate was performed at the Department of Chemistry and Biochemistry, University of Wisconsin, Milwaukee, USA. SH-053-2'F-S-CH3 has been demonstrated to have a greater relative affinity for α5 (Ki = 19.2), α2 (Ki = 33.3), and α3 (Ki = 291.5) compared with the relative affinity for α1 (Ki = 468.2) (Fischer *et al*, 2010; Savic *et al*, 2010). Moreover, SH-053-2'F-S-CH3 has been shown to have a greater efficacy at α5 (218/389), α2 (170/348), and α3 (138/301) compared with the relative efficacy at α1 (116/164) (efficacy expressed as percentage of control current at 100nM and 1µM)

(Fischer *et al*, 2010; Savic *et al*, 2010). SH-053-2'F-S-CH3 was dissolved with the aid of sonication in a solvent containing 85% deionized water, 14% propylene glycol, and 1% Tween 80. Corresponding vehicle (VEH) solution consisted of the solvent only. SH-053-2'F-S-CH3 was administered at a dose of 15 or 30 mg/kg (i.p.) according to dose ranges reported previously (Savic *et al*, 2010). All solutions were freshly prepared on the day of administration and were injected using an injection volume of 5ml/kg (i.p.). The solutions (drug or vehicle) were administered 20 minutes before each behavioural test.

3.5 Statistical Analyses

3.5.1 Neonatal Cross-Fostering Project

All gene expression (real-time PCR) data were analysed using a two-way ANOVA. In the dry maze matching-to-position paradigm of working memory, the distance moved and latency to locate the reward served as the critical test read-out and was analysed using a 2 × 2 × 2 × 3 (prenatal treatment × postnatal rearing × trial × day) repeated-measures ANOVA. In addition, a 2 × 2 × 3 (prenatal treatment × prenatal treatment × postnatal rearing day) repeated-measures ANOVA of the improvement scores (i.e., [time or distance in trial 1] – [time or distance in trial 2]) was conducted in the dry maze matching-to-position paradigm of working memory. In the Y-maze working memory test, the relative time spent in the novel arm and distance moved during the choice phase were analysed using a 2 × 2 (prenatal treatment × postnatal rearing) ANOVA. In the test of AMPH sensitivity, the distance travelled in the open field was expressed as a function of 5 min bins and analysed using a 2 × 2 × 6 (prenatal treatment × postnatal rearing × bins) repeated-measures ANOVA for the acclimatization and saline treatment phases, and by a 2 × 2 × 18 (prenatal treatment × postnatal rearing × bins) repeated-measures ANOVA for the AMPH treatment phase. All gene expression data were analysed using 2 × 2 (prenatal treatment × postnatal rearing) ANOVAs. The initial ANOVAs were followed by Fisher's least significant difference (LSD) post hoc comparisons or restricted ANOVAs whenever appropriate. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the statistical software StatView (version 5.0) implemented on a PC running the Windows XP operating system.

3.5.2 GABAergic Transcriptome Project

All gene expression (real-time PCR) and protein expression (Western blot) data were analysed using independent Student's t tests (two-tailed). In the dry maze matching-to-position paradigm of working memory, the distance moved and latency to locate the reward served as the critical test read-out and was analysed using a 2 × 2 × 5 (prenatal treatment × trial × day) repeated-measures ANOVA, followed by Fisher's least significant difference (LSD) post-hoc comparisons or restricted ANOVAs whenever appropriate. In addition, a 2 × 5 (prenatal treatment × day) repeated-measures ANOVA of the improvement scores (i.e., [time or distance in trial 1] – [time or distance in trial 2]) was conducted in the dry maze matching-to-position paradigm of working memory. All data were separately analysed for peri-pubertal and adult subjects. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using the statistical software StatView (version 5.0) implemented on a PC running the Windows XP operating system.

3.5.3 GABA Modulator Project

All gene expression (real-time PCR) data were analysed using independent Student's t tests (two-tailed). In the Y-maze working memory test, the relative time spent in the novel arm and distance moved during the choice phase were analysed using a 2 × 3 (prenatal treatment × drug treatment) ANOVA. The data obtained in the social interaction test were separately analysed for the two successive phases. In the first phase, a 2 × 3 (prenatal treatment × drug treatment) ANOVA of the percent time spent with the live mouse was used to assess relative exploration time between a congenic mouse and an inanimate dummy object. In the second phase, a 2 × 3 (prenatal treatment × drug treatment) ANOVA of the percent time spent with the unfamiliar mouse was used to assess the relative exploration time between a novel congenic mouse and a familiar congenic mouse. In the AMPH sensitivity test, the distance moved was expressed as a function of 5-min bins and analysed using a 2 × 3 × 6 (prenatal treatment × drug treatment × bins) repeated-measure ANOVA for the initial pre-AMPH phase, and using a 2 × 3 × 12 (prenatal treatment × drug treatment × bins) repeated-measure ANOVA for the subsequent AMPH phase. All ANOVAs were followed by Scheffe's post-hoc comparisons whenever appropriate. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the statistical software StatView (version 5.0) implemented on a PC running the Windows XP operating system.

3.5.4 Aging Project

All data were analysed using parametric analysis of variance (ANOVA), except for the data obtained in the food hoarding test, which were analysed using non-parametric tests (see below). In the Y-maze spatial recognition test, the relative time spent in the novel arm and distance moved during the choice phase were analysed using 2 × 3 (prenatal treatment × age) ANOVAs. In the conditioning phase of contextual fear test, the amount of percent time freezing during each 30-s post-shock period was analysed using a 2 × 3 × 3 (prenatal treatment × age × trial) repeated-measure ANOVA. Expression of conditioned fear during the second phase of the contextual fear test was analysed using a 2 × 3 × 6 (prenatal treatment × age × bins) repeated-measure ANOVA. In the acquisition phase of the Morris water maze test, the latency to find the platform across trials was analysed using a 2 × 3 × 15 (prenatal treatment × age × trial) repeated-measure ANOVA. The probe test of spatial memory retention in the water maze was analysed using a 2 × 3 × 4 (prenatal treatment × age × quadrant) ANOVA. In the open field test, all dependent measures (total distance moved in the entire arena, total distance moved in the centre zone, and time spent in the centre zone) were analysed using 2 × 3 (prenatal treatment × age) ANOVAs. All immunohistochemical data and serum cytokine levels were also analysed using 2 × 3 (prenatal treatment × age) ANOVAs, whereas the gene expression data were analysed using 2 × 2 (prenatal treatment × age) ANOVAs. All ANOVAs were followed by Fisher's least significant difference (LSD) post-hoc comparisons whenever appropriate. The dependent measures in the food hoarding test (i.e., food hoarded and food eaten during the 24-h test period) were first subjected to logarithmic transformation (base e) to reduce data skewness and then analysed using non-parametric Mann–Whitney U tests. Non-parametric tests were used in the food hoarding test in view of the non-normal distribution of the primary dependent measures (amount of food hoarded). Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the statistical software StatView (version 5.0) implemented on a PC running the Windows 7 operating system.

3.5.5 Microarray Analysis

Affymetrix CEL files were imported into Partek Genomics Suite version 6.6 for data visualization and statistical testing. Quality control assessment was performed using

Partek Genomic Suite 6.6. All samples passed the criteria for hybridization controls, labelling controls and 3'/5' Metrics. Background correction was conducted using Robust Multi-strip Average (RMA) (Irizarry *et al*, 2003) to remove noise from auto fluorescence. After background correction, normalization was conducted using Quantiles normalization (Bolstad *et al*, 2003) to normalize the distribution of probe intensities among different microarray chips. Subsequently, a summarization step was conducted using a linear median polish algorithm (Tukey, 1977) to integrate probe intensities in order to compute the expression levels for each gene transcript. Upon data upload, pre-processing of CEL data for the complete data set [total of twelve samples; six biological replicates per sample for vehicle, six biological replicates for GD17 Poly(I:C)] was performed using ANOVA to assess treatment effects. Differential gene expression across treatment was assessed by applying a p-value filter (for treatment) of $p < 0.05$ to the ANOVA results. To investigate the effect of Poly(I:C), a linear contrast was performed [Poly(I:C) versus vehicle]. In this comparison, a maximum filter of $p < 0.05$ and a minimum absolute fold change cut-off of 1.2 were applied. Subsequent validation analysis performed by quantitative Real-Time PCR was analysed with independent Student's t tests (two-tailed).

4 RESULTS AND DISCUSSION

4.1 Prenatal Immune Activation Induces Maturation-Dependent Alterations in the Prefrontal GABAergic Transcriptome

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Prenatal exposure to maternal infection has been associated with increased risk of developmental neuropsychiatric disease, most notably schizophrenia (Brown *et al*, 2010; Mortensen *et al*, 2007). This epidemiological link has also been supported by a plethora of experimental work in animals demonstrating schizophrenia-relevant behavioural, cognitive and neuroanatomical alterations following prenatal exposure to infectious pathogens or immune-activating agents (Boksa, 2010; Meyer *et al*, 2010a; Meyer *et al*, 2009b). Accumulating evidence suggests that cytokine-associated inflammatory events (Meyer *et al*, 2008b; Smith *et al*, 2007), together with downstream pathophysiological processes such as oxidative stress (Lante *et al*, 2008), hypoferremia (Aguilar-Valles *et al*, 2010), and zinc deficiency (Coyle *et al*, 2009), are critical in mediating the negative influences of prenatal infection on brain and behavioural development.

The central γ-aminobutyric acid (GABA) system is one of the neuronal networks that is highly sensitive to the disrupting effects of prenatal immune challenge. Indeed, prenatal exposure to viral infection or maternal immune activation has been repeatedly shown to cause GABAergic abnormalities at the cellular and neurochemical levels (Bitanihirwe *et al*, 2010a; Fatemi *et al*, 1999; Harvey and Boksa, 2012; Meyer *et al*, 2006b; Meyer *et al*, 2008d; Nyffeler *et al*, 2006), some of which are directly implicated in the pathophysiology of schizophrenia (Lewis *et al*, 2005). It is important to note that prenatal immune activation is capable of changing cellular GABAergic markers in the offspring's brain without concomitantly inducing marked neurodegeneration and reactive gliosis(Nyffeler *et al*, 2006). One implication is that such GABAergic abnormalities are unlikely to be the result of persistent necrotic and/or apoptotic processes, but they may rather be caused by changes in the expression of the corresponding genes. However, the extent to which prenatal immune activation can cause long-lasting changes in GABAergic gene expression remains essentially unexplored.

The present study sought evidence for this hypothesis by measuring the transcription levels of various pre- and post-synaptic GABAergic genes in offspring subjected to prenatal immune activation relative to controls. We used a well characterized mouse model of prenatal immune challenge induced by maternal gestational treatment with the viral mimetic poly(I:C), which is known to capture a variety of cellular GABAergic abnormalities and parallel behavioural dysfunctions

relevant to schizophrenia and autism (Meyer *et al*, 2010a; Meyer *et al*, 2009b). Poly(I:C) is a synthetic analogue of double-stranded RNA that induces a cytokine-associated viral-like acute phase response in maternal and fetal compartments, including the fetal brain (Meyer *et al*, 2010a; Meyer *et al*, 2009b).

Various GABAergic markers were assessed in the medial prefrontal cortex (mPFC), a brain region known to be sensitive to the disrupting effects of prenatal immune challenge (Fatemi *et al*, 1999; Meyer *et al*, 2010a; Meyer *et al*, 2009b; Meyer *et al*, 2006b; Meyer *et al*, 2008d). The examination of pre-synaptic GABAergic markers included the two isoforms of the rate limiting enzyme for GABA biosynthesis, GAD_{65} and GAD_{67}, as well as the vesicular GABA transporter (VGAT) that is responsible for uptake and storage of GABA by synaptic vesicles (Farrant and Kaila, 2007). In addition to these pre-synaptic markers, we ascertained the expression of the five distinct alpha-subunits (α1-α5) of the $GABA_{(A)}$ receptor because of their suggested pathophysiological relevance to schizophrenia (Beneyto *et al*, 2011). We further measured the expression levels of the sodium-potassium-chloride co-transporter 1 (NKCC1) and the potassium-chloride co-transporter 2 (KCC2), which mediate chloride uptake and extrusion, respectively, thereby regulating the extent to which GABA signalling is predominately excitatory or inhibitory (Farrant *et al*, 2007). All gene expression analyses were conducted in peri-puberty and adulthood so as to assess possible maturation-dependent effects of prenatal immune activation.

4.1.1 Prenatal Immune Activation Induces Age-Dependent Changes in Prefrontal Expression of Presynaptic GABAergic Genes

First, we studied the effects of prenatal immune challenge on the transcription levels of presynaptic GABAergic genes in the mPFC of peri-pubertal and adult offspring. Using 36B4 as internal normalizing gene, we found that adult offspring born to immune-challenged mothers displayed a marked deficit in mRNA expression levels of the enzymes regulating GABA biosynthesis (GAD_{65} and GAD_{67}) and vesicular GABA storage (VGAT) (**Fig. 2a**). Notably, these effects were not manifest when the offspring reached the peri-pubertal stage of development (**Fig. 2a**), suggesting that prenatal immune activation induces age-dependent changes in prefrontal expression of these presynaptic GABAergic genes. Importantly, the direction and magnitude of the observed changes between poly(I:C)-exposed and control animals remained stable when we analysed

mRNA expression of GAD_{65}, GAD_{67}, and VGAT using two other internal house-keeping genes, namely β-actin and 18S rRNA (**Fig. 3** and **Fig. 4**). These additional investigations highlight that the effects of prenatal immune activation on GABAergic gene expression are readily attributable to significant alterations in the expression of the target genes and not biased by possible effects on internal standard genes. Furthermore, we also confirmed the age-dependent effects of prenatal immune activation on presynaptic GABAergic markers at the protein level using Western blot analyses: Adult but not peri-pubertal offspring born to immune-challenged mothers displayed a significant reduction in $GAD_{65/67}$ and VGAT protein in the mPFC (**Fig. 2b**).

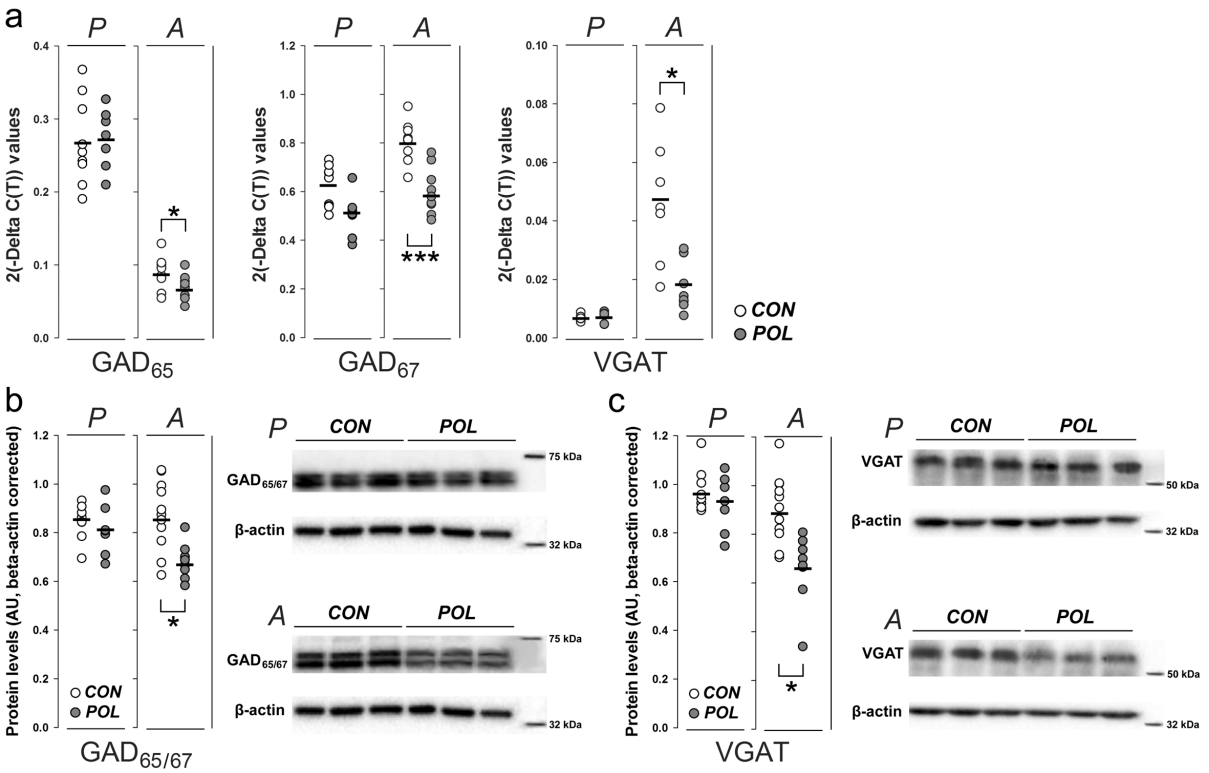

Figure 2. Gene and protein expression profiling of presynaptic GABAergic markers in prefrontal cortex following prenatal immune activation. (a) Levels of mRNA expression in prenatally poly(I:C)-exposed (POL) and control (CON) offspring at peri-pubertal or adult age assessed using quantitative real-time PCR. **P* < 0.05 and ***P* < 0.01, based on independent Student's *t* tests (two-tailed). *N*(CON/peri-puberty) = 10, *N*(POL/ peri-puberty) = 7, *N*(CON/adulthood) = 11, *N*(POL/ adulthood) = 9. All values are means±SEM. **(b)** Levels of protein expression in the mPFC of POL and CON offspring at peri-pubertal or adult age using Western blot analysis. The photomicrographs show Western blot samples for $GAD_{65/67}$ and VGAT protein in the mPFC of three representative CON and POL offspring. β-actin is shown as control for comparison. **P* < 0.05, based on independent Student's *t* tests (two-tailed). *N*(CON/peri-puberty) = 10, *N*(POL/ peri-puberty) = 7, *N*(CON/adulthood) = 11, *N*(POL/ adulthood) = 9. All values are means±SEM.

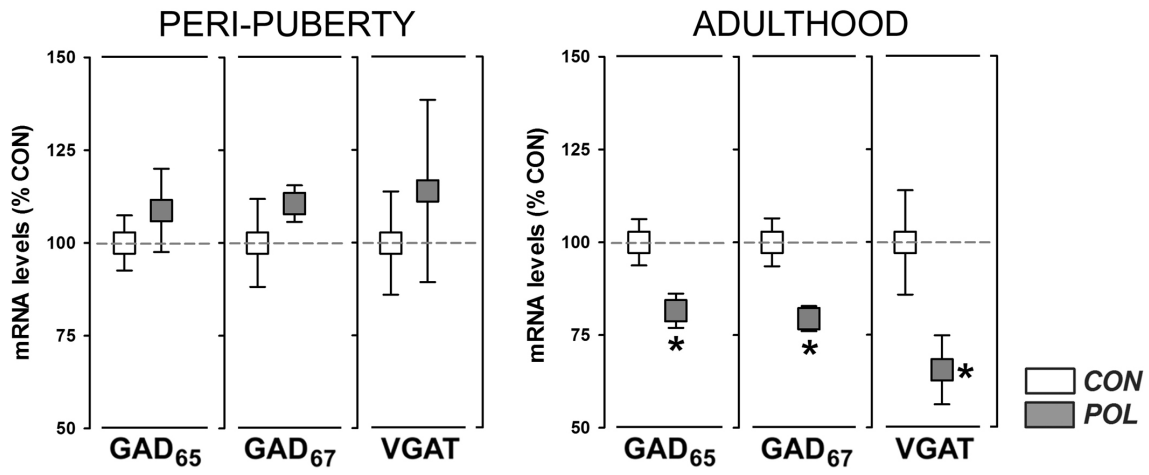

Figure 3. Gene expression profiling of presynaptic GABAergic markers in prefrontal cortex following prenatal immune activation using β-actin as internal normalizing gene. The graph depicts the levels of normalized mRNA expression in prenatally poly(I:C)-exposed (POL) and control (CON) offspring at peri-pubertal or adult age assessed using quantitative real-time PCR. *P < 0.05, based on independent Student's t tests (two-tailed). N(CON/peri-puberty) = 9, N(POL/ peri-puberty) = 7, N(CON/adulthood) = 11, N(POL/ adulthood) = 9. All values are means±SEM.

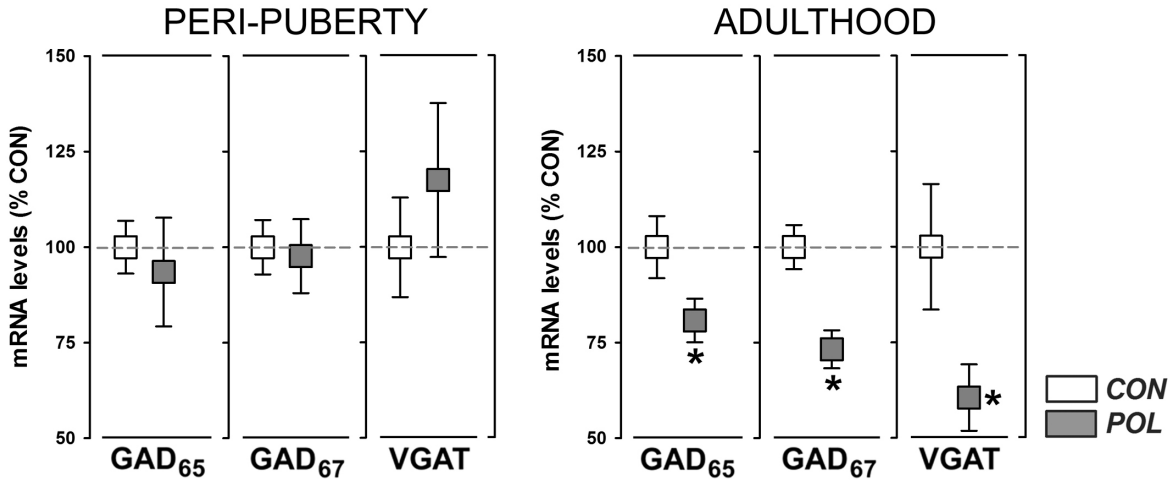

Figure 4. Gene expression profiling of presynaptic GABAergic markers in prefrontal cortex following prenatal immune activation using 18S rRNA as internal normalizing gene. The graph depicts the levels of normalized mRNA expression in prenatally poly(I:C)-exposed (POL) and control (CON) offspring at peri-pubertal or adult age assessed using quantitative real-time PCR. *P < 0.05, based on independent Student's t tests (two-tailed). N(CON/peri-puberty) = 9, N(POL/ peri-puberty) = 7, N(CON/adulthood) = 11, N(POL/ adulthood) = 9. All values are means±SEM.

4.1.2 Prenatal Immune Activation Induces Age-Dependent Changes in Prefrontal Transcription of $GABA_{(A)}$ Alpha-Receptor Subunit

We also sought evidence for the possibility that prenatal immune activation may affect the transcription of $GABA_{(A)}$ receptor genes. To this end, we focused on the expression of the five alpha subunits (α1-α5) of the $GABA_{(A)}$ receptor because previous investigations have detected cellular changes in the expression of these subunits following prenatal immune activation in mice (Meyer *et al*, 2008d; Nyffeler *et al*, 2006; Samuelsson *et al*, 2006). Here, we show that prenatal immune activation leads to age-dependent alterations in the expression of the alpha-subunits of $GABA_{(A)}$ receptor genes (**Fig. 5**): Whereas prefrontal mRNA levels of the α2 and α4 subunits were significantly increased in immune-challenged offspring at peri-pubertal age (**Fig. 5**), expression of the same genes was markedly down-regulated in immune-exposed offspring when they reached adulthood (**Fig. 5**). Furthermore, adult but not peri-pubertal offspring showed a significant increase and decrease in the expression of α3 and α5 mRNA levels, respectively (**Fig. 5**).

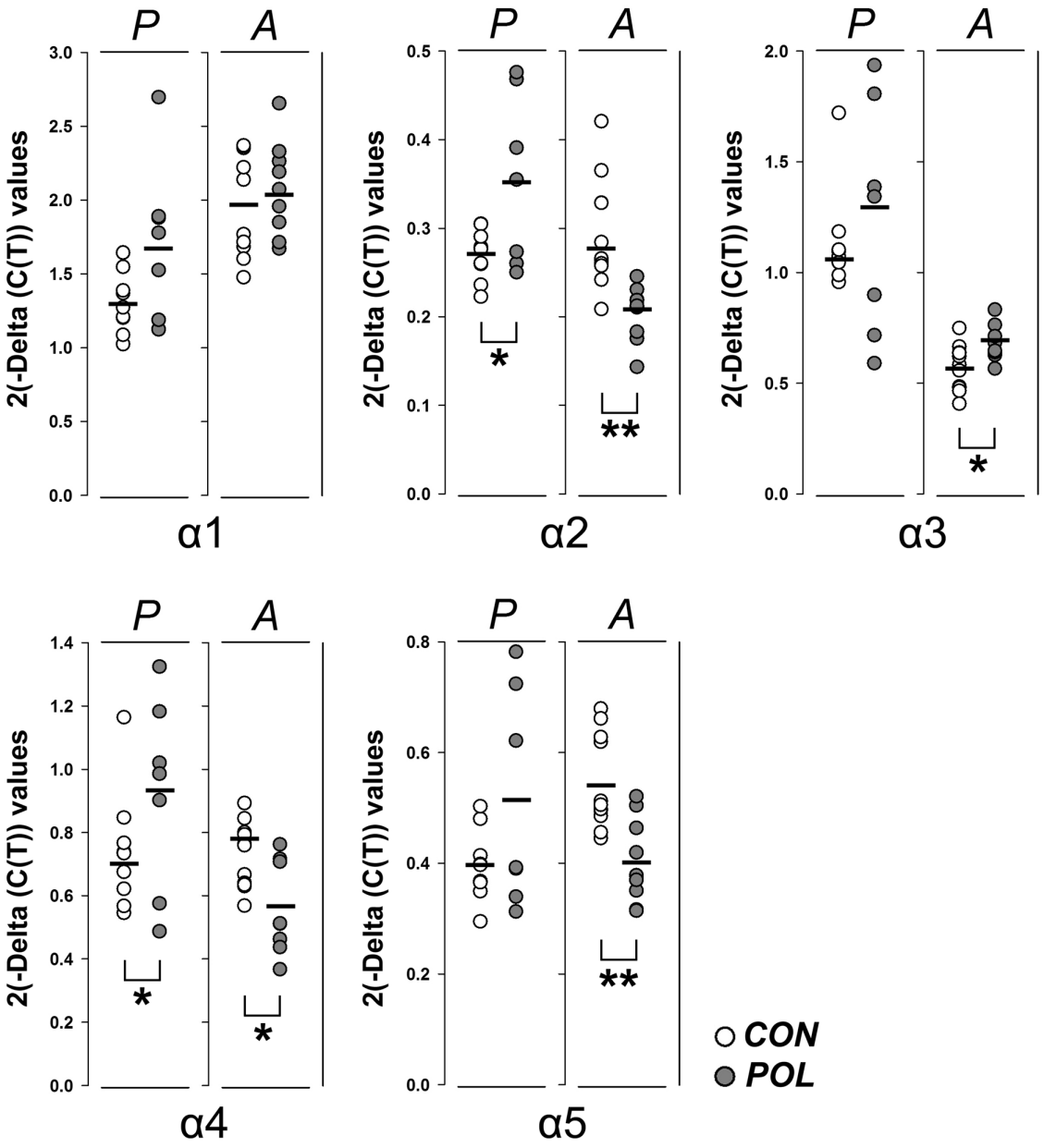

Figure 5. Analysis of $GABA_{(A)}$ receptor alpha-subunits mRNA levels in prefrontal cortex following prenatal immune activation. **(a)** The square symbols represent mRNA levels of the α1-5 subunits in prenatally poly(I:C)-exposed (POL) and control (CON) offspring at peri-pubertal age. **P* < 0.05, based on independent Student's *t* tests (two-tailed). *N*(CON) = 10, *N*(POL) = 7. **(b)** The square symbols show mRNA levels of α1-5 subunits in POL and CON offspring at adult age. **P* < 0.05 and ***P* < 0.01, based on independent Student's *t* tests (two-tailed). *N*(CON/peri-puberty) = 9, *N*(POL/ peri-puberty) = 7, *N*(CON/adulthood) = 11, *N*(POL/ adulthood) = 9. All values are means±SEM.

4.1.3 Prenatal Immune Activation Induces Age-Dependent Changes in Genes Regulating the GABA Excitatory/Inhibitory Shift

The age-dependent alterations of presynaptic and receptors-associated GABAergic gene expression manifest in immune-challenged offspring may be indicative of altered maturation of the prefrontal GABA system. To further seek evidence for this possibility, we measured the gene transcription levels of NKCC1 and KCC2, which regulate the extent to which GABA signalling is either excitatory or inhibitory (Farrant *et al*, 2007). KCC2 is a potassium-chloride co-transporter that mediates chloride extrusion, thereby facilitating the inhibitory actions of GABA (Farrant *et al*, 2007). It is expressed primarily in mature neurons and its expression therefore peaks only after full brain maturation in adulthood (Hashimoto *et al*, 2008b). In contrast, sodium-potassium-chloride co-transporter NKCC1 shows peak levels during early postnatal brain development, a time when GABA signalling is primarily excitatory (Ben-Ari *et al*, 2012). Therefore, the relative expression of NKCC1 *versus* KCC2 is typically taken as a molecular index of the functional maturation of the central GABA system.

We found that prenatal immune activation increased the transcription levels of both NKCC1 and KCC2 in the mPFC of peri-pubertal offspring (**Fig. 6a**). Because of this concomitant enhancement, the NKCC1:KCC2 ratio was highly similar between immune-challenged offspring ad controls at peri-pubertal age (**Fig. 6b**). In marked contrast, adult offspring born to immune-challenged mothers displayed a significant increase and decrease in the transcription of NKCC1 and KCC2, respectively (**Fig. 6a**). This imbalanced expression was further evident in the NKCC1:KCC2 ratio, which was significantly increased in immune-challenged offspring relative to controls at adult age (**Fig. 6b**). Taken together, these findings support the hypothesis that prenatal immune activation disrupts the normal maturation of the prefrontal GABA system, leading to molecular signs of an immature GABAergic system in adulthood.

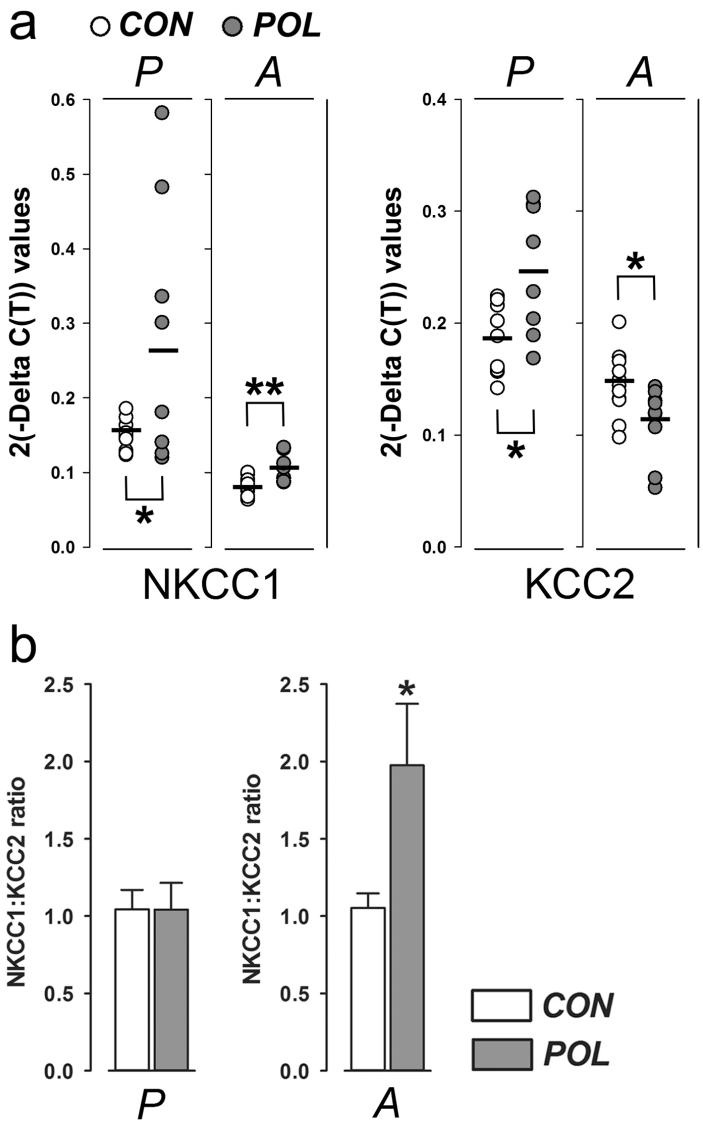

Figure 6. Analysis of NKCC1 and KCC2 mRNA levels in prefrontal cortex following prenatal immune activation. (a) The square symbols represent mRNA levels of NKCC1 and KCC2 in prenatally poly(I:C)-exposed (POL) and control (CON) offspring at peri-pubertal age, and the bar plot depicts the NKCC1:KCC2 ratio. *P < 0.05, based on independent Student's t tests (two-tailed). N(CON) = 10, N(POL) = 7. **(b)** The square symbols show mRNA levels of NKCC1 and KCC2 in prenatally poly(I:C)-exposed (POL) and control (CON) offspring at adult age, and the bar plot represents the NKCC1:KCC2 ratio. *P < 0.05 and **P < 0.01, based on independent Student's t tests (two-tailed). N(CON/peri-puberty) = 9, N(POL/ peri-puberty) = 7, N(CON/adulthood) = 11, N(POL/ adulthood) = 9. All values are means±SEM.

4.1.4 Prenatal Immune Activation Induces an Adult Onset of Working Memory Impairments

Because of its critical role in regulating neuronal synchronization, the prefrontal GABAergic system critically contributes to a variety of cognitive functions, including working memory (Lewis *et al*, 2005). Impairments in prefrontal GABAergic signalling are therefore believed to significantly contribute to working memory deficits in psychiatric disorders, especially in schizophrenia (Lewis *et al*, 2005). In view of our findings demonstrating maturation-dependent alterations in prefrontal GABAergic gene transcription following prenatal immune activation (**Figs. 2** to **6**), we further ascertained whether the presence of these molecular changes might be accompanied by cognitive dysfunctions in the form of working memory impairments. Because the prefrontal GABAergic transcriptome is arguably more affected in adult as compared to peri-pubertal offspring born to immune-challenged mothers (**Figs. 2** to **6**), one clear expectation would be that the negative influences of prenatal immune challenge on cognitive functions may be readily noticeable in adulthood as compared to earlier maturational stages.

The assessment of working memory performance in immune-challenged and control offspring confirmed this expectation: Using a matching-to-position paradigm, we found that adult but not peri-pubertal offspring born to immune-challenged mothers displayed a severe working memory deficit (**Fig. 7**). Indeed, at the peri-pubertal stage, both immune-challenged and control subjects showed a clear reduction in the latency and distance moved to find the rewarded hole in trial 2 relative to trial 1 (when the position of the rewarded hole was unknown to the animals) (**Fig. 7a**). In marked contrast, such improvement in performance from trial 1 to trial 2 was no longer evident in adult offspring subjected to prenatal immune activation, whilst at the same time, adult control offspring still displayed highly significant reduction in the latency and distance moved to find the rewarded hole in trial 2 relative to trial 1 (**Fig. 7b**).

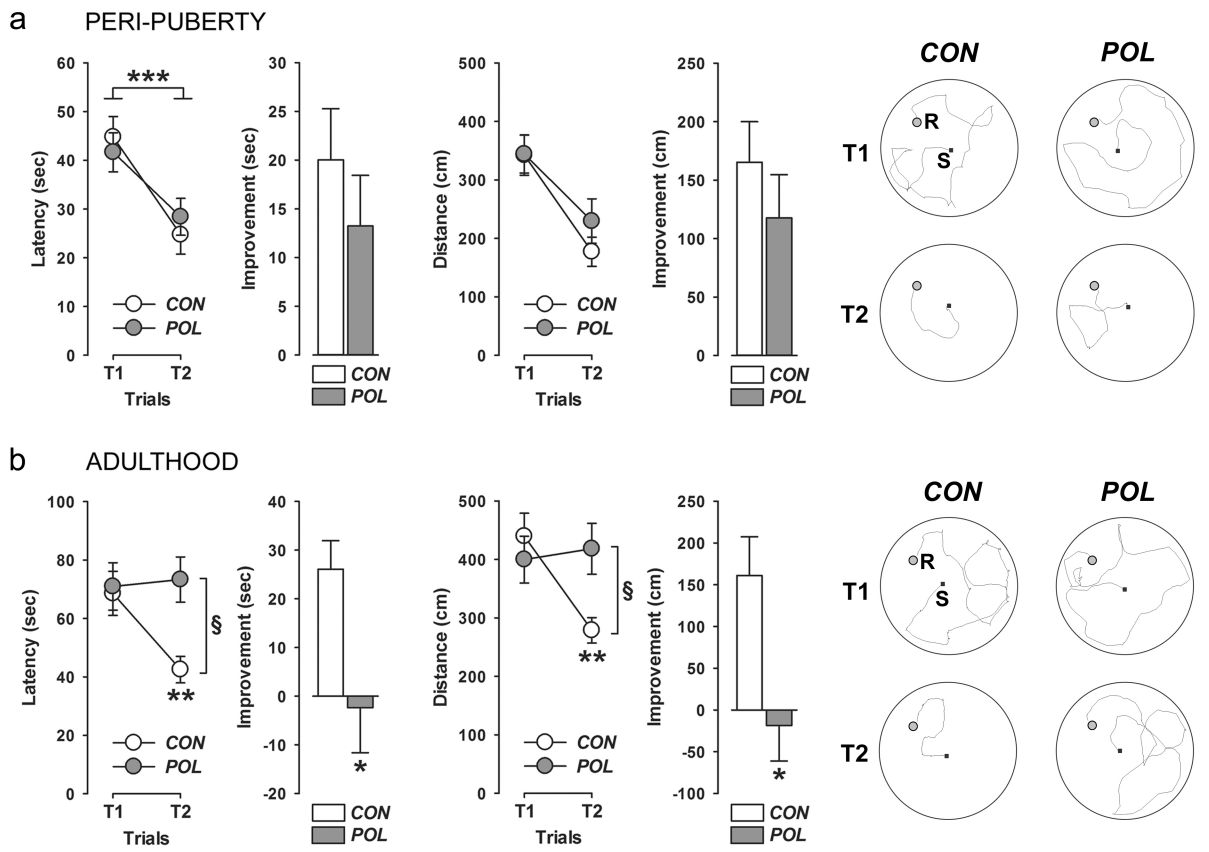

Figure 7. Working memory performance following prenatal immune activation. The line plots show the latency (sec) and distance moved (cm) to find the rewarded hole in trial 1 (T1) and T2, and the bar plots depicts the improvement in these measures from T1 to T2. The drawings illustrate computer-generated search path of representative CON and POL offspring in T1 and T2 of the working memory test. S, Starting position; R, location of the rewarded hole. **(a)** Working memory performance in prenatally poly(I:C)-exposed (POL) and control (CON) offspring at peri-pubertal age. ***P < 0.001, reflecting the significant main effect of trials [latency: $F_{(1,25)}$ = 14.63, P < 0.001; distance: $F_{(1,25)}$ = 11.75, P < 0.001] obtained by a 2 × 2 × 5 (prenatal treatment × trial × day) repeated-measures ANOVA. N(CON) = 13, N(POL) = 14. All values are means±SEM. **(b)** Working memory performance in POL and CON offspring at adult age. **P < 0.01, reflecting the significant main effect of trials [latency: $F_{(1,16)}$ = 7.92, P < 0.01; distance: $F_{(1,16)}$ = 8.95, P < 0.01] in adult CON animals following the presence of a significant prenatal treatment × trial interaction [latency: $F_{(1,29)}$ = 4.58, P < 0.05; distance: $F_{(1,29)}$ = 6.48, P < 0.05]; §P < 0.01, reflecting the significant difference between CON and POL animals in T2 based on post-hoc comparisons; *P < 0.05, reflecting the significant difference between CON and POL animals associated with the significant main effect of prenatal treatment [latency: $F_{(1,29)}$ = 5.01, P < 0.05; distance: $F_{(1,29)}$ = 5.82, P < 0.05] obtained by 2 × 5 (prenatal treatment × day) repeated-measures ANOVA of the improvement scores. N(CON) = 17, N(POL) = 14. All values are means±SEM.

4.1.5 Discussion

The present data provides the first line of experimental evidence showing that prenatal exposure to immune activation has long-lasting consequences on GABAergic mRNA expression in the mPFC. Some of the gene expression changes identified in adult immune-challenged offspring, including reduced prefrontal mRNA levels of GAD_{67}, α4 and α5, have been repeatedly noted in schizophrenia and other neurodevelopmental disorders with prenatal infectious aetiologies, including autism (Beneyto *et al*, 2011; Blatt and Fatemi, 2011; Hashimoto *et al*, 2008b; Lewis *et al*, 2005). On the other hand, our findings do not match the reports of decreased and increased α1 and α2 mRNA levels, respectively, in cortical areas of schizophrenic patients (Beneyto *et al*, 2011; Hashimoto *et al*, 2008b). Our experimental data can thus be taken to support the hypothesis that prenatal immune challenge is a significant environmental risk factor for some but not all long-term GABAergic abnormalities commonly found in neuropsychiatric disorders with developmental components.

However, we would like to emphasize that our experimental data may be more readily reminiscent of and relevant for neuropsychiatric disorders with a delayed onset in early adulthood, primarily schizophrenia. This is because the deleterious effects of prenatal immune activation on GABAergic pathology (and associated cognitive impairments) were found to be more pronounced when the offspring reached adulthood. Indeed, one of the major findings here was that the magnitude and/or direction of the prenatal infection-induced changes in mRNA expression were highly dependent on the postnatal age of the offspring. For example, deficits in the expression levels of enzymes regulating GABA biosynthesis (GAD_{65} and GAD_{67}) and vesicular GABA storage (VGAT) were only clearly manifest in immune-challenged offspring once they had reached adulthood. Furthermore, adult offspring subjected to prenatal immune activation also showed qualitatively (and quantitatively) more severe changes in mRNA expression of the $GABA_{(A)}$ receptor alpha-subunits compared to immune-challenged offspring at peri-pubertal age. Consistent with its maturational impact on dopaminergic development (Vuillermot *et al*, 2010), these findings emphasize that prenatal immune activation does not induce static effects on GABAergic gene expression. Rather, the prenatal immunological insult may change early neurodevelopmental trajectories that may then interact with maturational processes to precipitate the full spectrum of GABAergic abnormalities in adulthood. This proposition would also be congruent with

the complex temporal dynamics underlying the normal maturation of the central GABA system, in which even subtle changes during development can exert a profound impact on long-term GABAergic signalling (Levitt *et al*, 2004).

Our findings of altered NKCC1 and KCC2 mRNA expression in prenatally immune-challenged offspring further support the hypothesis of altered GABAergic maturation following exposure to the prenatal immunological insult. The expression of these two chloride transporters is developmentally regulated, which in turn is pivotal in shifting the functional properties of GABA from initially excitatory in the developing immature brain to its predominantly inhibitory actions in the adult nervous system (Ben-Ari *et al*, 2012). Whereas the mRNA expression levels of NKCC1 and KCC2 were concomitantly increased in peri-pubertal offspring born to immune challenged mothers, the relative expression of NKCC1 *versus* KCC2 was strongly shifted towards increased and decreased NKCC1 and KCC2 levels, respectively, when these offspring reached adult age. Owing to the normal developmental regulation of these chloride transporters, the presence of such imbalanced NKCC1 and KCC2 expression in the adult brain is typically taken as an index of an immature GABAergic phenotype (Ben-Ari *et al*, 2012). Interestingly, a similar pattern of imbalanced NKCC1 and KCC2 expression has recently been documented in the hippocampus of schizophrenic patients (Hyde *et al*, 2011). In addition, some of the kinases strongly regulating KCC2 and NKCC1 activity were found to be altered in the dorsolateral prefrontal cortex (DLPFC) of schizophrenic patients (Arion and Lewis, 2011). Hence, alterations in molecular mechanisms regulating the nature of GABA neurotransmission (inhibitory *versus* excitatory) are noticeable both in human subjects with schizophrenia and in the present experimental model system relevant to schizophrenia and related disorders.

The present study also confirms the deleterious impact of prenatal immune activation on working memory (Bitanihirwe *et al*, 2010b; Connor *et al*, 2012; Meyer *et al*, 2008d; Vuillermot *et al*, 2010). Working memory is a special short-term memory buffer used to hold relevant information temporarily active in order to guide on-going behaviour, and its disruption is a cardinal cognitive symptom in schizophrenia (Lewis *et al*, 2005). The profound disruption of working memory in adult offspring born to immune-challenged mothers is comparable with our own previous investigations using the same working memory paradigm (Vuillermot *et al*, 2010), and with other studies performed by other independent laboratories documenting significant working memory

deficits following prenatal poly(I:C) exposure on GD17 in mice (Connor *et al*, 2012). Intriguingly, Brown and colleagues have recently provided a first line of evidence showing that deficits in executive functions and working memory are more pronounced in schizophrenic cases with a positive history of prenatal infection compared to schizophrenic cases without such a history(Brown *et al*, 2011b). Hence, prenatal immune challenge seems to be a contributing factor for schizophrenia-associated cognitive functions both in the human clinical conditions as well as in-vivo animal model systems.

Our study further revealed a maturational correlation between multiple pre- and post-synaptic GABAergic deficits in the mPFC and working memory disruption, both of which appeared to be more pronounced in adult as compared to peri-pubertal offspring born to immune-challenged mothers. This association may not be unprecedented because the integrity of prefrontal GABAergic signalling is pivotal for normal working memory functions (Lewis *et al*, 2005). The age-dependent changes at the molecular and cognitive levels further highlight that the present prenatal immune activation model captures developmental aspects of abnormal brain structure and functions. This feature is particularly relevant to the neurodevelopmental perspective of schizophrenia, because the disorder's pathophysiological and neuropathological mechanisms are assumed to be progressive in nature (Insel, 2010). However, we acknowledge that our study fails short in dissecting the relative contribution of specific GABAergic abnormalities to the emergence of working memory impairments. Indeed, even though the temporal association between the prenatal infection-induced working impairments and multiple GABAergic changes is intriguing, we did not further attempt to delineate the functional contribution of GABAergic abnormalities to the induction of cognitive impairments. Additional work will be needed to further address this issue using pharmacological and/or genetic approaches, which could serve to mitigate the working memory deficiency by targeting distinct GABAergic abnormalities.

Another limitation of our study is that we did not confirm all of the identified gene expression changes to the protein level because of the limited availability of mPFC tissue. However, the successful verification of the effects of prenatal immune activation on $GAD_{65/67}$ and VGAT using Western blot protein analyses suggests that at least some of the identified GABAergic gene transcription changes are readily translatable to changes at the protein level. Furthermore, our study does not provide information about up-

stream molecular mechanisms modifying GABAergic gene transcription in offspring subjected to prenatal immune activation. For example, it remains uncharted whether the identified reductions in GAD_{65} and GAD_{67} protein and mRNA levels could be accounted for by reduced numbers of functionally intact interneurons and/or by altered transcriptional activity of the corresponding enzymes. On speculative grounds, one feasible and testable possibility would be that prenatal immune activation could induce epigenetic changes in the central GABA system, which is known to be highly accessible to epigenetic modifications (Guidotti *et al*, 2011).

In conclusion, our experimental data suggests that prenatal immune activation induces maturation-dependent alterations in the prefrontal GABAergic transcriptome. The importance of GABAergic changes present in schizophrenia and related disorders, together with our experimental data here, highlights the critical impact of prenatal immune-related insults on long-term GABAergic changes relevant to neuropsychiatric disorders with prenatal infectious aetiologies. It is also highly interesting to note that a variety of pre- or perinatal manipulations that negatively affect early brain development are capable of inducing similar long-term GABAergic pathology, including lesions of the neonatal ventral hippocampus (Tseng *et al*, 2008) and prenatal exposure to the antimitotic agent methylazoxymethanol acetate (MAM)(Penschuck *et al*, 2006). Hence, alterations in the adult central GABA system may represent a critical pathological convergence point for various early-life adversities.

4.2 Prenatal versus Postnatal Maternal Factors in the Development of Infection-Induced Working Memory Impairments in Mice

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Prenatal exposure to maternal infection represents a risk factor for the development of several neuropsychiatric disorders with a neurodevelopmental origin, most notably schizophrenia and autism (Brown, 2011). A number of translational rodent models support this epidemiological association by demonstrating multiple disease-relevant behavioural, cognitive and neuroanatomical alterations following prenatal exposure to infectious pathogens or immune-activating agents (reviewed in (Meyer *et al*, 2009b)). Importantly, such translational research has been extended successfully to species with more advanced prenatal development, including rhesus monkeys (Short *et al*, 2010; Willette *et al*, 2011) and spiny mice (Ratnayake *et al*, 2012), which further helps to verify the relevance of animal model findings to the human condition. According to a prevalent hypothesis, the maternal cytokine-associated response (Gilmore *et al*, 1997; Meyer *et al*, 2008a; Smith *et al*, 2007), together with downstream pathophysiological processes such as oxidative stress (Lante *et al*, 2008), hypoferremia (Aguilar-Valles *et al*, 2010), and zinc deficiency (Coyle *et al*, 2009), are critical in mediating the negative influences of prenatal infection on brain and behavioural development.

It has been widely acknowledged that the long-term brain dysfunctions manifest in prenatally immune-challenged offspring may stem from alterations in early prenatal brain development (Garbett *et al*, 2012; Meyer *et al*, 2008d; Stolp *et al*, 2011; Vuillermot *et al*, 2010). In contrast, the role of the postpartum milieu, in which offspring of infected mothers are raised, has received somewhat less attention. This seems surprising because disruption of the intricate mother-infant relationship resulting from immune activation in late pregnancy has been shown to confer additional risk for the offspring to develop brain pathology in later life (Schwendener *et al*, 2009). We have previously shown in a mouse model of maternal viral-like immune activation that being reared by a gestationally immune-challenged surrogate mother is sufficient to increase the fostered offspring's sensitivity to psychostimulant drugs (Meyer *et al*, 2008c) and to impair conditioned learning (Schwendener *et al*, 2009). These findings are congruent with the notion that exposure to reduced parental care in the course of postnatal brain maturation can exert a negative impact on behavioural functions in later life (Kaffman and Meaney, 2007).

In the present study, we sought to delineate the relative prenatal and postnatal maternal contributions to working memory impairments emerging following prenatal immune activation. Working memory is a special short-term memory buffer used to

hold relevant information temporarily active in order to guide on-going behaviour (Baddeley, 2003), and its disruption is a cardinal cognitive symptom in neuropsychiatric disorders with prenatal infectious aetiologies, including schizophrenia (Goldman-Rakic, 1994) and autism (Hill, 2004). Offspring of gestationally immune-challenged mothers seem to be highly vulnerable to cognitive impairments in the form of working memory deficiency. For example, whereas numerous studies consistently documented working memory deficits following prenatal viral-like immune activation (Bitanihirwe *et al*, 2010b; Connor *et al*, 2012; Meyer *et al*, 2008a; Richetto *et al*, 2014; Vuillermot *et al*, 2012), the same manipulation does not appear to affect (spatial) reference memory or basic forms of classical fear conditioning, active avoidance learning, and left-right discrimination learning (Meyer *et al*, 2006b; Meyer *et al*, 2006c; Zuckerman and Weiner, 2005). In addition to its negative influence on working memory, however, prenatally immune-challenged offspring also display abnormalities in selective or sustained attention (Meyer *et al*, 2005; Vuillermot *et al*, 2012; Zuckerman *et al*, 2003a; Zuckerman *et al*, 2005). Using mouse models of prenatal viral-like immune challenge, we have dissected the relative contribution of prenatal versus postnatal maternal factors to such attentional abnormalities before (Meyer *et al*, 2006c). In contrast, a delineation of the prenatal versus postnatal maternal factors in the development of working memory deficiency still waits verification.

Here, we used a well-characterized mouse model of prenatal immune challenge induced by maternal gestational treatment with the viral mimetic poly(I:C) (= polyriboinosinic-polyribocytidilic acid) (Meyer *et al*, 2009b). We further implemented a neonatal cross-fostering design in which offspring born to poly(I:C)- or vehicle-treated dams were simultaneously cross-fostered to surrogate rearing mothers that had either experienced the immunological or control manipulation during pregnancy. In addition to the assessment of working memory, we also included a test of amphetamine (AMPH) sensitivity. Our primary rationale for the AMPH sensitivity test was to include a positive control readout based on our previous findings showing that adoption by a gestationally immune-challenged surrogate mother is sufficient to increase AMPH-induced locomotor activity in the fostered offspring (Meyer *et al*, 2008c). A secondary rationale for the inclusion of this test was related to human studies showing that neuropsychiatric disorders with prenatal infections aetiologies such as schizophrenia are characterized

by elevated behavioural and neurochemical sensitivity to acute treatment with AMPH (Laruelle, 2000).

In addition the behavioural and cognitive testing, we performed a series of molecular investigations aiming to explore potential mechanisms underlying the anticipated prenatal and postnatal maternal effects on the offspring. More specifically, we measured the transcription levels of the two isoforms of the rate-limiting enzyme for γ-aminobutyric acid (GABA) biosynthesis, glutamic acid decarboxylase 65-kDa (GAD65) and 67-kDa (GAD67), in prefrontal and hippocampal areas of the fostered offspring. Converging evidence points to an important role of impaired presynaptic GABAergic mechanisms such as deficient GAD65 and GAD67 synthesis in the pathophysiology of neurodevelopmental brain disorders, including schizophrenia and autism (Blatt *et al*, 2011; Lewis *et al*, 2005). Furthermore, impaired GABAergic signalling in prefrontal and/or hippocampal areas is likely to contribute to working memory deficiency as seen in patients with schizophrenia (Lewis *et al*, 2005).

4.2.1 Infection-Induced Working Memory Impairments in the Matching-to-Position Paradigm Are Mediated by Prenatal but not Postnatal Maternal Effects

We first evaluated the impact of the prenatal and postnatal manipulations on spatial working memory in the dry maze matching-to-position paradigm. The critical measure of working memory in this task is the reduction in distance moved and time needed (latency) to find the location of the rewarded hole from trial one (when the location of the reward is unknown to the subjects) to trial two.

- **Pubescence**

As shown in **Fig. 8a**, offspring from all four experimental groups showed a noticeable reduction in the latency and distance moved in trial 2 relative to trial 1 of the working memory test, leading to a highly significant main effect of trials [latency: $F(1,53) = 30.27$, $p < 0.001$; distance: $F(1,53) = 24.01$, $p < 0.001$]. The analysis of latency further revealed a significant interaction between prenatal treatment and trials [$F(1,53) = 5.78$, $p < 0.05$]. This interaction appeared to arise because prenatally poly(I:C)-exposed offspring displayed a mild (and non-significant, $p = 0.0734$) reduction in the time needed to find the rewarded hole in trial 1 compared with prenatally vehicle-treated offspring, whereas the four groups exhibited highly similar latencies in trial 2 of the test (**Fig. 8a**). As a consequence, the improvement of latencies from trial 1 to 2 was

reduced in prenatally poly(I:C)-exposed offspring relative to offspring born to vehicle-treated mothers irrespective of the postnatal rearing conditions. This pattern of results was also mirrored in the analysis of the latency improvement scores yielding a significant main effect of prenatal treatment [$F(1,53) = 5.78$, $p < 0.05$]. The analysis of the distance improvement scores did not reveal any significant effects involving the between-subjects factors of prenatal treatment and postnatal rearing.

- **Adulthood**

Offspring born to vehicle-treated mothers displayed a marked improvement from trial 1 (when the location of the rewarded hole was unknown to the animals) to trial 2, indicating intact working memory in these animals (**Fig. 8b**). The postnatal rearing condition exerted no noticeable influence on working memory performance in prenatal control offspring. Hence, offspring born to gestationally vehicle-treated mothers displayed a highly significant improvement to find the rewarded hole in trial 2 relative to trial 1 regardless of whether they were raised by a poly(I:C)-exposed or vehicle-treated surrogate mother (**Fig. 8b**). In marked contrast, prenatal immune challenge markedly disrupted working memory: Prenatally poly(I:C)-exposed offspring displayed no reduction in the time spent or distance moved in trial 2 relative to trial 1. Notably, the disrupting effects of prenatal poly(I:C) exposure on working memory were not influenced by the postnatal rearing condition, so that prenatally poly(I:C)-exposed subjects displayed working memory impairments regardless of whether they were reared by a poly(I:C)-exposed or vehicle-exposed surrogate mother (**Fig. 8b**).

Statistical support for these impressions was obtained by the ANOVA of the latency and distance moved across the two trials and testing days, which yielded a significant interaction between prenatal treatment and trials [latency: $F(1,53) = 5.35$, $p < 0.05$; distance: $F(1,53) = 4.14$, $p < 0.05$]. Additional ANOVAs restricted to each prenatal treatment condition confirmed a significant effect of trials in prenatally vehicle-exposed offspring [latency: $F(1,31) = 8.17$, $p < 0.01$; distance: $F(1,31) = 12.24$, $P<0.01$], indicating intact working memory in these animals. In contrast, the main effect of trials was far from being significant in prenatal poly(I:C) offspring ($F < 1$), suggesting that the prenatal manipulation impaired working memory. These interpretations were further supported by the ANOVAs of the improvement scores revealing a significant main effect of prenatal treatment [latency: $F(1,53) = 5.35$, $p < 0.05$; distance: $F(1,53) = 4.14$, $p < 0.05$].

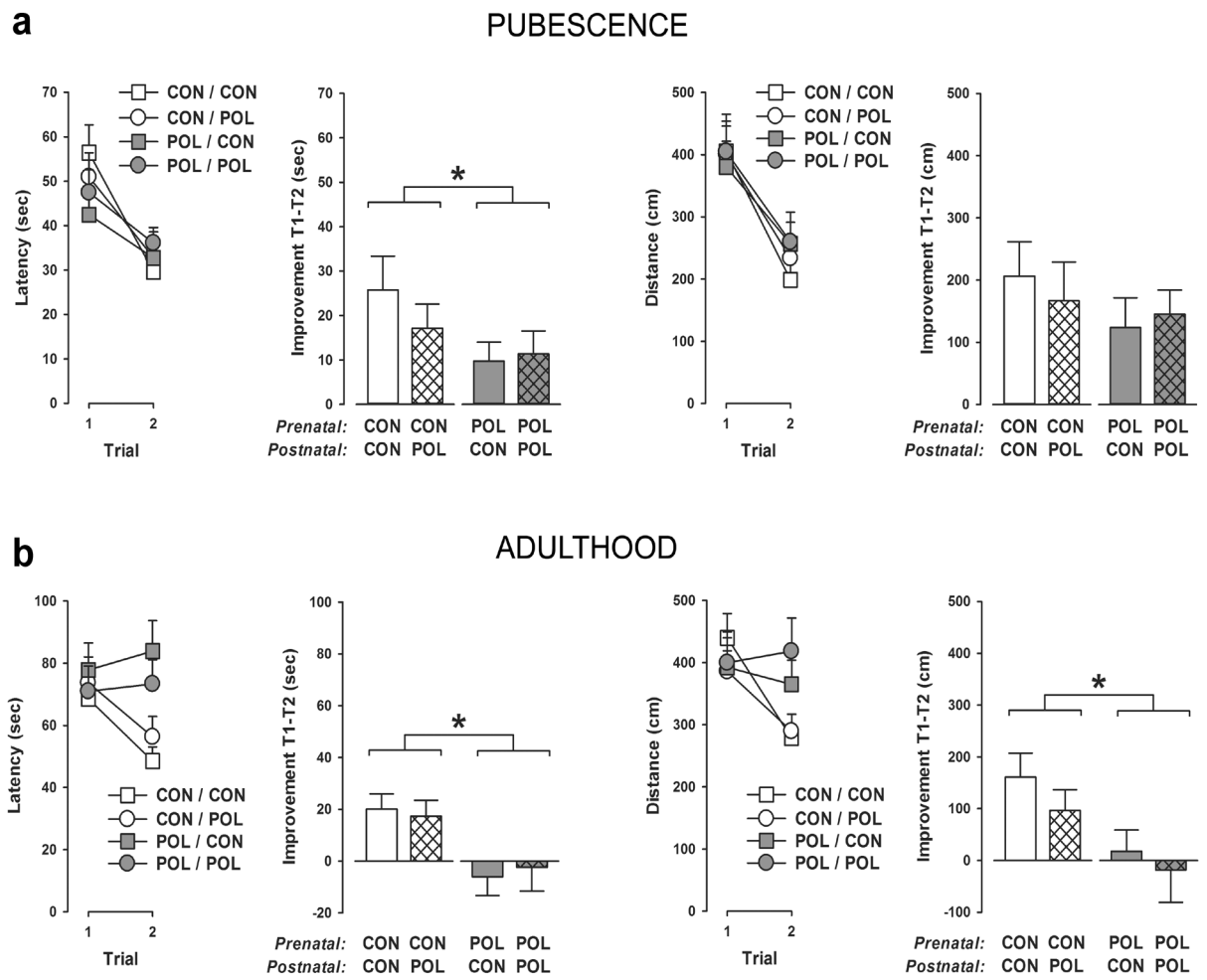

Figure 8. Effects of the prenatal and postnatal manipulations on spatial matching-to-position working memory in the dry maze paradigm. The line plots show the latency (sec) and distance moved (cm) to find the rewarded hole in trial 1 (T1) and T2, and the bar plots depicts the improvement in these measures from T1 to T2. (A) Working memory performance of fostered offspring at pubescent age. **P* < 0.05, reflecting the significant main effect of prenatal treatment. (B) Working memory performance of fostered offspring at adult age. **P* < 0.05, reflecting the significant main effect of prenatal treatment. All values are means±SEM. CON = vehicle control; POL = poly(I:C).

4.2.2 Infection-Induced Working Memory Impairments in the Spatial Novelty Preference Y-Maze Paradigm Are Mediated by Prenatal but not Postnatal Maternal Effects

In a next step, we explored the impact of the prenatal and postnatal manipulations on spatial working memory using a spatial novelty preference paradigm in the Y-maze.

The critical measure of spatial recognition memory in the Y-maze test is the relative time spent in the novel (previously unexplored) arm during the choice phase of the test.

- Pubescence

Pubescent offspring born to vehicle-treated mothers displayed a clear preference for the novel arm regardless of the postnatal rearing conditions (**Fig. 9a**). Hence, they performed markedly above the chance level of 33.3% (**Fig. 9a**). In contrast, pubescent offspring exposed to prenatal poly(I:C) exposure showed no obvious preference for the novel arm, indicating impaired spatial novelty preference in these animals (**Fig. 9a**). The prenatal poly(I:C)-induced impairment in spatial novelty preference emerged independently of the postnatal rearing conditions. Hence, prenatal immune activation led to significant Y-maze working memory impairment via prenatal but not postnatal maternal factors.

The interpretations were statistically supported by the presence of a significant main effect of prenatal treatment [$F(1,53) = 6.71$, $p < 0.05$] in the analysis of the novelty preference index (% time novel arm). No other main effects or interactions attained statistical significance. There were also no significant group differences with respect to the distance moved during the choice phase (**Fig. 9a**) or during the initial sample phase (data not shown). Hence, the prenatal poly(I:C)-induced working memory deficits observed in the spatial novelty preference Y-maze task were not confounded by possible differences in basal locomotor activity and maze exploration per se.

- Adulthood

Consistent with the outcomes in pubescent animals, adult offspring born to vehicle-treated mothers displayed a marked preference for the novel arm regardless of the postnatal rearing conditions (**Fig. 9b**). On the other hand, adult offspring subjected to prenatal poly(I:C) exposure showed no obvious preference for the novel arm, so that performance in these animals was only slightly above chance level and significantly different from prenatal control offspring (**Fig. 9b**). Again, these effects emerged independently of the postnatal rearing conditions, suggesting that the prenatal

poly(I:C)-induced impairments in spatial novelty preference are mediated by prenatal but not postnatal maternal effects on the offspring. The ANOVA of % time novel arm revealed a significant main effect of prenatal treatment [$F(1,53) = 7.11$, $p < 0.05$], while no other main effects or interactions attained statistical significance. There were also no significant group differences with respect to the distance moved during the choice phase (**Fig. 9b**), indicating that adult offspring born to poly(I:C)-treated mothers displayed a genuine impairment in spatial novelty preference.

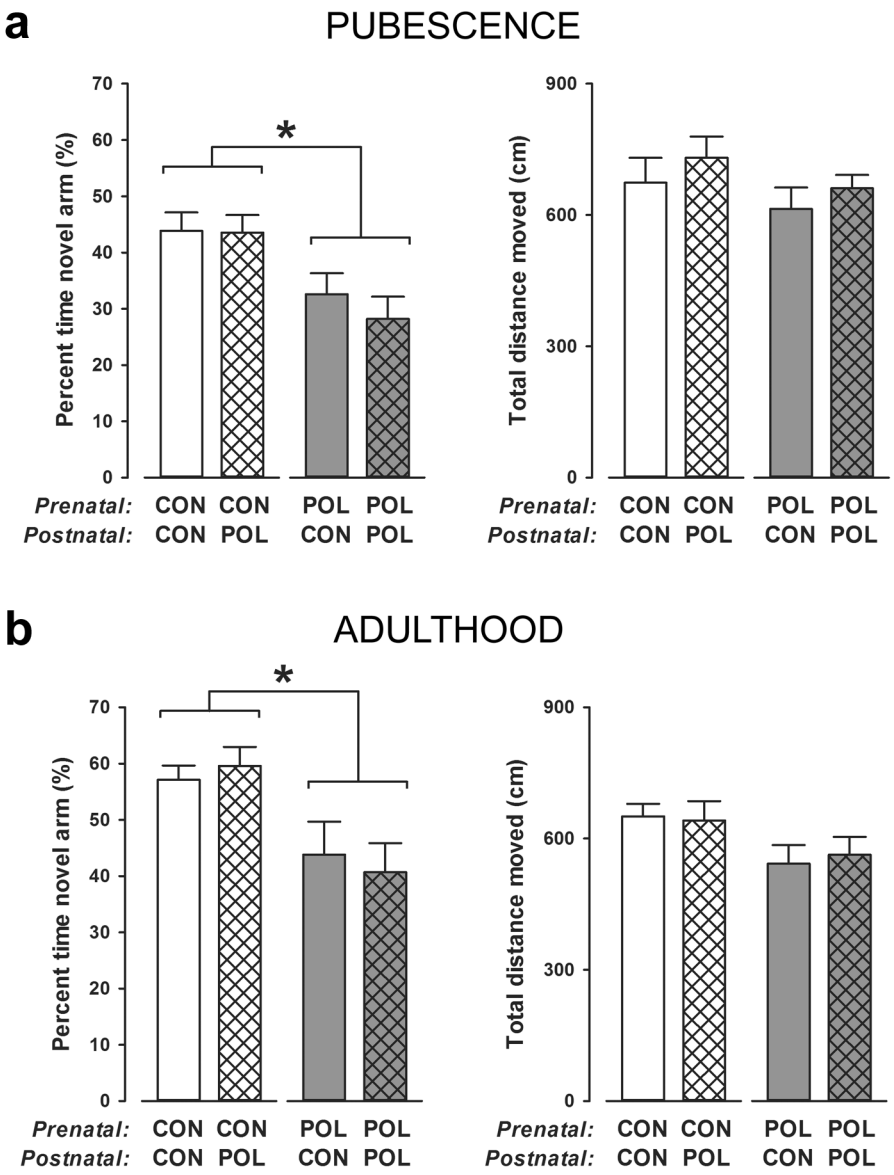

Figure 9. Effects of the prenatal and postnatal manipulations on spatial novelty preference in the Y-maze paradigm. The bar plots depicts the percent (%) time spent in the novel arm and total distance moved (cm) during the choice phase. **(A)** Spatial novelty preference in fostered offspring at pubescent age. **P* < 0.05, reflecting the significant main effect of prenatal treatment. **(B)** Spatial novelty preference in fostered offspring at adult age. **P* < 0.05, reflecting the significant main effect of prenatal treatment All values are means±SEM. CON = vehicle control; POL = poly(I:C).

4.2.3 Prenatal and Postnatal Maternal Factors Affect Amphetamine Sensitivity Following Prenatal Exposure to Infection

We also evaluated the effects of the prenatal and postnatal manipulations on the offspring's sensitivity to acute AMPH challenge. For this purpose, we exposed the animals to acute systemic AMPH (2.5 mg/kg, i.p.) following an initial habituation and vehicle (saline) administration phase and measured the animals' locomotor reaction to the drug challenge in a standard open field.

- **Pubescence**

As depicted in **Fig. 10a**, the prenatal and postnatal manipulations did not affect basal locomotor activity during the initial habituation or subsequent vehicle administration phase. Acute treatment with AMPH generally led to an increase in the locomotor activity, leading to a significant main effect of bins [$F(17,884) = 11.21$, $p < 0.001$]. The locomotor-enhancing effects of AMPH were significantly potentiated in pubescent offspring raised by gestationally infected mothers regardless of their prenatal immune treatment histories (**Fig. 10a**). The presence of this postnatal maternal effect was statistically supported by the significant main effect of postnatal rearing bins [$F(1,52) = 3.99$, $p < 0.05$] and its interaction with bins [$F(17,884) = 1.99$, $p < 0.01$] in the ANOVA of distance moved following AMPH administration. No other main effects or interactions attained statistical significance.

- **Adulthood**

Consistent with the results in pubescent animals, the prenatal and postnatal manipulations did not significantly influence basal locomotor activity during the initial habituation or subsequent vehicle administration phase (**Fig. 10b**). Administration of AMPH generally increased the animals' locomotor response, leading to a significant main effect of bins [$F(17,884) = 67.47$, $p < 0.001$]. Most interestingly, both the prenatal and postnatal manipulations affected the locomotor-enhancing effects of AMPH, as indicated by the significant interaction between prenatal treatment and postnatal rearing [$F(1,52) = 4.30$, $p < 0.05$] as well as by the three-way interaction between prenatal treatment, postnatal rearing, and bins [$F(17,884) = 2.31$, $p < 0.01$]. Subsequent post hoc comparisons confirmed that the locomotor-enhancing of AMPH were significantly ($p< 0.01$) increased in adult offspring prenatally exposed to poly(I:C) (i.e., in

POL-CON and POL-POL animals) and in offspring that were raised by gestationally poly(I:C)-treated mothers (i.e., in CON-POL animals) (**Fig. 10b**).

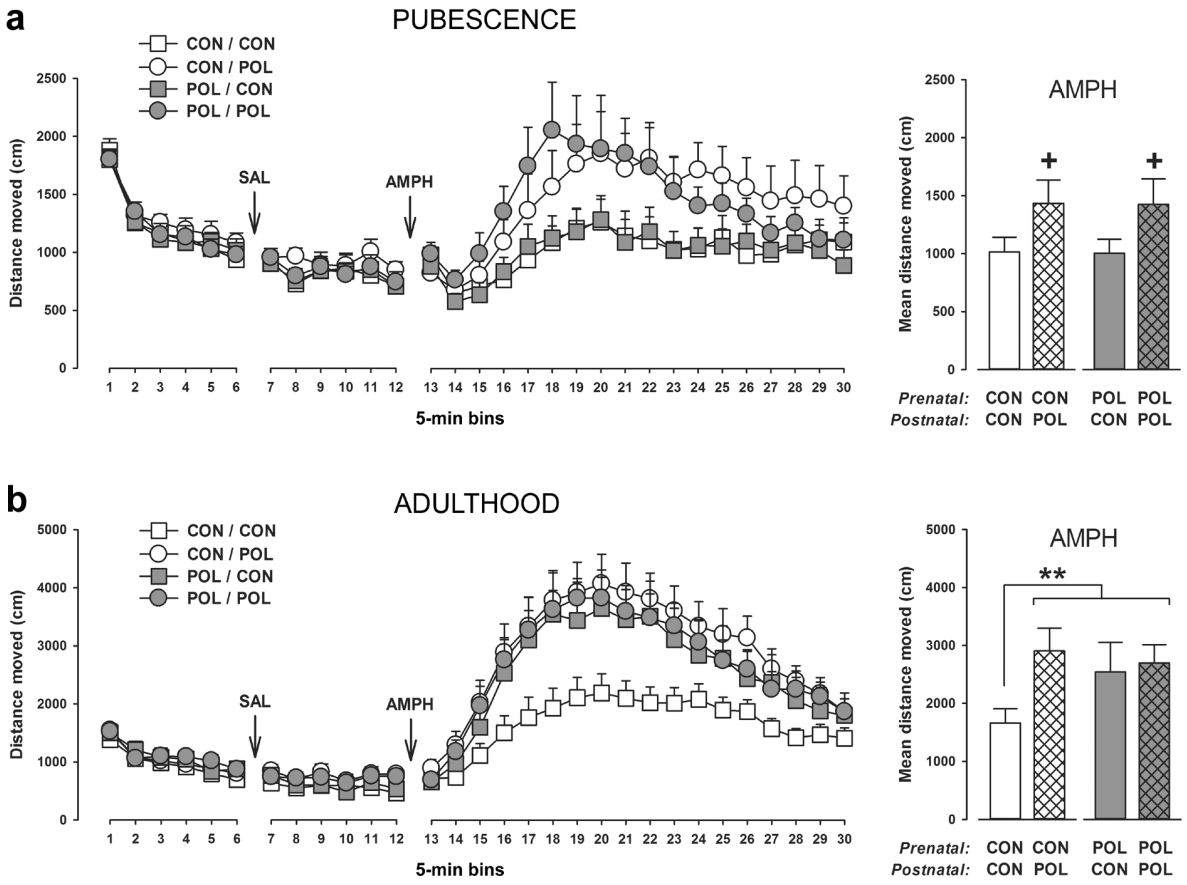

Figure 10. Effects of the prenatal and postnatal manipulations on the locomotor response to acute amphetamine treatment. The line plots depict the distance moved in the open field arena as a function of 5-min bins during the initial habituation phase and following subsequent saline (SAL) or amphetamine (AMPH) administration. The bar plots depict the mean distance moved after the acute AMPH challenge. (A) Locomotor activities in fostered offspring at pubescent age. $^{+}P < 0.05$, reflecting the significant main effect of postnatal rearing. (B) Locomotor activities in fostered offspring at adult age. $^{**}P < 0.01$, reflecting the significant increase in CON-POL, POL-CON, and POL-POL offspring relative to CON-CON offspring based on post hoc comparisons. All values are means±SEM. CON = vehicle control; POL = poly(I:C).

4.2.4 Prenatal and Postnatal Maternal Effects Influence GAD_{65} and GAD_{67} Gene Expression Depending on the Offspring's Age

The transcription levels of GAD_{65} and GAD_{67} were measured in the prefrontal (mPFC) and hippocampal (dHPC) areas of the fostered offspring using RT-PCR.

- **Pubescence**

The gene transcription levels of GAD_{65} and GAD_{67} in the mPFC and dHPC of pubescent offspring were affected by both the prenatal and postnatal manipulations. As depicted in **Fig. 11a**, being raised by a gestationally poly(I:C)-treated surrogate mother significantly increased GAD_{65} mRNA levels in the dHPC (main effect of postnatal rearing: $F(1,30) = 5.39$, $p < 0.05$), whereas prenatal poly(I:C) exposure significantly elevated GAD_{67} mRNA levels in the dHPC independently of the postnatal rearing condition (main effect of prenatal treatment: $F(1,30) = 5.12$, $p < 0.05$). In addition, offspring that were exposed to prenatal control treatment but raised by gestationally poly(I:C)-exposed surrogate mothers displayed a reduction in mPFC GAD_{65} mRNA levels relative to offspring born to and raised by vehicle-treated mothers, and relative to offspring born to and raised by poly(I:C)-exposed mothers (**Fig. 11a**). No significant changes were detected in the analysis of mPFC GAD_{67} mRNA levels (**Fig. 11a**).

- **Adulthood**

At adult age, the levels of GAD_{65} and GAD_{67} mRNA in the dHPC were significantly decreased in prenatally poly(I:C)-exposed offspring regardless of whether they were raised by control or immune-challenged surrogate mothers (main effect of prenatal treatment for GAD_{65}: $F(1,38) = 13.53$, $p < 0.001$; main effect of prenatal treatment for GAD_{67}: $F(1,38) = 13.82$, $p < 0.001$; **Fig. 11b**). Likewise, the levels of GAD_{67} mRNA in the mPFC were significantly reduced by prenatal immune activation regardless of the postnatal rearing condition (main effect of prenatal treatment: $F(1,38) = 20.11$, $p < 0.001$). On the other hand, being raised by a gestationally poly(I:C)-exposed surrogate mother caused a significant reduction in the mPFC mRNA levels of GAD_{65} (main effect of postnatal rearing: $F(1,38) = 4.19$, $p < 0.05$).

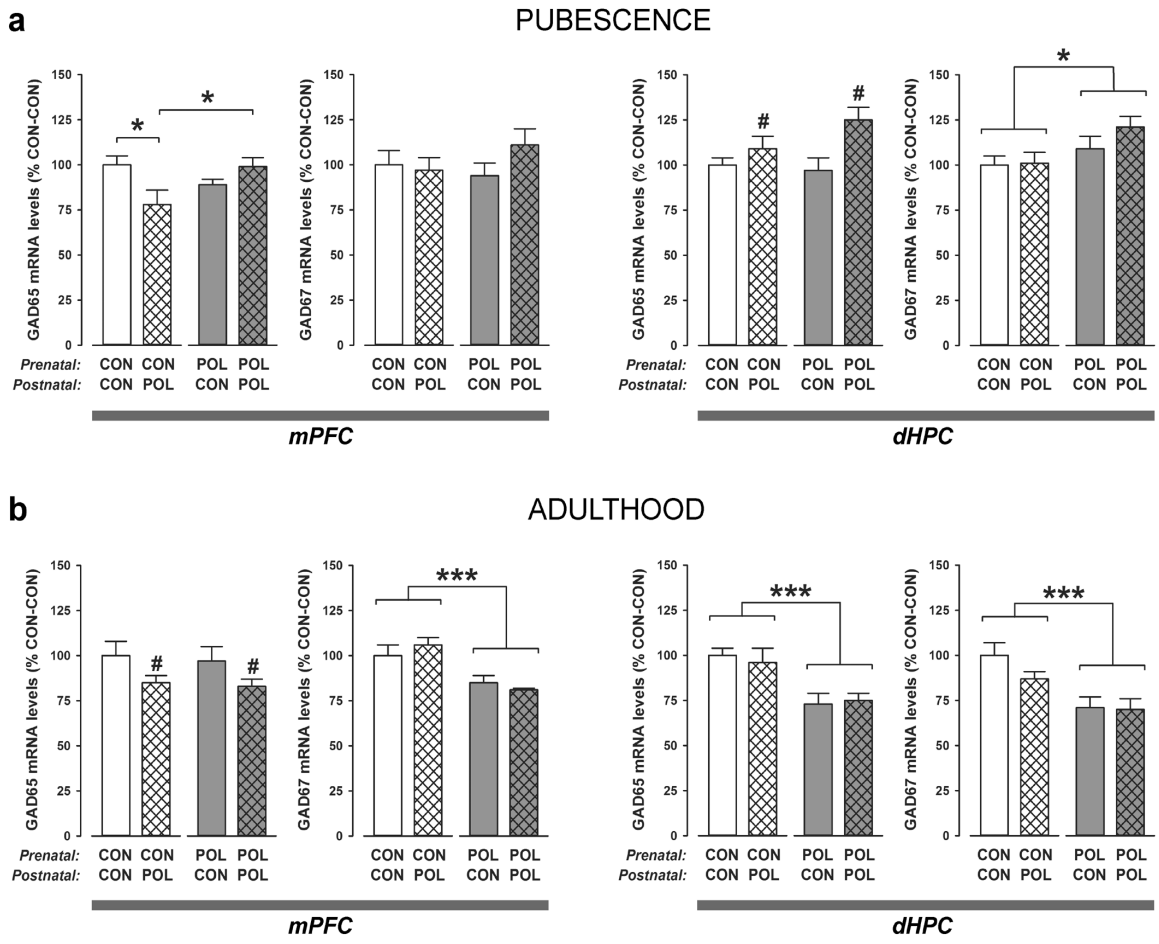

Figure 11. Effects of the prenatal and postnatal manipulations on GAD_{65} and GAD_{67} gene expression. The graphs depict the levels of normalized mRNA expression in the medial prefrontal cortex (mPFC) and dorsal hippocampus (dHPC) assessed using quantitative RT-PCR. (A) GAD_{65} and GAD_{67} gene expression in fostered offspring at pubescent age. $^{\#}P < 0.05$, reflecting the significant main effect of postnatal rearing; $^{*}P < 0.05$. (B) GAD_{65} and GAD_{67} gene expression in fostered offspring at adult age. $^{\#}P < 0.05$, reflecting the significant main effect of postnatal rearing; $^{***}P < 0.001$. All values are means±SEM. CON = vehicle control; POL = poly(I:C).

4.2.5 Discussion

The present study confirms that prenatal immune activation in mice induces long-lasting impairments in working memory (Bitanihirwe *et al*, 2010b; Connor *et al*, 2012; Meyer *et al*, 2008a; Richetto *et al*, 2014; Vuillermot *et al*, 2012). Here, we went on to show for the first time that such cognitive abnormalities are mediated by prenatal but not postnatal maternal factors on the offspring. Indeed, adult offspring prenatally exposed to poly(I:C) displayed significant impairments in both the matching-to-position dry maze paradigm and the Y-maze spatial novelty preference task regardless of whether they were raised by gestationally immune-challenged or non-challenged control surrogate mothers. Another implication of these manifestations is that the adoption of prenatally immune challenged neonates by control rearing mothers does not rescue the later appearance of cognitive deficits. Together, these findings are supportive of the hypothesis that prenatal immune challenge precipitates long-term cognitive abnormalities by directly interfering with fetal brain development (Garbett *et al*, 2012; Meyer *et al*, 2008d; Stolp *et al*, 2011; Vuillermot *et al*, 2010).

We further identified some forms of cognitive abnormalities in pubescent offspring born to poly(I:C)-exposed mothers. At this earlier stage of maturation, the poly(I:C)-induced cognitive impairments were mostly evident in the Y-maze test of spatial novelty preference, in which pubescent offspring that were prenatally exposed to poly(I:C) did not show the typical preference towards the novel arm (Fig. 2A). In contrast, working memory performance in the dry maze matching-to-position test was largely intact in pubescent offspring born to poly(I:C)-exposed mothers (Fig. 1A). This is consistent with our recent longitudinal study in mice showing that prenatal poly(I:C)-induced immune challenge leads to a post-pubertal onset of spatial working memory deficits in an identical dry maze matching-to-position test (Richetto *et al*, 2014).

The spatial novelty preference (Y-maze) and matching-to-position (dry maze) tests have typically been considered to involve elements of working memory, which in turn is often conceptualized as a special short-term memory buffer used to hold relevant information temporarily active in order to guide on-going behaviour (Baddeley, 2003). However, recent work performed by Sanderson and Bannerman (Sanderson and Bannerman, 2011, 2012; Sanderson *et al*, 2010) highlights that deficits in spatial novelty preference in the Y-maze task may reflect an inability to show stimulus-specific, short-term habituation to recently experienced stimuli rather than deficient working memory.

This novel theoretical account of the differential behavioural/cognitive processes involved in spatial novelty preference in the Y-maze on the one hand, and in spatial matching-to-position working memory tasks on the other hand, may provide a parsimonious explanation as to why pubescent offspring born to poly(I:C)-exposed mothers displayed significant deficits in the former but not the latter test.

The neuronal processes underlying the emergence of these cognitive deficits remain to be fully elucidated. Consistent with our recent investigations (Richetto *et al*, 2014), we found that prenatal poly(I:C)-induced immune activation led to an age-dependent decrease in GAD_{65} and GAD_{67} expression in the mPFC and dHPC, two brain areas critically involved in (spatial) working memory (Baddeley, 2003; Lewis *et al*, 2005). With the exception of GAD_{65} expression in the mPFC, these presynaptic GABAergic deficits were mediated by prenatal but not postnatal maternal effects on the offspring. It is of further note that the prenatal poly(I:C)-induced decrease in GAD_{65} and GAD_{67} expression temporally coincided with the adult onset of working memory deficiency in the matching-to-position test. Converging evidence indicates that impaired presynaptic mechanisms such as reduced GAD_{67} transcription and deficient GABA-mediated inhibitory control of pyramidal cell activity may contribute to working memory deficiency as seen in patients with schizophrenia (Lewis *et al*, 2005). Our findings are congruent with this hypothesis, even though further studies will be needed to bolster this putative link with causal evidence.

Interestingly, we found that being reared by gestationally immune-challenged surrogate mothers was sufficient to increase GAD_{65} transcription in the fostered offspring at pubescent age. This postnatal maternal effect on the offspring is congruent with previous findings highlighting that immune activation in late pregnancy can confer additional risk for the offspring to develop brain pathology in later life (Meyer *et al*, 2008c; Meyer *et al*, 2006c; Schwendener *et al*, 2009). One limitation of the present study is that we did not directly ascertain the negative influences of late gestational poly(I:C) exposure on postpartum maternal behaviour. Based on our previous finings (Schwendener *et al*, 2009), however, we expected such changes in maternal behaviour to occur. Indeed, we have previously documented the negative effects of late gestational poly(I:C) treatment on postpartum maternal behaviour (especially in the domains of pup licking/grooming and nest building) using an identical prenatal immune activation protocol in C57BL/6 mice (Schwendener *et al*, 2009). We therefore deem it likely that

the observed postnatal maternal effects on the offspring are associated with alterations in postpartum maternal behaviour.

Consistent with previous findings (Meyer *et al*, 2008c), we found that adoption by gestationally immune-challenged surrogate mothers exert a significant influence on AMPH sensitivity in the offspring. Interestingly, these postnatally mediated effects on AMPH potentiation emerged already in pubescent offspring and persisted into adulthood. In addition, increased behavioural responsiveness to systemic AMPH also emerged as a consequence of prenatal exposure to immune challenge and was therefore also present in prenatally poly(I:C) offspring raised by control surrogate mothers. However, the prenatally mediated effect on AMPH sensitivity was only manifest once the offspring reached the adult stage of development, suggesting that they are dependent on post-pubertal maturational processes. It thus follows that both prenatal and postnatal maternal factors can affect the offspring's sensitivity to dopaminergic drug challenges following gestational immune challenge, but the onsets of these effects critically differ depending on whether the maternal mediating factor is acting prenatally or postnatally. Furthermore, the long-term consequences of the prenatal immunological insult and of being reared by immune challenged surrogate mothers on AMPH sensitivity may be mediated by different neuronal mechanisms. In the context of maternal immune activation, it has been speculated that reduced expression of the AMPA receptor subunit GluR1 in the nucleus accumbens shell might represent one mechanism by which cross-fostering to a gestationally immune-challenged surrogate mother can increase AMPH-induced locomotor activity (Meyer *et al*, 2008c). In contrast, the prenatally mediated effects on AMPH-induced hyperactivity may be more directly related to altered dopaminergic mechanisms such as increased presynaptic dopamine synthesis and enhanced signalling at postsynaptic dopamine receptors (Meyer *et al*, 2010a; Meyer *et al*, 2008c; Vuillermot *et al*, 2010).

In conclusion, the present study provides novel insight into the ontogeny of working memory impairments, changes in $GAD_{65/67}$ gene transcription, and altered AMPH sensitivity following late prenatal immune challenge in mice. Our data show that prenatal infection-induced deficits in matching-to-position working memory and spatial novelty recognition are mediated by prenatal but not postnatal maternal effects on the offspring. Even though the full-spectrum of such cognitive abnormalities emerge only in adulthood, specific forms of cognitive impairments are already noticeable at earlier

maturational time points. The pubescent onset of (attenuated) cognitive changes observed in our model system is strikingly similar to recent human studies suggesting that fetal exposure to serologically determined influenza infection leads to an early onset of cognitive impairments that are present before the onset of full-blown schizophrenic disease (Ellman *et al*, 2009). At the same time, our study adds further weight to the notion that being reared by a surrogate mother that experienced immune activation during late pregnancy may constitute a risk factor for specific behavioural and molecular abnormalities.

4.3 Behavioural Effects of the Benzodiazepine Positive Allosteric Modulator SH-053-2'F-S-CH_3 in an Immune-Mediated Neurodevelopmental Disruption Model

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The central γ-aminobutyric acid (GABA) system is strongly implicated in cognitive processes. Accumulating evidence suggests that GABAergic interneurons critically regulate neuronal oscillatory activity (Gonzalez-Burgos *et al*, 2012), which in turn is believed to serve various complex functions, including perception, cognition, and memory (Gonzalez-Burgos *et al*, 2011; Lewis *et al*, 2012). On these bases, various cognitive deficits found in psychiatric disorders with neurodevelopmental components may, at least in part, stem from a dysregulated inhibitory GABAergic interneuron network (Gonzalez-Burgos *et al*, 2011; Lewis *et al*, 2012; Volk and Lewis, 2013). Altered pre- and post-synaptic markers of cortical and hippocampal GABAergic neurotransmission are, in fact, among the most consistently observed abnormalities in developmental psychiatric disorders, most notably schizophrenia (Benes and Berretta, 2001; Volk *et al*, 2013). Post-mortem studies conducted in schizophrenic patients report reduced expression levels of specific GABAergic interneuron markers, including parvalbumin and somatostatin (Fung *et al*, 2010{Konradi, 2011 #527; Hashimoto *et al*, 2008a), along with deficient expression of various presynaptic regulators of GABA neurotransmission such as the 67 kDA isoform of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD_{67}) and the GABA transporter 1 (Hashimoto *et al*, 2008b). These changes are further accompanied by altered levels of $GABA_A$ receptor subunits, including increased α2 subunits and decreased α1, α4 and α5 subunits in cortical layers of patients with schizophrenia (Beneyto *et al*, 2011; Duncan *et al*, 2010; Hashimoto *et al*, 2008a; Hashimoto *et al*, 2008b).

In addition to their involvement in cognitive processes, GABA-mediated inhibitory networks are also believed to critically regulate subcortical dopaminergic functions. According to a recent hypothesis (Grace, 2012; Lodge and Grace, 2011), disinhibition of the (ventral) hippocampus resulting from intrahippocampal impairments in GABAergic signalling could lead to a pathological hyperactivity of the (ventral) hippocampus and subsequent increase in mesoaccumbal dopamine system function. Such hippocampal abnormalities and states of subcortical hyperdopaminergia are prominent features of schizophrenia and related psychotic disorders (Lodge *et al*, 2011; Zierhut *et al*, 2010).

Against these backgrounds, it has been proposed that pharmacological interventions targeting GABA abnormalities may prove useful in correcting both cognitive impairments and dopaminergic dysfunctions present in patients with

schizophrenia (Guidotti *et al*, 2005; Stan *et al*, 2012). A first line of evidence supporting this hypothesis was derived from a small placebo-controlled clinical trial suggesting that treatment with a benzodiazepine-like agent with preferential activity at the α2/α3 subunit of $GABA_A$ receptors can improve cognitive and electrophysiological measures of prefrontal functions in individuals with chronic schizophrenia (Lewis *et al*, 2008). Such pro-cognitive effects associated with positive allosteric modulation of α2/α3 subunit, however, could not be replicated in a larger randomized clinical trial (Buchanan *et al*, 2011). In contrast, a novel α5 $GABA_A$ receptor positive allosteric modulator has been shown to reverse hyperactivation of the dopamine system in the methylazoxymethanol acetate (MAM)-based neurodevelopmental disruption model of schizophrenia (Gill *et al*, 2011), indicating that such GABAergic modulation may be useful in targeting positive symptoms of schizophrenic disease.

In the present study, we used an established immune-mediated neurodevelopmental disruption model to test the behavioural effects of SH-053-2'F-S-CH_3, a relatively novel benzodiazepine positive allosteric modulator (PAM) with partial selectivity at the α2, α3, and α5 subunits (Fischer *et al*, 2010; Savic *et al*, 2010). The chosen model is based on prenatal administration of the viral mimetic poly(I:C) in mice, which is known to capture a wide spectrum of behavioural and cognitive abnormalities relevant to neurodevelopmental psychiatric disorders (reviewed in (Meyer, 2014; Meyer *et al*, 2010a)). The prenatal poly(I:C) model has been established in relation to epidemiological studies showing increased risk of schizophrenia and related disorders following prenatal maternal exposure to infection or inflammation (Brown *et al*, 2010). Importantly, prenatal poly(I:C) treatment in mice is capable of inducing a wide range of schizophrenia-relevant prefrontal and hippocampal GABAergic abnormalities in adult offspring, including reduced mRNA and/protein expression of GAD_{65}, GAD_{67}, and parvalbumin (Meyer *et al*, 2008b; Piontkewitz *et al*, 2012; Richetto *et al*, 2013a; Richetto *et al*, 2013b). Prenatally poly(I:C)-exposed mice also show diminished prefrontal expression of the α4 and α5 subunits of $GABA_A$ receptors (Richetto *et al*, 2014) and increased α2-$GABA_A$ receptor immunoreactivity at axon initial segments (Meyer *et al*, 2008c; Nyffeler *et al*, 2006) akin to post-mortem findings in schizophrenia (Beneyto *et al*, 2011; Hashimoto *et al*, 2008a; Volk *et al*, 2002). These GABAergic changes are further paralleled by schizophrenia-relevant behavioural and cognitive dysfunctions, including impaired working memory and cognitive flexibility, reduced social approach behaviour,

and increased amphetamine (AMPH) sensitivity (Bitanihirwe *et al*, 2010b; Connor *et al*, 2012; Meyer *et al*, 2005; Richetto *et al*, 2013a; Richetto *et al*, 2014; Zuckerman *et al*, 2003a). Based on these findings, we tested whether positive allosteric modulation of the α2, α3, and α5 $GABA_A$ receptor subunits by systemic SH-053-2'F-S-CH_3 treatment may mitigate working memory deficiency, social interaction deficits, and AMPH hypersensitivity in adult offspring prenatally exposed to poly(I:C).

4.3.1 Effects of Prenatal Immune Activation on the Gene Expression Profile of the $GABA_A$ Receptor $\alpha_{(1\text{-}5)}$ Subunits

We first determined the gene expression levels of the α1-5 subunits of the $GABA_A$ receptor in the mPFC, Str, dHPC, and vHPC of prenatally poly(I:C)-exposed and control offspring. As summarized in **Fig 12**, poly(I:C) offspring displayed a significant reduction in the expression levels of the α2 (-15%; $p < 0.01$, $t_{18} = 3.76$), α4 (-20%; $p < 0.01$, $t_{18} = 2.91$) and α5 (-15%; $p < 0.05$, $t_{18} = 2.73$) subunits in the mPFC compared to controls. Moreover, prenatal poly(I:C) treatment significantly increased the expression of α3 in the mPFC by 23% ($p < 0.05$, $t_{18} = 2.70$; **Fig 12**). Prenatal poly(I:C) exposure did not significantly affect the expression levels of the α1-5 subunits in the Str or dHPC (**Fig 12**). Poly(I:C) offspring displayed, however, a significant increase in the expression levels of the α1 (+20%; $p < 0.01$, $t_{18} = 2.93$) and α2 (+25%; $p < 0.01$, $t_{18} = 2.96$) subunits in the vHPC relative to controls (**Fig 12**).

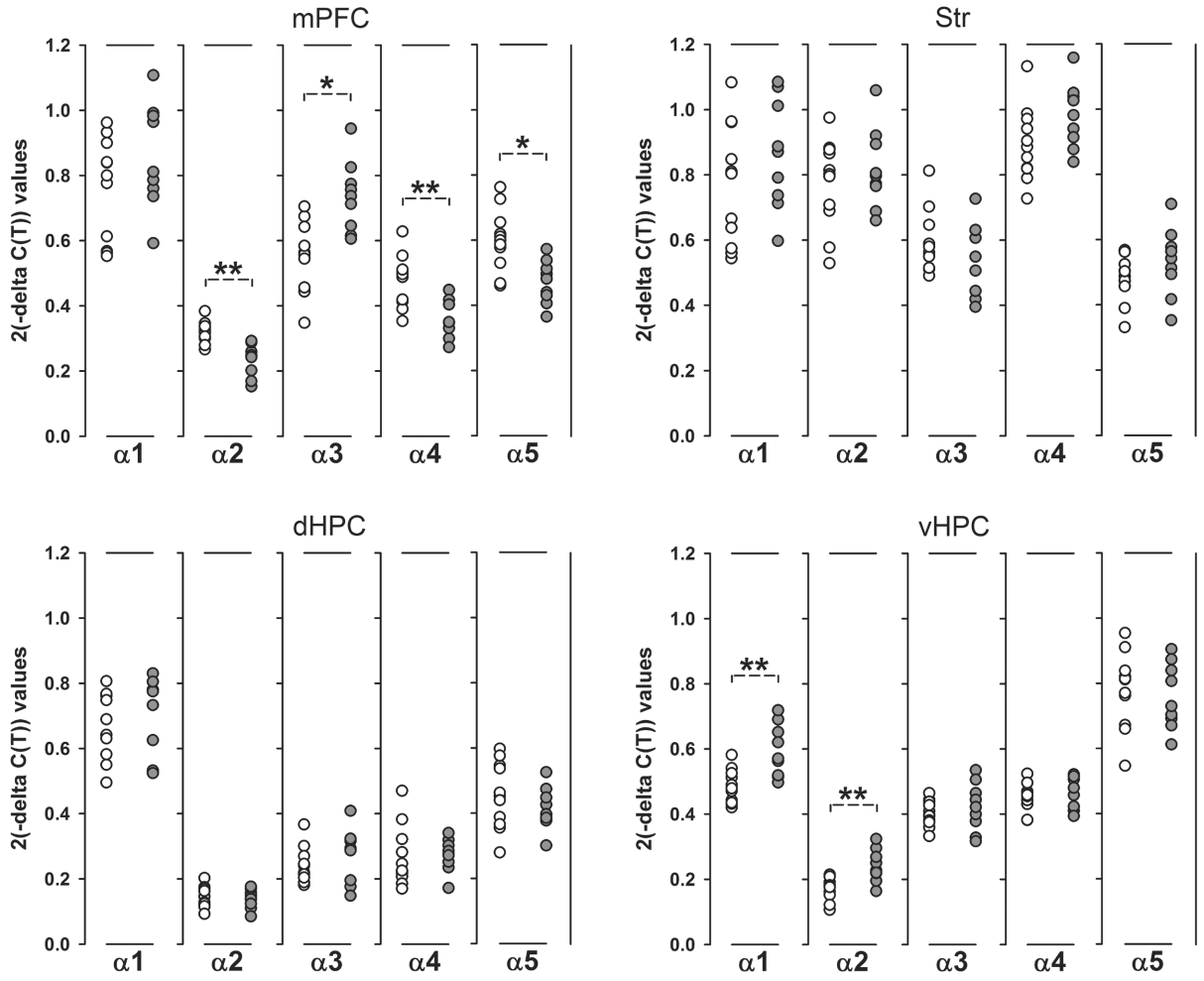

Figure 12. Prenatal immune activation alters the gene expression profile of the $GABA_A$ receptor $\alpha_{(1\text{-}5)}$ subunits in the medial prefrontal cortex and ventral hippocampus. The graphs depict the individual 2(-Delta C(T)) values for the medial prefrontal cortex (mPFC), striatum (Str), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC) of control (CON) and poly(I:C)-exposed (POL) offspring. *N*(CON) = 11 and *N*(POL) = 9 for each region and subunit. *$p < 0.05$ and **$p < 0.01$. All values are means ± SEM.

4.3.2 Effects of SH-053-2'F-S-CH_3 on Working Memory Deficits Induced by Prenatal Immune Activation

We evaluated possible pro-cognitive effects of SH-053-2'F-S-CH_3 using a spatial recognition working memory test in the Y-maze. The critical measure of spatial recognition memory is the relative time spent in the novel (previously unexplored) arm during the choice phase of this test. As expected, VEH-treated control offspring displayed a noticeable preference towards the novel arm, indicating intact working memory in these animals (**Fig 13**). Poly(I:C) offspring exhibited a significant reduction in this measure regardless of whether they were treated with VEH or SH-053-2'F-S-CH_3 (**Fig 13a**), suggesting that the PAM failed to restore working memory deficits in poly(I:C) offspring. Moreover, administration of the higher dose of SH-053-2'F-S-CH_3 (30 mg/kg) in prenatal control offspring also impaired working memory (**Fig 13a**), with both groups regressing to chance level of performance after treatment with the PAM. ANOVA of relative time spent in the novel arm revealed a significant main effect of prenatal treatment ($F(1,62) = 7.51$, $p < 0.01$), drug treatment ($F(2,62) = 14.45$, $p < 0.001$), and their interaction ($F(2,62) = 4.16$, $p < 0.05$). Subsequent *post-hoc* analysis confirmed the significant difference between VEH-treated control and poly(I:C) offspring ($p < 0.01$); between VEH-treated control offspring and poly(I:C) offspring treated with SH-053-2'F-S-CH_3 (*p's* < 0.001); and between VEH-treated control offspring and control offspring treated with SH-053-2'F-S-CH_3 at 30 mg/kg ($p < 0.001$) (see **Fig 13a**).

Prenatal poly(I:C) exposure did not significantly affect the distance moved during the Y-maze test (**Fig 13b**). Administration of SH-053-2'F-S-CH_3, however, led to an increase in the total distance moved independent of the prenatal treatment histories (**Fig 13b**), as supported by the significant main effect of drug treatment in the ANOVA of total distance moved ($F(2,62) = 5.67$, $p < 0.01$). Subsequent *post-hoc* tests verified the significant difference between animals treated with VEH and SH-053-2'F-S-CH_3 at 15 mg/kg ($p < 0.05$), as well as between animals treated with VEH and SH-053-2'F-S-CH_3 at 30 mg/kg ($p < 0.01$) (see **Fig 13b**).

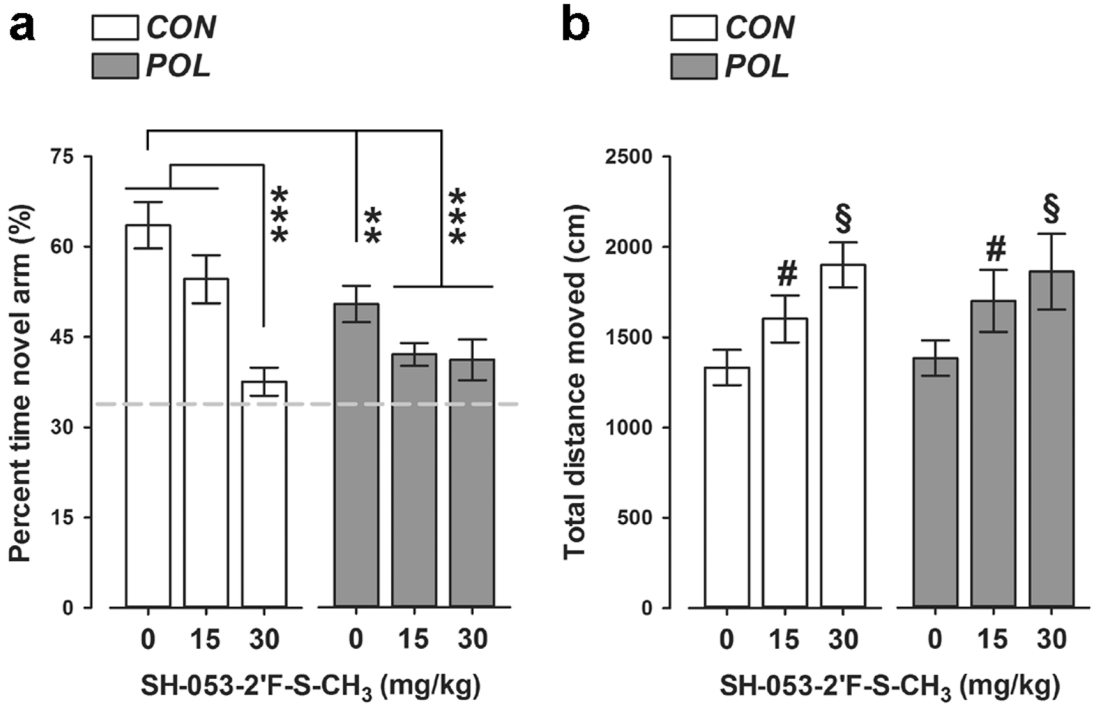

Figure 13. SH-053-2′F-S-CH_3 administration produces working memory deficits in the Y-maze spatial recognition paradigm in both control and poly(I:C) offspring. (a) The bar plot depicts the percent time spent in the novel (previously unexplored) arm during the choice phases of the test following vehicle (= 0 mg/kg SH-053-2′F-S-CH_3) or SH-053-2′F-S-CH_3 (at 15 or 30 mg/kg) treatment in control (CON) and poly(I:C) (POL) offspring. The dashed line represents the chance level. *p < 0.05, **p < 0.01 and ***p < 0.001, based on Scheffe's *post-hoc* tests. (b) The graph shows the total distance moved during the choice phases of the test. $^{\#}p$ < 0.01 and $^{\S}p$ < 0.001, reflecting the increase in distance moved displayed by animals treated with 15 mg/kg and 30 mg/kg SH-053-2′F-S-CH_3, respectively, relative to vehicle treatment (= 0 mg/kg SH-053-2′F-S-CH_3); p values are based on Scheffe's *post-hoc* tests. N(CON, 0 mg/kg) = 12, N(CON, 15 mg/kg) = 11, N(CON, 30 mg/kg) = 11, N(POL, 0 mg/kg) = 12, N(POL, 15 mg/kg) = 11, and N(POL, 30 mg/kg) = 11. All values are means ± SEM.

4.3.3 Effects of SH-053-2'F-S-CH_3 on Social Interaction and Recognition Deficits Induced by Prenatal Immune Activation

In a next step, we investigated whether SH-053-2'F-S-CH3 would be effective in normalizing social interaction deficits that are typically seen following prenatal immune challenge. The relative exploration time between an unfamiliar congenic mouse and an inanimate dummy object was used to assess social approach behaviour in the first phase of the social interaction test. As shown in **Fig 14a**, prenatal control offspring displayed a clear preference towards the unfamiliar live mouse regardless of whether they were treated with VEH or SH-053-2'F-S-CH3. Such social approach behaviour was significantly disrupted in poly(I:C) offspring independent of whether they received VEH or SH-053-2'F-S-CH3 treatment. Indeed, poly(I:C) offspring did not display a clear preference towards the unfamiliar live mouse such that the percent time spent with the live mouse was approximately at the 50%-chance level in these animals (see **Fig 14a**). ANOVA of the percent time spent with the live mouse revealed only a significant main effect of prenatal treatment ($F(1,62) = 7.05$, $p < 0.01$).

In the second phase of the test, the relative exploration time between the previously explored live mouse and a novel unfamiliar mouse was then used to assess social recognition memory. Both control and poly(I:C) offspring treated with VEH displayed a clear preference towards the novel unfamiliar mouse relative to the previously explored mouse, suggesting that prenatal poly(I:C) exposure did not affect social recognition memory (see **Fig 14b**). Administration of SH-053-2'F-S-CH3, however, significantly impaired social recognition memory regardless of the prenatal treatment histories (**Fig 14b**). Indeed, the percent time spent with the novel unfamiliar mouse decreased with increasing doses of SH-053-2'F-S-CH3, leading to a significant main effect of drug treatment in the ANOVA of this measure ($F(2,62) = 5.42$, $p < 0.01$). Subsequent post-hoc analyses confirmed the significant difference between animals treated with VEH and SH-053-2'F-S-CH3 at 15 mg/kg ($p < 0.05$), as well as between animals treated with VEH and SH-053-2'F-S-CH3 at 30 mg/kg ($p < 0.01$) (**Fig 14b**).

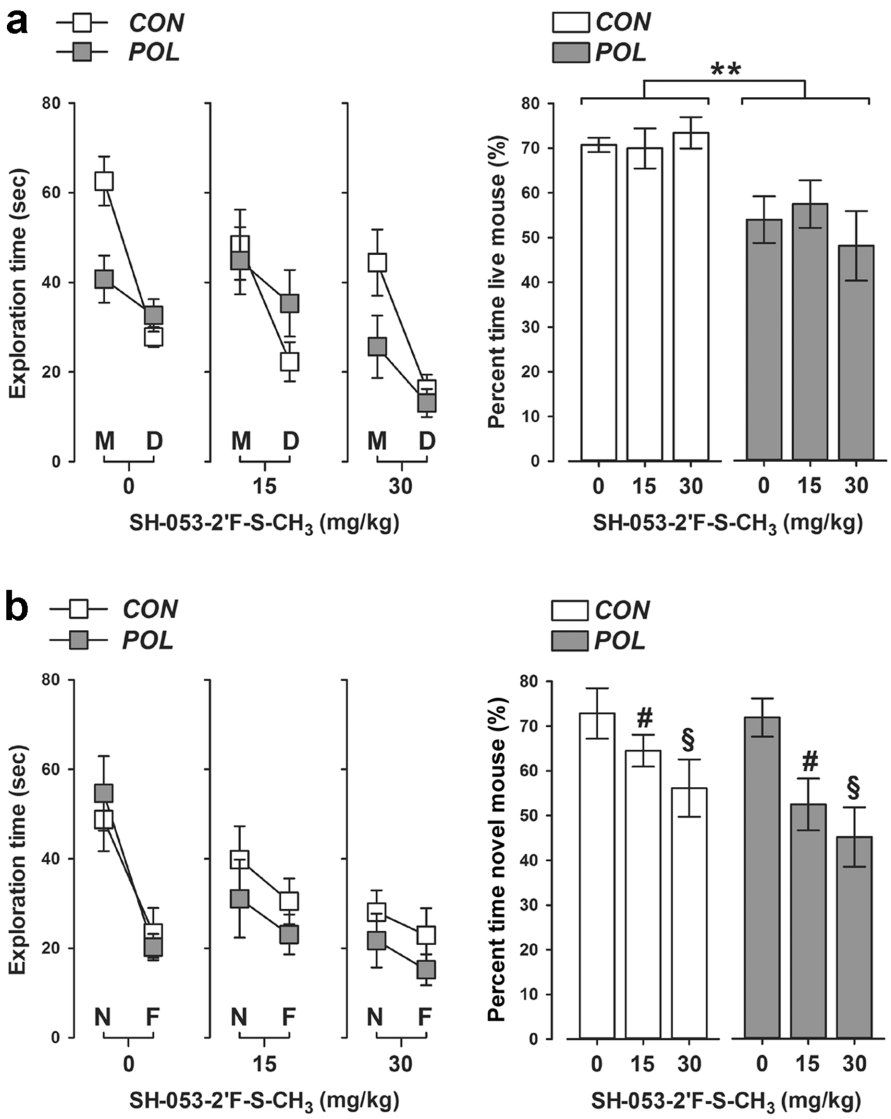

Figure 14. SH-053-2′F-S-CH$_3$ does not impair social approach behaviour but interferes with social recognition performance in both control and poly(I:C) offspring. (a) Behavioural outcomes in the social approach test ("dummy phase"). The line plot shows absolute exploration time of an unfamiliar live mouse (M) and inanimate dummy object (D) separately for control (CON) and poly(I:C) (POL) offspring treated with vehicle (= 0 mg/kg SH-053-2′F-S-CH$_3$) or SH-053-2′F-S-CH$_3$ (at 15 or 30 mg/kg). The bar plot depicts the percent time spent with the live mouse in the social approach test. $^{**}p < 0.01$, reflecting the significant main effect of prenatal poly(I:C) exposure. (b) Behavioural outcomes in the social recognition test ("novelty phase"). The line plot shows absolute exploration time of a novel live mouse (N) and the familiar live mouse (F) separately for CON and POL offspring treated with vehicle (= 0 mg/kg SH-053-2′F-S-CH$_3$) or SH-053-2′F-S-CH$_3$ (at 15 or 30 mg/kg). The bar plot depicts the percent time spent with the novel mouse in the social recognition test. $^{\S}p < 0.01$, reflecting the significant decrease displayed by animals treated with 30 mg/kg SH-053-2′F-S-CH$_3$ relative to vehicle-treated animals. N(CON, 0 mg/kg) = 11, N(CON, 15 mg/kg) = 12, N(CON, 30 mg/kg) = 11, N(POL, 0 mg/kg) = 11, N(POL, 15 mg/kg) = 11, and N(POL, 30 mg/kg) = 12. All values are means ± SEM, and all p values are based on Scheffe's *post-hoc* tests.

4.3.4 Effects of SH-053-2'F-S-CH_3 on AMPH Hypersensitivity Induced by Prenatal Immune Activation

Finally, we also explored whether SH-053-2'F-S-CH_3 may be effective in normalizing potentiated AMPH sensitivity typically emerging following prenatal immune activation. For this purpose, we assessed the effects of the PAM on AMPH-induced hyperactivity in the open field test.

Neither prenatal poly(I:C) exposure nor SH-053-2'F-S-CH_3 pretreatment significantly affected the total distance moved during the initial 30-min period of the open field test (**Fig 15**). Poly(I:C) and control offspring treated with the higher dose of SH-053-2'F-S-CH_3 (30 mg/kg) tended to show higher locomotor activity scores during this initial testing period, but this effect did not attain statistical significance (**Fig 15**). ANOVA of the total distance moved only revealed a significant main effect of bins ($F(5,310) = 9.04$, $p < 0.001$), reflecting the overall changes in locomotor activity resulting from habituation to the open field.

Subsequent administration of AMPH led to a general increase in the total distance moved, as supported by the main effect of bins ($F(11,682) = 77.78$, $p < 0.001$) in the AMPH phase of the test. VEH-treated poly(I:C) offspring showed a marked potentiation of the AMPH-induced hyperactivity response compared with the locomotor-enhancing effects of AMPH in VEH-treated control offspring (**Fig 15**). Most interestingly, SH-053-2'F-S-CH_3 pretreatment fully normalized the potentiation of the AMPH-induced hyperactivity response in poly(I:C) offspring without significantly affecting the locomotor-enhancing effects of AMPH exposure in control offspring (**Fig 15**). These impressions were supported by ANOVA revealing a significant interaction between prenatal treatment and drug treatment ($F(2,62) = 3.56$, $p < 0.05$), and a significant three-way interaction between prenatal treatment, drug treatment and bins ($F(22,682) = 1.60$, $p < 0.05$). Subsequent post-hoc analyses of the mean distance moved across the 60-min AMPH test period confirmed a significant difference between VEH-treated control and poly(I:C) offspring ($p < 0.05$), as well as between VEH-treated poly(I:C) offspring and poly(I:C) offspring treated with SH-053-2'F-S-CH_3 (p's < 0.05).

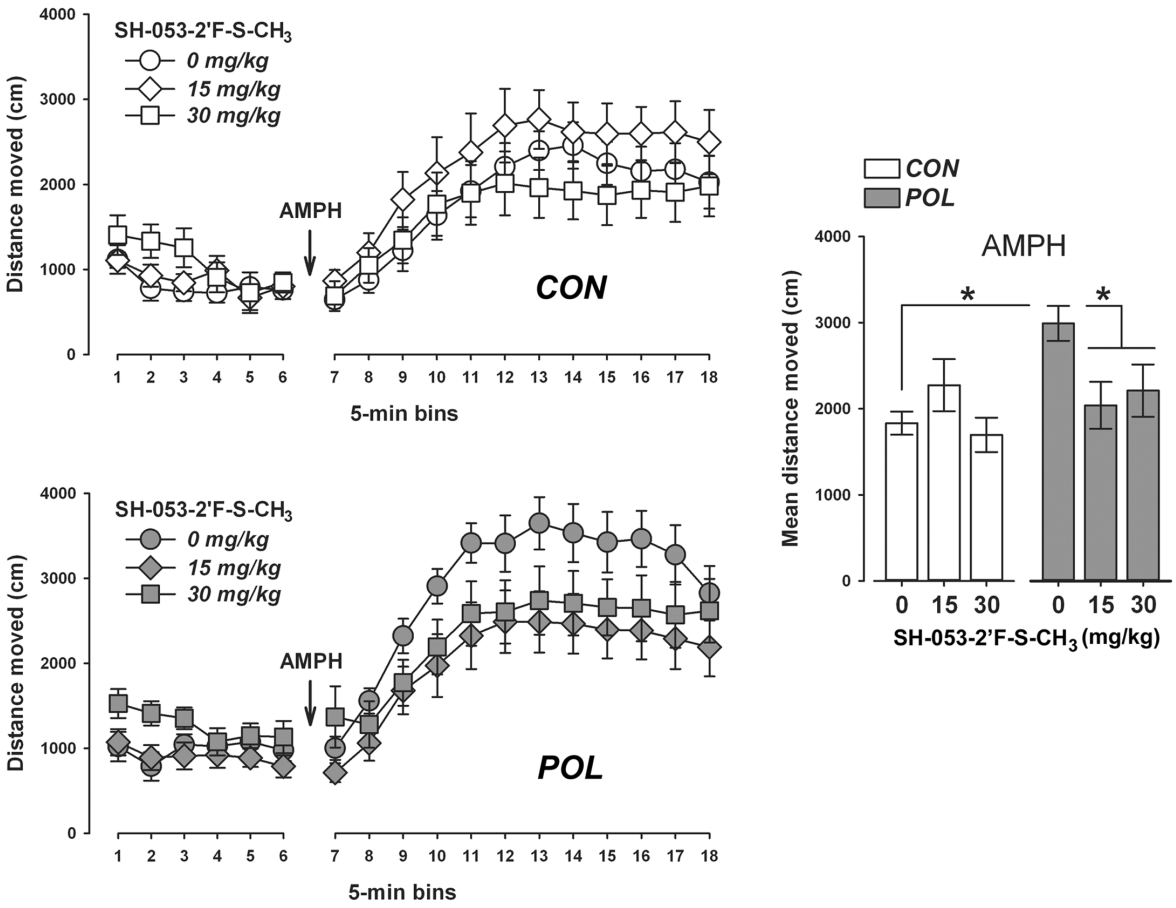

Figure 15. SH-053-2′F-S-CH_3 selectively reverses the potentiated amphetamine locomotor response in poly (I:C) offspring. The line plots show the distance moved in the open field as a function of 5-min bins during the initial habituation phase and the subsequent amphetamine (AMPH; 2.5 mg/kg, i.p.) exposure phase separately for control (CON) and poly(I:C) (POL) offspring treated with vehicle (= 0 mg/kg SH-053-2′F-S-CH_3) or SH-053-2′F-S-CH_3 (at 15 or 30 mg/kg). The bar plot depicts the mean distance moved during the AMPH exposure phase for all groups. *p < 0.05 and **p < 0.01, based on Scheffe's *post-hoc* analyses. N(CON, 0 mg/kg) = 11, N(CON, 15 mg/kg) = 11, N(CON, 30 mg/kg) = 12, N(POL, 0 mg/kg) = 11, N(POL, 15 mg/kg) = 12, and N(POL, 30 mg/kg) = 11. All values are means ± SEM.

4.3.5 Discussion

The present study confirms that prenatal immune activation by the viral mimic poly(I:C) alters GABAergic gene expression in the adult CNS (Richetto *et al*, 2013a; Richetto *et al*, 2014). Here, we replicated our initial findings of impaired α2, α4, and α5 gene expression in the mPFC of adult poly(I:C) offspring (Richetto *et al*, 2014). Similar reductions in cortical α4 and α5 gene expression have been found in schizophrenia and other neurodevelopmental disorders with prenatal infectious aetiologies, including autism (Beneyto *et al*, 2011; Blatt *et al*, 2011; Duncan *et al*, 2010). On the other hand, our cortical findings do not parallel the reports of decreased and increased α1 and α2 mRNA levels, respectively, in cortical areas of schizophrenia patients (Clancy *et al*, 2007; Hashimoto *et al*, 2008a). Adult poly(I:C) offspring, however, displayed a significant increase in α1 and α2 gene expression in the vHPC, the latter being consistent with previous immunohistochemical studies showing increased α2 protein expression in the vHPC of poly(I:C)-exposed offspring (Meyer *et al*, 2008b). Interestingly, the dHPC was largely spared by the prenatal manipulation with respect to $GABA_A$ receptor alterations, and similar region-specific effects have been reported for other GABAergic markers such as parvalbumin and reelin (Meyer *et al*, 2008b). It thus appears that the vHPC may be more susceptible to the disrupting effects of prenatal immune challenge compared to the dHPC. This notion fits well with accumulating evidence supporting a pivotal role of ventral hippocampal (GABAergic) abnormalities in developmental psychiatric disorders such as schizophrenia (Grace, 2010, 2012; Lodge *et al*, 2011; Steullet *et al*, 2010).

Based on the GABAergic effects reported here and previously (Meyer *et al*, 2008d; Richetto *et al*, 2014), we speculated that administration of SH-053-2'F-S-CH_3, a positive allosteric modulator with high affinity for the α2 and α5 (and to a lesser extent for α3) subunits of the $GABA_A$ receptor, may exert beneficial effects against prenatal infection-induced behavioural abnormalities. Consistent with previous studies (Bitanihirwe *et al*, 2010a; Bitanihirwe *et al*, 2010b; Connor *et al*, 2012; Richetto *et al*, 2013a; Richetto *et al*, 2014), poly(I:C) offspring were found to display impaired working memory in the Y-maze spatial recognition test and reduced social approach behaviour in the social interaction test. Furthermore, they exhibited increased sensitivity to the locomotor-enhancing effects of the indirect dopamine receptor agonist AMPH compared to control offspring, as reported before (Meyer *et al*, 2005; Meyer *et al*, 2008d; Zuckerman *et al*,

2003a). In contrast to our hypothesis, the PAM SH-053-2'F-S-CH_3 did not mitigate the poly(I:C)-induced working memory and social interaction deficits, two behavioural abnormalities commonly found in people with schizophrenia and related disorders (Foussias and Remington, 2010; Lewis and Moghaddam, 2006). In fact, SH-053-2'F-S-CH_3 administration to prenatal control offspring impaired performance in the Y-maze working memory and social interaction tests. In the latter, SH-053-2'F-S-CH_3 pretreatment led to a selective impairment in social recognition but not social approach behaviour, suggesting that the drug negatively affected short-term retention of social cues rather than social approach behaviour *per se*. Several previous studies have shown that prenatal poly(I:C)-induced cognitive deficits in rats and mice can be restored or even prevented by atypical antipsychotic drugs such as clozapine and risperidone (Meyer *et al*, 2010b; Ozawa *et al*, 2006; Piontkewitz *et al*, 2009; Zuckerman *et al*, 2003a). Therefore, one implication is that a normalization of prenatal poly(I:C)-induced spatial and social recognition deficits would require a modulation of neurotransmitter systems that go beyond those primarily mediated by $GABA_A$ receptors. However, one clear limitation of our study is that the assessment of cognitive functions was performed using a test that is primarily based on spontaneously motivated behaviour. Additional investigations are thus warranted to extend our findings to other cognitive domains.

The detrimental effects of SH-053-2'F-S-CH_3 on cognitive functions revealed here are in contrast to previous findings in rats suggesting that this PAM does not negatively affect spatial reference memory as assessed in the Morris water maze test (Savic *et al*, 2010). We have no parsimonious explanation for this discrepancy, but it could be related to differential cognitive processes involved (short-term working memory *versus* long-term reference memory) and/or potentially important species differences (mice *versus* rats). The amnesic effects of SH-053-2'F-S-CH_3 on short-term spatial and social recognition memory may also seem surprising in view of previous findings indicating that GABAergic agonists acting at the at the α2/α3 subunits induce pro-cognitive effects (Castner *et al*, 2010; Lewis *et al*, 2008). It needs to be pointed out, however, that the pro-cognitive effects of α2/α3 agonists remain controversial (Buchanan *et al*, 2011). Furthermore, accumulating evidence suggests that reduced and increased activity of the α5 subunit of the $GABA_A$ receptor facilitates and impairs cognitive functions, respectively (Collinson *et al*, 2002; Crestani *et al*, 2002; Dawson *et al*, 2006; Milic *et al*, 2013; Redrobe *et al*, 2012; Tan *et al*, 2011). Hence, the negative consequences of SH-

053-2′F-S-CH_3 on short-term memory revealed here may also be explained by positive modulation of the α5 subunit, given that the drug is characterized by strong α5 activity (Fischer *et al*, 2010; Savic *et al*, 2010). One clear possibility to further test this hypothesis would be to evaluate whether compounds with selective affinity for the α5 subunit would share such cognitive disruptive effects. Intriguingly, the R-isomer of the PAM used here (namely the SH-053-2′F-R-CH_3 isomer) shows such selectivity for the α5 subunit (Fischer *et al*, 2010; Gill *et al*, 2011; Savic *et al*, 2010), and therefore, it would provide a reasonable pharmacological tool to address these issues. In such attempts, it would be important to compare various dose ranges because the S-isomer (SH-053-2′F-S-CH_3) and R-isomer (SH-053-2′F-R-CH_3) differ in their affinity for α5 (Fischer *et al*, 2010; Gill *et al*, 2011; Savic *et al*, 2010). Hence, administration of the S-isomer (SH-053-2′F-S-CH_3) and R-isomer (SH-053-2′F-R-CH_3) at the same dose may differentially influence cognitive functions due to distinct α5 affinities.

Despite the inability of SH-053-2′F-S-CH_3 to correct prenatal infection-induced cognitive impairments, it was highly effective in mitigating AMPH hypersensitivity in adult poly(I:C) offspring without concomitant effects in prenatal control offspring. AMPH exposure can produce psychosis-like states in healthy human subjects and exacerbate existing psychoses in patients with schizophrenia (Laruelle, 2000). Moreover, potentiation of AMPH-induced dopamine release in schizophrenia patients tends to correlate with the severity of positive symptoms (Laruelle and Abi-Dargham, 1999). The efficacy of SH-053-2′F-S-CH_3 to correct the poly(I:C)-induced potentiation of AMPH sensitivity may thus be relevant especially for attempts to establish and validate GABA-based treatments targeting positive symptoms.

Our data are also highly congruent with previous findings showing that acute treatment with a selective α5 $GABA_A$-receptor PAM (namely the SH-053-2′F-R-CH_3 isomer) can fully reverse AMPH hypersensitivity and hyperactivity of ventral midbrain dopamine neurons in the MAM-based neurodevelopmental model of schizophrenia (Gill *et al*, 2011). The fact that prenatal MAM-induced AMPH hypersensitivity can be normalized by a selective α5 $GABA_A$-receptor PAM indicates that the beneficial effects of SH-053-2′F-S-CH_3 revealed here may also be largely attributable to the drug's activity at the α5 subunit. The congruent findings obtained in the prenatal poly(I:C) and MAM administration models emphasize the possibility that alterations in the adult central GABA system may represent a critical pathological convergence point for various

prenatal adversities implicated in the aetiology of neurodevelopmental brain abnormalities (Volk *et al*, 2013). These models also support the hypothesis that there is a causal link between altered signalling at α subunits of the $GABA_A$ receptor and the emergence of schizophrenia-relevant dysfunctions in neurodevelopmentally compromised offspring (Stan *et al*, 2012; Volk *et al*, 2013), at least with respect to AMPH hypersensitivity and related hyperdopaminergic functions. Consistent with this notion, reduced GABAergic signalling at the α5 subunit has previously been implicated in other dopamine-dependent behaviours: Genetically induced deficits in α5 expression impair prepulse inhibition (PPI) of the acoustic startle reflex (Hauser *et al*, 2005) and selective associative learning as assessed by the latent inhibition (LI) paradigm (Gerdjikov *et al*, 2008).

According to a prevalent hypothesis (Grace, 2012; Lodge *et al*, 2011), disinhibition of the ventral hippocampus resulting from intrahippocampal impairments in GABAergic signalling could lead to a pathological hyperactivity of the (ventral) hippocampus and subsequent increase in mesoaccumbal dopamine system function. It needs to be evaluated further whether or not similar mechanisms may underlie the prenatal poly(I:C)-induced AMPH hypersensitivity. Likewise, the mechanisms by which SH-053-2′F-S-CH_3 can ameliorate altered AMPH sensitivity in immune-exposed offspring still await exploration. Related to this, it is possible that behavioural functions critically regulated by ventral hippocampal activity could benefit from preferential α5 $GABA_A$-receptor PAMs, whereas such pharmacological compounds may lack therapeutic efficacy for cognitive functions that are more directly linked to neuronal activity in the prefrontal cortex, a site with lower α5 $GABA_A$ receptor expression. It also conceivable that region-specific increases in the expression of distinct GABAA receptor α subunits (e.g. increased prefrontal α3 or ventral hippocampal α2 expression) may occlude potential effects of SH-053-2′F-S-CH3, at least in offspring with immune-mediated neurodevelopmental abnormalities.

In conclusion, our study provides preclinical support for the use of benzodiazepine-positive allosteric modulators in the symptomatic treatment of AMPH hypersensitivity that emerges following (immune-mediated) neurodevelopmental disruption. Together with the recent findings obtained in the prenatal MAM administration model (Gill *et al*, 2011), our data suggest that positive allosteric modulation of the $GABA_A$ receptor α5 subunit may be particularly useful in mitigating

pathological overactivity of the dopaminergic system. At the same time, however, our study falls short in detecting possible pro-cognitive effects of PAM treatment with selective activity at the α2, α3, and α5 subunits of the $GABA_A$ receptor. The lack of such pro-cognitive effects may raise concerns regarding the effective use of some types of GABA subunit selective compounds with the aim to target multiple pathological domains that involve the co-existence of psychotic, social, and cognitive dysfunctions.

4.4 Prenatal Immune Activation Exacerbates Hippocampus-Regulated Cognitive Aging

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Prenatal exposure to infectious pathogens or inflammatory stimuli is increasingly recognized to play an important aetiological role in neurodevelopmental brain disorders, including schizophrenia (Brown *et al*, 2010), autism (Atladottir *et al*, 2010), and bipolar disorder (Canetta *et al*, 2014). A number of translational rodent models support such epidemiological associations by demonstrating multiple behavioural, cognitive, and neuroanatomical alterations following prenatal exposure to infectious or immune-activating agents (Harvey, 2012; Meyer, 2014; Meyer *et al*, 2010a). Many of the behavioural and neuronal changes induced by prenatal immune activation are dependent on maturational processes and thus only emerge once the offspring reach a certain age, typically adolescence or early adulthood (Piontkewitz *et al*, 2011a; Richetto *et al*, 2014; Vuillermot *et al*, 2010; Zuckerman *et al*, 2003a). The delayed onset of such prenatal infection-induced abnormalities suggests that the brain and behavioural consequences of this early-life insult are, at least in part, progressive in nature.

Whilst the importance of immune-related prenatal adversities has been widely acknowledged in the fields of developmental neuropsychiatry (Bale *et al*, 2010; Brown *et al*, 2010; Meyer *et al*, 2010a), less attention has been given to the possibility that such early-life environmental insults could also negatively influence brain aging (Krstic *et al*, 2012). Such early-life programming of altered brain aging would indeed seem possible in view of the progressive nature of neuronal dysfunctions associated with prenatal exposure to immune challenge (Piontkewitz *et al*, 2011a; Richetto *et al*, 2014; Vuillermot *et al*, 2010). Against these backgrounds, we tested the hypothesis that prenatal immune challenge may predispose the offspring to an exacerbation of behavioural and cognitive abnormalities during aging.

For this purpose, we used a well-established mouse model of maternal gestational exposure to the viral mimetic poly(I:C) (= *polyriboinosinic-polyribocytidilic acid*), which has been frequently used to stimulate a cytokine-associated inflammatory response in maternal and fetal units (Meyer, 2014). Using this model, we compared cognitive functions between prenatally immune-challenged and control offspring from the early pubescent to the aged stage of life. A special emphasis was placed on distinct forms of learning and memory that are known to be critically regulated by the hippocampal formation. Structural and functional abnormalities in hippocampal areas have been repeatedly associated with aging-related neurological disorders that are characterized by cognitive decline (Driscoll and Sutherland, 2005; Lister and Barnes, 2009; Small *et al*,

2011). Hippocampal deficits have also been noted in pubescent and/or adult animals born to immune-challenged mothers (reviewed in (Boksa, 2010; Meyer *et al*, 2009b)), emphasizing that the hippocampus is sensitive to immune-related prenatal adversities.
In addition to the cognitive assessment, we measured pre- and postsynaptic proteins and neuroplasticity-related genes to identify possible neuronal correlates of cognitive aging in immune-challenged and control offspring. Based on the idea that prenatal immune activation may exacerbate neuronal aging by inducing persistent systemic inflammation and accompanied neuroinflammatory reactions (Krstic and Knuesel, 2013; Krstic *et al*, 2012), we also compared peripheral and central cytokine levels as well as microglia and astroctye cell populations between prenatally immune-exposed and control offspring at different ages of life.

4.4.1 Prenatal Immune Activation Induces a Pubescent Onset of Spatial Short-Term Memory Impairments

First, we compared short-term spatial recognition memory in pubescent, adult and aged mice that were born to immune-challenged or control mothers. Spatial recognition memory is critically dependent on the hippocampus, as confirmed by lesion studies in rats and mice (Bannerman *et al*, 1999; Deacon *et al*, 2002a). Consistent with previous findings (Bitanihirwe *et al*, 2010b; Richetto *et al*, 2013a), pubescent and adult control mice showed a clear preference towards the novel arm during the choice phase of Y-maze test (**Fig. 16a**), suggesting that these animals displayed robust short-term spatial recognition memory. Such short-term memory declined as a function of normal aging in animals born to control mothers, so that aged control offspring showed a marked (~ 40%) reduction in the percent time spent in the novel arm compared with pubescent or adult control offspring (**Fig. 16a**). Significant impairments in short-term spatial recognition memory were also evident in mice born to poly(I:C)-exposed mothers. Most interestingly, these poly(I:C)-induced deficits emerged already at pubescent age and persisted into the aged stage of life (**Fig. 16a**). These data thus show that prenatal immune activation causes an early pubescent onset of spatial short-term memory impairment, which in control offspring typically emerges as a result of aging.

General locomotor activity indexed by the distance moved during the choice phase of the Y-maze test was not affected by prenatal immune activation or aging (**Fig. 16b**). The deficits in short-term spatial recognition memory emerging in immune-exposed

offspring or aged control mice thus seem to represent genuine cognitive effects rather than alterations in exploratory behaviour per se.

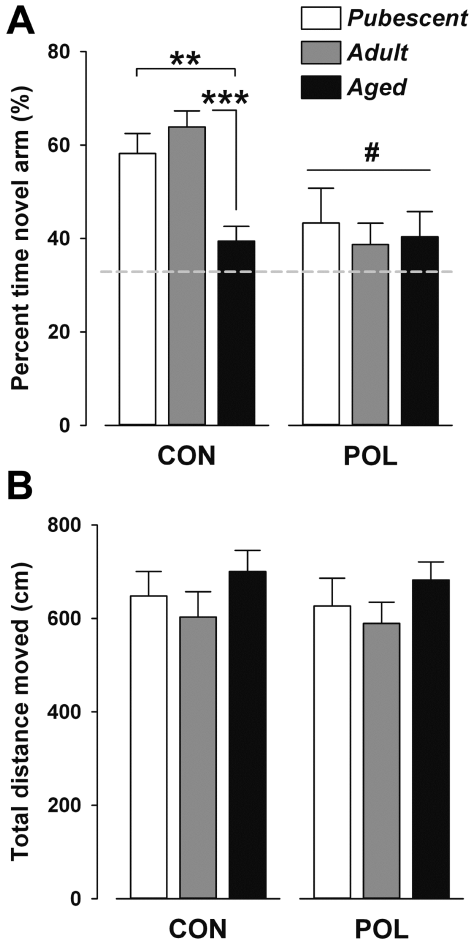

Figure 16. Short-term spatial recognition memory in pubescent, adult, and aged offspring born to poly(I:C)-exposed (POL) or control (CON) mothers as assessed using the Y-maze test. (A) Percent time spent in the novel arm during the choice phase of the test. The dashed line represents the chance level of 33.3%. $^{**}p < 0.01$ and $^{***}p < 0.001$, reflecting the significant difference between aged CON and pubescent CON offspring, and between aged CON and adult CON offspring, respectively. $^{\#}p < 0.001$, reflecting the significant difference between POL and CON offspring. p values are based on post-hoc analyses following the presence of significant main effects of age ($F_{(2,68)} = 3.65$, $p < 0.05$) and prenatal treatment ($F_{(1,68)} = 9.05$, $p < 0.01$), and of its interaction ($F_{(2,68)} = 4.18$, $p < 0.05$). **(B)** Total distance moved during the choice phase of the test. N(pubescent CON) = 9, N(pubescent POL) = 9, N(adult CON) = 12, N(adult POL) = 12, N(aged CON) = 16, N(aged POL) = 16. All values are means±SEM.

4.4.2 Prenatal Immune Activation Induces Age-Dependent Deficits in Contextual Fear Memory

In a next step, we explored whether prenatal immune activation might induce deficits in another cognitive domain known to be regulated by the hippocampus, namely contextual processing (Maren *et al*, 1997; Otto *et al*, 2006). To this end, we studied the acquisition and expression of contextual fear in pubescent, adult and aged mice born to poly(I:C)-exposed or control mothers.

During the conditioning phase of the contextual fear test, the amount of percent time freezing generally increased across the 3 successive post-shock periods (**Fig. 17a**), indicating that the animals developed a noticeable fear response as a result of shock exposure. The development of such fear responses was largely comparable between all experimental groups (**Fig. 17a**). Hence, neither prenatal immune activation nor aging significantly affected the acquisition of contextual fear.

Contextual memory was then evaluated 24 h following conditioning and was indexed by the expression of conditioned fear towards the context, in which aversive conditioning took place. Pubescent, adult and aged control mice displayed largely comparable levels of conditioned fear towards the context (**Fig. 17b**), suggesting that aging did not affect contextual memory in mice born to control mothers. On the other hand, prenatal immune challenge caused an age-dependent reduction in conditioned fear expression, which emerged only once poly(I:C)-exposed offspring reached the aged stage of life (**Fig. 17b**). Against the background of intact acquisition of learned fear (**Fig. 17b**), these data thus demonstrate that prenatal immune activation selectively impairs the retention of contextual fear memories at the aged stage of life.

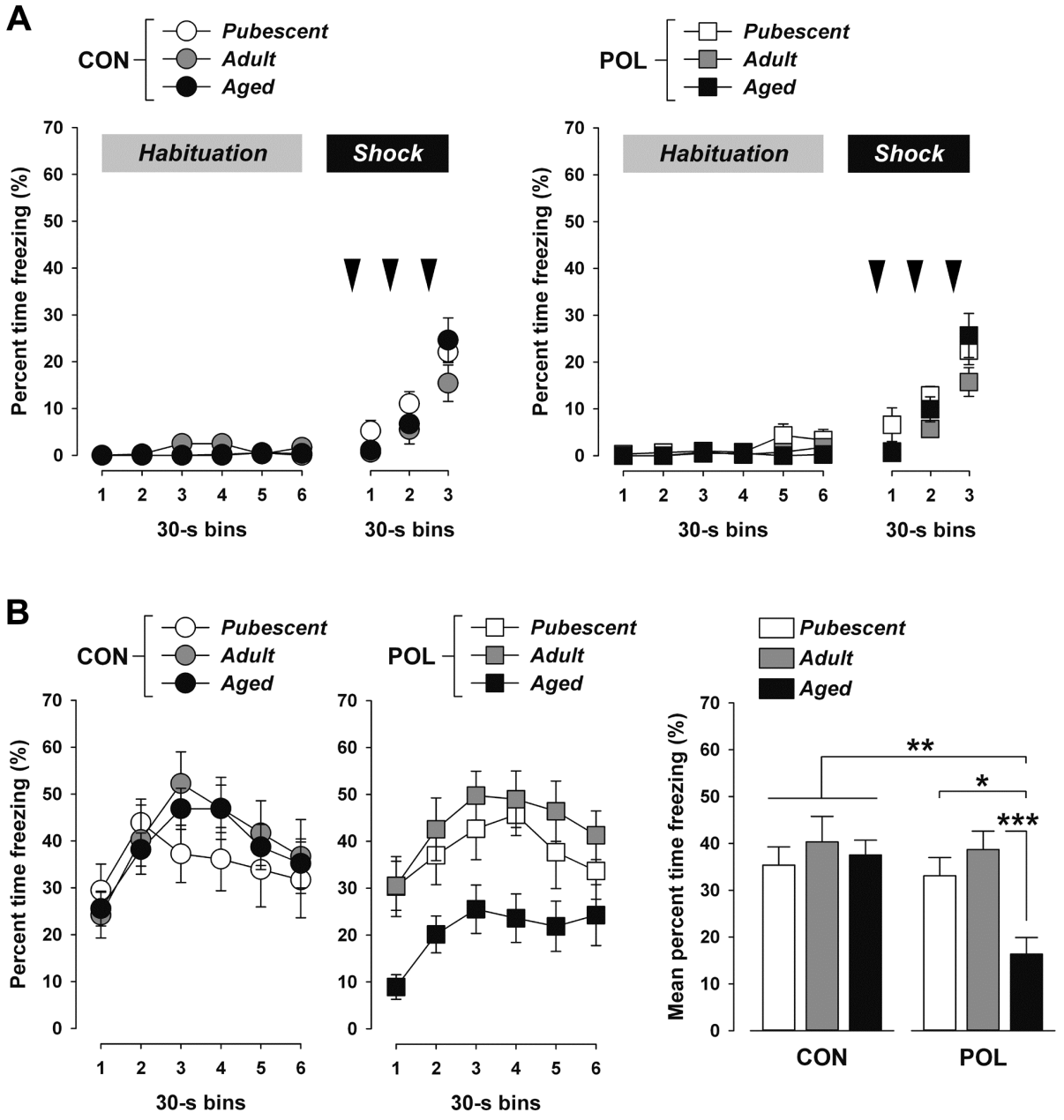

Figure 17. Contextual learning and memory in pubescent, adult, and aged offspring born to poly(I:C)-exposed (POL) or control (CON) mothers as assessed using a contextual fear condition test. (A) The line plots show percent time freezing as a function of 30-sec bins for the initial habituation and subsequent shock exposure periods during the conditioning phase of the test. The black arrow heads indicate the occurrence of the foot-shock stimulus. **(B)** The line plots display percent time freezing as a function of 30-sec bins during the contextual memory test phase, and the bar plots show the mean percent time freezing across the entire 3-min test period of the contextual memory test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, based on post-hoc analyses following the presence of a significant interaction between prenatal treatment and age ($F_{(2,64)} = 3.62$, $p < 0.05$). N(pubescent CON) = 9, N(pubescent POL) = 9, N(adult CON) = 12, N(adult POL) = 12, N(aged CON) = 16, N(aged POL) = 16. All values are means±SEM.

4.4.3 Prenatal Immune Activation Causes Severe Deficits in Spatial Reference Learning and Memory during Aging

We extended the investigations of cognitive functions to the domain of spatial reference learning and memory using a place navigation task in the Morris water maze. Optimal performance in this task depends on the functional integrity of the hippocampus (Morris *et al*, 1982), especially on its dorsal part (Moser *et al*, 1993).

First, the animals underwent pretraining using a visible cued platform to ascertain the integrity of sensorimotor functions. Animals from all groups displayed a significant reduction in the latency to locate the cued escape platform across the 3 trials, suggesting that neither prenatal immune activation nor aging affected essential sensorimotor functions such as vision and motor coordination (Vorhees and Williams, 2006). The overall means±S.E.M of escape latencies during the 3 cued platform trials were 45.6±2.3 s for trial 1, 24.1±2.5 s for trial 2, and 17.6±2.2 s for trial 3.

Following cued platform training, spatial reference learning was assessed by measuring the latency to find an invisible platform across 15 learning trials. As shown in **Fig. 18a**, pubescent mice showed shorter escape latencies especially during the first 3 learning trials compared to adult or aged mice, regardless of whether they stemmed from immune-challenged or control mothers. Adult offspring of immune-challenged mothers and adult control offspring both displayed significant improvement in spatial reference learning across successive training trials, so that their escape latencies decreased with increasing learning trials (**Fig. 18a**). Such improvement in spatial reference learning was also clearly evident in aged control offspring (**Fig. 18a**). In marked contrast, aged offspring born to immune-challenged mothers displayed a severe deficit in spatial reference learning: they did not improve as training progressed, so that their escape latencies largely remained unchanged across trials (**Fig. 18a**). This deficit became significant starting from trial 6 and persisted until completion of spatial reference training on trial 15 (**Fig. 18a**). This impairment in spatial reference learning was not attributable to excessive floating behaviour. Indeed, highly similar results were obtained when distances swum to find the escape platform were used as the dependent variables, showing that aged poly(I:C) offspring swum longer distances in attempts to find the escape platform (data not shown). Moreover, the swim speed (cm/s) was highly comparable between groups, indicating that motor coordination and swim capacity were not affected by prenatal immune activation or aging. The overall mean±S.E.M of

swim speed during spatial reference training was 17.3±5.6 cm/s in pubescent offspring, 17.9±4.9 cm/s in adult offspring, and 16.7±5.4 cm/s in aged offspring.

Consistent with the preceding learning phase, aged offspring born to immune-challenged mothers also displayed a severe deficit in spatial reference memory retention, which was assessed during the probe test 1 day after completion of reference training (**Fig. 18b**). Indeed, in contrast to all other groups, aged poly(I:C) offspring failed to show a preference for the target quadrant, in which the platform was previously positioned during spatial reference training (**Fig. 18c**). Even though less severe compared to aged poly(I:C) offspring, aged control offspring also displayed a significant decrease in the time spent in the target quadrant compared with adult control offspring. Together, these results suggest that aging can generally impair spatial reference memory retention, but this effect is strongly exacerbated by exposure to prenatal immune challenge.

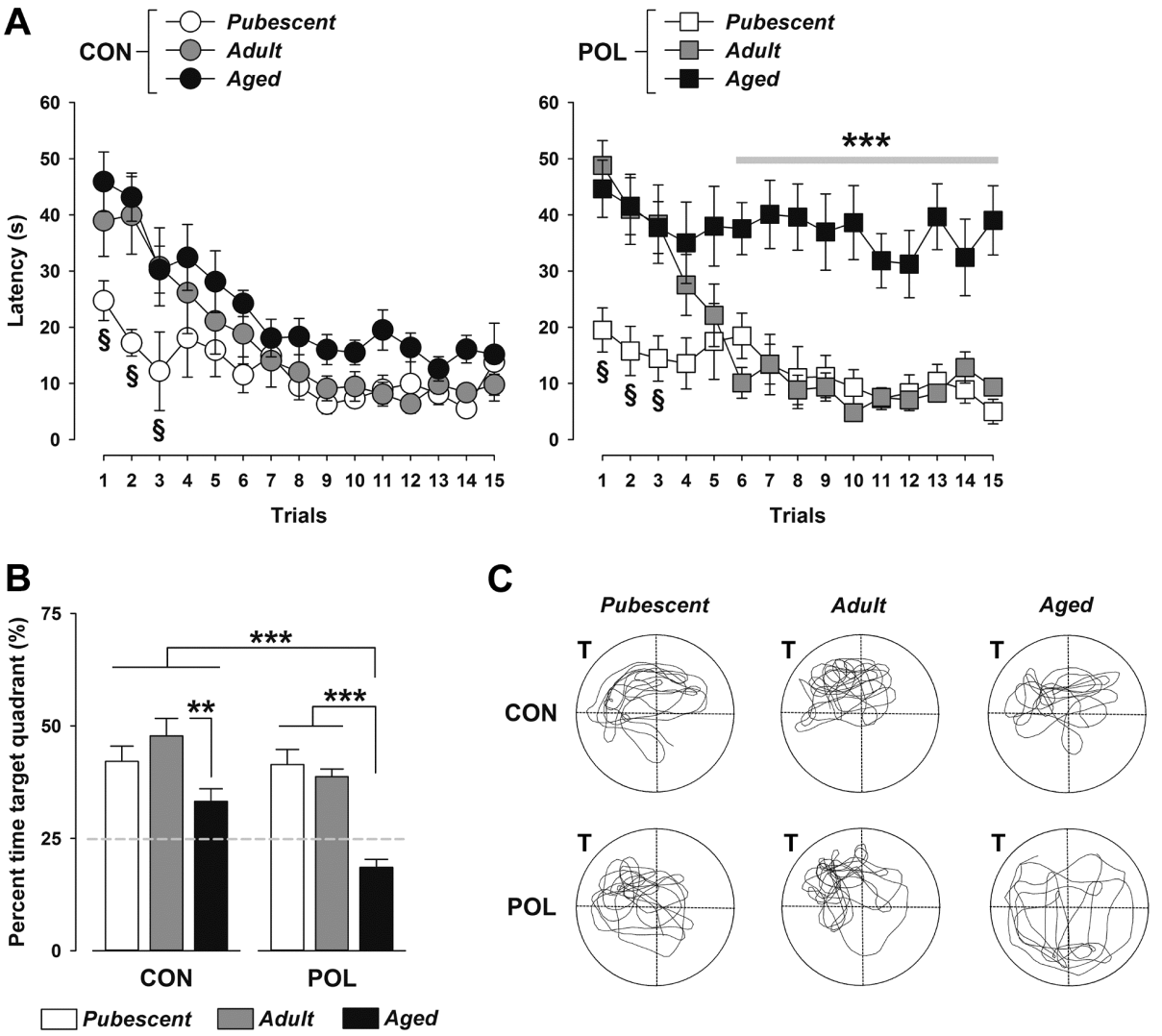

Figure 18. Spatial reference learning and memory in pubescent, adult, and aged offspring born to poly(I:C)-exposed (POL) or control (CON) mothers as assessed using the Morris water maze. (A) The line plots show the latency to find the invisible escape platform as a function of trials during the acquisition phase of the test. ***$p < 0.001$, reflecting the significant learning impairment displayed by aged POL offspring relative to all other groups between trials 6 and 15 of the acquisition phase; §$p < 0.001$, reflecting the significant reduction in the latency to find the platform displayed by pubescent offspring relative to adult and aged offspring. p values are based on post-hoc analyses following the presence of significant main effects of age ($F_{(2,68)}$ = 64.33, $p < 0.001$), prenatal treatment ($F_{(1,68)}$ = 11.84, $p < 0.001$) and trials ($F_{(14,952)}$ = 18.83, $p < 0.001$), as well as of a two-way interaction between prenatal and age ($F_{(2,68)}$ = 13.07, $p < 0.001$) and a three-way interaction between age, prenatal treatment, and trials ($F_{(28,952)}$ = 1.67, $p < 0.05$). **(B)** The bar plot shows the percent time spent in the target quadrant during the probe test of spatial reference memory retention as assessed 1 day after completion of reference training. **$p < 0.01$ and ***$p < 0.001$, based on post-hoc tests following the presence of a significant main effect of quadrant ($F_{(3,204)}$ = 58.70, $p < 0.001$), a significant two-way interaction between quadrant and age ($F_{(6,204)}$ = 12.29, $p < 0.001$), and a significant three-way interaction between quadrant, age, and prenatal treatment ($F_{(6,204)}$ = 5.13, $p < 0.001$). **(C)** Computer-generated search paths of representative CON and POL offspring during the probe test of spatial reference memory retention at the pubescent, adult, and aged stages of life. T, target quadrant. N(pubescent CON) = 9, N(pubescent POL) = 9, N(adult CON) = 12, N(adult POL) = 12, N(aged CON) = 16, N(aged POL) = 16. All values are means±SEM.

4.4.4 Prenatal Immune Activation Reduces Food Hoarding Behaviour during Aging

Based on the preceding findings, we were further interested in exploring whether prenatal immune activation might cause deficits in species-typical behaviours that are known to be regulated by the hippocampus. Therefore, we went on to assess the consequences of prenatal immune challenge on food hoarding, which has been shown to be disrupted by hippocampal lesions (Deacon *et al*, 2002a).

In agreement with previous studies in mice (Deacon, 2012), the extent to which mice displaced food from the distant food source to their home cages was found to be variable, even within individual experimental groups (**Fig. 19a**). Despite this, we found that aged offspring of immune-challenged mothers displayed a significant reduction in the amount of food hoarded compared to aged control offspring (**Fig. 19a**). Similar to mice with hippocampal lesions (Deacon *et al*, 2002b), aged immune-exposed offspring hoarded virtually no food (**Fig. 19a**). On the other hand, immune-challenged and control offspring exhibited comparable amounts of food hoarding when they were tested at the pubescent or adult ages (**Fig. 19a**). Hence, prenatal immune activation induces a long-term negative impact on food hoarding behaviour that emerges specifically during aging. Importantly, this impairment is unlikely to be attributable to possible differences in general food intake because no group differences were detected in the analysis of total food eaten during the test period (**Fig. 19b**).

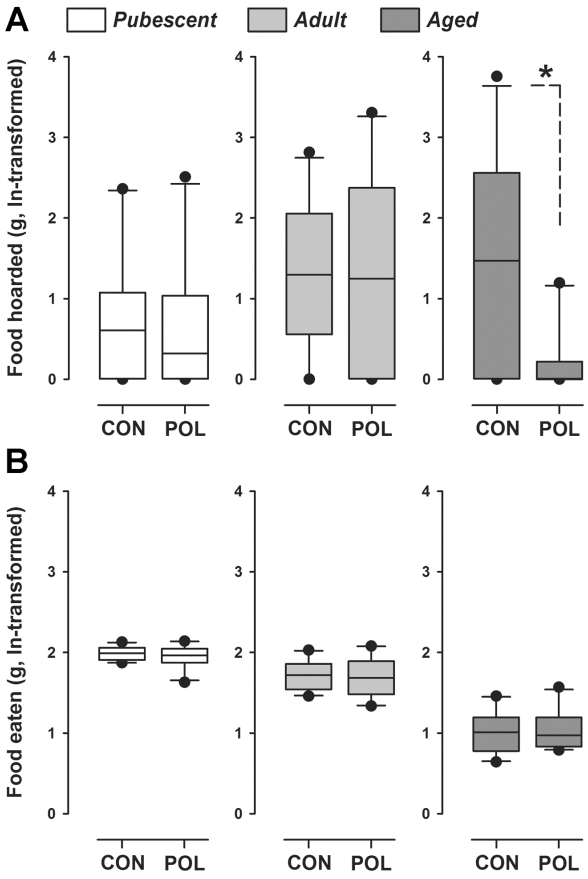

Figure 19. Food hoarding behaviour in pubescent, adult, and aged offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. **(A)** The box plots represent the amount of food hoarded (g, ln-transformed). *$p < 0.05$, reflecting the significant reduction in food hoarding displayed by aged POL offspring relative to aged CON offspring based on non-parametric Mann–Whitney analysis. **(B)** The box plots represent the amount of food eaten (g, ln-transformed). $N = 10$ in each group.

4.4.5 Prenatal Immune Activation does not Affect Innate Anxiety-Like Behaviour or Basal Locomotor Activity in a Novel Environment

Using the open field test, we further investigated whether prenatal immune activation and aging would alter innate anxiety-like behaviour and/or locomotor activity, which in turn could confound the interpretation of the findings obtained in the preceding cognitive tests of primary interest (Holmes *et al*, 2002; Huang *et al*, 2012). In line with previous findings (Schwendener *et al*, 2009), we found that prenatal immune challenge did not change the primary indices of innate anxiety-like behaviour in the open field test (i.e., time spent or distance moved in the central area of the open field) at pubescent or adult age (**Table 5**). Similarly, there were no group differences with respect to these measures when the offspring reached the aged stage (**Table 5**).

The prenatal immunological insult did also not affect basal locomotor activity as indexed by the total distance moved in the entire open field arena (**Table 5**). The total distance moved in the open field test decreased with increasing age, and this age-associated effect on basal locomotor activity was not influenced by prenatal immune activation (**Table 5**). Together, these data thus suggest that the cognitive consequences of prenatal immune activation (Fig. 1-4) are unlikely to be confounded by possible changes in innate anxiety-like behaviour and/or locomotor activity.

Age	Prenatal treatment	Total distance moved (m)	Distance moved in centre zone (m)	Time spent in centre zone (s)
Pubescent	CON	76.27±4.57[a]	5.92±1.01	196.26±35.61
	POL	73.04±3.61[a]	6.79±1.49	234.58±47.07
Adult	CON	65.81±2.69[b]	8.41±0.92	242.34±47.88
	POL	61.51±5.21[b]	7.02±0.69	195.94±18.51
Aged	CON	55.44±4.64	5.99±1. 40	143.22±41.52
	POL	52.04±3.55	5.28±1.05	155.36±47.59

Table 5. Summary of the effects of age and prenatal treatment on behavioural measures in the open field test. Separate cohorts of prenatally poly(I:C)-treated (= POL) and control (= CON) offspring were tested when they reached the pubescent, adult, or aged stage of life. [a]$p < 0.001$, reflecting the significant difference between pubescent and adult or aged offspring based on post-hoc analyses; and [b]$p < 0.05$, reflecting a significant difference between adult and aged offspring based on post-hoc analyses following presence of a significant main effect of age ($F_{(2,54)} = 12.82$, $p < 0.001$). All values are means±SEM; $N = 10$ in each group.

4.4.6 Prenatal Immune Activation Affects Synaptic Proteins and Neurotrophins Gene Expression during Aging

The presence of age-related deficits in hippocampus-regulated cognitive functions prompted us to explore whether prenatal immune activation might affect hippocampal synaptic integrity across aging (Morrison *et al*, 2012). To this end, we first assessed the hippocampal protein levels of PSD95, a postsynaptic density protein that is highly enriched at close apposition to the presynaptic active zone (Kennedy, 2000). Based on previous studies (Migaud *et al*, 1998), we speculated that the hippocampal levels of PSD95 would be decreased in prenatally immune-challenged offspring especially when they reach the aged stage of life. Contrary to our expectations, however, we found that aged offspring born to poly(I:C)-exposed mothers showed a significant increase in the hippocampal density of PSD95, both in the pyramidal cell layer of the CA1 and CA3 areas, as well as in the granule cell layer of the DG, compared to aged control offspring (**Fig. 20a,b,c**).

This elevation of hippocampal PSD95 levels in aged immune-exposed offspring might represent a compensatory postsynaptic reaction to impaired presynaptic input (Nyffeler *et al*, 2007). We explored this hypothesis by assessing the hippocampal levels of SYN, a vesicle-associated protein that is rapidly recruited to presynaptic terminals in response to presynaptic neuronal activity (Jin *et al*, 2011). Consistent with previous studies in C57BL6 and CD-1 mice (Benice *et al*, 2006; Himeda *et al*, 2005), SYN immunoreactivity in the CA1 and CA3 pyramidal cell layers generally increased with increasing age. Most interestingly, however, aged offspring born to immune-challenged mothers displayed a significant reduction in the relative density of SYN in the pyramidal cell layer of the hippocampal CA1 and CA3 regions compared with aged control offspring (**Fig. 21a,b**). Prenatal immune activation also decreased SYN density in the granule cell layer of the DG (**Fig. 21c**). This latter effect was present already at pubescence and persisted into the aged stage (**Fig. 21c**), suggesting that prenatal immune activation can lead to age-independent effects in the DG in addition to its age-dependent effects emerging in the CA1 and CA3 regions.

Since presynaptic vesicle-associated proteins are downstream targets of signalling by neurotrophic factors such as BDNF, NGF, and NT-3 (Poo, 2001; Pozzo-Miller *et al*, 1999), impaired expression of these neurotrophins might provide a molecular mechanism underlying the reduction in hippocampal SYN levels in aged immune-

exposed offspring. Consistent with this hypothesis, we found that the dorsal hippocampal mRNA levels of BDNF and NT-3 were significantly reduced in aged offspring born to immune-challenged mothers (**Fig. 22**). On the other hand, no significant effects were obtained in the analysis of hippocampal NGF expression (**Fig. 22**).

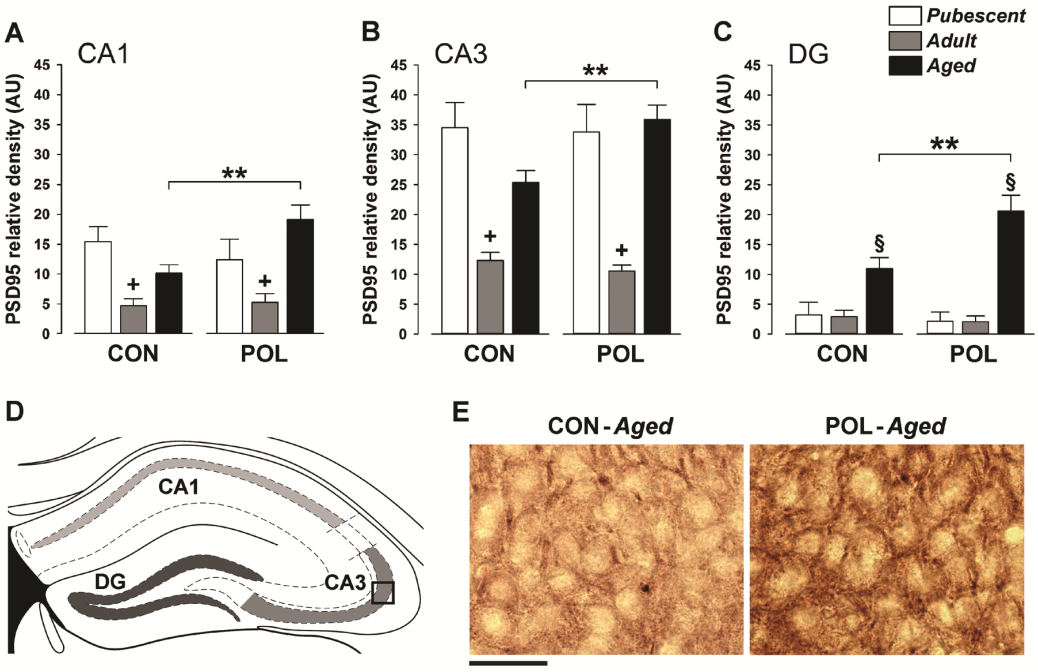

Figure 20. Hippocampal postsynaptic density protein 95 (PSD95) in pubescent, adult, and aged offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. (A) Relative optical density (in arbitrary units, AU) of PSD95 in the pyramidal cell layer of the cornu ammonis area 1 (CA1). $^{+}p < 0.001$, reflecting the significantly lower PSD95 density in adult CON and POL offspring relative to pubescent or aged CON and POL offspring; $^{**}p < 0.01$, reflecting the significant difference between aged POL and aged CON offspring. *p* values are based on post-hoc analyses following the presence of a significant main effect of age ($F_{(2,44)} = 8.99$, $p < 0.001$) and its interaction with prenatal treatment ($F_{(2,44)} = 3.49$, $p < 0.05$). **(B)** Relative optical density of PSD95 in the pyramidal cell layer of the CA3 area. $^{+}p < 0.001$, reflecting the significantly lower PSD95 density in adult CON and POL offspring relative to pubescent or aged CON and POL offspring; $^{**}p < 0.01$, reflecting the significant difference between aged POL and aged CON offspring. *p* values are based on post-hoc analyses following the presence of a significant main effect of age ($F_{(2,44)} = 33.20$, $p < 0.001$) and its interaction with prenatal treatment ($F_{(2,44)} = 3.08$, $p < 0.05$). **(C)** Relative optical density of PSD95 in the granule cell layer of the dentate gyrus (DG). $^{\S}p < 0.001$, reflecting the significantly higher PSD95 density in aged CON and POL offspring relative to pubescent or aged CON and POL offspring; $^{**}p < 0.01$, reflecting the significant difference between aged POL and aged CON offspring. *p* values are based on post-hoc analyses following the presence of a significant main effect of age ($F_{(2,44)} = 21.57$, $p < 0.001$) and its interaction with prenatal treatment ($F_{(2,44)} = 3.92$, $p < 0.05$). For **A-C**, *N*(pubescent CON) = 8, *N*(pubescent POL) = 8, *N*(adult CON) = 8, *N*(adult POL) = 7, *N*(aged CON) = 9, *N*(aged POL) = 10; all values are means±SEM. **(D)** Schematic illustration of the hippocampal cell layers, in which the relative optical density of PSD95 was measured (as highlighted by the boundaries shown in grey). **(E)** Representative examples of PSD95 staining in the CA3 pyramidal cell layer (as indicated by the square in **D**) of aged CON and POL offspring. Scale bar: 20 µm.

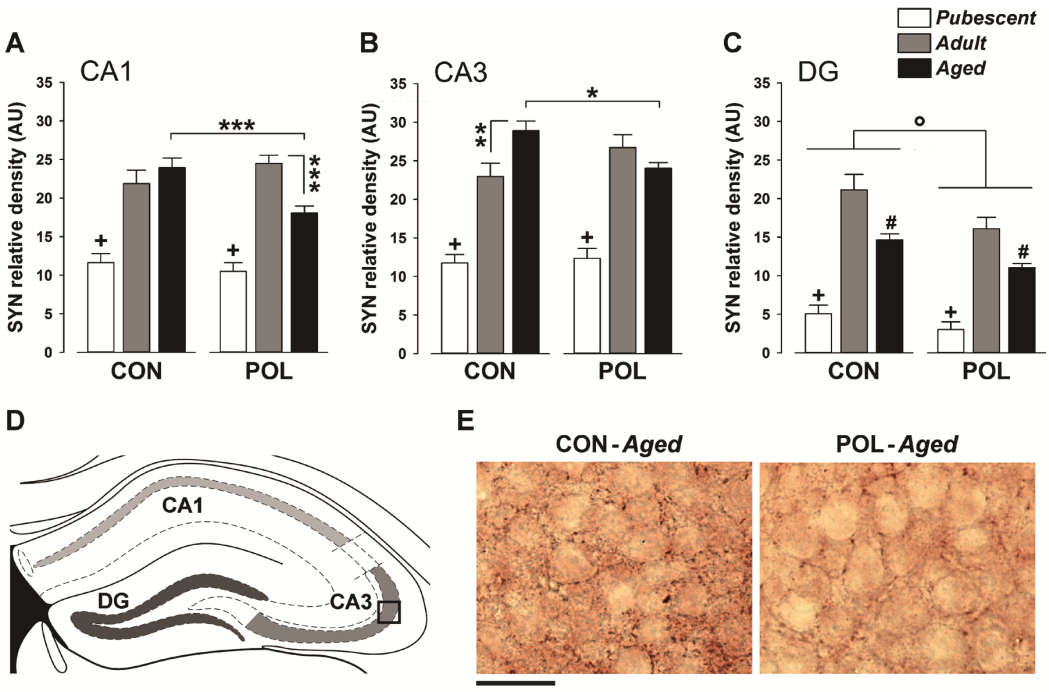

Figure 21. Hippocampal synaptophysin (SYN) density in pubescent, adult, and aged offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. (A) Relative optical density (in arbitrary units, AU) of SYN in the pyramidal cell layer of the cornu ammonis area 1 (CA1). $^{+}p < 0.001$, reflecting the significantly lower SYN density in pubescent CON and POL offspring relative to adult or aged CON and POL offspring; $^{***}p < 0.001$, reflecting the significant difference between aged POL offspring and adult or aged CON offspring. *p* values are based on post-hoc analyses following the presence of a significant main effect of age ($F_{(2,44)} = 52.48$, $p < 0.001$) and its interaction with prenatal treatment ($F_{(2,44)} = 6.03$, $p < 0.01$). **(B)** Relative optical density of SYN in the pyramidal cell layer of the CA3 area. $^{+}p < 0.001$, reflecting the significantly lower SYN density in pubescent CON and POL offspring relative to adult or aged CON and POL offspring; $^{*}p < 0.05$ and $^{**}p < 0.01$, reflecting the significant difference between aged POL and CON offspring, and between adult and aged CON offspring, respectively. *p* values are based on post-hoc analyses following the presence of a significant main effect of age ($F_{(2,44)} = 79.14$, $p < 0.001$) and its interaction with prenatal treatment ($F_{(2,44)} = 4.57$, $p < 0.05$). **(C)** Relative optical density of SYN in the granule cell layer of the dentate gyrus (DG). $^{+}p < 0.001$, reflecting the significantly lower SYN levels in pubescent CON and POL offspring relative to adult or aged CON and POL offspring; $^{\#}p < 0.001$, reflecting the significantly lower SYN density in aged CON and POL offspring relative to adult CON and POL offspring; $^{\circ}p < 0.05$, reflecting the significant overall difference between POL and CON offspring. *p* values are based on post-hoc analyses following the presence of a significant main effects of age ($F_{(2,44)} = 52.\ 59$, $p < 0.001$) and prenatal treatment ($F_{(1,44)} = 6.59$, $p < 0.05$). For **A-C**, *N*(pubescent CON) = 8, *N*(pubescent POL) = 8, *N*(adult CON) = 8, *N*(adult POL) = 7, *N*(aged CON) = 9, *N*(aged POL) = 10; all values are means±SEM. **(D)** Schematic illustration of the hippocampal cell layers, in which the relative optical density of SYN was measured (as highlighted by the boundaries shown in grey). **(E)** Representative examples of SYN staining in the CA3 pyramidal cell layer (as indicated by the square in **D**) of aged CON and POL offspring. Scale bar: 20 µm.

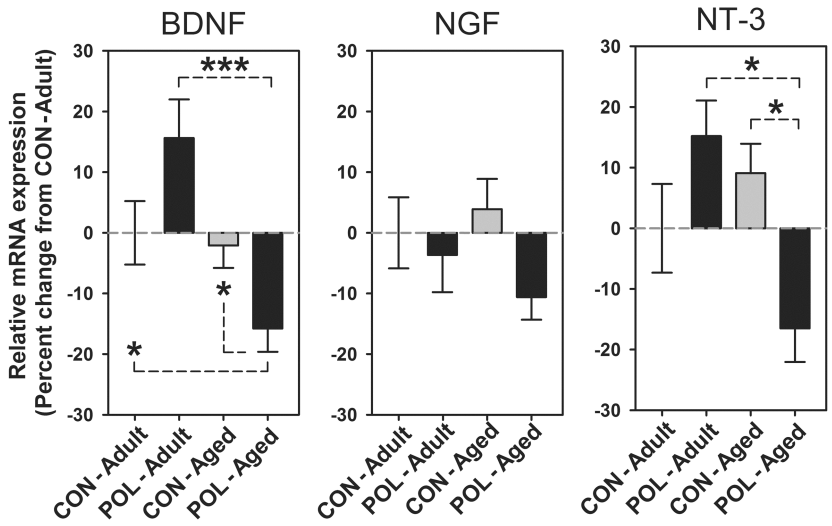

Figure 22. Expression of neuroplasticity-related genes in the dorsal hippocampus of adult and aged offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. The bar plots show normalized mRNA expression of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3) as assessed using quantitative RT-PCR. *p < 0.05 and ***p < 0.001, based on post-hoc analyses following the presence of a significant interaction between prenatal treatment and age (BDNF: $F_{(1,48)}$ = 5.66, p < 0.05; NT-3: $F_{(1,48)}$ = 5.53, p < 0.05). N(adult CON) = 13, N(adult POL) = 13, N(aged CON) = 14, N(aged POL) = 12; all values are means±SEM.

4.4.7 Prenatal Immune Activation does not Induce Signs of Persistent Systemic or Hippocampal Inflammation

It has been proposed that prenatal immune activation may exacerbate neuronal aging by inducing persistent systemic inflammation and accompanied neuroinflammatory reactions throughout brain maturation and aging (Krstic *et al*, 2013; Krstic *et al*, 2012). To explore whether systemic inflammatory abnormalities might be involved in the precipitation of aging-related cognitive and synaptic impairments following prenatal poly(I:C) exposure, we assessed the serum levels of cytokines with pro-inflammatory (IL-1β, IL-6, TNF-α, and IFN-γ) and anti-inflammatory (IL-4 and IL-10) properties (Curfs *et al*, 1997). In agreement with the concept of "inflammaging" (Franceschi *et al*, 2007), the serum levels of the pro-inflammatory cytokines IL-6 and TNF-α generally increased during aging (**Fig.23**). On the other hand, IFN-γ serum levels were markedly recued in aged relative to pubescent or adult offspring (**Fig. 23**), which may reflect attenuated production of cytokines by T helper type 1 (Th1) cells at aged stages of life (Baruch *et al*, 2013). Notably, none of these aging-associated effects were influenced by prenatal immune activation. The serum levels of the anti-inflammatory cytokine IL-10 also varied as a result of aging, so that aged mice showed higher IL-10 levels compared to adult or pubescent animals (**Fig. 23**). Interestingly, this effect was potentiated in offspring exposed to prenatal immune challenge: Aged poly(I:C) offspring displayed significantly higher serum IL-10 levels compared to aged control offspring (**Fig. 23**). Moreover, aged offspring born to immune-challenged mothers showed a marked and selective increase in the serum levels of the anti-inflammatory cytokine IL-4 (**Fig. 23**). Together, these results demonstrate that the direction and magnitude of aging-associated changes in the production of prototypical pro-inflammatory cytokines such as IL-6 and TNF-α are not affected by the prenatal immunological insult. At the same time, however, our findings show that prenatal immune activation leads to an age-specific increase in the production of serum cytokines with anti-inflammatory properties.

We also did not find any evidence for the hypothesis that prenatal viral-like immune activation might cause signs of persistent neuroinflammation in the hippocampus. First, our stereological estimations of CD68-positive microglia cells in the hippocampal formation did not reveal any numeric differences between prenatally immune-challenged and control offspring at any age investigated (**Fig. 24**). The number of CD68-immunoreactive microglia cells was generally lower in the CA1-CA3 (but not DG) region

of adult relative to pubescent or aged offspring, independently of whether they stemmed from immune-challenged or control mothers (**Fig. 24**). Similarly, no significant changes were detected in the stereological analyses of GFAP-immunoreactive cells, suggesting that the numbers of astrocytes in the hippocampus was not affected by prenatal immune activation and/or aging (**Fig. 24**). In line with these results, quantitative RT-PCR analyses of hippocampal cytokine mRNAs showed that these cytokine genes were expressed at relatively low levels in adult and aged offspring, leading to high cycle threshold (Ct) values ranging between 32 and 37 cycles (Livak and Schmittgen, 2001). A summary of the average Ct values for the selected cytokine and house-keeping genes is provided in **Table 6**. Taken together, our results show that prenatal immune activation does not cause long-lasting numeric changes in glia cells known to be major sources of inflammatory stimuli, nor does it alter the levels of inflammatory cytokine genes in the hippocampus across aging.

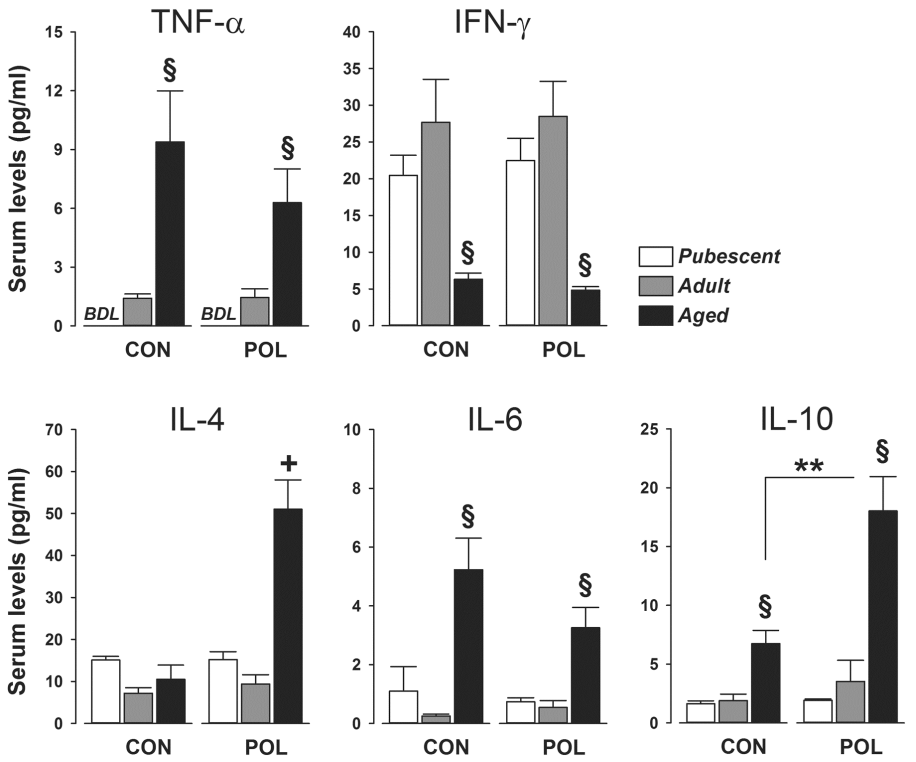

Figure 23. Cytokine serum levels in pubescent, adult, and aged offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. The graphs depict the levels of tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-4, IL-6, and IL-10. §$p < 0.001$, reflecting the significant difference between aged CON and POL offspring and pubescent or adult CON and POL offspring, based on post-hoc comparisons following the presence of a significant main effect of age (TNF-α: $F_{(1,48)} = 4.74$, $p < 0.05$; IFN-γ: $F_{(2,44)} = 13.67$, $p < 0.001$; IL-6: IFN-γ: $F_{(2,44)} = 5.65$, $p < 0.01$; IL-10: $F_{(2,44)} = 15.32$, $p < 0.001$). $^{+}p < 0.01$, reflecting the selective increase in aged POL offspring relative to all other groups, based on post-hoc comparisons following the presence of a significant interaction between prenatal treatment and age ($F_{(2,44)} = 6.15$, $p < 0.01$). **$p < 0.01$, reflecting the significant difference between aged CON and aged POL offspring, based on post-hoc comparison following the presence of a significant interaction between prenatal treatment and age ($F_{(2,44)} = 3.30$, $p < 0.01$). The serum levels of TNF-α were below detection limit (BDL) in pubescent offspring, and so were the serum levels of IL-1β (not depicted) at all ages investigated. N(pubescent CON) = 9, N(pubescent POL) = 9, N(adult CON) = 8, N(adult POL) = 8, N(aged CON) = 8, N(aged POL) = 8; all values are means±SEM.

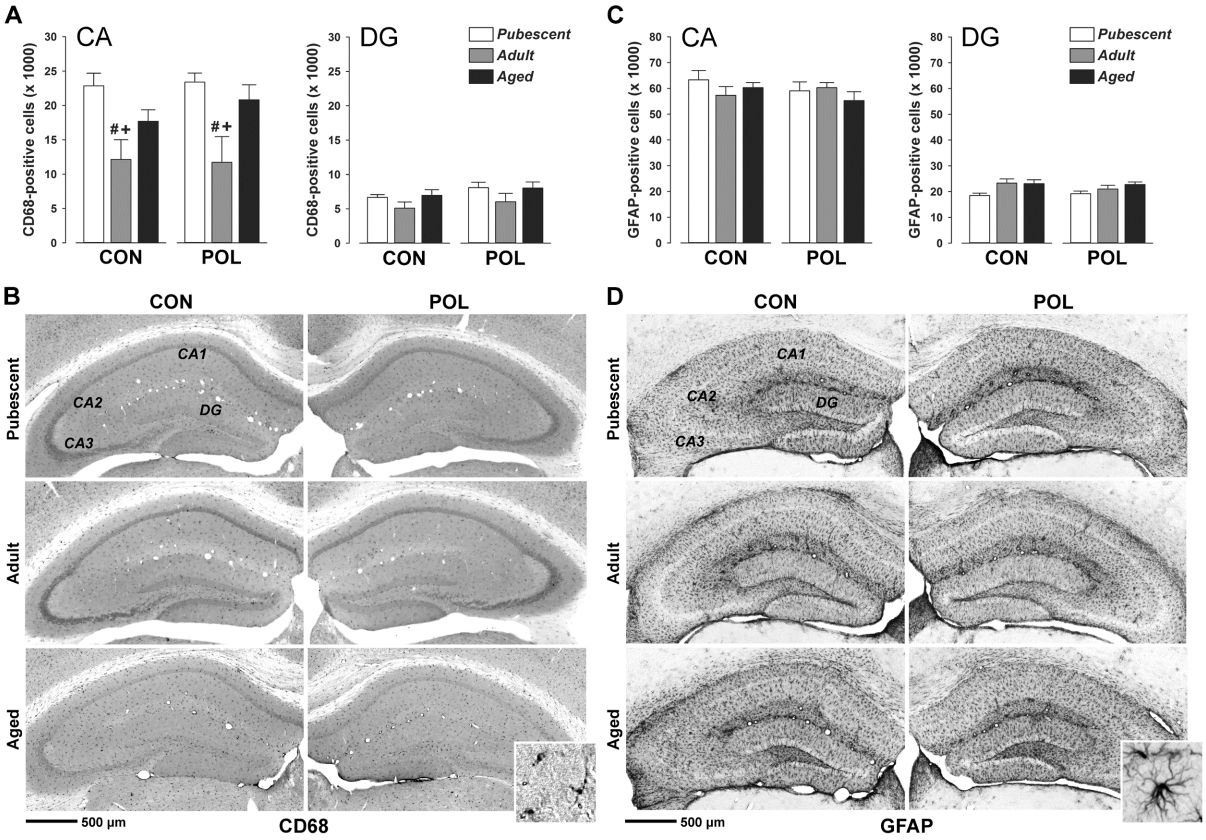

Figure 24. Hippocampal microglia and astroctye numbers in pubescent, adult, and aged offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. (A) Stereological estimates of CD68-immunoreactive microglia in the entire cornu ammonis (CA) area (i.e., CA1-CA3 areas) and dentate gyrus (DG). $^{\#}p < 0.001$ and $^{+}p < 0.01$, reflecting the significant difference between pubescent CON and POL offspring and adult CON and POL offspring, and between pubescent CON and POL offspring and aged CON and POL offspring, respectively. *p* values are based on post-hoc analyses following the presence of a significant main effect of age ($F_{(2,44)}$ = 10.33, $p < 0.001$). *N*(pubescent CON) = 8, *N*(pubescent POL) = 8, *N*(adult CON) = 8, *N*(adult POL) = 7, *N*(aged CON) = 9, *N*(aged POL) = 10; all values are means±SEM. **(B)** Representatives hippocampal sections of pubescent, adult, and aged CON and POL offspring stained with anti-CD68 antibody. The insert photomicrograph depicts CD68-positive microglia cells at higher magnification. **(C)** Stereological estimates of GFAP-immunoreactive astrocytes in the entire CA and DG regions. *N*(pubescent CON) = 8, *N*(pubescent POL) = 8, *N*(adult CON) = 8, *N*(adult POL) = 7, *N*(aged CON) = 9, *N*(aged POL) = 10; all values are means±SEM. **(D)** Representatives hippocampal sections of pubescent, adult, and aged CON and POL offspring stained with anti-GFAP antibody. The insert photomicrograph shows GFAP-positive astrocytes at higher magnification.

	CON-Adult	POL-Adult	CON-Aged	POL-Aged
IL-1β/36B4	35.5/27.0	36.6/26.9	35.3/27.1	32.6/27.1
IL-4/36B4	35.6/26.0	35.3/26.1	36.0/26.0	35.3/26.2
IL-6/36B4	32.2/26.2	32.1/25.9	32.3/26.5	32.8/26.6
TNF-α/36B4	35.6/26.3	35.0/25.1	36.3/25.9	35.2/25.0

Table 6. Summary of the average cycle threshold (Ct) values for the cytokines genes (IL-1β, IL-4, IL-6, and TNF-α) and house-keeping gene (36B4) measured in the hippocampi of adult and aged offspring born to poly(I:C)-exposed (POL) and control (CON) mothers using real-time PCR. *N*(adult CON) = 13, *N*(adult POL) = 13, *N*(aged CON) = 14, *N*(aged POL) = 12.

4.4.8 Discussion

Our study demonstrates that prenatal exposure to viral-like immune activation negatively influences hippocampus-related cognitive and synaptic functions during aging. Based on its relevance to neurodevelopmental disorders (Atladottir *et al*, 2010; Brown *et al*, 2010; Canetta *et al*, 2014), early-life immune insults such as maternal infection have been mostly studied with respect to (hippocampus-related) neuronal and behavioural abnormalities that emerge in the offspring when they mature from pubescence to adulthood (Harvey, 2012; Meyer, 2014; Meyer *et al*, 2010a). Although some developmental dysfunctions induced by prenatal immune challenge can be detected as early as the fetal and early neonatal stages (Baharnoori *et al*, 2012; Escobar *et al*, 2011; Meyer *et al*, 2008d; Vuillermot *et al*, 2010), many of the behavioural and neuronal changes induced by prenatal immune activation are dependent on maturational processes and only emerge once the offspring have reached a certain stage of age, typically adolescence or early adulthood (Piontkewitz *et al*, 2011a; Richetto *et al*, 2014; Vuillermot *et al*, 2010; Zuckerman *et al*, 2003a). These latter observations have led to the hypothesis that the neuropathological consequences of prenatal immune challenge are, at least in part, progressive in nature. The present findings are consistent with this hypothesis and suggest that the progression of hippocampus-related brain dysfunctions continues throughout aging. Indeed, the full spectrum of prenatal infection-induced cognitive deficits only emerged once the offspring reached the aged stage of life. In agreement with previous studies in rats (Vorhees *et al*, 2012; Zuckerman *et al*, 2005), contextual fear memory and spatial reference learning and memory were not different between immune-exposed and control offspring at pubescent or adult age. It thus seems

that the subsequent aging process is required to unmask prenatal infection-induced deficits in these cognitive domains.

These age-dependent effects, however, contrast the consequences of prenatal immune activation on short-term recognition memory, which was impaired already in pubescent offspring of immune-exposed mothers. One implication of these findings is that prenatal immune challenge can cause multiple cognitive abnormalities that differ in their temporal onsets: in offspring with prenatal infectious histories, deficits in short-term memory (as indexed by the disruption of spatial recognition memory) appear to precede the subsequent emergence of impairments in intermediate- to long-term forms of learning and memory (as indexed by the deficits in contextual fear memory and spatial reference learning and memory). Similar age-dependent versus age-independent effects of prenatal viral-like immune activation have been observed before with respect to dopamine-regulated behavioural alterations (Meyer *et al*, 2008a; Vuillermot *et al*, 2010). The present study extends these findings by drawing attention to early- versus late-onset cognitive deficits that develop following prenatal immune challenge.

It should also be noted that aging itself, that is, in offspring born to control mothers, caused a certain degree of cognitive decline, which manifested primarily as attenuated short-term recognition memory and reduced retention of spatial reference memory. These aging-associated effects are in line with the observation that hippocampus-regulated cognitive capabilities generally decline during the process of normative aging (Driscoll *et al*, 2005; Lister *et al*, 2009; Morrison *et al*, 2012). Against these backgrounds, our data thus support the hypothesis that prenatal immune activation may represent an early-life environmental risk factor for exacerbated cognitive aging (Krstic *et al*, 2013; Krstic *et al*, 2012), at least with respect to cognitive processes that are known to critically involve the hippocampal formation.

A similar (albeit more tentative) conclusion can also be drawn when considering the age-dependent manifestation of synaptic abnormalities in prenatally immune-exposed offspring. Even though the characterization of such neuronal effects is far from complete, we were able to identify significant alterations in key proteins and neuroplasticity-promoting genes that are pivotal for the integrity of synaptic functions (Morrison *et al*, 2012; Poo, 2001). Immune-exposed offspring displayed significantly decreased SYN levels compared with aged control offspring, and this presynaptic deficit

temporally coincided with impaired hippocampal expression of the neurotrophic factors BDNF and NT-3. The age-dependent effects on BDNF may be of particular importance because this neurotropic factor can effectively mobilize SYN-positive synaptic vesicles to presynaptic active zones (Lu and Chow, 1999; Pozzo-Miller *et al*, 1999). Impaired BDNF expression in the hippocampus of aged immune-exposed offspring may thus represent a contributing factor for decreased hippocampal SYN levels at this stage of life.

Our study further showed that aged offspring born to immune-challenged mothers displayed increased hippocampal levels of PSD95 relative to aged control offspring. PSD95 is a key postsynaptic density protein that is highly enriched at close apposition to the presynaptic active zone, especially at *N*-methyl-D-aspartate (NMDA)-type glutamatergic synapses (Kennedy, 2000). Reductions in (hippocampal) PSD95 levels have typically been associated with the emergence of impaired spatial learning and memory (Louveau *et al*, 2013; Migaud *et al*, 1998). Therefore, our findings of increased PSD95 levels in aged immune-exposed offspring may seem counterintuitive given that these animals displayed marked deficits in spatial learning and memory. It should be noted, however, that abnormally elevated hippocampal PSD95 levels are likely to reflect disturbed synaptic functions as well, and such abnormalities may be particularly relevant for aging-related learning impairments (Nyffeler *et al*, 2007). Indeed, previous aging studies in rats demonstrated a concomitant increase and decrease in hippocampal PSD95 and presynaptic vesicle-associated proteins, respectively, emerging selectively in aged learning-impaired relative aged learning-unimpaired subjects (Nyffeler *et al*, 2007). These findings have been taken as support for the hypothesis that aging-induced deficits in presynaptic morphology and functions can lead to compensatory postsynaptic changes. Our findings are in line with this notion and further suggest that prenatal immune activation aggravates such aging-related synaptic dysfunctions.

The emergence of (age-dependent) cognitive and synaptic changes in prenatally immune-exposed offspring were not paralleled by signs of systemic or hippocampal inflammation. Whilst the acute maternal and fetal pro-inflammatory effects of maternal viral-like immune activation seem robust (Abazyan *et al*, 2010; Arrode-Bruses and Bruses, 2012; Meyer *et al*, 2006b), the extent to which this prenatal insult can cause inflammatory responses that persist into adult life remains controversial. Hence, rodent models of maternal gestational immune activation have produced both positive and

negative findings that either support (Borrell *et al*, 2002; Krstic *et al*, 2012; Mattei *et al*, 2014) or refute (Pacheco-Lopez *et al*, 2013; Willi *et al*, 2013)this hypothesis. Related to this, it is important to emphasize that the consequences of prenatal immune activation on postnatal inflammatory processes are likely to be influenced by various factors, including prenatal timing and immune stimulus identity and/or severity. It is also likely that the postnatal environment, in which the offspring mature, can significantly influence their immune parameters, including the levels of perceived stress (Giovanoli *et al*, 2013) and pathogen load of the animal holding facilities (Doom *et al*, 2009; Montalvo *et al*, 2013). Whatever reasons for these differential outcomes, our study highlights that persistence of inflammatory reactions is not a prerequisite for hippocampus-related cognitive and synaptic abnormalities to develop in offspring with a history of prenatal infection. Therefore, our data do not support the hypothesis that prenatal immune activation may exacerbate neuronal aging by inducing persistent systemic inflammation and accompanied neuroinflammatory reactions throughout brain maturation and aging (Krstic *et al*, 2013; Krstic *et al*, 2012). If anything, our findings suggest that the aging-related aggravation of hippocampal dysfunctions following prenatal immune activation are associated with increased systemic levels of two cytokines with potent anti-inflammatory activities, namely IL-4 and IL-10 (Curfs *et al*, 1997). Current attempts to delineate the relative contribution of systemic or brain inflammation to cognitive impairments following prenatal immune challenge have mostly focused on pro-inflammatory markers, but have thus far largely neglected the anti-inflammatory system (Krstic *et al*, 2012; Mattei *et al*, 2014). This seems surprising because soluble factors with anti-inflammatory actions such as IL-4 are known to exert a significant impact on neuronal and cognitive functions as well (Baruch *et al*, 2013; Chakrabarty *et al*, 2012; Gadani *et al*, 2012).

We acknowledge that our study did not directly address whether and to what extent the aging-related changes in serum IL-4 and IL-10 could relate to the emergence of exacerbated cognitive and synaptic abnormalities in aged immune-exposed offspring. There is initial evidence, however, suggesting that increased peripheral levels of IL-4 and IL-10 positively correlate with the rate of cognitive decline in elderly people with dementia (Leung *et al*, 2013). This raises the question whether abnormal anti-inflammatory signalling may be detrimental to brain aging as well, especially in subjects with a predisposition to aging-related neuronal and cognitive impairments.

In conclusion, our study provides converging evidence that prenatal immune challenge exacerbates hippocampus-related cognitive aging in the absence of persistent systemic or hippocampal inflammation. Our findings may be particularly relevant for the identification of early-life risk factors for cognitive disorders with onsets during late stages of life. It has been suggested that prevention of maternal infection during pregnancy may be effective in reducing neuropsychiatric disorders with neurodevelopmental components (Brown and Patterson, 2011a). Our findings may be taken to encourage similar considerations in the context of preventive practice against prenatal infection-induced exacerbation of cognitive aging.

4.5 Late Prenatal Immune Activation Induces Alterations in Myelination: Insight from a Genome-Wide Approach

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Unpublished

In order to find new potential mechanisms underlying the association between prenatal infection and psychiatric disorders, such as schizophrenia, we were interested in investigating the long-term molecular alterations emerging after gestational treatment with the viral mimetic polyriboinosinic-polyribocytidilic acid [Poly(I:C)]. For this purpose, we used an unbiased genome-wide approach. This approach may readily provide new insight into the precise pathways and systems altered by the prenatal manipulation, which in turn may unravel novel targets for therapeutic intervention in subjects with (immune-mediated) neurodevelopmental disturbances..

We treated pregnant dams with Poly(I:C) on gestation day 17 (GD17). This period corresponds roughly to the second trimester of human pregnancy, which is crucial for nervous system development and neuronal proliferation and differentiation (Clancy *et al*, 2007). Once the offspring reached adulthood, they were first subjected to behavioural testing in order to verify typical behavioural phenotypes associated with prenatal Poly(I:C) exposure (see previous sections). Specifically, we probed the cognitive functioning of these animals in the spatial novelty preference Y-maze paradigm, that is related to working memory and learning, and went on to assess their social behaviour in the social interaction and recognition test. Lastly, a separate cohort of animals was subjected to an acute challenge with systemic amphetamine, in order to uncover possible functional imbalances in dopaminergic transmission.

We then proceeded to investigate the underlying molecular phenotypes that could contribute to the behavioural deficits following prenatal Poly(I:C) exposure. To this end, we implemented an unbiased genome-wide based approach to analyse the transcription profile of different brain regions implicated in psychiatric disorders. In particular, we performed Microarray analysis of the prefrontal cortex and the nucleus accumbens. These brain areas were selected based on their functional importance in various neuropsychiatric conditions and experimental models of immune-mediated neurodevelopmental disruption. Besides other functions, the prefrontal cortex is critical for the planning of goal-directed behaviour, decision making and controlling social behaviour, whereas the nucleus accumbens plays a fundamental role in the reward circuit, including reinforcement learning, and is involved in several of the positive symptoms of schizophrenia ((Csernansky and Bardgett, 1998; Del Arco and Mora, 2009; Floresco *et al*, 2009; Grace, 2000)).

4.5.1 Prenatal Immune Activation Induces Behavioural Alterations in the Form of Impaired Spatial Working Memory and Altered Social Approach and Recognition

First, we investigated the impact of prenatal exposure to Poly(I:C) on spatial working memory using a spatial novelty preference paradigm in the Y-maze. In line with our previous studies, offspring born to vehicle-treated mothers displayed a clear preference for the novel arm instead of the familiar one (**Fig. 25a**); in contrast, Poly(I:C) offspring showed no obvious preference for the novel arm, indicating impaired spatial novelty preference in these animals. Statistical support for these observations was obtained in the *t*-test of relative time spent in the novel arm that yields a significant main effect of prenatal treatment ($p < 0.05$).

We next performed the social interaction test. As shown in **Figs. 25b,c**, prenatal immune challenge leads to reduced social interaction in the social interaction test, both in the form of social approach and social recognition. In the first phase of the test, control offspring born to vehicle-treated mothers displayed a clear preference towards the mouse, while offspring exposed to prenatal Poly(I:C) treatment showed no such preference (**Fig. 25b**). In the second phase of the test, control offspring again showed a clear preference for the novel mouse, while Poly(I:C) offspring did not discriminate between the familiar and novel mouse (**Fig. 25c**). The relative exploration time was recorded during a 5-min test period and analysed as % time spent with the live mouse and with the novel mouse, respectively. Statistical support for these impressions was obtained by a *t*-test of percent exploration time, which yielded a significant main effect of treatment on the interaction with the live and novel mouse ($p < 0.05$), respectively.

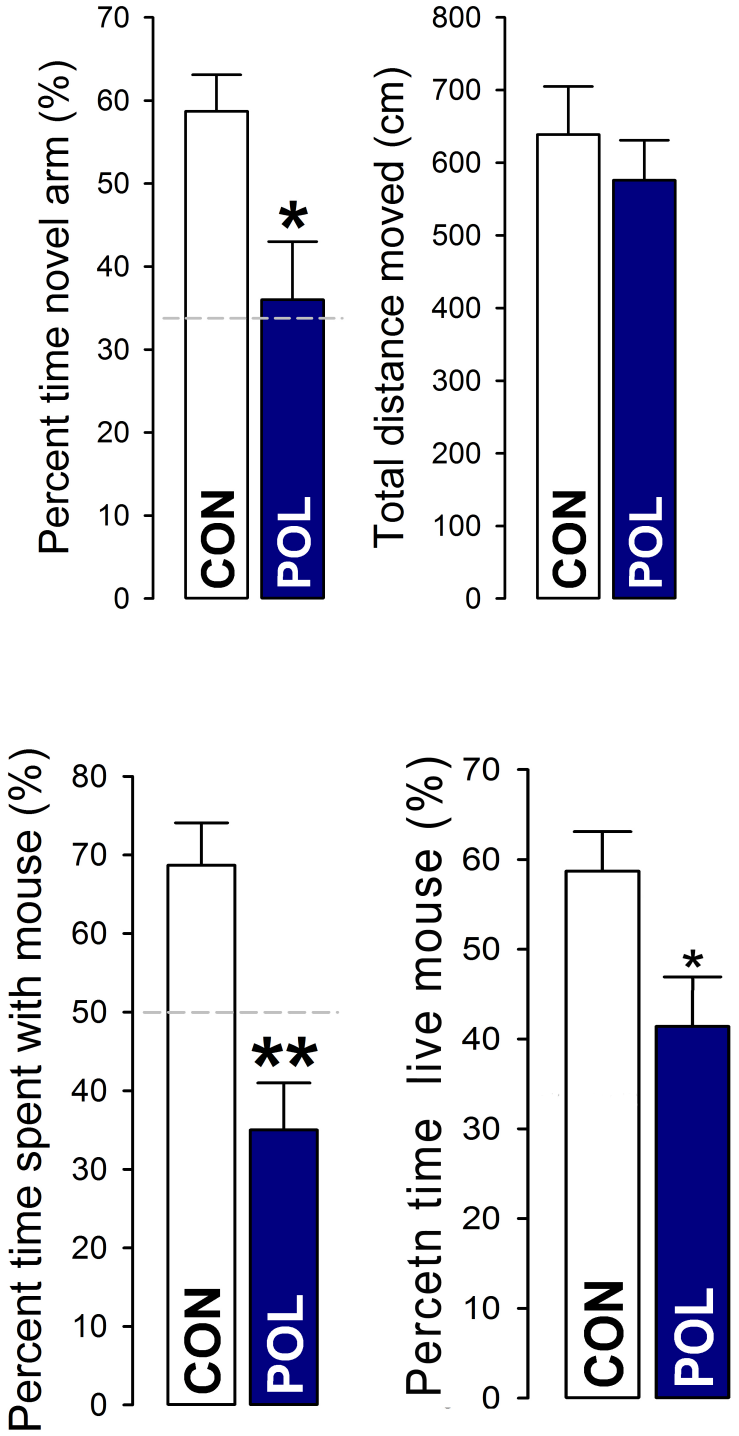

Figure 25. Prenatal poly(I:C) exposure impairs spatial working memory, social approach and social recognition. The bar plots depicts the percent (%) time spent in the novel arm during the choice phase of the test, the % time spent exploring the live mouse and the % time spent with the novel mouse, respectively *p < 0.05, reflecting the significant main effect of prenatal treatment in the analysis of percent time spent in the novel arm based on T-TEST. All values are means ± SEM.

4.5.2 Prenatal Immune Activation Induces Behavioural Alterations in the Form of Increased Amphetamine Sensitivity

Lastly, we investigated whether this cohort of animals presented heightened sensitivity to amphetamine, a consistent behavioural trait observed after prenatal Poly(I:C) exposure. Thus, we compared the locomotor-enhancing effects of acute AMPH exposure in Poly(I:C) and control offspring. For this purpose, we exposed the animals to acute systemic AMPH (2.5 mg/kg, i.p.) following an initial saline (vehicle) administration phase and measured the animals' locomotor response to the drug challenge in a standard open field. The prenatal manipulation did not affect basal locomotor activity (**Fig. 26**) during the initial vehicle administration phase. On the other hand, acute amphetamine administration led to a general increase in locomotor activity, as demonstrated by the significant main effect of bins. Consistently with our previous findings, the locomotor-enhancing effects of acute exposure to amphetamine were strongly potentiated in Poly(I:C) animals, suggesting increased sensitivity to the drug. Statistical support for these observations was provided by the ANOVA of distance moved which yielded a significant main effect of prenatal treatment [$F(1,27) = 12.78$, $p < 0.01$], bins [$F(11,297) = 62.44$, $p < 0.001$] and its interaction [$F(11,297) = 3.16$, $p < 0.001$]. No other effects attained statistical significance.

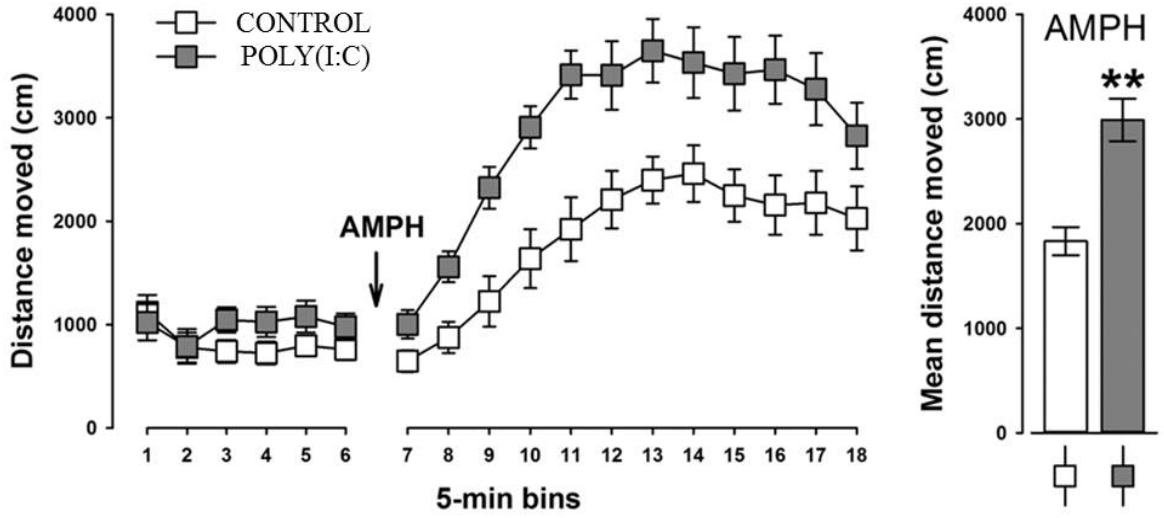

Figure 26. Prenatal exposure to Poly(I:C) leads to increased sensitivity to amphetamine. The line plot depicts the distance moved (cm) as a function of 5-min bins during the initial habituation period and after systemic treatment with AMPH, and the bar plots shows the mean distance moved across the entire post-AMPH treatment phase. The distance moved during the initial saline phase was analysed using a 2 x 6 (prenatal treatment x bins) repeated-measure ANOVA. This analysis yielded no significant main effects or interactions, suggesting that control and Poly(I:C)-exposed offspring displayed similar basal locomotor activities. The distance moved following AMPH treatment was analysed using a 2 x 12 (prenatal treatment x bins) repeated-measure ANOVA. **p < 0.01, reflecting the significant main effect of prenatal treatment. N(CON) = 18 and *N*(POL) = 11.

4.5.3 Microarray Analysis of the Prefrontal Cortex from Mice Exposed to Prenatal Immune Activation

As shown in **Fig 27**, which represents the hierarchical clustering of expression changes induced by prenatal infection with Poly(I:C), we found 185 genes that were differentially expressed in the prefrontal cortex (fold change cutoff: ± 1.2; $p < 0.05$). In the hierarchical clustering, the genes are grouped together with the aim to deduce the relationships between the different clusters. It represents, for each single sample, the expression levels of a given gene and how this gene is expressed in the analysed subjects. Of the 185 genes that were differentially expressed, 71 were down-regulated and 114 were up-regulated. The top twenty most up-regulated genes in terms of fold change are presented in the **Table 7**, while the top twenty most down-regulated genes are listed in **Table 8.**

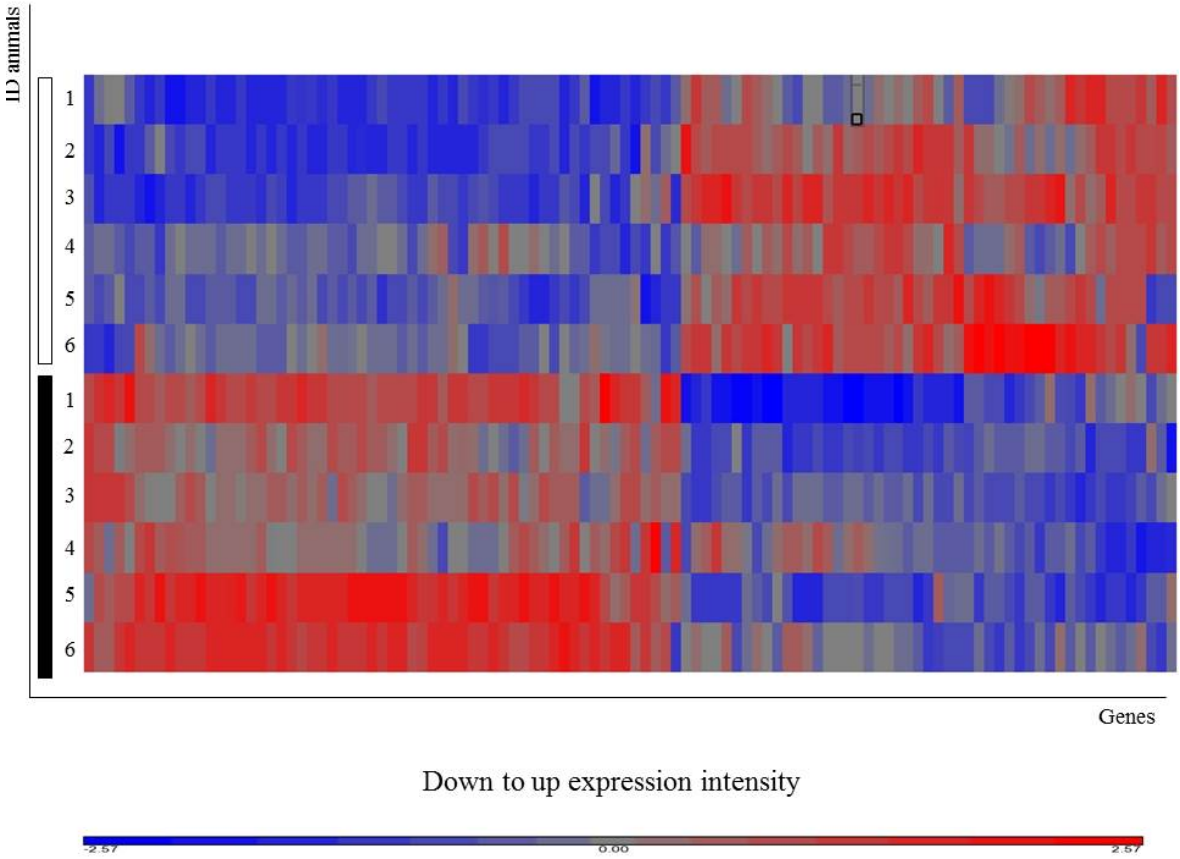

Figure 27. Hierarchical clustering of gene expression changes in the prefrontal cortex after prenatal infection with Poly(I:C). Each rectangle on the x axis corresponds to a single gene, while the rectangles on y axis represent each control and Poly(I:C) animal. Red indicates genes that are up-regulated, while blue indicates genes that are down regulated. In the image it is clear how the differentially expressed genes are, for example, up-regulated in control animals and down-regulated in Poly(I:C) exposed animals.

Top 20 Up-Regulated Genes

Gene symbol		p-value	Fold-Change
Npas4		0,0138476	1,59753
Vmn2r87		0,00098056	1,54698
Mir128-1		0,00000971088	1,51712
Mir382		0,00469434	1,48828
BC030499		0,000214599	1,48167
Fam36a		0,001944	1,46433
Snord14c		0,00537517	1,44522
Mir679		0,00176899	1,44017
Snord14c		0,00567927	1,43686
Mir410		0,0000284079	1,41920
Snord14e		0,00674074	1,40522
C230004F18Rik		0,000918765	1,39085
Mir344		0,00126448	1,38651
Leng8		0,00259674	1,37652
Mir377		0,00154195	1,37340
Mir344		0,00185084	1,36772
Mir376b		0,00559728	1,36103
Gm10759		0,000588411	1,35760
Ttc14		0,000611469	1,35582
Uggt2		0,000499779	1,35536

Table 7. Top twenty up-regulated genes in the mPFC.

Top 20 Down-Regulated Genes

Gene symbol		p-value	Fold-Change
Snord47		0,0000513461	-2,07249
Snord32a		0,000000109466	-1,98017
Snord34		0,0000000331084	-1,83875
Snord33		0,00000165274	-1,81872
Snord87		0,0000018579	-1,80696
Snord49a		0,0000958707	-1,78046
Gas5		0,0000019195	-1,72427
Snord82		0,0000236971	-1,65563
Rpl13		0,0000127176	-1,64535
Cryge		0,0163217	-1,63784
Rny1		0,00240824	-1,57444
Snord35b		0,00000263206	-1,54314
Gm11362		0,00000027052	-1,53481
Snord104		0,000271503	-1,52484
Drd2		0,0298378	-1,45221
Gpr6		0,0118314	-1,43112
Thbs4		0,0015016	-1,41704
Mobp		0,000436516	-1,41191
Snord35a		0,00000543427	-1,40553
5530400C23Rik		0,00157834	-1,36654

Table 8. Top twenty down-regulated genes in the mPFC.

4.5.3.1 Prenatal immune activation impacts different biological functions important for nervous system development and function

After having performed the microarray analysis, we ran a pathway analysis using Ingenuity Pathways Analysis software (IPA), as this analysis best captures the diverse and complex mechanisms possibly altered by prenatal immune activation (Thomas and Bonchev, 2010). In particular, Ingenuity Pathway Analysis (IPA) software facilitates the interpretation of microarray results, allowing one to address the biological and functional impact of the gene expression changes uncovered by the microarray analysis. As shown in **Table 9**, we identified 5 pathways that were significantly regulated by prenatal immune activation ($p < 0.05$). Of particular relevance is the G protein Coupled Receptor signalling, which incorporates important signalling pathways involved in an extensive variety of physiological processes, as regulation of behavioural and mood, immune system activity regulation and cell density detecting. Moreover, prenatal immune activation also alters Nerve growth factor (NGF) signalling, which has a trophic function on nervous cells and other cells associated with the nervous system, such as endocrine and immune cells. Ultimately, the cAMP-dependent pathway is necessary for many living organisms and life processes and mediates many different cell responses, while abnormalities in sphingomyelin metabolism can impact membrane biology and cell function. The network's score, or p score, is the exponent of the p value determined using Fisher's exact test. We used $p < 0.05$ as enrichment p-value cutoff for pathway analysis.

Finally, we investigated specific biological processes that could be affected by prenatal Poly(I:C) by analysing the networks that could be impacted by prenatal Poly(I:C)- induced changes in gene expression. The main networks associated with the gene expression changes we observed are cellular development, cellular growth and proliferation and nervous system development and function (**Table 10**). The most relevant network, namely nervous system development and function, is also graphically represented in **Fig. 28**.

Top Canonical Pathways

Name	p-value	Ratio
G-Protein Coupled Receptor Signalling	1.14E-02	5/276
		(0.018)
NGF Signalling	1.88E-02	3/122
		(0.025)
Gα12/13 Signalling	2.4E-02	3/127
		(0.024)
cAMP-mediated Signalling	2.83E-02	4/226
		(0.018)
Sphingomyelin Metabolism	4.11E-02	1/16
		(0.062)

Table 9. Top affected canonical pathways in the Poly(I:C) prefrontal cortex. The top pathways are ranked together with p-value, which specifies the statistical significance, and the ratio, the number of altered genes compared to the total number of genes for that specific pathway.

Top Networks

ID Associated Network Functions	*Score*
Cellular development, Cellular growth and proliferation, Nervous System development and function	42
Cell signalling, Nucleic Acid metabolism, Small molecule biochemistry	36
Connective tissue disorders, Developmental disorder, Hereditary disorder	27
Connective tissue disorders, Developmental disorder, Hereditary disorder	25
Nutritional disease, Molecular transport, Small molecule biochemistry	23

Table 10. Selected top five implicated networks in the prefrontal cortex and the related level of involvement (score).

Nervous System Development and Function

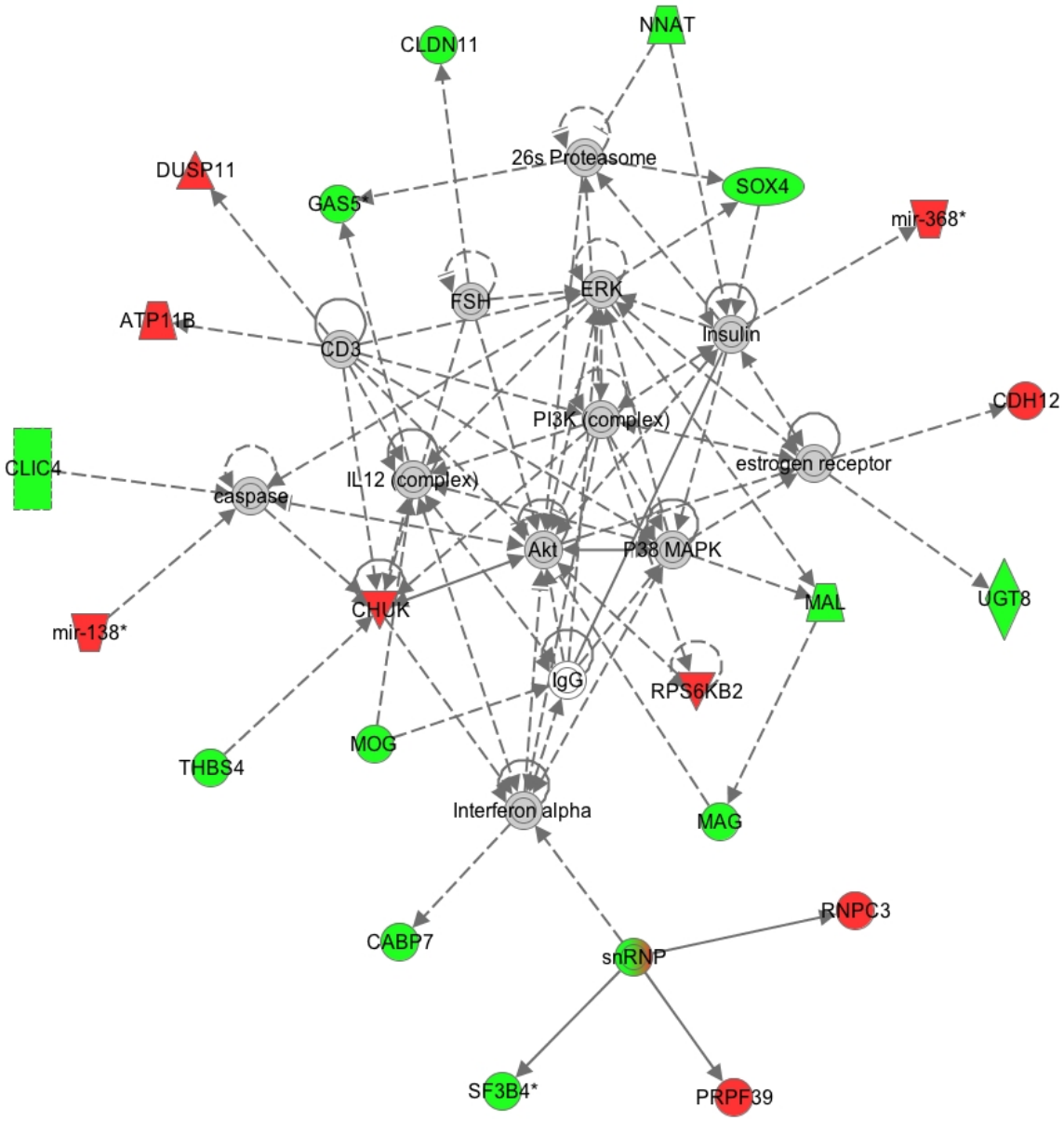

Figure 28. Graphical representation of nervous system development network in the Poly(I:C) prefrontal cortex by Ingenuity system. In green down-regulated genes, in red the up-regulated ones.

4.5.4 Microarray Analysis of the Nucleus Accumbens from Mice Exposed to Prenatal Immune Activation

After having analysed the prefrontal cortex, we performed microarray analysis of the nucleus accumbens. Once again, in **Fig 29,** we report the hierarchical clustering of expression changes induced by prenatal infection with Poly(I:C), and we observed that 325 genes were differentially expressed in the NAc following prenatal infection (fold change cutoff: ± 1.2; $p < 0.05$).

Of the 325 genes that were differentially expressed, 159 were up-regulated while 166 were down-regulated. The top twenty most up-regulated genes in terms of fold change are presented in the **Table 11**, while the top twenty most down-regulated genes are listed in **Table 12.**

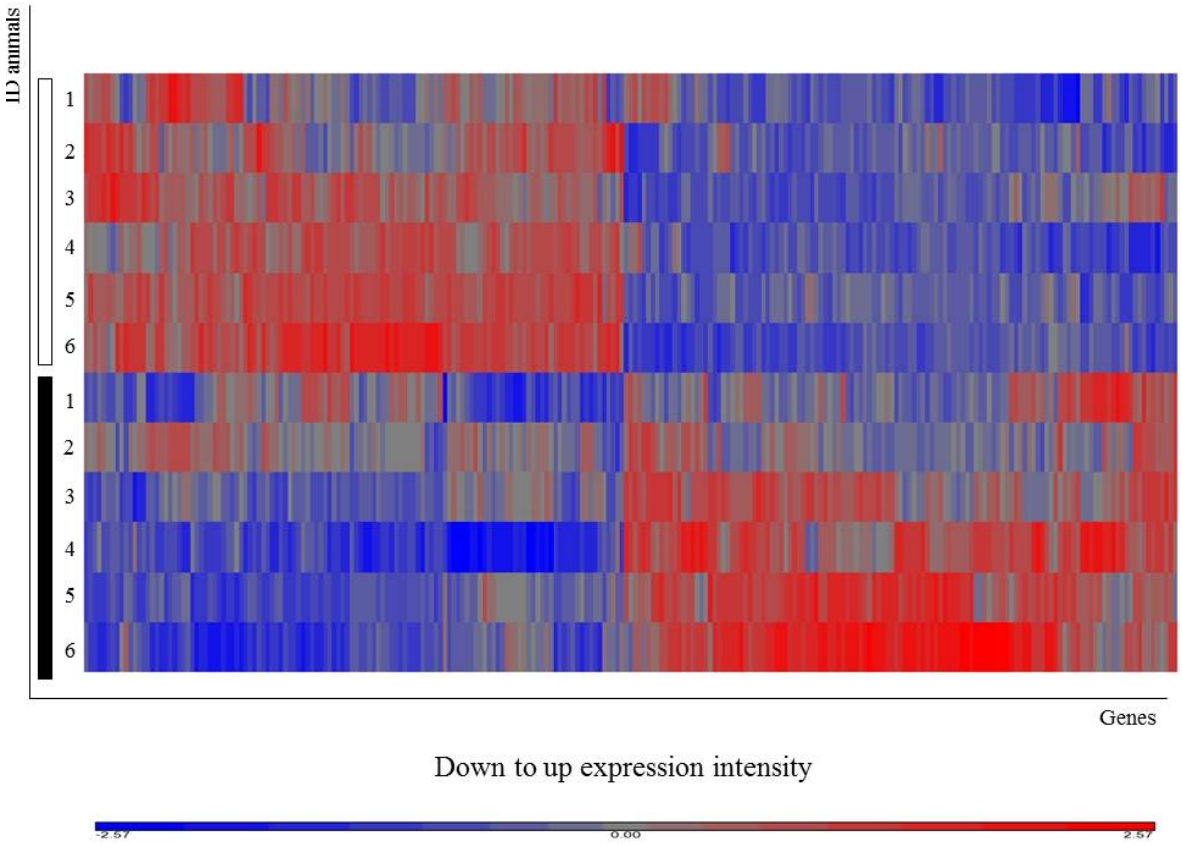

Figure 29. Hierarchical clustering of gene expression changes in the nucleus accumbens after prenatal infection with Poly(I:C). Each rectangle on the x axis corresponds to a single gene, while the rectangles on y axis represent each control and Poly(I:C) animal. Red indicates genes that are up-regulated while blue indicates genes that are down regulated. In the image it is clear how the differentially expressed genes are, for example, up-regulated in control animals and down-regulated in Poly(I:C) exposed animals.

Top 20 Up-Regulated Genes in the Nucleus Accumbens

Gene symbol		p-value	Fold-Change
Slc17a7		0,0290275	2,85106
Nov		0,00894434	2,66046
3110035E14Rik		0,00841871	2,29052
Nmbr		0,0209392	1,74568
Stard5		0,00453269	1,69014
Nptx1		0,00896167	1,65287
Ctgf		0,0282534	1,64784
St3gal1		0,0000882596	1,58939
Npas4		0,00953208	1,54037
Drd3		0,000182858	1,5163
Prss12		0,0271643	1,49249
Cnih3		0,0184109	1,46211
Mir382		0,0107333	1,45844
Npsr1		0,0143958	1,4527
Sidt1		0,0127782	1,43759
B3galt2		0,0134754	1,43701
Islr		0,0321029	1,41723
Htr1a		0,0243749	1,40838
Fam131a		0,00111559	1,40558
Prdm8		0,041371	1,40056

Table 11. Top twenty up-regulated genes in the NAc.

Top 20 Down-Regulated Genes in the Nucleus Accumbens

Gene symbol		p-value	Fold-Change
Ttr		0,00282672	-7,21783
Snora3		0,0000480909	-3,02317
Tac2		0,00268061	-2,34182
Gal		0,00221473	-2,31403
Prlr		0,00737445	-2,07634
Cryge		0,017578	-1,85538
Snora44		0,000595043	-1,83929
Lbp		0,0302795	-1,82342
Rnu2-10		0,00177219	-1,79227
Rnu2-10		0,00177219	-1,79227
Rnu2-10		0,00177219	-1,79227
Rnu2-10		0,00177219	-1,79227
Rnu2-10		0,00173195	-1,78354
Rnu2-10		0,00269842	-1,72149
Enpp2		0,00898319	-1,6902
Rnu2-10		0,00276675	-1,68464
Nr2f2		0,00307328	-1,67239
Stoml3		0,0336009	-1,66704
Snora16a		0,0000892206	-1,64797
Rnu12		0,00437322	-1,63890

Table 12. Top twenty down-regulated genes in the NAc.

4.5.4.1 Prenatal immune activation impacts different biological functions important for nervous system development and function

Once again, after the microarray analysis, we ran a pathway analysis using Ingenuity Pathways Analysis software (IPA). In **Table 13**, we report the 5 most affected pathways that were significantly regulated by prenatal immune activation ($p < 0.05$). They include G protein-coupled receptor signalling and cAMP-dependent signalling, which we also found enriched in the prefrontal cortex. This could be particularly relevant as these signalling pathways are considered necessary for many living organisms and life processes and mediate many different cell responses. The changes we observed are also associated with serotonin receptor signalling and ERK5 signalling. Extracellular signal-regulated kinase 5 (ERK5) is the most recently identified member of the mitogen-activated protein kinase (MAPK) family and it is activated by a variety of extracellular stimuli, for instance cellular stresses and growth factors, and regulates processes such as cell proliferation and differentiation.

Secondly, we analysed our results also in terms of the functional networks affected by the gene expression changes induced by prenatal immune activation. Interestingly, the main affected networks were again relevant to developmental processes important for nervous system development and function, cell and tissue development and embryonic development (**Table 14**). The most relevant network, namely nervous system development and function, is also graphically represented in **Fig. 30**.

Top Canonical pathways

Name	p-value	Ratio
G-Protein Coupled Receptor Signalling	2.23E-04	12/276
		(0.043)
cAMP-mediated Signalling	8.83E-04	10/226
		(0.044)
Serotonin Receptor Signalling	1.00E-02	3/49
		(0.061)
ERK5 Signalling	1.12E-02	4/68
		(0.059)
Acyl Carrier Protein Metabolism	1.36E-02	1/6
		(0.167)

Table 13. Top affected canonical pathways in the Poly(I:C) Nucleus Accumbens. The top pathways are ranked together with p-value, which specifies the statistical significance, and the ratio, the number of altered genes compared to the total number of genes for that specific pathway.

Top Networks

ID Associated Network Functions	Score
Nervous System development and function, Tissue development, Neurological disease	46
Cell development, Embryonic development, Nervous System development and function	44
Cellular assembly and organization, Cellular compromise, Cardiovascular disease	36
Gastrointestinal disease, Lipid metabolism, Small molecule biochemistry	34
Neurological disease, Psychological disorders, Behaviour	27

Table 14. Selected top five implicated networks in the prefrontal cortex and the related level of involvement (score).

Nervous System Development and Function

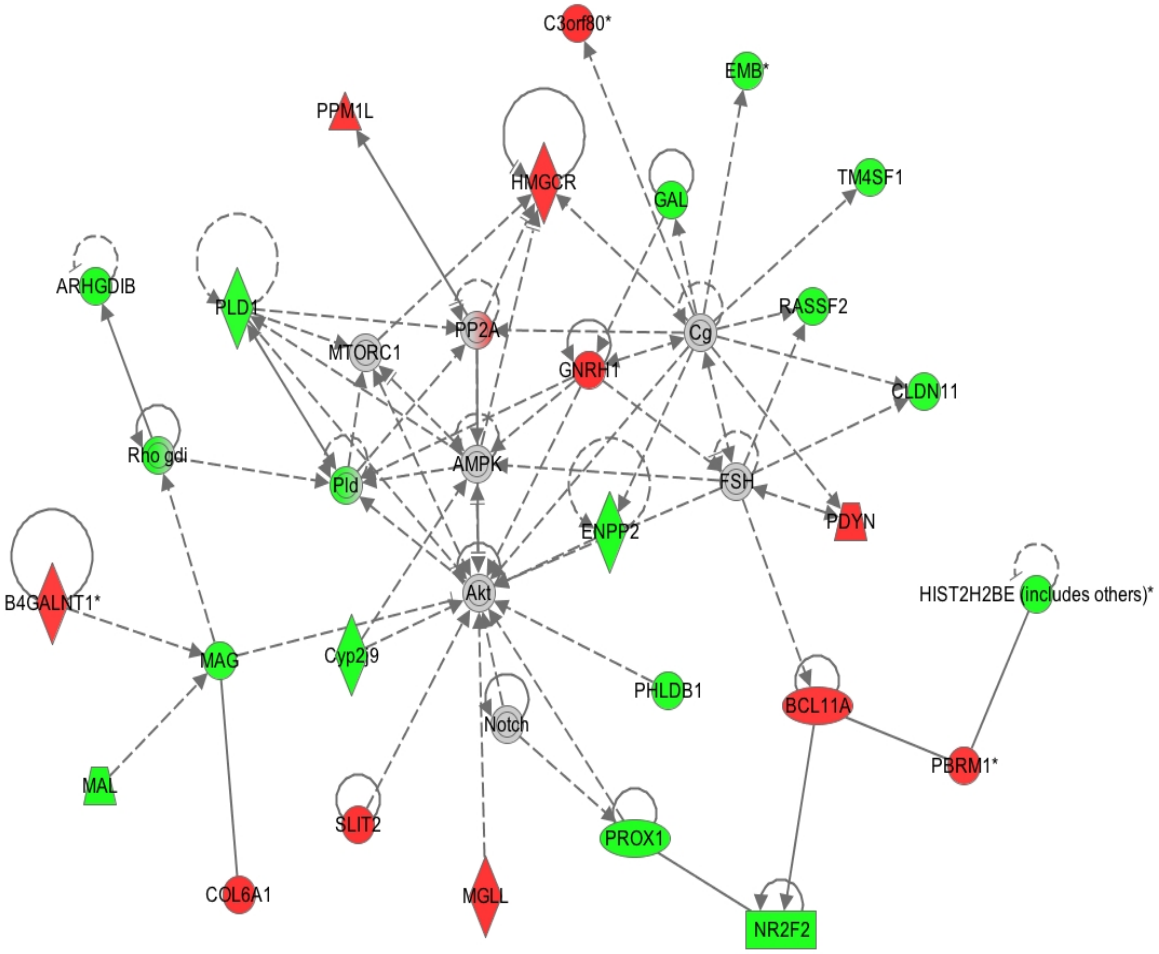

Figure 30. Graphical representation of nervous system development network in the Poly(I:C) Nucleus Accumbens by Ingenuity system. In green down-regulated genes, in red the up-regulated ones.

4.5.5 Analysis of the Overlap between Prefrontal Cortex and Nucleus Accumbens

After having analysed the prefrontal cortex and the nucleus accumbens separately, we performed an overlap of the gene expression changes in the two brain areas, in order to short list the wide array of information obtained with the microarray analysis and to focus on the most relevant functions altered by prenatal infection. As shown in **Fig. 31**, the Venn diagram of the overlap highlights 27, 20 of which are modulated in the same direction in both brain areas (**Table 15**).

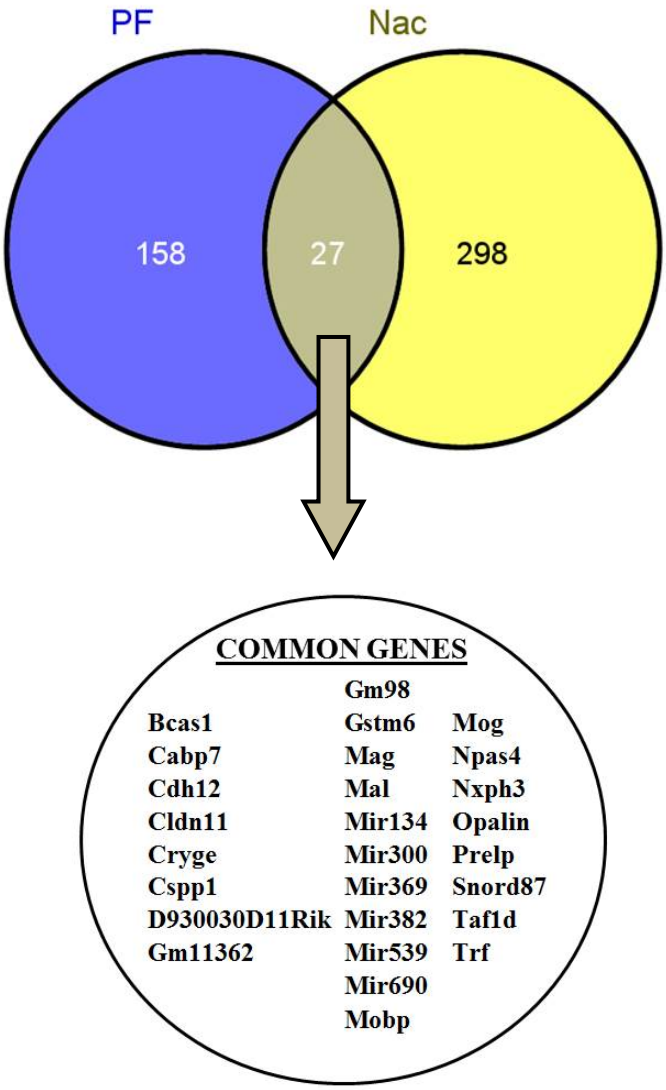

Figure 31. Venn diagram of the overlap of changes observed in the prefrontal cortex and nucleus accumbens. The diagram shows all possible logical relations between a finite collection of sets. In particular, this involves two sets, PF and Nac, represented here as colored circles. The blue circle, set PF, represents all genes modified in the prefrontal cortex; the yellow circle, set Nac, represents the genes changed in the nucleus accumbens. Each gene can be imagined as a point somewhere in the diagram, but the genes which are involved in both regions correspond to points in the area where the blue and yellow circles overlap.

Gene	Fold Change in PFC	Fold Change in NAc
Snord87	↓	↓
Cryge	↓	↓
Gm11362	↑	↓
Mobp	↓	↓
Cldn11	↓	↓
Opalin	↓	↓
Mal	↓	↓
Mag	↓	↓
Mog	↓	↓
Cabp7	↑	↓
Gstm6	↓	↓
Trf	↓	↓
Gm98	↓	↓
Bcas1	↓	↓
Prelp	↓	↓
Nxph3	↑	↓
Cspp1	↓	↑
Taf1d	↓	↑
Cdh12	↑	↑
Mir690	↓	↑
Mir539	↑	↑
Mir134	↑	↑
Mir369	↑	↑
D930030D11Rik	↓	↑
Mir300	↑	↑
Mir382	↑	↑
Npas4	↑	↑

Table 15. Regulation of the 27 common modulated genes in the PFC and NAc.

After having performed the overlap, we ran an Ingenuity Pathways Analysis and reported the canonical pathways over represented in these 27 common genes. Different pathways are significantly regulated in both areas. As shown in **Table 16**, the first one is the Notch signalling pathway, important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life. Notch signalling also plays a role in neuronal function and development. Moreover, the common genes were enriched in the G protein subunit receptor signalling, and in Liver X receptors (LXR) activation, which play a critical role in gene transcription, lipid synthesis and barrier formation. We then performed a network analysis to study the common biological processes affected in the Poly(I:C) model. As shown in **Table 17**, neurological disease (graphically represented in **Fig 32**) and cellular dysfunction are the most significantly altered networks.

Lastly, we analysed in detail the precise cellular functions and networks that were associated with neurological disease. Among others, myelination resulted as the most affected function associated with neurological disease. In particular, we observed that Poly(I:C) exposure affects the expression of three main genes involved in myelination both in the prefrontal cortex and in the nucleus accumbens: myelin and lymphocyte protein (MAL), myelin-associated glycoprotein (MAG) and myelin-associated oligodendrocytic basic protein (MOBP). Because of this evidence and the importance of myelination for the correct functioning of the nervous system, we validated these genes, together with other genes associated with myelination, with qRT-PCR analysis.

Top Canonical Pathways

Name	p-value	Ratio
Notch Signalling	2.35E-02	1/43
		(0.023)
Gα12/13 Signalling	7.15E-02	1/127
		(0.008)
LXR/RXR Activation	7.32E-02	1/139
		(0.007)
Tight Junction Signalling	9.17E-02	1/167
		(0.006)
Acute Phase Response Signalling	1.01E-01	1/181
		(0.006)

Table 16. Top affected canonical pathways common to the PFC and NAc. The top pathways are ranked together with p-value, which specifies the statistical significance, and the ratio, the number of altered genes compared to the total number of genes for that specific pathway.

Top Networks

ID Associated Network Functions	Score
Cellular compromise, Renal dysfunction, Neurological disease	30
Neurological disease, Drug metabolism, Molecular transport	9

Table 17. Selected top implicated networks common to the PFC and NAc and the related level of involvement (score).

Neurological Disease

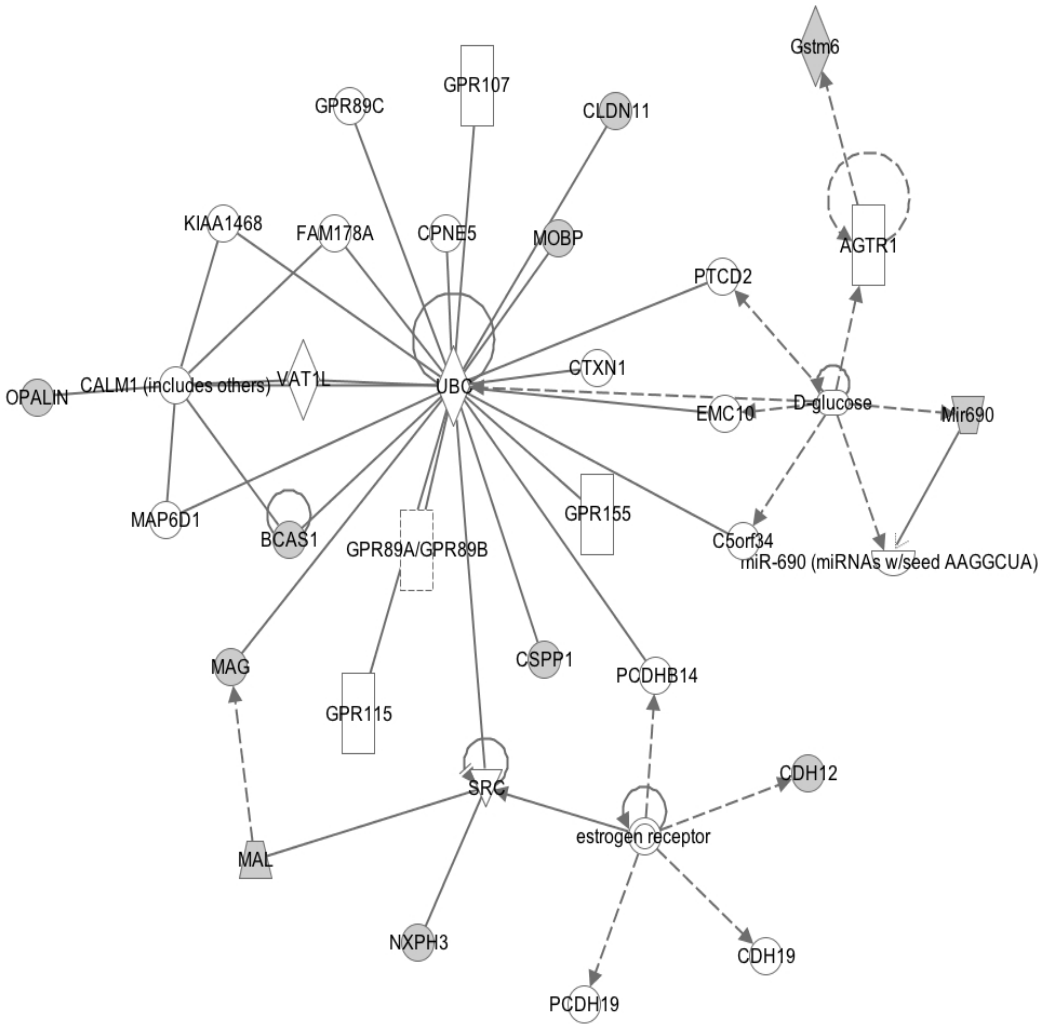

Figure 32. Neurological disease network common to the prefrontal cortex and nucleus accumbens by Ingenuity system.

4.5.6 Validation of the Microarray Results with Real-Time-qRT-PCR

In order to validate some of the results obtained in the Microarray analysis we performed real-time qRT-PCR on individual mice to quantify and substantiate the observed changes in gene expression in the prefrontal cortex and nucleus accumbens. We focused on the gene expression levels of MAG, MOG, MOBP and MAL, as they highlight in the list of genes that are commonly affected in both brain areas. Moreover, these genes are all implicated in myelination, one of the main biological processes that resulted affected in Poly(I:C) offspring according to IPA analysis . All four genes, which were found to be down-regulated by Poly(I:C) in the gene array analysis, resulted decreased by the prenatal manipulation also when analysed with real-time RT-PCR. The results of this analysis are shown in **Fig. 33** and **Fig. 34**. Statistical support for these observation was provided by Student's t-test that yielded a significant effect of prenatal treatment for all four targets investigated ($p< 0.05$, $p< 0.01$ or $p<0.001$).

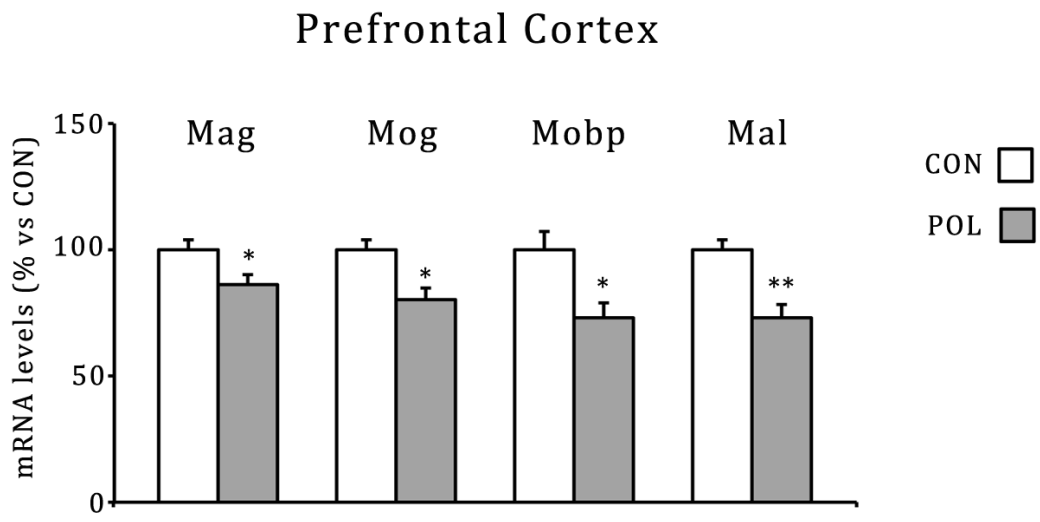

Figure 33. Effects of the prenatal manipulation on myelination genes expression in the prefrontal cortex. The graphs depict the levels of normalized mRNA expression in the prefrontal cortex (PF) assessed using qRT-PCR. *p < 0.05, **p < 0.01 based on T-TEST. All values are means ± SEM of at least 11 animals per group and are expressed as % vs control animals.

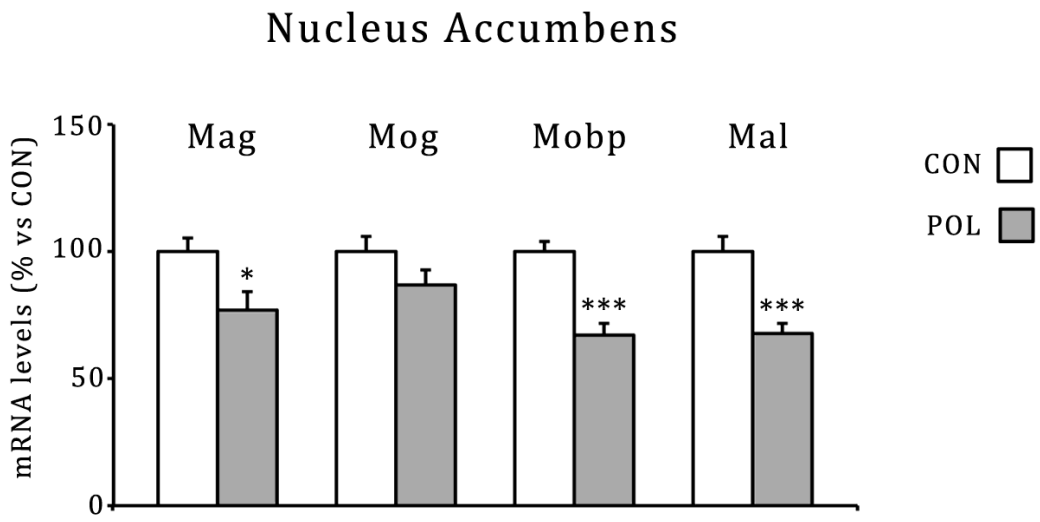

Figure 34. Effects of the prenatal manipulation on myelination genes expression in the nucleus accumbens. The graphs depict the levels of normalized mRNA expression in the prefrontal cortex (PF) assessed using qRT-PCR. *p < 0.05, **p < 0.01 based on T-TEST. All values are means ± SEM of at least 11 animals per group and are expressed as % vs control animals.

4.5.7 Pearson's Correlation between Behavioural Testing and Molecular Analysis

In order to establish a potential relationship between the molecular changes affecting myelin and the behavioural abnormalities observed in Poly(I:C) mice, we performed a correlation analysis between social behaviour, evaluated in the social interaction test, and the mRNA levels of some of the main genes involved in the myelination processes: MAG, MAL, MOBP and MOG.

First of all, we analysed the prefrontal cortex. For each animal (control and GD17), we took into account the total time spent with the mouse (expressed in % time mouse) and the value of gene expression levels in the prefrontal cortex (expressed as fold change) at PND 114, after the behavioural tests performed at PDN 92-106. As shown in **Fig. 35**, there is a somewhat linear correlation between the mRNA levels of each gene and the time spent with the live mouse, although these correlations do not reach statistical significance (MAG r = 0,349, n = 18, p > 0.10; MAL r = 0,233, n = 19, p > 0.10; MOBP R = 0,390, n = 19, p > 0.10; MOG e = 0,310, n = 20, p > 0.10 Pearson's correlation). Next, we investigated the correlation between the recognition phase of the social interaction test and the mRNA levels of the same four genes. For each animal

(control and GD17), we considered the total time spent with the novel mouse (expressed in % time mouse) and the value of gene expression levels in the prefrontal cortex (expressed as fold change) at PND 114, after the behavioural test performed at PDN 92-106. As shown in **Fig. 36**, the correlation between the mRNA levels of each gene and the time spent with the novel mouse is linear but it does not reach statistical significance (MAG r=0,089, n = 18, p > 0.10; MAL r = 0,372, n = 19, p > 0.10; MOBP r = 0,055, n = 19, p > 0.10; MOG r = 0,368, n = 20, p > 0.10 Pearson's correlation).

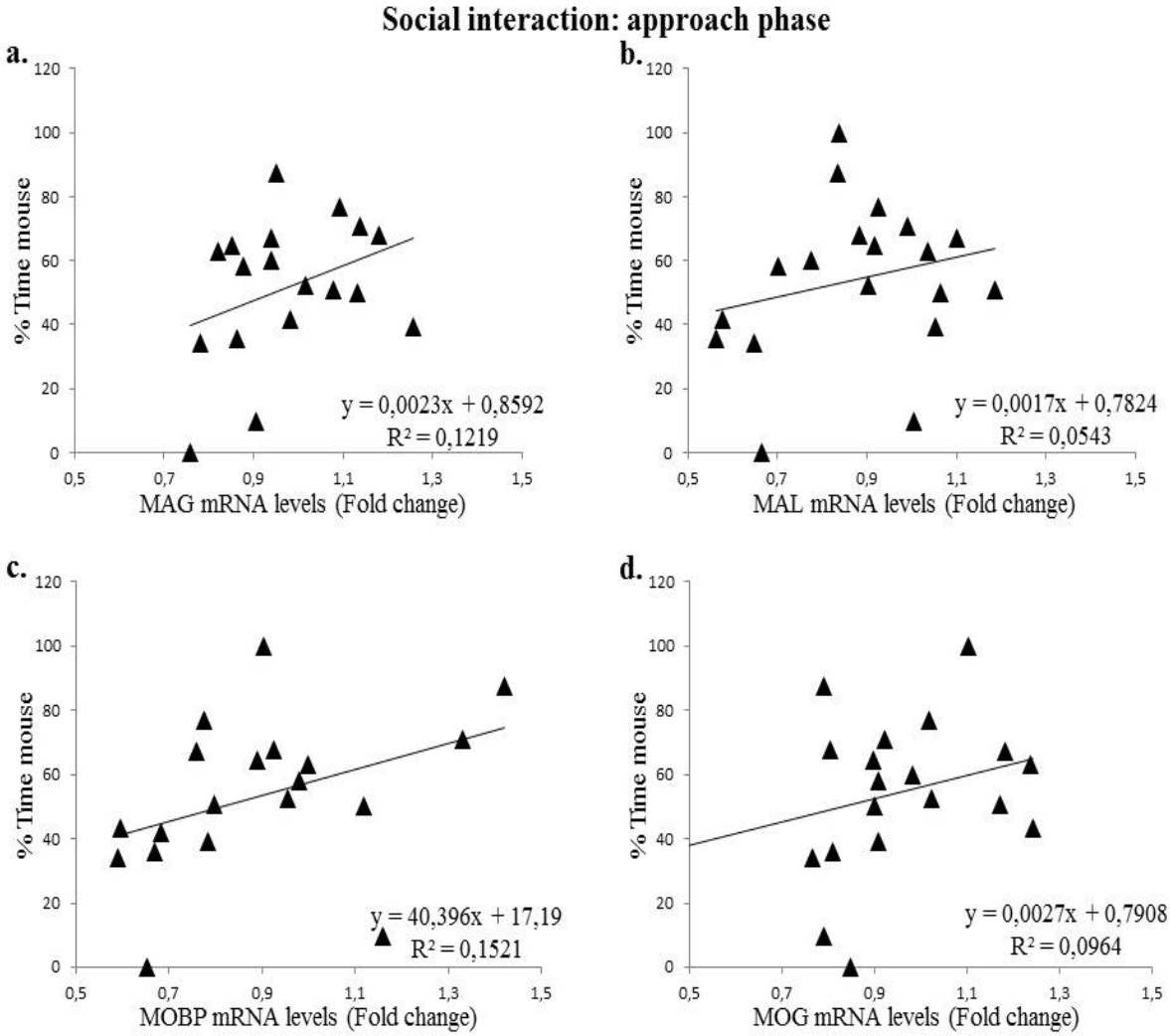

Figure 35. Pearson's correlation between the social approach phase of the social interaction test and the gene expression in the prefrontal cortex. The figure shows the correlation analysis between the social approach phase of the social interaction test and MAG, MAL, MOBP, MOG mRNA expression levels in the prefrontal cortex of control and Poly(I:C) exposed mice. Each dot represents the intersection between the % time spent with the live mouse and the level of mRNA expression levels (as fold change).

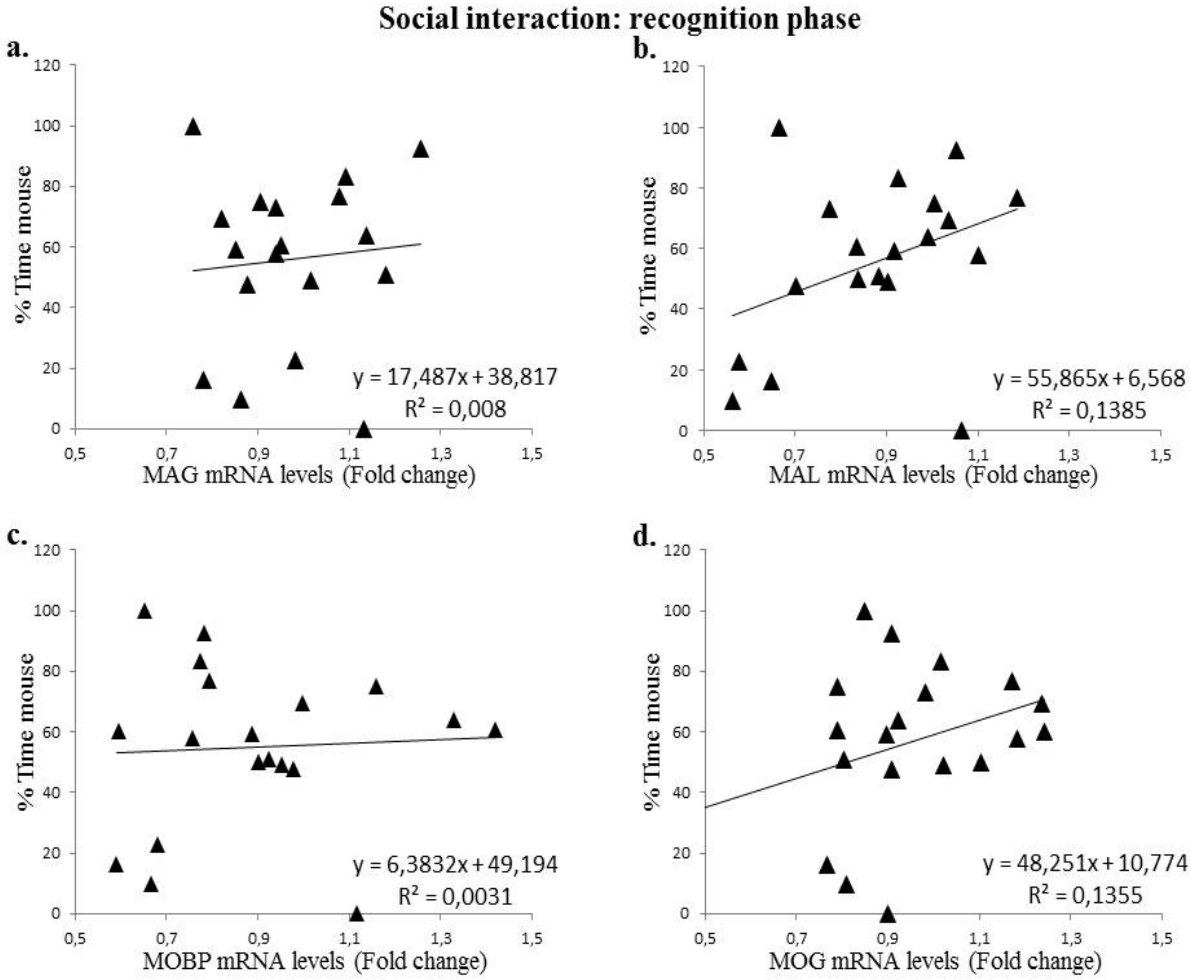

Figure 36. Pearson's correlation between recognition phase of the social interaction and the gene expression in the prefrontal cortex. The figure shows the correlation analysis between the social approach phase of the social interaction test and MAG, MAL, MOBP, MOG mRNA expression levels in the prefrontal cortex of control and Poly(I:C) exposed mice. Each dot represents the intersection between the value of % time spent with the novel mouse and the level of mRNA expression (as fold change).

Subsequently, we analysed the correlation between the approach phase of the social interaction test with the mRNA levels of MAG, MAL MOBP and MOG in the nucleus accumbens. For each animal (control and GD17), we took into account the total time spent with the mouse (expressed in % time mouse) and the value of gene expression levels in the nucleus accumbens (expressed as fold change) at PND 114, after the behavioural test performed at PDN 92-106. As shown in **Fig. 37**, there is a linear correlation between the mRNA levels of each gene and the time spent with the live mouse. Regarding MAL and MOG, the Pearson's correlation is not significant (MAL R =

0,303, n = 20, p > 0.10; MOG r = 0,149, n = 19, p > 0.10 Pearson's correlation); while MAG and MOBP gene expression showed a statistically significant direct correlation with the % time spent with the live mouse (MAG r = 0,465, n = 19, p < 0.05; MOBP r = 0,474, n = 19, p < 0.05 Pearson's correlation). These findings suggest that reduced expression of these genes in animals exposed to prenatal maternal immune activation could be associated with impaired social behaviour. Lastly, we explored the correlation between the recognition phase of the social interaction test with the mRNA levels of the four myelination genes in the nucleus accumbens. **Fig. 38** depicts this correlation. In particular, the Pearson's correlation is statistically significant only when considering MOBP gene expression levels (MOBP r = 0,476, n = 19, p < 0.05 Pearson's correlation). The direct correlation suggests that reduced expression of this gene in animals exposed to prenatal maternal immune activation could be directly associated with alterations in social recognition. The others correlation do not reach statistical significance (MAG r = 0,285, n = 19, p > 0.10; MAL r = 0,342, n = 20, p > 0.10; MOG r = 0,185, n = 19, p > 0.10 Pearson's correlation).

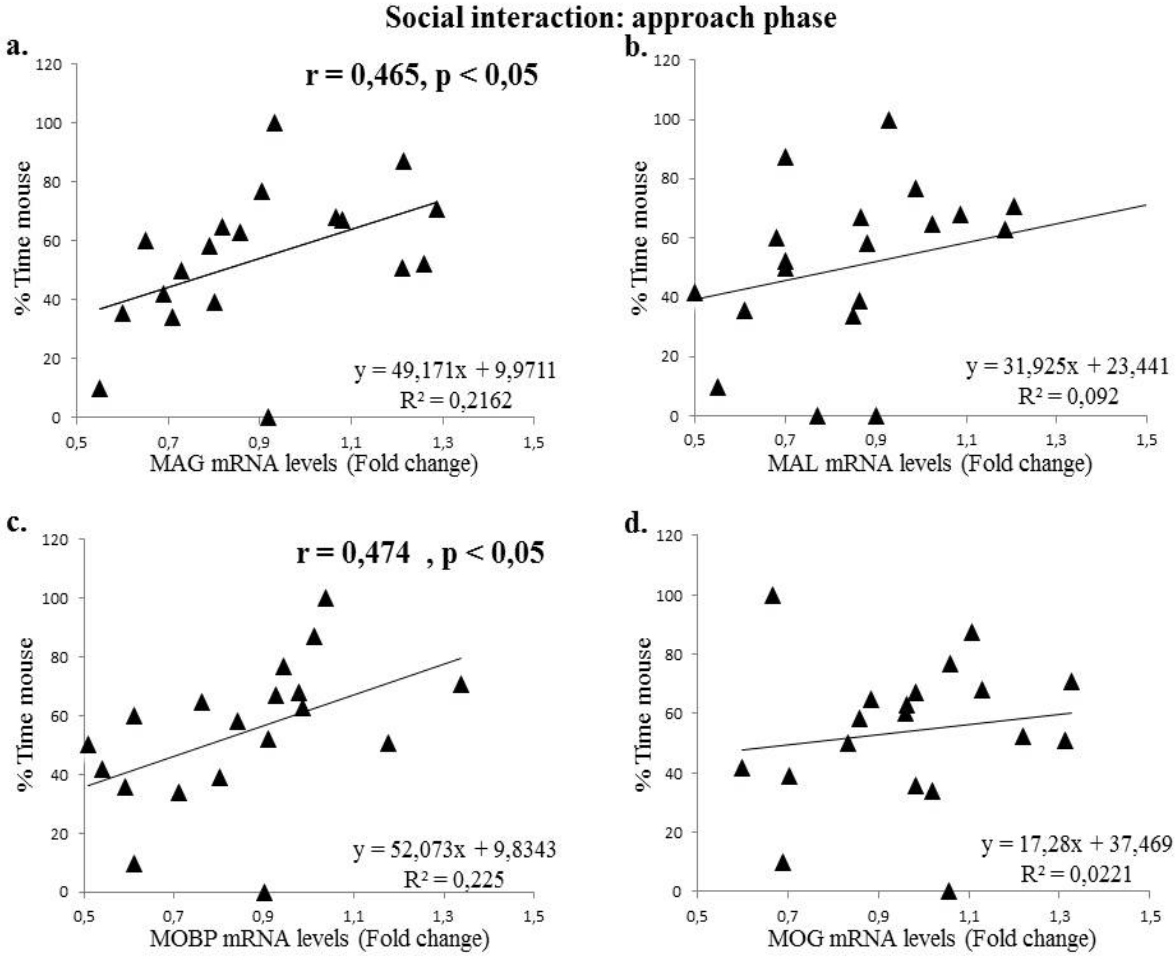

Figure 37. Pearson's correlation between approach phase of the social interaction and the gene expression in the nucleus accumbens. The figure shows the correlation analysis between the social approach phase of the social interaction test and MAG, MAL, MOBP, MOG mRNA expression levels in the nucleus accumbens of control and Poly(I:C) exposed mice. Each dot represents the intersection between the value of % time spent with the live mouse and the level of mRNA expression levels (expressed as fold change).
Pearson's correlation coefficient: MAG r = 0.465, $p < 0.05$; MOBP r = 0,474, $p < 0.05$.

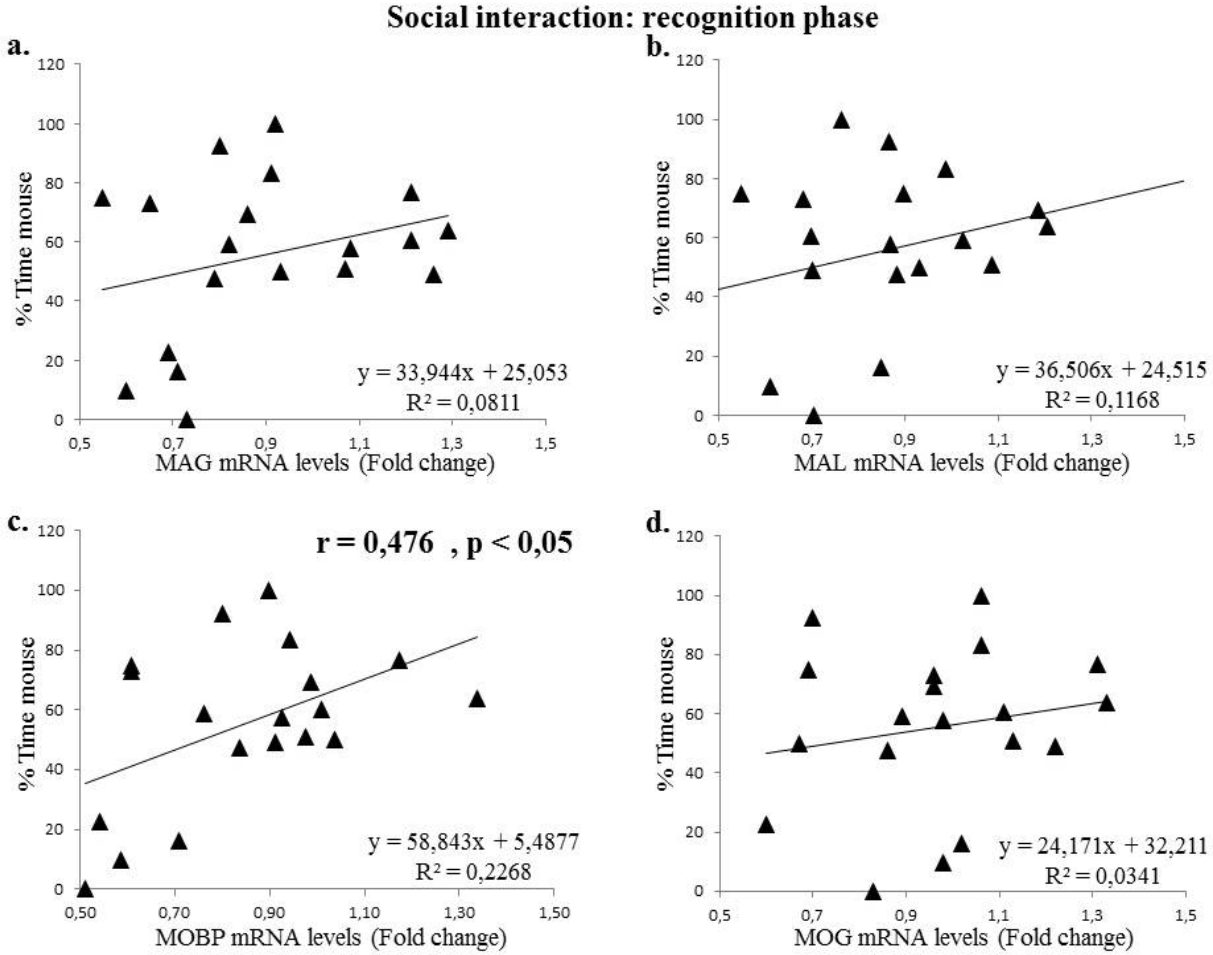

Figure 38. Pearson's correlation between recognition phase of the social interaction and the gene expression in the nucleus accumbens. The figure shows the correlation analysis between the social approach phase of the social interaction test and MAG, MAL, MOBP, MOG mRNA expression levels in the nucleus accumbens of control and Poly(I:C) exposed mice. Each dot represents the intersection between the value of % time spent with the novel mouse and the level of mRNA expression levels (expressed as fold change). Pearson's correlation coefficient: MOBP r = 0,476, n = 19, $p < 0.05$.

4.5.8 Discussion

The aim of this study was to investigate the underlying molecular phenotypes that could mediate the association between prenatal Poly(I:C) exposure and long-term behavioural abnormalities, and to do so we chose an unbiased genome-wide based approach to analyse the transcription profile of different brain regions implicated in psychiatric disorders. In particular, we performed Affimetrix microarray analysis on the prefrontal cortex and the nucleus accumbens of adult GD17 Poly(I:C) offspring, two brain that are pivotal in mediating many aspects of psychiatric disease. Moreover, before performing the microarray analysis, we subjected the animals to a battery of behavioural tests to confirm the effectiveness of the prenatal manipulation. Consistent with our previous results, Poly(I:C) exposed offspring displayed impairments in spatial recognition working memory (Richetto *et al*, 2013a), social approach and recognition (Bitanihirwe *et al*, 2010a) and increased sensitivity to the psychostimulant amphetamine (Meyer *et al*, 2008d). Thus, having ascertained the validity of this lot of animals, we proceeded with the whole genome transcriptome analysis.

First of all, we analysed the transcription profile the prefrontal cortex. Interestingly, we observed a significant long-term dysregulation of 185 genes (71 of which were down-regulated, while 114 were up-regulated) induced by prenatal exposure to the immune activating agent. Among these, several have been implicated in the aetiopathology of schizophrenia, so we performed a pathway analysis with Ingenuity Software to investigate the precise biological functions that could be impacted by these changes and ultimately affected by prenatal infection. We identified five top pathways that were significantly regulated by prenatal immune activation; in particular, the most implicated pathway was the G protein coupled receptor signalling, that incorporates important signalling pathways involved, for example, in the regulation of behaviour and mood. Prenatal manipulation also seemed to alter trophic Nerve Growth Factor (NGF) signalling, cAMP-mediated signalling and sphingomyelin metabolism. Moreover, Ingenuity Pathway Analysis identified cellular development, cellular growth and proliferation, nervous system development and function, cell signalling, nucleic acid metabolism and small molecule biochemistry as the main biological functions affected by the observed gene expression alterations.

The same approach was used to analyse the nucleus accumbens, where we observed that 159 genes were up-regulated, and 166 down-regulated, by prenatal

infection (for a total of 325 affected genes). These genes are enriched in various pathways identified by IPA, including G protein-coupled receptor signalling and cAMP-dependent signalling, that were also found enriched in the prefrontal cortex, serotonin receptor signalling and ERK5 signalling. Moreover, the main networks impacted by prenatal Poly(I:C) manipulation were nervous system development and function, which remains the most affected biological process, cell, tissue and embryonic development, neurological disease, cellular assembly and organization, lipid metabolism and small molecule biochemistry, psychological disorder and behaviour. The large magnitude of changes seen in Poly(I:C) offspring suggests that infection during late pregnancy may determine many effects in the exposed animals, which may contribute to the enhanced risk for the development of neurodevelopmental psychiatric disorders. In particular, our findings point to an effect of prenatal infection on the correct development and functioning of the nervous system, which is in line with the neurodevelopmental nature of this model.

Our results are also in line with previous studies investigating alterations in the brain transcriptome that is manifested after prenatal immunological activation. Fatemi and colleagues investigated the effects of prenatal infection with influenza virus on GD9 in mice on the transcription profile of newborn offspring. Interestingly, they also observed that the impacted genes were involved in signal transduction, cell communication, protein metabolism, cell growth and immune response, leading to permanent changes in brain structure and functions and increasing the risk for schizophrenia (Fatemi *et al*, 2005). These initial findings were confirmed and amplified by more microarray analyses on the hippocampus and the prefrontal cortex of mice prenatally exposed to the human influenza virus at embryonic day 7, 16 and 18 (Fatemi *et al*, 2009b; Fatemi *et al*, 2008). Our results are thus consistent with these findings and point to the notion that maternal infection causes a variety of postnatal neuropathological molecular changes in the offspring, which could underlie the behavioural alterations that ensue after this prenatal manipulation. Moreover, the similarities between our findings and those reported by Fatemi et al. support the idea that the detrimental effects of prenatal infection are not mediated by the particular infective agent involved, but by the maternal immune response to the infection. Oskvig et al. performed microarray analysis in a rat model of maternal infection with the bacterial endotoxin lipopolysaccharide (LPS) 4h after the maternal injection, and the

results clearly indicated that also maternal LPS infection can affect the fetal brain by altering the expression of genes that are critically important to nervous system development, such as genes which regulate neuronal migration of GABAergic interneurons (Oskvig *et al*, 2012). These studies underline and emphasize the importance of prenatal immune activation in the development of long-lasting impairments that are considered typical characteristics of schizophrenia.

Due to the huge array of information obtained with the microarray analysis, we performed an overlap of the gene expression changes in the prefrontal cortex and the nucleus accumbens with the aim to identify genes commonly affected in these brain regions as a long-lasting consequence of prenatal infection. We identified a substantial overlap of 27 genes that were altered in the same way in the two brain areas we examined. Then, we proceeded to establish the molecular signalling pathways implicated in the common effects of prenatal Poly(I:C). Among these, Notch signalling resulted to be the mainly affected pathway and this is particularly important because it is linked to neuronal function and development. G-protein coupled receptor signalling also highlighted both in the prefrontal cortex and in the nucleus accumbens. This pathway has high significance because dopamine, which binds to G protein-coupled receptors and activates numerous downstream protein kinase and phosphatase signalling pathways (Greengard *et al*, 1999), is one of the neurotransmitters involved in schizophrenia. In addition, we performed network analysis to study the biological processes that could be altered by the common changes induced by Poly(I:C) in the prefrontal cortex and nucleus accumbens. Neurological disease, cellular compromise and renal dysfunction the most significantly altered networks, together with drug metabolism and molecular transport.

We then investigated the precise molecular functions altered in the context of neurological disease, and we observed that myelination and the myelin-related processes were highlighted as the most significant functions involved in this network. In particular, we observed that prenatal infection with Poly(I:C) down-regulated the expression levels of various genes associated with myelination, in particular MAG, MAL, MOBP and MOG, both in the prefrontal cortex and nucleus accumbens. This finding is indeed interesting because the expression of these genes is physiologically enriched in myelin-forming oligodendrocytes, while it is down-regulated in schizophrenic subjects. In recent years, in fact, other authors also demonstrated the potential role of myelin-

related pathways in the pathological mechanisms of schizophrenia, contributing to the understanding that genes like MAL, MAG, MOG and MOBP are implicated in the aetiology of the disorder due to their altered regulation (Aston *et al*, 2004; Hakak *et al*, 2001; Katsel *et al*, 2005; Le-Niculescu *et al*, 2009). Interestingly, our results are consistent with previous reports of alterations in the myelination network following prenatal immune challenge. Fatemi and colleagues, indeed, using offspring born to human influenza virus (H1N1)-infected mothers at day 16, observed significant changes in the expression of these genes in the hippocampus at postnatal day 0, 14 and 56 (birth, childhood and adulthood). These alterations included the down-regulation of the myelination genes MAG, MOG, MOBP and MAL at PND0 and MAG down-regulation at PND14 (Fatemi *et al*, 2009b; Fatemi *et al*, 2009c).

There are also several genetic studies that establish an association between polymorphisms in oligodendrocyte-related genes and specific populations of schizophrenia patients (Liu *et al*, 2013; Wan *et al*, 2005; Yang *et al*, 2005; Zai *et al*, 2005). In line with these findings, our results highlight that the relationship between prenatal infection and neurodevelopmental psychiatric disorders could be mediated by differential expression of myelination-related genes. Our findings further suggest that prenatal immunological manipulation could bring to the disruption in oligodendrocyte function seen in many schizophrenic patients, once again strengthening the hypothesis that sees prenatal infection as an aetiological factor in the development of such disorders.

Myelin provides the basis for rapid impulse conduction in the central nervous system and acts as electrical insulation for the unsheathed axon, which both helps to preserve the amplitude and increase the conduction velocity of the propagating axonal potential. Assuming a part as the primary infrastructure for long distance communication in the nervous system and for the development and long-term survival of axons, it is not surprising that damage to the myelin structure has been implicated in schizophrenia. In particular, abnormal myelin and oligodendroglia could create a functional obstacle to corticocortical and corticosubcortical interactions that, in turn, would clearly be compatible with some of the symptoms of schizophrenia, particularly with the cognitive deficits. For example, Davis and others demonstrated that age-related changes in white matter, morphologic abnormalities in oligodendroglia and myelin-related gene abnormalities contribute to schizophrenia (Davis et al. 2014). Moreover, according to

works by Sanfilipo, Brier and their research team, volume reductions in white matter of the prefrontal cortex have been repeatedly found in schizophrenia and seem to be related with negative and cognitive symptoms of this disorder (Sanfilipo *et al*, 2000). Recently, Connor et al. have investigated the increased density and altered spatial distribution of subcortical white matter, which represents another consistent cellular alteration found in schizophrenia and related disorders. These alterations have been proposed to reflect disturbances of neurodevelopmental processes, including neuronal migration, neurogenesis and cell death (Connor *et al*, 2011). At present, the mechanisms underlying the molecular and cellular changes in white matter in psychosis remain unclear, even if the disruption of early developmental events, including migration of cortical neurons during the second or early third trimester of pregnancy and apoptosis of embryonic neurons, appears to play an important role in these abnormalities (Ayoub and Kostovic, 2009). In this context, it seems clear that prenatal insults, such as maternal infection during pregnancy, which alter correct neurodevelopment, could have a chief role in the correct development of the myelin structure. Accordingly, Fatemi found a decrease in hippocampal volume in adolescent mice prenatally exposed to human influenza virus on GD16 (Fatemi *et al*, 2009c). Additional support for the involvement of oligodendrocytes and myelin in schizophrenic disorders derives from microarray analysis in the prefrontal cortex of postmortem brain samples from schizophrenic patients (Arnold and Trojanowski, 1996; Foong *et al*, 2002). In particular, MAG and MAL have been highlighted in microarray studies, and subsequent single-gene quantitative RT-PCR studies confirmed their down-regulation in different schizophrenia cohorts. Myelin and lymphocyte protein (MAL) is expressed in oligodendrocytes and localized in compact myelin. It plays a role in the interaction with glycosphingolipids to decrease membrane plasticity and stabilize myelinating cells (Frank, 2000). Myelin-associated glycoprotein (MAG), instead, regulates the membrane-membrane interaction between cells and axons. In particular, it acts in two directions: as ligand for an axonal receptor and as a receptor for an axonal signalling, which has a trophic effect on oligodendrocytes enhancing a variety of other functions. These functions implicate MAG in signal transduction pathways taking place in myelin-forming oligodendrocytes or Schwann cells, on the one hand, and in the axoplasm of myelinating axons, on the other, to promote the maintenance of the internodal axon–glial junction as well as tight junctions in the paranodal regions (Quarles, 2007). Indeed, a variety of ultrastructural

defects have been observed in the central nervous system of MAG-deficient mice (Montag *et al*, 1994; Schachner and Bartsch, 2000), including delayed myelination and hypomyelination (Bartsch *et al*, 1997). This mouse model has morphologically atypical myelin sheaths, lacks a well-developed cytoplasmic collar, contains redundant myelin and uncompact areas of myelin and has periaxonal zones of degeneration and dystrophy of distal oligodendryte processes. Further support to the notion that myelination could be implicated in the pathophysiology of schizophrenia is derived by the fact that the temporal onset of psychotic disorders in late adolescence or early adulthood coincides with the concluding myelination of the prefrontal cortex (Tamnes *et al*, 2010) and other areas. Benes observed that preexisting defects, like genetic predisposition or acquired prenatal insults, could remain dormant until changes in myelination mechanisms become manifest during the late adolescent period (Benes, 1989). Such changes could involve, for example, increased myelination of certain pathways that mediate performance of delayed response tasks. Our study also supports the association between social impairments in schizophrenia and alterations in myelination. In particular, our correlation analysis revealed a significant association between gene expression levels of MOBP and MAG in the nucleus accumbens and the animals' performance during the social interaction tests. In detail, the down regulation of these genes is correlated with reduced social approach and social recognition, respectively. A similar association was observed by Makinodan et al, who investigated if and how life experiences influence central nervous system myelination and function (Makinodan *et al*, 2012). In detail, male mice, isolated from postnatal day 21 to 35, showed impairment in working memory and social behaviour tasks if tested during adulthood (PND65). These results coincided with alterations in white matter, especially in the prefrontal cortex, where the morphology of oligodendrocytes was less diverse than in control animals, with short processes, less branching and fewer internodes. Moreover, MAG expression was reduced and myelination mechanisms altered. These findings demonstrate that early negative social experiences influence oligodendrocyte maturation and myelination in the prefrontal cortex if they occur during a 'critical period' between PND21 and PND35. An explanation could be that altered myelin changes the conduction velocity of myelinated axons, which leads to abnormal information processing with consequent aberrant social behaviours and working memory; otherwise, myelin defects may change dopaminergic function, which could

contribute to the deficits in many behavioural functions (Vijayraghavan *et al*, 2007). In line with these results, our study points to the possibility that insults during another critical period, namely neurodevelopment, could also alter correct myelination and consequently social behaviour. Regarding the association between social impairments and MOBP expression levels, a myelin-associated oligodendrocytic basic protein that plays a role in the stabilization of myelin sheaths (Montague *et al*, 2006), little is yet known, and to our knowledge this is the first report of such correlation. Future studies are thus warranted to dissect these findings.

In summary, our study provides support to the notion that prenatal infection is capable of inducing alterations in the expression levels of various genes implicated in myelination, strengthening the hypothesis of a causal link between this prenatal insult and the emergence of neurodevelopmental disorders such as schizophrenia. Additionally, the alterations we observed regarding myelination correlate, at least in part, with the behavioural alterations we observed in our animals, underlining the possible implication of myelinations deficits in many symptoms observed in schizophrenic patients. The Poly(I:C) maternal manipulation model could thus be considered as an important tool for investigating these myelin-related changes. Indeed, our molecular results are consistent with the gene expression alterations changes observed human schizophrenic subjects (Hakak *et al*, 2001). Confirmation of our findings, together with the analysis of different brain regions and different phases of nervous system development in follow-up studies, may increase the knowledge of the disease pathogenesis and advance causative and diagnostic understanding of this psychotic disorder. Investigating the role of disrupted myelination processes in depth could thus provide new insights into the pathophysiology of schizophrenia, leading to the identification of novel targets for therapeutic intervention aimed at reversing underlying molecular abnormalities and eventually improving clinical outcomes.

5. SUMMARY AND CONCLUSIONS

All in all, the studies conducted during my PhD add substantial preclinical evidence to the hypothesis that prenatal infection can confer increased risk for the development of psychiatric disease by interfering with normal brain and behavioural development. In particular, we demonstrated that prenatal treatment with poly(I:C) during the late phase of gestation (GD17) induces a variety of molecular and behavioural abnormalities that are relevant to various psychiatric disorders, and in particular schizophrenia.

Indeed, we found that late prenatal infection induces a wide spectrum of abnormalities in the GABAergic system that are more pronounced in adult compared with peripubertal offspring born to immune-challenged mothers. These included altered mRNA expression levels of enzymes regulating γ-aminobutyric acid (GABA) biosynthesis (glutamic acid decarboxylase 65-kDa [GAD65] and GAD67), vesicular GABA transporter (VGAT), alpha-subunits of the GABA(A) receptor (α2, α3, α4, and α5), and the chloride transporters sodium-potassium-chloride cotransporter 1 and potassium-chloride cotransporter 2. Intriguingly, these age-dependent changes in GABAergic gene expression were paralleled by an adult onset of working memory deficiency, emphasizing a critical impact of prenatal immune-related insults on long-term GABAergic changes relevant to neuropsychiatric disorders with prenatal infectious aetiologies, especially for those with delayed onset in early adulthood.

We then went on to show that offspring prenatally exposed to poly(I:C) displayed significant impairments in spatial matching-to-position working memory and spatial novelty presence regardless of whether they were raised by gestationally immune-challenged or non-challenged control surrogate mothers, suggesting that prenatal infection-induced deficits in spatial short-term memory are mediated by prenatal maternal effects on the offspring. Moreover, we demonstrated that being raised by a gestationally immune-challenged surrogate mother is sufficient to increase the offspring's locomotor response to systemic amphetamine treatment, adding further weight to the notion that being reared by a surrogate mother that experienced immune activation during pregnancy may constitute a risk factor for specific dopaminergic abnormalities.

When considering pharmacological interventions aimed at ameliorating the behavioural phenotype induced by prenatal poly(I:C), we observed that systemic administration of SH-053-2'F-S-CH_3 failed to normalize the poly(I:C)-induced deficits in working memory and social interaction, but instead impaired performance in these cognitive and behavioural domains both in control and poly(I:C) offspring. In contrast, SH-053-2'F-S-CH_3 was highly effective in mitigating the poly(I:C)-induced amphetamine hypersensitivity phenotype without causing side effects in control offspring. Our preclinical data thus suggest that benzodiazepine-like positive allosteric modulators with activity at the α2, α3, and α5 subunits of the $GABA_A$ receptor may be particularly useful in correcting pathological overactivity of the dopaminergic system, but they may be ineffective in targeting multiple pathological domains that involve the co-existence of psychotic, social, and cognitive dysfunctions.

Another aspect that was evaluated in my PhD thesis relates to the effects of prenatal immune activation on the behavioural and cognitive functions during the process of aging. Interestingly, our data showed that prenatal immune activation causes an early pubescent onset of spatial short-term memory impairment, which in control offspring typically emerges as a result of normal aging. Moreover, our findings demonstrate that this early-life adversity induces age-dependent deficits in the retention of contextual fear memories, spatial reference learning and memory, and food hoarding behaviour, all of which emerged only once prenatally immune-exposed reached the aged stage of life. These cognitive abnormalities were further paralleled by aging-related alterations in the hippocampal expression of pre- and postsynaptic proteins and neuroplasticity-related genes. The prenatal immunological insult, however, did not induce signs of persistent systemic or hippocampal inflammation, microgliosis, or astrogliosis. Taken together, these findings provide converging evidence that prenatal immune challenge exacerbates hippocampus-related cognitive aging in the absence of persistent systemic or hippocampal inflammatory responses. Furthermore, these results support the idea that the effects of prenatal infection may be neuropathologically relevant beyond the adult stage of life, thus extending into the periods of aging.

Lastly, we investigated the long-term molecular alterations emerging after gestational treatment poly(I:C) using an unbiased genome-wide approach. We observed that poly(I:C) induces a wide range of long term alterations in gene expression profile of the prefrontal cortex and nucleus accumbens, and that these changes are implicated in

various functional processes that could underlie the behavioural phenotype induced by the prenatal immunological manipulation. Interestingly, when performing an overlap between the changes observed in the prefrontal cortex and nucleus accumbens, myelination highlights as the main function impacted by these common alterations, suggesting that abnormalities in this process could be of pivotal importance in mediating the deleterious effects of prenatal poly(I:C). In line with this hypothesis, we show that gene expression changes in myelin markers positively correlate with the severity of the behavioural alterations observed in our animals.

In conclusion, the results obtained during my PhD thesis broaden the knowledge regarding the association between prenatal immune activation and increased risk of developing psychopathology later in life. In particular, we focused on dissecting the molecular consequences of such manipulation, as information on this aspect of the model is still somewhat lacking. Our results point to a strong implication of GABAergic system abnormalities in the behavioural and cognitive alterations induced by late prenatal immune activation, even if pharmacological interventions directly targeting the GABAergic system proved useful in correcting only some of such abnormalities. Interestingly, the genome-wide approach shed light onto a wide array of different molecular mechanism associated with prenatal poly(I:C) exposure, and subsequent studies are definitely warranted to follow up and characterize these findings. In particular, our results point to a possible role of myelination in mediating the negative effects of prenatal infection, and this aspect definitely needs to be pursued with appropriate MRI and imaging studies.

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