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Characterization of a rat model of Maternal Immune Activation with relevance to schizophrenia in terms of maternal and foetal cytokine responses, adult behavioural outcome and chronic brain inflammation

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Abstract

Introduction: Schizophrenia is a serious psychiatric disorder with a neurodevelopmental origin. One of the most important environmental risk factors for schizophrenia is maternal immune activation (MIA). Induction of pro-inflammatory cytokines in the foetal brain is thought to underlie the neurodevelopmental insult, resulting in a disturbed development of the brain which ultimately leads to the emergence of behavioural abnormalities in late adolescence. It has been suggested that schizophrenia patients have persistent immunological disturbances such as a hyperactive pro-inflammatory immune system. Microglia activation has been observed in the brains of adult schizophrenia patients and might be related to the behavioural deficits.

Aims: The aims of this study were to implement a rat model of MIA (poly I:C model) with relevance to schizophrenia in our lab and to characterize this model in terms of maternal and foetal cytokine responses, adult behavioural outcome and adult brain pathology. In addition, we wanted to test whether poly I:C offspring are more sensitive to a systemic inflammatory challenge.

Materials and methods: 1) The effect of several doses of poly I:C (2, 4 & 8 mg/kg) on the induction of cytokines in the maternal (n=4-5 per group) and foetal compartments (n=6-8 per group) was tested on two gestational days (GD9 & GD15) in the rat through relative quantification of inflammatory cytokine mRNAs by real-time qPCR. **2)** We evaluated the effect of two low doses (0.1 & 0.5 mg/kg) of LPS on sickness behaviour, anhedonia and microglia activation in normal adult rats (n=3 per group) and the time-course of the response (6h, 24h, 3d & 7d) in order to determine the most optimal LPS dose and time point to assess microglia activation in the final study. Sucrose preference and total liquid consumption were assessed in a sucrose preference test. Microglia were detected by immunohistochemistry against the CD11b-receptor. **3)** Based on the first experiments, pregnant rats were injected with vehicle (n=5) or 4 mg/kg poly I:C (n=7) on GD15 and body weight change was assessed. Adult offspring were subjected to a number of schizophrenia-related behavioural tests: prepulse inhibition (PPI), spontaneous locomotion, amphetamine- and MK-801-stimulated locomotion, and sucrose preference. The immune system was challenged with LPS in a subgroup of control and MIA offspring. Microglia activation was assessed on brain sections at 3d post-injection.

Results: 1) The 4 mg/kg and 8 mg/kg poly I:C doses induced the highest increases of the mRNA expression of inflammatory cytokines in the maternal and foetal compartments. However, not all pregnant dams and foetuses seemed to respond to the MIA challenge. **2)** In normal adult rats, a dose as low as 0.1 mg/kg LPS induced global microglia activation in the brain, which peaked at 3 days post-injection ($p \leq 0.05$). The LPS injection induced acute sickness behaviour in all animals ($p \leq 0.05$) and persisting low levels of sucrose preference and total liquid consumption in a subgroup of rats. **3)** The offspring of dams that had lost weight following MIA had the worst behavioural outcome in nearly every test. Firstly, they showed a trend for a PPI disruption, indicative of a sensorimotor gating deficit. Secondly, poly I:C (weight loss) offspring had a reduced responsiveness to the dopamine agonist amphetamine and a slower and generally lower response to the NMDA-R antagonist MK-801, indicating a dysfunctioning of the dopaminergic and glutamatergic pathways in the brain. Poly I:C (weight loss) offspring exhibited lower basal levels of sucrose preference than controls ($p \leq 0.05$), reflecting anhedonia. When challenged with LPS, poly I:C (weight loss) offspring lost the most weight ($p \leq 0.05$). The offspring of dams that gained weight following MIA did however show some behavioural abnormalities as well, such as a trend for an increased response to MK-801. Poly I:C (weight loss) offspring had a higher level of microglia activation than poly I:C (weight gain) offspring ($p \leq 0.05$). The LPS injection failed to induce global microglia activation in the final study and failed to reproduce the results from the second study in controls, questioning the reliability of the LPS test.

Conclusion: The individual response of the dams to MIA in terms of body weight change determined the behavioural outcome and brain pathology in adult offspring with offspring from weight loss dams displaying more schizophrenia-like symptoms and a higher microglia activation, suggesting a link between chronic brain inflammation and the behavioural deficits**.** The difference in body weight change after MIA could be related to a different cytokine response in these dams, resulting in a distinctly different neurodevelopmental insult in the foetuses.

Samenvatting

Introductie: Schizofrenie is een ernstige psychiatrische aandoening met een neuro-ontwikkelingsbasis. Een van de belangrijkste risicofactoren voor schizofrenie is maternale immuun-activatie (MIA). Inductie van pro-inflammatoire cytokines in de foetale hersenen zou aan de basis liggen van de verstoorde ontwikkeling van de hersenen, welke uiteindelijk leidt tot het ontstaan van gedragsafwijkingen in de late adolescentie. Men heeft gesuggereerd dat schizofrenie patiënten aanhoudende immuunstoornissen vertonen zoals een hyperactief pro-inflammatoir immuunsysteem. Microglia activatie is geobserveerd geweest in de hersenen van volwassen schizofrenie patiënten en zou gerelateerd kunnen zijn aan de gedragsafwijkingen in deze patiënten.

Doelstellingen: Het doel van deze studie was om een ratmodel voor MIA (het poly I:C model) met relevantie voor schizofrenie in ons lab te implementeren en dit model te karakteriseren betreffende de maternale en foetale cytokine respons, de gedragsafwijkingen en neuropathologie in de volwassen nakomelingen, en om na te gaan of deze dieren gevoeliger zijn voor een immuunstimulus.

Materiaal en methoden: 1) Het effect van verschillende dosissen poly I:C (2, 4 & 8 mg/kg) op de inductie van cytokines in moeders (n=4-5 per groep) en foetussen (n=6-8 per groep) werd getest op twee tijdspunten van de zwangerschap (GD9 & GD15) in de rat door relatieve kwantificatie van inflammatoire cytokine gentranscripten m.b.v. *real-time* qPCR. **2)** We evalueerden het effect van twee lage LPS dosissen (0.1 & 0.5 mg/kg) op ziektegedrag, anhedonie en microglia activatie in normale ratten (n=3 per groep) en het tijdsverloop van de respons (6h, 24h, 3d & 7d), om de optimale dosis en tijdspunt te bepalen voor de meting van microglia activatie in de finale studie. Sucrose preferentie en totale vloeistofinname werden gemeten in een sucrose preferentie test. Microglia werden aangetoond met een immunohistochemische kleuring tegen de CD11b-receptor. **3)** Op basis van de eerste experimenten werden zwangere vrouwtjes geïnjecteerd met *vehicle* (n=5) of 4 mg/kg poly I:C (n=7) op GD15 en verandering in lichaamsgewicht werd gemeten. De volwassen nakomelingen werden onderworpen aan een aantal schizofrenie-gerelateerde gedragstesten: prepuls inhibitie (PPI), spontane locomotie, amfetamine- en MK-801-gestimuleerde locomotie, en sucrose preferentie. Een subgroep van de controle- en MIA-nakomelingen kreeg een LPS injectie. Microglia activatie werd onderzocht op weefselcoupes 3 dagen na injectie.

Resultaten: 1) De 4 en 8 mg/kg poly I:C dosissen resulteerden in de grootste stijgingen van de mRNA expressie van inflammatoire cytokines in de moeders en foetussen. Niet alle moeders en foetussen leken echter te reageren op de immuunstimulus. **2)** Een LPS dosis zo laag als 0.1 mg/kg induceerde een globale activatie van microglia in de hersenen van normale ratten, welke een piek vertoonde op 3d na injectie ($p \leq 0.05$). De LPS injectie induceerde acuut ziektegedrag in alle dieren ($p \leq 0.05$) en aanhoudende lage niveaus van sucrose preferentie en totale vloeistofinname in een subgroep van de ratten. **3)** De nakomelingen van de vrouwtjes die gewicht verloren na MIA vertoonden de grootste gedragsafwijkingen. Ze vertoonden een trend voor een PPI stoornis, welke in schizofrenie voorkomt. Ze vertoonden ook een verminderde gevoeligheid voor de dopamine agonist amfetamine en de NMDA-receptor antagonist MK-801, wat wijst op een verstoorde werking van de dopaminerge en glutamaterge banen in de hersenen. Ze vertoonden ook lagere sucrose preferentie levels dan controles ($p \leq 0.05$), wat wijst op anhedonie. Na LPS injectie verloren deze dieren ook het meeste gewicht ($p \leq 0.05$). De nakomelingen van vrouwtjes die gewicht bijkwamen na MIA vertoonden ook een aantal gedragsafwijkingen bv. een trend voor een verhoogde respons op MK-801. Poly I:C (gewichtsverlies) dieren vertoonden meer microglia activatie dan poly I:C (gewichtstoename) dieren ($p \leq 0.05$). De LPS injectie kon geen verhoogde microglia activatie induceren in de finale studie en de resultaten van de 2^{e} studie niet reproduceren, wat de betrouwbaarheid van de LPS test in vraag stelt.

Conclusie: De individuele respons van de moeders op MIA m.b.t. gewichtsverandering bepaalde de ernst van de gedragsafwijkingen en de neuropathologie in de volwassen nakomelingen. Poly I:C (gewichtsverlies) dieren vertoonden meer schizofrenie-gerelateerde symptomen en meer microglia activatie, wat een link suggereert tussen de herseninflammatie en gedragsafwijkingen. Het verschil in gewichtsverandering na MIA zou gerelateerd kunnen zijn aan een verschillende cytokine respons in de zwangere vrouwtjes, resulterend in een verschillende neuro-ontwikkelingsinsult in de foetussen.

List of abbreviations

α7nACh	α7 nicotinic acetylcholine (receptor)
CD11b	cluster of differentiation 11b
CD68	cluster of differentiation 68
cDNA	complementary DNA
CNS	central nervous system
CR3	complement receptor 3
Ct	cycle threshold
dB	decibel
EDTA	ethylenediaminetetraacetic acid
GD	gestational day
HPA axis	hypothalamic-pituitary-adrenal axis
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IFN-γ	interferon-γ
IL-1	interleukin-1
IL-1β	interleukin-1β
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IL-12	interleukin-12
IL-13	interleukin-13
i.p.	intraperitoneal
LPS	lipopolysaccharide
MIA	maternal immune activation
mRNA	messenger RNA
MZ	monozygotic
NMDA(-R)	N-methyl D-aspartate (receptor)
NSAID	Nonsteroidal anti-inflammatory drug
PET	positron emission tomography
PND	postnatal day
poly I:C	polyinosinic: polycytidylic acid
PPI	prepulse inhibition
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
TRYCAT	tryptophan catabolite
qPCR	quantitative polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4

Introduction

1 Schizophrenia

Schizophrenia is a serious psychiatric disorder that is clinically characterised by positive symptoms (e.g. hallucinations, delusions), negative symptoms (e.g. social withdrawal, anhedonia) and cognitive symptoms (e.g. attention deficits, working memory deficits). It affects approximately 1% of the population (Kneeland and Fatemi, 2012) and has a higher incidence in males with a male to female risk ratio of 1.4 (McGrath, 2005). Monozygotic (MZ) twins have a concordance rate for schizophrenia of approximately 45-60% while this is only 10-15% in dizygotic twins (Brown, 2011). This observation indicates an important genetic component in the development of schizophrenia. It should however be noted that the concordance rate for schizophrenia in MZ twins that shared a placenta - and therefore a foetal blood circulation - is about 60%, while in MZ twins that did not share a placenta (18-36% of MZ twins) the concordance rate is only 10.7% (Davis et al., 1995). This observation suggests that the shared prenatal environment, rather than the shared genes, accounts for the high concordance rate in MZ twins. There is indeed a large amount of evidence for a variety of prenatal environmental factors that increase the risk of developing schizophrenia. Pre-, peri- and postnatal environmental risk factors are extensively reviewed in (Brown, 2011).

The current hypothesis states that schizophrenia is a neurodevelopmental disorder in which a brain insult early in life disturbs normal brain development leading to abnormalities that are expressed in the young adult brain. Although several genes have been identified to play an important role in the etiology of schizophrenia, the effect sizes of these risk genes have always been small and many proposed risk genes could not be replicated in other studies. A possible explanation is that these genes do not confer an increased risk of schizophrenia on their own, but make an individual more susceptible to certain environmental risk factors. An interaction between genetic and environmental risk factors is then required to increase the risk of developing schizophrenia in an individual (Brown, 2011). Environmental risk factors might also interact with genes by altering epigenetic signals such as DNA methylation and histone modifications. Environmental exposures that alter transcriptional activity of genes during a critical time period in development can result in permanent changes in epigenetic regulation and lead to abnormal phenotypes (Waterland and Michels, 2007). With regard to the delayed onset of the disease, the two-hit hypothesis of schizophrenia is often mentioned. In this model, genetic or environmental factors disrupt early central nervous system (CNS) development, which constitutes the first hit and produces long-term vulnerability to a second hit later in life (induced by e.g. childhood trauma, social stress, cannabis abuse) that leads to the development of schizophrenia (Maynard et al., 2001).

Schizophrenia has a severe impact on the quality of life of patients and they often experience difficulties in functioning in our society. The current treatment with antipsychotics is unsatisfying as it has many side effects, resulting in low therapy compliance. These facts contribute to a high economic cost for the society. More research into the field of schizophrenia is consequently needed. To improve the diagnosis, treatment and prevention of schizophrenia, there is a need for a better insight into the disease as well as animal models with an objective and reproducible outcome to test new hypotheses and treatment strategies.

2 Prenatal and perinatal environmental risk factors

Epidemiologic studies have revealed several prenatal and perinatal environmental exposures as potential risk factors for schizophrenia. The first and foremost one is prenatal infection. Many infections during pregnancy have been associated with an increased risk of schizophrenia in the offspring, including viral (rubella, influenza), parasitic (T. gondii) and bacterial infections (C. diphteriae, N. gonorrhoeae) (Brown, 2011). The greatest increase in risk has been observed with prenatal influenza exposure. Brown et al. found that influenza exposure during the first half of pregnancy was related to a three-fold increase in risk of schizophrenia. (Brown et al., 2004a).

A second environmental risk factor is prenatal malnutrition. Both macronutrient (famine) and micronutrient deficiency (folate, iron, Vitamin D) have been associated with an increased risk of schizophrenia in offspring. Subjects exposed to severe food deprivation in early gestation showed a two-fold increase in risk of schizophrenia (Brown, 2011). It is worth mentioning that macro- and micronutrient deficiencies can suppress immune functions by affecting both the cellular and humoral immune responses, which increases the susceptibility to infections (Wintergerst et al., 2007).

Another factor that is associated with an increased risk of schizophrenia is an advanced paternal age (Malaspina et al., 2001). The children born to fathers over age 50 have a 1.66-fold increase in risk of schizophrenia compared to children born to fathers between 25 and 29 years old (Miller et al., 2011a). An increase in *de novo* mutations and epigenetic aberrations in the spermatozoa of older fathers has been proposed to explain this observation (Malaspina, 2001).

Obstetric complications also pose a risk for schizophrenia. Subjects exposed to obstetric complications during early life have a two-fold increase in risk of schizophrenia (Geddes and Lawrie, 1995). These include complications of pregnancy and delivery such as abnormal foetal growth and emergency caesarean section (Cannon et al., 2002). Foetal hypoxia, which is often associated with obstetric complications, has been proposed as a common underlying mechanism that results in an increased risk of schizophrenia.

Maternal stress is another risk factor for schizophrenia. Offspring born to mothers who were exposed to high levels of stress during pregnancy (e.g. death of a relative) have a ±1.5-2.0-fold increased risk of schizophrenia (Brown, 2011). It is known that prenatal exposure to excessive stress and concomitant high glucocorticoid levels permanently elevates basal glucocorticoid levels in offspring as well as basal hypothalamic-pituitary-adrenal (HPA) axis activity. These individuals are consequently more vulnerable to stressful events. The molecular mechanisms that underlie these changes include epigenetic changes in certain gene promoters (Harris and Seckl, 2011).

Being born and raised in an urban area also increases the risk of developing schizophrenia in later life compared with individuals that are born and raised in a rural area (Marcelis et al., 1999). A 2.4-fold increase in risk has been recorded for people born in a capital versus rural areas (Mortensen et al., 1999). This observation might just reflect environmental exposures that are typical of cities such as pollutants and certain toxins like leaded gasoline. The increased risk of being raised in an urban area might also be related to an increased experience of social stress. The higher population density in cities might also facilitate the spread of infectious agents and thus lead to an increased risk of prenatal infection.

Finally there is also an association between the season of birth and schizophrenia. Being born during the winter or early spring is associated with a 5-15% increased risk of developing schizophrenia (Torrey et al., 1997). This seasonal effect may be related to the risk of prenatal infection as many infections have seasonally varying patterns. It might also be related to a relative prenatal Vitamin D deficiency due to decreased sun exposure during the winter months.

3 Common mediating pathways

3.1 *General*

Although originally much attention has been paid to the second trimester of pregnancy in humans as a sensitive period for brain development disruption, more recent data suggest that environmental insults may act at multiple time points in pregnancy and postnatal life (up to adolescence) to increase the risk of schizophrenia. Environmental insults probably act by interfering with neurodevelopmental processes that occur within restricted time intervals, representing as such windows of vulnerability of the brain. These processes include neurogenesis, neuronal migration, differentiation, formation of neuronal circuits and networks, synaptogenesis, synaptic pruning and myelination (Brown, 2011).

The various environmental risk factors that have been described above might act through common pathways that interact with developmental events to increase the risk of schizophrenia. As such

oxidative stress, apoptosis, inflammatory mechanisms and HPA axis-mediated mechanisms have been hypothesized to be common mechanisms through which different environmental factors such as infections, malnutrition, obstetric complications and psychosocial stress can act (Brown, 2011).

Firstly, the mentioned environmental risk factors might mediate their effect by increasing oxidative and nitrosative stress, which involves an imbalance between overproduction of reactive oxygen and nitrogen species and a deficiency of antioxidants such as glutathione. This results in harmful (per)oxidation of DNA, proteins and lipids. Redox dysregulation has been shown to induce N-methyl D-aspartate (NMDA) receptor hypofunction as well as deficient myelination (Do et al., 2009).

Several environmental risk factors such as pro-inflammatory cytokines and oxidative stress are pro-apoptotic. Apoptosis has been proposed to contribute to the observed reduction in cortical grey matter volume seen in adult schizophrenia patients. Interestingly, the levels of Bcl-2, an anti-apoptotic protein, were found to be decreased in the temporal cortex of schizophrenia patients. The protection of neurons against pro-apoptotic insults is consequently reduced (Jarskog et al., 2004).

Inflammatory processes, mediated by an imbalance of cytokines, are not only induced by prenatal infection (see §3.2), but also by a number of other environmental risk factors such as chronic foetal hypoxia (Guo et al., 2010).

The environmental stressors mentioned above also lead to activation of the HPA axis (Brown, 2011). Early hyperactivation of the HPA axis can result in a permanent elevation of its basal activity, which makes these individuals more vulnerable to stressful events later in life (see §2).

A number of these environmental risk factors might also exert their influence by increasing the tryptophan catabolite (TRYCAT) pathway. Two enzymes can convert tryptophan into TRYCATs: indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase. Indoleamine 2,3-dioxygenase is primarily activated by interferon-γ (IFN-γ) and pro-inflammatory cytokines and leads to the production of the neurotoxic and excitotoxic 3-hydroxykynurenine and quinolinic acid. Quinolinic acid can result in overactivation of neurons via activation of the NMDA receptor and is associated with excitotoxic neurodegeneration. Tryptophan 2,3-dioxygenase on the other hand is mainly induced by the stress hormone cortisol and converts tryptophan into kynurenine and kynurenic acid. Kynurenic acid is an inhibitor of the α7 nicotinic acetylcholine (α7nACh)-receptor and results in a decreased release of many neurotransmitters. At high concentrations it can also decrease NMDA receptor activity (Anderson and Maes, 2012; Anderson et al., 2012). Increased levels of kynurenic acid in rats have been associated with cognitive deficits, which could be prevented by the administration of the α7nACh-receptor positive allosteric modulator galantamine (Alexander et al., 2012).

3.2 *Cytokine-mediated effects of prenatal infection*

Because many different infections (viral, parasitic and bacterial) have been associated with an increased risk for schizophrenia, it has been proposed that they exert their effect by acting through a common mechanism, namely by the induction of the maternal immune response. This hypothesis is supported by the observation that maternal immune activation (MIA) or prenatal immune challenge in the absence of an infectious agent suffices to evoke abnormal brain development in offspring. MIA results in the production of pro-inflammatory cytokines that can cross the placenta and enter the brain of the foetus (Gilmore and Jarskog, 1997). A number of mechanisms have been proposed on how the imbalance of cytokines might influence the brain. Induction of neuroinflammation in the brain by direct stimulation of microglia and astrocytes is one possible mechanism (Na et al., 2012). Cytokines can also disturb the normal maturation of oligodendrocytes and thus contribute to white matter abnormalities seen in schizophrenia patients (Davis et al., 2003; Do et al., 2009). Given the important and diverse roles of many cytokines and other immune proteins (such as MHC I molecules) in normal CNS development (Deverman and Patterson, 2009), it is not surprising that a disturbance of the normal cytokine balance in the foetal brain might lead to neurodevelopmental abnormalities (Meyer et al., 2009). Cytokines are involved in neurogenesis, neuronal migration, differentiation and

axon pathfinding. They are also involved in the regulation of cell survival and synaptic refinement (Deverman and Patterson, 2009).

Epidemiologic studies have revealed associations between excess maternal cytokines during pregnancy and the development of schizophrenia in offspring. Increased maternal serum levels of interleukin (IL-)8, a pro-inflammatory cytokine, during pregnancy were significantly associated with an increased risk of schizophrenia in the offspring (Brown et al., 2004b). Maternal serum levels of tumor necrosis factor-α (TNF-α), another pro-inflammatory cytokine, were also found to be significantly elevated in pregnancies of which the offspring would develop a psychotic disorder (Buka et al., 2001).

3.3 *Additional mechanisms of prenatal infection*

There are a number of additional mechanisms by which prenatal infections such as influenza might increase the risk of schizophrenia in offspring. These include fever, prenatal hypoxia and malnutrition. Fever or hyperthermia during pregnancy has been shown to be able to cause a number of structural and functional defects in the foetus, especially of the CNS (Edwards, 2006). It has been proposed as an environmental risk factor for schizophrenia (Edwards, 2007). The pathogenic mechanisms of hyperthermia include induction of the heat shock response, death of dividing neuroblasts, disruption of neuronal migration and microvascular damage (Edwards, 2007). Inflammation has been shown to sensitize the foetal brain to greater damage due to hypoxia by lowering the threshold at which hypoxia initiates apoptosis of neurons (Ugwumadu, 2006). Infections are often associated with malnutrition because of a reduced intake of nutrients and an altered utilization of nutrients by the body. As already mentioned above, prenatal malnutrition is an important risk factor for schizophrenia (Wintergerst et al., 2007). A well-functioning placenta is of great importance for a normal development and growth of the foetus. Structural abnormalities of the placenta as well as other changes might have a profound impact on the foetus. A well-known animal model of prenatal infection with relevance to schizophrenia is the pregnant mouse infected with human influenza virus. In a particular study the placenta of these influenza-infected pregnant mice was investigated. The placentas showed signs of cytoarchitectural disorganization, immune cell recruitment and thrombosis (Fatemi et al., 2012). The occlusion of blood vessels by thrombi can induce hypoxia of the foetus, which has been proposed as a risk factor for schizophrenia.

4 Animal models of MIA with relevance to schizophrenia

4.1 *General*

Various animal models of MIA with relevance to schizophrenia have been developed. As prenatal infection also poses an increased risk for autism, these animal models have been shown to have some relevance for this disorder as well (Malkova et al., 2012). First of all, pregnant animals such as mice and rhesus monkeys have been infected with human influenza virus. The offspring of virally infected mice showed behavioural (Shi et al., 2003), structural brain and neurochemical abnormalities as well as gene expression and protein expression changes that are reminiscent of schizophrenia. The offspring of virally infected rhesus monkeys also replicated some structural brain abnormalities of schizophrenia patients, including ventriculomegaly and reduced cortical grey matter volume (Short et al., 2010). MIA in the absence of an infectious agent however seems sufficient to replicate schizophrenia-like abnormalities in the offspring. Administration of polyinosinic: polycytidylic acid (poly I:C), a synthetic analogue of double-stranded RNA, elicits an immune response by activating Toll-like receptor (TLR) 3. Poly I:C acts as a viral mimetic that induces the acute phase antiviral immune response and leads to an inflammatory cascade (Fortier et al., 2004b; Meyer et al., 2005; Watanabe et al., 2011). The offspring of poly I:C-treated rodents have been shown to exhibit many molecular, neurochemical and structural abnormalities similar to those seen in schizophrenia patients (Kneeland and Fatemi, 2012) i.e. this model has good construct validity, as well as behavioural abnormalities reminiscent of schizophrenia patients i.e. good face validity. The model also has a good predictive validity as it has a similar responsiveness to antipsychotic drugs as

schizophrenic patients (Ozawa et al., 2006; Piontkewitz et al., 2011; Piontkewitz et al., 2009; Piontkewitz et al., 2012; Richtand et al., 2011; Roenker et al., 2011; Zuckerman et al., 2003). The model might therefore also be used to validate new drugs and other therapies for schizophrenia. This model will be discussed in §4.2.

On the other hand, animal models have been developed that investigate the influence of an immune response against a bacterial pathogen. To this end, pregnant rats and rhesus monkeys have been administered lipopolysaccharide (LPS), a bacterial endotoxin that elicits strong immune responses in animals by acting on TLR4. Rhesus monkeys prenatally exposed to LPS showed behavioural disturbances such as sensorimotor gating deficits (Willette et al., 2011). Rats exposed to LPS during gestation also showed behavioural abnormalities like sensorimotor gating deficits, that could be reversed by typical and atypical antipsychotics (Borrell et al., 2002), and enhanced locomotor activity (LMA) after amphetamine challenge (Fortier et al., 2004a) as well as structural and neurochemical abnormalities associated with schizophrenia (Cui et al., 2009; Paintlia et al., 2004; Romero et al., 2007).

It is clear from all these examples that schizophrenia-like phenotypes can evolve after prenatal exposure to MIA in the absence of an infectious agent (Shi et al., 2003). An immune response in the mother, characterised by pro-inflammatory cytokines, seems sufficient to induce impaired brain development in the offspring.

Surprisingly, a single injection of pregnant mice with just IL-6 could cause deficits of prepulse inhibition (PPI) and latent inhibition in the adult offspring (Smith et al., 2007). When poly I:C was administered to pregnant mice in combination with an anti-IL-6 antibody, the usual PPI-, latent inhibition-, exploratory and social deficits were prevented in the offspring. MIA in IL-6 knockout mice did not result in behavioural abnormalities in the offspring either while these are present in the offspring of wild-type mice after MIA (Smith et al., 2007). This indicates an important role for IL-6 but does not necessarily exclude the involvement of other pro-inflammatory cytokines.

4.2 *Poly I:C model of MIA*

4.2.1 *Behavioural changes*

Many behavioural deficits in the poly I:C model are reminiscent of clinical symptoms of schizophrenia patients (i.e. face validity). These include deficits in sensorimotor gating (reduced PPI of the acoustic startle response), deficits in social interaction, working memory, object(-in-place) recognition, exploratory behaviour and executive functioning as well as attentional deficits, learning deficits, emotional disturbances, increased anxiety, rapid reversal learning, disrupted latent inhibition, anhedonia and novelty- and psychostimulant-induced stereotypic behaviour.

The acoustic startle response is an involuntary contraction of the muscles in response to an unexpected strong acoustic stimulus and can be reduced by giving a weak acoustic prepulse shortly before the strong stimulus (50-500 ms apart). This phenomenon is known as PPI and reflects sensorimotor gating: the ability to filter incoming sensory information. PPI is impaired in schizophrenia patients as well as in groups at high risk of schizophrenia. This sensorimotor gating deficit is thought to result in excessive processing of stimuli leading to cognitive overload and ultimately thought disorder. PPI deficit of the acoustic startle response has become a very important test to validate animal models of schizophrenia (Wolff and Bilkey, 2008, 2010). PPI deficits have been observed in the offspring of poly I:C-treated rodents (Dickerson et al., 2010; Yee et al., 2011). MIA in general and poly I:C treatment in particular lead to a significant reduction of PPI in juvenile and adult rat offspring (Wolff and Bilkey, 2008, 2010). PPI levels depend on the prepulse intensity and the prepulse-pulse interval as well as on the degree of weight loss of the mother following MIA (Howland et al., 2012; Vorhees et al., 2012). There are however also studies that failed to find disruption of the PPI response in poly I:C offspring (Fortier et al., 2007; Giovanoli et al., 2013; Li et al., 2009).

The altered sensitivity of poly I:C-treated offspring compared with control offspring to amphetamine (an indirect dopamine agonist) and MK-801 (a non-competitive NMDA receptor antagonist) is another widely replicated test (Zuckerman et al., 2003). In healthy humans, these agents induce psychotic symptoms while in rodents hyperactivity and stereotypies can be induced, depending on the dose of the drugs (Richtand et al., 2011). Amphetamine and MK-801 respectively challenge the dopaminergic and glutamatergic systems in the brain, both of which have been implicated in the pathology of schizophrenia. Results of studies concerning the sensitivity of poly I:C-treated offspring to the locomotion-stimulating effects of amphetamine and MK-801 seem to be contradictory. Some studies found an increased response to amphetamine and MK-801 compared with controls, while others observed a decreased response. The outcome is partly dependent on the timing of prenatal exposure to poly I:C (e.g. at early or late gestation), the age at testing (e.g. before puberty or in adulthood) and the individual response of the mother to the immune stimulus (e.g. weight loss or not). The increased or decreased locomotor response to amphetamine in adulthood could be prevented by treatment with atypical antipsychotics in adolescence i.e. this model has also good predictive validity (Piontkewitz et al., 2011; Piontkewitz et al., 2009; Richtand et al., 2011). A decreased sensitivity to MK-801 is consistent with the NMDA receptor hypofunction hypothesis. This hypothesis states that a hypofunction of the glutamatergic system (by hypofunction of the NMDA receptors) underlies the dysfunction of the mesolimbic and mesocortical dopaminergic systems, resulting in positive, negative and cognitive symptoms. In case of a hypofunction of the NMDA receptors, the response to MK-801 could be expected to decrease.

Most studies did not find an alteration in spontaneous locomotor activity in the offspring of poly I:C-treated rats (Ozawa et al., 2006; Zuckerman et al., 2003; Zuckerman and Weiner, 2005). One study found an elevated spontaneous activity in young adult offspring when compared with controls, an effect that was absent in prepuberty (Howland et al., 2012). Our lab recently reported a decrease in spontaneous locomotor activity in poly I:C offspring compared to control offspring (Van den Eynde et al., submitted).

An important negative symptom that has been observed in schizophrenia patients is anhedonia, the inability to enjoy nice things. The sucrose preference test has been developed to assess anhedonic behaviour in rodents. The test is based on the natural preference of rodents for a sweet solution when they can freely choose between a sucrose solution and water. A reduction of this preference is indicative of anhedonia and has indeed been observed in the offspring of poly I:C-treated mice (Bitanihirwe et al., 2010).

4.2.2 *Cytokines and microglia activation*

As mentioned above, administration of poly I:C to a pregnant dam induces a systemic acute and innate antiviral immune response with the concomitant production of pro-inflammatory cytokines. These cytokines can cross the placenta and seem to influence the foetal brain, resulting in an abnormal development of the brain and ultimately leading to neuroanatomical and behavioural abnormalities in the adult offspring of the poly I:C-treated dams. MIA leads to acute cytokine changes in the foetal brain, hours after MIA challenge. These alterations in cytokine levels are complex and change over time. They also depend on the timing of the MIA challenge in the pregnancy (Meyer et al., 2006). A recent study found that MIA also induces long-lasting changes in brain cytokines which are region-specific and which change during postnatal development (Garay et al., 2012). They found that many cytokines (mainly pro-inflammatory) were elevated at birth in the frontal and cingulate cortices, after which they decreased during periods of synaptogenesis and plasticity before increasing again in the adult poly I:C offspring. They also observed alterations in cytokine levels in the hippocampus and in serum (Garay et al., 2012).

Many different doses of poly I:C have been used in the literature to induce the MIA model, as well as different time points during pregnancy. Surprisingly, even though the model is based on the induction of pro-inflammatory cytokines by activation of the maternal immune system, there is no

comprehensive dose-response study available that describes the effect of different doses of poly I:C and different time points during pregnancy on the induction of cytokines in the mother and foetus.

One proposed mechanism through which pro-inflammatory cytokines could act to increase the risk of schizophrenia is by triggering uncontrolled brain inflammation, resulting in detrimental effects on neurogenesis and neurodevelopment (Na et al., 2012). Microglia and astrocytes play an important role in brain inflammation. Microglia are the resident macrophages of the CNS and the most important players of the immune response in the CNS. They constitute about 10-20% of the total glial cell population in the adult brain. Under normal conditions these cells are in a resting state in which they survey the microenvironment. They monitor the wellbeing of cells in their immediate environment and contribute to the maintenance of homeostasis in the brain (Fetler and Amigorena, 2005). Microglia can be activated by a number of stimulatory signals. Depending on the nature of the stimulus, the molecular context and the time frame, they can acquire different phenotypes. Danger signals such as pro-inflammatory cytokines, ATP release etc. can induce microglia to switch to an activated state in which they secrete a number of inflammatory mediators such as pro-inflammatory cytokines, reactive oxygen and nitrogen species, and proteases such as matrix metalloproteinase-9 and tissue-type plasminogen activator (Dheen et al., 2007). These microglia are known as the classically activated M1 cells and are associated with brain injury and inhibition of neurogenesis, neuronal migration and differentiation (Hagberg et al., 2012). Other cytokines like IL-4 and IL-13 can activate microglia so that they produce anti-inflammatory cytokines. This phenotype is known as the alternatively activated M2 microglia and they are associated with tissue repair, remodeling of the extracellular matrix and an increased cell survival and neurogenesis. Finally, transforming growth factor-β (TGF-β), IL-10 and glucocorticoids can induce microglia to differentiate to a phenotype in which they promote immunosuppression. This is called acquired deactivation and is sometimes termed the M2-deactivated phenotype (Hagberg et al., 2012; Michelucci et al., 2009). It is clear that activated microglia can have both beneficial and detrimental effects on neurons and glial cells, mainly depending on the balance of pro-inflammatory and anti-inflammatory signals in their microenvironment (Ekdahl et al., 2009). A similar division of M1 and M2 phenotypes applies to macrophages (Kigerl et al., 2009). Neuroinflammation does not only involve activation of resident microglia, but also of astrocytes and infiltrating blood monocytes.

As MIA in general and poly I:C treatment in particular mainly induce the production of pro-inflammatory cytokines, it is to be expected that microglia will differentiate to the classically activated pro-inflammatory M1 phenotype, at least in the acute stage of the model. It is however possible that microglia that initially adopted the M1 phenotype switch to the neuroprotective anti-inflammatory M2 phenotype in a later stage of the disease.

4.2.3 *Persistent immune alterations*

There is extensive evidence in the literature that schizophrenia patients have persistent immunological disturbances. It has been proposed that schizophrenia patients might have a hyperactive pro-inflammatory immune system. High levels of pro-inflammatory cytokines have been observed in the sera and cerebrospinal fluid of schizophrenia patients (Kim et al., 2009; McAllister et al., 1995; O'Brien et al., 2008). A meta-analysis of cytokine alterations in the sera of schizophrenia patients showed that some of these vary with clinical status and thus act as state markers of the disease: IL-1β, IL-6 and TGF-β serum levels were found to increase during acute exacerbations and to normalize following antipsychotic treatment. In contrast, the serum levels of IL-12, IFN-γ, TNF-α and soluble IL-2 receptor remained unaltered during acute exacerbations and after antipsychotic treatment. They appear to be trait markers of the disease (Miller et al., 2011b). Peripheral cytokines however do not necessarily reflect inflammation of the CNS. Neuroinflammation as indicated by microglia activation has also been observed in the brains of adult schizophrenic patients, both in post-mortem and positron emission tomography (PET) studies (Bayer et al., 1999; Doorduin et al., 2009; Radewicz et al., 2000), especially in the frontotemporal regions (Radewicz et al., 2000). Microglia activation has also been shown in the hippocampus and striatum of the adult offspring of

poly I:C-treated rodents by the group of Juckel and by our group (Van den Eynde et al., submitted). The microglia in these offspring showed a reduced arborisation compared with the microglia found in control offspring, which indicates an activated state of these microglia (Juckel et al., 2011). Recently we performed an immunohistochemical staining against the CD68 receptor, which is expressed on lysosomes, endosomes and to a lesser extent on the cell surface of macrophages, neutrophils and reactive microglia. Although increases in the number and activation of CD11b-positive microglia had been found by our group in adult poly I:C offspring, almost no CD68-positive microglia were detected (Van den Eynde et al., submitted). However, this does not necessarily mean that no activated microglia are present in poly I:C offspring. CD68 has recently been proposed to be a marker for the pro-inflammatory M1 microglia (Kobayashi et al., 2013) and as discussed above, microglia that initially adopted the M1 phenotype may switch to the anti-inflammatory M2 phenotype in a later stage of the disease. However, CD68 has also been proposed to be a marker of active phagocytosis (Perego et al., 2011), which is typically believed to be more associated with the M2 phenotype (Fricker et al., 2012; Neher et al., 2011). It can therefore not be excluded whether the activated microglia in adult poly I:C offspring are of the M1 or the M2 phenotype. Other markers of M1 and M2 should be tested and not only in the chronic stage of the model, but in the acute stage as well. Good M1 and M2 markers are however lacking thus far.

Besides microglia, astrocytes may also play a role in the neuroinflammatory response in MIA offspring. Astrogliosis has not yet been described in the poly I:C model of MIA. It has however been observed in other models of MIA, namely in the hippocampus of adult offspring of IL-6-treated rats (Samuelsson et al., 2006) and in white matter structures of offspring of LPS-treated rats (Rousset et al., 2006). The data on astrogliosis in schizophrenia patients are however more ambiguous and inconclusive than those on microgliosis (Schnieder and Dwork, 2011).

It is unknown whether MIA offspring possess a hyperactive pro-inflammatory immune system as has been proposed for schizophrenia patients. This could be tested by administering LPS. LPS is widely used to systemically and acutely trigger the peripheral innate immune system of rats. Activation of the peripheral immune system leads to increased levels of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. These immune signals are relayed to the brain through neural and humoral pathways. Circulating LPS and pro-inflammatory cytokines make use of the breach in the blood-brain barrier at the circumventricular organs and activate microglia and other CNS immune cells which consequently produce brain pro-inflammatory cytokines (Dantzer, 2009; Hines et al., 2013). These brain cytokines act on neuronal substrates and trigger besides fever a behavioural response in the host to the inflammatory challenge. This adaptive response is known as sickness behaviour and serves to help the organism in overcoming the infection by reorganizing the host's priorities and motivations. It is characterized by depression-like behaviour such as anhedonia, lethargy, anxiety, reduced locomotor activity and explorative behaviour, reduced social interaction, sleepiness and loss of motivation for food and drink (Bassi et al., 2012; Dantzer, 2009; Henry et al., 2008; Soncini et al., 2012; Yirmiya, 1996).

5 Hypotheses and aims of the study

5.1 *Hypotheses*

We hypothesize that

1) MIA with poly I:C results in an acute increase of the mRNA levels of inflammatory cytokines in the maternal compartment (particularly in the maternal blood) and in the foetal compartment (especially in the foetal brain)
2) persisting brain inflammation, as measured by microglia activation, underlies the behavioural deficits in adult poly I:C offspring
3) adult poly I:C offspring are more sensitive to an inflammatory challenge than control offspring

5.2 *Aims*

The aims of this study are to

1) implement a model of MIA in our lab
2) evaluate the dose-response relationship between different doses of poly I:C and the induction of inflammatory cytokines in the maternal and foetal compartments at two different gestational periods in the rat: early/mid gestation (gestational day (GD)9) and mid/late gestation (GD15)
3) characterize the behavioural deficits in the poly I:C offspring and to link these with brain pathology i.e. microglia activation
4) evaluate the effect of an immune challenge on sickness behaviour and brain inflammation in adult control and poly I:C offspring

5.3 *Study design*

Three experiments were designed to meet these aims.

First of all, a poly I:C dose-response study is performed to assess the dose of poly I:C and the timing of exposure during pregnancy that result in the highest and most stable increases in pro-inflammatory cytokine mRNAs in the pregnant dams and the foetuses. Three doses of poly I:C are evaluated on two different time points during gestation in the rat: one in early/mid gestation (GD9) and one in mid/late gestation (GD15). We investigate whether there is a dose-response relationship between the examined poly I:C doses (2 mg/kg, 4 mg/kg and 8 mg/kg) and the mRNA expression of pro-inflammatory and anti-inflammatory cytokines in serum and spleen samples of the mother, as well as in the brains and peripheral tissues of the foetuses, and whether there is a different response to the poly I:C doses at different time points in the pregnancy.

Secondly, an LPS time-course and dose-response study is performed to assess the dose of peripherally administered LPS that induces overt yet not maximal brain inflammation in control animals, as well as the timing after LPS injection when brain inflammation peaks. Many different high doses of LPS have been used in the literature to induce massive brain inflammation in rodents. The ultimate goal of our final study is however to investigate whether poly I:C offspring are more sensitive to an inflammatory challenge than control offspring. Therefore we should be able to differentiate between the two groups. This study is set-up using lower doses of LPS (0.1 and 0.5 mg/kg) than those typically found in literature (Chen et al., 2012; Fan et al., 2012; Ha et al., 2012; Park et al., 2012; Tokes et al., 2011) to assess the dose that still is able to induce overt microglia activation in controls (as a proof of concept), but does not result in maximal microglia activation. Many different time points have been reported in literature of when microglia activation is apparent after intraperitoneal (i.p.) LPS administration, ranging from a few hours till one week (Fan et al., 2012; Ha et al., 2012; Park et al., 2012; Tokes et al., 2011). We choose to assess microglia activation at 6 hours, 24 hours, 3 days and 7 days after i.p. LPS injection to decide on the optimal time point for sacrificing the animals in the final study. As a low dose of LPS is typically used to induce sickness behaviour and depression-like behaviour in rodents, we investigate at the same time the evolution of anhedonia, a core symptom of sickness behaviour and depression, in the rats following LPS administration.

Finally, after having chosen the optimal dose of poly I:C and time point during pregnancy for induction of the MIA model, pregnant rats are injected with poly I:C or vehicle. Male adult offspring of poly I:C- and vehicle-treated dams are subjected to a number of behavioural tests to assess schizophrenia-like behavioural abnormalities, namely PPI disruption of the acoustic startle response, altered spontaneous locomotor activity, altered responsiveness to the locomotion-stimulating drugs amphetamine and MK-801, and anhedonia. To evaluate whether adult poly I:C offspring are more sensitive to an inflammatory challenge than control offspring, some of the animals receive an LPS challenge at the end of the behavioural assays. It is to our knowledge the first time that a similar test is performed in the poly I:C model. The animals challenged with LPS are subjected to a final sucrose

preference test and are expected to display an increased level of anhedonia, as well as a reduced total fluid intake and other behavioural symptoms of sickness. It is to be expected that poly I:C offspring are more affected than controls. We also expect poly I:C offspring to exhibit more brain inflammation due to the LPS challenge than control offspring. However, as poly I:C offspring are known to exhibit persisting brain inflammation due to the prenatal poly I:C challenge, these two factors might confound each other. Therefore animals are divided into two groups, a challenged group and a non-challenged group. Poly I:C and control offspring in the challenged group receive amphetamine and MK-801 challenges during the locomotion testing and an LPS challenge during a final sucrose preference test. Poly I:C and control offspring in the non-challenged group do not receive these challenges. LPS is administered at the end of all the behavioural experiments as the LPS-induced brain inflammation could have confounding effects on the behavioural assays. The optimal dose of LPS to induce microglia activation and time point to assess microglia activation in control and poly I:C offspring is deduced from the LPS time-course and dose-response study.

Materials and Methods

1 Animal ethics

All animals were treated in accordance with the European Ethics Committee (decree 86/609/CEE), the Animal Welfare Act (7 USC 2131) and the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003). Animal experiments were approved by the ethical committee of the University of Antwerp (approval number: 2011-23).

2 Poly I:C dose-response study

2.1 *Timed mating and MIA induction*

Thirty-six male and thirty-six female Wistar-Hannover rats of about 10 weeks old were purchased from Charles River Laboratories (France) and were used for in-house breeding. We opted for in-house breeding rather than purchasing pregnant females to avoid stress-related influences from transport during pregnancy. A timed mating procedure was employed in order to know the exact gestational age of the foetuses. Upon arrival, the rats were housed individually under standard laboratory conditions in a temperature- (22 $\pm$ 2°C) and humidity- (55 $\pm$ 10%) controlled room on a 12h-12h light/dark cycle (lights on at 8:00am) with standard food and water available *ad libitum*, and they were allowed to acclimatise for one week. During partitution the individually housed male and female rats switched cages and were put close together for two days in order to bring the female into estrus through olfactory stimulation by the male. At the end of the second day, the rats were allowed to copulate for one night. The following morning, a vaginal smear sample was taken from the female rat using a sterilised cotton swab. In case of successful coitus, sperm cells were found under microscopic magnification. This was termed day 1 of the pregnancy: GD1. Pregnant rats were either treated in early/mid pregnancy (GD9, n=17) or in mid/late pregnancy (GD15, n=19). On the day of treatment, pregnant rats were injected subcutaneously between 9h00 and 11h00 in the morning with either vehicle (n=4 for GD9, n=5 for GD15), 2 mg/kg (n=4 for GD9, n=5 for GD15), 4 mg/kg (n=4 for GD9, n=4 for GD15) or 8 mg/kg (n=5 for GD9, n=5 for GD15) poly I:C (Polyinosinic-polycytidylic acid sodium salt, Sigma-Aldrich, USA) dissolved in nuclease-free water (Sigma-Aldrich, USA). The administration route was based on a previous study in the lab that compared subcutaneous, intraperitoneal and intravenous administration of poly I:C regarding the induction of cytokines. Subcutaneous injection was considered the most optimal route. Nuclease-free water was used as vehicle to ensure that there was no immediate breakdown of the double-stranded RNA.

2.2 *Sample collection*

Six hours after injection, the pregnant rats were sacrificed by decapitation and exsanguination. Blood and spleen samples were collected from the mother, and brain and peripheral tissue samples were collected from the foetuses (four per litter). About 1 ml of blood was collected in an ethylenediaminetetraacetic acid (EDTA)-coated tube (BD Vacutainer, USA) that prevents the blood

from clotting, after which 0.5 ml was transferred to a 2 ml collection tube containing 1.3 ml of RNA*later* (Ambion, Life Technologies, USA). RNA*later* is an RNA stabilization reagent that immediately stabilizes and protects RNA from breakdown in blood and tissue samples ensuring reliable gene expression data. Next, the uterus containing the foetuses was dissected under sterile conditions out of the rat's abdominal cavity and put in dissection fluid on ice (4.5 g glucose/liter PBS) to minimize further molecular changes. Then the mother's spleen was isolated and a small part of it was transferred to an eppendorf tube containing RNA*later*. Following this, four foetuses per mother were dissected out of their amniotic sacs and further dissected to obtain samples of CNS tissue and peripheral tissue. Depending on the gestational age, this meant either cutting the embryo into two parts (one part giving rise to the brain and the other giving rise to the remainder of the body) in case of a small embryo of 9 days old, or dissecting the upper part of the head containing the brain, and the liver, in case of a foetus of 15 days old. These samples were also preserved in RNA*later*-containing eppendorf tubes. All of the samples were stored at -80°C.
Part of the study was performed before my arrival at the lab, according to a slightly different protocol. No spleen samples were collected from the mothers in this subgroup of rats (GD9: n=8; GD15: n=11).

2.3 *Sample processing and cytokine analysis*

RNA was extracted out of whole blood using the RiboPure™-Blood Kit (Ambion, Life Technologies, USA). The blood samples stored in RNA*later* were first centrifuged in a microcentrifuge after which the supernatans was discarded. The remaining pellet consists of blood cells and plasma proteins. Blood cells were lysed by vortexing the pellet in lysis solution and sodium acetate solution. RNA was then extracted in an initial purification step by adding acid-phenol:chlorophorm to the cell lysate and mixing it by vortexing. The aqueous and organic phases were separated by centrifugation. RNA partitions in the aqueous phase, which was transferred to a new tube. About one-half volume of 100% ethanol was added to the sample. Final RNA purification was achieved by passing the sample through a filter cartridge. The sample was applied to the filter and the assembly was centrifuged. The flow-through was discarded. The filter was washed three times by applying wash solutions to the filter, centrifuging the assembly and discarding the flow-through. Finally, the RNA was eluted from the filter by applying heated elution solution to the filter, centrifuging the assembly and recovering the elution solution containing the RNA in a collection tube. RNA was then quantified using a NanoDrop spectrophotometer (NanoDrop, USA), based on its property to have a peak absorption of ultraviolet light at 260 nm. The more UV light of 260 nm is absorbed by the sample, the higher the concentration of nucleic acid in the sample. The quality of the RNA purification was evaluated by determining the ratio of absorption at 260 nm and 280 nm (260/280 ratio) to assess contamination of the RNA solution by protein, phenol and other contaminants that absorb strongly at or near 280 nm, as well as the ratio of absorption at 260 nm and 230 nm (260/230 ratio) to assess contamination by certain organic compounds such as phenol that absorb at or near 230 nm. Ideally, the 260/280 ratio should be 2.0, and the 260/230 ratio should be in the range of 2.0-2.2. The RNA samples were stored at -80°C.

RNA was extracted out of spleen samples and foetal tissue samples (one or two foetuses per litter) in an initial purification step by homogenizing the samples in QIAzol (containing phenol and the chaotropic agent guanidinium thiocyanate - Qiagen, the Netherlands) using a homogenizer (Qiagen, the Netherlands) and mixing the resulting cell lysates with chloroform by vortexing. The aqueous and organic phases were separated by centrifugation. The RNA-containing aqueous phase was recovered and one and a half volume of 100% ethanol was added. Final RNA purification was achieved using the RNeasy Mini Kit (Qiagen, the Netherlands). The sample was loaded onto an RNeasy Mini spin column, the whole assembly was centrifuged and the flow-through discarded. The filter was washed three times and finally the RNA was eluted in RNase free water. The quantity and quality of the RNA was determined using the NanoDrop spectrophotometer as described above. The RNA samples were stored at -80°C.

Messenger RNA (mRNA) was converted to complementary DNA (cDNA) in a reverse transcriptase-polymerase chain reaction (RT-PCR). For each sample, 500 ng of RNA was used in a reaction, based on the concentration of the RNA sample as measured with the NanoDrop spectrophotometer. One reaction also contained 0.5 µl RNaseOUT (Invitrogen, Life Technologies, USA), 1 µl Oligo-dT primer (Invitrogen, Life Technologies, USA), 10 µl RT buffer (Promega, USA), 1 µl RT enzyme (Promega, USA) and 5 µl of a 2.5 mM mixture of deoxynucleoside triphosphates (dNTPs - Invitrogen, Life Technologies, USA). Finally, the reaction mixture was diluted to an end volume of 50 µl with Depe-Bidi water. All reaction mixtures were then placed in a PCR machine (Applied Biosystems, Life Technologies, USA) and the following program was run: 60min at 42°C, 15min at 75°C and 30min at 4°C. RNaseOUT inhibits ribonuclease activity and thus RNA breakdown. Oligo-dT anneals to the poly-adenosine tail of mature eukaryotic mRNA and allows for the selective conversion of mRNA amidst other RNA molecules such as tRNAs and rRNAs. The reverse transcriptase enzyme synthesizes cDNA starting from the oligo-dT primer onwards making use of the dNTP building blocks. The dNTP mixture consists of dATPs, dGTPs, dCTPs and dTTPs. The resulting cDNA was diluted 1:5 with Depe-Bidi water.

All cDNA samples were then subjected to real-time qPCR for the relative quantification of the most important pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and of the anti-inflammatory cytokine IL-10. For the quantification of one gene transcript in the cDNA samples, a mastermix was prepared to include per well 12.5 µl SYBR Green-mix (which contains besides SYBR Green I also the Taq DNA polymerase, dNTPs and buffer solution - Bioline, USA), 0.875 µl forward primer (Eurogentec, Japan), 0.875 µl reverse primer (Eurogentec, Japan) and 8.25 µl Depe-Bidi water. The primers define which sequence of DNA is replicated during each cycle (see Appendix for an overview of the used primers). 2.5 µl of each cDNA sample was added to different wells on a 96-well plate, as well as 22.5 µl of the mastermix. Each well contained thus 25 µl as an end volume. Each cDNA sample was tested *in triplo* on a 96-well plate for each gene transcript. The PCR plate was shortly centrifuged and placed into the qPCR machine (ABI 7300, Applied Biosystems, Life Technologies, USA). Forty amplification-cycles were run. A dissociation step was added to evaluate the binding specificity and thus the quality of the primers. The DNA is quantified during amplification (in real-time) by means of the intercalating fluorescent dye SYBR Green I, that binds to the double-stranded DNA and absorbs blue light while emitting light of a longer wavelength (green light). The amount of target nucleic acid in a sample (e.g. of IL-1β) is measured by assessing the Ct value or cycle threshold, which is defined as the number of amplification cycles required for the fluorescent signal of SYBR Green I to exceed the background fluorescence. The more amplification cycles are needed to cross this threshold, the lower the amount of target nucleic acid in the sample and thus the lower the expression of the target transcript (provided that this expression is normalized to the expression of one or more housekeeping genes in the sample). The qPCR results were analyzed using the 2^{-ddCt} method, which is a convenient method for the relative quantification of gene expression changes. First, the Ct values of a target transcript of a sample are normalized to the expression of one or more internal control genes in that sample, which are known to have a stable expression throughout all samples (housekeeping genes). As the expression of housekeeping genes might be more variable during an immune response in pregnancy, the Ct values of a target transcript were normalized to the geometrical mean of the Ct values of three housekeeping genes (β-actin, 28S rRNA and hypoxanthine-guanine phosphoribosyltransferase - HPRT). The geometrical mean indicates the central tendency of a set of values.

> dCt (target transcript) = Ct (target transcript) – geometrical mean of the Ct values of β-actin, 28S rRNA and HPRT

Next, the normalized expression of a target transcript (e.g. IL-1β) in the treated samples (e.g. 2 mg/kg, 4 mg/kg and 8 mg/kg poly I:C on GD15) was compared to the normalized expression of the target transcript in the reference sample or calibrator (e.g. 0 mg/kg poly I:C on GD15) i.e. relative quantification.

> ddCt (treated sample) = dCt (treated sample) – median of the dCt values of the reference samples

Because there was a lot of variation in the qPCR results, we calculated with medians rather than averages, as to reduce the influence of outliers. Finally, the logarithmic scale was transformed into a linear scale by a simple transformation: 2^{-ddCt}. This value expresses the fold change in gene expression, normalized to a number of internal control genes and relative to the untreated reference sample. For the reference sample, ddCt is near to zero and 2^{-ddCt} near to one. The gene expression changes in the treated samples are therefore relative to one, with one indicating no change in the gene expression of the target transcript. A 2^{-ddCt} value greater than one indicates an upregulation of the target transcript, while a 2^{-ddCt} value smaller than one reflects a downregulation of the target transcript.

3 LPS time-course and dose-response study

3.1 *Treatment of animals*

Twenty-one male Wistar rats of about 12 weeks old were purchased from Charles River Laboratories and were housed individually upon arrival under standard laboratory conditions in a temperature- (22 $\pm$ 2°C) and humidity- (55 $\pm$ 10%) controlled room on a reversed 12h-12h light/dark cycle (lights out at 10:00am) with standard food and water available *ad libitum*, and they were allowed to acclimatise for one week. The rats were then subjected to a sucrose preference test according to a protocol adapted from (Vloeberghs et al., 2007). This consisted of an initial habituation period of 48h to habituate the animals to i) the presence of two drinking bottles in the home cage, ii) the presence of a sweet sucrose solution in one of the bottles. One bottle contained 500 ml of either tap water or 2% sucrose solution (2 g of sucrose dissolved in 100 ml tap water). The bottles were weighed before placing them in the cages and again after the 48h habituation period. The sucrose preference was calculated according to the following formula:

$$\%\ sucrose\ preference = \frac{sucrose\ solution\ consumption}{total\ liquid\ consumption} \times 100$$

The sucrose and water bottles were placed randomly in the cages so that the sucrose bottle was sometimes on the right side and sometimes on the left side. This was done to exclude confounding effects of place preference. The position of the bottles was switched after 24h. After the 48h habituation period, all rats received an intraperitoneal injection with a low dose of the bacterial endotoxin LPS (Lipopolysaccharides, from Escherichia coli O111:B4, Sigma-Aldrich, USA) dissolved in saline. Twelve rats received 0.1 mg/kg LPS and nine rats 0.5 mg/kg LPS. Rats were returned to their home cages and again subjected to a sucrose preference test (the actual test period). Sucrose preference and total liquid consumption were measured at 6 hours, 24 hours, 3 days and 7 days after LPS injection as described above. Animals were followed-up until they were sacrificed. At each of the different time points, three rats per dose were sacrificed, except for the last time point (7 days) where only rats of the 0.1 mg/kg dose were sacrificed. Three age-matched control animals of another study were included in the data set.

3.2 *Brain collection*

Rats were sacrificed by decapitation, after which the brain was dissected out of the skull. The brain was then cut in half with a scalpel. One hemisphere was directly snap-frozen in 2-methylbutane on dry ice at -35°C for 3 min (“fresh tissue”). The other hemisphere was first fixated overnight at 4°C in freshly made 4% paraformaldehyde (PFA), then impregnated overnight at 4°C with the cryoprotectant sucrose (20% sucrose containing 0.1% of the bacteriostatic sodium azide) and finally snap-frozen in 2-methylbutane on dry ice at -50°C for 2 min (“fixated tissue”). All samples were stored at -80°C.

3.3 *Histology*

The PFA-fixated brain hemispheres were used for histology. Serial 20 µm-thick sagittal cryosections were cut at 2.10 mm from bregma (Paxinos and Watson rat brain atlas) using a cryostat (Thermo

Scientific Microm, Germany). Tissue sections were mounted on glass microscope slides that were previously coated with the positively charged poly-L-lysine in order to achieve better adhesion of the tissue. Sections were then dried for at least two hours in an incubator at 37°C to achieve still better adhesion of the tissue sections. Finally they were stored at -80°C.

Microglia activation was detected through immunohistochemistry by making use of OX-42 antibodies that recognize the CR3 complement receptor CD11b that is expressed by microglia in the rat. Tissue sections were dried at room temperature for at least two hours prior to staining. Sections were fixated in ice-cold acetone for 10 min and dried again at room temperature for 20 min. Sections were washed with 0.01 M phosphate buffered saline (PBS) and treated with 3% hydrogen peroxide (Sigma-Aldrich, USA) in distilled water (dH_2O) for 5 min and with 3% normal horse serum (NHS) in PBS for 10 min to respectively block endogenous peroxidase and endogenous proteins in order to reduce aspecific background activity. Sections were incubated overnight with primary antibody (mouse anti-rat CD11b, AbD Serotec, UK) diluted 1:1000 in antibody diluent containing 0.1% bovine serum albumin (BSA), 0.2% Triton X-100 and 2% NHS in PBS. The next morning, sections were washed with PBS and incubated for 1h with biotinylated secondary antibody (donkey anti-mouse IgG, Jackson Immunoresearch, USA) diluted 1:500 in antibody diluent. Following this step, sections were washed with PBS and incubated for 1h with ExtrAvidin-Horse Radish Peroxidase (Sigma-Aldrich, USA), diluted 1:1000 in PBS. Avidin has a strong affinity and specificity for biotin, which is conjugated to the secondary antibody. Sections were washed with PBS and the reaction was visualized by shortly adding the colorimetric substrate 3,3'-diaminobenzidine (1 drop of DAB per ml buffer solution - Dako, Denmark), which is oxidized in the presence of hydrogen peroxide (present in the buffer) and the peroxidase enzyme (Horse Radish Peroxidase), conjugated to the avidin, to give a dark-brown colour. This staining reaction was followed-up visually and stopped with dH_2O. A counterstaining with the basic dye cresyl violet was performed to visualize cell bodies of neurons for anatomical orientation in the tissue (5 min in dH_2O, 30 sec in cresyl violet). Cresyl violet stains the RNA-containing Nissl substance in neurons. Sections were progressively dehydrated in dH_2O (2 min), 70% ethanol (2 min), acid ethanol (1 ml glacial acetic acid per 100 ml 95% ethanol – 1 min), 100% ethanol (3 min), 100% ethanol (3 min), xylene (3 min) and xylene (3 min). Finally, a cover slip was mounted on the sections using Entellan (Merck, USA).

3.4 *Histological analysis*

The level of microglia activation in the rat brain was assessed by use of a visual scoring system (CD11b score). Two researchers scored the tissue slides independently and blinded for treatment. An average of these two scores was used for the analysis. Four brain regions per animal were scored under microscopic magnification (10x): frontotemporal cortex, corpus callosum, hippocampus and thalamus. Each brain region was scored for two parameters: the number of stained microglia and the activation state of the microglia, based on their morphological appearance. Resting-state microglia are highly ramified, while activated microglia show a reduced arborisation (thicker and shorter processes) and big cell bodies. A score between 0 and 3 was given for each of the two parameters per brain region per animal. We first looked at the entire data set and determined the average number of microglia and activation of microglia in each brain region. During scoring, a score of 2 was given to an animal with an average number of microglia or an average activation state of microglia. Animals with a lower than average number/activation of microglia, were assigned a score of 1. Animals with a higher than average number/activation of microglia, were assigned a score of 3. Scores of 0.5, 1.5 and 2.5 could also be given. Occasionally, a score of 0 can be given when no microglia are activated. The two parameters were regarded independently. An animal could have a high number of microglia (score 3 for number) with a low activation state (score 1 for activation). The scores for number and activation were added to give a total score per brain region per animal. These individual brain region scores were added to give a total score between 0 and 24 per animal.

4 Behavioural and histopathological study of poly I:C and control offspring

4.1 *Timed mating, MIA induction and follow-up of MIA response*

Male and female Wistar-Hannover rats of about 10 weeks old were purchased from Charles River Laboratories and were used for in-house breeding. Animals were housed individually as in §2.1 under standard laboratory conditions on a 12h-12h light/dark cycle (lights on at 8:00am). A timed mating procedure, as described in §2.1, was carried out on a weekly basis with max. five rat couples so that behavioural testing of adult offspring could be initiated every week and so that the number of animals to be tested in a week would not be higher than 12 (in case of a 60% chance of success and testing 4 littermates per mother). Based on the results of the poly I:C dose-response study, the pregnant dams' immune system was challenged with 4 mg/kg poly I:C on GD15 of the pregnancy. The rats were weighed and injected subcutaneously between 9h00 and 11h00 in the morning with either vehicle (nuclease-free water) or 4 mg/kg poly I:C. In total, five pregnant dams were injected with vehicle and seven dams with poly I:C.

Six hours after injection, the response of the pregnant rats to the immune challenge was followed-up by weighing them and taking their body temperature. Poly I:C is known to induce a reduced food intake and thus weight loss in rats (Fortier et al., 2004b). The body weight change was calculated by subtracting the rat's weight before injection from the rat's weight at 6 hours post-injection. Poly I:C is also known to induce an acute IL-1-dependent fever response in rats (Fortier et al., 2004b). Rats were anesthetized with isoflurane (5% for induction, 2.5% for maintenance) in medical oxygen for an approximate 10 min. The rectal temperature was measured as quickly as possible with a rectal probe because of the hypothermia that develops following induction of anesthesia. The rats were placed on a heating pad to counteract this hypothermia. In addition, we collected a blood sample under anesthesia. Approximately 15-20 drops of blood were drawn from the tail vein and collected in a clot-activator coated tube (BD Vacutainer, USA). These blood samples were centrifuged (4°C, 3000rpm, 10 min) and the resulting serum samples were collected. These samples will be used for future evaluation of the circulating cytokines.

On the day of birth, the nests were culled to eight pups per nest, retaining as many male pups as possible. In this way the nests were more similar to each other, which promotes the standardization of results. When offspring reached the age of 3 weeks (21 days), male offspring were weaned and group-housed and left undisturbed until they reached adulthood (12 weeks). Maximum four male rats per litter were included in the behavioural assays: two rats were assigned to the challenged group, the other two to the non-challenged group. This was done in order to prevent that too many littermates make up the data of one group. Their close genetic relationship might confound data otherwise. The remainder of the male offspring were included in a cross-sectional study, in which offspring of vehicle- and poly I:C-treated dams were sacrificed at 5 different ages between birth and adulthood for the collection of brain and blood samples (see Future perspectives).

We decided to use male pups in this study to avoid confounding effects of hormonal variation in females on certain behavioural tests. PPI for instance can be influenced by the estrous cycle (Fortier et al., 2007). The offspring was group-housed after weaning, as to avoid confounding factors of single housing. Indeed, it has been shown that social isolation rearing of rats leads to behavioural deficits related to schizophrenia (Moller et al., 2012). Offspring was subjected to behavioural testing in adulthood (12 weeks), as the behavioural deficits in this model have been most consistently reported in adulthood (Zuckerman and Weiner, 2003). In total, 19 male control offspring and 25 male poly I:C offspring were included in the study.

4.2 *Behavioural assessment*

When the animals reached adulthood, behavioural testing started. One week prior to testing, animals were transferred to a reversed 12h-12h light/dark cycle (lights out at 10:00am) so that all behavioural tests could be performed during the active (dark) phase of the animals.

4.2.1 *Prepulse inhibition of the acoustic startle response*

First of all, the PPI of the acoustic startle response was determined on postnatal day (PND)81-82 according to a protocol adapted from (Wolff and Bilkey, 2008). The PPI was measured in standard startle boxes (Kinder Scientific, USA). Rats were placed in the startle boxes and were allowed to acclimatise for 5 min under a 65 dB background noise level. Following acclimatisation, rats received a train of 5 startle pulses to normalize the startle response of the rat. A startle pulse had an intensity of 120 dB and a duration of 40 ms. The startle response of the rat was measured by a piezoelectric sensor, which transduces force and pressure into an electrical charge. The actual experiment was then initialized and consisted of 8 blocks of 10 trials. During a trial, a stimulus is given to the animal and its startle response is recorded. Different stimuli were delivered to an animal in semi-random order: no stimulus (=background noise of 65 dB), a startle pulse (120 dB, 40 ms), a weak prepulse, and the combination of a weak prepulse and the startle pulse with a fixed time interval of 100 ms. Three prepulses of different intensities were tested: 70 dB, 75 dB and 80 dB, each with a duration of 20 ms. The intertrial interval varied between 10 and 20 seconds. The %PPI for each of the three prepulses was then calculated according to the following formula:

%PPI=[1-(startle response at prepulse+pulse-trial/ startle response at pulse alone-trial)]x100

4.2.2 *Spontaneous and psychotomimetic-induced locomotor activity*

Locomotor activity was measured using the home cage - 4x8 photobeam activity system (San Diego Instruments, USA) around PND82-84. The protocols for measuring the psychotomimetic-induced hyperactivity were adapted from (Zuckerman et al., 2003) and (Zuckerman and Weiner, 2005). Animals were allowed to acclimatise for at least 15 min, after which an initial test period of 60 min began. During this period, spontaneous locomotor activity was recorded in 5-min bins. All beam interruptions were counted, but only beam-to-beam crossings were taken into account during data analysis, as they reflect true ambulatory movement of the rat. Sequential interruption of the same beam without interruption of another beam, reflecting fine movements such as grooming, were not taken into account. Ambulatory beam crossings in the x and y direction were summated for data analysis. After the 60 min test period, poly I:C- and control offspring in the challenged groups (respectively n = 12 and n = 8) received a subcutaneous injection with 1 mg/kg of the indirect dopamine agonist amphetamine (Dextroamphetamine sulfate, Janssen Pharmaceuticals, Belgium) dissolved in saline. Poly I:C- and control offspring in the non-challenged groups received a subcutaneous injection with the vehicle saline instead (respectively n = 12 and n = 10). Following injection, locomotor activity was recorded for an additional 120 min, again in 5-min bins. In the rats that received saline, spontaneous activity was recorded, while in the amphetamine-treated rats, psychotomimetic-induced hyperactivity was registered. The poly I:C- and control offspring in the challenged groups underwent the same experiment again one or two days later, but were now challenged with the NMDA-receptor antagonist MK-801 ((+)-MK-801 hydrogen maleate, Sigma-Aldrich, USA) dissolved in saline. So now again, 60 min of spontaneous activity was recorded, after which the rats received a subcutaneous injection with 0.2 mg/kg MK-801. Psychotomimetic-induced hyperactivity was then recorded for a subsequent 120 min.

4.2.3 *Sucrose preference test and LPS-induced sickness behaviour*

Finally, the animals were subjected to a sucrose preference test according to the protocol described in §3.1. For this test, animals were housed individually. The test started usually on PND89. Following the 48h-habituation period, a test period of 24h was initiated to assess the basal sucrose preference levels of poly I:C and control offspring. After this 24h-test period, the poly I:C- and control offspring in the challenged groups received an intraperitoneal LPS injection (0.1 mg/kg, dissolved in saline) to challenge the immune system. The dose was deduced from the LPS pilot study. The offspring in the non-challenged groups received an i.p. saline injection. The

rats were then subjected to another sucrose preference test. The sucrose preference and total liquid consumption were measured at 6 hours and 3 days after LPS or saline injection. A reduced sucrose preference and a reduced total fluid intake are both indicative of sickness behaviour, as well as a reduction in the weight of the rats. To assess weight loss, rats were weighed before LPS or saline injection and again at 6 hours and 3 days after injection. The body weight change was calculated by subtracting the rat's weight before injection from the rat's weight at 6 hours and 3 days post-injection.

4.3 *Histopathological analysis*

4.3.1 *Brain collection*

Animals were sacrificed by decapitation 3 days post-injection and brains were collected and stored as described in §3.2. Samples were stored at -80°C.

4.3.2 *Histology*

The histology was performed analogous to §3.3. This time however, sagittal cryosections of 10 µm were cut and used for immunohistochemical staining of microglia by OX-42 antibodies and subsequent DAB visualization. Sections were now cut and stained by a lab technician. We opted for 10 µm sections instead of 20 µm sections in order to reduce the background of the staining.

4.3.3 *Histological analysis*

The degree of microglia activation in the rat brain was assessed blinded by two researchers using a visual scoring system as described in §3.4. Six schizophrenia-related brain regions per animal were scored under microscopic magnification (10x): striatum, frontotemporal cortex, corpus callosum, hippocampus, thalamus and pons. Because in two animals one of the six brain structures could not be scored, we opted to present an average CD11b score for each animal (an average of the CD11b scores of the 6 or 5 brain regions) instead of a total summated score.

5 Statistics

The qPCR data of both mothers and foetuses were analysed using a Kruskal-Wallis test. Post-hoc testing was performed using Dunn's multiple comparisons test.

The data on sucrose preference, total liquid consumption and microglia activation (CD11b scores) were analysed using a Kruskal-Wallis test with Dunn's multiple comparisons test for post-hoc testing. The evolution of the sucrose preference in three 0.1 mg/kg-treated rats and three 0.5 mg/kg-treated rats that were followed-up from 6 hours up to 3 days post-injection, was analysed using a Friedman test with Dunn's multiple comparisons test. Correlations between sucrose preference, total fluid intake and microglia activation were investigated using Spearman's correlation test.

The weight change data of the mothers were analysed using a Mann-Whitney test. The PPI data and locomotor activity data over time were analysed using a repeated measures two-way ANOVA test. Post-hoc testing was performed using Tukey's multiple comparisons test. The total locomotor activity data were also analysed in a repeated measures two-way ANOVA test. Post-hoc testing was done using Sidak's multiple comparisons test. The data on basal sucrose preference, basal total liquid consumption, acute weight change following LPS or saline injection and microglia activation (CD11b scores) were all analysed using Kruskal-Wallis tests with Dunn's multiple comparisons test for post-hoc testing. The data on sucrose preference and total fluid intake before and after LPS or saline injection were analysed in a Friedman test with Dunn's multiple comparisons test to evaluate changes over time within a group, as well as with a Kruskal-Wallis test with Dunn's multiple comparisons test to evaluate changes between groups.

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., USA).

Results

1 Poly I:C dose-response study

1.1 *Maternal compartment*

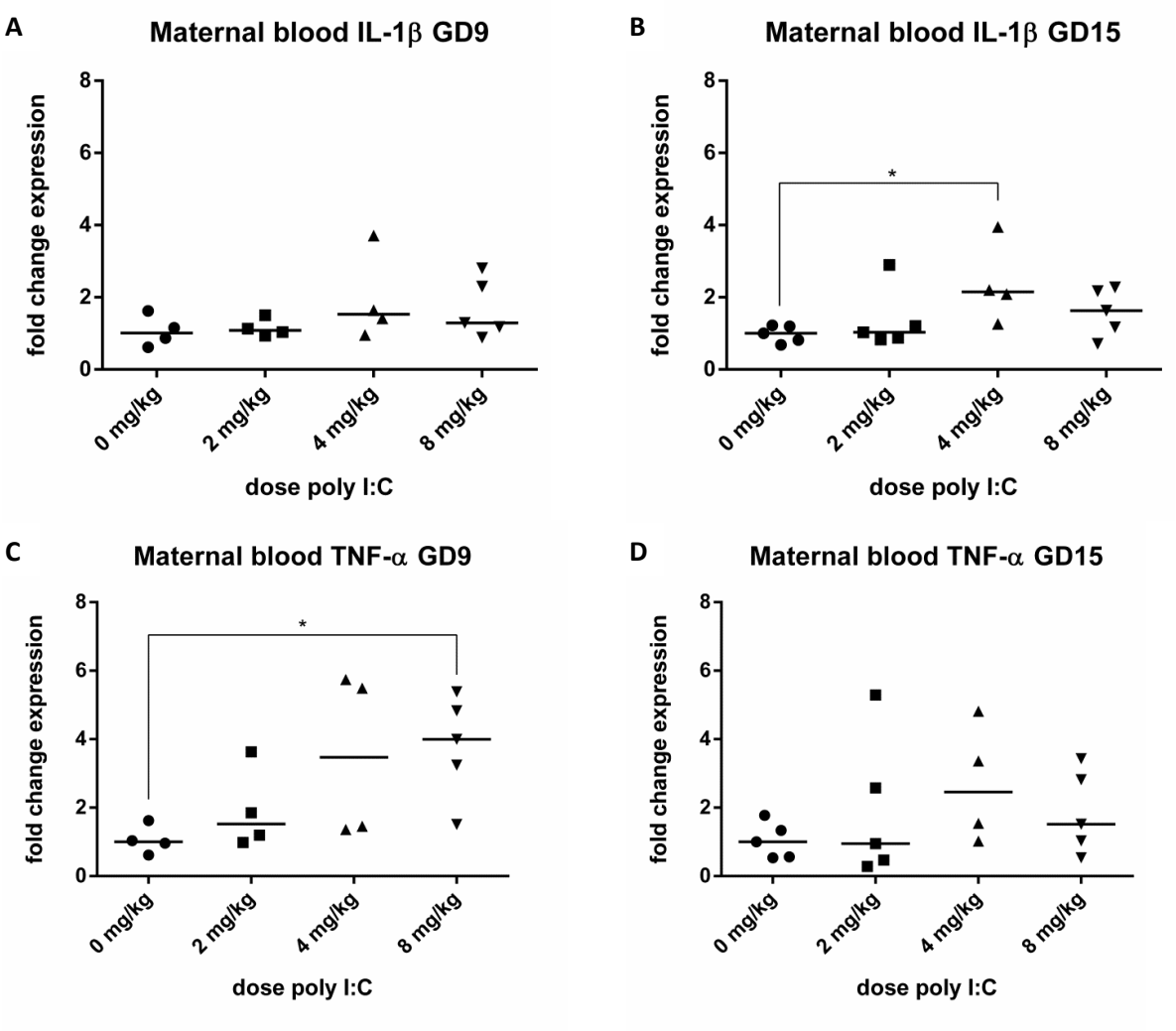

Fig 1. Induction of pro-inflammatory cytokines IL-1β (panels A and B) and TNF-α (panels C and D) in the maternal blood 6 hours after subcutaneous injection with either vehicle (nuclease-free water) or one of three doses of poly I:C (2 mg/kg, 4 mg/kg or 8 mg/kg) on gestational days GD9 (panels A and C) and GD15 (panels B and D) of the pregnancy. All data are represented as separate data points in the plot, with a line representing the median of the group. (Panel A and C. 0 mg/kg: n=4, 2 mg/kg: n=4, 4 mg/kg: n=4, 8 mg/kg poly I:C: n=5; Panels B and D. 0 mg/kg: n=5, 2 mg/kg: n=5, 4 mg/kg: n=4, 8 mg/kg poly I:C: n=5) * = p≤0.05 (Kruskal-Wallis test with Dunn's multiple comparisons test)

Significant increases in the mRNA levels of inflammatory cytokines could be found in the blood of the pregnant dams 6 hours after subcutaneous injection with poly I:C compared to vehicle-treated controls. IL-1β mRNA was the highest upregulated after injection with the 4 mg/kg poly I:C dose in the maternal blood (Fig 1A and B). This increase in IL-1β mRNA was significant in rats that were in their mid/late pregnancy (GD15) compared to vehicle-treated controls (p≤0.05 - Fig 1B). TNF-α also had the highest mRNA expression after the 4 mg/kg dose in the blood of GD15-mothers (Fig 1D). mRNA levels of TNF-α were also very high after the 4 mg/kg and 8 mg/kg poly I:C doses in the blood of mothers that were in their early/mid pregnancy (GD9). The increase in TNF-α mRNA expression after the 8 mg/kg dose in GD9-mothers was significant compared to the TNF-α mRNA expression in vehicle-treated dams (p≤0.05 - Fig 1C).

However, when we look at the individual data of the pregnant dams, it becomes apparent that not all dams responded to the immune challenge with an increase in cytokine mRNA levels.

In parallel with the maternal blood, the 4 mg/kg and 8 mg/kg poly I:C doses induced the highest increases in mRNA expression of inflammatory cytokines in the spleen of the pregnant mother, which is rich in immune cells. The highest increases were found in IL-6 mRNA after the 8 mg/kg dose, in the spleens of both the GD9- and G15-mothers (data not shown).

When comparing the cytokine response of rats in early/mid pregnancy and rats in mid/late pregnancy to the poly I:C immune challenge, no statistically significant differences were found between GD9- and GD15-mothers for any of the treatments (2 mg/kg, 4 mg/kg and 8 mg/kg poly I:C).

1.2 *Foetal compartment*

The foetuses seemed to be divided into responders and non-responders regarding the induction of inflammatory cytokines following MIA. Some foetuses exhibited a moderate to high increase in the mRNA levels of inflammatory cytokines compared to controls, while others showed no increase at all. This makes it difficult to find statistically significant differences between groups. Results will therefore be described in a descriptive way.

In the foetal compartment, the 4 mg/kg poly I:C dose most often resulted in the largest induction of cytokines. Two GD9-old foetuses and one GD15-old foetus were particularly interesting as they showed high increases in the mRNA levels of all the investigated pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in their brains after MIA with the 4 mg/kg poly I:C dose (Fig 2A-D, indicated in red). The mRNA expression of the anti-inflammatory cytokine IL-10 was also highly induced in the brains of these two GD9-old foetuses after injection of the mother with the 4 mg/kg dose (Fig 2E). The induction of IL-10 mRNA was by comparison much lower in the brain of the GD15-old foetus after MIA with the 4 mg/kg dose (Fig 2F). It is interesting to note that the two above-mentioned GD9-old foetuses that showed a very similar cytokine response to the MIA challenge were littermates and that the mentioned GD15-old foetus had a littermate with a much lower cytokine response (also indicated in red in Fig 2B, D and F).

The changes in mRNA expression of inflammatory cytokines in the peripheral tissue of the foetuses were often parallel with the changes in the brains of the foetuses, but could also deviate from the alterations in the CNS (data not shown). These results will not be discussed in detail as they are of less importance to the neurodevelopmental insult than the MIA-induced changes in brain cytokine expression.

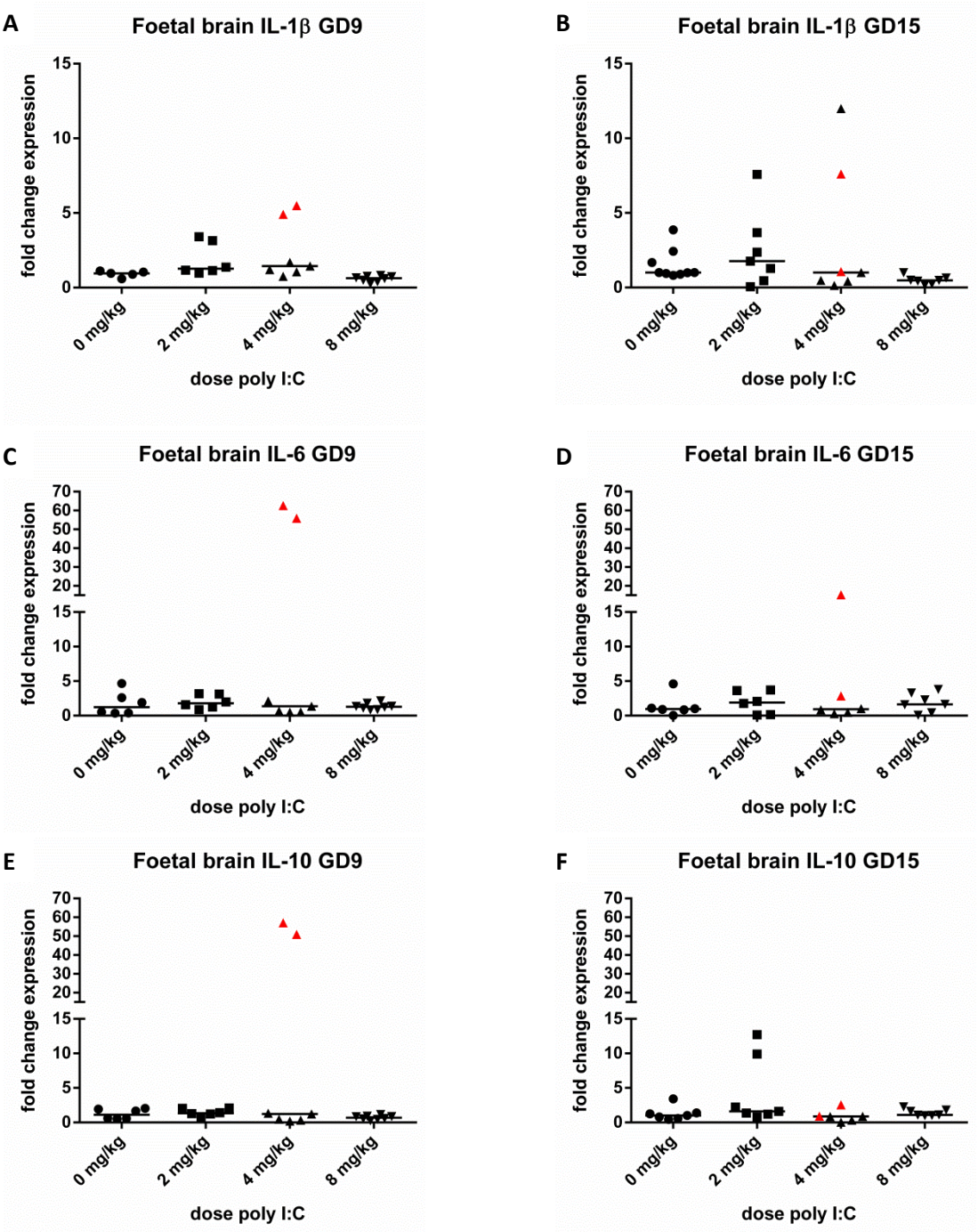

Fig 2. Induction of pro-inflammatory cytokines IL-1β (panels A and B) and IL-6 (panels C and D) and of the anti-inflammatory cytokine IL-10 (panels E and F) in the foetal brain 6 hours after subcutaneous injection of the mother with either vehicle (nuclease-free water) or one of three doses of poly I:C (2 mg/kg, 4 mg/kg or 8 mg/kg) on gestational days GD9 (panels A, C and E) and GD15 (panels B, D and F) of the pregnancy. All data are represented as separate data points in the plot, with a line representing the median of the group. Panels A, C and E: The two data points in red are littermates. They showed a very similar response to the MIA challenge: high increases in the mRNA levels of every investigated pro-inflammatory cytokine and of the anti-inflammatory cytokine as well. Panels B, D and F: The two data points in red are littermates. They exhibited a different response to the MIA challenge. One of them showed high increases in the mRNA levels of all the investigated pro-inflammatory cytokines and a moderate increase in the mRNA levels of the anti-inflammatory cytokine. The other one showed almost no increases in the mRNA levels of any investigated inflammatory cytokine. (Panel A. 0 mg/kg: n=5, 2 mg/kg: n=6, 4 mg/kg: n=7, 8 mg/kg poly I:C: n=8; Panel B. 0 mg/kg: n=9, 2 mg/kg: n=7, 4 mg/kg: n=7, 8 mg/kg poly I:C: n=7; Panel C. 0 mg/kg: n=6, 2 mg/kg: n=6, 4 mg/kg: n=7, 8 mg/kg poly I:C: n=8; Panel D. 0 mg/kg: n=6, 2 mg/kg: n=6, 4 mg/kg: n=6, 8 mg/kg poly I:C: n=7; Panel E. 0 mg/kg: n=6, 2 mg/kg: n=6, 4 mg/kg: n=7, 8 mg/kg poly I:C: n=8; Panel F. 0 mg/kg: n=7, 2 mg/kg: n=7, 4 mg/kg: n=6, 8 mg/kg poly I:C: n=7) (Kruskal-Wallis test with Dunn's multiple comparisons test)

2 LPS time-course and dose-response study

2.1 *Behavioural findings*

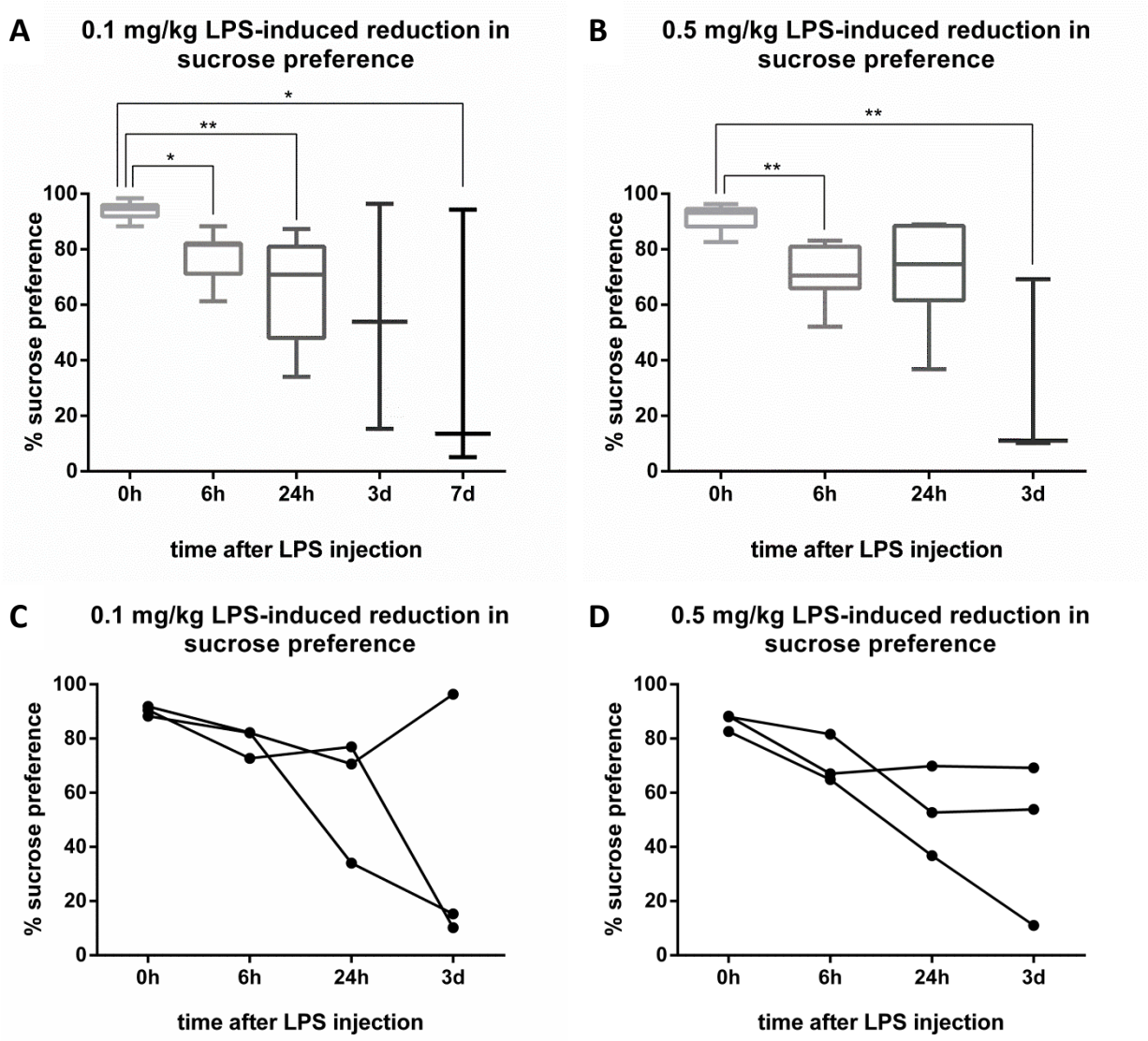

Fig 3. Reduction of the sucrose preference following peripheral (i.p.) administration of a low dose of LPS, 0.1 mg/kg (panels A and C) and 0.5 mg/kg (panels B and D). In panels A and B, the sucrose preference of a group of animals is plotted as a boxplot with the outer bars representing the min and max values of that group and the midline at the median of the group (panel A: n=12 at 0h, n=9 at 6h, n=6 at 24h, n=3 at 3d, n=3 at 7d, panel B: n=9 at 0h, n=9 at 6h, n=6 at 24h, n=3 at 3d). In panels C and D, the evolution of the sucrose preference within three individual animals is represented (panel C: n=3, panel D: n=3). * = p≤0.05, ** = p≤0.01 (panels A and B: Kruskal-Wallis test with Dunn's multiple comparisons test, panels C and D: Friedman test with Dunn's multiple comparisons test)

Peripheral administration of a low dose of LPS induced an acute decrease of the sucrose preference in all rats, which can be observed at 6 hours after treatment with both 0.1 and 0.5 mg/kg LPS (Fig 3). This reduction in sucrose preference at 6 hours was significant in 0.1 mg/kg LPS-treated rats compared to the sucrose preference before LPS injection, measured at 0h (p≤0.05 - Fig 3A) and was even highly significant after treatment with 0.5 mg/kg LPS (p≤0.01 - Fig 3B). The sucrose preference at 24 hours post-injection was more variable. While in some animals the sucrose preference still decreased, in others the sucrose preference increased again, as can be seen in Fig 3, panels C and D. In these panels, the evolution of the sucrose preference is represented for three animals per dose that were followed-up from 48 hours before LPS injection (sucrose preference measured at 0 hours) until 3 days after injection (panel C: 0.1 mg/kg LPS, panel D: 0.5 mg/kg LPS). Overall, the sucrose preference was highly significantly reduced at 24 hours after injection with 0.1 mg/kg LPS compared

to the sucrose preference before injection (p≤0.01 - Fig 3A) and showed a trend toward significance in 0.5 mg/kg-treated rats (p=0.0627 - Fig 3B). The sucrose preference at 6 hours did not differ significantly from the sucrose preference at 24 hours post-injection, neither for the 0.1 nor the 0.5 mg/kg LPS treatments (Fig 3A and B). The sucrose preference levels at 3 days and 7 days post-injection were still more variable than at 24h. In some animals, the sucrose preference had returned to normal levels (around 95%) at these time points, as for instance in one rat in Fig 3C. In another group of animals, the sucrose preference seemed to have reached stable low levels between 50 and 70%, as for example in two rats in Fig 3D. Finally in a third group the sucrose preference kept on decreasing and reached very low levels (around 10%) at 3 days and 7 days post-LPS injection, e.g. in two rats in Fig 3C and one rat in Fig 3D. Overall, the sucrose preference was decreased significantly one week after injection with 0.1 mg/kg LPS compared to the sucrose preference before injection (p≤0.05 - Fig 3A) and highly significantly at 3 days post-injection with 0.5 mg/kg LPS compared to pre-injection (p≤0.01 - Fig 3B). There was no statistically significant difference in sucrose preference reduction between the two doses at any time point.

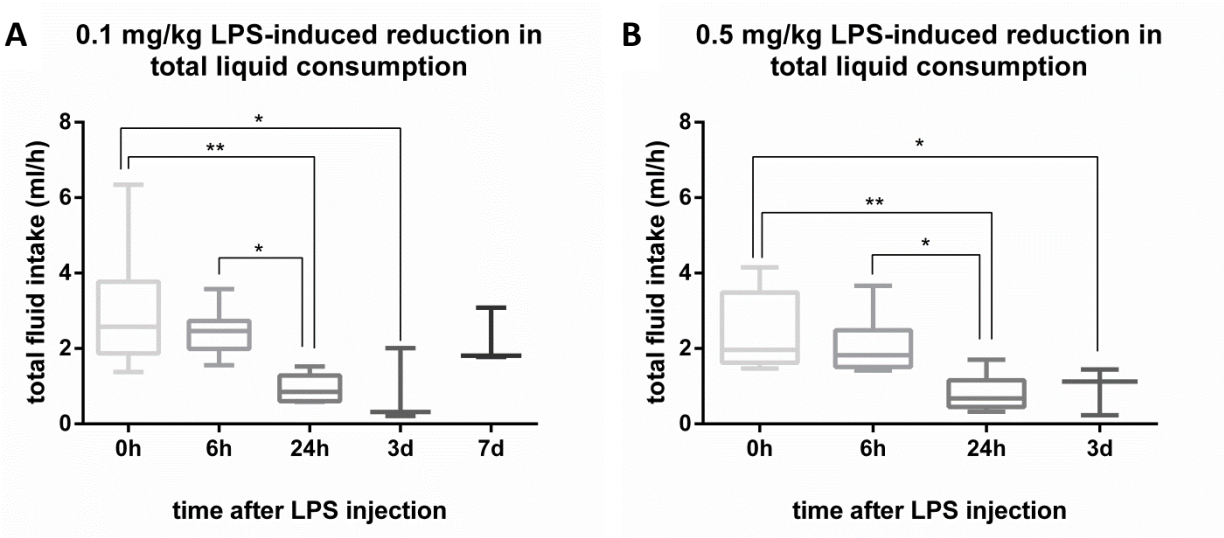

Fig 4. Reduction of the total liquid consumption following peripheral (i.p.) administration of a low dose of LPS, 0.1mg/kg (panel A) and 0.5 mg/kg (panel B). The total fluid intake per hour of a group of animals is plotted as a boxplot with the outer bars representing the min and max values of that group and the midline at the median of the group (panel A: n=12 at 0h, n=9 at 6h, n=6 at 24h, n=3 at 3d, n=3 at 7d, panel B: n=9 at 0h, n=9 at 6h, n=6 at 24h, n=3 at 3d). * = p≤0.05, ** = p≤0.01 (Kruskal-Wallis test with Dunn's multiple comparisons test)

Although it is difficult to compare the average total liquid consumption (water + sucrose solution) per hour measured over short time spans, because of a differential contribution of active and non-active time, the average total fluid intake per hour over long time spans can be readily compared. Rats had a lower total liquid consumption per hour at 3 days post-LPS injection than before LPS injection, which was significant after treatment with both 0.1 mg/kg LPS (p≤0.05 - Fig 4A) and 0.5 mg/kg LPS (p≤0.05 - Fig 4B). As the total fluid intake at 6 hours post-injection was measured solely during the active (dark) phase of the animals, a higher total liquid consumption per hour than before LPS injection (measured over the total dark-night cycle) would be expected when unaffected. As the total fluid intake per hour measured over these 6 hours after injection is about the same as the total fluid intake per hour before LPS injection, one could imagine that there is a significant reduction of the total fluid intake at 6 hours post-injection. As the total fluid intake per hour at the 24h-time point is measured mainly during the non-active (light) phase of the rats, the total liquid consumption per hour is expected to be low in any case and masks therefore a reduction in this parameter due to the LPS challenge at this time point. There was no statistically significant difference in total fluid intake reduction between the two doses at any time point.

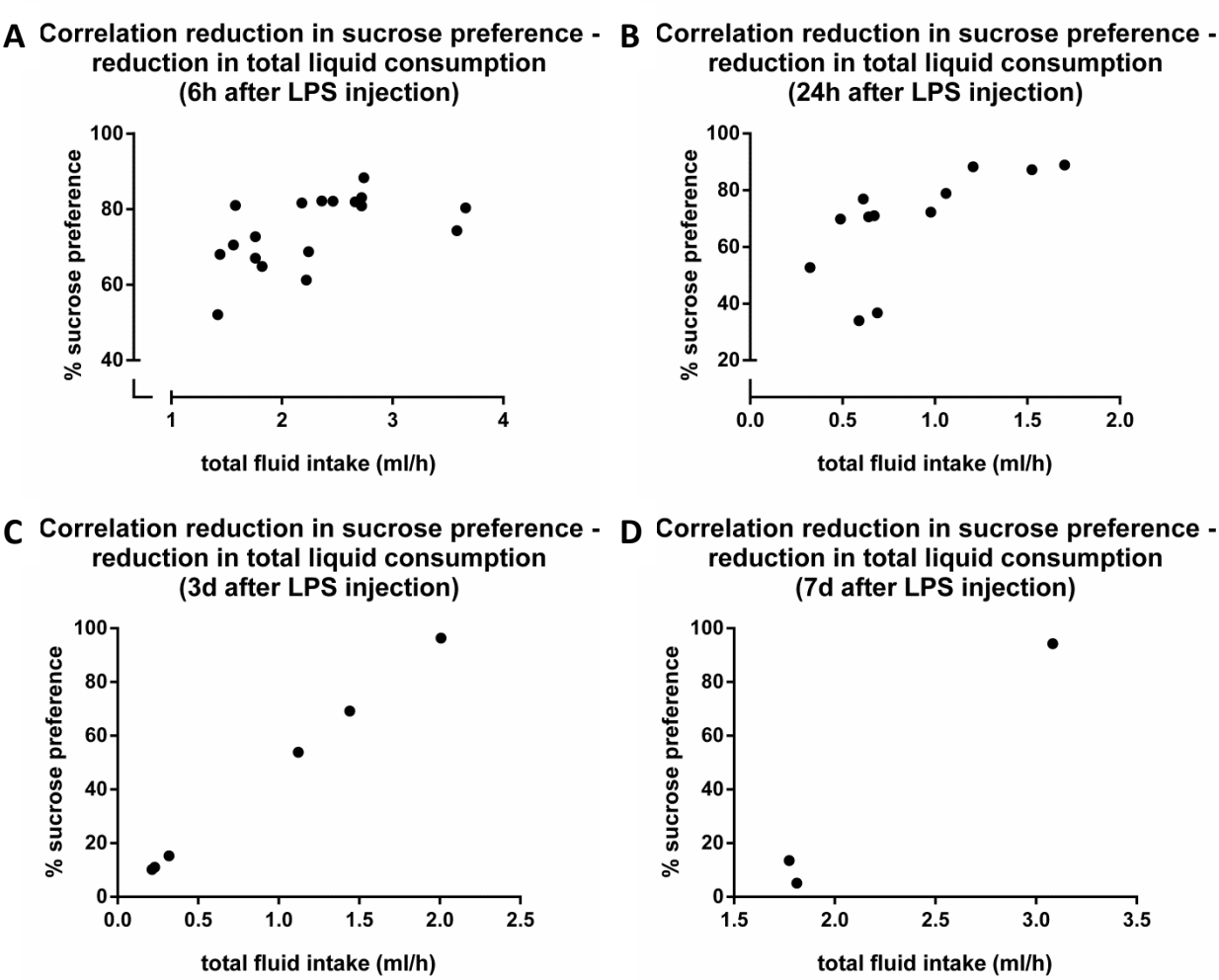

Fig 5. Positive correlation of the reduction in sucrose preference and the reduction in total liquid consumption per hour following peripheral (i.p.) administration of a low dose of LPS, 0.1 mg/kg and 0.5 mg/kg. As there was never a significant difference in response to the two LPS doses, neither in sucrose preference reduction nor in total fluid intake reduction, the data from the two groups (0.1 and 0.5 mg/kg LPS) were pooled to increase the number of data in the correlation analysis. Each time point was analysed separately. Panel A: 6h post-LPS injection (n=18, Spearman's $\rho = 0.5981$, $p \leq 0.01$). Panel B: 24h post-LPS (n=12, Spearman's $\rho = 0.8042$, $p \leq 0.01$). Panel C: 3d post-LPS (n=6, Spearman's $\rho = 1.000$, $p \leq 0.01$). Panel D: 7d post-LPS (n=3). (Spearman's correlation test)

To investigate whether the animals that had a reduced sucrose preference at a certain time point also had a reduced total fluid consumption at that time point, a correlation was performed between the two parameters for each animal. But to avoid the above mentioned problem of comparing the total fluid intake per hour measured over different time periods, we compared the sucrose preference and total fluid consumption of animals at each time point separately. As there was never a significant difference in response to the two LPS doses, neither in sucrose preference reduction nor in total fluid intake reduction, the data from the two groups (0.1 and 0.5 mg/kg LPS) were pooled to increase the number of data in the correlation analysis (Fig 5). It is obvious that there is a strong positive correlation between the two parameters at each time point. Animals with a decreased sucrose preference also had a decreased total fluid intake, as acutely as 6 hours post-injection and up to one week after LPS treatment. The correlation is highly significant at 6 hours post-LPS (Spearman's $\rho = 0.5981$, $p \leq 0.01$ - Fig 5A), at 24 hours post-LPS (Spearman's $\rho = 0.8042$, $p \leq 0.01$ - Fig 5B) and 3 days post-LPS (Spearman's $\rho = 1.000$, $p \leq 0.01$ - Fig 5C).

2.2 *Histological findings*

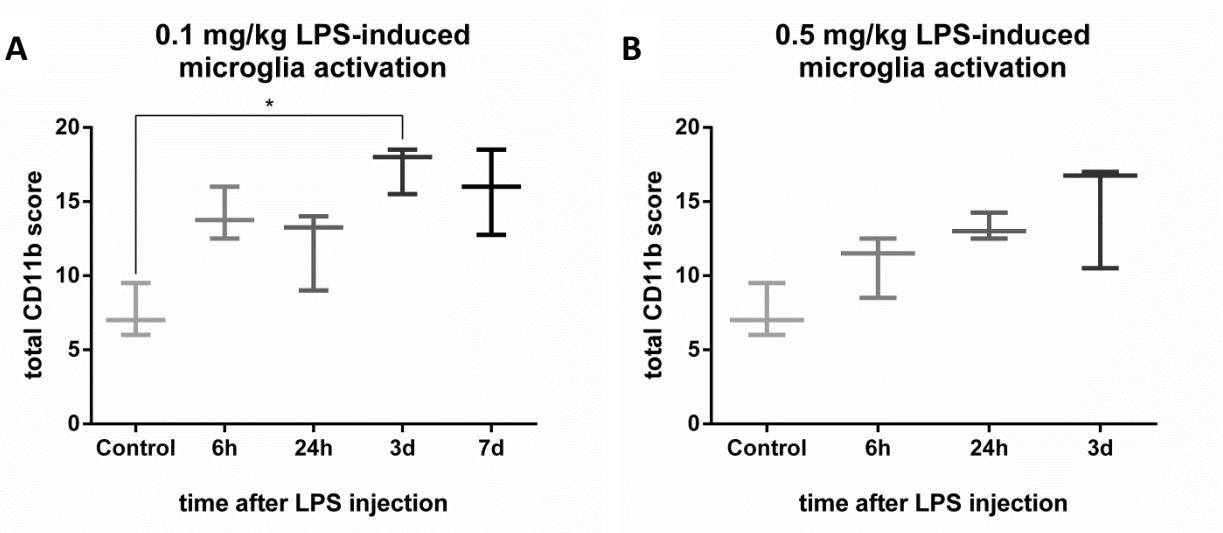

Fig 6. Induction of microglia activation in the brain by peripheral (i.p.) administration of a low dose of LPS, 0.1 mg/kg (panel A) and 0.5 mg/kg (panel B). Microglia were detected by immunohistochemical staining of the CD11b receptor. The level of microglia activation was subsequently determined by use of a visual scoring system that considered both increases in the number and the activation state of microglia. A high CD11b score indicates the presence of numerous, amoeboid microglia with big cell bodies. The total CD11b score depicted in the graphs is the sum of the scores of four different brain regions (frontotemporal cortex, corpus callosum, hippocampus and thalamus). The three individual data per group are plotted (median with min and max values). The medians varied significantly over time, both in panels A and B. Post-hoc testing showed that the microglia activation at 3 days after treatment with 0.1 mg/kg LPS was significantly increased compared to controls. (n=3 per time point per dose) * = p≤0.05 (Kruskal-Wallis test with Dunn's multiple comparisons test)

A single intraperitoneal injection with a low dose of LPS resulted in a global activation of microglia in the brain, which is already apparent at 6 hours after the injection (n.s.). The total CD11b score per animal was higher after LPS injection than after saline injection (control), both for the 0.1 and 0.5 mg/kg LPS treatments (see Fig 6, panels A and B). The microglia activation increased with time up to 3 days after LPS injection; the medians varied significantly over time (p≤0.05) (Fig 6 and 7).The microglia activation was the highest at 3 days after injection with 0.1 mg/kg LPS, which was significantly higher compared to control (p≤0.05 - Fig 6A). Post-hoc testing did not reveal significant differences between control and the high dose at any of the time points investigated. Because we were interested to know when the brain inflammation peak occurs, three additional rats were injected with the low LPS dose only and sacrificed at a later time point, namely 7 days after injection. One week after treatment with LPS, the microglia activation seemed to have reached a plateau compared to the previous time point (Fig 6A).

No significant differences were found in the microglia response between the two doses at any of the time points investigated.

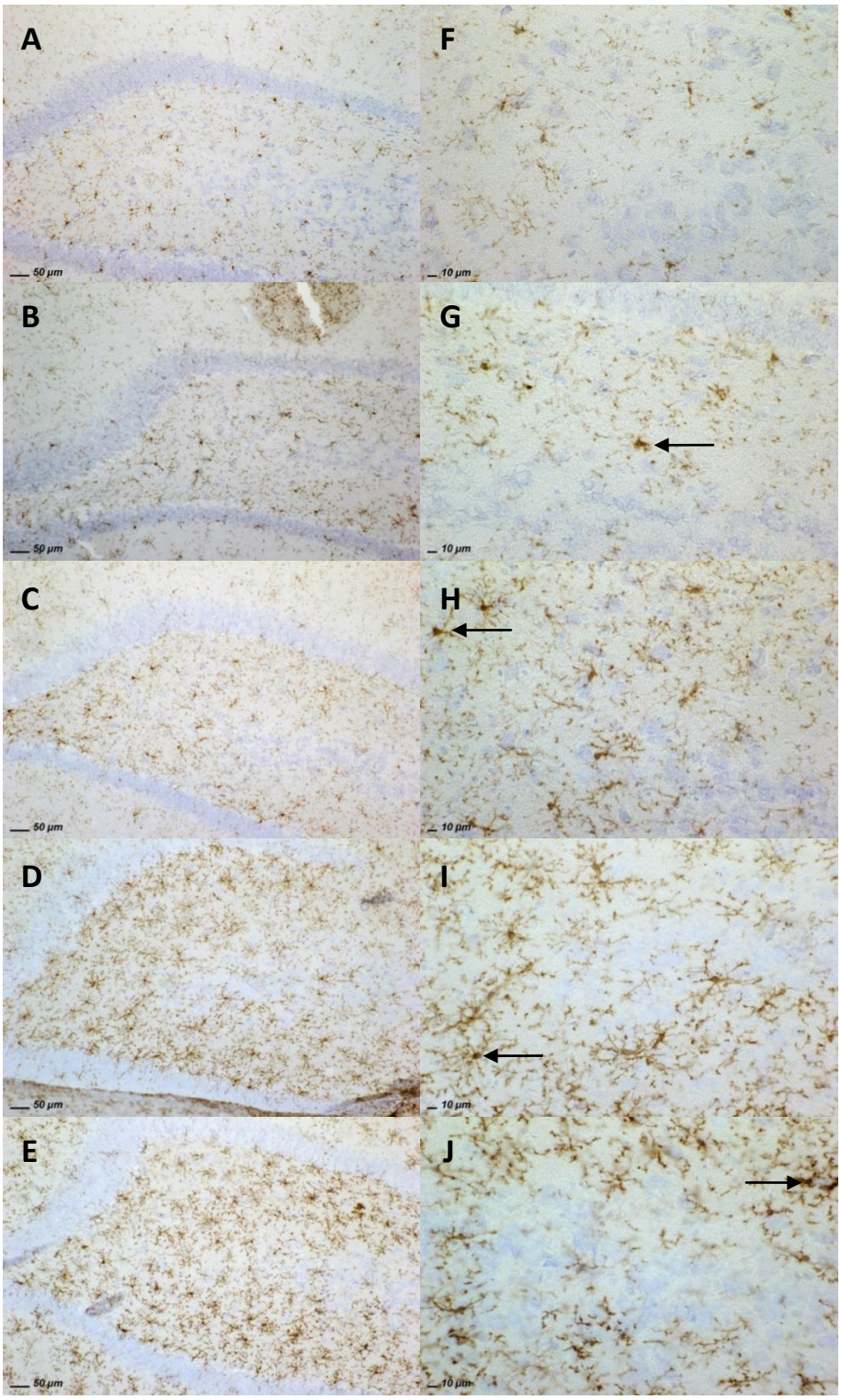

Fig 7. Induction of microglia activation in the hippocampus by peripheral (i.p.) administration of a low dose of LPS, 0.1 mg/kg. Microglia were detected by immunohistochemical staining of the CD11b receptor. Activated microglia have an amoeboid shape with large cell bodies and thicker and shorter processes. Examples of activated microglia are indicated by arrows. Panels A-E: Representative micrographs of the hippocampus with a 10x magnification. Panels F-J: Representative micrographs of the hippocampus with a 25x magnification. Panels A and F: Micrographs of a rat injected with saline and sacrificed at 3 days post-injection. Panels B and G: Micrographs of a rat injected with 0.1 mg/kg LPS and sacrificed at 6 hours post-injection. Panels C and H: Micrographs of a rat injected with 0.1 mg/kg LPS and sacrificed at 24 hours post-injection. Panels D and I: Micrographs of a rat injected with 0.1 mg/kg LPS and sacrificed at 3 days post-injection. Panels E and J: Micrographs of a rat injected with 0.1 mg/kg LPS and sacrificed at 7 days post-injection. An increase in microglia activation can be seen from 6 hours post-LPS up to 3 days post-LPS when it seems to reach a plateau that lasts at least up to 7 days post-injection.

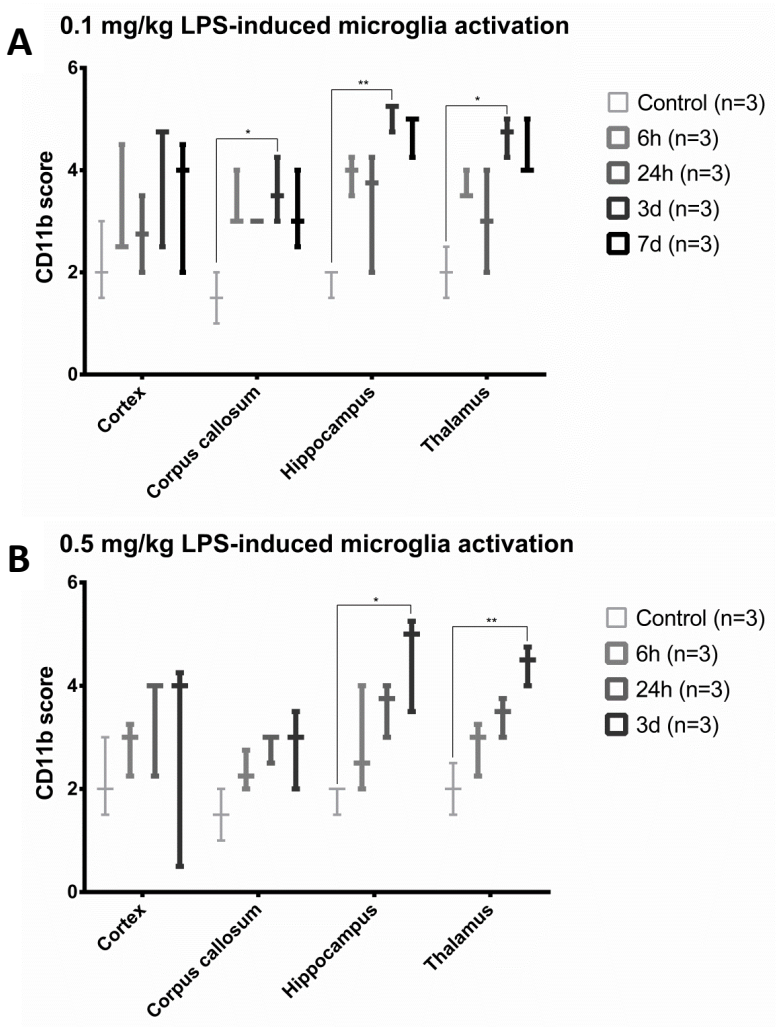

Fig 8. Induction of microglia activation in four different brain regions (frontotemporal cortex, corpus callosum, hippocampus and thalamus) by peripheral (i.p.) administration of a low dose of LPS, 0.1 mg/kg (panel A) and 0.5 mg/kg (panel B). Microglia were detected by immunohistochemical staining of the CD11b receptor. The level of microglia activation was subsequently determined by use of a visual scoring system that considered both increases in the number and the activation state of microglia. A high CD11b score indicates the presence of numerous, amoeboid microglia with big cell bodies. The CD11b score per brain region is depicted in the graphs. The three individual data per group are plotted (median with min and max values). (n=3 per brain region per time point per dose) * = p≤0.05, ** = p≤0.01 (Kruskal-Wallis test with Dunn's multiple comparisons test)

When we investigate the level of microglia activation in the separate brain regions, we can conclude that the CD11b score for each of the four investigated brain regions (frontotemporal cortex, corpus callosum, hippocampus and thalamus) shows a similar time-course as the overall brain score, both for the 0.1 and 0.5 mg/kg LPS treatments (see Fig 8, panels A and B).

Microglia activation was the highest at 3 days after treatment with LPS. At this time point, the microglia activation was significantly higher compared to control in the corpus callosum (p≤0.05), hippocampus (p≤0.01 - Fig 7) and thalamus (p≤0.05) of 0.1 mg/kg LPS-treated rats. (Fig 8A). In 0.5 mg/kg LPS-treated rats the microglia activation was significantly higher compared to control at 3 days post-LPS in hippocampus (p≤0.05) and thalamus (p≤0.01) (Fig 8B).

When comparing the two doses of LPS, no significant differences were found in the microglia response at any time point in any of the four brain regions.

In conclusion, the most optimal LPS dose to induce microglia activation and time point to assess microglia activation in the follow-up study in vehicle and poly I:C offspring were considered to be the low dose (0.1 mg/kg) at 3 days post-LPS injection, because a similar microglia response could be

achieved with the low dose as with the high dose and the response reached a peak at 3 days post-injection. We decided to evaluate the difference in microglia activation due to an LPS immune challenge between poly I:C and control offspring at the peak of the response, as the difference might be most pronounced at this time. The microglia activation was not maximal in this study, as this would mean a total CD11b score of 24 while the maximal given total score in this study was well under 20. Therefore we considered it safe to choose for the peak of the brain inflammation, because even if the poly I:C offspring would exhibit a higher degree of microglia activation, this difference could probably be identified.

2.3 *Correlation of behavioural and histological findings*

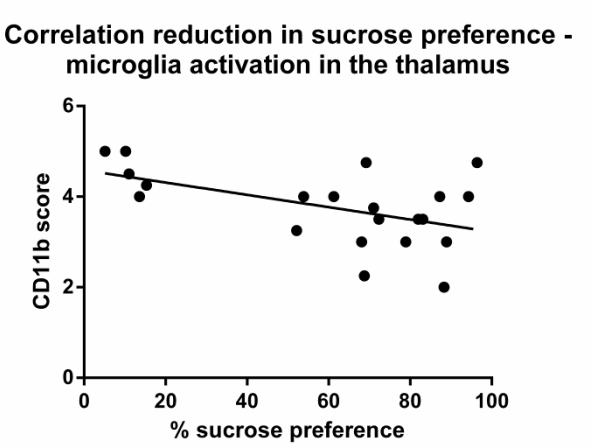

Fig 9. Negative correlation between the sucrose preference and the microglia activation following peripheral (i.p.) administration of a low dose of LPS, 0.1 and 0.5mg/kg. As there was never a significant difference in response to the two LPS doses, neither in sucrose preference reduction nor in microglia activation, the data from the two groups (0.1 and 0.5mg/kg LPS) were pooled to increase the number of data in the correlation analysis. (n=21, Spearman's ρ = -0.4125, p=0.0631) (Spearman's correlation test)

Finally we have investigated whether there was a correlation between the sucrose preference of a rat at a certain time point following peripheral administration of LPS and the level of microglia activation in the brain at the same time point. We have found a trend toward significance for a negative correlation for one brain region i.e. the thalamus (Spearman's ρ = -0.4125, p=0.0631 - Fig 9). Rats with a very low sucrose preference exhibited at the same time a high level of microglia activation in their thalamus.

3 Behavioural and histopathological study of poly I:C and control offspring

3.1 *Follow-up of MIA induction*

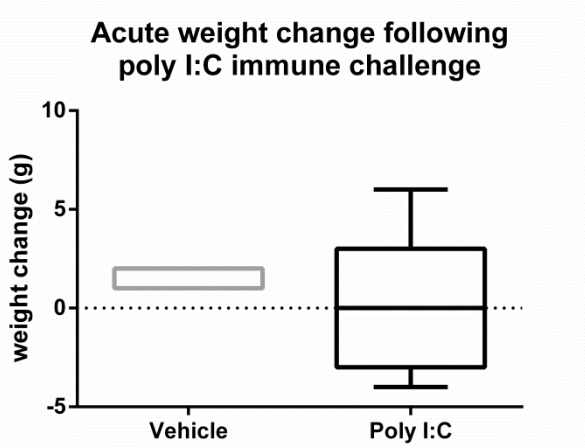

Fig 10. Acute body weight change following subcutaneous injection with 4 mg/kg poly I:C (dissolved in 10 ml/kg nuclease-free water) or vehicle. Positive values indicate weight gain, negative values indicate weight loss. Overall, there was no significant difference in body weight change between the poly I:C- and vehicle treated dams. Body weight change is plotted as a standard boxplot. (vehicle-treated dams: n=5, poly I:C-treated dams: n=7) (Mann-Whitney test)

The pregnant dams responded quite differently to the poly I:C immune challenge in terms of body weight change. Three out of seven rats exhibited a body weight reduction 6 hours after subcutaneous injection with poly I:C. Another three rats had gained body weight after the immune challenge, while one rat had neither gained nor lost weight (Fig 10). Overall, there was no significant difference in body weight change in the immune-challenged rats compared to the controls.

Six hours after injection with poly I:C, the mean ± SEM rectal temperature of the dams was 37.2±0.1°C compared to 36.9±0.2°C in vehicle injected dams (n.s.).

For the analysis of the behavioural and histopathological results, the offspring of poly I:C-treated dams were divided into two groups based on the response of the mother to the immune challenge, as has been done in some recent studies (Bronson et al., 2011; Vorhees et al., 2012). The first group contained the offspring of dams (n=3) that exhibited a body weight reduction following poly I:C immune challenge: poly I:C (weight loss) offspring. The second group comprised the offspring of dams (n=3) that gained body weight after the immune challenge: poly I:C (weight gain) offspring. The offspring of the rat that neither lost nor gained weight following poly I:C injection was not included in the analysis, because these animals showed a response in between the two differential responses of the poly I:C (weight loss) and the poly I:C (weight gain) offspring, and we wished to dichotomize between the two offspring groups.

Results will be discussed in a descriptive manner when no significant changes have been found.

3.2 *Behavioural findings*

3.2.1 *Prepulse inhibition of the acoustic startle response*

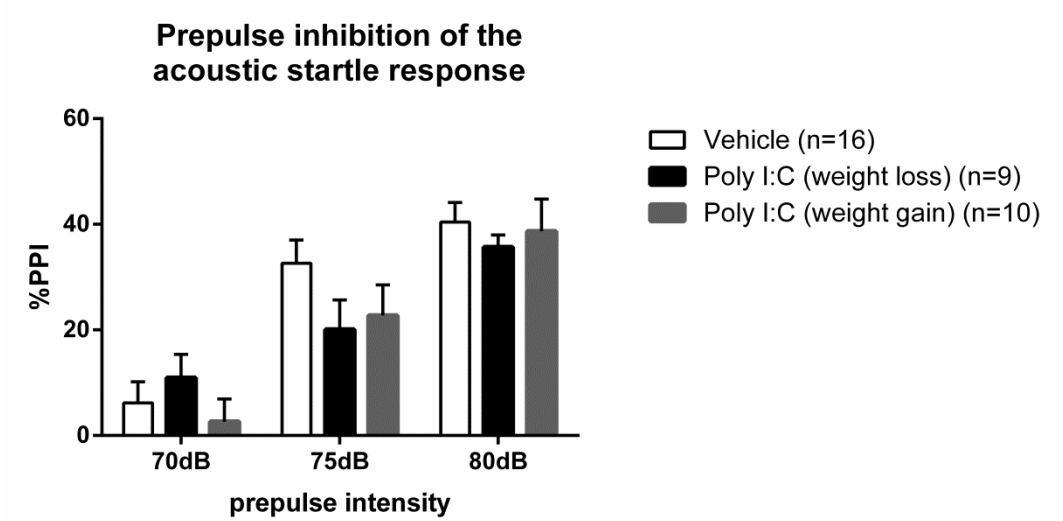

Fig 11. PPI of the acoustic startle response in control offspring, poly I:C (weight loss) offspring and poly I:C (weight gain) offspring. Three different prepulses were tested: 70 dB, 75 dB and 80 dB. There were no significant differences in %PPI between the three offspring groups. There was a slight reduction of the %PPI in poly I:C (weight loss) offspring after the 75 dB prepulse, which shows a trend toward significance (one-sided p=0.0704). The mean ± SEM %PPI is plotted. (vehicle-treated control offspring: n=16, poly I:C (weight loss) offspring: n=9, poly I:C (weight gain) offspring: n=10) (Repeated measures two-way ANOVA with Tukey's multiple comparisons test)

Overall, there were no significant differences in %PPI between the three offspring groups (Fig 11). The poly I:C (weight loss) offspring had a lower mean %PPI compared to controls after the 75 dB and 80 dB prepulses with a trend toward significance after the 75 dB prepulse (one-sided p=0.0704). In all three groups, the %PPI increased with increasing intensity of the prepulse ($p \leq 0.0001$). The startle response to a startle stimulus without preceding prepulse did not differ between the three groups (data not shown).

3.2.2 *Spontaneous and psychotomimetic-induced locomotor activity*

No significant differences have been found in spontaneous locomotor activity between control offspring, poly I:C (weight loss) offspring and poly I:C (weight gain) offspring (Figs 12 and 13A). In the rats that were challenged with amphetamine and later with MK-801, differences in the response to these drugs could be observed between the three offspring groups, although overall not significant.

A subcutaneous injection with 1 mg/kg amphetamine induced hyperlocomotion in all three offspring groups, but to a different extent (Figs 12 and 13B). Poly I:C (weight loss) offspring showed a reduced responsiveness to amphetamine. The total locomotor activity per hour was significantly increased in control offspring ($p \leq 0.01$) and poly I:C (weight gain) offspring ($p \leq 0.05$) after challenge with amphetamine compared to pre-injection, while this was not significant in poly I:C (weight loss) offspring (Fig 12). The mean locomotor activity per time interval in poly I:C (weight loss) offspring was constantly lower than in the control offspring after amphetamine challenge, over the full recorded 120 minutes (Fig 13B). This reduction in sensitivity to amphetamine in poly I:C (weight loss) offspring versus control offspring showed a trend toward significance ($p \leq 0.1$) at a number of time intervals: 95 min, 100 min and 105 min (Fig 13B). The amphetamine-stimulated locomotion did not reach a peak in the poly I:C (weight loss) offspring, while the control offspring exhibited a clear peak in the amphetamine-induced hyperlocomotion. The poly I:C (weight gain) offspring on the other hand showed a response to amphetamine which was not different from the response of the control offspring (Fig 13B). At one time interval (i.e. 115 min), the difference between poly I:C (weight loss) offspring and poly I:C (weight gain) offspring was significant ($p \leq 0.05$) and approached significance ($p \leq 0.1$) at a number of other time intervals: 95 min, 100 min, 105 min and 135 min (Fig 13B).

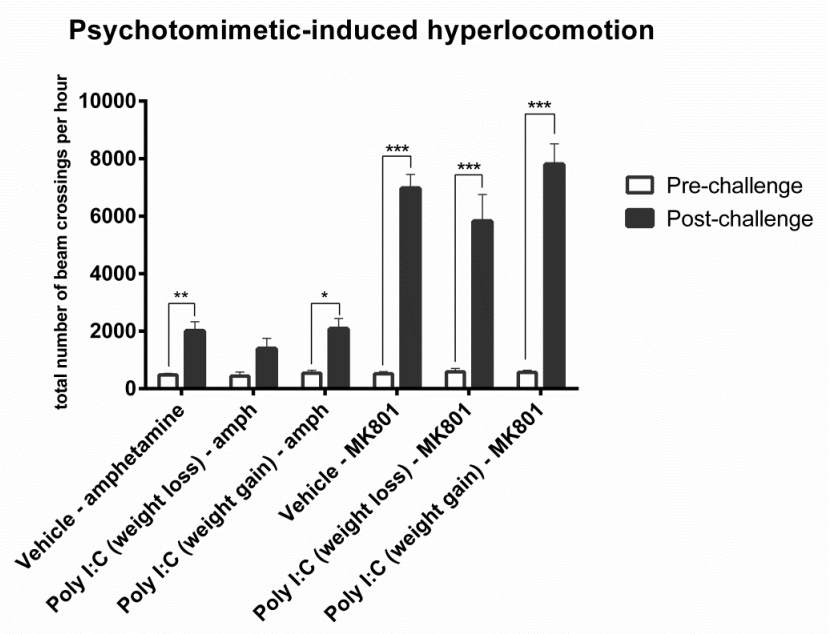

Fig 12. Amphetamine- and MK-801-stimulated locomotor activity in control offspring, poly I:C (weight loss) offspring and poly I:C (weight gain) offspring. The locomotor activity of the pre-challenge test periods and post-challenge test periods was summated over time (60 min pre-challenge and 120 min post-challenge) and the total locomotor activity per hour was calculated. The mean ± SEM total number of beam crossings per hour is plotted. Administration of 1 mg/kg amphetamine resulted in a highly significant increase of the total locomotor activity in control offspring and a significant increase of the total locomotor activity in poly I:C (weight gain) offspring compared to pre-challenge. Poly I:C (weight loss) offspring exhibited a decreased response to amphetamine compared to control offspring and poly I:C (weight gain) offspring. The total locomotor activity was not significantly increased in poly I:C (weight loss) offspring compared to pre-challenge. Administration of 0.2 mg/kg MK-801 resulted in a very highly significant increase of the total locomotor activity in all three offspring groups compared to pre-challenge. (Control offspring with amphetamine injection: n=8, poly I:C (weight loss) offspring with amphetamine injection: n=5, poly I:C (weight gain) offspring with amphetamine injection: n=5, control offspring with MK-801 injection: n=8, poly I:C (weight loss) offspring with MK-801 injection: n=5, poly I:C (weight gain) offspring with MK-801 injection: n=5) * = p≤0.05, ** = p≤0.01, *** = p≤0.001 (Repeated measures two-way ANOVA with Sidak's multiple comparisons test)

A subcutaneous injection with 0.2 mg/kg MK-801 induced an enormous increase in the locomotor activity in all three offspring groups compared to pre-injection (p≤0.001 - Fig 12). The poly I:C (weight loss) offspring however seemed to respond more slowly to the drug than the control and poly I:C (weight gain) offspring, as indicated by a slower rise in the curve following injection (Fig 13C, the arrow indicates the time of injection). This resulted in significant differences between poly I:C (weight loss) offspring on the one hand and control and poly I:C (weight gain) offspring on the other hand in the first ten minutes after injection with MK-801. Poly I:C (weight loss) offspring had a significantly lower locomotor activity than control offspring at 65 min (p≤0.05), which was even highly significant at 70 min (p≤0.01). The locomotor activity in poly I:C (weight loss) offspring was also significantly lower than in poly I:C (weight gain) offspring at 70 min (p≤0.05) (Fig 13C). The mean MK-801-stimulated locomotor activity in poly I:C (weight loss) offspring was continually lower over time than in control offspring. This difference was however not significant over all time points. The poly I:C (weight gain) offspring initially followed the same response as control offspring to MK-801. After 50 minutes however, the two groups diverged. The mean MK-801-induced hyperlocomotion was from then on continually higher than in control offspring. The difference was however not significant over all time points. Due to the opposite responses of poly I:C (weight loss) offspring and poly I:C (weight gain) offspring to MK-801, significant differences could be found between the two groups at some time intervals: 135 min, 155 min, 160 min and 165 min (p≤0.05) as well as many trends toward significance (p≤0.1): at 145 min, 150 min, 170 min and 175 min (Fig 13C).

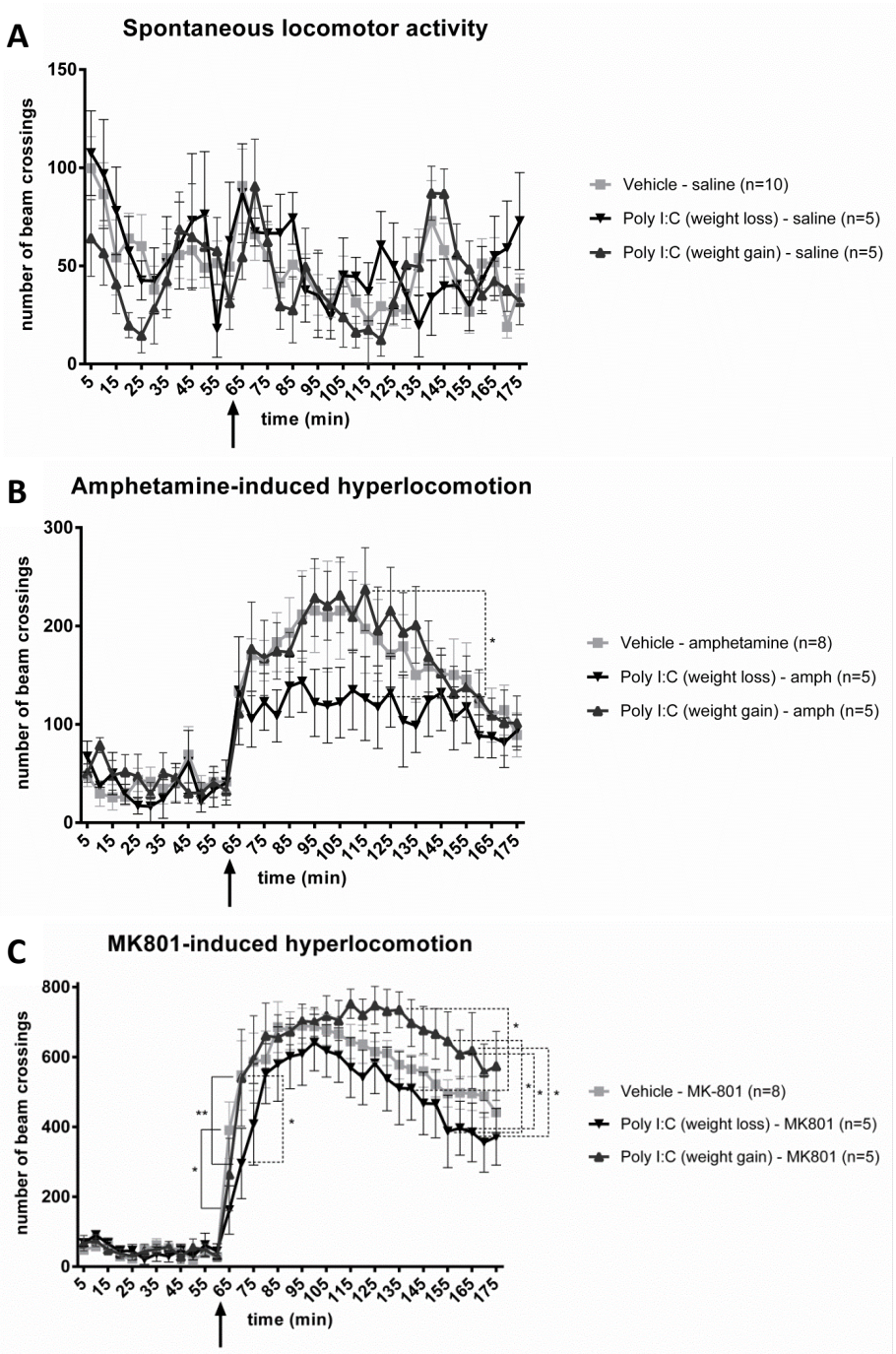

Fig 13. Spontaneous and psychotomimetic-stimulated locomotor activity in control offspring, poly I:C (weight loss) offspring and poly I:C (weight gain) offspring. Spontaneous locomotor activity was recorded during the first 60 min for all animals, after which the rats received a subcutaneous injection with either saline (panel A), 1 mg/kg amphetamine (panel B) or 0.2 mg/kg MK-801 (panel C). The arrow indicates the time of injection. Locomotor activity was recorded for an additional 120 min. Data were recorded in 5-min bins. The mean ± SEM number of beam crossings per 5 min is plotted. Panel A. No significant differences were found in spontaneous locomotor activity between control offspring, poly I:C (weight loss) offspring and poly I:C (weight gain) offspring. Panel B. Poly I:C (weight loss) offspring exhibited a trend for a decreased response to amphetamine compared to control offspring. Poly I:C (weight gain) offspring showed no difference in response to amphetamine compared to controls. Panel C. Poly I:C (weight loss) offspring responded more slowly and showed a trend for a decreased response to MK-801 compared to control offspring, while poly I:C (weight gain) offspring showed a trend for an increased response to MK-801 compared to controls. (Panel A. Control offspring: n=10, poly I:C (weight loss) offspring: n=5, poly I:C (weight gain) offspring: n=5; Panel B. Control offspring: n=8, poly I:C (weight loss) offspring: n=5, poly I:C (weight gain) offspring: n=5; Panel C. Control offspring: n=8, poly I:C (weight loss) offspring: n=5, poly I:C (weight gain) offspring: n=5) * = p≤0.05, ** = p≤0.01 (Repeated measures two-way ANOVA with Tukey's multiple comparisons test)

3.2.3 *Sucrose preference, total liquid consumption and LPS-induced sickness behaviour*

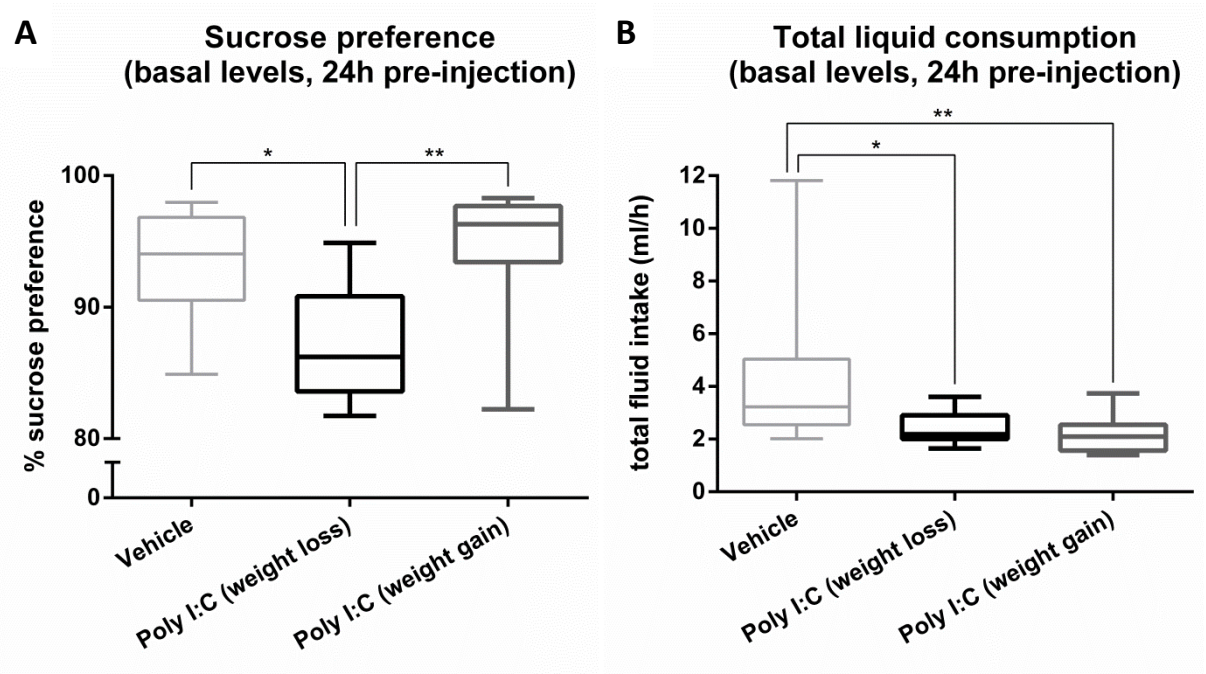

Fig 14. Basal levels of sucrose preference (panel A) and total liquid consumption per hour (panel B) in control offspring, poly I:C (weight loss) offspring and poly I:C (weight gain) offspring. The data are plotted as standard boxplots. Panel A. Poly I:C (weight loss) offspring had a significantly lower sucrose preference than control offspring and poly I:C (weight gain) offspring. Panel B. Poly I:C offspring had a significantly lower total fluid intake per hour than control offspring. (control offspring: n=17, poly I:C (weight loss) offspring: n=7, poly I:C (weight gain) offspring: n=9-10) * = p≤0.05, ** = p≤0.01 (Kruskal-Wallis test with Dunn's multiple comparisons test)

Poly I:C (weight loss) offspring had a slightly but significantly lower sucrose preference than control offspring (p≤0.05 - Fig 14A). The sucrose preference of poly I:C (weight loss) offspring was also highly significantly lower than the sucrose preference of poly I:C (weight gain) offspring (p≤0.01 - Fig 14A). Poly I:C offspring also had a lower total liquid consumption than control offspring, which was significant for poly I:C (weight loss) offspring (p≤0.05 - Fig 14B) and highly significant for poly I:C (weight gain) offspring (p≤0.01 - Fig 14B).

Intraperitoneal injection with 0.1 mg/kg LPS induced a highly significant reduction in sucrose preference compared to pre-injection in control offspring (p≤0.01 - Fig 15A), but not in poly I:C (weight loss) offspring. In poly I:C (weight gain) offspring, there was an acute decrease in the sucrose preference compared to pre-injection that just missed significance (p=0.0578). In each of the three LPS-challenged groups there was one rat with a persisting decrease in sucrose preference at 3 days after LPS injection.

As discussed in §2.1, it is difficult to compare the total fluid intake per hour measured over different time spans because of the differential contribution of active and non-active time. Nevertheless, there was a significant reduction in the total liquid consumption per hour of control offspring at 6 hours and 3 days post-LPS injection compared to pre-injection (p≤0.05 - Fig 15B). In control offspring that received a saline injection, the total fluid intake was significantly increased at 6 hours post-injection compared to pre-injection (p≤0.05 - Fig 15B). Poly I:C (weight loss) offspring showed a significantly lower total fluid intake at 6 hours post-LPS injection compared to poly I:C (weight loss) offspring that received a saline injection (p≤0.05 - Fig 15B). The poly I:C (weight loss) and poly I:C (weight gain) offspring showed a trend toward significance for a reduced total liquid consumption at 3 days post-LPS compared to pre-injection (p≤0.1).

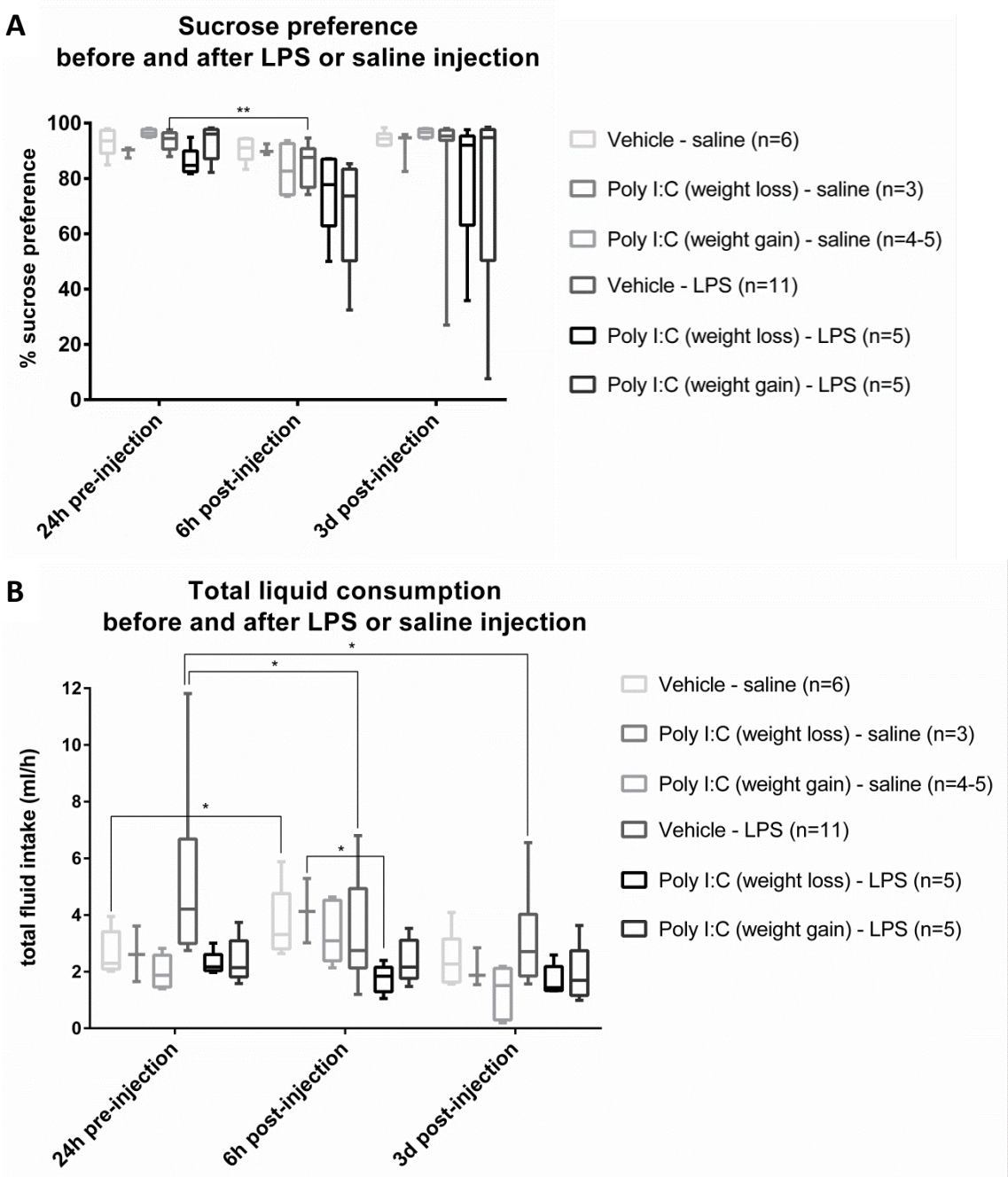

Fig 15. Sucrose preference (panel A) and total liquid consumption per hour (panel B) before and after intraperitoneal injection with 0.1mg/kg LPS or saline in control offspring, poly I:C (weight loss) offspring and poly I:C (weight gain) offspring. The data are plotted as standard boxplots. Panel A. LPS induced a highly significant decrease in sucrose preference in control offspring at 6 hours after injection compared to pre-injection. Panel B. LPS induced a significant decrease in total fluid intake per hour in control offspring at 6 hours and 3 days post-injection compared to pre-injection, while the total fluid intake per hour significantly increased in control offspring 6 hours after saline injection compared to pre-injection. Poly I:C (weight loss) offspring showed a significantly lower total fluid intake at 6 hours post-LPS injection compared to poly I:C (weight loss) offspring that received a saline injection. (Control offspring with saline injection: n=6, poly I:C (weight loss) offspring with saline injection: n=3, poly I:C (weight gain) offspring with saline injection: n=4-5, control offspring with LPS injection: n=11, poly I:C (weight loss) offspring with LPS injection: n=5, poly I:C (weight gain) offspring with LPS injection: n=5) * = $p \leq 0.05$,** = $p \leq 0.01$ (Friedman test with Dunns's multiple comparisons test; Kruskal-Wallis test with Dunn's multiple comparisons test)

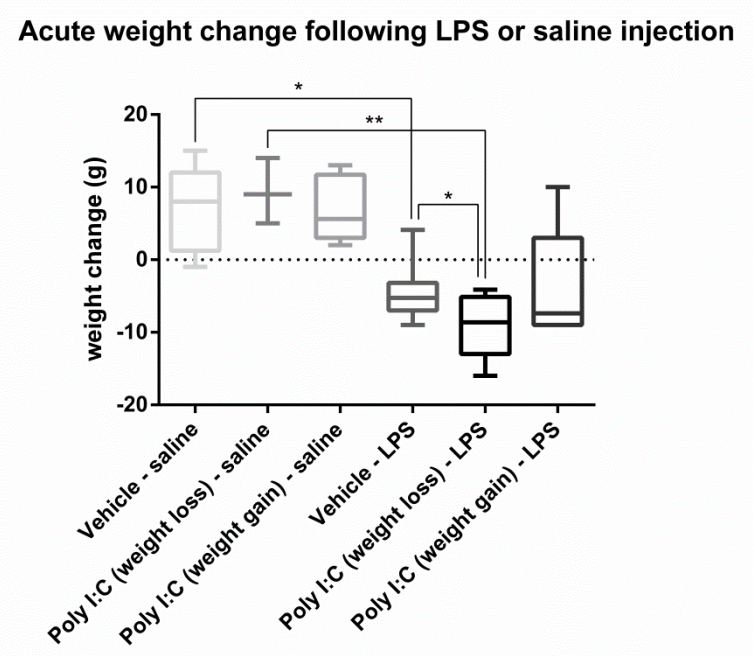

Fig 16. Acute body weight change following intraperitoneal injection with 0.1 mg/kg LPS or saline in control offspring, poly I:C (weight loss) offspring and poly I:C (weight gain) offspring 6 hours after injection. Positive values indicate weight gain, negative values indicate weight loss. LPS injection caused a significant body weight reduction in control offspring and a highly significant reduction in poly I:C (weight loss) offspring compared to saline injection. The body weight reduction in poly I:C (weight loss) offspring is significantly lower than the weight reduction in control offspring after LPS injection. Body weight change is plotted as a standard boxplot. (Control offspring with saline injection: n=6, poly I:C (weight loss) offspring with saline injection: n=3, poly I:C (weight gain) offspring with saline injection: n=5, control offspring with LPS injection: n=12, poly I:C (weight loss) offspring with LPS injection: n=7, poly I:C (weight gain) offspring with LPS injection: n=5) * = $p \leq 0.05$, ** = $p \leq 0.01$ (Kruskal-Wallis test with Dunn's multiple comparisons test)

Intraperitoneal injection with 0.1 mg/kg LPS induced a significant body weight reduction in control offspring ($p \leq 0.05$) and a highly significant body weight reduction in poly I:C (weight loss) offspring compared to saline injection ($p \leq 0.01$ - Fig 16). The body weight reduction in poly I:C (weight loss) offspring 6 hours after LPS injection was significantly lower than in control offspring (Fig 16). Animals regained their pre-injection weight (measured immediately before injection) at 3 days post-LPS (data not shown).

3.3 *Histological findings*

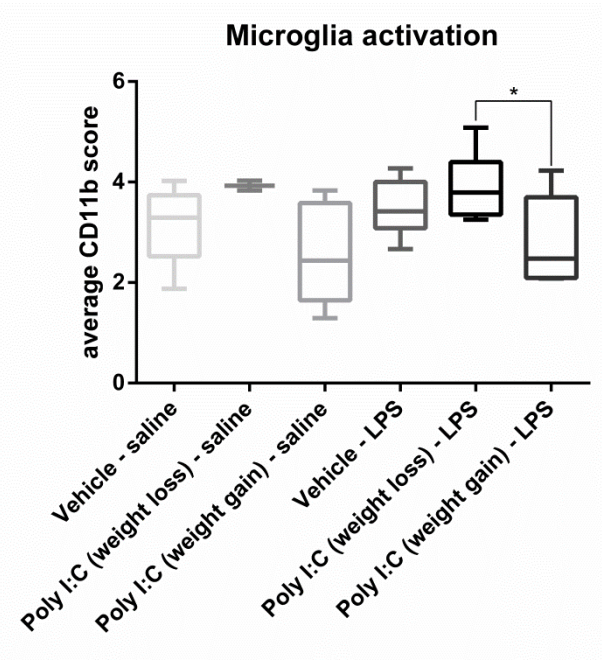

Fig 17. Microglia activation in control offspring, poly I:C (weight loss) offspring and poly I:C (weight gain) offspring 3 days after injection with either 0.1 mg/kg LPS or saline. There was a significantly higher microglia activation in poly I:C (weight loss) offspring compared to poly I:C (weight gain) offspring after LPS injection. A similar observation was made in the poly I:C (weight loss) offspring compared to the poly I:C (weight gain) offspring without LPS challenge (saline injection) but the poly I:C (weight loss) offspring group with saline injection contained a too small number of rats (n=2) to make this statistically reliable. The CD11b score is the average of the scores of six investigated brain regions: frontotemporal cortex, corpus callosum, hippocampus, thalamus, striatum and pons. In two animals, one brain region was not scored (1x hippocampus, 1x striatum). The average CD11b score in these two animals is the average of only five brain regions. For this reason, we opted to present an average CD11b score instead of a total summated score as in the LPS time-course dose-response study. CD11b scores are plotted as standard boxplots. (Control offspring with saline injection: n=5, poly I:C (weight loss) offspring with saline injection: n=2, poly I:C (weight gain) offspring with saline injection: n=5, control offspring with LPS injection: n=11, poly I:C (weight loss) offspring with LPS injection: n=7, poly I:C (weight gain) offspring with LPS injection: n=5) (Kruskal-Wallis test with Dunn's multiple comparisons test)

Poly I:C (weight loss) offspring had a significantly higher microglia activation compared to poly I:C (weight gain) offspring after LPS injection (Fig 17). A similar observation was made in the poly I:C (weight loss) offspring compared to the poly I:C (weight gain) offspring without LPS challenge (saline injection) but the poly I:C (weight loss) offspring group with saline injection contained a too small number of rats (n=2) to make this observation statistically reliable. More data are currently on their way.

Surprisingly, there were no significant differences in microglia activation after LPS injection compared to pre-injection in any of the offspring groups.

Discussion

1 Poly I:C dose-response study

1.1 Maternal compartment

As expected, subcutaneous injection of the pregnant dams with the viral mimetic poly I:C resulted in increases of the mRNA expression of a number of pro-inflammatory cytokines in the blood. The mRNA levels of IL-1β were increased significantly 6 hours after the 4 mg/kg poly I:C treatment in the blood of mothers in mid/late pregnancy (GD15) compared to vehicle-treated dams. TNF-α mRNA was increased significantly 6 hours after treatment with the 8 mg/g poly I:C dose in the blood of mothers in early/mid pregnancy (GD9) compared to controls. A clear dose-response relationship between the tested poly I:C doses and the induction of cytokine mRNA expression was absent. Treatment with the 4 mg/kg poly I:C dose most often led to the highest increases in mRNA levels of the pro-inflammatory cytokines. The main reasons for not finding significance in other treatment-groups are probably the low number of animals in each group (and thus a lack of power in the study) as well as the apparent presence of non-responders among the dams. When we look at the individual data, it becomes apparent that not all dams responded to the immune challenge with an increase in cytokine mRNA levels. It could be possible that we missed the increase in mRNA levels in these rats by sampling at only one time point i.e. 6 hours after administration of poly I:C. The immune response to an acute immune challenge is strongly time-dependent; different cytokines can show different time-courses. It is possible that the cytokine mRNA levels of some rats in our study had returned to their basal levels by 6 hours post-treatment. There is however also the possibility that these rats had an altogether different response to the acute immune challenge and showed no increase in cytokine mRNA expression at all. This could be linked to the differential response in body weight change that we observed in pregnant dams challenged with poly I:C. While some dams responded with a clear weight loss to the immune challenge, others clearly gained weight. This differential response of the mothers coincided with a distinctly different behavioural and histological brain pathology in the respective offspring. The difference in body weight change following MIA could be related to a different immune response of the mothers to the poly I:C immune challenge, resulting in a distinctly different neurodevelopmental insult in the offspring. Peripheral pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α are thought to be the prime inducers of sickness behaviour, including a decreased food intake and subsequent body weight loss (Buchanan and Johnson, 2007; Dantzer, 2009; Kent et al., 1996). One could hypothesize that a larger immune response to poly I:C, resulting in higher levels of circulating pro-inflammatory cytokines, induces a more pronounced sickness behaviour and thus higher weight loss. On this assumption, body weight change could be regarded as an indirect measure of the maternal responsiveness to poly I:C, as has been done by (Bronson et al., 2011; Vorhees et al., 2012). The different levels of circulating pro-inflammatory cytokines in the dams could have a direct relation with the severity of the neurodevelopmental insult in the foetal brain. Further studies are needed to investigate the (direct) association between pro-inflammatory cytokine levels and mRNA expression and body weight change following an immune response.

Because the immune system of a pregnant dam alters throughout pregnancy (Sargent, 1993), one could expect that dams in their early/mid pregnancy (GD9) respond differently to an immune challenge than dams in their mid/late pregnancy (GD15). There were however no statistically significant difference in the cytokine expression response between the two groups for any of the tested doses of poly I:C.

1.2 Foetal compartment

Maternal cytokines can cross the placenta and influence the expression of cytokines by the immature neurons and glial cells in the foetal brain. As many cytokines (including IL-1β, TNF-α, IL-6 and IL-10) play crucial and diverse roles in CNS development, it is believed that an alteration in their expression patterns can have important and deteriorating effects on the normal development of the brain (Gilmore and Jarskog, 1997). In our study, there seemed to be both responders and non-responders

among the foetuses. Some foetuses responded with a very high increase in inflammatory cytokine mRNAs in the brain following MIA, while others showed no increase at all compared to controls. It should however be noted that there was a lot of variation in the cytokine mRNA values of the control groups, which makes it difficult to estimate the relevance of smaller changes of cytokine mRNAs in MIA-exposed foetuses. It might also be possible that we missed the alterations in foetal brain cytokine mRNA expression in some foetuses because we sampled at only one time point i.e. 6 hours after treatment. The changes in foetal brain cytokine expression could be more pronounced at an earlier or later time point. Another possible explanation for the observed differences in cytokine mRNA expression between foetuses is that each of them has its own placenta and thus maternal-foetal blood circulation, and might therefore be exposed to a different amount of maternal cytokines. Moreover, each foetus also has its own unique genetic background that might render it more or less susceptible to respond to fluctuating cytokine changes by regulating the foetal brain cytokine mRNA expression.

Of all the doses that were tested, the 4 mg/kg poly I:C dose was most consistent in inducing increases of cytokine mRNAs, in both the maternal and foetal compartments. Two GD9-old foetuses and one GD15-old foetus were particularly interesting as they showed consistently high increases in all the investigated pro-inflammatory cytokines after MIA with the 4 mg/kg dose. They only differed in the expression of the anti-inflammatory cytokine. While the GD9-old foetuses exhibited a very high increase in the mRNA levels of IL-10, the GD15-old foetus showed only a modest increase by comparison. This is interesting in light of the foetal brain cytokine balance hypothesis as put forward by Meyer and colleagues. They state that especially a shift in the normal balance between pro-inflammatory and anti-inflammatory cytokines in the foetal brain underlies the neurodevelopmental insult, ultimately leading to the emergence of schizophrenia-related brain pathology following MIA (Meyer et al., 2009; Meyer et al., 2006). Meyer and colleagues found that an overexpression of the anti-inflammatory cytokine IL-10 could prevent the emergence of behavioural deficits in a poly I:C-induced MIA mouse model, but that in the absence of a prenatal immune challenge, the excess levels of IL-10 in itself could result in behavioural deficits in the offspring. They thus hypothesized that besides a shift of the cytokine balance towards increased pro-inflammatory signaling, a shift towards anti-inflammatory signaling can affect foetal brain development as well, and that this effect is cancelled out when both pro- and anti-inflammatory cytokines are induced (see Fig 18). The possibility might arise that the very high increases in pro-inflammatory cytokine mRNAs in the GD9-old foetal brains following the 4 mg/kg poly I:C treatment in our study are compensated by the very high increase in anti-inflammatory cytokine mRNA, thereby cancelling out the effects that the induction of pro-inflammatory cytokines might have on the development of the brain. In the GD15-old foetal brain on the other hand, the increase in IL-10 mRNA was much lower following MIA with 4 mg/kg poly I:C than the increases in pro-inflammatory mRNAs. One should however be careful when comparing cytokine levels in this way. It would be better to compare the protein levels, as large increases in mRNA expression do not necessarily translate to large increases in protein expression (due to post-transcriptional processing). Our results differ from those of Meyer and colleagues in mice. They found a significant reduction in IL-10 mRNA expression in the foetal brain 6 hours after treatment with 5 mg/kg poly I:C on GD9 compared to control, and a significant increase in IL-10 mRNA expression (as well as of IL-6 and TNF-α) in the foetal brain after treatment with poly I:C on GD17 compared to control. They did not find any alterations in the mRNA expression of pro-inflammatory cytokines in the foetal brain on GD9 after poly I:C treatment, although they did find significant increases in the protein levels of pro-inflammatory cytokines in the foetal brain at both GD9 and GD17. They concluded that the cytokines in the GD9-foetal brain must originate from the maternal compartment or the placenta and that the cytokines in the GD17-foetal brain could, at least partly, originate from endogenous production in the foetus (Meyer et al., 2006). Here, we demonstrate that the GD9-old foetal rat brain is able to produce inflammatory cytokines of its own.

1.3 Link between maternal and foetal responses

In our study there was no clear association between the cytokine induction in the mother and the cytokine induction in the foetuses. While a positive correlation could exist between the response of the mother and the response of the foetuses, one could also find examples of mothers with a high cytokine induction having offspring with a low cytokine mRNA expression and of mothers without cytokine induction where the offspring showed increases in the cytokine mRNA expression. Secondly, the offspring of one mother could be very similar in their cytokine mRNA expression responses to the MIA event (e.g. the two above-mentioned GD9-old foetuses), but could also have quite different cytokine responses. Likewise, we had litters in the behavioural study where all the littermates showed similar behavioural abnormalities and some litters where one of the littermates showed a normal response (data not shown). The concordance and discordance of this response in littermates can be explained by many factors. Littermates share about 50% of their genes as well as a similar environment, but at the same time they have each their own placenta and amniotic sac and therefore the prenatal environment can differ in many aspects. Probably both genetic and environmental factors are needed to explain the whole picture. This underlines again the importance and relevance of the neurodevelopmental hypothesis of schizophrenia that postulates that an interaction between genetic and environmental risk factors is necessary to explain the increased risk of an individual to develop schizophrenia due to exposure to common environmental factors such as prenatal infection (Brown, 2011).

1.4 Choice of dose and time point of induction

Finally we decided to use the 4 mg/kg poly I:C dose on GD15 for the induction of the MIA model in our lab. The dose of poly I:C and time point of induction were not only based on the results of this study, but on the literature as well. GD15 is the most commonly used time point for induction of the MIA model in the rat (Dickerson et al., 2010; Howland et al., 2012; Wolff and Bilkey, 2008; Yee et al., 2011; Zuckerman et al., 2003). Contrary to the mouse, the poly I:C MIA model in the rat has almost always been induced during mid/late pregnancy (around GD15). GD15 in rats is equivalent to the late first trimester in humans with regard to brain development (Bayer et al., 1993; Clancy et al., 2001). Meyer and colleagues identified the early/mid and late gestational periods as two distinct windows of vulnerability in the mouse in terms of foetal brain cytokine responses, adult behavioural outcome and brain pathology (Meyer et al., 2006). Data on the vulnerability of the early/mid gestational period in the rat are however largely lacking. Only one study looked at the effect of MIA with poly I:C during early/mid gestation in the rat (GD10-11). In this study, only PPI was investigated. Prenatal treatment with poly I:C on GD10 and GD11 had no significant effect on the PPI of male adult offspring, but nor did they find PPI disruptions in the adult offspring when poly I:C was administered at other stages of pregnancy (GD15-16 and GD18-19) (Fortier et al., 2007). To be able to compare our findings in the MIA rat model with other rat studies in the literature, it is the most relevant to choose the GD15 time point for induction. It would however be interesting to repeat our follow-up study in the rat, challenged with an immune stimulus in early/mid gestation (GD9) and compare the offspring with rats that were exposed to MIA on GD15.

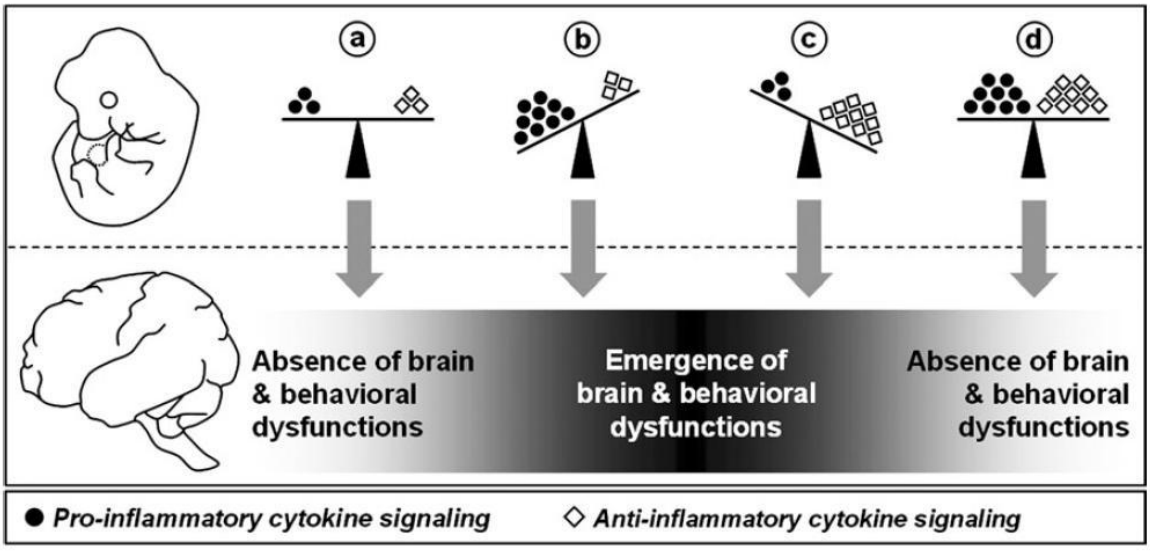

Fig 18. Illustration of the foetal brain cytokine balance hypothesis. A shift of the normal cytokine balance (a) in the foetal brain towards either increased pro-inflammatory signaling (b) or anti-inflammatory signaling (c) will result in adult brain and behavioural dysfunctions. Induction of both pro-inflammatory and anti-inflammatory cytokines cancels their respective negative effects out (from (Meyer et al., 2009)).

2 LPS time-course and dose-response study

2.1 Sickness behaviour and depression-like behaviour

Administration of a single low dose of LPS to adult rodents is a commonly used model to investigate the role of inflammation in depression. Depressive symptoms immediately ensue following LPS administration such as anhedonia, lack of motivation for food and drink, reduced exploratory locomotor activity and lethargy. These symptoms constitute the transient sickness behaviour, an adaptive behavioural response that reorganizes the motivations and priorities of an organism to better overcome the infection. The sickness behaviour usually resolves within 24 hours after LPS injection, as measured by a normalization in body weight loss and exploratory locomotor activity, while depression- and anxiety-like behaviours such as anhedonia persist in the model (Salazar et al., 2012). Both peripheral (i.p.) and central (intracerebroventricular) administration of LPS have been shown to cause transient sickness behaviour and persisting depressive-like behaviour. However, especially systemic inflammation has been implicated in the pathogenesis of depression and therefore it is more interesting to study the effects of peripherally administered LPS. These observations have been replicated with poly I:C, indicating that the sickness behaviour and persisting depression- and anxiety-like behaviours do not depend on the nature of the inflammatory challenge, bacterial (LPS) or viral (poly I:C) (Gibney et al., 2012). It should however be noted that it is quite difficult to make a clear distinction between the two behaviours, as sickness behaviour and depression-like behaviour share many symptoms. Some argue for example that a decreased sucrose preference following immune challenge is an expression of sickness behaviour when accompanied by a decrease in total fluid consumption, while a decreased sucrose preference with an unaffected total fluid consumption would reflect pure anhedonia and thus depression. In one of these studies the LPS-induced diminished total fluid consumption levels returned to basal levels within 24 hours post-injection while the reduction in sucrose preference persisted up to 39 hours (Henry et al., 2008). In another study that made use of poly I:C to challenge the immune system, the total fluid consumption was not even affected after immune challenge while saccharin preference was decreased up to 3 days post-injection (Gibney et al., 2013).

In our study all animals showed a significant decrease in sucrose preference at 6 hours post-LPS compared to the sucrose preference before injection. This observation can easily be attributed to the sickness behaviour of these rats. At this time point, almost all the animals exhibited symptoms of

sickness: squinting eyes, a hunched posture, porphyrin around the eyes and nose, and piloerection. Twenty-four hours post-injection, these symptoms had resolved, but all 0.1 mg/kg LPS-treated animals still showed significant decreases in sucrose preference and total liquid consumption compared to pre-injection measurements. At 3 days and 7 days post-injection, the sucrose preference was overall significantly decreased in respectively 0.5 and 0.1 mg/kg LPS-treated rats, as was the total fluid intake at 3 days post-injection in all animals. Overall, there was no significant difference in response between the two doses. When we look at the individual data, it becomes apparent that there were in fact three groups to be distinguished among the rats. In one group, the sucrose preference returned to normal levels (around 95%) at 3 days and 7 days post-injection. In the second group, the sucrose preference reached stable low levels between 50 and 70%. In the third group, the sucrose preference kept decreasing and reached very low levels (around 10%) at these late time points. At every time point, there was a very strong positive correlation between the sucrose preference of an animal and its total fluid consumption. Rats with a very low sucrose preference had a very low total fluid intake and vice versa. It is difficult to conceive that the persisting reduction in total fluid consumption in these animals is due to a persisting sickness behaviour, which is known for its transient nature. The reduction in total fluid intake could of course be an expression of depression-like behaviour itself. A loss of motivation for drinking may accompany the loss of appetite which is an important symptom of depression (Yirmiya, 1996). To be certain that the sickness behaviour has resolved at these late time points, other parameters should be investigated in the future such as body weight change and locomotor activity. For this reason, we decided to measure body weight changes after LPS in the follow-up study of poly I:C- and control offspring. Still a better way to measure anhedonia would be through an intracranial self-stimulation paradigm in which rodents learn how to deliver electrical stimulations to the reward system in their brain (van Heesch et al., 2013). Rodents that tend to do less so experience anhedonia. The great advantage of this method is that the anhedonia measurements are not biased by LPS-induced reductions in food and water consumption.

2.2 Taste avoidance

While the rats that exhibited a decreased sucrose preference between 50 and 70% could be thought of as depressed rats, another phenomenon seems to present itself in the third group of rats, where the sucrose preference is extremely low (around 10%). There seems to be an aversion for the sucrose solution in these rats or a taste avoidance. It is known that when a favoured beverage such as a sweet sucrose solution is paired with a disease-provoking immune stimulus (such as LPS or poly I:C), a taste aversion can ensue for the sucrose solution in later trials (Vidal and Chamizo, 2010). Something similar could be going on in our rats. They could have linked the new situation of two drinking bottles in the home cage and the presence of a sucrose solution with the LPS-induced sickness and therefore avoid the sucrose solution altogether. Normally however, this kind of conditioning is very acute, while in our study a 48h-habituation period preceded the LPS injection. We can however not exclude a similar effect.

2.3 Microglia activation

Our study represents the first study to extensively characterize the time-course of LPS-induced microglia activation in the brain from a few hours up to one week post-injection. Many studies have reported different time points of when the microglia activation is obvious after i.p. LPS injection, both of low (Corona et al., 2010; Hu et al., 2012; Viana et al., 2010) and high doses of LPS (Fan et al., 2012; Ha et al., 2012; Park et al., 2012; Tokes et al., 2011). Corona et al. reported an increase in activated microglia in prefrontal cortex, hippocampus, but not amygdala 3 days after treatment with 0.5 mg/kg LPS in mice (Corona et al., 2010). Viana et al. reported an increase in the number of activated microglia in the hippocampus 24 hours after i.p. injection with 0.45 mg/kg LPS in mice (Viana et al., 2010). Hu et al. reported that there was no change in the morphology of microglia in any of the investigated brain regions 6 hours after i.p. injection with 0.1 mg/kg LPS in mice. They did however see changes in microglial morphology at 24 hours after injection with 0.1 mg/kg LPS, but

restricted to the circumventricular organs. After treatment with 1 mg/kg LPS, activated microglia were already apparent at 6 hours post-LPS, but still only in the circumventricular organs. Twenty-four hours after treatment with 1 mg/kg LPS, activated microglia were found in other brain regions as well (Hu et al., 2012). We report here that treatment with both 0.1 and 0.5 mg/kg LPS resulted in a widespread activation of microglia in the brain, which was already apparent at 6 hours after LPS, although not significant. The main reason for not attaining significance was probably the low number of animals in each group (n=3). The microglia activation increased in time and reached a peak at 3 days post-LPS, which was significant in the corpus callosum, hippocampus and thalamus of 0.1 mg/kg LPS-treated rats and in the hippocampus and thalamus of 0.5 mg/kg LPS-treated rats compared to controls. The microglia activation seemed to have reached a plateau that lasted at least up to 7 days post-treatment. It should however be noted that we investigated the 7 days-time point only in 0.1 mg/kg LPS-treated rats. We can therefore not exclude that the microglia activation does not increase beyond the 3 days-time point in 0.5 mg/kg LPS-treated rats. There was however no significant difference in the microglia response to the 0.1 and 0.5 mg/kg LPS doses from 6 hours up to 3 days post-treatment.

2.4 Link between LPS-induced microglia activation and anhedonia

There is strong evidence for a direct link between the activation of microglia following LPS administration and the LPS-induced anhedonia. Minocycline, an antibiotic with anti-inflammatory properties, is thought to be an inhibitor of microglia and was shown to reduce the LPS-induced expression of TLR2 on microglia in the brain of mice. In the same study, pre-treatment with minocycline could prevent LPS-induced anhedonia and improve the recovery from sickness behaviour. At the same time, mRNA levels of indoleamine 2,3-dioxygenase and a number of pro-inflammatory cytokines were decreased in the brain (Henry et al., 2008). Indoleamine 2,3 dioxygenase has been recognized as an important mediator of LPS-induced anhedonia. Pre-treatment with an inhibitor of indoleamine 2,3-dioxygenase has been shown to diminish the LPS-induced anhedonia in mice (Salazar et al., 2012). Inhibition of indoleamine 2,3-dioxygenase also reduced the microglia activation in the prefrontal cortex and hippocampus of mice 3 days post-LPS injection (Corona et al., 2013). In our study we found a trend toward significance for a negative correlation between the microglia activation in the thalamus and the sucrose preference in rats. The higher the microglia activation in the thalamus, the lower the sucrose preference in a rat. The thalamus has become more and more important in psychiatric disorders, particularly in mood disorders (Dham and Alexander, 2013). As it connects the mood-related limbic system (including amygdala and hippocampus) with the frontal cortex, the thalamus is of particular interest for potential involvement in depression-like behaviours.

3 Behavioural and histopathological study of poly I:C and control offspring

3.1 Maternal body weight change following MIA induction

As discussed earlier in §1.1, the pregnant dams responded quite differently to the 4 mg/kg poly I:C immune challenge. Some of the dams lost weight following the immune challenge, while others gained weight. Body weight change might serve as an indicator of the pregnant dam's individual responsiveness to the poly I:C immune stimulus. Bronson et al. and later Vorhees et al. (members of the same lab) found significant differences in schizophrenia-related behaviour between the offspring of dams that had lost weight after intraperitoneal injection with 8 mg/kg poly I:C on GD14 and the offspring of dams that had gained weight (Bronson et al., 2011; Vorhees et al., 2012). Bronson et al. (2011) were the first group to report a significant variability in the body weight response to poly I:C in rodents and identified this response as having a bimodal distribution. These findings are consistent with our own observations. About half of the dams had a mean weight loss of approximately 3 grams, while the other half had a mean weight gain of approximately 4 grams. One dam showed neither loss nor gain of weight. Accordingly to Bronson et al. and Vorhees et al., we decided to analyze the behavioural (and histopathological) data of the poly I:C offspring based on the individual response of the mother to the immune challenge, leading up to two poly I:C offspring groups: poly I:C

(weight loss) offspring and poly I:C (weight gain) offspring. The offspring of the dam that had neither lost nor gained weight consequently had behavioural and histopathological responses in between the responses of the two poly I:C offspring groups. As we wanted to dichotomize between poly I:C (weight loss) and poly I:C (weight gain) offspring, this nest was left out of the data analysis.

3.2 Behavioural findings

3.2.1 Prepulse inhibition of the acoustic startle response

Disruption of the PPI response is considered as one of the most important behavioural deficits to validate animal models of schizophrenia (Swerdlow and Geyer, 1998; Weiss and Feldon, 2001). PPI disruption has been found in the offspring of poly I:C-treated mice (Smith et al., 2007) and rats (Dickerson et al., 2010; Howland et al., 2012; Wolff and Bilkey, 2008, 2010; Yee et al., 2011). There are however also studies that failed to find a significant effect of prenatal treatment with poly I:C on PPI in adult offspring, both in mice (Giovanoli et al., 2013) and rats (Fortier et al., 2007). Li et al. found a disruption of the sensorimotor gating in mice that were treated prenatally with poly I:C in early gestation (GD9) but not in mice that were treated in late gestation (GD17) (Li et al., 2009). Giovanoli et al. did not find an effect of prenatal treatment with poly I:C during early gestation (GD9) on PPI in adult mice. However, when they combined this prenatal immune challenge with peripubertal stress, another environmental risk factor for developing schizophrenia, they did find a disruption of the PPI response, supporting evidence for the theory that psychiatric disorders with a delayed onset require multiple (environmental) hits to develop i.e. the two-hit hypothesis (Giovanoli et al., 2013). In our study, we have found a trend toward significance for a PPI disruption in poly I:C (weight loss) offspring but not in poly I:C (weight gain) offspring, and only after the 75 dB prepulse. These findings are inconsistent with the observations of Vorhees and colleagues. They also found differences between poly I:C (weight loss) offspring and poly I:C (weight gain) offspring, but the other way around. They observed a reduced PPI in poly I:C (weight gain) offspring but not in poly I:C (weight loss) offspring. It should however be noted that they observed this PPI defect only in female offspring and not in male offspring, while we investigated only male offspring. They also divided the poly I:C offspring according to a different rule. The offspring of dams that lost or gained the least weight after poly I:C treatment were assigned to the poly I:C (weight loss) group while the offspring of dams that gained the most weight after MIA were assigned to the poly I:C (weight gain) group (Vorhees et al., 2012). Wolff and Bilkey also investigated PPI deficits in poly I:C offspring as a function of maternal weight change following the immune challenge. They stated that the PPI deficits were independent of the maternal weight change. It should however be noted that the poly I:C treatment in this study did not cause weight loss in the rats (Wolff and Bilkey, 2010). It is therefore possible that only a clear distinction in the maternal response to the poly I:C immune challenge (weight loss versus weight gain), as in our study and Vorhees' study, results in a distinct behavioural pathology in the respective offspring.

3.2.2 Spontaneous and psychotomimetic-induced locomotor activity

We have found no significant differences in spontaneous locomotor activity between any of the offspring groups despite the long period tested. This is consistent with most other studies that investigated spontaneous locomotor activity in poly I:C offspring (Ozawa et al., 2006; Zuckerman et al., 2003; Zuckerman and Weiner, 2005), though there are also studies that did find a significant difference ((Howland et al., 2012) and (Van den Eynde et al., submitted)). These two studies tested a far greater number of rats than we did. Howland et al. tested 35 control rats and 30 poly I:C rats, while Van den Eynde et al. tested 37 control rats and 56 poly I:C rats. Both studies investigated both male and female rats, while we only tested male offspring. We did observe however differences between poly I:C (weight loss)-, poly I:C (weight gain)- and control offspring in the responsiveness to the locomotion-stimulating drugs amphetamine and MK-801.

There is extensive evidence in the literature for an abnormal development of the dopaminergic system in the MIA model, which is already apparent in the foetus and progresses throughout life. The

main structures to be affected are the mesoaccumbal and nigrostriatal dopaminergic pathways and the medial prefrontal cortex (Meyer et al., 2008c; Vuillermot et al., 2010). Rodents are known to respond to a low dose of the indirect dopamine agonist amphetamine (e.g. 1 mg/kg) with an increase in their locomotor activity, which is at least partly mediated by the mesoaccumbal dopaminergic pathways (Heidbreder and Feldon, 1998). Dopamine-mediated behaviour such as the amphetamine-induced hyperlocomotion can be used to evaluate the presence of dopaminergic abnormalities in the MIA model. In this study, we have found that adult poly I:C (weight loss) offspring were less sensitive to amphetamine challenge compared to control offspring and poly I:C (weight gain) offspring, as the total locomotor activity of poly I:C (weight loss) offspring was not significantly elevated after treatment with amphetamine compared to before treatment, while this was the case in control offspring and poly I:C (weight gain) offspring. There was no difference in the response to amphetamine between control offspring and poly I:C (weight gain) offspring. A decreased sensitivity to the locomotion-stimulating effects of amphetamine in adult poly I:C (weight loss) offspring has previously been described by Bronson and colleagues. They also found no difference between control offspring and poly I:C (weight gain) offspring (Bronson et al., 2011). Most studies however have described an increased sensitivity of adult poly I:C offspring to amphetamine compared to controls, which could be prevented by treatment with atypical antipsychotics in adolescence (Meyer et al., 2008a; Meyer et al., 2008b; Ozawa et al., 2006; Piontkewitz et al., 2011; Piontkewitz et al., 2009; Zuckerman et al., 2003). These observations are more in line with the classical dopamine hypothesis of schizophrenia, in which a hyperdopaminergic activity in the mesolimbic system is thought to account for the psychotic symptoms in schizophrenia patients and as a result, blocking of dopamine receptors relieves psychotic symptoms in patients. The decreased sensitivity to amphetamine in adult poly I:C offspring as observed by Bronson et al. and Richtand et al. (members of the same lab) could however also be reversed by treatment with atypical antipsychotics in adolescence (Richtand et al., 2011). Surprisingly, in a later study performed by this lab, poly I:C (weight loss) offspring showed an increased responsiveness to amphetamine compared to the other offspring groups (Vorhees et al., 2012). An important difference between the two studies was that Bronson et al. tested the offspring on postnatal day PND90, while Vorhees et al. tested the offspring at PND63-65. We tested our offspring on PND82 or even later for some rats, which can account for the consistency in results between our study and Bronson's study. The age at testing seems to be an important determinant of the behavioural outcome in this particular test. Whatever the precise nature of the dopaminergic abnormalities may be, it is clear that the dopaminergic system is involved in the pathology of the MIA model, as it is in schizophrenia. Further studies are however needed to identify the exact nature of the dopaminergic dysfunction in this model, in terms of the dopamine receptor and transporter expression and function, dopamine synthesis and metabolism.

There is also evidence for glutamatergic abnormalities in MIA offspring, which are largely consistent with the NMDA receptor hypofunction model of schizophrenia. In this model, a decreased function of NMDA-type glutamate receptors would account for the dysfunction of the mesolimbic and mesocortical dopaminergic pathways, explaining not only the positive symptoms of schizophrenia, but the negative and cognitive symptoms as well. A reduced expression of the NR1/GluN1 subunit of the NMDA receptor has been described in the adult offspring of poly I:C-treated mice (Meyer et al., 2008c) and rats (Forrest et al., 2012). The involvement of the glutamatergic system (and specifically, the NMDA receptor) in the pathology of the MIA model can be tested by assessing the response of MIA offspring to the non-competitive NMDA receptor antagonist MK-801, which is known to induce hyperlocomotion in rodents when administered in a low dose e.g. 0.2 mg/kg (Andine et al., 1999). As with amphetamine, both an increased responsiveness (Meyer et al., 2008a; Meyer et al., 2008b; Zuckerman and Weiner, 2005) and a decreased responsiveness (Howland et al., 2012; Vorhees et al., 2012) to MK-801 have been described in the MIA model. The age at testing as well as the timing of prenatal immune challenge are important determinants of the response to MK-801 (Meyer et al., 2008a; Meyer et al., 2008b). We have found that both poly I:C (weight loss)- and poly I:C (weight gain) offspring show a different response compared to controls and that the responses of the poly I:C

offspring groups were in the opposite direction compared to controls. These differences did not reach overall statistical significance, probably due to the low number of rats in each group (i.e. lack of power). Poly I:C weight loss offspring responded slower to MK-801, which resulted in significant differences between this group and the control group for the first 10 minutes of the measurement. This suggests different pharmacokinetics and/or pharmacodynamics of this drug in poly I:C (weight loss) offspring. Poly I:C (weight loss) offspring also had in general a lower responsiveness to MK-801, though not significant. Poly I:C (weight gain) offspring initially showed a similar response as controls to the drug, but after approximately 50 minutes the response started to deviate and was from then on generally higher than in controls. These results are consistent with the observations by Bronson et al. who reported a strongly reduced sensitivity to MK-801 in poly I:C (weight loss) offspring and a modestly increased response in poly I:C (weight gain) offspring that only reached significance in the third hour after administration of the drug (Bronson et al., 2011). Vorhees et al. found no difference in the response to MK-801 in poly I:C (weight gain) offspring versus controls, but also reported a decreased response in poly I:C (weight loss) offspring (Vorhees et al., 2012). A decreased responsiveness to MK-801 is consistent with the NMDA receptor hypofunction hypothesis of schizophrenia. Further evidence for the NMDA receptor hypofunction in the MIA model came from a study by Roenker et al. who measured the extracellular glutamate levels in the brain of poly I:C offspring. Basal extracellular glutamate levels were significantly increased in the prefrontal cortex, and MK-801-induced elevations in extracellular glutamate levels were significantly lower in poly I:C offspring, which was prevented by pre-treatment with atypical antipsychotics (Roenker et al., 2011). Further studies are needed to explain the increased sensitivity to MK-801 in poly I:C offspring.

3.2.3 Sucrose preference, total liquid consumption and LPS-induced sickness behaviour

An important negative symptom of schizophrenia is anhedonia, not being able to experience pleasure, which is linked to an inability to respond to reward. In our study, the poly I:C (weight loss) offspring showed a significantly lower sucrose preference than control offspring before LPS injection. There was no difference between poly I:C (weight gain)- and control offspring. The difference in mean sucrose preference between poly I:C (weight loss) and control offspring was 6%, which was similar to the decrease in sucrose preference that Bitanihirwe et al. found in poly I:C mice offspring (Bitanihirwe et al., 2010). The anhedonia-like behaviour in the poly I:C (weight loss) offspring may be linked to a reduced dopaminergic status in these animals. Dopamine is one of the most important neurotransmitters involved in reward. The reduced ability to respond to reward in these rats might therefore be associated with a reduced basal dopaminergic tone. The same animals showed a reduced response to the dopamine-inducer amphetamine in the locomotion test. Contrary to Bitanihirwe's findings, the total fluid consumption was also significantly decreased in the poly I:C offspring in our study, both of poly I:C (weight loss) and poly I:C (weight gain) offspring. As argued in §2.1, the reduction in total fluid intake might also be an expression of depression-like behaviour. A loss of motivation for drinking may accompany the loss of appetite which is an important symptom of depression (Yirmiya, 1996).

Next we challenged the immune system of the rats with an intraperitoneal low-dose LPS injection, the dose being deduced from the LPS time-course and dose-response study (0.1 mg/kg). As in the mentioned study, there was a significant decrease in the sucrose preference in normal rats (control offspring) at 6 hours post-injection. In the poly I:C (weight gain) offspring, there was an acute decrease in the sucrose preference that just missed significance ($p=0.0578$). In the poly I:C (weight loss) offspring, the decrease in sucrose preference was not significant, perhaps due to the fact that the basal levels in these animals were significantly lower than those of the other offspring groups to begin with. At 3 days post-injection, the sucrose preference levels of all animals but one in every offspring group had returned to normal high levels. Remarkably, the poly I:C (weight loss) offspring had no decreased sucrose preference levels anymore after LPS injection compared to controls. Only one rat in every offspring group exhibited a persistent decrease in sucrose preference at 3 days post-injection. The control offspring showed a significant decrease in total liquid consumption at 6 hours

and 3 days post-LPS injection compared to the pre-injection period. Poly I:C (weight loss) offspring showed a significantly lower total fluid intake at 6 hours post-LPS injection compared to poly I:C (weight loss) offspring that received a saline injection. The poly I:C (weight loss) and poly I:C (weight gain) offspring showed a trend toward significance for a reduced total liquid consumption at 3 days post-LPS compared to pre-injection ($p \leq 0.1$). We also followed-up the acute weight change following LPS injection in the three offspring groups as another measure of the sickness behaviour. LPS injection induced a significant body weight reduction in control offspring ($p \leq 0.05$) and a highly significant body weight reduction in poly I:C (weight loss) offspring ($p \leq 0.01$) compared to saline injection. Poly I:C (weight loss) offspring showed a significantly greater weight loss at 6 hours post-LPS compared to LPS-treated control offspring. There was no difference between poly I:C (weight gain) and control offspring. Based on this parameter, one could argue that the poly I:C (weight loss) offspring were most sensitive to the peripheral immune challenge of all three offspring groups. This might reflect a hyperactive pro-inflammatory immune system, as has been proposed to exist in schizophrenia patients. The other measured parameters of sickness behaviour, the sucrose preference and total liquid consumption, give however no conclusive evidence for a heightened sensitivity of these rats to a peripheral immune challenge. It should however be noted that changes in these two parameters due to LPS are difficult to compare across the three offspring groups, as the basal pre-injection levels of sucrose preference and/or total liquid consumption are already lower in poly I:C offspring compared to controls. Finally, it is also remarkable that all the offspring of the dams that had lost weight following immune challenge, consistently showed the highest weight loss after LPS injection. This response may be genetically predisposed and be unrelated to the MIA pathology.

3.3 Histological findings

A significantly increased microglia activation compared to control has been observed at PND30 in the hippocampus and striatum of poly I:C mice offspring by Juckel and colleagues (Juckel et al., 2011) as well as in the pons, corpus callosum, hippocampus and thalamus of adult poly I:C rat offspring by a member of our group (Van den Eynde et al., submitted). We report here a higher microglia activation in poly I:C (weight loss) offspring compared to poly I:C (weight gain) offspring after LPS injection. The same observation was made in poly I:C (weight loss) offspring versus poly I:C (weight gain) offspring without LPS challenge (saline injection) but the number of animals in the poly I:C (weight loss) offspring group was too low (n=2) to make reliable statements about this. Additional data are on their way to verify this observation (see Future perspectives).

There was no significant difference in microglia activation between poly I:C and control offspring, neither after saline nor LPS injection. It should be noted that some of the control offspring showed rather high levels of microglia activation.

The LPS injection seems to have failed to induce significant increases in microglia activation in all three offspring groups, and could not even reproduce the findings from the previous study in control offspring. We therefore believe that the LPS injection did not work, at least not to the same extent as in the LPS time-course and dose-response study. The significantly higher microglia activation in poly I:C (weight loss) offspring compared to poly I:C (weight gain) offspring after LPS injection might just reflect higher basal levels of microglia activation in the poly I:C (weight loss) offspring.

There could be a number of reasons for the lack of effect by the LPS injection. First of all, the intraperitoneal injection may not be well performed. If the LPS was injected in the colon, it is possible that it didn't reach systemic circulation. If the LPS was administered subcutaneously, it would result in a much lower microglia activation than an intraperitoneal dose, as we observed in an earlier experiment performed at the lab. Secondly, the LPS could have degraded during storage. We prepared a stock solution of the LPS and stored it at -20°C. Resuspended LPS should be stable for 6 months at -20°C and for 1 month at 4°C. We worked with the resuspended LPS for an approximate 6 months, but we repeatedly thawed and froze the stock solution, which might have accelerated the degradation process. Although the LPS dose was able to affect sucrose preference, total fluid intake

and body weight of the animals, its effects seem to have been less severe in the final study as the other sickness symptoms of the rats (squinting eyes, a hunched posture, porphyrin around the eyes and nose, piloerection) that were present in the previous study, were absent in the final study. Due to these or other problems, we have been unable to verify our hypothesis that poly I:C offspring are more vulnerable for LPS-induced brain inflammation than controls.

4 Future perspectives

First of all, we have still two litters of rats to test. One of the litters contains poly I:C (weight loss) offspring (n=4), the other one control offspring (n=4). These rats will be used to 1) verify the observation that poly I:C (weight loss) offspring have a higher microglia activation than poly I:C (weight gain) offspring without LPS challenge, by providing additional data points for the statistical analysis described above; and 2) determine whether intraperitoneal injection with freshly dissolved LPS can induce increased microglia activation in these offspring and thus to test our hypothesis that the LPS failed to induce microglia activation in the final study because of (partial) degradation. Secondly, we will investigate whether the dams that lost weight following MIA showed the highest cytokine response in the blood and if the dams that gained weight had the lowest cytokine induction. We have collected blood samples of these dams at the same time as we detected the weight change and can therefore investigate the association between cytokine induction and body weight change following MIA. Thirdly, we will investigate the role of microglia activation in the MIA-induced pathology in earlier stages of the model. We have performed a cross-sectional study in which we sacrificed male pups of poly I:C- and vehicle-treated dams and collected their brains and blood samples at five different ages between birth and adulthood: PND1, PND7, PND14, PND30 and PND56. These samples will be used in the future to further examine the role of brain inflammation in MIA-induced pathology. More attention will be paid to the actual phenotype of the microglia. Activated microglia can be pro-inflammatory (M1 phenotype) or anti-inflammatory (M2 phenotype) in nature and be involved in either brain injury (M1) or tissue repair (M2). This will be investigated by use of M1/M2 markers or pro-inflammatory markers such as iNOS. Additionally, the role of astrocytes in MIA-induced pathology can also be investigated. Finally, a more mechanistic approach will be taken to gain insight into the mechanisms through which MIA exerts its effects. Particular attention will be paid to the TRYCAT pathway. TRYCATs are known agonists and antagonists of NMDA receptors and have been implicated in the NMDA receptor hypofunctioning in schizophrenia patients (Muller et al., 2011). One of the most important enzymes in the TRYCAT pathway is indoleamine 2,3-dioxygenase. Microglia activation has been associated with increased levels of indoleamine 2,3-dioxygenase and both have been implicated in schizophrenia and depression. This pathway provides an interesting link between immune activation and NMDA receptor hypofunctioning.

5 Conclusion

We have characterized a rat model of MIA with relevance to schizophrenia in terms of maternal and foetal cytokine responses, adult behavioural outcome and adult brain histopathology. In addition, we have evaluated the time-course of LPS-induced sickness behaviour, anhedonia and microglia activation in normal adult rats.

MIA with poly I:C induced acute increases of the mRNA expression of inflammatory cytokines in the maternal and foetal compartments, however, not all pregnant dams and foetuses seemed to respond to the MIA challenge. Likewise, not all pregnant dams in the follow-up study lost weight after immune challenge. This differential response in body weight change of pregnant dams following MIA has previously been observed. The different response of pregnant dams to MIA in terms of cytokine induction has however not been investigated. Our observations suggest that these responses may be associated, which will be further investigated. If dams with a different weight change response also have different cytokine responses, then this may result in a distinctly different neurodevelopmental insult in the respective offspring.

The individual maternal response to the poly I:C immune challenge determined the behavioural outcome in adult offspring. The offspring of dams that had lost weight following immune challenge seemed to have the worst behavioural outcome in nearly every test compared to the offspring of dams that gained weight following MIA which showed an overall better outcome in most of the behavioural tests. First of all, poly I:C (weight loss) offspring showed a trend for a PPI disruption of the acoustic startle response, indicating a sensorimotor gating deficit. This is contrary to published data, where only poly I:C (weight gain) offspring showed a PPI deficit. Secondly, poly I:C (weight loss) offspring had a reduced responsiveness to amphetamine and MK-801, which is consistent with previous findings. Poly I:C (weight loss) offspring exhibited lower basal levels of sucrose preference than controls, reflecting anhedonia, a negative symptom observed in schizophrenia patients. Both poly I:C groups had lower basal levels of total fluid consumption than controls. These observations have not been reported previously in the literature. When challenged with LPS, poly I:C (weight loss) offspring lost the most weight. However, there were no differences in microglia activation between vehicle and LPS-treated groups. This was very surprising as our initial LPS challenge study in controls demonstrated clear effects of LPS on sucrose preference and microglia activation. A dose as low as 0.1 mg/kg LPS could induce a global microglia activation in the brain of normal adult rats, which peaked at 3 days post-injection. This LPS injection induced acute sickness behaviour in all animals and persisting low levels of sucrose preference (anhedonia) and total liquid consumption in a subgroup of rats. For some reason or other, the LPS injection failed to induce global microglia activation in the final follow-up study in poly I:C and control offspring, which makes it unfortunately impossible to infer whether poly I:C offspring are more sensitive to an inflammatory challenge than control offspring.

Our data suggest that poly I:C (weight loss) offspring have a higher degree of microglia activation than poly I:C (weight gain) offspring. This study represents the first time that this observation is made. It suggests a link between chronic brain inflammation and behavioural abnormalities in MIA offspring. Poly I:C offspring with an overall worse behavioural outcome have a higher level of microglia activation than poly I:C offspring with an overall better behavioural outcome.

Our results confirm the general observation that prenatal immune activation can disturb the normal brain development and lead to schizophrenia-related behavioural deficits in the adult offspring. This underlines the relevance of this environmental risk factor for schizophrenia. However, the behavioural abnormalities seem to be rather subtle, as the deviant behaviour was often not overall significantly different from the control offspring. This could be due to the rather low number of animals tested, but one should also be reminded of the fact that the effect of only one environmental risk factor for schizophrenia was investigated. It is known that many risk factors for schizophrenia, both genetic and environmental, exist and probably interact to give rise to the disorder. It would be interesting to investigate the interaction of MIA with other risk factors of schizophrenia in our model, by combining MIA for instance with peripubertal stress or cannabis exposure and to investigate whether the behavioural deficits observed in the MIA model become more pronounced.

Only one other lab working on the MIA model has made the distinction between poly I:C offspring of dams that lost weight following MIA and offspring of dams that gained weight. Yet there is an important difference in behavioural outcome for the two offspring groups, as we now reproduced in this study. This observation should urge other labs to make a similar distinction in the MIA offspring and investigate the differences between the two groups. Our data suggest that there is not only a difference in behavioural outcome between the two groups, but in chronic brain inflammation as well. Our observations suggest a link between chronic brain inflammation in the model and the behavioural deficits. This underlines the involvement of an inflammatory component in the pathophysiology of schizophrenia, which might constitute a viable target for treatment of schizophrenia. As it is, anti-inflammatory agents such as NSAIDs have been shown to work synergistically with antipsychotic drugs to reduce symptoms in schizophrenia patients. Antipsychotics

have also been proposed to exhibit anti-inflammatory properties of their own and it has been suggested that they exert their anti-inflammatory effects on microglia activation (Kato et al., 2011). We now want to further investigate the precise role of microglia in the MIA model, not only in the chronic stage of the model, but in earlier stages of development as well, to examine their involvement in both the pathophysiology and pathogenesis of the disease and to identify them possibly as targets for treatment and even prevention. We believe the MIA model to be the ideal model for testing this hypothesis, because of its immunological basis and its relevance to schizophrenia.

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Appendix

Overview of the used qPCR primers (rat):

IL-1β (forward primer)	`tctggagatgacaccaagctc`
IL-1β (reverse primer)	`gcgcttgtcttcttctttgttc`
TNF-α (forward primer)	`tgaacttcggggtgatcg`
TNF-α (reverse primer)	`gggcttgtcactcgagtttt`
IL-6 (forward primer)	`cccttcaggaacagctatgaa`
IL-6 (reverse primer)	`acaacatcagtcccaagaagg`
IL-10 (forward primer)	`agtggagcaggtgaagaatga`
IL-10 (reverse primer)	`tcatggccttgtagacacctt`
β-actin (forward primer)	`cccgcgagtacaaccttct`
β-actin (reverse primer)	`cgtcatccatggcgaact`
28S rRNA (forward primer)	`agttctggcccgatcactc`
28S rRNA (reverse primer)	`gccgcacggtacttttca`
HPRT (forward primer)	`gaccggttctgtcatgtcg`
HPRT (reverse primer)	`acctggttcatcatcactaatcac`