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Evolution of a maternal immune activation (mIA) model in rats: early developmental effects

Katie N. Murray[1#*], Michelle E. Edye[1+*], Maurizio Manca[1$], Anthony C. Vernon[3, 4], Joanna M. Oladipo[1], Victoria Fasolino[1], Michael K. Harte[1], Varsha Mason[1], Ben Grayson[1], Patrick C. McHugh[6], Irene Knuesel[5], Eric P. Prinssen[5], Reinmar Hager[2], Joanna C. Neill[1].

[1]Division of Pharmacy and Optometry, School of Health Sciences, [2]Division of Evolution and Genomic Sciences, School of Biological Sciences, Faculty of Medicine, Biology and Health, University of Manchester, Manchester, M13 9PT, UK

[3]King's College London, Institute of Psychiatry, Psychology and Neuroscience, Department of Basic and Clinical Neuroscience, Maurice Wohl Clinical Neuroscience Institute, 5 Cutcombe Road, London, SE5 9RT, United Kingdom

[4]King's College London, MRC Centre for Neurodevelopmental Disorders, New Hunt's House, Guy's Hospital Campus, London SE1 1UL, United Kingdom.

[5]Roche Innovation Center Basel, 124 Grenzacherstrasse, Basel, CH 4070, Switzerland

[6] Centre for Biomarker Research and Department of Pharmacy, School of Applied Sciences, University of Huddersfield, HD1 3DH, UK

* Joint 1st authors

[#] Present address: Departments of Neurology and Neuroscience, Yale University, New Haven, Connecticut, 06510.

+ Present address: Wolfson Centre for Age-Related Disease, King's College London, London, SE1 1UL, United Kingdom

$ Present address: Centre for Biomarker Research and Department of Pharmacy, School of Applied Sciences, University of Huddersfield, HD1 3DH, UK

Corresponding authors: Reinmar Hager and Jo Neill, Reinmar.Hager@manchester.ac.uk, joanna.neill@manchester.ac.uk: Faculty of Medicine, Biology and Health, University of Manchester, Manchester, M13 9PT, UK

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Abstract

Maternal immune activation (mIA) in rodents is rapidly emerging as a key model for neurodevelopmental disorders such as autism spectrum disorder (ASD) and schizophrenia. Here, we optimise a mIA model in rats, aiming to address certain limitations of current work in this area. Specifically, the lack of clear evidence for methodology chosen, identification of successful induction of mIA in the dams and investigation of male offspring only. We focus on gestational and early juvenile changes in offspring following mIA as detailed information on these critical early developmental time points is sparse.

Following strain (Wistar, Hooded Lister, Sprague Dawley) comparison and selection, and polyriboinosinic-polyribocytidylic acid (poly I:C) dose selection (2.5-15 mg/kg single or once daily for 5 days), mIA was induced in pregnant Wistar rats with 10 mg/kg poly I:C i.p. on gestational day (GD) 15. Early morphometric analysis was conducted in male and female offspring at GD21 and postnatal day (PD) 21, eight dams for each treatment at each time point were used, 32 in total. Subsequent microglia analysis was conducted at PD21 in a small group of offspring.

Poly I:C at 10 mg/kg i.p. induced a robust, but variable, IL-6 response and reduced body weight at 6 h and 24 h post-injection in two separate cohorts of Wistar rats at GD15. IL-6 was not elevated at PD23 in offspring or dams. Poly I:C-induced mIA did not affect litter numbers, but resulted in PD21 pup, and GD21 placenta growth restriction. Poly I:C significantly increased microglial activation at PD21 in male hippocampi.

We have identified 10 mg/kg poly I:C i.p on GD15 as a robust experimental approach for inducing mIA in Wistar rats and used this to identify early neurodevelopmental changes. This work provides a framework to study the developmental trajectory of disease-relevant, sex-specific phenotypic changes in rats.

1. Introduction

There is accumulating evidence for the role of neuroinflammatory processes in the aetiology of neuropsychiatric disorders. Epidemiological evidence initially directed researchers towards prenatal infection as an explanation for increased incidence of psychiatric disorders following an influenza epidemic (Mednick, 1988). More recent genome-wide association studies and transcriptome analysis provide genetic support for these observations (Lombardo et al., 2017; Ripke et al., 2014; Voineagu et al., 2011). To fill the gap between epidemiological studies and clinical trials requires appropriate developmental *in vivo* model systems to identify tractable drug targets (Bespalov et al., 2016). Numerous studies have induced maternal immune activation (mIA) in rodents, mostly in mice, and more recently in rats, using a range of inflammatory stimuli including influenza virus (Shi et al., 2003), lipopolysaccharide (LPS; Urakubo et al., 2001), interleukin IL-6 (Smith et al., 2007) and turpentine (Fortier et al., 2007). The viral-mimetic polyriboinosinic-polyribocytidylic acid (poly I:C) has been most widely used with its administration on specific gestational days (GD; i.e. 9-17) inducing a discrete inflammatory response in the dam, in addition to eliciting behavioural and neurochemical abnormalities in the offspring (Meyer, 2014; Meyer et al., 2006; see Estes and McAllister, 2016 for an excellent recent overview). Differences in strain of rodent, dose and route of administration are apparent in the literature (de Souza et al., 2015; Giovanoli et al., 2015; Vernon et al., 2015; Estes and McAllister, 2016), which may contribute to the wide variation seen in different behavioural and neurochemical effects observed across studies. One key issue emerging is the need to establish an early outcome measure to identify offspring likely to show a robust phenotype, essential for enhancing reproducibility across laboratories (Estes and McAllister, 2016). Initial mIA studies predominantly used mice, despite the benefits of using rats in complex, cognitively-demanding or social behaviour tasks and neuroimaging read-outs, to which their size and lack of conspecific-directed aggression

confers significant advantage (Abbott, 2004; Yao et al., 2012). More work has now been conducted in rats and no doubt work in both species will bring advantages to this field, but it is unlikely that the same methodology will be appropriate. Experimental studies often only use males owing to their lack of oestrous cycle (suggested to produce less variable data, a common misconception, see in-depth analysis by Prendergast et al., 2014) and the increased prevalence of neurodevelopmental disorders (NDD) in males (Werling and Geschwind, 2013). However, NDDs are not exclusively a male phenomenon and, with known sex differences in neuroanatomy, neurochemistry and behaviour in rodents (Hughes, 2007), the lack of female data presents an important caveat which may delay our understanding of NDDs and thus the development of effective treatments (Hill, 2015).

A long-term goal of our work is to explore the developmental trajectory of mIA from pre-birth to mature adulthood. Here we start by exploring effects very early in this process, i.e. prior to birth, gestational day (GD)21 and just prior to weaning, post-natal day (PD)21. Studying early development is not only important for ASD, which is typically diagnosed by the age of three, but also for investigating pre-morbid and prodromal states in other disorders such as schizophrenia (Knuesel et al., 2014; Lauritsen, 2013). Data from young offspring will be beneficial to further our understanding of impaired developmental processes and thereby provide additional information on potential therapeutic targets to normalise the trajectory (Knuesel et al., 2014). Indeed a very recent, most elegant study describes the evolution of pyramidal cell induced excitation of interneurons in mice, from as early as post-natal day 2, known to be integral to circuitry controlling cognitive function, a clinical unmet need in psychiatry (Wong et al. 2018). With such information emerging about “normal” brain development, we can explore how this might be disturbed in disease models such as the one we describe here. Furthermore, with global enhanced emphasis on early intervention in psychosis, development of an animal model to study the early stage of the illness and its

progression is of particular clinical importance. England and Australia have been developing services based on a stratified model of care for psychosis and early intervention since the mid- 1990s (e.g. see McGory et al. 2008) and early intervention in first episode psychosis is now standard practice in many European countries and the UK (NHS England, 2015). More recently, numerous states in the USA have also begun clinical as well as research pathways focused on early intervention (Dixon et al. 2018), albeit current pharmacological interventions are still not being developed within this staged approach (Marwaha et al. 2016).

Our work therefore aims to establish a rat model of mIA by conducting a comprehensive dose-response study in three strains of rat and examination of a poly I:C dosing schedule prior to investigating the morphological effects of poly I:C in both male and female offspring at two early developmental time points. We provide a preliminary analysis of neuroinflammation induced by mIA by looking for evidence of microglial activation in the offspring and IL-6 in plasma at PD21, an early juvenile stage of development, from dams and offspring,

2. Methods

2.1. Animals

See Figure 1 for detailed description of the 3 studies conducted. Adult non-pregnant rat experiments were performed using female Wistar, Lister Hooded and Sprague Dawley rats (Charles River Laboratories, UK) housed in groups of 4-5. Rats were housed in individually ventilated cages (IVCs) with two levels (GR1800 Double-Decker Cage, Tecniplast, UK) under a standard 12 hour light: dark cycle (lights on 7:00 am). The environment was maintained at 21 ± 2 ^{o}C, 55 ± 5 % humidity. Animals had *ad lib* access to standard rat chow (Special Diet Services, UK) and water. All procedures adhered to the Animals (Scientific

Procedures) Act 1986 and were approved by the University of Manchester AWERB (Animal Welfare and Ethical Review Body). A total of 76 non-pregnant Wistars (44 for the strain selection study and 32 for the repeated dosing study), 44 Lister Hooded and 44 Sprague-Dawley rats were used, i.e. 8-11 per group. Uneven group sizes occur when tail vein blood could not be collected from an animal for technical reasons, which was thus excluded from the IL-6 analysis, see Figure 2. In pregnancy, rats were housed in pairs or threes before being singly housed from GD19. Thirty two pregnant Wistar dams were used in total (16 poly I:C and 16 vehicle treated; 8 of each for each time point, GD21 and PD21). See table 1 for further details of dams, offspring and their experimental pathway. Our studies were conducted in two separate cohorts each with GD21 and PD21 time points, and data pooled. The cohort effect was taken into account in our statistical analysis (see below).

Experimental procedure and drug administration

See Figure 1 for experimental design.

Acute systemic inflammation was induced in female Wistar, Lister Hooded and Sprague Dawley rats (n=8-11 per group; 182-281 g; approximately 10-weeks old) intraperitoneally (i.p.) with saline or poly I:C at 5, 10 or 15 mg/kg; Sigma P9582 (base equivalent weight; Sigma, UK) and blood sampling was performed 3 h post-injection (Choi et al., 2016). We chose the i.p. route to avoid the need to anaesthetise pregnant animals in subsequent studies for intravenous poly I:C administration.

Repeated systemic inflammation was induced in female Wistar rats (n=8 per group; 195-281 g) by poly I:C (2.5, 5 or 10 mg/kg; i.p) or saline once daily over 5 consecutive days and blood sampling was performed 3 h post-injection on days 1, 3 and 5.

Maternal immune activation Female Wistar rats were mated when 3 months old. GD 1 was confirmed by the appearance of a vaginal plug. Poly I:C treatment at GD15 has been shown in rats to induce an inflammatory response and altered behaviour in offspring reminiscent of NDDs, in several previous studies (Mattei et al., 2014; Piontkewitz et al., 2012, 2011, 2009; Vernon et al., 2015; Zuckerman et al., 2003). Thus on GD15, pregnant Wistar rats (293.0-428.7 g) were injected with 10 mg/kg poly I:C (i.p.) or saline and blood sampled at 3 h post-injection via the tail vein (n=32 pregnant females in total, 16 for each treatment, 8 for each time point i.e. 8 poly I:C and 8 vehicle treated dams for GD21 and the same for PD21). Offspring development was assessed at GD21 and PD21. Tail tips from GD21 pups were analysed for the sex specific gene, SRY1, to determine sex. The gene encoding the sex determining region Y (Sry) protein was used to positively identify male fetuses. Primer sequences for rat Sry gene were taken from An et al. (1997). Lack of Sry gene amplification was taken to infer female sex. DNA was extracted from fetal tail tips using DNeasy kit (Qiagen) and genotype was determined by PCR using the following primers: F: 5' – CATCGAAGGGTTAAAGTGCCA – 3'; R: 3' – ATAGTGTGTAGGTTGTTGTCC – 5'. PCR was performed in a total volume 20 µL containing 2 µL 10x PCR buffer, 2 µL 2 µM dNTPs 12.1 µL PCR water, 1.2 µL 1.5 mM MgCl2, 0.8 µL 0.2 µM forward and reverse primers and 0.1 µL 5 U/µL Taq DNA polymerase (all from Invitrogen Life Sciences, Paisley, UK) with 1 µL gDNA. PCR was carried out in a DNA Thermal Cycler (Perkin Elmer, UK), starting with a single denaturing step for 5 min at 94 °C. Amplification was performed for 30 cycles, each cycle consisting of 3 steps for 30 s each: denaturation at 94 °C, annealing at 60 °C and extension at 72 °C. An additional extension step was carried out after 30 cycles at 72 °C for 5 min. Sry amplicon (104 bp) was visualised by agarose gel electrophoresis and a UV transilluminator (Gel Doc 2000 system, BioRad, SnapGene software, UK) was used to visualise PCR products. On PD1, pups were sex typed based on anogenital distance and then

culled to litters of 4 males and 4 females. Animals were randomised for experiments where appropriate and assessments were performed in a blinded manner.

2.3 Core body temperature

Core body temperature was recorded prior to poly I:C or vehicle injection, and at 3 h and 6 h post-injection, prior to blood sampling using a rectal thermister probe (TES, China). The temperature of the room where this measurement was conducted was 20.7-20.8^{o}C. Non-pregnant rats were habituated to measurement of rectal temperature for 7 days to minimise any effects of stress caused by this procedure. Pregnant rats were not habituated to this procedure in order to minimise any distress during pregnancy caused by excessive handling.

2.4. Tissue processing

At GD21, pregnant female rats were anaesthetised (4%) and maintained (1.5–2%) with isoflurane delivered in 70:30 N_2O/O_2. Pups and respective placentas were removed and weighed and pup brains were removed and weighed, this was conducted for pups from both cohorts. At PD21, pups were sacrificed, body weights recorded and brain weights recorded from some of these animals.

2.5 Enzyme-linked immunosorbent assay

IL-6 has been shown to be an important mediator of mIA (Choi et al., 2016; Smith et al., 2007) thus IL-6 concentration in blood plasma was determined using a rat-specific ELISA DuoSet (R&D Systems, Abingdon, UK. R&D systems do not provide intra and inter-assay coefficient of variability (CV) for this assay. Our intra-assay CV was below the recommended 10% (2.4-4%). We ran all samples from each experiment on one plate and once only as the amount of sample collected was small, so we could not calculate inter-assay

CV. Absorbance was measured using a plate reader (MRX, Dynatech, UK) at room temperature, and results were calculated from the standard curve using Prism 6 software (GraphPad, USA). We measured plasma IL-6 in dams from both cohorts, these data are shown in Figure 4A. IL-6 was also measured in plasma from dams and pups at PD21 from the offspring of cohort 1.

2.6 Tissue preparation and methods for immunohistochemistry

Rats were culled by overdose of anaesthetic (5% isoflurane in O2), brains were perfused with phosphate buffered saline (PBS) 0.1 M, rapidly extracted and half brains from offspring were immerse-fixed in 4% paraformaldehyde, stored in 30% sucrose overnight before being snap-frozen in isopentane and stored at -80°C until use. Coronal sections (30μm) through the hippocampus (~Bregma -3.3 to -4.8mm) were cut and stained for Iba1 with a goat polyclonal antibody for 24 hours (1:2000, Abcam UK). Sections were transferred to biotinylated horse anti-Goat IgG antibody (1:200 for 2 hours at room temperature) and revealed by ABC (2 hours at room temperature) and DAB (10 minutes at room temperature) kits (Vector Laboratories, PK-6100 and SK4100) as described previously (McKibben et al., 2010). Sections were mounted on slides, coded, and analysed blind to treatment. Stained sections were scanned at 4x magnification using an Olympus BX51 microscope interfaced to an Image ProPlus (version 6.3) analysis system (Media Cybernetics, USA) via a JVC 3-CCD video camera. Estimations of microglia density (cells/mm^2) were carried out in every 6th section, with a minimum of six sections per animal counted. The region of interest was highlighted and Iba-1 positive cells were counted and analysed live at a higher magnification (20x) using randomly generated points and a 2D counting frame. The total number of microglia and the numbers of ramified, activated and amoeboid were counted manually.

Morphological differentiation was based on a previous study (Cotel et al., 2015). There was no staining in sections where the primary antibody was omitted.

2.7 Statistical analysis

Data are presented as mean ± SEM. To illustrate multiple comparisons for non-pregnant rat data we used Bonferroni correction where appropriate for parametric data, and Kruskal-Wallis test followed by Dunn's multiple comparison where appropriate for non-parametric data. We used ANOVA, and univariate General Linear Models (GLM) to analyse dam traits in SPSS (version 22) with cohort as a fixed factor and other covariates where appropriate, e.g. maternal baseline body weight to account for prior differences between females not caused by treatment (e.g. Ashbrook et al, 2015, 2017). For comparisons between offspring of poly I:C and saline-treated dams and repeated measures in dams, we used General Linear Mixed Models (GLMM) using Restricted Maximum Likelihood (REML) estimation, repeated where appropriate, and with dam as a random variable. Here, the Satterthwaite estimation is used to estimate the degrees of freedom.

3. Results

3.1. Strain differences exist in the rat response to poly I:C

Our first experiment was conducted to optimise a model of mIA in rats by determining whether strain may have an effect on response to mIA and enable choice of the most suitable strain of rat. Vehicle (saline), or poly I:C at 5, 10 or 15 mg/kg was injected via the intraperitoneal route to adult female non-pregnant Wistar, Lister Hooded and Sprague Dawley rats (8-11 females per strain per dose). Elevations in core body temperature (Figure 2A-C) were observed to a different extent across rat strains (GLMM, $F_{3,96} = 16.15$, $p < 0.0001$). IL-6 is a key mediator of mIA (Smith et al., 2007) and at 10 mg/kg, poly I:C induced a significant elevation in plasma IL-6 in all three strains tested (GLMM, $F_{3, 80} = 8.338$, $p < 0.0001$; Figure 2D-F). Elevated IL-6 was most consistently observed with the lowest spread of data in Wistar rats (standard deviation 121.8 Wistar; 208.3 Lister Hooded; 307.0 Sprague Dawley, for a 10 mg/kg dose of poly I:C) and fewest non-responders (7 in Wistars over the 3 doses of poly I:C; 12 in Lister Hoodeds and 15 in Sprague Dawleys; Figure 2D-F), We consider an IL6 level of 50 pg/ml or less to be a non-responder. Therefore, the Wistar rat strain was used for all subsequent experiments.

3.2 Repeated poly I:C only elicits a robust immune response on day 1 of poly I:C treatment

To further optimise the poly I:C dosing regimen, the effect of 5 days of dosing was investigated using a lower dose range. Body temperature was elevated at 3 h and 6 h post poly I:C to a similar extent on each day of dosing, showing both a main effect of dose and an interaction between day and dose (repeated GLMM, $F_{3,26} = 30.510$, $p < 0.0001$ and $F_{12,91} = 2.281$, $p = 0.014$; (Figure 3A). However, plasma IL-6 levels were only elevated on the first day of poly I:C dosing (ANOVA, $F_{2,93} = 6.155$, $p = 0.003$; Figure 3B).

3.3 mIA on GD15 elicits a maternal immune response in pregnant Wistar rats, reduced dam body weight without affecting litter numbers or body temperature.

Following determination of best rat strain and protocol optimisation in non-pregnant animals, Wistar rats were mated in house and 10 mg/kg poly I:C injected i.p. in pregnant dams at GD15 (n = 16 poly I:C, 16 vehicle). As described above for non-pregnant female Wistar rats, 10 mg/kg poly I:C induced a significant elevation in plasma IL-6, 3h post-injection (GLM; $F_{1,28}$=18.10; p<0.001; Figure 4A). Unlike in non-pregnant rats, core body temperature was not significantly affected at either 3h, 6h or 24h (GLM; $F_{1,28}$=3.35, p=0.078; $F_{1,28}$=1.25, p=0.273 and $F_{1,17}$=3.13, p=0.095; Figure 4B) post-injection. In contrast, poly I:C treatment negatively affected maternal body weight 6h and 24h post-injection (GLM, $F_{1,27}$=9.94; p=0.004; and $F_{1,27}$=6.19, p=0.019, Figure 4C), but not at 3h post-injection (GLM, $F_{1,28}$=1.05, p=0.314). Poly I:C had no effect on litter size (GLM, $F_{1,29}$=0.33; p=0.57, Figure 4D).

3.4 mIA on GD15 reduces placenta weight at GD21, but not body or brain weight

Poly I:C induced changes in the offspring of poly I:C treated dams at GD21. Placenta weight was significantly reduced following poly I:C treatment (GLMM, $F_{1,14}$=21.20; *P*<0.001; Figure 5A) with a sex-specific effect that closely approached significance (male placentas were heavier; GLMM, $F_{1,201}$=3.83; p=0.052). However, neither pup body weight nor pup brain weight was significantly reduced in either male or female offspring (body weight: GLMM, $F_{1,14}$=0.82; p=0.381; Figure 5B, brain weight: GLMM, $F_{1,13}$=0.033; p=0.859, Figure 5C), resulting in reduced placenta to brain weight ratio in poly I:C-treated offspring (GLMM, $F_{1,11}$=11.10; p=0.006; Figure 5D).

3.5 mIA on GD15 results in reduced male offspring body weight at PD21 and increases brain to body weight ratio

On PD21 we observed a significant treatment effect on pup body weight (GLMM, $F_{1,111}$=7.43, p=0.007, Figure 6A) but not on pup brain weight (GLMM, $F_{1,3}$=1.535, p=0.304, Figure 6B). Not all pups were assessed for brain weight as some went on to imaging and other studies, see above. However, when brain weight data were available, the ratio of brain to body weight was significantly affected by treatment with pups born to poly I:C treated dams showing a higher ratio than vehicle control pups (GLMM, $F_{1,4}$=9.692, p=0.036; Figure 6C). Further, there was no significant effect of treatment on PD21 pup IL-6 levels (GLMM, $F_{1,8}$=0.86, p=0.776) nor on dam IL-6 levels (GLM, $F_{1,8}$=0.98, p=0.349); these levels were just within the level of ELISA detection, data not shown.

3.6 mIA on GD15 results in increased activated microglia in male hippocampi

We analysed seven microglia traits from the hippocampus of male offspring at PD21: the proportion of two stages (resting and intermediate) and that of amoeboid cells in the hippocampus as well as the total cell count. We found that both the proportion (shown as percentage) and number of amoeboid cells were significantly affected by treatment, Figure 7. The proportion of amoeboid cells was significantly higher in poly I:C treated hippocampi (GLM, $F_{1,15}$=7.959, p=0.013) as was the number of ameboid cells (GLM, $F_{1,15}$=8.953, p=0.009). Both traits remained significant after correction for multiple testing of seven traits following Benjamini & Hochberg (1995).

4. Discussion

This work presents the establishment of robust methods in our laboratory for inducing mIA in rats. We present an assessment of the effects of poly I:C-induced immune activation first in adult non-pregnant rats of three strains and then in pregnant female Wistar rats and their offspring of both sexes. An acute rise in plasma IL-6 was observed following poly I:C injection in pregnant and non-pregnant female rats which is similarly observed following influenza infection in humans (Kaiser et al., 2001) and has been proposed to drive the mIA-induced changes in offspring (Smith et al., 2007). Additionally, changes in poly I:C-treated offspring, such as placenta and pup growth restriction, reveals morphometric delays in both male and female offspring at early developmental time points. Detection of an increase in activated microglia supports the hypothesis of neuroinflammation induced by mIA, at least in male offspring in hippocampus, however more work is needed to confirm this, and to investigate the time course, other brain regions and of course, sex dependent effects.

First we demonstrate that, although poly I:C increased plasma IL-6 levels and body temperature in all three rat strains, these effects were most robust and least variable in Wistar rats. Further validation experiments to test repeated versus acute poly-I:C exposure revealed that repeated administration of poly I:C lowered rather than increased plasma IL-6 levels above acute levels, in spite of consistently raised body temperature. Indeed plasma IL-6 was almost undetectable after day 1. IL-6 was not elevated in the offspring at PD21 or the dams at this time-point. One of the reasons for using Poly I:C and not a live virus is the induction of a discrete and short-lived immune activation following acute administration, therefore this result was expected but important to confirm, it is unclear the significance of elevated body temperature in the absence of IL-6 elevation. Sub-populations of schizophrenia and depressed patients have elevated cytokine levels representing an important step towards patient stratification (see below and Pariante 2017 for review in depression and Upthergrove et al.,

2014 for schizophrenia). This may be precipitated by further insults/infection/stressors during development which can be modelled by a two-hit model, currently being attempted by several research groups, and a distinct advantage of this neurodevelopmental model (see Estes and McAllister, 2016 for review).

Strain dependent effects have long been observed in experimental research. Behavioural tests measuring learning, memory, anxiety and regional volumetric measurements have shown clear strain and sex dependent differences in rats (Keeley et al., 2015). Furthermore, strain dependent variations in brain cytokine induction and offspring gene expression have also been observed in response to stressful stimuli (Neeley et al., 2011; Porterfield et al., 2011). Our results show noticeable differences amongst strains regarding animals that "respond" versus "non-responders" with Wistar rats showing the most consistent elevation of plasma IL-6 in response to poly I:C treatment.

High versus low inflammatory responses have been observed in patients with schizophrenia and may suggest the need to stratify patients when considering treatment options (Fillman et al., 2013). In the depression field there is growing evidence to support the hypothesis that non-responders to standard medication have a pro-inflammatory profile (see (Pariante, 2017) for a recent update). Most likely this will also be the case for schizophrenia as suggested by Pariante. Indeed, Mondelli and colleagues have shown that, in first episode psychosis, non-responders to treatment have elevated serum IL-6 and interferon-γ (Mondelli et al. 2015). This essential area of research is expanding. Indeed, Carol Tamminga and colleagues have recently demonstrated three distinct biotypes of psychosis patients who will likely require differential therapeutic strategies (Clementz et al., 2016). Our pregnant rats show an inflammatory profile, not sustained to PD21 in pups or dams, however microglial activation

was observed at PD21 indicative of neuroinflammation. Identification of individual differences between animals and linking this to phenotype will be important in future studies.

Despite noticeable differences amongst strains regarding "responders" versus "non-responders", all three strains exhibited a bell shaped dose response to increasing concentrations of poly I:C. A number of studies have seen similar responses in which higher doses of inflammatory stimuli paradoxically result in an attenuated immune response (Scheller et al., 2011). Furthermore, following repeated administration of poly I:C to Wistar rats, we observed a significantly attenuated response to poly I:C as measured by IL-6 plasma levels. This phenomenon, known as immune tolerance, in which an immune-stimulating agent has diminished efficacy to induce a pro-inflammatory cytokine reaction compared to first exposure has previously been seen in response to LPS (Fan and Cook, 2004; Medvedev 2006).

It should also be considered that alternative methods of inducing mIA may be used, such as influenza virus or LPS (Shi et al., 2003; Urakubo et al., 2001), producing different outcomes. Smolders and colleagues demonstrate that LPS, but not poly I:C or IL-6, led to direct microglial activation in brain slices (Smolders et al., 2015). Whilst using a live virus such as influenza may represent a more translational model of mIA, poly I:C mimics many of the effects of the live virus but allows more precise control over the timing and the degree of the immune response (Reisinger et al., 2015). Additionally, alternative readouts of poly I:C-induced immune activation could be measured including type-1 interferons (Murray et al., 2015) and IL-17 (Choi et al., 2016) in addition to further cytokine panels (Hsiao and Patterson, 2011). However, the increase in IL-6 expression following poly I:C administration has been robust and consistent across multiple rodent and human studies (Patterson, 2009) and IL-6 acts upstream of IL-17a and downstream of IFNβ (Choi et al., 2016; Murray et al., 2015).

Following identification of optimal dose, strain and administration regimes of poly I:C, a significant reduction in offspring body weight was observed in male pups of poly I:C treated pregnant Wistar rats at PD21, but not GD21. Reduced placenta to brain weight was also observed at GD21, and increased brain to body weight at PD21, possibly consistent with the macrocephaly sometimes observed in early phases of ASD (Courchesne et al., 2011). However an important consideration with our data is the lower number of offspring included in brain weight analysis at PD21, due to offspring brains being used for other studies, eg imaging. Further investigation is required to determine the mechanisms by which mIA results in impaired growth. Most importantly, we observed a large and significant reduction in placental weight post-poly I:C. Previous work has highlighted a role for the placenta in mediating the effects of mIA (Choi et al., 2016; Hsiao and Patterson, 2011). Hsiao and Patterson demonstrate that an elevation in maternal IL-6 reduces placental levels of growth hormone and insulin-like growth factor; key hormones for embryonic developmental (Hsiao and Patterson, 2011). We have begun to analyse placental changes in some detail and are starting to reveal very interesting and sex specific effects induced by poly I:C, although reduced placenta weight was clearly observed in both sexes here. However, it is interesting to note that, despite a reduced placental weight, fetal body and brain weight was maintained in the poly I:C group, resulting in a reduced placental:fetal brain weight ratio, implying that placental function may have been adapted to preserve this. Multiple transport pathways may be affected, but examination of amino acid transport as a major determinant of fetal weight (protein) accrual is an attractive candidate for further study. Recent work by Weinberger and colleagues has identified risk factors genes for schizophrenia that are involved in early life consequences and placentation (Ursini et al. 2017; 2018). This suggests that understanding mechanisms of reduced placenta weight, and likely function, in the model could be of particular importance for schizophrenia research. Our results are also consistent with

epidemiological data demonstrating that low birth and placental weight increase the risk of developing schizophrenia (Wahlbeck et al., 2001), albeit we did not observe reduced body weight in our pups until PD21. Additional studies are needed to fully elucidate the effects of mIA on the maternal/foetal interface and how this in turn affects subsequent development of the offspring, work we are currently undertaking.

Post-mortem immunohistochemical analysis in the hippocampus on PD21 using anti-Iba1 antibody revealed that the total number of microglial cells remain unaffected between the two groups, however there was a significant increase in the density of amoeboid cells in male pups born to poly I:C treated mothers, accompanied by a non-significant decline in the intermediate cell phenotype. Due to the high numbers of receptors for inflammatory mediators, the hippocampus is a region that is particularly vulnerable to inflammatory insult (Seki et al., 2013). In a previous study, Manitz et al., 2013 investigated microglial cell numbers in pups at PD10, PD30 and PD100 in several regions, including the hippocampus. Maternal immune activation was induced at GD9. Results showed an increase in microglial cells at PD30 in all regions, as well as an increase in the frontal cortex at PD100, with no significant increase at PD10. Despite an initial lack of response at PD10, the significant increase at adolescence (PD30) indicates the dynamic nature of microglial activation. This increase demonstrates their ability to proliferate and migrate to the site of immune response, as well as alter morphological states when activated. At adulthood, the immunological activation remains only within frontal association cortex, which was suggested to be linked with the cognitive deficits and negative symptoms present in schizophrenia. Various other laboratories have replicated the rodent model of mIA with Poly I:C, reporting both increased, no change or even a decrease in numbers of activated microglia within different regions of the brain (See review by Smolders et al., 2018). There are variations in the rodent species used, the timing and intensity of mIA, the age of the offspring at culling and the analysis

method used (some reporting density and not morphological analysis of activation state) making overall conclusions difficult. Our initial finding in male offspring emphasizes the impact and importance of investigating early developmental time-points to identify and establish any potential disease specific biomarkers as well as detection of any important changes that may be masked later in adulthood. There are of course limitations to our study, we only analysed males, only one brain region, and offspring are taken from a small number of dams. However, our statistical analysis takes this into account and we believe this is a fair representation of microglial activation in male offspring at PD21 in spite of these experimental limitations.

5. Conclusion

This study provides a robust and systematic assessment of poly I:C-induced plasma IL-6, body temperature and weight alterations in pregnant rats and explores the consequences of this immune challenge on morphology and neurobiology in male and female offspring at two developmentally distinct time points. These findings, which we will confirm by functional studies, add to the growing body of evidence that this model is a useful tool to explore the effect of mIA. This model has clear advantages as we have outlined above. Of course, it is important to emphasise that these conditions in Wistar rats were optimal in our laboratory, this may not be the case for all other laboratories. Indeed, as discussed by a recent working group (at ACNP, 2018) certain methodological issues remain unresolved. These include variability between Poly I:C batches, and contamination of poly I:C with LPS, the importance of comparing both sexes, housing conditions (i.e. Individually Ventilated Caging-IVCs) compared with open caging (Mueller et al. 2018) and, very importantly, the establishment of a reliable way to identify affected offspring at an early developmental stage for phenotypic evaluation from adolescence through to adulthood and potentially old age, combined with the

study of resilience. Once these issues have been overcome, the model will be extremely valuable in investigating the developmental trajectory of appropriate and ethologically relevant behavioural deficits in conjunction with electrophysiological and neuroimaging readouts. This work will contribute to improved understanding of NDDs and ultimately to the development of enhanced therapeutic and potentially even preventative strategies for patients.

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Disclosure

Irene Knuesel and Eric Prinssen are full-time employees and shareholders (EP) of Roche. Other authors declare no competing financial interests.

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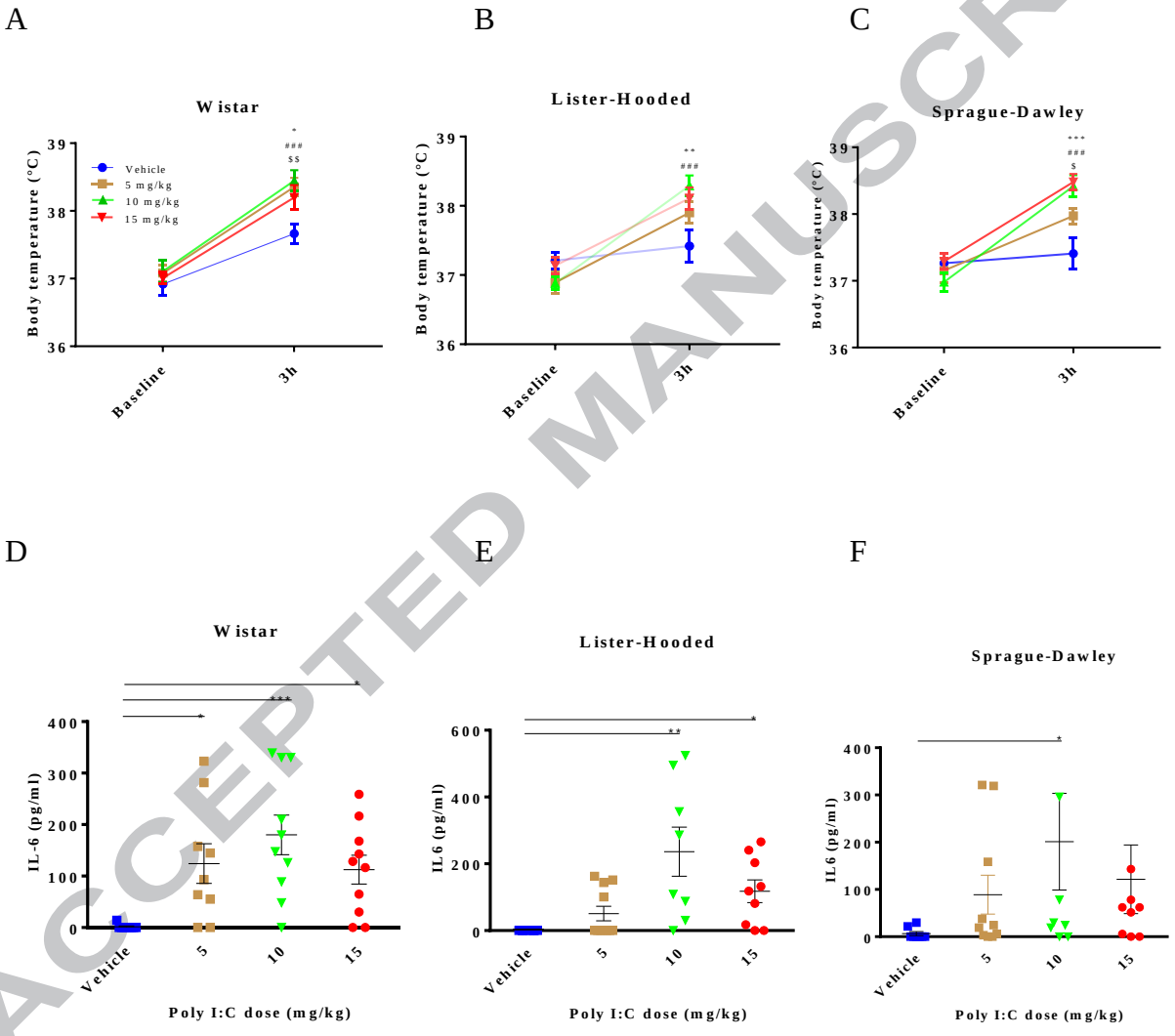
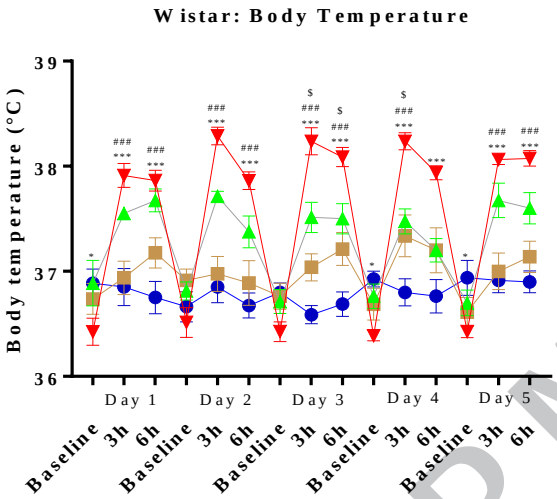


Figure 2

A



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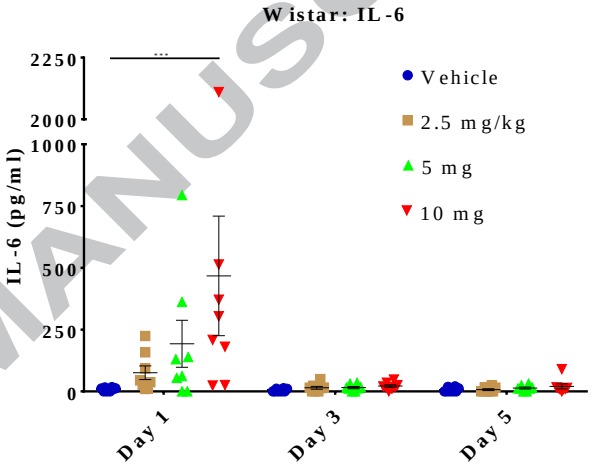
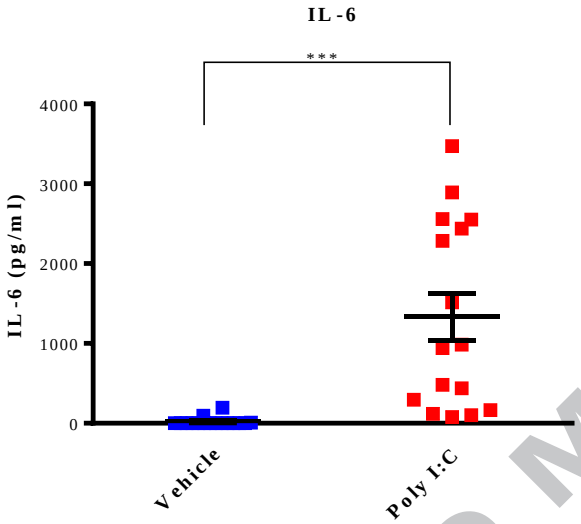


Figure 3

A



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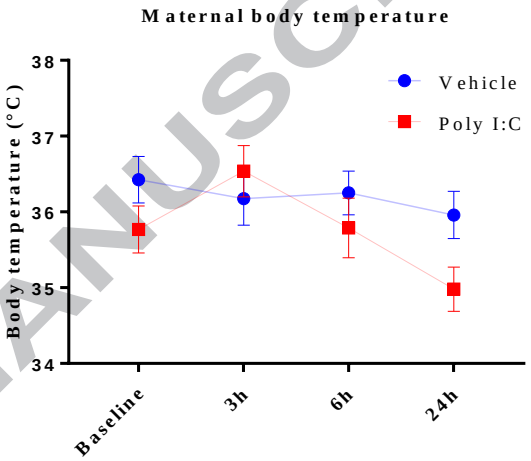

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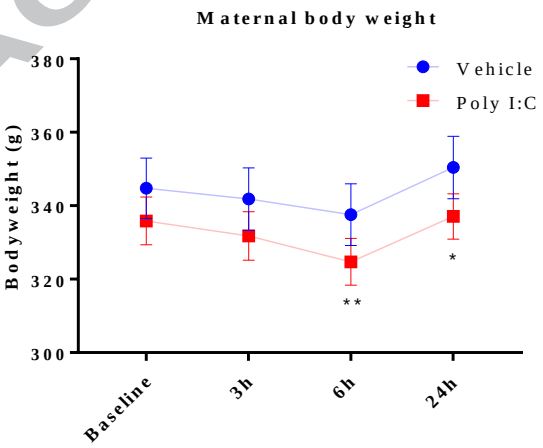

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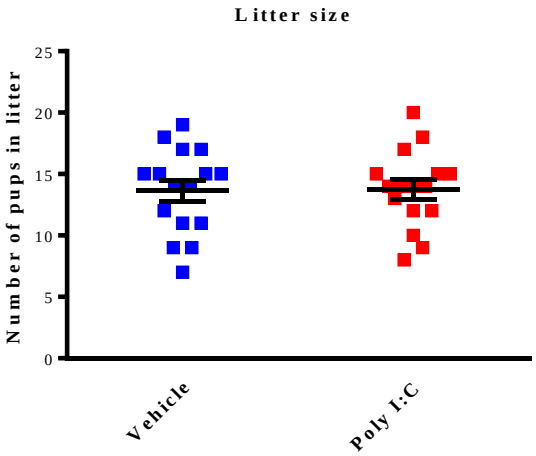


Figure 4

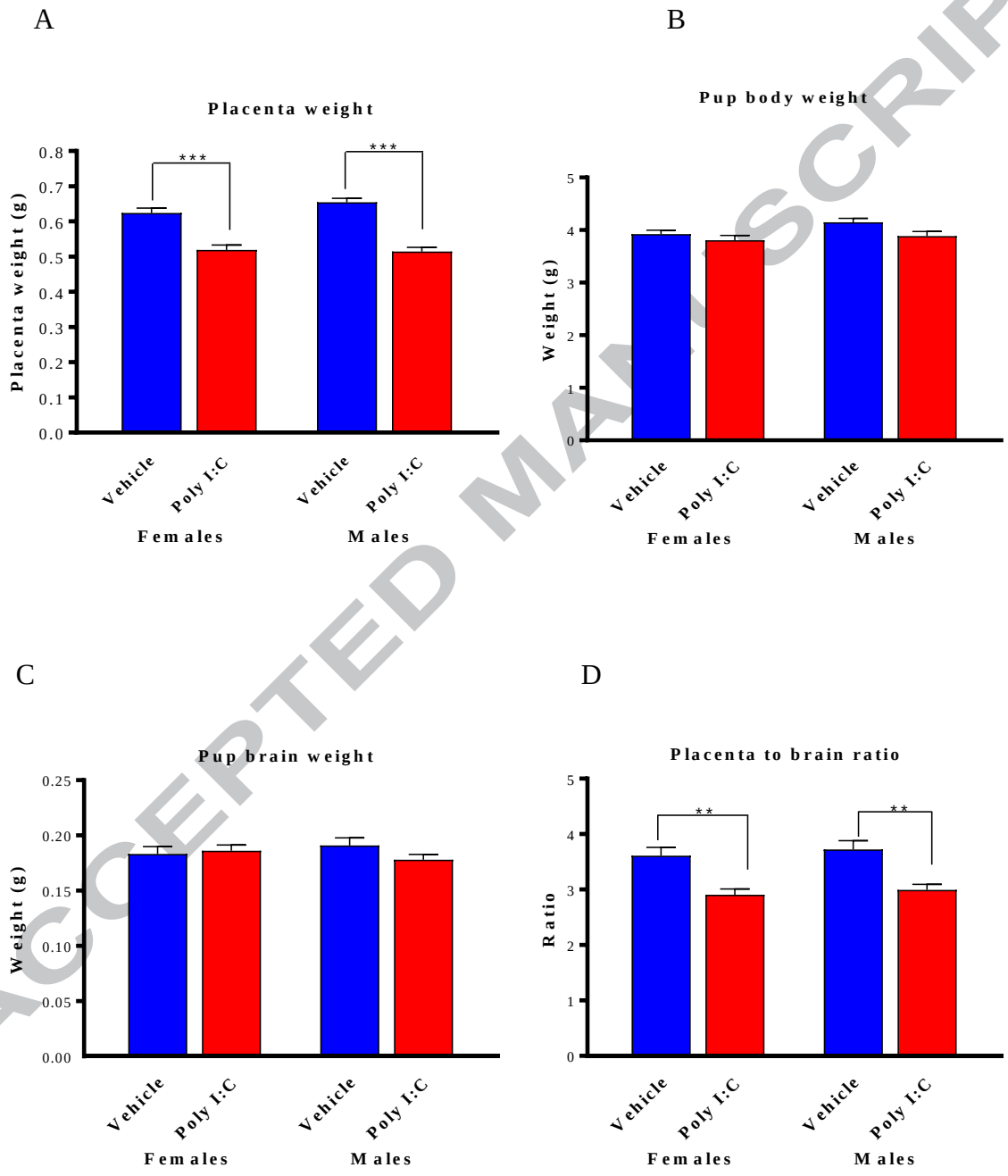
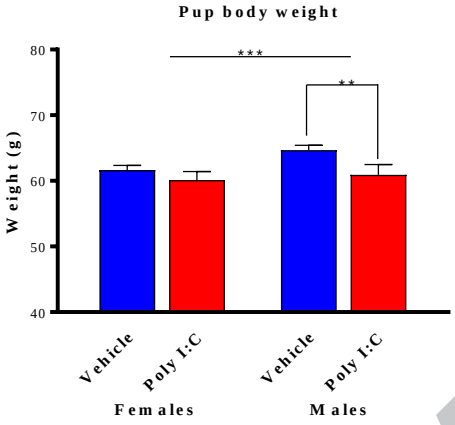
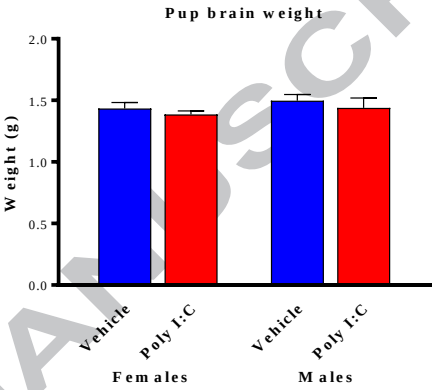


Figure 5

A



B



C

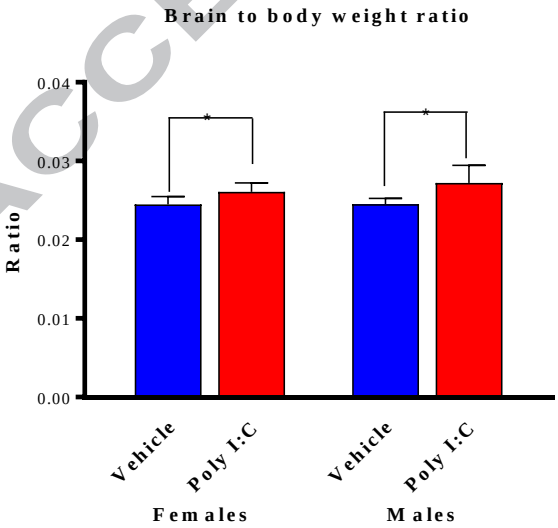


Figure 6

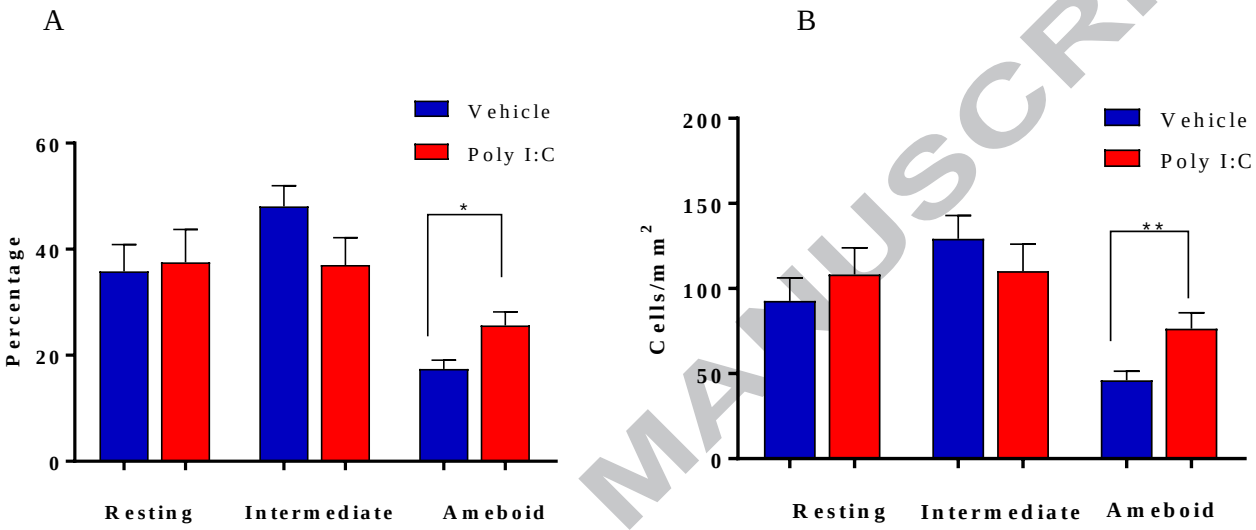


Figure 7A-B

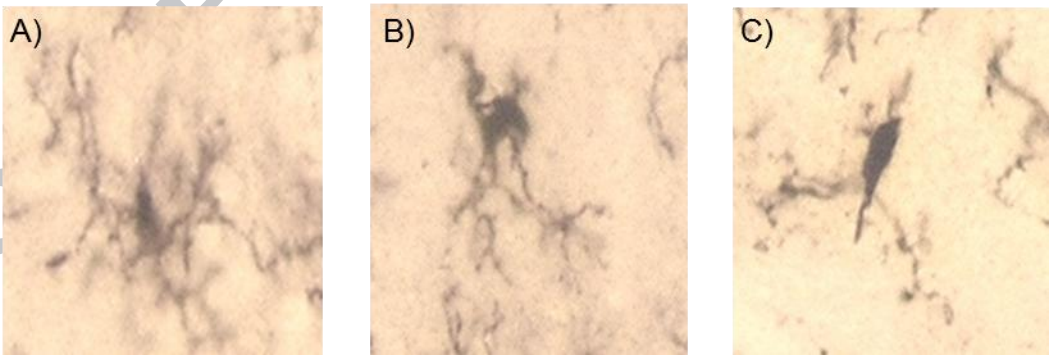


Figure 7C: Representative images of microglia staining in the hippocampus.

Figure legends

Figure 1. Experimental design. Experimental measures/procedures appear in the order taken/conducted.

Figure 2. Core body temperature (**A-C**) was recorded at baseline and 3 h post-injection (Wistar, **A;** Lister Hooded, **B;** Sprague Dawley, **C**) rats (N=8-11). IL-6 (**D-F**) levels in plasma were measured at 3 h post-injection (i.p) in female non-pregnant Wistar (**D**), Lister Hooded (**E**) and Sprague Dawley (**F**) rats (n=8-11). Data are presented as mean ± SEM. Statistical analysis was performed using repeated measures two-way ANOVA followed by Bonferroni's multiple-comparison test (**A-C**). $ P<0.05-$$ P<0.01 = 5 mg; ###P<0.001 = 10 mg; * P<0.05-***P<0.001 = 15 mg, all compared with vehicle. Kruskal-Wallis test followed by Dunn's multiple comparison test (**D-F)** *P<0.05, **P<0.01, ***P<0.001 compared with vehicle.

Figure 3. Core body temperature was measured prior to (baseline), and at 3 h and 6 h post-poly I:C injection (i.p) in female Wistar rats over 5 days (**A**). Plasma IL-6 (**B**) concentration was measured at 3 h post poly I:C injection (i.p) on days 1, 3 and 5 of dosing. N=8. Data are presented as mean ± SEM. Statistical analysis was performed using a two-way repeated measures ANOVA, followed by Bonferroni post hoc test was performed. In **A**, $, P<0.05 = 2.5 mg vs vehicle; ###, P<0.001 = 5 mg vs vehicle; *P<0.05-***P<0.001 = 10 mg all compared with vehicle. In **B** ***P<0.001.

Figure 4. In pregnant Wistar rats, plasma IL-6 was measured at 3 h post-injection (**A),** maternal body temperature recorded at baseline, 3 h, 6 h, and 24 h post-injection (**B**), maternal body weight recorded at baseline, 3 h, 6 h, and 24 h post-injection (**C**), number of pups per litter (**D**) were recorded in response to maternal vehicle or poly I:C (GD15; i.p; n=16 per treatment). Data are presented as mean ± SEM. Data were analysed by ANOVA, and univariate General Linear Models (GLM). *P<0.05, ** P<0.01, ***P<0.001 poly I:C compared with vehicle.

Figure 5. Effects of vehicle or poly I:C administered (i.p) at GD15 in female Wistar rats on female and male: pup placenta weight (**A**), body weight (**B**), brain weight (**C**) and placenta to brain ratio (**D**) at GD21. Pup and placenta numbers (vehicle female n=46, poly I:C female n=54; vehicle male n=59;

poly I:C male n=55) are derived from 8 litters/dams per treatment group with an average of 13.5 pups per litter. Data are presented as mean ± SEM. For comparisons between offspring, General Linear Mixed Models (GLMM) using Restricted Maximum Likelihood (REML) estimation was used. **P<0.01, ***P<0.001, comparing male to females, and poly I:C with vehicle.

Figure 6. Effects of vehicle or poly I:C administered (i.p) at GD15 in female Wistar rats on male and female offspring body weight (**A,** n=32 vehicle female, n-28 poly I:C female; n=32 vehicle males, n=24 poly I:C males), brain weight (**B** vehicle female n=12; poly I:C female n=8; n=11 vehicle males; n=8 poly I:C males) and brain weight as a proportion of body weight (**C**) at PD21. Pup numbers are derived from 8 litters/dams per treatment group culled down to 4 male and 4 female pups per litter. Data are presented as mean ± SEM. For comparisons between offspring, General Linear Mixed Models (GLMM) using Restricted Maximum Likelihood (REML) estimation was used. *P<0.05, **P<0.01, ***P<0.001, comparing male to females, and poly I:C with vehicle.

Figure 7: Microglia in male hippocampi by immunohistochemistry. Data are shown for 17 male offspring, 7 poly I:C and 10 vehicle treated from 2 and 3 dams respectively The total number of microglia and the numbers of ramified, activated and amoeboid were counted manually. Morphological differentiation was based on a previous study (Cotel et al., 2015). Data are presented as mean ± SEM. For comparisons between offspring, General Linear Mixed Models (GLMM) using Restricted Maximum Likelihood (REML) estimation was used. *p<0.05-p<0.01 significant increase in amoeboid cell number and percentage in poly I:C compared with vehicle pups, **A** percentage of cell type and **B** number /mm^3 of cell type.

C shows representative images of microglia staining in the hippocampus. A) Resting/Ramified, B) Intermediate and C) Amoeboid/Activated Iba-1 immunoreactive microglia. Image: 20x magnification.

Number of dams Fig. 4 Section 3.3	Treatment	GD21-214 offspring Fig. 5 Section 3.4	PD21-223 offspring born Fig. 6 Section 3.5	PD21 body weight Fig. 6 Section 3.5	PD21 brain weight Fig. 6 Section 3.5	PD21 microglia Fig. 7 Section 3.6
16	Poly I:C	109 (54f, 55m)	110 (52f, 58m)	52 (28f, 24m)	16 (8f, 8m)	7m
16	vehicle	105 (46f, 59m)	113 (43f, 70m)	64 (32f, 32m)	23 (12f, 11m)	10m

Table 1. Details of dams, offspring and their experimental pathway. For PD21 studies, at PD1 we culled to 8 pups per litter, 4 of each sex, resulting in 128 potential offspring for testing at PD21, 64 for each treatment group, 32 of each sex. For the PD21 body weight analysis, we had N=52 poly I:C treated and N=64 vehicle treated, but for brain weight analysis, only 16 poly I:C treated and 23 vehicle treated. This is because at PD21, the brains of pups were used for several different outcome measures, specifically PD21 offspring from cohort 2 were used for imaging and other studies. For microglia analysis, we used 17 male offspring, 7 poly I:C and 10 vehicle treated from 2 and 3 dams respectively. F=female, m=male. GD21 is gestational day 21 and PD21 is post-natal day 21.

Highlights

Poly I:C at 10mg/kg i.p. produced the most robust IL6 response in non-pregnant Wistar rats compared with Lister Hooded and Sprague-Dawley strains.

Poly I:C at 10mg/kg given i.p. to pregnant Wistar rats at gestational day 15 increased plasma IL-6 3h post injection and reduced body weight 6h and 24h post-injection.

Maternal immune activation resulted in placenta at GD21 and pup at PD21 growth restriction.

Maternal immune activation resulted in increased microglia activation in male pup hippocampi at PD21.