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Full-length Article

Maternal behaviours and adult offspring behavioural deficits are predicted by maternal TNFα concentration in a rat model of neurodevelopmental disorders

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ABSTRACT

Exposure to inflammatory stressors during fetal development is a major risk factor for neurodevelopmental disorders (NDDs) in adult offspring. Maternal immune activation (MIA), induced by infection, causes an acute increase in pro-inflammatory cytokines which can increase the risk for NDDs directly by inducing placental and fetal brain inflammation, or indirectly through affecting maternal care behaviours thereby affecting postnatal brain development. Which of these two potential mechanisms dominates in increasing offspring risk for NDDs remains unclear. Here, we show that acute systemic maternal inflammation induced by the viral mimetic polyinosinic:polycytidylic acid (poly I:C) on gestational day 15 of rat pregnancy affects offspring and maternal behaviour, offspring cognition, and expression of NDD-relevant genes in the offspring brain. Dams exposed to poly I:C elicited an acute increase in the pro-inflammatory cytokine tumour necrosis factor (TNF; referred to here as TNFα), which predicted disruption of key maternal care behaviours. Offspring of poly I:C-treated dams showed early behavioural and adult cognitive deficits correlated to the maternal TNFα response, but, importantly, not with altered maternal care. We also found interacting effects of sex and treatment on GABAergic gene expression and DNA methylation in these offspring in a brain region-specific manner, including increased parvalbumin expression in the female adolescent frontal cortex. We conclude that the MIA-induced elevation of TNFα in the maternal compartment affects fetal neurodevelopment leading to altered offspring behaviour and cognition. Our results suggest that a focus on prenatal pathways affecting fetal neurodevelopment would provide greater insights into the mechanisms underpinning the TNFα-mediated genesis of altered offspring behaviour and cognition following maternal inflammation.

Abbreviations: 5mC, 5-methylcytosine; ASST, attentional set-shifting task; CD, complex discrimination; DHipp, dorsal hippocampus; DI, discrimination index; EDS, extradimensional shift; EPM, elevated plus maze; FCtx, frontal cortex; GABA, gamma amino butyric acid; GD, gestational day; GLM, general linear model; GLMM, general linear mixed model; i.p., intraperitoneal; ID/ED, intradimensional/extradimensional; IDS, intradimensional shift; IL-6, interleukin-6; LPS, lipopolysaccharide; MIA, maternal immune activation; NDD, neurodevelopmental disorder; NOR, novel object recognition; PD, postnatal day; PFC, prefrontal cortex; poly I:C, polyinosinic:polycytidylic acid; PVALB, parvalbumin; R1-3, reversal 1–3; RAM, radial arm maze; SD, simple discrimination; SI, social interaction; TLR, toll-like receptor; TNFα, tumor necrosis factor; USV, ultrasonic vocalisation.

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1. Introduction

Maternal immune activation (MIA) is a well-established risk factor for neurodevelopmental disorders (NDDs) such as schizophrenia in the adult offspring (Mednick et al., 1988). Much of the research investigating MIA has focussed on developing models in rodents (Kowash et al., 2019; Murray et al., 2019) and non-human primates (Bauman et al., 2019) to elucidate causal mechanisms and determine potential therapeutic biomarkers (Conway and Brown, 2019). Such models involve administration of an immunogen, such as the viral mimetic polyinosinic: polycytidylic acid (poly I:C) or bacterial lipopolysaccharide (LPS; also referred to as endotoxin), to pregnant animals to mimic activation of the innate immune system. Poly I:C or LPS administration causes activation of the Toll-like receptors (TLR) 3 or TLR4, respectively, resulting in downstream activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) with the associated production of pro-inflammatory cytokines (Alexopoulou et al., 2001; Park and Lee, 2013). MIA in rodents has repeatedly been shown to induce developmental and cognitive changes in the adult offspring, including sex- and age-specific deficits in a novel object recognition cognitive task (Dabbah-Assadi et al., 2019), social discrimination deficits (Núñez Estevez et al., 2020), and impairments in cognitive flexibility related to dysfunctional prefrontal gamma aminobutyric acid (GABA)-ergic signalling (Canetta et al., 2016). The gestational timing of MIA induction has a significant impact on neurodevelopmental and behavioural outcomes. For example, poly I:C administration on GD15 has a strong association with developmental and cognitive deficits related to schizophrenia in the offspring (Murray et al., 2019; Knuesel et al., 2014) as well as cortical neuronal migration in the fetal rat brain which occurs during mid-late gestation in humans (Sarkar et al., 2019). Furthermore, such behavioural changes have often been associated with molecular markers of cognitive dysfunction, such as remodelling of the DNA methylation patterns in the promoter region of the GABAergic signalling genes *glutamic acid decarboxylase-1* (*Gad1*) and *Gad2* (Labouesse et al., 2015) and decreased release probability in parvalbumin (PVALB)-expressing interneurons in the offspring prefrontal cortex (PFC) (Canetta et al., 2016). The evidence that multiple types of pathogens, such as viral (Mednick et al., 1988; Buka et al., 2008; Kneeland and Fatemi, 2015), bacterial (Sorensen et al., 2019), or parasitic (Brown et al., 2005) infections induce MIA (Silasi et al., 2015) resulting in molecular and cognitive deficits in the adult offspring, implicates downstream inflammatory responses as a possible causal link to the developmental programming of NDDs in the offspring. However, the mechanism by which acute maternal induction of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF; referred to here as TNFα) results in offspring developmental and cognitive deficits is not fully understood. In principle, MIA may affect offspring brain development directly *in utero*, indirectly through affecting maternal behaviour and thus postnatal brain development, or a combination of both. Our aim was to establish evidence for both pre- and postnatal effects of MIA.

1.1. Prenatal inflammatory effects on fetal brain development

Exposure to poly I:C *in utero* has demonstrated gene expression changes and cognitive deficits in adult mice (Smith et al., 2007). IL-6 and TNFα play critical roles in the developing mammalian nervous system, such as during the establishment and maintenance of synaptic connections (Borsini et al., 2015), PVALB + interneuron survival (Dugan et al., 2009); and cognitive function in children (Ghassabian et al., 2018). The extent to which maternally-derived pro-inflammatory cytokines access the fetal compartment is unclear, but there is accumulating evidence that MIA causes persistent activation of microglia in the offspring brain (Murray et al., 2019; Hadar et al., 2017; Mattei et al., 2017) which may then increase cytokine production (Garay et al., 2013; Barichello et al., 2020). Hence, there is accumulating evidence that maternal or placental pro-inflammatory cytokines may contribute to fetal neuroinflammation in the context of NDDs.

1.2. Postnatal maternal effects on offspring brain development

The postnatal maternal environment may also impact offspring cognition in MIA models. Maternal care involves investment of resources (nutrients, time) to offspring (Woodroffe and Vincent, 1994; Hager and Johnstone, 2003) and is critical for normal neurodevelopment in humans (Curly 0000) and rodents (Peña and Champagne, 2013). In rodents, maternal care includes nest-building, nursing and suckling to provide water, nutrients, and immune factors (e.g. immunoglobulins), and licking/grooming. Aberrations in maternal care during critical periods of development have been associated with cognitive impairments. For example, Champagne et al. (Champagne et al., 2008) demonstrated that reductions in maternal licking/grooming impair basal synaptic plasticity, fear-conditioning-associated memory, dendritic morphology, and hippocampal glucocorticoid responsiveness in adulthood (Champagne et al., 2008). Licking/grooming also affects midbrain dopaminergic development, affecting social interaction and reward-directed behaviours (Peña et al., 2014). Clinically, children with higher exposure to adverse childhood experiences have an increased risk of developing schizophrenia and associated white matter defects (Hughes et al., 2017; Poletti et al., 2015).

Recently, MIA studies have investigated changes in maternal care in rodent dams treated with poly I:C. Ronovsky et al. investigated the effect of poly I:C treatment in mice on gestational day (GD) 12.5 on maternal behaviours and transgenerational inheritance of offspring traits (Ronovsky et al., 2017). Poly I:C exposure modulated maternal care in a behaviour-specific manner with a reduction in licking/grooming and an increase in nest-building. This pattern of maternal care was replicated in a second mouse strain and extended to show increases in F_1 hippocampal microRNA expression (Berger et al., 2018). Zhao et al. (Zhao et al., 2021) demonstrate that poly I:C treatment on GD12 in mice impairs maternal nest-building quality at 15 days postnatally in standard housed litters, but that this is ameliorated with environmental enrichment (Zhao et al., 2021). Similarly, Zambon et al. (Zambon et al., 2022) have shown that exposure to poly I:C on GD12.5 in mice promotes exploratory behaviour in dams but reduces pup retrieval and does not affect pup USVs (Zambon et al., 2022). However, these studies have not investigated the pre- and postnatal effects of MIA on maternal care alongside offspring development, cognition, and molecular effects in offspring brain as a model for NDDs.

Here, we present the first study, to our knowledge, to examine the joint effects of the pre- and postnatal maternal environments on offspring cognitive development in both sexes in a rat model of MIA. Based on the accumulating evidence that both fetal responses to poly I:C-induced MIA and maternal care behaviours have a critical impact on GABAergic neurodevelopment, we examined candidate gene expression in female and male offspring brains, as well as global DNA methylation as a potential mechanism for developmental programming of cognitive deficits. Understanding the link between adverse maternal environments and impaired offspring neurodevelopment and cognition will provide important mechanistic insight to enable target identification for drug discovery relating to prevention and rescuing of such deficits in clinical populations.

2. Results

2.1. Maternal response to poly I:C

To validate the use of poly I:C as a viral mimetic, the concentrations of pro-inflammatory cytokines in maternal plasma were measured before treatment (to assess baseline inflammation) and at 3 h post-treatment. All dams had low baseline plasma concentration of IL-6 (general linear model; GLM, $F_{1,14} = 1.59$, $p = 0.228$; Fig. S1A) and TNFα (GLM, $F_{1,13} = 0.45$, $p = 0.512$; Fig. S1B; p-values denoting

comparison between treatment groups). At 3 h post-treatment, poly I:C did not significantly increase maternal plasma IL-6 compared to vehicle (GLM, $F_{1,15} = 4.12$, $p = 0.060$; Fig. 1A; seven of nine vehicle samples had an interpolated IL-6 concentration of 0 pg/mL; four of eight poly I:C samples were higher than the vehicle population) but did significantly increase maternal plasma TNFα concentration (GLM, $F_{1,15} = 17.92$, $p = 0.001$; Fig. 1B; with seven of eight poly I:C samples being higher than the vehicle population) compared to vehicle. Interestingly, both the baseline (pre-pregnancy) and stimulated (3 h post poly I:C treatment) maternal plasma TNFα concentration were higher than that of IL-6. Based on this validation of MIA induction, and the important roles of TNFα in fetal brain development (McCoy and Tansey, 2008), this trait was used as a covariate in subsequent data analyses. Following administration of poly I:C on GD15, there was a significant main effect of treatment (general linear mixed model; GLMM, $F_{1,56} = 4.44p = 0.040$) and the treatment group*gestational day interaction (GLMM, $F_{1,106} = 1498.01p < 0.001$) that predicted a reduction in maternal weight gain in poly I:C-treated dams (Fig. 1C). Postnatal maternal weight gain was also significantly predicted by the treatment group*postnatal day interaction (GLMM, $F_{1,245} = 21.31$, $p < 0.001$; Fig. S1C).

2.2. Litter characteristics and offspring weight

We then recorded offspring litter characteristics including litter size and offspring weight to understand whether prenatal poly I:C treatment affected survival and development. Prenatal poly I:C treatment did not affect litter size (GLM, $F_{1,13} = 0.08$, $p = 0.789$; Fig. 1D), offspring sex distribution (GLM, $F_{1,13} = 1.88$, $p = 0.193$; Fig. 1E), or offspring bodyweight (GLMM, $F_{1,60} = 0.03$, $p = 0.868$; Fig. S1D) or organ weight (brain weight GLMM, $F_{1,49} = 0.54$, $p = 0.467$, liver weight GLMM $F_{1,40} = 1.09$, $p = 0.303$; Fig. S1E-F) on PD1, as compared to vehicle control. Female offspring exposed to poly I:C had reduced weight gain postnatally (GLMM, $F_{1,330} = 13.96$, $p < 0.001$; Fig. 1F), which was not observed in males (GLMM, $F_{1,283} = 1.04$, $p = 0.308$; Fig. 1H). As expected, on PD1 male offspring were significantly heavier with respect to bodyweight (GLMM, $F_{1,204} = 49.62$, $p < 0.001$; Fig. S1D) and brain weight (GLMM, $F_{1,43} = 5.98$, $p = 0.019$; Fig. S1E). Male liver weight was lower in the vehicle group and higher in the poly I:C group compared to females (GLMM, $F_{1,45} = 4.48$, $p = 0.040$; Fig. S1F).

Offspring bodyweight on the early postnatal behavioural testing days (PD6, 10 and 14) was further analysed to establish differences in bodyweight, which may affect behavioural traits (e.g. larger pups soliciting a higher proportion of maternal resources). There was a significant main effect of postnatal day for both females (GLMM, $F_{1,165} = 8000.84$, $p < 0.001$) and males (GLMM, $F_{1,179} = 5010.22$, $p < 0.001$). However, in line with the previous data, there was a sex-specific reduction in female poly I:C offspring bodyweight gain, evidenced by the significant treatment*postnatal day interaction (GLMM, $F_{1,239} = 4.14$, $p = 0.043$; Fig. 1G), which, again, was not seen in males (GLMM, $F_{1,221} = 1.88$, $p = 0.172$; Fig. 1I).

2.3. Ultrasonic vocalisations (USVs)

Offspring USVs were recorded on three postnatal days, corresponding to the peak of maternal care behaviours in rodents (Hager and Johnstone, 2003; Peña and Champagne, 2013; Yin et al., 2016), to assess whether prenatal MIA affects offspring solicitation of maternal resources.

In female offspring, there was no significant effect of treatment group on the number of syllables emitted on PD6 (repeated measures general linear model; RM-GLM, $F_{1,80} = 0.01$, $p = 0.933$) or PD10 (RM-GLM, $F_{1,80} = 0.52$, $p = 0.474$). However, on PD14 female offspring exposed to

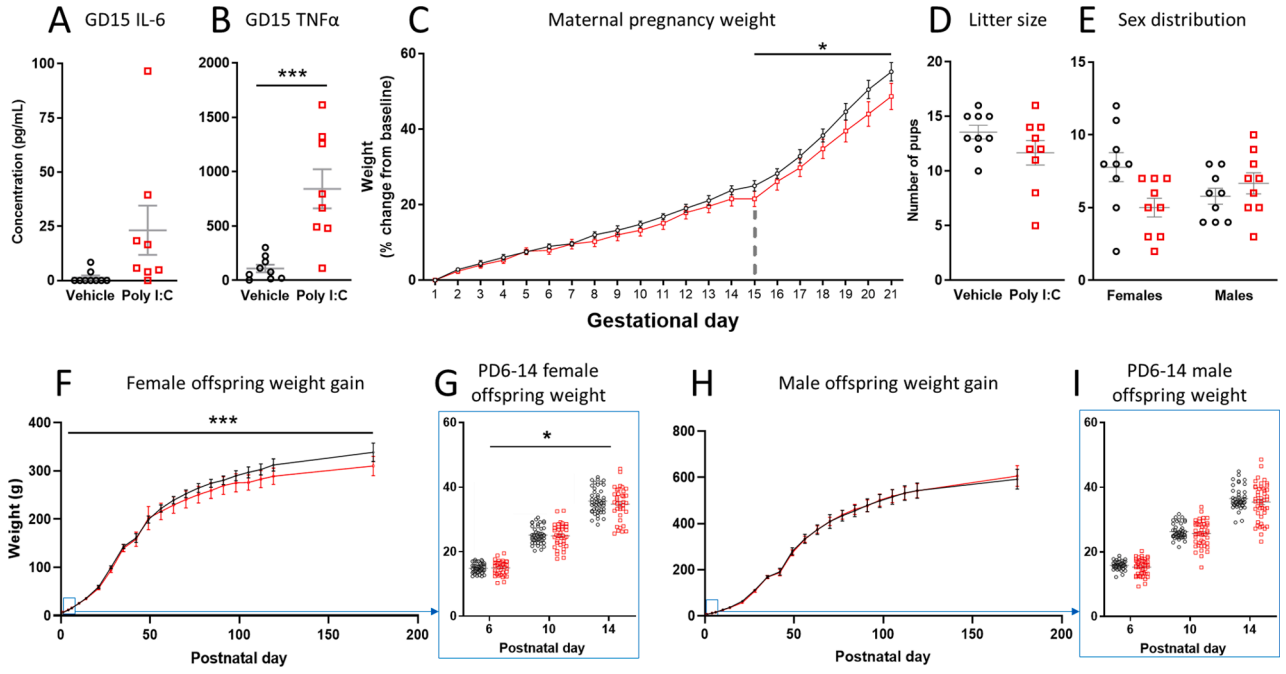

Fig. 1. Maternal, litter, and offspring developmental response to poly I:C. Maternal plasma **A)** IL-6 and **B)** TNFα concentration at 3 h post-treatment on GD15. **C)** Maternal weight gain during gestation from GD1-21 (vertical dashed grey line denotes treatment on GD15). **D)** Litter size and **E)** number of females and males on PD1. Bodyweight was recorded on PD1, 4, 6, 10, 14 and every week after until adulthood with data showing **F)** overall female weight gain (female vehicle n = 12–69; female poly I:C n = 8–44), **G)** female weight gain on PD6, 10 and 14 (female vehicle n = 46; female poly I:C n = 37; each data point represents an individual pup), **H)** overall male weight gain (male vehicle n = 7–50; male poly I:C n = 6–58) and **I)** male weight gain on PD6, 10 and 14 (male vehicle n = 44; male poly I:C n = 46; each data point represents an individual pup). Vehicle shown in black circles/lines; poly I:C shown in red squares/lines. For A, B, D, and E, each data point represents a dam/litter; for C, each data point represents the dam group mean; for F and H, each data point represents the group mean for individual offspring; for G and I, each data point represents an individual offspring. Data shown as mean ± SEM, as depicted in grey. Key: *p < 0.05; ***p < 0.001. Offspring sample size is shown as a range because of different postnatal tissue harvesting timepoints. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

poly I:C emitted significantly fewer syllables compared to vehicle (RM-GLM, $F_{1,78} = 4.43$, $p = 0.039$; Fig. 2A). There was no significant effect of treatment group on male pup syllables on PD6 (RM-GLM, $F_{1,87} = 0.80$, $p = 0.375$), PD10 (RM-GLM, $F_{1,87} = 0.19$, $p = 0.663$), or PD14 (RM-GLM, $F_{1,87} = 0.72$, $p = 0.398$; Fig. 2B). There were no significant main effects of treatment or sex on USV syllable power (Fig. S1G-H) or mean syllable frequency (Fig. S1I-J; statistics not shown).

Overall, syllable duration (Fig. 2C-D) was significantly affected by pup weight (GLMM, $F_{1,264} = 21.87$, $p < 0.001$), postnatal day (GLMM, $F_{1,318} = 23.46$, $p < 0.001$), sex (GLMM, $F_{1,169} = 4.65$, $p = 0.032$), and the sex*treatment group interaction (GLMM, $F_{2,168} = 5.33$, $p = 0.006$). In females, the treatment group*postnatal day predicted a significant increase in syllable duration (Fig. 2C) in poly I:C animals (GLMM, $F_{1,243} = 14.62$, $p < 0.001$; Fig. 2C) when corrected for the significant main effect of weight (GLMM, $F_{1,243} = 18.66$, $p < 0.001$) and postnatal day (GLMM, $F_{1,243} = 17.53$, $p < 0.001$). TNFα concentration also had a significant positive main effect on female syllable duration (GLMM, $F_{1,234} = 7.19$, $p < 0.001$; Fig. 2C). Pup weight (GLMM, $F_{1,145} = 5.95$, $p = 0.016$) and postnatal day (GLMM, $F_{1,178} = 7.89$, $p = 0.006$) were the only factors with significant main effects on male syllable duration (Fig. 2D). Treatment group (GLMM, $F_{1,88} = 0.01$, $p = 0.905$) and TNFα (GLMM, $F_{1,83} = 0.37$, $p = 0.546$) did not have significant main effects on male syllable duration (Fig. 2D).

2.4. Maternal-offspring interactions

Immediately following USV measurements, offspring were returned to their home cage where maternal-offspring interactions were recorded for 30 min using a behavioural ethogram (Figure S3) to assess the effects of poly I:C on maternal resource provisioning. Treatment group had no significant effect on suckling (GLMM, $F_{1,16} = 0.49$, $p = 0.496$; Fig. 2E) or nursing (GLMM, $F_{1,16} < 0.01$, $p = 0.983$; Fig. 2G). Interestingly, the maternal TNFα response significantly predicted a reduction in suckling behaviour (GLMM, $F_{1,14} = 5.03$, $p = 0.042$; Fig. 2F). Neither treatment

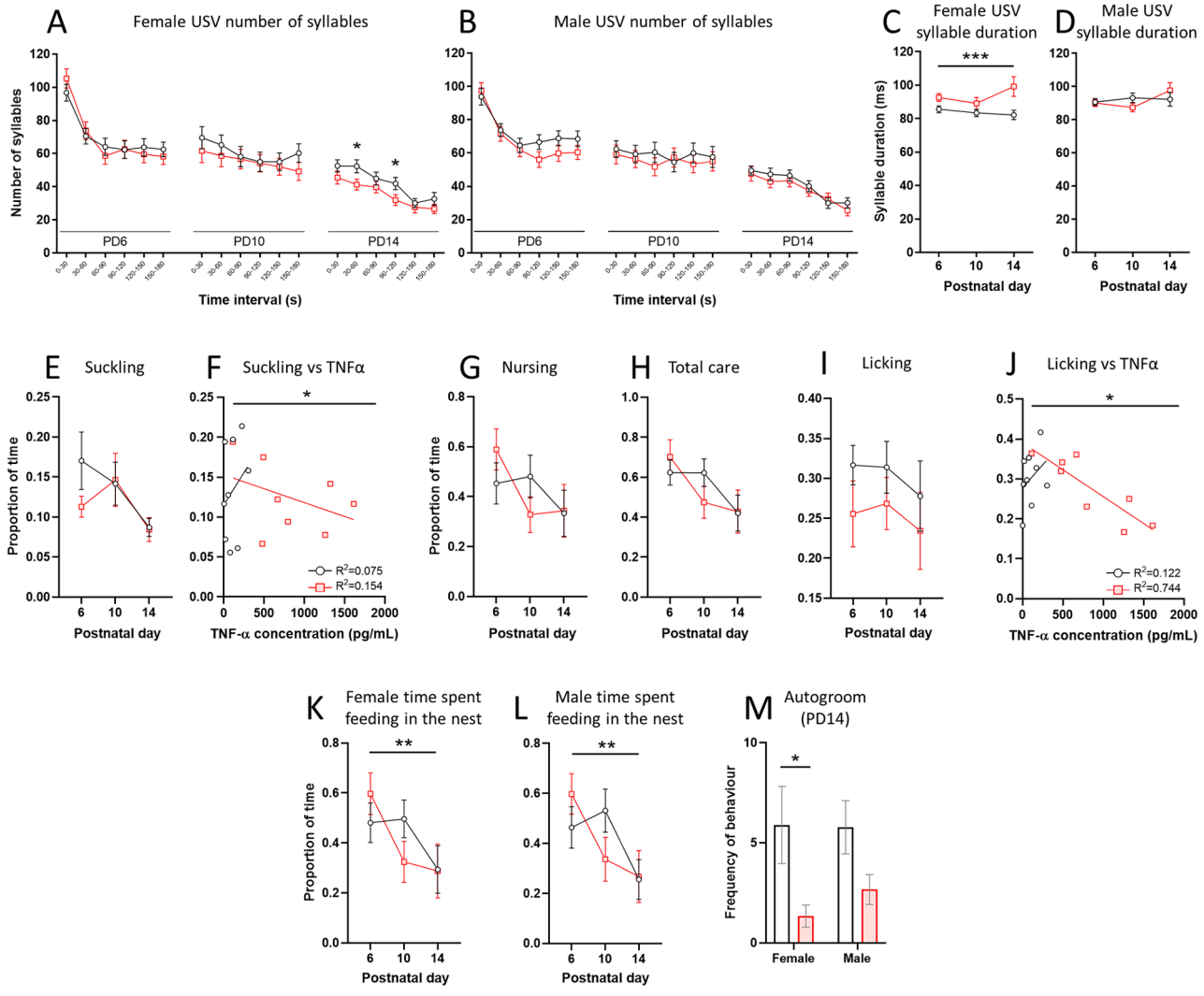

Fig. 2. Effect of poly I:C treatment in pregnant dams on offspring USVs and maternal offspring interactions. Pup maternal separation-induced USVs and maternal-offspring interactions were recorded across the three postnatal testing days (PD6, 10, and 14). Number of USV syllables emitted by **A)** female and **B)** male pups, mean syllable duration for **C)** female and **D)** male pups (female vehicle n = 46; female poly I:C n = 35–37; male vehicle n = 44; male poly I:C n = 46 for all; for all USV data, data points show group mean ± SEM). Proportion of time dams spent engaged in **E)** suckling and **F)** the relationship between maternal suckling and maternal TNFα concentration (linear regression shown for each group). Proportion of time dams spent engaging in **G)** nursing, **H)** the sum of suckling and nursing ('total care'), **I)** licking, and **J)** the relationship between maternal licking and maternal TNFα concentration (linear regression shown for each group). Proportion of time spent feeding in the nest for **K)** female and **L)** male pups, and **M)** the observed frequency of pup autogroom behaviour on PD14 (only data from PD14 was used due to the low number of litters with pups that engaged in these behaviours at earlier timepoints; N = 9 per treatment group for all maternal-offspring interactions; for all other maternal-offspring interaction data in G-L, data points represent an individual dam/litter). For all graphs, vehicle shown in black circles/bars; poly I:C shown in red squares/bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

group (GLMM, $F_{1,16} = 0.04$, $p = 0.836$) nor maternal TNFα (GLMM, $F_{1,15} = 0.03$, $p = 0.873$) had a significant main effect on the total time dams spent engaging in maternal behaviour (defined as the sum of the time spent nursing and suckling previously reported (Ashbrook et al., 2015); Fig. 2H). Whilst treatment group had no significant main effect on maternal licking (GLMM, $F_{1,16} = 1.45$, $p = 0.247$; Fig. 2I), the maternal TNFα*postnatal day interaction significantly predicted maternal licking (GLMM, $F_{1,26} = 7.28$, $p = 0.012$; Fig. 2J). There were no significant effects of treatment group or maternal TNFα on maternal care behaviours including sniffing, pup retrieval, resting, drinking, feeding, digging, or autogrooming (Fig. S1K-Q).

In line with the reduction in maternal nursing and licking behaviours, there was a significant reduction in time offspring spent feeding in the nest, most notably on PD10 (GLMM, $F_{3,34} = 5.39$, $p = 0.004$), which was independent of sex (GLMM, $F_{1,89} = 0.02$, $p = 0.903$; Fig. 2K-L). Interestingly, there was a sex-specific effect of poly I:C exposure whereby females engaged in autogroom behaviour less frequently on PD14 (GLMM, $F_{1,16} = 5.17$, $p = 0.037$; Fig. 2M) but did not reach statistical significance in males (GLMM, $F_{1,16} = 4.16$, $p = 0.058$; Fig. 2M).

When analysing whether pup USV traits affected maternal care behaviours, we found that the proportion of time dams spent engaged in suckling was predicted by both the number of syllables emitted (GLMM, $F_{1,169} = 8.12$, $p = 0.005$) and total vocalisation time (GLMM, $F_{1,169} = 11.31$, $p < 0.001$) on PD6, by the number of syllables only on PD10 (GLMM $F_{1,170} = 4.65$, $p = 0.032$), and subsequently by the total time spent vocalising on PD14 (GLMM, $F_{1,171} = 22.32$, $p < 0.001$). In addition, the proportion of time dams spent engaging in licking behaviour (which was previously shown to be reduced by the maternal TNFα response) as well as the total time spent engaging in maternal care behaviours on PD10 were significantly predicted by the time pups spent vocalising (GLMM licking, $F_{1,170} = 7.02$, $p = 0.009$; GLMM total care, $F_{1,169} = 5.51$, $p = 0.020$).

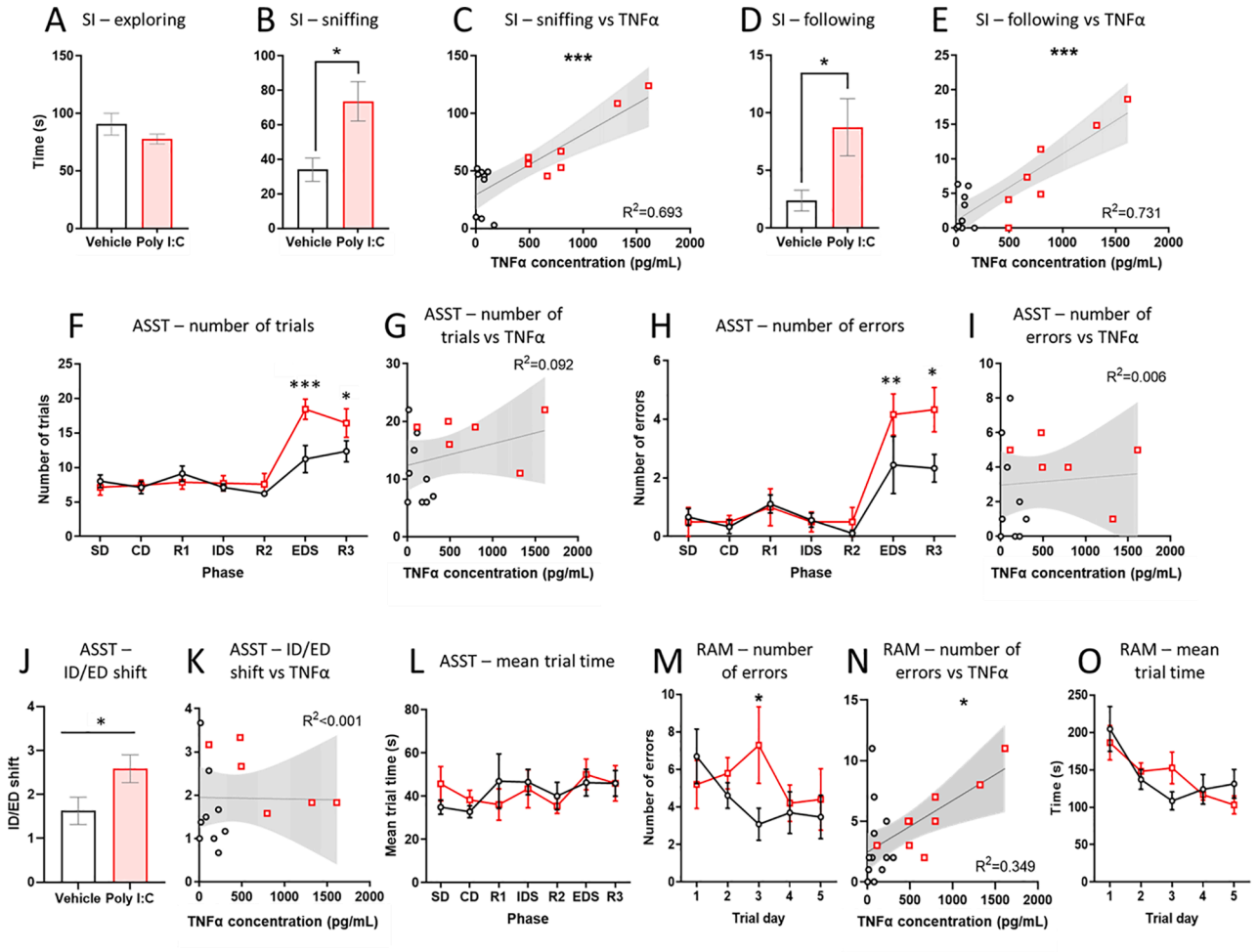

Fig. 3. Effect of poly I:C treatment in pregnant dams on offspring cognitive performance in adult female offspring. Time adult female offspring spent engaging in **A)** exploration of an inanimate object, **B)** sniffing of the conspecific animal, **C)** the relationship between sniffing and maternal TNFα concentration, and **D)** time spent following the conspecific animal, and **E)** the relationship between following and maternal TNFα concentration in the social interaction (SI) task (for A-E vehicle n = 9; poly I:C n = 7). **F)** Number of trials needed to complete each phase of the attentional set-shifting task (ASST) and **G)** the relationship between number of trials in the EDS phase and maternal TNFα concentration. **H)** Number of errors needed to complete each phase of the ASST and **I)** the relationship between number of errors made in the EDS phase and the maternal TNFα concentration. **J)** The ratio of number of trials needed to complete the IDS and EDS phases (ID/ED shift) and **K)** the relationship between the ID/ED shift and the maternal TNFα concentration. **L)** Mean time taken to complete each test phase (for F-L vehicle n = 9; poly I:C n = 6–7). **M)** Number of working memory errors across the five testing days of the radial arm maze (RAM) in adult female offspring and **N)** the relationship between working memory errors on day three with the maternal TNFα concentration. **O)** Mean trial time in the RAM (for Q-S vehicle n = 13; poly I:C n = 10). Vehicle shown in black circles/bars/lines; poly I:C shown in red squares/bars/lines; all error bars denote mean ± SEM. For A, B, D, and J, data bars represent group means; for C, E, G, I, K, and N, data points represent individual offspring and their corresponding dam cytokine response fitted to a linear function (dashed line) with goodness of fit (R^2) shown for each and 95 % confidence intervals in grey; for F, H, L, M, and O, data points represent group means. Key: *p < 0.05; **p < 0.01; ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Offspring cognition

An array of clinically relevant tasks, selected according to the Measurement and Treatment Research to Improve Cognition in Schizophrenia Consensus (Cognitive Battery) (Young et al., 2009), were used to test whether prenatal poly I:C treatment impacted offspring cognitive development. First, the novel object recognition (NOR) task was used to assess visual learning as a measure of hippocampal function. During both adolescence and adulthood, there were no significant effects of treatment group, maternal TNFα, or sex on NOR performance (Fig. S2A-H), including number of line crossings (Fig. S2I) and total exploration time (Fig. S2J). Anxiety-like behaviour was assessed using the elevated plus maze (EPM) due to the high comorbidity of anxiety with NDDs like schizophrenia (Jones et al., 2016). There was no effect of treatment group on the time spent in the open arms of the EPM during adolescence (GLMM, $F_{1,17} = 0.28$, $p = 0.605$; Fig. S2K) or adulthood (GLMM, $F_{1,14} = 0.43$, $p = 0.521$; Fig. S2L). These behaviours were therefore considered to be normal based on the ability of adolescent and adult offspring to discriminate between the novel and familiar object in the NOR task and increased time spent in the closed arm of the EPM.

The social interaction (SI) task was used to measure social cognition of offspring when exposed to a novel conspecific animal. In adult female offspring (PD123) there was no effect of treatment on object exploration in the SI task (GLMM, $F_{1,10} = 1.70$, $p = 0.222$; Fig. 3A). However, there was a significant main effect of both treatment group (GLMM, $F_{1,9} = 8.65$, $p = 0.017$; Fig. 3B) and the maternal TNFα response (GLMM, $F_{1,14} = 31.61$, $p < 0.001$; Fig. 3C) on the pro-social behaviour of sniffing an unfamiliar conspecific such that offspring of poly I:C-treated dams had an increased trait value. A similar effect was observed for the pro-social behaviour of following a conspecific where treatment group (GLMM, $F_{1,10} = 7.20$, $p = 0.023$; Fig. 3D) and the maternal TNFα concentration (GLMM, $F_{1,12} = 36.91$, $p < 0.001$; Fig. 3E) had significant effects. Interestingly, of the maternal behaviours analysed as predictors for offspring cognitive deficits (suckling, licking, total maternal care), the total amount of maternal care significantly predicted the increase in prosocial following behaviour in adult female offspring of poly I:C-treated dams (GMM, $F_{1,13} = 7.85$, $p = 0.015$).

Reasoning and problem solving as a measure of cognitive flexibility was assessed using the attentional set-shifting task (ASST) in female offspring only. There was a significant main effect of phase on the number of trials taken to complete the ASST in adult female offspring at (PD130 ± 4 days to accommodate testing of staggered litters; GLMM, $F_{6,84} = 17.58$, $p < 0.001$). Furthermore, the significant phase*treatment group interaction (GLMM, $F_{7,43} = 3.28$, $p = 0.007$) demonstrated an increase in the number of trials needed to complete the extradimensional shift (EDS) phase (GLMM, $F_{1,85} = 4.03$, $p < 0.001$) and reversal 3 (*R*3) phase (GLMM, $F_{1,85} = 2.29$, $p = 0.024$) phases for female offspring exposed to poly I:C (Fig. 3F). There was no significant main effect of maternal TNFα concentration on number of trials in the EDS phase (GLMM, $F_{1,12} = 1.13$, $p = 0.309$; Fig. 3G). There was also no significant main effect of maternal TNFα concentration on the number of errors made, where phase (GLMM, $F_{6,84} = 18.74$, $p < 0.001$) and, marginally, the phase*treatment group interaction (GLMM, $F_{7,43} = 2.10$, $p = 0.063$), predicted an increase in the EDS (GLMM, $F_{1,83} = 2.89$, $p = 0.005$) and *R*3 (GLMM, $F_{1,83} = 2.43$, $p = 0.017$) phases (Fig. 3H). There was no significant main effect of maternal TNFα concentration on number of errors in the EDS phase (GLMM, $F_{1,12} = 0.04$, $p = 0.838$; Fig. 3I). Treatment group also predicted an increased intradimensional/extradimensional (ID/ED) shift in offspring of poly I:C animals (GLMM, $F_{1,13} = 7.20$, $p = 0.019$; Fig. 3J), but similarly this was not predicted by the maternal TNFα concentration (GLMM, $F_{1,13} = 0.01$, $p = 0.909$). This was not related to any effect of treatment (GLMM, $F_{6,85} = 1.00$, $p = 0.432$), phase (GLMM, $F_{6,85} = 0.61$, $p = 0.719$), or their interaction (GLMM, $F_{1,13} = 0.00$, $p = 0.975$) on mean trial time (Fig. 3L).

Finally, the radial arm maze (RAM) was used to assess spatial working memory. All tested animals entered all eight arms on each of the five test days, with the first testing day starting between PD128-137 to allow testing of staggered litters. There was a significant main effect of treatment group on number of working memory errors such that animals exposed to poly I:C made more errors on the third testing day (RM-GLM, $F_{1,19} = 5.61$, $p = 0.029$; Fig. 3M). Maternal TNFα concentration also predicted an increase in working memory errors on the third testing day (RM-GLM, $F_{1,19} = 5.61$, $p = 0.010$; Fig. 3N). There was no significant main effect of treatment group (RM-GLM, $F_{1,21} < 0.01$, $p = 0.988$) or maternal TNFα (RM-GLM, $F_{1,20} = 1.06$, $p = 0.316$) on mean trial time (Fig. 3O).

2.6. Gene expression in the offspring brain

We assessed the impact of prenatal MIA exposure on offspring brain development through expression of candidate (NDD-related) genes and global DNA methylation in target regions. In the PD35 DHipp, there was a significant main effect of sex (GLMM, $F_{1,5} = 36.41$, $p = 0.003$) and a treatment group*sex interaction (GLMM, $F_{2,6} = 12.45$, $p = 0.007$) that predicted a sex-specific increase in *Gad1* expression in poly I:C-exposed males (Fig. 4A). In the PD35 frontal cortex (FCtx), there was an increase in expression of *Pvalb* in female (GLMM, $F_{1,10} = 5.25$, $p = 0.045$) and *Gad1* in male offspring (GLMM, $F_{1,12} = 4.87$, $p = 0.048$) of poly I:C-treated dams (Fig. 4B). There was a further significant main effect of treatment group on *Gad2* expression in the FCtx (GLMM, $F_{1,22} = 8.27$, $p = 0.009$), which was driven by a sex-specific increase in males exposed to poly I:C (GLMM, $F_{1,12} = 5.19$, $p = 0.042$; Fig. 4B). There were no significant effects of treatment group on gene expression in the PD35 PFC (Fig. 4C). There was a significant increase in *Pvalb* expression in the ventral hippocampus (VHipp) of female offspring of dams of poly I:C-treated (GLMM, $F_{1,10} = 7.09$, $p = 0.024$; Fig. 4D).

In adult (PD175) offspring, we found an overall effect of treatment that predicted a reduction in *reelin* (*Rln)* expression in the DHipp which was independent of sex (GLMM, $F_{1,16} = 5.00$, $p = 0.040$; Fig. 4E). The significant treatment group*sex effect predicted an increase in DHipp expression of *somatostatin* (*Sst)* in female offspring and concurrent reduction in *Sst* in male offspring of poly I:C-treated dams (GLMM, $F_{1,15} = 5.54$, $p = 0.03$; Fig. 4E). There were no significant effects of treatment group on gene expression in the PD175 FCtx (Fig. 4F). In the PD175 PFC, there was a female-specific increase in *Sst* expression in offspring exposed to poly I:C (GLMM, $F_{1,18} = 5.54$, $p = 0.030$; Fig. 4G). There was a significant main effect of treatment which predicted a reduction in *Gad2* expression in the VHipp, independent of sex (GLMM, $F_{1,10} = 5.43$, $p = 0.042$; Fig. 4H). Of note, we found no effect of maternal TNFα concentration on gene expression in any brain region of offspring from poly I:C-treated dams.

2.7. Global DNA methylation in the offspring brain

Adolescent female offspring exposed to poly I:C had significantly reduced global DNA methylation in the DHipp (GLMM, $F_{1,7} = 6.22$, $p = 0.041$; Fig. 4I). Whilst there were no other significant main effects of treatment group on global DNA methylation, maternal TNFα concentration significantly predicted a decrease in DHipp DNA methylation (GLMM, $F_{1,7} = 9.30$, $p = 0.019$) and concurrent increase in PFC DNA methylation (GLMM, $F_{1,8} = 7.45$, $p = 0.026$) in adolescent (PD35) female offspring (Fig. 4I). There were no significant main effects of either treatment group or the maternal TNFα in any adult brain region analysed (Fig. 4J).

3. Discussion

Previous studies have shown that MIA induction by poly I:C affects both maternal care behaviours and offspring cognition in mice, with translational relevance across a range of NDDs including schizophrenia (Ronovsky et al., 2017; Berger et al., 2018; Zhao et al., 2021; Zambon et al., 2022). Here, we tested the hypothesis that MIA induced by poly I:

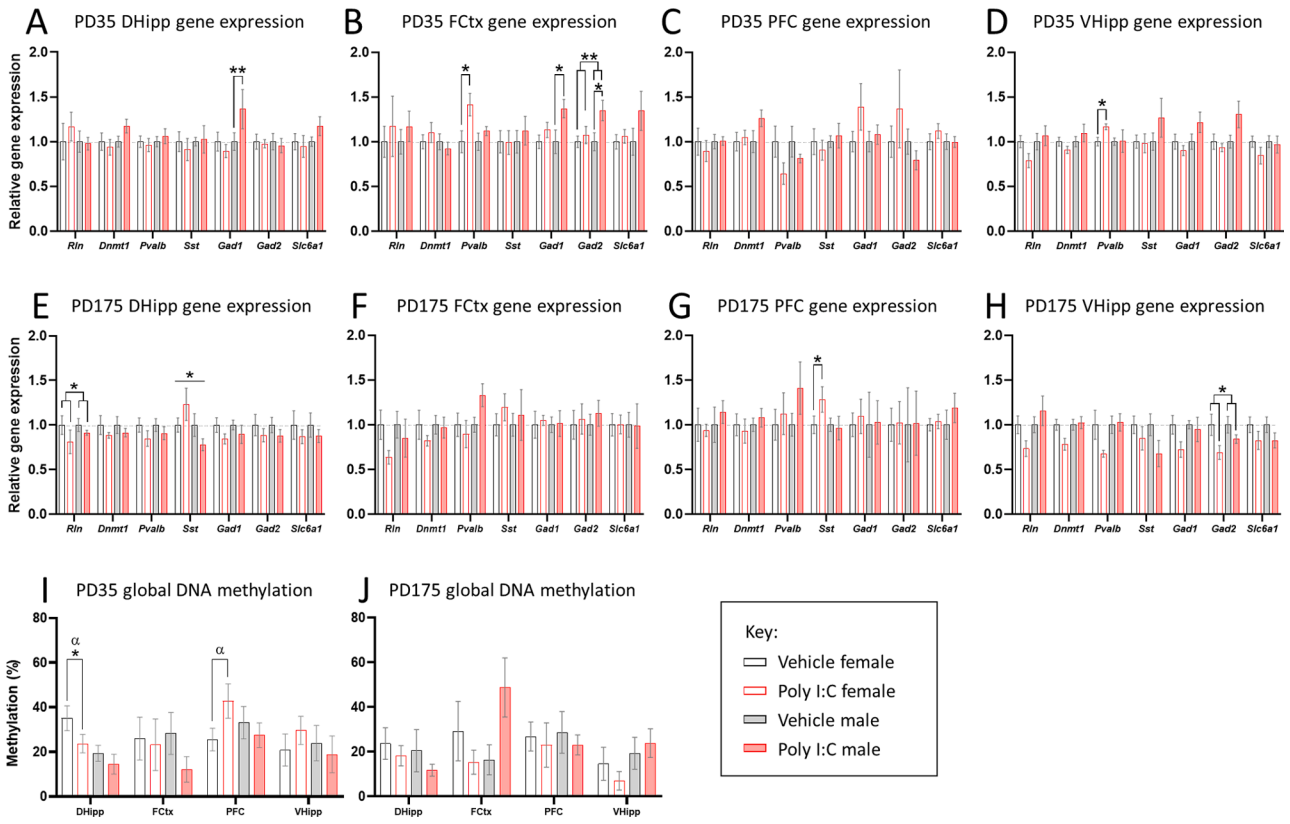

Fig. 4. Effect of poly I:C treatment in pregnant dams on gene expression and global DNA methylation in the offspring brain. Relative gene expression of candidate genes *Rln*, *Dnmt1*, *Pvalb*, *Sst*, *Gad1*, *Gad2*, and *Slc6a1* in the adolescent (PD35) **A)** DHipp, **B)** FCtx, **C)** PFC, and **D)** VHipp and adult (PD175) **E)** DHipp, **F)** FCtx, **G)** PFC, and **H)** VHipp. Global DNA methylation of CpG residues in the DHipp, FCtx, PFC, and VHipp of **I)** PD35 and **J)** PD175 offspring (for all PD35 female vehicle n = 7; female poly I:C n = 6; male vehicle n = 7; male poly I:C n = 5; for all PD175 female vehicle n = 5; female poly I:C n = 6; male vehicle n = 7; male poly I:C n = 5). Vehicle shown in black bars, poly I:C shown in red bars, females in open bars, males in filled bars. Data is presented as mean ± SEM. Key: *$p < 0.05$ and **$p < 0.01$ for significant effects of treatment group; $^{\alpha}p < 0.01$ for significant main effect of maternal TNFα. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

C in the rat jointly affects maternal care and offspring cognition and sought to investigate the role of the prenatal maternal inflammatory response in predicting such outcomes. To our knowledge, this is the first exploration of the collective effects of MIA in rats on 1) the maternal inflammatory response, 2 litter size and offspring somatic development, 3) offspring solicitation and maternal care behaviours, 4) offspring cognitive development, and 5) NDD-related gene expression and DNA methylation in offspring brain. We show that maternal treatment with poly I:C on GD15 affects both maternal and offspring outcomes, including an increase in maternal plasma TNFα concentration, attenuating offspring solicitation and maternal care behaviours postnatally, and impairing adult female offspring cognition (social interactions, spatial working memory, and cognitive flexibility). Our data supports the role for the pro-inflammatory effects of MIA disrupting normal GABAergic development in the PFC and hippocampus.

A major finding of our study is that MIA, and specifically the maternal plasma TNFα response, alters both the early postnatal maternal environment (i.e. maternal and offspring behaviour) and cognitive deficits seen in adult female offspring. This finding agrees with previous cross-fostering studies in mice, where it is largely prenatal exposure to pro-inflammatory mediators that affects cognitive and behavioural deficits in adult offspring (Meyer et al., 2006; Meyer et al., 2008; O'Leary et al., 2014; Richetto et al., 2013; Schwendener et al., 2009). We extend these findings in the rat and also provide novel insights as to how the prenatal (MIA) and postnatal (i.e. maternal care) environments relate to offspring solicitation behaviours, somatic and cognitive development, and NDD-related gene expression in offspring brain. We also demonstrate that the total amount of maternal care exhibited significantly predicts an increase in pro-social following behaviours in adult female offspring. Of the behaviours analysed, this was the only significant effect seen whereby the postnatal maternal environment, through maternal care behaviours, showed significant effects on offspring cognition. However, due to our study design we were unable to establish the relative contributions of these prenatal (i.e. maternal inflammation) and postnatal (i.e. maternal care behaviours) pathways on programming offspring cognition. Indeed, our findings do not rule out the possibility that the postnatal maternal environment impacts offspring development in the MIA model used here – for example, several MIA cross-fostering studies have implicated the postnatal maternal environment in deficits induced in *adolescent* offspring, including context-induced freezing (Schwendener et al., 2009), locomotor response to amphetamine challenge (Richetto et al., 2013), and sociability and social novelty preference (O'Leary et al., 2014). This may suggest that in MIA models, attenuated maternal care in early life induces transient effects on offspring cognition which may abate or be masked in adulthood. The use of a split-litter cross-fostering design in the model used here would allow for an in-depth analysis of the relative contributions of the pre- and postnatal maternal environments in the pathological programming of cognitive deficits, such as that seen in the ASST. Interestingly, transgenerational studies have demonstrated that maternal care, offspring cognition, and offspring brain gene expression (e.g. mineralocorticoid and glucocorticoid receptors) deficits are propagated until at least the F_2 generation through intricate patterns of maternal and paternal poly I:C heritage (Ronovsky et al., 2017; Berger et al., 2018). These studies indicate that whilst maternal care behaviours may not directly impact offspring cognition, they are likely to be critical

for the intergenerational transfer of poly I:C-induced deficits, warranting further studies into the developmental and molecular effects in these paradigms. Such effects may have origins in immune-mediated development of sexual behaviours, recently shown to be modulated by mast cells in the central nervous system which are liable to programming by inflammatory processes (Lenz et al., 2018).

3.1. MIA increases maternal TNFα concentration

A pro-inflammatory maternal response to poly I:C treatment was evidenced by a significant increase in TNFα plasma concentration measured at 3 h post-treatment, in agreement with previous studies (Hollins et al., 2016; Verdurand et al., 2014; Missault et al., 2014) and was a predictor of several behavioural and neurodevelopmental outcomes measured. TNFα is released by monocytic cells and transduces various inflammatory signalling pathways resulting in pleiotropic functions relating to immunity, cell survival/death, and inflammation (Hayden and Ghosh, 2014). Functional and genetic knockout studies have demonstrated a critical role for TNFα in neuronal development (Neumann et al., 2002) and fetal survival in MIA models (Carpentier et al., 2011). Therefore, alongside other pro-inflammatory cytokines such as IL-6 (Smith et al., 2007); TNFα is likely to play an important role in the altered neurodevelopmental programming in the rat poly I:C model. For example, whilst we found there was no main effect of treatment group on maternal behaviours, maternal TNFα concentration predicted a significant decrease in maternal suckling and licking, two key behaviours that have been shown to be critical for neurodevelopment (Champagne et al., 2008). Similarly, the maternal TNFα response was a significant predictor of deficits in adult female social interaction and spatial working memory in the RAM, but not cognitive flexibility deficits in the ASST. Maternal pro-inflammatory cytokines may affect fetal/offspring neurodevelopment by several mechanisms. Firstly, maternal cytokines could cross the placenta and affect the developing brain directly to affect microglial functions (Murray et al., 2019) such as synaptic pruning, which, in turn, could impact on the pool of developing neural progenitor cells (Squarzoni et al., 2015). This scenario seems unlikely based on the common finding that TNFα does not appear to cross the rodent placenta (Carbó et al., 1998). Alternatively, maternal inflammatory cytokines may be sensed by the placenta, causing secondary inflammation as previously shown (Hsiao and Patterson, 2011), which may then propagate to the fetal brain. This may then induce lasting endocrine dysfunction which impacts upon postnatal maternal care behaviours or by affecting placental transport mechanisms (McColl and Piquette-Miller, 2019). Based on the evidence for prenatal programming presented here and elsewhere (Meyer et al., 2006; Meyer et al., 2008; O'Leary et al., 2014; Richetto et al., 2013; Schwendener et al., 2009), placental dysfunction seems a plausible mechanism underpinning altered fetal neurodevelopmental programming and warrants further investigation. It was recently demonstrated that amino acid transport across the placenta is downregulated 24 h after poly I:C treatment (GD16) followed by an adaptive response with upregulated transport at GD21 (Kowash et al., 2022). The function of the visceral yolk sac in rodents, which supports fetal growth by expression of nutrient transporters (Owaydhah et al., 2020), as well as seeding the developing fetal brain with haematopoietic progenitors of tissue-resident macrophages (microglia) (Ginhoux et al., 2010; Magalhaes et al., 2022), should also be investigated as a key site for prenatal programming of NDDs.

3.2. MIA induced sex differences in early postnatal offspring development and behaviour

Our data shows that female offspring exposed to poly I:C prenatally exhibit significant changes in their weight gain and behaviour during the first 2 weeks postnatally, which are not seen in males. Female offspring had a significantly reduced weight gain at PD6-14 which was matched with an increase in USV syllable duration and decreased autogroom behaviour. It is well established that female and male fetuses have differing survival strategies, dependent on placental function (Clifton, 2010), in response to prenatal stressors such as MIA which may help to explain these differences reported here. Therefore, it may be that in our study, female offspring experienced a reduction in growth prenatally, causing postnatal behavioural adaptations to increase maternal resource solicitation behaviours such as USVs. Indeed, data from this model has recently demonstrated sex-specific effects in placental function which would support this, including a more pronounced reduction in amino acid transport in female placentas at GD16, compared to males (Kowash et al., 2022).

3.3. MIA elicited deficits in social behaviours and cognitive flexibility in adult female offspring

Prenatal MIA induced a range of cognitive deficits in the adult female offspring, some of which were predicted by the maternal TNFα response, whereas none were predicted by the frequency of maternal care offspring were exposed to postnatally. Surprisingly, there were no effects of MIA on NOR or EPM performance. A recent study has suggested evidence for distinct clustering of cognitive phenotypes in our MIA model, indicating that effects on NOR performance in 'high-risk' individuals may be masked by those with resilience factors born to poly I:C dams (Lorusso et al., 2022). It may be important for future analyses to consider clustering of phenotypes, rather than limiting analysis by treatment group, to identify such effects as well as risk and resilience factors in MIA models. In contrast, both poly I:C and the maternal TNFα response predicted an increase in pro-social sniffing and following, in contrast to the body of literature that suggests MIA by poly I:C has an antisocial effect on offspring (Labouesse et al., 2015; Mueller et al., 2018; Osborne et al., 2019). It may be that the prosocial effect of poly I:C observed here is a compensatory mechanism for the maternal separation experienced during the three postnatal maternal-offspring interaction test days, which could be considered as a 'second-hit'. Indeed, it was recently demonstrated that dual-hit paradigms do not simply combine as an additive stressor to induce effects on offspring social behaviour but interact in more complex ways (Goh et al., 2020). For example, gestational poly I:C exposure followed by isolation rearing appears to ameliorate the antisocial (aggression) effect of isolation rearing alone (Goh et al., 2020). It may be that gestational poly I:C acts as a priming mechanism to subsequent environmental stressors (such as isolation), acting as an evolutionary counterbalance to promote normal cognition during postnatal development. It is important to consider the methodological factors that may have impacted our data. For example, following local ethical guidance there were sex differences in post-weaning cage group sizes used here (3 males vs 5 females), which may have contributed to increased pro-social behaviours in females. There is evidence to suggest that the number of cage mates has a sex-differential effect on corticosterone levels in Wistar rats, which is known to affect cognitive performance (Brown and Grunberg, 1995). Similarly, all animals were tested on cognitive tasks in the same order and as such, whilst the interval between tasks remained constant, their observed traits may have been impacted by exposure to the previous task.

In contrast, poly I:C induced a clinically relevant and expected deficit in cognitive flexibility (ASST) and spatial working memory (RAM). Cognitive flexibility, as assayed using the ASST, is thought to be underpinned by GABAergic interneurons expressing the calcium-binding protein parvalbumin in the PFC, with impaired function being linked to molecular and functional deficits of these populations (Canetta et al., 2016). It is well-established that inflammatory signals have pleiotropic roles in coordinating fetal neurodevelopment, acting as molecular signals for the migration of cortical neurons and their integration into target networks. This may, in part, be mediated by aberrant microglial function, including their role in developmental synaptic

pruning and the regulation of neural progenitor cell pools (Squarzoni et al., 2015), and may explain the link between maternal peripheral inflammation and fetal neurodevelopment by the mechanisms previously discussed. Furthermore, the role of maternal peripheral inflammation in offspring neurodevelopmental and cognitive outcomes has been investigated recently showing that third trimester C-reactive protein inversely correlates with cognitive flexibility in 4–6-year-olds (Morgan et al., 2020). This provides clinical evidence for the pathological programming of cognitive deficits by maternal inflammatory factors established in MIA models, in agreement with observations reported here.

A key limitation of our study is the lack of evidence for a causal relationship between maternal TNFα production and alterations in maternal care and offspring cognition. To address this, future studies should employ pharmacological approaches to block maternal TNFα signalling, such as by employing anti-TNFα neutralising monoclonal antibodies, to establish whether such deficits remain. Further studies may then explore the effects of emerging non-pharmacological TNFα inhibitors on cognition as a more accessible and inexpensive dietary intervention in MIA models, such as diferuloylmethane (curcumin), the active component of turmeric (Aggarwal et al., 2013). A second limitation is the group sizes for some of the measures reported which fall short of the N = 9 indicated by our power analysis. Due to the need to cull animals for molecular analysis at several timepoints, we were unable to maintain this sample size e.g. for social interaction, ASST, and molecular analyses. Further studies should aim to increase these sample sizes in an attempt to replicate our findings. It may be that this has either masked some true biological effects by reducing the power of our analyses, or that some of our significant findings are underpowered. Further studies should aim to increase these sample sizes in an attempt to replicate our findings.

3.4. MIA causes region-, age-, and sex-specific NDD-related gene expression changes in offspring brain

We finally investigated expression of candidate genes and global DNA methylation in the adolescent and adult offspring brain as a proposed molecular mechanism by which cognitive deficits could occur, as recently reviewed systematically (Woods et al., 2021). Whilst showing no difference in adulthood, poly I:C treatment as well as the maternal TNFα response predicted a decrease in female DHipp global DNA methylation during adolescence. These data primarily implicate upregulated GABAergic gene expression during adolescence, which is then dysregulated in adulthood in a sex- and region-specific manner. It is worth noting that GABAergic signalling modulates immune function through the expression of GABA receptors on immune cells including macrophages, B and T lymphocytes, and dendritic cells, such as by modulating pro-inflammatory cytokine release (Bhandage and Barragan, 2021). Whilst we were unable to demonstrate any significant effects of maternal TNFα on GABAergic gene expression in the offspring brain, future studies may benefit from combined analysis of offspring immune perturbations (e.g. peripheral or central cytokine expression) and GABAergic function to establish a causal link. Combined with change in global DNA methylation during adolescence, this suggests that MIA may affect the development of GABAergic circuits during early postnatal life which then predispose offspring to related cognitive deficits during adulthood, similar to previous reports in mice (Canetta et al., 2016). Whilst providing an insight into these mechanisms, future studies would benefit from first validating the importance of these circuits in MIA-induced cognitive changes (e.g. by single-cell isolation from MIA-exposed offspring brain tissue followed by transcriptomic, proteomic, or metabolomic sequencing). Next, the mechanistic relevance of these populations should be experimentally tested (e.g. by genetic manipulation of parvalbumin-expressing interneurons at various neurodevelopmental stages) in order to identify the molecular and cellular hallmarks of such cognitive deficits.

In summary, our study provides evidence that the prenatal maternal inflammatory response to poly I:C affects offspring neurodevelopment, which, however, is not affected by postnatal maternal care behaviours. Delineating the relative contributions of the pre- and postnatal maternal environments on offspring cognition in rat MIA models, for example by full- or split-litter cross fostering designs, is a critical next step in understanding the mechanisms by which such effects occur. Cognitive flexibility deficits are canonical features of both the clinical presentation of NDDs and those established in several animal models of MIA (e.g. (Canetta et al., 2016) which have been extended here, to our knowledge for the first time, in a rat model of prenatal poly I:C exposure. Future work should seek to extend this by asking the following: 1) to what extent does the prenatal maternal environment programme cognitive flexibility deficits in adult offspring? 2) is maternal inflammation causal or correlative of cognitive flexibility deficits, and if causal, what factors (e.g. cytokines) are necessary for such effects (e.g. by the use of knockout models or by co-administration of anti-cytokine neutralising antibodies, as in (Smith et al., 2007)? 3) is this deficit reproducible both across studies and in both female and male offspring? 4) when does this deficit appear in offspring and how does age affect its progression? and 5) can it be modulated by 'dual/second-hit' models (Goh et al., 2020), or rescued by pharmacological or environmental (e.g. exercise, dietary) interventions?

3.5. Methods

An experimental timeline is shown which outlines the gestational timings of blood sampling and poly I:C treatment (Fig. 5A) as well as postnatal maternal-offspring behavioural recordings, offspring cognitive tasks, and tissue collection (Fig. 5B).

3.5.1. Animals

Adult Wistar nulliparous female and male rats were obtained from Charles River Laboratories (UK). All animals were housed in environmentally enriched, individually ventilated cages (GR1800 Double-Decker Cage, Tecniplast, UK). All animal experiments were carried out in the Biological Services Unit at the University of Manchester under a standard 12 h light:dark cycle (lights on at 07:00 h) with the holding room maintained at 21 ± 2 °C and 55 ± 5 % humidity. Unless otherwise stated, animals had *ad libitum* access to standard rat chow (Special Diet Services, UK) and water. All procedures adhered to the Animals (Scientific Procedures) Act 1986, were performed under the authority of project licence number P473EC3B1 (approved by the University of Manchester Animal Welfare and Ethical Review Body).

Sample size was calculated using the statistical package G*Power v3.1.9.2 (G*Power, Germany) (Faul et al., 2017) based on published behavioural data (NOR) from Wistar rats exposed to poly I:C-induced MIA on GD15 (Mattei et al., 2014). An effect size (d) = 1.446 was calculated which predicted N = 9 dams per treatment group to be sufficient for power (1-β) = 0.8 with a type 1 error rate (α) = 0.05. Hence, a total of 18 nulliparous adult female Wistar rats were used in the final study (N = 9 vehicle, N = 9 poly I:C) with a mean weight ± SEM of 264.4 ± 2.2 g.

3.5.2. Maternal immune activation

To assess baseline (pre-pregnancy) plasma cytokine concentrations, non-pregnant females had a blood sample taken from the lateral tail vein from which plasma was taken. Females were then timed-mated with adult male Wistar rats in monogamous pairs and the appearance of a vaginal plug was designated as GD1.

The design and reporting of this study have been carefully considered to improve the reproducibility of MIA models, as highlighted recently (Kentner et al., 2018). The MIA induction protocol was determined based on a series of validation and optimisation studies indicating a variety of factors (e.g. cage systems (Mueller et al., 2018), gestational timing (Knuesel et al., 2014; Meyer et al., 2006), biomolecular

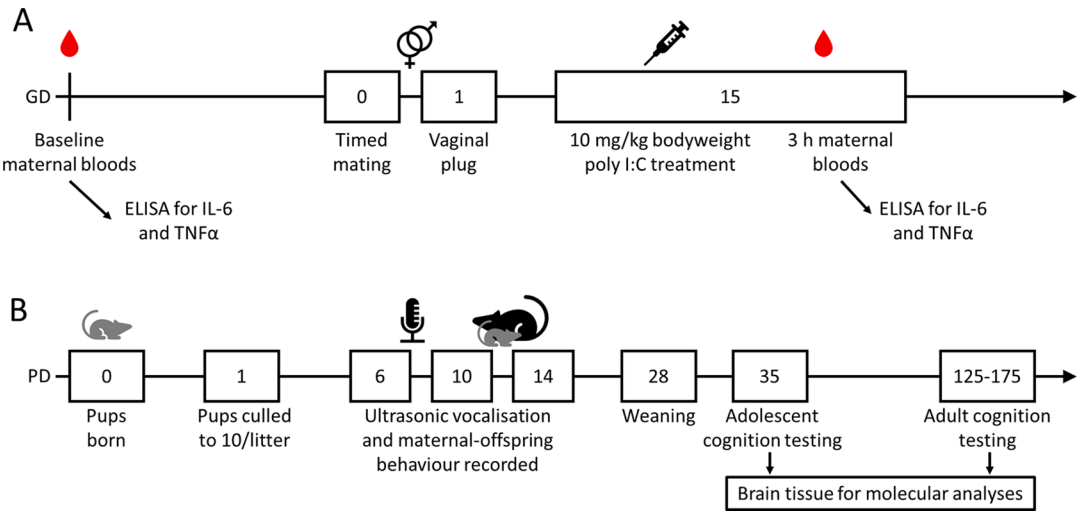

Fig. 5. Experimental timelines. Methods used across the study **A)** during gestation and **B)** postnatally.

characteristics of poly I:C (Kowash et al., 2019; Kentner et al., 2018; Careaga et al., 2018; Zhou et al., 2013) can affect phenotypic outcomes. First, the rat strain and poly I:C dose were determined with respect to the optimal and most reproducible pro-inflammatory cytokine response, physiologically confirmed by microglial activation in the offspring hippocampus (Murray et al., 2019). Second, the commercial source of poly I:C was investigated with regards to molecular weight and endotoxin contamination, both of which are known to affect the magnitude of the maternal pro-inflammatory cytokine response and fetal outcomes (Kowash et al., 2019), independently replicated by others (Mueller et al., 2019). Such methodological analyses have been shown to have a significant impact on fetal and offspring neurodevelopmental outcomes – for example, the maternal and fetal phenotypic variability induced by unpredictable poly I:C molecular weight and endotoxin contamination has recently been extended to variability in cognitive (working memory) deficits in adult mice (Mueller et al., 2019; McGarry et al., 2021). In addition, we are in full support of recent efforts to improve the transparent and rigorous reporting of methodological parameters used in MIA models, and as such we provide details of experimental conditions (Table S1), as advocated by by Kentner et al. (Kentner et al., 2018).

Dams were pseudo-randomised resulting in N = 9 for vehicle and poly I:C. All experimenters involved in treatment administration and data collection were blinded to treatment group until all data had been collected. Poly I:C was reconstituted by an independent experimenter who did not otherwise take part in data collection. Low molecular weight poly I:C (InvivoGen, France; catalogue number tlrl-picw) reconstituted in endotoxin-free physiological water (0.9 % saline) according to the manufacturer's instructions. We have previously shown that the poly I:C batch used here (PIW-38–04) has a consistent molecular weight profile (176–186 kDa) and low endotoxin contamination (1.2–5.8 endotoxin units/mL (Kowash et al., 2019). A single treatment of 10 mg/kg bodyweight poly I:C or vehicle solution (endotoxin-free physiological water) was administered to pregnant Wistar rats by intraperitoneal (i.p.) injection GD15 between 09:00 and 10:00 to limit circadian variation of immune function (Scheiermann et al., 2013). At 3 h post-treatment, which has previously been shown to correspond to the peak of the maternal inflammatory response to this source of poly I:C by us (Kowash et al., 2022) and others (Clark et al., 2019), dams had a second tail vein blood sample taken as described above. Maternal weight was recorded daily from GD1-21 and then daily from PD1-14.

3.5.3. *Maternal cytokines*

Both IL-6 and TNFα have been shown to be induced during the maternal response to poly I:C and mediate downstream offspring developmental deficits (Murray et al., 2019; Smith et al., 2007). Recent data from our own validation of this model has demonstrated that both maternal IL-6 and TNFα plasma concentration peak at 2–3 h post-treatment with this source of poly I:C in pregnant Wistar rats, which is not seen for other canonical pro-inflammatory cytokines such as IL-1β (Kowash et al., 2022). Therefore, their concentration was measured in maternal plasma at baseline (pre-pregnancy) and 3 h post-treatment using rat-specific ELISAs for IL-6 and TNFα (ab100772 and ab100784 respectively, Abcam, UK). The intra- and inter-assay coefficients of variation values were 3.0 % and 15.0 % for IL-6, respectively, and 7.9 % and 19.0 % for TNFα, respectively. One dam in the poly I:C group did not yield sufficient plasma to assay for either cytokine at 3 h post-treatment.

3.5.4. *Early postnatal behaviour*

The day that pups were first seen was designated PD0. On PD1, litters were culled to a maximum of 10 pups to standardise the effect of offspring solicitation behaviours on maternal care (Suvorov and Vandenberg, 2016). Culled pup bodyweight, brain weight, and liver weight was recorded. For the remaining offspring, bodyweight was recorded on the early postnatal testing days (PD6, 10 and 14) and subsequently every week until offspring were sacrificed (PD35 or PD175).

3.5.5. *Ultrasonic vocalisations*

USVs were recorded using a BatScan Duet bat detector (Batbox ltd, UK), suspended 15 cm above the test chamber floor from individual pups during a 1 h maternal separation on PD6, 10 and 14. Maternal separation is known to induce USVs at around 40 kHz in rodent pups, and we selected recording timepoints to correspond with the period of maximal maternal resource allocation (approximately PD8; (Hager and Johnstone, 2003; Peña and Champagne, 2013; Yin et al., 2016). Throughout all maternal separation and USV recordings, pups were kept on heat pads in a separate room to their dams to minimise the effect of audial and olfactory cues. During the USV recording protocol, pups were selected at random throughout the 1 h maternal separation period and recorded for 3 min. The number of syllables (calls), mean syllable duration, total vocalisation time, syllable power, and mean syllable frequency were automatically calculated using the open-source script for Mouse Ultrasonic Profile ExTraction (MUPET) (Van Segbroeck et al., 2017).

3.5.6. *Maternal-offspring interactions*

Following the 1 h maternal separation maternal-offspring interactions were recorded *in situ* using an ethogram (as previously described (Hager and Johnstone, 2007); Figure S3). Testing always started at 10.00 h and litters were counterbalanced by maternal treatment group (i.e. testing vehicle and poly I:C litters in turn) to minimise

circadian effects on maternal-offspring interactions. Two pups in the female poly I:C died between PD6 and PD14 and so do not have datapoints for USV or maternal-offspring interaction analyses.

Offspring were weaned on PD28 and dams sacrificed by exposure to a rising concentration of CO_2. Offspring were housed in mixed litter cages of up to either five females or three males.

3.5.7. Offspring cognition

For cognitive tasks, we focused on phenotyping female adult offspring based on evidence that females are typically underrepresented in scientific studies and that females experience more severe cognitive deficits in cognitive domains such as working memory (Leger and Neill, 2016). All behaviours were recorded and scored by experimenters who were blinded with respect to treatment group. Testing began at 10.00 h and the order of testing was counter-balanced for sex and treatment group to minimise the effects of circadian fluctuations on behaviour. The experimental setup for offspring cognitive tasks is shown in Figure S2M-P.

3.5.8. Novel object recognition (NOR)

Visual recognition memory was tested using the NOR task during adolescence (PD35) and adulthood (PD121) in both female and male offspring as previously described (Neill et al., 2016). The objects used (Figure S2M) were selected based on previous data showing no exploration bias in adult female rats (Neill et al., 2016). Exploration of objects was recorded for 3 min. A 30 min inter-trial interval was selected based on previous evidence suggesting impaired hippocampal glutamatergic transmission and impairments in genetic models of NDDs (Dere et al., 2007; Yang et al., 2012). The number of line crossings made (etched into the NOR arena floor) and total object exploration time were recorded as a measure of locomotor. The discrimination index (DI) was calculated as $(E_{Novel} - E_{familiar})/(E_{Novel} + E_{Familiar})$. Due to time constraints following staggering of timed-matings, some animals were not tested on the NOR, detailed in Table S5.

3.5.9. Elevated plus maze (EPM)

The EPM was used to measure anxiety-like behaviour in adolescent (PD35, 3 h after NOR testing) and adult (PD122) offspring. Anxiety is an established symptom of schizophrenia with shared genetic risk factors and has been demonstrated to precede psychotic symptoms (Jones et al., 2016). Due to size restrictions of the apparatus, adult males were not tested on the task. Animals were placed at the point of convergence of the arms (centre) facing the open arm and exploration of open and closed arms (Figure S2N) was recorded for 5 min. The proportion of time spent in the open arms as a proportion of the time spent in all arms was measured.

3.5.10. Social interaction (SI)

Social behaviour was measured following introduction to an unfamiliar weight matched conspecific in a SI task as previously described (Wilson and Koenig, 2014) in adult (PD123) female offspring. Animals were placed in the NOR arena with a single object in the centre (Figure S2M) and were joined by an unfamiliar age and sex-matched conspecific. Behaviour was recorded from above for 10 min. The amount of time spent sniffing or following the conspecific was used as a measure of social behaviour, whereas time spent avoiding the conspecific or exploring the object was used as a measure of asocial behaviour.

3.5.11. Attentional set-shifting task (ASST)

Reasoning and problem solving was assessed using the ASST (PD126-134) based on a modified protocol and arena as previously described (Birrell and Brown, 2000) and optimised within our group. This protocol has previously been used to demonstrate a phencyclidine-induced selective cognitive deficit in female Lister-Hooded rats in the EDS phase which was reversed by the antipsychotics clozapine and risperidone (McLean et al., 2008). To encourage food reward-motivated behaviour, rats were food-restricted for three days prior to testing up to a maximum weight loss of 15 % free-feeding weight. Honey Nut Cheerios (Nestlé, Switzerland) were placed at the bottom of ceramic bowls as food rewards allowing adult female rats to perform a series of discriminations across seven test phases (McLean et al., 2008). The position and cue dimensions of the baited bowls were pseudo-randomised according to a Gellerman schedule (Figure S2O) and shown in Table S2, respectively. The number of trials, number of errors, and mean trial time to complete each phase was recorded and used to assess task performance.

3.5.12. Radial arm maze (RAM)

Spatial working memory was tested using an eight-arm RAM (PD128-137) first described by Olton and Samuelson (Olton and Samuelson, 1976) based on similar published protocols (Dubreuil et al., 2003) optimised for use here. The RAM consisted of an octagonal central platform (side length 10.0 cm) from which animals were able to select and enter eight platform arms (70.0 × 10.0 cm) raised 100.0 cm from the ground. Starting on the central platform, rats were initially prevented from entering the arms by clear doors with visual cues that had unique and distinguishable characteristics (Fig. S2P). EthoVision tracking software (Noldus, Netherlands) which was also used to record movement. For five test days, animals were placed in the centre of the maze and were able to select each arm in sequence to collect a food reward – the trial ended when all eight arms had been selected. Trial time and number of working memory errors (re-entries into a previously selected arm) were recorded.

3.5.13. Molecular analyses

3.5.13.1. Tissue preparation. Animals were sacrificed at PD35 or PD175 by exposure to 2 % CO_2 for up to 10 min followed by cervical dislocation. The left hemisphere was stored at −80 °C in RNAlater Stabilization Solution (Sigma-Aldrich, UK) for RNA and DNA assays and the right hemisphere was stored immediately at −80 °C. The Rat Brain Atlas was used as a guide to dissect the FCtx, PFC, DHipp, and VHipp (Paxinos and Watson, 2007). Tissue was analysed for global DNA methylation and candidate gene expression using the DNeasy Blood and Tissue and RNeasy Plus Mini kits (both Qiagen, UK) according to the manufacturer's instructions. The tissue homogenate was split at a 2:1 ratio and used to allow dual extraction of RNA and DNA, respectively.

3.5.13.2. Gene expression quantification by qPCR. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, UK) including a genomic DNA (gDNA) elimination step according to the manufacturer's instructions. RNA was stored at −80 °C. RNA concentration and purity were assessed using a Nanodrop (Thermo Scientific, UK). RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription kit (Qiagen, UK) according to the manufacturer's instructions, and stored at −20 °C.

To identify a suitable reference gene, or combination of reference genes, expression stability across experimental conditions (treatment group, sex), was performed by geNorm analysis. A geNorm kit (PrimerDesign, UK) was used that included a panel of six rat-specific primers for commonly used reference genes (Table S3). Cycle threshold (Ct) values were entered into qBase + software v3.2 (Biogazelle, Belgium) which uses an algorithm based on pair-wise analysis to establish the most stable reference genes (PD35 DHipp, *Gapdh/Mdh1*; PD35 FCtx, *Mdh1/Gapdh*; PD35 PFC, *Mdh1/Gapdh*; PD35 VHipp, *Gapdh/Mdh1*; PD175 DHipp, *Mdh1/B2m*; PD175 FCtx, *Actb/B2m*; PD175 PFC, *Gapdh/Mdh1*; PD175 VHipp, *Mdh1/Gapdh*.

Candidate gene primers (Table S4) were selected based on relevance to schizophrenia, neuronal development, and GABAergic function, as recently systematically reviewed (Woods et al., 2021). All amplification cycles were performed on an AriaMx qPCR machine (Agilent, USA): one cycle at 95 °C for 5 min; 40 cycles at 95 °C for 10 s followed by primer

annealing at 60 °C for 30 s. Amplification efficiency was 90.4–104.1 %.

3.5.13.3. Global CpG methylation quantification by ELISA. Extraction of gDNA was performed using the DNeasy Blood and Tissue kit (Qiagen, UK). The QuantiFluor dsDNA System (Promega, UK) was used to accurately quantify sample DNA concentration. Global DNA methylation was quantified using the 5-methylcytosine DNA ELISA kit (Enzo Life Sciences, UK). An additional standard of 0.05 % 5-methylcytosine (5mC) was added to the recommended standard range to account for the relatively low proportion of methylated DNA in rat brain tissue (Mega et al., 2018). The inter- and intra-assay coefficients of variation for the 5mC ELISAs were 8.2 % and 14.8 %, respectively.

3.5.13.4. Statistical analysis. Data are presented as individual data points with mean ± SEM shown in grey, unless otherwise stated. Vehicle animals/offspring are presented as black and poly I:C as red. 'N' refers to the number of dams and 'n' refers to the number of offspring, shown in figure legends and also summarised in Table S5. Graphs were produced using GraphPad Prism v7.04 (GraphPad, USA). Statistical tests were performed using SPSS v23 (IBM, USA). Dam and litter traits were analysed by univariate general linear models (GLMs) with treatment as a fixed factor and covariates where appropriate (maternal weight, cytokine response). For offspring-related traits, general linear mixed models (GLMMs) were used with dam as a random factor to account for reduced trait variance within litters. Where possible, outcome measures included offspring from each litter, but in some cases, this was not possible due to low animal numbers available at a given timepoint following culling for molecular analyses. For GLMM analyses the Satterthwaite estimation was used to estimate the degrees of freedom.

CRediT authorship contribution statement. **Harry G. Potter:** Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Writing – review & editing. **Hager M. Kowash:** Validation, Investigation. **Rebecca M. Woods:** Investigation. **Grace Revill:** Investigation. **Amy Grime:** Investigation. **Brendan Deeney:** Investigation. **Matthew A. Burgess:** Investigation. **Toby Aarons:** Investigation. **Jocelyn D. Glazier:** Conceptualization, Funding acquisition, Investigation, Supervision, Project administration, Writing – review & editing. **Joanna C. Neill:** Conceptualization, Funding acquisition, Supervision, Project administration, Resources, Writing – review & editing. **Reinmar Hager:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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