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Full-length Article

Intrauterine position effects in a mouse model of maternal immune activation

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ABSTRACT

Rodent models of maternal immune activation (MIA) are increasingly used as experimental tools in preclinical research of immune-mediated neurodevelopmental disorders and mental illnesses. Using a viral-like MIA model that is based on prenatal poly(I:C) exposure in mice, we have recently identified the existence of subgroups of MIA-exposed offspring that show dissociable behavioral, transcriptional, brain network and inflammatory profiles even under conditions of genetic homogeneity and identical MIA. Here, we tested the hypothesis that the intrauterine positions of fetuses, which are known to shape individual variability in litter-bearing mammals through variations in fetal hormone exposure, may contribute to the variable outcomes of MIA in mice. MIA was induced by maternal administration of poly(I:C) on gestation day 12 in C57BL/6N mice. Determining intrauterine positions using delivery by Cesarean section (C-section), we found that MIA-exposed offspring developing between female fetuses only (0M-MIA offspring) displayed significant deficits in sociability and sensorimotor gating at adult age, whereas MIA-exposed offspring developing between one or two males *in utero* (1/2M-MIA offspring) did not show the same deficits. These intrauterine position effects similarly emerged in male and female offspring. Furthermore, while MIA elevated fetal brain levels of pro- and anti-inflammatory cytokines independently of the precise intrauterine position and sex of adjacent fetuses during the acute phase, fetal brain levels of TNF-α remained elevated in 0M-MIA but not 1/2M-MIA offspring until the post-acute phase in late gestation. As expected, 1/2M offspring generally showed higher testosterone levels in the fetal brain during late gestation as compared to 0M offspring, confirming the transfer of testosterone from male fetuses to adjacent male or female fetuses. Taken together, our findings identify a novel source of within-litter variability contributing to heterogeneous outcomes of short- and long-term effects in a mouse model of MIA. In broader context, our findings highlight that individual differences in fetal exposure to hormonal and inflammatory signals may be a perinatal factor that shapes risk and resilience to MIA.

1. Introduction

Rodent models of maternal immune activation (MIA) are widely used as experimental tools to study neuronal and behavioral dysfunctions in relation to immune-mediated neurodevelopmental disorders and mental illnesses (Brown and Meyer, 2018). These models were originally developed to provide experimental support for human epidemiological studies implicating MIA in the etiology of neuropsychiatric illnesses, including schizophrenia, autism spectrum disorder (ASD), depression, and bipolar disorder (Zuckerman et al., 2003; Meyer et al., 2005; Patterson, 2011; Meyer, 2014; Careaga et al., 2017). As such, they provide preclinical platforms allowing to explore causal relationships, identify underlying neurobiological mechanisms, and, ultimately, develop novel therapeutic interventions and preventative strategies against MIA-induced brain disorders (Brown and Meyer, 2018).

Despite the increasing evidence for significant health consequences, the effects of MIA on the offspring are heterogeneous in both male and female offspring (Meyer, 2019). This heterogeneity is also mirrored by the variable effects of MIA in animal models (Weber-Stadlbauer and Meyer, 2019; Meyer, 2023). Like in many other model systems (Kafkafi et al., 2018; Richter et al., 2010; Voelkl et al., 2020), the specificity of the effects induced by MIA in laboratory animals is influenced by a

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number of factors, including immunogen specificity and dosing (Meyer et al., 2005; Mueller et al., 2019; Kowash et al., 2019; Estes et al., 2020; Bao et al., 2022,), prenatal timing (Meyer et al., 2006, 2008b; Meehan et al., 2017; Richetto et al., 2017), genetic background (Meyer et al., 2008a; Abazyan et al., 2010; Vuillermot et al., 2012; Lipina et al., 2013, Schwartzer et al., 2013), age and sex of the offspring (Garay et al., 2013; Richetto et al., 2014; Giovanoli et al., 2015; Gogos et al., 2020, Missig et al., 2020), and rearing environment (Connors et al., 2014; Mueller et al., 2018; Zhao et al., 2021). All these factors represent a certain source of intended or unintended variability in rodent models of MIA, which provides both opportunities and challenges for preclinical MIA research (Kentner et al., 2019; Weber-Stadlbauer and Meyer, 2019; Meyer, 2023).

Even under identical experimental conditions, however, noticeable variability exists in rodent models of MIA. For example, using a mouse model of MIA that was based on prenatal administration of the viral mimetic, poly(I:C) (=*polyriboinosinic-polyribocytidylic* acid), we recently identified the existence of subgroups of MIA-exposed offspring that show dissociable behavioral, transcriptional, brain network and inflammatory profiles even under conditions of genetic homogeneity, identical MIA, and constant laboratory conditions (Mueller et al., 2021). Remarkably, in this large-scale study involving a whole-litter phenotyping of over 150 offspring of multiple MIA-exposed and control dams, we further revealed that the variable outcomes of MIA were largely driven by within-litter rather than between-litter variability (Mueller et al., 2021). These findings are consistent with another study using the same MIA model in mice, which identified larger within-litter than between-litter variability in the effects of MIA on cortical interneuron development (Vasistha et al., 2020).

One plausible source of within-litter variability in multiparous species such as mice relates to intrauterine position effects. In litter-bearing mammals, multiple fetuses develop *in utero* and are subjected to differing hormonal microenvironments based upon the sex of neighboring fetuses (Clemens and Coniglio, 1971; vom Saal, 1989; Ryan and Vandenbergh, 2002). These intrauterine position effects mostly result from the transfer of male sex hormones, such as testosterone, from male fetuses to adjacent fetuses, which in turn shapes the variability of various physiological, anatomical and behavioral traits throughout life (Ryan and Vandenbergh, 2002; Kawata, 2013). In the mouse, fetal testosterone levels typically rise from gestational day (GD) 13 to a peak level at GD 17 and are higher in male than female fetuses (Pointis et al., 1979). Being a steroid hormone, testosterone can diffuse through the amniotic fluid between fetuses and influence the microenvironment of the neighboring fetuses (Ryan and Vandenbergh, 2002). As a result, both male and female fetuses that are flanked by either one (1M) or two (2M) male fetuses have higher concentrations of fetal testosterone than fetuses that are flanked by female fetuses only (0M) (vom Saal and Bronson, 1980; vom Saal et al., 1990).

While intrauterine positions have been shown to contribute to variability in other mouse models of prenatal adversities, including models of prenatal maternal stress and fetal growth restriction (vom Saal et al., 1990; D'Errico et al., 2021), it remains unknown whether they also contribute to variability in models of MIA. Therefore, the present study examined whether the intrauterine positions of fetuses influence the nature and/or severity of pathological traits induced by MIA in mice. We used the poly(I:C)-based MIA model in mice and determined the intrauterine positions of male and female offspring through near-term Cesarean section (C-section), after which we investigated social behavior and sensorimotor gating in adulthood in order to identify possible long-term influences of the intrauterine positions on behaviors relevant to neurodevelopmental disorders (Meyer et al., 2009; Brown and Meyer, 2018). In addition, we measured the levels of pro- and anti-inflammatory cytokines and testosterone in fetal brains after determining the precise intrauterine position and sex of the offspring.

2. Methods

2.1. Animals

C57BL6/N mice were used throughout the study. Female and male breeder mice were obtained from Charles River Laboratories (Sulzfeld, Germany) at the age of 12 weeks. Upon arrival, they were housed in individually ventilated cages (IVCs; Allentown Inc., Bussy-Saint-Georges, France) as described in detail before (Mueller et al., 2018). The cages were kept in a specific-pathogen-free (SPF) holding room, which was temperature- and humidity-controlled (21 ± 3 °C, 50 ± 10 %) and kept under a reversed light–dark cycle (lights off: 09:00 AM–09.00 PM). All animals had *ad libitum* access to standard rodent chow (Kliba 3336, Kaiseraugst, Switzerland) and water throughout the entire study. All procedures had been previously approved by the Cantonal Veterinarian's Office of Zurich, Switzerland.

2.2. Breeding and maternal manipulations

Timed-pregnant mice were generated via on-site breeding, which began two weeks after the animals were acclimatized to our facility. To this end, female and male breeders were subjected to a timed-mating procedure as previously described (Mueller et al., 2018, 2019). Successful mating was verified by the presence of a vaginal plug, upon which dams were housed individually throughout gestation. The presence of a vaginal plug was referred to as gestational day (GD) 0. A dam showing a vaginal plug at GD 0 and a weight gain of ≥ 3 g from GD 0 to GD 12 was considered as undergoing successful pregnancy (Mueller et al., 2019).

On GD 12, pregnant mice were randomly assigned to a single injection of poly(I:C) (potassium salt, P9582, Sigma–Aldrich, Buchs, St Gallen, Switzerland) or treatment with endotoxin-free 0.9 % NaCl (B. Braun, Melsungen, Switzerland) vehicle solution. To avoid possible batch-to-batch variation (Mueller et al., 2019), the same batch of poly(I:C) (batch #117M4005V) was used in all experiments included in this study. Based on our previous molecular and functional characterization of this batch (Mueller et al., 2019), poly(I:C) was administered intravenously (i.v.) into the tail vein at a dose of 2.5 mg/kg. The dose of poly(I:C) was calculated based on the pure form and was dissolved in glass vials with vehicle kept at room temperature. Control dams received vehicle solution (i.v.) only. All solutions were freshly prepared on the day of their administration and injected using an injection volume of 5 ml/kg. The tail vein injections were performed under mild physical constraint using a semi-restrictive rodent injection cone (model 561-RC; Plas-Labs Inc., Lansing, USA). Immediately after poly(I:C) or vehicle administration, the dams were placed back to their home cages and left undisturbed until delivery of offspring via C-section or vaginal birth (cohorts 1 and 2), dissection of fetuses on GD 12 (cohort 3), or dissection of fetuses on GD 17 (cohort 4) (Table 1).

Offspring of poly(I:C)- or vehicle-treated mothers were weaned on postnatal day (PND) 21, and littermates of the same sex were caged separately and maintained in groups of 3 to 5 animals per cage. Additional methodological details regarding the maternal manipulations are summarized in the reporting guideline checklist for the MIA model (Kentner et al., 2019), as provided in Supplementary **Table S1**.

2.3. Delivery by C-section

All offspring in cohort 2, and a subgroup of offspring in cohort 1 (Table 1), were delivered by a near-term C-section delivery protocol modified from Chiesa et al. (2019). Offspring delivered by C-section were raised by genetically identical foster mothers with identical treatment histories, whereby each foster mother kept two of its own (vaginally born) pups and received 6 C-sectioned pups. Poly(I:C)- or vehicle-treated mothers were prepared as described above and were assigned to C-section or foster mother conditions. For C-sectioning, near-

Table 1
Summary of the animal cohorts used in this study.

Cohort	Number of litters per group	Number of offspring per group	Age of testing
1	*N*(VD) = 7 *N*(CS) = 8	*n*(VD) = 38 (21 m, 17f) *N*(CS) = 41 (20 m, 21f)	Adulthood (12 weeks onwards)
2	*N*(CON) = 6 *N*(MIA) = 6	*n*(0M-CON) = 14 (7 m, 7f) *n*(1/2M-CON) = 18 (9 m, 9f) *n*(0M-MIA) = 15 (7 m, 8f) *n*(1/2M-MIA) = 18 (9 m, 9f)	Adulthood (12 weeks onwards)
3	*N*(CON) = 7 *N*(MIA) = 7	*n*(0M-CON) = 18 (11 m, 7f) *n*(1/2M-CON) = 17 (9 m, 8f) *n*(0M-MIA) = 19 (10 m, 9f) *n*(1/2M-MIA) = 18 (9 m, 9f)	Fetal stage (gestational day 12)
4	*N*(CON) = 4 *N*(MIA) = 5	*n*(0M-CON) = 9 (4 m, 5f) *n*(1/2M-CON) = 10 (5 m, 5f) *n*(0M-MIA) = 12 (4 m, 8f) *n*(1/2M-MIA) = 7 (4 m, 3f)	Fetal stage (gestational day 17)

Independent cohorts (cohorts 1–4) of timed-pregnant mice and their offspring were generated via on-site breeding. For each cohort, the table summarizes the number of litters per group (corresponding to the number of dams in each birth condition or treatment group), the number of offspring (m, males; f, females) in each experimental group, and the offspring's age of testing. Offspring in cohort 1 were used to examine possible effects of the C-section (CS) delivery *per se*, and therefore, this cohort encompassed a comparison of offspring that were born vaginally to non-treated dams (VD) *versus* offspring that were delivered by near-term C-section (CS) of non-treated dams. Offspring in cohort 2 were all delivered by near-term C-section after poly(I:C)-induced maternal immune activation (MIA) or vehicle control treatment (CON) on gestational day 12. Animals in cohort 3 and 4 were generated to quantify fetal brain levels of inflammatory cytokines on gestation day 12 and 17, that is, 3 hrs and 5 days after MIA, respectively.

term dams on GD 20 were killed by cervical dislocation, after which they were immediately placed on a heating pad (34–35 °C). The abdomen was then carefully opened using surgical scissors, and the uterus was rapidly disconnected from the maternal blood supply (within 20–30 s after cervical dislocation). Each pup was gently squeezed out of the opened yolk sac and immediately placed on the heating pad, where the umbilical cord was removed. While remaining on the heating pad, each pup was softly massaged for 15 min by using cotton swabs to remove liquid from their lungs and to stimulate breathing. Afterwards, the pups were brought together with foster mothers of the same treatment. C-sectioned offspring were weaned on PND 21 as described above.

A total of 8 animals died or were euthanized within 7 days after delivery by C-section (3 in cohort 1; 5 in cohort 2), which corresponded to less than 8 % of all animals delivered by C-section.

2.4. Collection of fetal brain tissue

The dissection of fetuses on GD 12 (cohort 3) or GD 17 (cohort 4) followed protocols described in detail elsewhere (Mueller et al., 2019). In brief, pregnant mice were decapitated using surgical scissors while being anesthetized with isoflurane, and the abdominal cavity was exposed to collect the uterus, which was placed into a petri dish filled with ice-cold PBS. Decidual tissue and yolk sac were then removed from individual fetuses, and fetuses were further dissected to obtain fetal brain tissue. Subsequently, the isolated fetal brain tissue was placed into Eppendorf tubes, snap-frozen and stored at −80 °C until further use. Individual fetuses from both sides of the uterine horns were collected, thereby noting the intrauterine position of each fetus (see below).

The GD 12 sampling interval for fetal brain collection was chosen in order to measure pro- and anti-inflammatory cytokine responses shortly after MIA. For this interval, fetal brain samples were collected 3 hrs after poly(I:C) or vehicle treatment on GD 12. In addition, fetuses were collected on GD17, that is, 5 days after poly(I:C) or vehicle treatment, in order to measure post-acute cytokine responses in fetal brains.

2.5. Determination of sex and intrauterine positions

The sex of each of the fetal sample was determined using PCR of fetal tail samples to detect male-specific sequence (Sry) according to previously established protocols (Lambert et al., 2000). The sex of the near-term offspring that were delivered by C-section was determined by the anogenital distance index (Hotchkiss and Vandenbergh, 2005). We used the 0M-2M classification system (Ryan and Vandenbergh, 2002) to determine the intrauterine positions of male and female offspring *in utero*. As schematically illustrated in Fig. 1, this classification system refers to the number of males flanking a given male or female fetus in the uterus. Hence, a male or female fetus that was flanked by one and two male fetuses was referred to as 1M and 2M, respectively, whereas a male or female fetus that was flanked by female fetuses only was referred to as 0M (Fig. 1).

In our study, we included the animals in the intrauterine end positions as well. Because animals positioned at either end of the uterine horns cannot be assigned to the 2M condition, our sampling strategy reduced and increased the likelihood of sampling 2M and 0M animals in the entire litter, respectively (Vandenbergh and Huggett, 1994). We combined 1M and 2M offspring into a 1/2M condition for comparison with the 0M condition, as we were primarily interested in exploring possible differences between conditions in which fetuses were flanked (1/2M) or not flanked (0M) by any males.

2.6. Behavioral testing

Behavioral testing commenced when the offspring (cohorts 1 and 2) reached 12 weeks of age and included tests for social interaction and

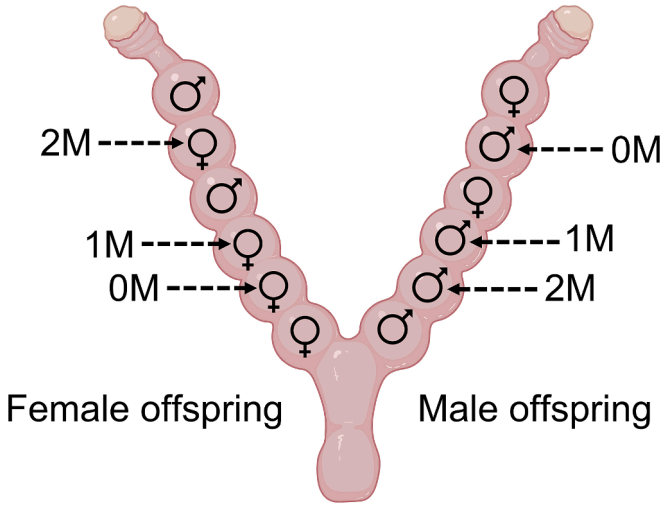

Fig. 1. Schematic illustration of the 0M-2M classification system used to determine the intrauterine positions of male and female offspring. This classification system refers to the number of males flanking a given female (left) or male (right) fetus in the uterus. Hence, a male or female fetus that is flanked by one or two male fetuses is referred to as 1M or 2M, respectively, whereas a male or female fetus that is flanked by female fetuses only is referred to as 0M.

prepulse inhibition (PPI) of the acoustic startle reflex. These tests were selected because of their relevance to neurodevelopmental disorders (Meyer et al., 2009) and because of their wide use in animal models of MIA (Brown and Meyer, 2018, Kentner et al., 2019; Weber-Stadlbauer and Meyer, 2019). All animals were first tested in the social interaction test, followed by the PPI test. A testing-free resting period of 4 days was interposed between the two tests.

2.6.1. Social interaction test

Social interaction was assessed by analyzing the relative exploration time between an unfamiliar congenic mouse and an inanimate dummy object using methods established before (Weber-Stadlbauer et al., 2017; Mueller et al., 2018). The test apparatus was made of transparent Plexiglas and consisted of three identical arms (50 cm × 9 cm; length × width) surrounded by 10-cm high Plexiglas walls. The three arms radiated from a central triangle (8 cm on each side) and spaced 120° from each other. Two out of the three arms contained a rectangular wire grid cage (13 cm × 8 cm × 10 cm, length × width × height; bars horizontally and vertically spaced 9 mm apart). The third arm did not contain a metal wire cage and served as the start zone (see below).

All animals were first habituated to the test apparatus on the day before social interaction testing. This served to familiarize the test animals with the apparatus and to reduce novelty-related locomotor hyperactivity, which may potentially confound social interaction during the critical test phase. The rectangular wire cages (located at the end of two arms) were left empty during the habituation phase. During habituation, each test mouse was gently placed in the start arm and allowed to explore the apparatus for 5 min.

The test phase took place one day after the habituation day. During the test phase, one metal wire cage contained an unfamiliar C57BL6/N mouse of the same sex (10–12 weeks of age), whereas the other wire cage contained an inanimate dummy object. The latter was a black scrunchie made of velvet material. The allocation of the unfamiliar live mouse and inanimate dummy object to the two wire cages was counterbalanced across experimental groups. To start a test trial, the test mouse was gently placed in the start arm and allowed to explore freely for 5 min. Behavioral observations were made by an experimenter who was blinded to the experimental conditions, and social interaction was defined as nose contact within a 2-cm interaction zone. For each animal, a social preference index was calculated by the formula ([time spent with the mouse] / [time spent with the inanimate object + time spent with the mouse]) - 0.5. The social preference index was used to compare the relative exploration time between the unfamiliar mouse and the inanimate dummy object, with values > 0 signifying a preference towards the unfamiliar mouse. In addition, the absolute times spent with the unfamiliar mouse and the inanimate dummy object were analyzed.

2.6.2. Prepulse inhibition test

PPI of the acoustic startle reflex refers to the reduction in startle reaction in response to a startle-eliciting pulse stimulus when it is shortly preceded by a weak prepulse stimulus. The apparatus consisted of four startle chambers for mice (San Diego Instruments, San Diego, CA, USA) and has been fully described elsewhere (Weber-Stadlbauer et al., 2017; Mueller et al., 2018). In the demonstration of PPI, the animals were presented with a series of discrete trials comprising a mixture of four trial types. These included pulse-alone trials, prepulse-plus-pulse trials, prepulse-alone trials, and no-stimulus trials in which no discrete stimulus other than the constant background noise was presented. The pulse and prepulse stimuli used were in the form of a sudden elevation in broadband white noise level (sustaining for 40 and 20 ms, respectively) from the background (65 dB_A), with a rise time of 0.2–1.0 ms. In all trials, three different intensities of pulse (100, 110, and 120 dB_A) and three intensities of prepulse (71, 77, and 83 dB_A, which corresponded to + 6, +12, and + 18 dB_A above background, respectively) were used. The stimulus-onset asynchrony of the prepulse and pulse stimuli on all prepulse-plus-pulse trials was 100 ms (onset-to-onset).

The protocol used for the PPI test was extensively validated before (Weber-Stadlbauer et al., 2017; Mueller et al., 2018). A session began with the animals being placed into the Plexiglas enclosure. They were acclimatized to the apparatus for 2 min before the first trial began. The first 6 trials consisted of 6 startle-alone trials; such trials served to habituate and stabilize the animals' startle response and were not included in the analysis. Subsequently, the animals were presented with 10 blocks of discrete test trials. Each block consisted of the following: three pulse-alone trials (100, 110, or 120 dB_A), three prepulse-alone trials (71, 77, or 83 dB_A), nine possible combinations of prepulse-plus-pulse trials (3 levels of pulse × 3 levels of prepulse), and one no stimulus trial. The 16 discrete trials within each block were presented in a pseudorandom order, with a variable interval of 15 s on average (ranging from 10 to 20 s). For each of the three pulse intensities (100, 110, or 120 dB_A) and three prepulse intensities (71, 77, or 83 dB_A), PPI was indexed by percent PPI calculated as % PPI = [(pulse-alone) − (prepulse-plus-pulse)/(pulse-alone) × 100 %]. In addition to PPI, the reactivity to pulse-alone trials was analyzed in order to examine possible group differences in acoustic startle reactivity *per se*.

2.7. Measurements of cytokines and testosterone in fetal brains

Fetal brain samples were thawed in ice-cold Roche complete lysis buffer (cOmpleteTM Lysis-M, Sigma-Aldrich, Switzerland). Upon complete thawing, the brain samples were lysed with a tissue lyser (Tissue Lyser II, Qiagen) for 3 min at a frequency of 20/s. After centrifugation (12,000 rpm at 4 °C for 20 min), the supernatant was removed and frozen at −80 °C until the cytokine and testosterone assays were performed.

Protein levels of IL-1β, IL-6, IL-10, and TNF-α were quantified using a customized Meso-Scale Discovery (MSD) V-Plex electrochemoluminescence assay (MSD, Rockville, Maryland, USA) for mice as previously described (Mueller et al., 2018, 2019). The plates were read using the SECTOR PR 400 (MSD) imager and analyzed using MSD's Discovery Workbench analyzer and software package. All assays were run in duplicates according to the manufacturer's instructions. The detection limits were 0.04 pg/ml for IL-1β, 0.80 pg/ml for IL-6, 1.25 pg/ml for IL-10, and 0.21 pg/ml for TNF-α. All cytokines measured in supernatants were expressed and analyzed as pg/mg total protein in fetal brains.

The levels of testosterone in fetal brains were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Mouse/Rat Testosterone ELISA Kit, Abcam plc, Cambridge, UK; catalog nr. Ab285350). The assay was run in duplicates according to the manufacturer's instructions, with minor modifications. In brief, brain samples were diluted 2-fold in the assay diluent provided with the kit. The goat anti-rabbit IgG-precoated wells were incubated at room temperature for 60 min with 25 µl of testosterone standards, negative control solutions, or diluted samples, along with 100 µl of testosterone-horseradish peroxidase conjugate and 50 µl of rabbit anti-testosterone reagent. After washing, 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to each well. 50 µl of stop solution was added after 15 min of incubation at room temperature. The samples were read with a Spark® multimode microplate reader (Tecan group AG, Männedorf, Switzerland) set to a wavelength of 450 nm. The detection limit of the assay was 0.10 ng/ml.

2.8. Statistical analyses

All statistical analyses were performed using SPSS Statistics (version 29.0, IBM, Armonk, NY, USA) and Prism (version 10.0; GraphPad Software, La Jolla, California), with statistical significance set at $p < 0.05$. The social preference index of offspring in cohort 1 was analyzed using 2 × 2 (mode of delivery × sex) analysis of variance (ANOVA), whereas absolute exploration times in the social interaction test were analyzed by 2 × 2 × 2 (mode of delivery × sex × object) ANOVA.

Acoustic startle reactivity and % PPI scores obtained from cohort 1 offspring were analyzed using 2 × 2 × 3 (mode of delivery × sex × pulse intensity) and 2 × 2 × 3 × 3 (mode of delivery × sex × prepulse intensity × pulse intensity) repeated-measures ANOVA, respectively. The social preference index of offspring in cohort 2 was analyzed using 2 × 2 × 2 (prenatal treatment × sex × intrauterine position) ANOVA, whereas absolute exploration times in the social interaction test were analyzed by 2 × 2 × 2 × 2 (prenatal treatment × sex × intrauterine position × object) ANOVA. In addition, one sample t-tests with a hypothetical value of 0 were used to ascertain whether the social preference scores were significantly above the chance level of 0. Acoustic startle reactivity and % PPI scores obtained from cohort 2 offspring were analyzed using 2 × 2 × 2 × 3 (prenatal treatment × sex × intrauterine position × pulse intensity) and 2 × 2 × 2 × 3 × 3 (prenatal treatment × sex × intrauterine position × prepulse intensity × pulse intensity) repeated-measures ANOVA, respectively. Cytokine and testosterone levels in fetal brains were analyzed using 2 × 2 × 2 (prenatal treatment × sex × intrauterine position) ANOVAs. Tukey's post-hoc test for multiple comparisons were used after ANOVA whenever appropriate. Because the ANOVAs did not detect any significant interaction between sex and prenatal treatment and/or intrauterine position, the data were graphically depicted with the two sexes combined, whereby different graphical symbols were used for males and females. Correlative analyses between levels of testosterone and TNF-α in the fetal brains of male and female fetuses were conducted separately for MIA and control conditions using Pearson's product moment correlations.

3. Results

3.1. Comparison of adult behaviors after vaginal birth or delivery by C-section

We first examined whether the C-section protocol used in the present study has lasting effects on behaviors *per se*. To this end, we compared adult male and female mice that were born vaginally or delivered by C-section in the two tests of primary interest, namely social interaction and PPI of the acoustic startle reflex.

Regardless of sex, there were no significant differences between mice that were born vaginally or delivered by C-section in terms of the social preference index or absolute exploration times in the social interaction test (Fig. 2**A**). ANOVA of absolute exploration times only revealed a significant main effect of object ($F_{(1,75)} = 132.72$, $p < 0.001$), reflecting the overall preference of mice to spend more time interacting with an unfamiliar mouse as compared to an inanimate dummy object. Hence, C-section *per se* did not alter sociability at adult age.

There were also no significant group differences in the PPI test. As shown in Fig. 2**B**, the acoustic startle reactivity to pulse-alone trials was highly comparable between mice that were born vaginally and mice delivered by C-section. Acoustic startle reactivity increased as a function of pulse intensities, as supported by the main effect of pulse intensity ($F_{(2,150)} = 180.70$, $p < 0.001$). Likewise, the % PPI scores were equivalent between mice that were born vaginally and mice delivered by C-section (Fig. 2**C**). Repeated-measure ANOVA of % PPI only revealed a significant main effect of prepulse ($F_{(2,308)} = 151.82$, $p < 0.001$) and pulse ($F_{(2,308)} = 7.79$, $p < 0.01$), reflecting increasing levels of % PPI as a function of prepulse and pulse intensities, respectively. These findings show that the C-section protocol used here did not influence % PPI or acoustic startle responses in adult mice.

3.2. Intrauterine position influences behavioral outcomes after MIA

We investigated whether the intrauterine position of fetuses influences the nature and/or severity of MIA-induced behavioral changes at adult age. To this aim, offspring of poly(I:C)- or vehicle-treated dams were delivered by C-section in order to determine the intrauterine positions of male and female offspring *in utero*. They were then subjected to social interaction and PPI tests when they reached adult age.

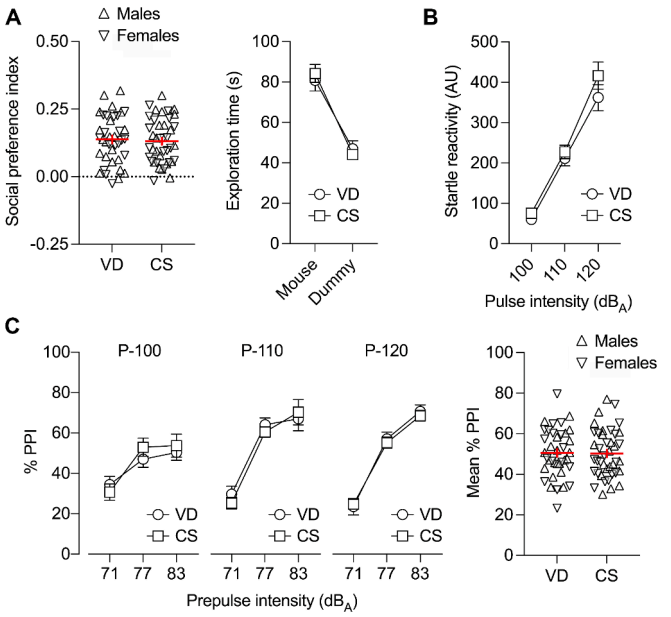

Fig. 2. Comparison of adult behaviors in mice after vaginal delivery (VD) or delivery by C-section (CS). (A) The scatter plot shows the social preference index (values > 0 represent a preference toward the unfamiliar mouse) in the social interaction test for individual male and female mice (with means ± s.e.m overlaid in red color). The line plot depicts the means ± s.e.m of absolute exploration times for the unfamiliar mouse (mouse) and inanimate dummy object (dummy). (B) The line plot depicts the acoustic startle reactivity (in arbitrary units, AU; means ± s.e.m) to 100-, 110- and 120-dB$_A$ pulse stimuli. (C) The line plots show % PPI (means ± s.e.m) as a function of prepulse intensity (71, 77 and 83 dB$_A$) for each of the three pulse conditions (P-100, P-110 and P-120, which correspond to pulse intensities of 100, 110 and 120 dB$_A$). The scatter plots depict the mean % PPI for individual male and female mice (with means ± s.e.m overlaid in red color) across all prepulse and pulse conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Regardless of sex, the social preference index was influenced by intrauterine position and MIA, as supported by the significant main effects of intrauterine position ($F_{(1,57)} = 36.85$, $p < 0.001$) and prenatal treatment ($F_{(1,57)} = 19.94$, $p < 0.001$), as well as their interaction ($F_{(1,57)} = 6.71$, $p < 0.05$) in the ANOVA of the social preference index. As shown in Fig. 3**A**, MIA-exposed offspring developing between female fetuses only (i.e., 0M-MIA offspring) displayed the lowest social preference index and were significantly different from MIA-exposed offspring developing between one or two male fetuses (i.e., 1/2M-MIA offspring; $p < 0.001$), or from control offspring developing under either *in utero* condition (0M-CON or 1/2M-CON offspring; both $p < 0.001$). The social preference index of 1/2M-MIA offspring did not differ from CON conditions, demonstrating that the presence of one or two flanking male fetuses attenuated the adult emergence of social interaction deficits after MIA (Fig. 3**A**). Interestingly, there was also a small but significant ($p < 0.05$) difference between 0M-CON and 1/2M-CON offspring, with the latter showing an increase in the social preference index as compared to the former (Fig. 3**A**). There were no significant interactions involving the between-subjects variable of sex, suggesting that intrauterine position influenced the outcomes of sociability similarly in male and female offspring. Additional one sample t-tests with a hypothetical value of 0 were used to ascertain whether social preference scores were significantly above the chance level of 0. These analyses confirmed that the social preference scores were significantly above the chance level of 0 in the 0M-CON ($t_{(15)} = 7.86$, $p < 0.001$), 1/2M-CON ($t_{(17)} = 11.\ 60$, $p < 0.001$), and 1/2M-MIA ($t_{(15)} = 11.76$, $p < 0.001$) conditions, but not in the 0-MIA condition ($t_{(14)} = 0.09$, $p = 0.93$).

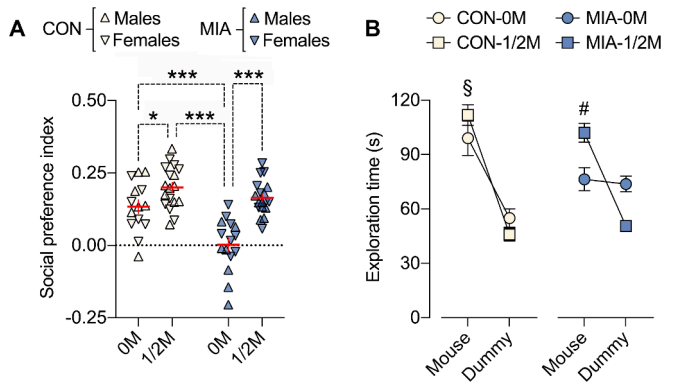

Fig. 3. Influence of intrauterine position on sociability after maternal immune activation (MIA) or control treatment (CON). Sociability was compared between adult CON and MIA offspring developing between female fetuses only (0M) or between one or two male fetuses (1/2M). (A) The scatter plot shows the social preference index in the social interaction test for individual male and female mice, with means ± s.e.m overlaid in red color. *$p < 0.05$ and ***$p < 0.001$, reflecting significant group differences based on Tukey's post-hoc test. (B) The line plot depicts the means ± s.e.m of absolute exploration times for the unfamiliar mouse (mouse) and inanimate dummy object (dummy) in the social interaction test. $^{§}p < 0.001$, reflecting the significant difference between mouse and dummy exploration times in the CON-0M and CON-1/2M groups, based on Tukey's post-hoc tests; $^{\#}p < 0.001$, reflecting the significant difference between mouse and dummy exploration times in the MIA-1/2M group, based on Tukey's post-hoc test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Consistent results were also obtained when comparing the absolute times spent interacting with the unfamiliar mouse or the inanimate dummy object in the social interaction test. ANOVA of absolute exploration times revealed a significant main effect of object ($F_{(1,57)} = 152.41$, $p < 0.001$), significant 2-way interactions between prenatal treatment and object ($F_{(1,57)} = 18.44$, $p < 0.001$) and between intrauterine position and object ($F_{(1,57)} = 29.25$, $p < 0.001$), as well as a significant 3-way interaction between prenatal treatment, intrauterine position, and object ($F_{(1,57)} = 4.47$, $p < 0.05$). Subsequent post-hoc tests confirmed that 0M-CON and 1/2M-CON offspring spent significantly ($p < 0.001$) more time with the unfamiliar mouse relative to the dummy object (Fig. 3**B**). A significant ($p < 0.001$) preference towards the unfamiliar mouse was also observed for 1/2M-MIA offspring, but not for 0M-MIA offspring (Fig. 3**B**), confirming that MIA disrupted sociability specifically in offspring developing between female fetuses only.

We also identified intrauterine position effects in the modulation of PPI of the acoustic startle reflex after MIA. ANOVA of % PPI revealed a significant main effect of intrauterine position ($F_{(1,57)} = 5.30$, $p < 0.05$) and treatment ($F_{(1,57)} = 11.69$, $p < 0.001$), as well as a significant 2-way interaction between prenatal treatment and intrauterine position ($F_{(1,57)} = 8.63$, $p < 0.01$). Post-hoc comparisons confirmed that MIA-exposed offspring developing between female fetuses only (i.e., 0 M-MIA offspring) displayed a significant reduction in mean % PPI compared to all other groups (all $p < 0.01$; Fig. 4**B**). This PPI deficit mainly stemmed from effects emerging under the 100- and 110-dB_A pulse conditions (Fig. 4**A**), as indicated by the significant 3-way interaction between prenatal treatment, intrauterine position, and pulse ($F_{(2,228)} = 3.18$, $p < 0.05$). Post-hoc tests confirmed that 0M-MIA offspring significantly differed from 1/2M-MIA offspring in the 100-dB_A ($p < 0.01$) and 110-dB_A ($p < 0.05$) pulse conditions. In these two pulse conditions, 0 M-MIA offspring also significantly differed from control offspring developing under either *in utero* condition (0M-CON or 1/2M-CON offspring; all $p < 0.01$). There were no significant interactions involving the between-subjects variable of sex in the analysis of % PPI, suggesting intrauterine position similarly influenced % PPI in male and female offspring.

As expected (Scarborough et al., 2019), the acoustic startle reactivity to pulse-alone stimuli generally increased with increasing pulse intensities, leading to a significant main effect of pulse intensity ($F_{(2,114)} = 177.38$, $p < 0.001$). 0M-MIA and 1/2M−MIA offspring did not differ with regards to acoustic startle reactivity (Fig. 4**C**). Interestingly, however, control offspring displayed reduced startle reactivity to pulse-alone trials when they developed in the absence of flanking male fetuses (i.e., 0M-CON offspring), as compared to control offspring developing between one or two male fetuses (i.e., 1/2M−CON offspring; Fig. 4**C**). Statistical support for this finding was obtained by the significant interaction between prenatal treatment and intrauterine position ($F_{(1,57)} = 4.11$, $p < 0.05$) in the ANOVA of startle reactivity, and by subsequent post-hoc comparisons confirming a significant difference between 0M-CON and 1/2M−CON offspring ($p < 0.05$; Fig. 4**C**).

3.3. Intrauterine position influences post-acute TNF-α responses to MIA

In a next step, we investigated whether the intrauterine positions of male and female offspring *in utero* influence the nature and/or severity of fetal cytokine responses to MIA. To this end, we quantified the fetal brain levels of IL-1β, IL-6, IL-10, and TNF-α at 3 hrs or 5 days after poly(I:C) or vehicle exposure, which reflected the acute and post-acute phases of MIA relative to control treatment, respectively.

As summarized in Fig. 5**A**, intrauterine position did not influence the cytokine responses in fetal brains during the acute phase of MIA (3 hrs post-treatment). At this sampling interval, MIA increased fetal brain levels of IL-6, IL-10, and TNF-α similarly in 0M-MIA and 1/2M−MIA offspring, as supported by the significant main effect of prenatal treatment in the ANOVA of IL-6 ($F_{(1,64)} = 94.54$, $p < 0.001$), IL-10 ($F_{(1,64)} = 13.85$, $p < 0.001$), and TNF-α ($F_{(1,64)} = 72.70$, $p < 0.001$). There were no significant main effects or interactions involving the between-subjects variables of intrauterine position and/or sex, indicating that the acute fetal cytokine responses were not influenced by these factors.

At 5 days post-treatment, however, we observed a significant interaction between prenatal treatment and intrauterine position ($F_{(1,30)} = 4.96$, $p < 0.05$) in the analysis of TNF-α, indicating that intrauterine position influences post-acute responses of this cytokine. Indeed, at this sampling interval, the fetal brain levels of TNF-α were still increased specifically in 0M-MIA offspring, as compared to 1/2M-MIA offspring and 0M-CON or 1/2M-CON offspring (Fig. 5**B**; all $p < 0.01$ in post-hoc tests). The elevation of TNF-α in the fetal brains of 0M-MIA offspring was not influenced by sex, as there was no significant interaction involving the between-subjects variable of sex in the analysis of this cytokine. There were no significant effects in terms of IL-1β, IL-6, and IL-10, indicating that MIA and/or intrauterine position did not cause lasting changes in these cytokines during the post-acute phase of MIA.

3.4. Intrauterine position influences testosterone levels in fetal brains

We also quantified testosterone levels in fetal brains 5 days after poly(I:C) or vehicle treatment (i.e. on GD 17), as this sampling interval coincides with the gestational phase showing peak levels of fetal testosterone in the mouse (Pointis et al., 1979). Irrespective of sex and intrauterine position, MIA did not affect fetal brain testosterone levels; and there were no significant main effects or interactions involving the between-subjects factor of prenatal treatment. Fetal brain testosterone levels were, however, influenced by intrauterine position and sex (Fig. 6**A**), as indicated by the significant main effect of intrauterine position ($F_{(1,30)} = 34.94$, $p < 0.001$) and sex ($F_{(1,30)} = 9.60$, $p < 0.01$), as well as their interaction ($F_{(1,30)} = 10.69$, $p < 0.01$). When restricting the analysis of fetal brain testosterone to a comparison between males and females in the vicinity of 1/2M fetuses, we found significantly increased testosterone levels in male fetuses with 1/2M neighbors as compared to female fetuses with 1/2M neighbors ($t_{(13)} = 3.91$, $p < 0.01$), confirming a significant sex effect in the 1/2M condition (**Supplementary Fig. S1**). When restricting the analysis to a comparison between males and

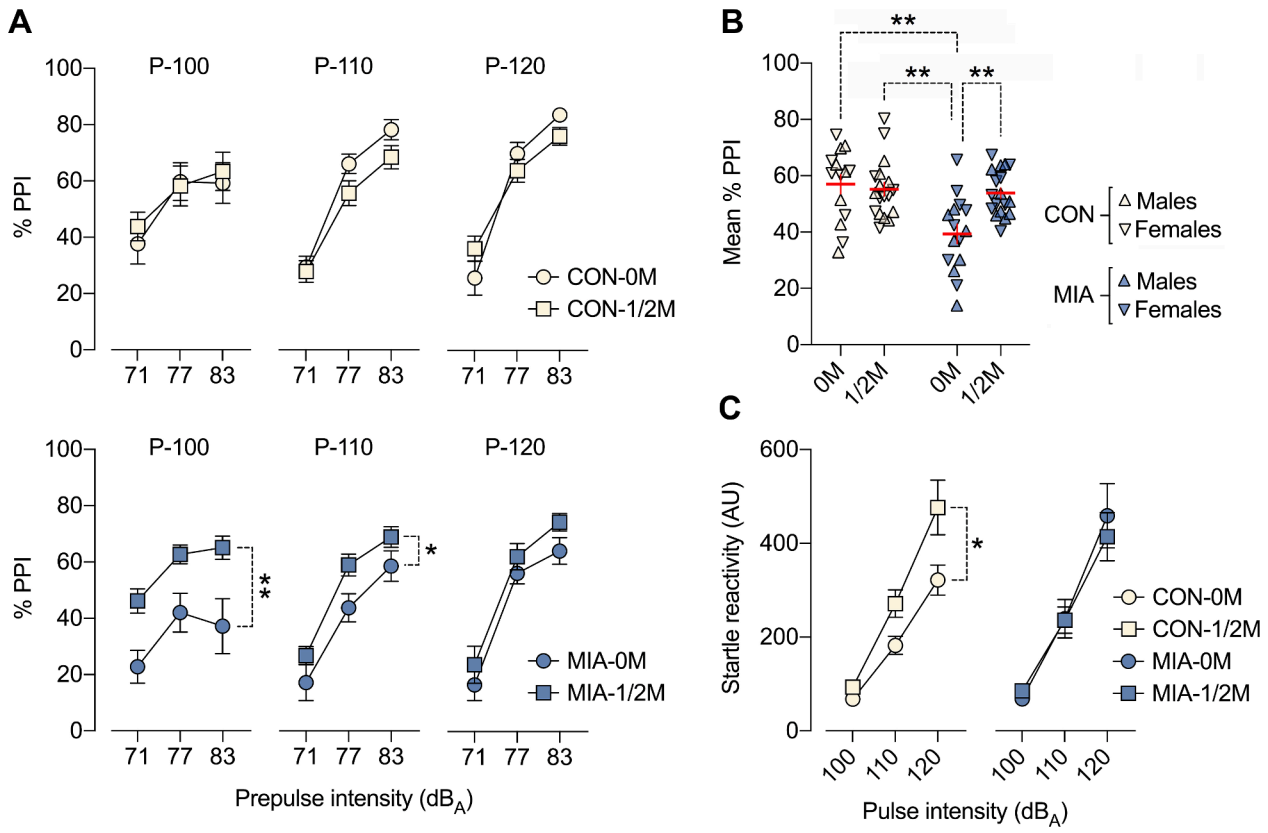

Fig. 4. Influence of intrauterine position on prepulse inhibition (PPI) after maternal immune activation (MIA) or control treatment (CON). PPI of the acoustic startle reflex was compared between adult CON and MIA offspring developing between female fetuses only (0M) or between one or two male fetuses (1/2M). (A) The line plots show % PPI (means ± s.e.m) as a function of prepulse intensity (71, 77 and 83 dB_A) for each of the three pulse conditions (P-100, P-110 and P-120, which correspond to pulse intensities of 100, 110 and 120 dB_A). *$p < 0.05$ and **$p < 0.01$, reflecting the significant difference between the MIA-0M and MIA-1/2M groups in the P-100 and P-110 conditions, based on Tukey's post-hoc test. (B) The scatter plot depicts the mean % PPI for individual male and female mice (with means ± s.e.m overlaid in red color) across all prepulse and pulse conditions. **$p < 0.01$, reflecting significant group differences based on Tukey's post-hoc test. (C) The line plot depicts the acoustic startle reactivity (in arbitrary units, AU; means ± s.e.m) to 100-, 110- and 120-dB_A pulse stimuli. *$p < 0.05$, reflecting the significant difference between the CON-0M and CON-1/2M groups, based on Tukey's post-hoc test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

females in the vicinity of 0M fetuses, however, there was no significant difference between males and females anymore ($t_{(19)} = 0.09$, n.s.; **Supplementary Fig. S1**). These data demonstrate that intrauterine position has a stronger influence on testosterone levels in the fetal brain as compared to the sex of the fetuses *per se* (Fig. 6**A**).

Since intrauterine position influenced fetal brain levels of TNF-α during the post-acute phase of MIA (Fig. 5**B**), we also investigated possible relationships between levels of testosterone and TNF-α in the fetal brains in late gestation (i.e., on GD 17) using Pearson's product moment correlations. As shown in Fig. 6**B**, there was a significant ($r = -0.51$, $p < 0.05$, $n = 19$) negative correlation between fetal brain testosterone and TNF-α in the MIA condition, but not in the CON condition ($r = 0.01$, $p > 0.9$, $n = 19$). These findings demonstrate that there is a significant association between fetal testosterone and TNF-α under inflammatory but not control conditions.

4. Discussion

Our study demonstrates that the intrauterine position of fetuses influences the experimental outcomes in a widely used mouse model of viral-like MIA. Determining intrauterine positions using delivery by near-term C-section, we found that MIA-exposed offspring developing between female fetuses only displayed significant deficits in sociability and sensorimotor gating at adult age, whereas MIA-exposed offspring developing between one or two males *in utero* did not show the same deficits. These intrauterine position effects similarly emerged in male and female offspring. Hence, developing adjacent to male fetuses *in utero* appeared to attenuate the effects of MIA on adult behaviors reported to be impaired in these models (Brown and Meyer, 2018; Kentner et al., 2019; Weber-Stadlbauer and Meyer, 2019; Mueller et al., 2021). Furthermore, while MIA elevated fetal brain levels of pro- and anti-inflammatory cytokines independently of the precise intrauterine position and sex of adjacent fetuses during the acute phase, fetal brain levels of TNF-α remained elevated in 0M-MIA but not in 1/2M−MIA offspring until the post-acute phase in late gestation. These findings suggest that intrauterine position can also influence the time course of MIA-induced fetal cytokine responses, such that fetuses developing adjacent to males *in utero* show faster recovery of post-acute TNF-α responses as compared to fetuses developing in-between females only. Taken together, our findings identify a novel source of within-litter variability contributing to heterogeneous outcomes of short- and long-term effects in a mouse model of MIA.

Our data are consistent with and extend previous studies showing that intrauterine positions contribute to variability in mouse models of other prenatal adversities, including prenatal maternal stress and fetal growth restriction (vom Saal et al., 1990; D'Errico et al., 2021). Heterogeneity in experimental outcomes can introduce challenges in terms of reproducing and comparing MIA models across independent research laboratories (Kentner et al., 2019; Weber-Stadlbauer and Meyer, 2019; Meyer, 2023). Even within well-designed and well-controlled MIA models, such heterogeneity can be quite large and minimizes or even nullifies simple group differences, especially when the experimental

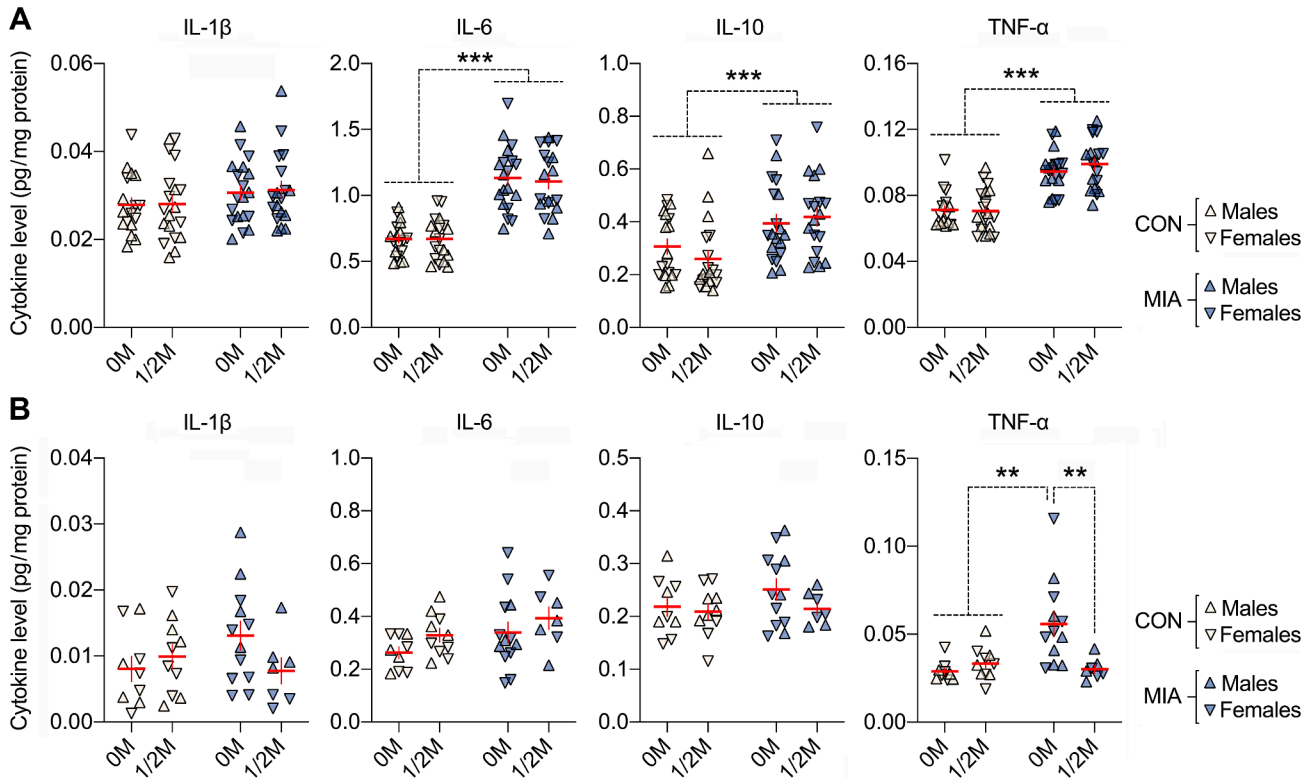

Fig. 5. Influence of intrauterine position on fetal cytokine responses to maternal immune activation (MIA) or control treatment (CON). The levels of fetal brain cytokines were compared between CON and MIA offspring developing between female fetuses only (0M) or between one or two male fetuses (1/2M). (A) Acute cytokine responses measured 3 hrs after CON or MIA exposure. The scatter plots show cytokine levels for individual male and female fetuses, with means ± s.e.m overlaid in red color. ***$p < 0.001$, reflecting the significant main effect of MIA based in ANOVA. (B) Post-acute cytokine responses measured 5 days after CON or MIA exposure. The scatter plots show cytokine levels for individual male and female fetuses, with means ± s.e.m overlaid in red color. **$p < 0.01$, reflecting the significant difference between 0M-MIA and all other groups, based on Tukey's post-hoc test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

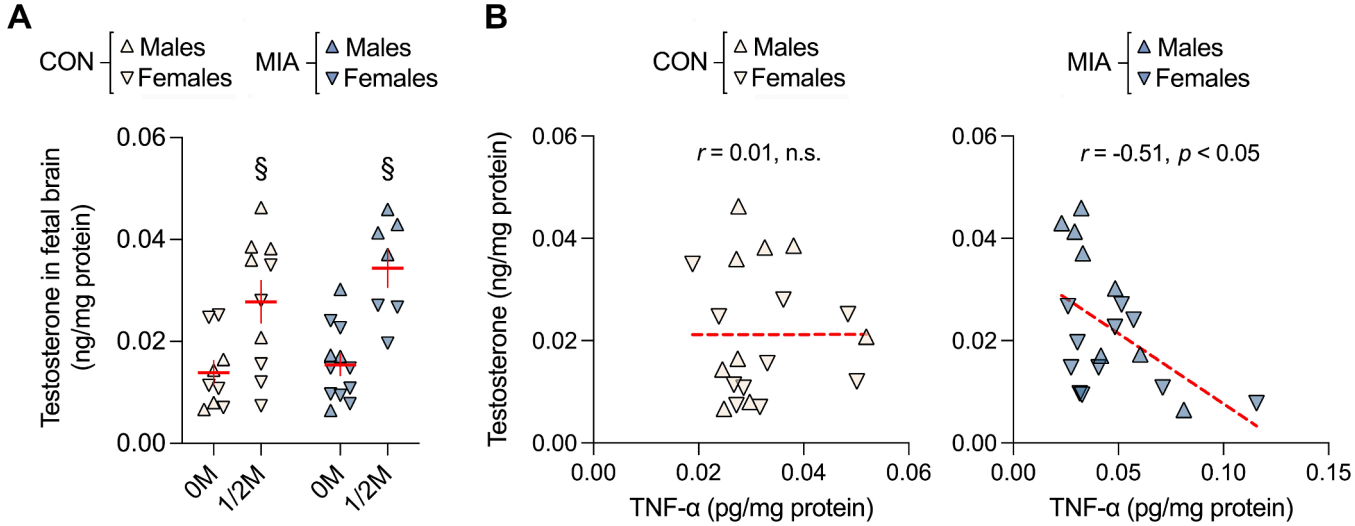

Fig. 6. Influence of intrauterine position on fetal testosterone levels after maternal immune activation (MIA) or control treatment (CON) and correlation with TNF-α in the fetal brains. (A) The scatter plot shows testosterone levels in the fetal brains of individual male and female fetuses developing between female fetuses only (0M) or between one or two male fetuses (1/2M), with means ± s.e.m overlaid in red color. §$p < 0.001$, reflecting the significant main effect of intrauterine position in ANOVA. (B) Pearson's product moment correlations between levels of testosterone and TNF-α in the fetal brains of male and female fetuses exposed to CON (left) or MIA (right); r = Pearson correlation coefficient. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

groups are considered as being homogenous entities (Estes et al., 2020; Haddad et al., 2020; Mueller et al., 2021; Lorusso et al., 2022). The challenges associated with outcome heterogeneity appear particularly pronounced when MIA, or any other prenatal manipulation, is implemented in multiparous species such as rats and mice, where both between- and within-litter effects can influence the experimental outcomes

of interest (Zorrilla, 1997; Meyer et al., 2009; Lazic and Essioux, 2013; Spencer and Meyer, 2017). Model variability does not, however, necessarily undermine the utility of MIA models for translational research, as it offers unique opportunities for new discoveries and developments in this field, including the identification of disease pathways and molecular mechanisms determining susceptibility and resilience to MIA (Estes et al., 2020; Mueller et al., 2021; Purves-Tyson et al., 2021). While different research groups may have their own strategies to handle the challenges (and make use) of variability in MIA models, continuous efforts are needed to determine the precise sources underlying such variability. By identifying intrauterine position effects in a widely used mouse model of MIA, our study adds to the existing knowledge on the factors impacting outcome variability in preclinical research of immune-mediated neurodevelopmental disorders. Moreover, our findings might provide an explanation as to why within-litter variation exists and contributes substantially to heterogenous outcomes in mouse models of MIA, as previously identified in terms of abnormal cortical and behavioral development (Vasistha et al., 2020; Mueller et al., 2021).

Interestingly, in addition to influencing MIA outcomes, the effects of intrauterine position were also noticeable in offspring of vehicle-treated control mothers. More specifically, we found that control offspring developing adjacent to one or two male fetuses displayed a higher social preference index and higher acoustic startle reactivity compared to control offspring developing exclusively between females *in utero*. While the precise biological significance of these effects remains to be established in future studies, these findings corroborate and extend previous studies demonstrating intrauterine position effects on adult behaviors, even in the absence of additional prenatal manipulations. For example, adult mice that were flanked by one or two males *in utero* were found to be more sensitive to the rewarding effects of opiates (Morley-Fletcher et al., 2003) and to display increased novelty seeking (Palanza et al., 2001), as compared to adult mice that developed between female fetuses only. Similar intrauterine position effects were also found with respect to saccharin preference in mice (Bushong and Mann, 1994). The fact that behavioral traits can be influenced by the precise fetal position even in the absence of specific prenatal manipulations adds an additional layer of complexity to research studying the effects of prenatal manipulations such as MIA in litter-bearing mammals. Hence, it is possible that prenatal manipulations in multiparous species interact with individual fetal differences that arise from pre-existing intrauterine position effects.

In agreement with previous findings in mice (vom Saal and Bronson, 1980; vom Saal et al., 1990), our study confirmed that fetal testosterone levels were higher in fetuses that were flanked by one or two males *in utero*, as compared to fetuses developing in-between females only. These differences are likely to stem from the transfer of testosterone from male fetuses to adjacent male or female fetuses, given that testosterone can diffuse through the amniotic fluid between fetuses (Ryan and Vandenbergh, 2002). Interestingly, clear sex differences in fetal brain testosterone were only seen in males *versus* females in the 1/2M condition, but not when they developed in the vicinity of 0M fetuses, suggesting that sex *per se* had only a restricted influence on testosterone levels in the fetal brain during late gestation. Thus, our data suggest that the influence of intrauterine position on fetal brain testosterone is larger than the sex of the fetuses. This notion is in agreement with studies suggesting that there are surprisingly small effects of sex on testosterone levels in brain tissue, especially during ontogeny. For example, Konkle and McCarthy (2011) and Karaismailoglu et al. (2017) showed that male and female rat fetuses do not differ in terms of cortical, hippocampal and hypothalamic testosterone levels during late gestation, which corresponds to the time window of fetal brain sampling in our study. The lack of sex differences in our analyses of fetal brain cytokines and adult behaviors readily mirrors the relatively small effects of sex on fetal brain testosterone reported here and before (Konkle and McCarthy, 2011; Karaismailoglu et al., 2017). At the same time, the sex-independent effects of intrauterine position on fetal brain testosterone matches the observed influence of intrauterine position on fetal brain cytokines and adult behaviors, both of which emerged independently of sex as well.

In keeping with the sex-independent influence of intrauterine position on behavioral outcomes after MIA, our findings may tentatively be taken as circumstantial evidence suggesting that higher levels of testosterone in the fetal brain may confer resilience against the development of MIA-induced behavioral alterations in both male and female offspring. While this hypothesis seems plausible in view of our findings reported here and by others before (Zheng et al., 2022), more research will be needed to test this hypothesis more thoroughly. It should also be emphasized that our study included testosterone measurements in fetal brains only. Hence, our findings should not be generalized to fetal or postnatal testosterone levels in peripheral tissues, such as blood or gonads, which are known to be associated with more pronounced sex differences as compared to testosterone levels in brain tissue (Southren et al., 1965; Courant et al., 2010; Konkle and McCarthy, 2011; Clarkson and Herbison, 2016).

In addition to its influence on sexual differentiation and individualization of reproductive, social and aggressive traits (Kinsley et al., 1986; Ryan and Vandenbergh, 2002; Correa et al., 2013; Kawata, 2013), testosterone also modulates certain immune functions, including inflammation (Bianchi, 2018). Of note, testosterone has been shown to attenuate TNF-α responses under inflammatory conditions (Bobjer et al., 2013; Mohamad et al., 2019) and after infection with Zika virus (Zheng et al., 2022). This anti-inflammatory effect of testosterone appears to involve the extracellular-signal-regulated kinase (ERK) pathway (Chen et al., 2016) and IL-10 signaling (Olmos-Ortiz et al., 2019). Our findings demonstrating an inverse relationship between fetal brain testosterone and TNF-α levels are consistent with previous studies showing a negative correlation between testosterone and TNF-α in umbilical vein serum from newborns (Olmos-Ortiz et al., 2019) or serum from young men (Bobjer et al., 2013). In our study, however, the negative correlation between testosterone and TNF-α was only noticeable after MIA exposure, suggesting that this relationship emerged specifically under inflammatory conditions. These findings align with the above-mentioned hypothesis suggesting that prenatal manipulations could interact with pre-existing variations in fetal development. This suggests that the impact of intrauterine position on inflammatory responses may manifest under certain conditions only, such as following MIA or infection with Zika virus (Zheng et al., 2022), rather than uniformly across all circumstances.

IL-6 and IL-17a are thought to be major immune mediators of the link between MIA and neurodevelopmental disturbances (Smith et al., 2007; Choi et al., 2016). The apparent importance of these cytokines does not, however, exclude the involvement of other immune mediators as well. For example, blocking IL-1 receptor signaling during pregnancy was shown to protect against neurodevelopmental defects induced by MIA in mice (Girard et al., 2010), similarly to what has been observed after blocking IL-6 and IL-17a in MIA models (Smith et al., 2007; Choi et al., 2016). Moreover, Potter et al. (2023) recently demonstrated that prenatal TNF-α concentrations (rather than IL-6) predicted the development of adult behavioral deficits in a rat model of poly(I:C)-induced MIA. Our findings are consistent with the latter data and suggest that non-resolving TNF-α responses to MIA may play a critical role in precipitating lasting effects on brain and behavior. This notion is also supported by findings showing that brain TNF-α is persistently elevated in a subgroup of MIA-exposed offspring until adult age (Purves-Tyson et al., 2021). Whether or not non-resolving TNF-α responses are key in mediating lasting effects on brain and behavior after MIA warrants further investigations in future studies.

Some researchers have suggested that female offspring may generally be more resilient to the effects of MIA compared to male offspring (e.g. Arnold and Saijo, 2021; Guma and Chakravarty, 2024), although significant long-term consequences of MIA on brain and behavior have repeatedly been documented in both male and female offspring (e.g. Gogos et al., 2020; Kobayashi et al., 2021; Tartaglione et al., 2022; Martz et al., 2024). Consistent with the latter findings, our own stratification

studies using the poly(I:C)-based MIA model in mice did not find females to be more resilient than males in general (Mueller et al., 2021; Herrero et al., 2023). In fact, our recent findings suggest that male and female offspring of MIA-exposed mice can be similarly stratified into resilient and susceptible subgroups based on the absence (resilient) or presence (susceptible) of overt behavioral alterations in adulthood (Mueller et al., 2021; Herrero et al., 2023). Here, we show that MIA-exposed offspring developing between female fetuses only (i.e., 0M-MIA offspring) display significant deficits in sociability and sensorimotor gating at adult age, whereas MIA-exposed offspring developing between one or two males *in utero* (i.e., 1/2M−MIA offspring) did not show the same deficits. Importantly, these intrauterine position effects similarly emerged in male and female offspring and were associated with intrauterine position effects on fetal brain testosterone, with 1/2M offspring generally showing higher testosterone levels in the fetal brain during late gestation as compared to 0M offspring. The latter finding may be taken as circumstantial evidence suggesting that higher levels of testosterone in the fetal brain may confer resilience against the development of MIA-induced behavioral alterations. While this hypothesis seems indeed plausible in view of our findings reported here and by others before (Zheng et al., 2022), more research will be needed to test this hypothesis more thoroughly.

Because we did not assess circulating testosterone levels in amniotic fluid, our findings offer only a weak translation to epidemiological and clinical studies in humans, where circulating testosterone levels in amniotic fluid are taken to assess disease risk in offspring (Auyeung et al., 2010). Interestingly, in the with the prenatal sex steroid theory of autism, some studies have found a positive correlation between circulating testosterone in prenatal life and ASD traits in early childhood (Auyeung et al., 2010; Auyeung et al., 2012), and to a lesser extent in adolescence (Dooley et al., 2022). Other studies did not, however, confirm this association (Kung et al., 2016; Baron-Cohen et al., 2020), suggesting that there is no consistent relationship between prenatal testosterone exposure and subsequent development of ASD (Coscini et al., 2021). Interestingly, while a large epidemiological study using the Danish Biobank did not find a significant difference in amniotic fluid testosterone between children with or without ASD, the same study identified high prenatal levels of estradiol, estrone and progesterone to be associated with increased risk of ASD (Baron-Cohen et al., 2020). Hence, prenatal steroids beyond the masculinizing testosterone may play a role in the etiology of neurodevelopmental disorders such as ASD. Future research will be needed to explore whether prenatal steroids other than testosterone might shape risk and resilience to MIA.

In our study, we did not observe any significant influence of C-section delivery *per se*, at least when considering social functions and sensorimotor gating in non-treated control animals. These results are consistent with the findings reported by Chiesa et al. (2019), who did not reveal lasting effects of term- or near-term delivery by C-section on numerous behaviors, including social interaction, in mice. Other studies in mice or rats have, however, observed enduring effects of C-section delivery on sociability (Nagano et al., 2021) and other behavioral and cognitive functions (El-Khodor and Boksa, 1998; Morais et al., 2020). The discrepancy between these findings likely stems from differences in the C-section protocols used. Unlike Nagano et al. (2021), who cross-fostered entire litters to genetically distinct foster mothers, our study used a protocol in which offspring delivered by C-section were raised by genetically identical foster mothers that kept two of their own (vaginally born) pups and received six C-sectioned pups. Hence, our C-section protocol enabled co-housing with vaginally born mice with the same prenatal treatment history, which has been shown to correct long-term behavioral alterations induced by C-section and whole-litter fostering in mice (Morais et al., 2020). Importantly, even if the absence of C-section effects in our control experiments cannot be generalized to the conditions of MIA, we deem it very unlikely that the C-section would have influenced, or even confounded, the developmental trajectories in MIA animals specifically. Indeed, the effects of MIA observed in C-sectioned animals are consistent with our previous reports showing that significant deficits in sociability and PPI are manifest only in a subgroup of adult offspring born vaginally (Mueller et al., 2021; Herrero et al., 2023). If the C-section procedure influenced the MIA outcomes, it would have been unlikely to obtain these consistent results. Therefore, we conclude that the identified intrauterine position effects were unlikely to be confounded by the C-section procedure.

Our study emphasizes that knowledge of the precise intrauterine position can help interpret and compare the outcomes of MIA in multiparous species such as mice. Arguably, the use of C-section protocols in determining the intrauterine position of offspring might not always be practically feasible in these models. Measuring the anogenital distance (AGD) would offer an alternative method to ascertain the influence of prenatal androgen exposure due to intrauterine position effects in mice, given that offspring developing between male neighbors *in utero* have longer AGDs compared to those developing next to female neighbors only (Vandenbergh and Huggett, 1995; Ryan and Vandenbergh, 2002; Hurd et al., 2008). Hence, even if less precise than determining the intrauterine position through C-section procedures, ascertaining the AGD of individual mice in MIA models could be used to estimate the relative intrauterine position of offspring retrospectively without implementing invasive techniques. Adding this factor to statistical analyses may optimize the ability to identify the genuine consequences of MIA on brain and behavior in multiparous species.

We acknowledge a number of limitations of our study. First, we did not assess indices of maternal behavior towards the fostered pups (delivered by C-section) and own pups (delivered vaginally). Hence, we do not know whether possible differences in maternal behavior could have influenced behavioral measures in C-sectioned offspring beyond those assessed here. Second, as we were primarily interested in exploring possible differences between conditions in which fetuses were flanked (1/2M) or not flanked (0M) by any males, our study was not designed to enable additional comparisons between the 1M and 2M conditions. Indeed, since we included the animals at either end of the uterine horns as well, our sampling strategy reduced the likelihood of sampling 2M animals in the entire litter (Vandenbergh and Huggett, 1994). Hence, a technically sound comparison between the 1M and 2M conditions was not possible because of low sample sizes in the 2M condition. Despite this limitation, however, we believe that the comparison between the 0M and combined 1/2M conditions provided a novel set of data that are important for the field.

In conclusion, our study shows that intrauterine positions contribute to the heterogeneous outcomes in a mouse model of MIA. Our findings add to the growing list of known factors that can influence the nature and/or severity of experimental outcomes in MIA models (Kentner et al., 2019; Weber-Stadlbauer and Meyer, 2019; Meyer, 2023). In a broader context, our findings highlight that individual differences in fetal exposure to hormonal and inflammatory signals may be a perinatal factor that shapes risk and resilience to MIA. While the importance of susceptibility and resilience factors in the context of MIA is increasingly recognized (Meyer, 2019), the underlying cellular and molecular mechanisms remain elusive and warrant further investigation. The identification of mechanisms mediating susceptibility and resilience to MIA in animal models requires a careful consideration of factors influencing their experimental outcomes. By identifying a novel source of within-litter variability, the results presented here provide new knowledge that can be leveraged to improve the reproducibility and comparability of MIA models.

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CRediT authorship contribution statement

Ron Schaer: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Flavia S.**

Mueller: Writing – review & editing, Methodology, Investigation, Formal analysis. **Tina Notter:** Writing – review & editing, Supervision, Investigation, Funding acquisition. **Ulrike Weber-Stadlbauer:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Urs Meyer:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization.

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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