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Maternal immune activation with high molecular weight poly (I:C) induces selective depressive-like phenotype in adult offspring

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Maternal immune activation (MIA) during pregnancy may increase the risk for neurodevelopmental disorders such as schizophrenia and autism in offspring. Preclinical and human evidence support a potential role for MIA in the development of depressive symptoms in offspring. Among animal models of MIA, prenatal treatment with the synthetic viral mimetic polyinosinic-polycytidylic acid [poly (I:C)] is well established in studying psychotic-like and autism-like phenotypes, while its validity for modeling depressive-like behaviors remains underexplored. In this study, we assessed whether MIA, induced in rats with an injection of high molecular weight (HMW) poly (I:C), at gestational day 15, leads to a depressive-like phenotype in the offspring. In male and female offspring during adolescence and adulthood, we evaluated i) behavioral despair and anhedonia using the forced swim test (FST) and sucrose preference test (SPT); ii) the electrophysiological properties of dorsal raphe nucleus (DRN) serotonin (5-HT) neurons in vivo; iii) serum cytokine profile. We found that MIA offspring exhibited increased immobility and reduced climbing and swimming in the FST, with more pronounced effects in males, while sucrose preference remained unaltered. In vivo recordings revealed a significant increase in 5-HT neuron firing rate in MIA adult males. Peripheral cytokine analysis showed elevated IL-1α in MIA males and decreased GRO/KC levels in MIA females. In conclusion, these findings indicate that prenatal exposure to HMW poly (I:C) selectively affects stress-coping mechanisms without inducing anhedonia, modulates serotonergic signaling in a sex- dependent manner in the absence of widespread inflammatory alterations.

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INTRODUCTION

The global impact of the COVID-19 pandemic has underscored the significant burden posed by infectious diseases. Indeed, there is a growing scientific interest in understanding the effects of prenatal COVID-19 infections, and the consequent maternal immune activation (MIA), on the neurodevelopmental trajectories of offspring [1, 2]. Maternal infections during pregnancy have emerged as a significant risk factor for the onset of neuropsychiatric disorders, such as autism spectrum disorder and schizophrenia [3]. Studying SARS-CoV-2 infection directly in pregnant animal models could offer crucial insights into its impact on offspring neurodevelopment. However, this approach presents significant challenges, as it requires strict adherence to biosafety standards and technical precautions, as pioneering studies have shown using prenatal exposure to live pathogens, such as the influenza virus [4, 5]. Thus, one of the most characterized MIA models is based on the exposure during pregnancy to the polyinosinic-polycytidylic acid [poly (I:C)], a double-stranded synthetic RNA, which triggers an innate immune response by mimicking a viral infection. MIA models reliably replicate neurodevelopmental disruptions observed in humans, inducing neurodevelopmental disorders in the offspring [6, 7]. In our laboratory, we previously characterized a poly (I:C)-induced MIA model, demonstrating that inflammation triggered by MIA disrupts endocannabinoid system signaling [8], ultimately impairing dopaminergic function and contributing to the emergence of a schizophrenia-like phenotype in adult offspring across generations [9–11]. Moreover, we demonstrated that activation of peroxisome proliferator-activated receptor-α (PPARα) with the clinically available agonist fenofibrate attenuates the neurodevelopmental disturbances induced by MIA in rat offspring [12] by reducing the cytokine imbalance during pregnancy [13]. While the MIA model is well-established in studying psychotic-like and autism-like phenotypes [14, 15], its validity for modeling depressive-like behaviors remains underexplored. While some studies have suggested an association between MIA and depressive-like phenotype in offspring [16–20], the extent to which this relationship reflects a direct causal mechanism remains uncertain. Moreover, among the various challenges in MIA models, experimental conditions represent a crucial factor, as the type of poly (I:C) used (e.g. low molecular weight, LMW vs. high molecular weight, HMW); [21], the gestational timing of administration [22], and laboratory

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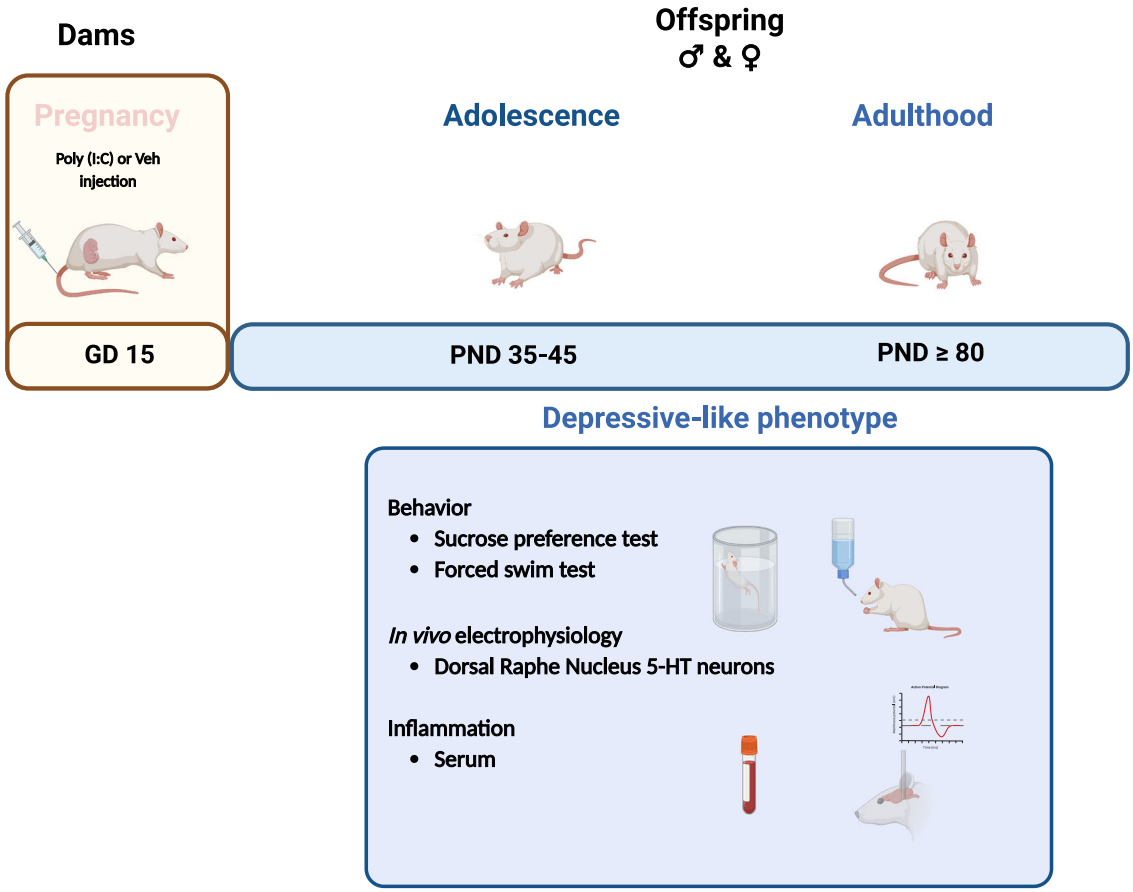

Fig. 1 Representation of the experimental protocol. Poly (I:C) injection during pregnancy consisted of a single i.v. injection of HMW poly (I:C) (4 mg/kg) or vehicle (sterile pyrogen-free saline) at GD15. Behavioral experiments, in vivo electrophysiology recordings were performed both during adolescence (PND 35-45) and adulthood (PND ≥ 80). A biochemical assay was performed during adulthood (PND 90). Figure 1 was created with BioRender.

environment coupled with the caging system [23, 24] play a key role in shaping developmental outcomes. Notably, a growing body of evidence highlights the importance of the molecular weight of poly (I:C) in modulating maternal and fetal immune responses, as well as pregnancy viability. Studies in mice have shown that HMW poly (I:C) induces stronger cytokine responses but is also associated with a higher rate of fetal loss, leading many laboratories to adopt LMW poly (I:C) as a refinement measure to reduce variability and ameliorate gestational outcomes [23, 24]. In contrast, our laboratory has consistently utilized HMW poly (I:C) in a rat model of MIA, with well-characterized and reproducible phenotypes [8–13, 25]. Moreover, rats appear less sensitive than mice to the effects of HMW poly(I:C) on abortion rate, allowing for robust and sustained maternal immune activation without compromising pregnancy viability.

Thus, we sought to determine whether MIA induced by HMW poly (I:C) (4 mg/kg, i.v.) at gestational day (GD) 15 contributes to the emergence of a depressive-like phenotype in male and female offspring. We investigated longitudinally the neurodevelopmental trajectories in male and female offspring from adolescence (postnatal days, PND 35-45) to adulthood (PND ≥ 80). To this end, we performed a behavioral characterization using the forced swim test and the sucrose preference test, two well-established paradigms for assessing depressive-like behavior, specifically behavioral despair and anhedonia, respectively [26, 27]. Additionally, we performed electrophysiological recordings of putative serotonin (5-HT) neurons in the dorsal raphe nucleus (DRN), critically involved in mood regulation, to investigate a neural correlate of depressive-like behavior [28, 29]. Due to the growing recognition of inflammation as a critical factor in the pathophysiology of depression [30–32], we further evaluated systemic inflammation by analyzing full cytokines/chemokines/growth factors profile in the serum of the male and female offspring. This study aims to elucidate whether prenatal exposure to HMW poly (I:C) at GD 15 in Sprague Dawley rats leads to a depressive-like phenotype in the offspring, providing novel insights into the long-term effects of MIA on mood-related behaviors and underlying neurobiological mechanisms.

METHODS

Animals

Female Sprague Dawley rats (Envigo, Italy) were mated at the age of 3 months. The first day after the copulation, which was confirmed through the presence of the vaginal plug, was defined as GD 0 [33]. Pregnant dams on GD 15 were randomly assigned to receive either a single injection of poly (I:C) or an equivalent volume of endotoxin-free saline solution in the lateral vein of the tail (vehicle). Offspring were weaned and sexed on PND 21. After weaning, offspring were housed with littermates and maintained undisturbed until experiments. Subsequently, rats were randomly assigned to experimental procedures and care was taken to avoid assigning more than three animals from the same litter to the same experimental group. In fact, for the experiments described here, a total of 25 dams were utilized [13 were treated with vehicle and 12 with poly (I:C)] (Fig. 1). The use of Sprague-Dawley rats ensures consistency with our prior works [8–10, 12, 13, 25, 34], enabling interpretation of the present findings within an established strain-specific phenotype. Behavioral tests and in vivo electrophysiological recordings were performed throughout

adolescence (PND 35-45) and during adulthood (PND ≥ 80). Blood for the analysis of cytokine levels was collected at PND 90. For cytokine measurements, rats were deeply anesthetized with isoflurane (5%) (Merial, Toulouse, France), sacrificed by decapitation, and trunk blood was collected into a 8 ml tube and allowed to clot at room temperature. The animals subjected to behavioral testing during adolescence were the same individuals later assessed at adulthood. This design allowed us to directly compare behavioral outcomes across developmental stages while minimizing inter-individual variability. Moreover, using the same cohort reduced potential experimental biases related to treatment effects or other factors that might have influenced pregnancy. In contrast, the animals used for electrophysiological recordings and molecular analyses were distinct from those included in the behavioral experiments.

All procedures were performed in accordance with the European legislation EU Directive 2010/63 and were approved by the Animal Ethics Committee of the University of Cagliari and by the Italian Ministry of Health (auth. n. 658/2015-PR; 631/2020-PR).

Drugs and treatments

Polyinosinic:polycytidylic acid (poly(I:C) (HMW) (InvivoGen, France; Italian InvivoGen supplier: Aurogene s.r.l); Catalogue number: trlc-pic-5; Lot number: 5935-44-05. Poly (I:C) was dissolved in endotoxin-free saline solution, and injected at 4.0 mg/kg, i.v in the lateral vein of the tail of pregnant dams. To assess the efficacy of poly (I:C) exposure, all pregnant rats were weighed on the day of the injection and on the first day after the administration of either poly (I:C) or saline to evaluate weight loss as underlined by previous investigations [35].

Behavioral tests

During adolescence, behavioral tests began on PND 35, with a two-day recovery interval between each testing condition. In adulthood, behavioral tests began on PND 80, employing the same inter-test recovery period as used during adolescence.

Forced-swim test. The forced-swim test was conducted as previously described [36]. Briefly, rats were placed in transparent Plexiglas cylinders (45.7 × 30 cm in diameter) filled with water to a depth of 30 cm, maintained at a temperature of 25 °C. The test lasted for 10 minutes, with environmental light set at 300 lux. The duration of immobility (defined as the minimal movement required to keep the head above water in the absence of the other two behaviors), climbing (upward movements of the forepaws directed toward the walls of the cylinder), and swimming (horizontal movement throughout the cylinder) was manually recorded. The behavioral tests were conducted under illumination of approximately 300 lux, consistent with the standard lighting conditions adopted in our facility. Moreover, performing the FST under comparable light intensities (≈ 300–500 lux) enhances reproducibility and maintains a consistent level of environmental stress. Thus, the chosen light level minimizes the risk of underestimating immobility or overestimating active behaviors. The FST was conducted during the light phase (between 9:00-17:00).

Sucrose preference test. Rats were exposed to a 1.5% or 1% sucrose solution for 48 h in order to acclimate them to the test procedure. On the test day, rats were isolated and provided for 24 h with two bottles, one containing the sucrose solution and the other containing tap water. The positions of the two bottles were changed every 12 h to control for position preference. Fluid (sucrose solution or water) consumption was calculated by weighing each bottle before and after animal exposure. Sucrose preference was calculated as 100% sucrose solution consumption (g)/total fluid consumption (g). During the sucrose preference test, the animals were isolated in a home cage within the housing room and had free access to bottles containing either water or sucrose solution for 24 hours, encompassing both the inactive and active phases.

In vivo electrophysiology

Rats were anesthetized with urethane (1.3 g/kg, i.p.) and placed in the stereotaxic apparatus (Kopf, Tujunga, CA, USA) with their body temperature maintained at 37 ± 1°C by a heating pad. For the positioning of a recording electrode, the scalp was retracted and one burr hole was drilled above the DRN according to the Paxinos and Watson stereotaxic rat brain atlas [37] (DRN coordinates adjusted in adolescence: 6.8 mm posterior from bregma, 0.0-0.1 mm lateral to the midline, 5.0-6.0 mm from the cortical surface; DRN coordinates in adulthood: 7.5 mm posterior from bregma, 0.0-0.1 mm lateral to the midline, 5.0-6.0 mm from the cortical surface). Extracellular single unit activity of putative 5-HT neurons was recorded with glass micropipettes filled with 2% Pontamine sky blue dissolved in 0.5 M sodium acetat with an impedance of 2.5-5 MΩ.

Spontaneous population activity of DRN 5-HT cells was determined in 4-6 predetermined tracks separated by 100 µm and the total number of active cells encountered in each brain area was divided by the number of tracks (cells/track). Putative serotoninergic neurons were selected if all identification criteria were met according to previously published criteria [38, 39]. The action potential is characterized by a duration of 2-5 ms and positive-negative or positive-negative-positive deflections. Spontaneous activity can be either regular or irregular, with a frequency range of 0.1-4.0 Hz [39].

Electrical activity from individual neurons was filtered using a bandpass filter of 0.1–10000 Hz. Individual action potentials were isolated and amplified (DAM80, WPI, Hertfordshire, UK, and Neurolog System, Digitimer, Hertfordshire, UK), displayed on a digital oscilloscope (DL708E, Yokogawa, Japan), and digitally recorded for a period of 2–3 minutes. Experiments were sampled online with Spike2 7.20 software by a computer connected to the CED 1401 interface (Cambridge Electronic Design, Cambridge, UK).

At the end of each recording session, direct current (7 µA for 10 minutes) from a constant current isolated stimulator (DS3, Digitimer, Hertfordshire, UK) was applied through the recording micropipette for ejecting PSB to mark the recording site. The position of the electrodes was identified microscopically on 60 µm sections. Only cells from subjects for which correct electrode placement was verified histologically were included in the study.

Cytokine measurements

Blood was collected from MIA adult rats (PND 90). Rats were deeply anesthetized with isoflurane (5%) and sacrificed by decapitation. Blood was collected from the trunk into a 10 ml tube containing spray-coated silica and a polymer gel for serum separation (Becton Dickinson, #367896) and allowed to clot at room temperature for 45 minutes. Samples were centrifuged at 1000 g for 15 min at 4°C, and the supernatant was collected and further centrifuged at 10,000 g for 10 min at 4°C. The resulting serum was aliquoted and stored at -80°C for subsequent cytokine analysis.

Cytokine assay. As previously described [8, 13] serum samples were analyzed for cytokines, chemokines and growth factors using a Luminex xMAP-based multiplex bead-based immunoassay, the Bio-Plex ProTM Rat Cytokine Group I Panel 23-Plex (Bio-Rad Laboratories, Inc., USA), which detects cytokines: [Interleukins (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL- 6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17A, IL-18, interferon (IFN)γ, tumor necrosis factor (TNF)α; chemokines: monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)1α, MIP-3α, regulated on activation normal T cells expressed and secreted (RANTES), keratinocyte derived chemokine (GRO/KC or CXCL1), and growth factors: granulocytes macrophage colony-stimulating factor (GM)-CSF, granulocyte (G)-CSF, macrophage (M)-CSF and vascular endothelial growth factor (VEGF)].

Assays were performed in 96-well plates. Each plate included 8 lyophilized cytokine standards in duplicate, two blank wells, and up to 32 serum samples (diluted 1:4 prior to assay). All samples were run in duplicate and control and MIA offspring at PND 90 were analyzed in the same plate. Wash steps were carried out at room temperature using Bio-Plex Pro wash station. A Bio- Plex MagPix Multiplex System by Luminex was used to read the plate and data were analyzed using BioPlex manager 4.1 software. Cytokine concentrations were calculated with a 5-parametric logistic regression (5 PL) curve fitting to determine the standard curve (pg/ml) from 8 reference standards in duplicate for each cytokine. Specifically, unknown sample cytokine concentrations were calculated by Bio-Plex Manager software using a standard curve derived from the known reference cytokine concentrations supplied by the manufacturer. Only standards and samples with coefficients of variance under 5% were included. Data below the assay's detection limit (i.e., background value) were excluded from analyses.

Statistical analysis

Data are analyzed by two-way ANOVA with treatment and sex as factors. Normality and homoscedasticity tests were carried out using the Shapiro-Wilk and the Brown-Forsythe test, respectively. Behavioral and electrophysiological data were analyzed with two-way ANOVAs followed by Tukey's test for post-hoc comparisons. When two-way ANOVA did not detect a significant interaction but only a significant effect of main factors,

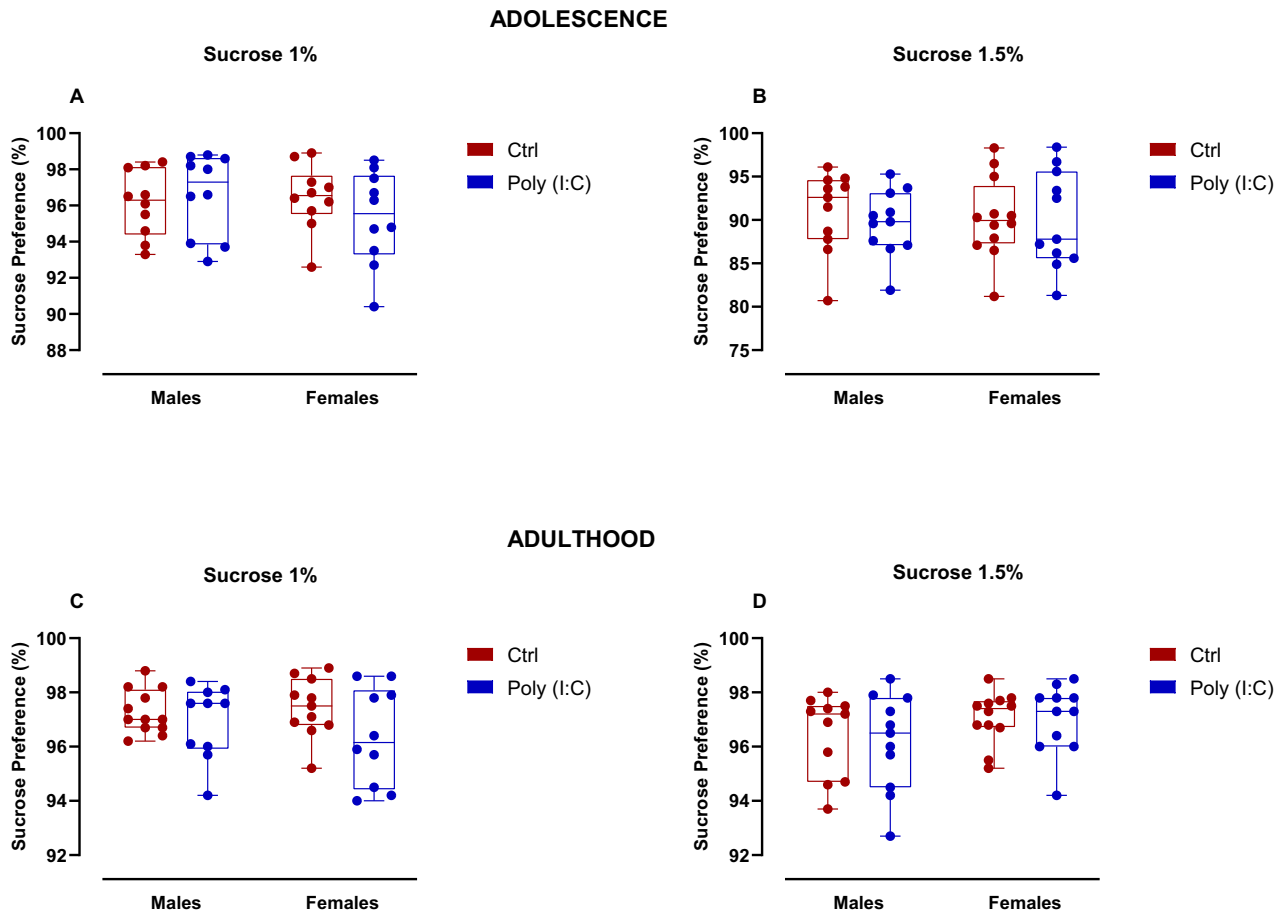

Fig. 2 Effects of MIA induced with gestational HMW poly (I:C) on anhedonic-like phenotype in adolescent and adult male and female offspring. Effects of MIA induced with gestational HMW poly (I:C) on anhedonic-like phenotype in adolescent (**A**, **B**) and adult (**C**, **D**) male and female offspring. Rats were exposed to two different concentrations of sucrose: 1% (A, C) and 1.5% (B, D). Gestational HMW poly (I:C) does not induce anhedonia-like phenotype. Sucrose concentration 1% $N = 10$ rats, control and poly (I:C) adolescent male and female offspring, and $N = 12$ control adult male offspring, $N = 11$ control adult female offspring and $N = 10$ poly (I:C) adult male and female offspring. Sucrose concentration 1.5% $N = 11$ control and poly (I:C) adolescent and adult male offspring, $N = 12$ control adolescent and adult female offspring and $N = 11$ poly (I:C) adolescent and adult female offspring. Data are expressed as a percentage of baseline and are the mean ± SEM.

a post-hoc pairwise comparisons for the main factors were performed by using a two-tailed Bonferroni's test with corrected alpha values. All biochemical data, expressed as pg/ml, are mean ± SEM calculated from one experiment performed in duplicate and analyzed using GraphPad Software Prism 10.0.

RESULTS

Effects of gestational HMW poly (I:C) on depressive-like behavior in male and female offspring during adolescence and adulthood

MIA has been shown to induce depressive-like phenotypes in offspring in a manner dependent on strain, behavioral task, and immunostimulant used [20, 40, 41]. Therefore, we explored whether gestational exposure to HMW poly (I:C) induces behavioral phenotypes associated with depressive-like states, including anhedonia and coping strategies to stress. The animals exposed to the 1% sucrose solution were different from those tested with the 1.5% solution. This design was aimed to prevent that prior sucrose exposure might influence subsequent intake. As previously reported by Strekalova et al. [42, 43], rodents (both rats and mice) exhibit distinct preferences depending on sucrose concentration. Our first cohort was exposed to a 1% solution; however, because no differences emerged, we questioned whether this concentration might have been too low for the animals to reliably discriminate. We therefore tested a second cohort with a slightly higher concentration (1.5%). We deliberately avoided using a 2% or higher sucrose solution to prevent introducing an excessively palatable stimulus that could mask more subtle group differences. For the dose of sucrose 1% we used $n = 10$ rats, control and poly (I:C) adolescent male and female offspring, and $n = 12$ control adult male offspring, $n = 11$ control adult female offspring and $n = 10$ poly (I:C) adult male and female offspring. For the dose 1.5% we used $n = 11$ control and poly (I:C) adolescent and adult male offspring, $n = 12$ control adolescent and adult female offspring and $n = 11$ poly (I:C) adolescent and adult female offspring.

After 24 h, rats were individually housed and re-exposed to both bottles. Two-way ANOVA did not detect any differences between male and female adolescent rats at either the 1% sucrose dose [treatment x sex: $F_{(1,36)} = 1.395$, $P = 0.245$] or the 1.5% sucrose dose [treatment x sex: $F_{(1,41)} = 0.137$, $P = 0.712$] (Fig. 2A-B). Likewise, no significant differences were found in the sucrose preference at both doses of 1% [treatment x sex: $F_{(1,39)} = 0.869$, $P = 0.356$] and 1.5% of sucrose [treatment x sex: $F_{(1,41)} = 0.072$, $P = 0.788$] during adulthood (Fig. 2C-D). Active and passive coping behaviors in response to acute stress were assessed using the forced swim test. For this experiment we used $n = 11$ rats, control and poly (I:C) adolescent male offspring, $n = 10$ control adolescent female offspring, $n = 11$ poly (I:C) female offspring. To test the

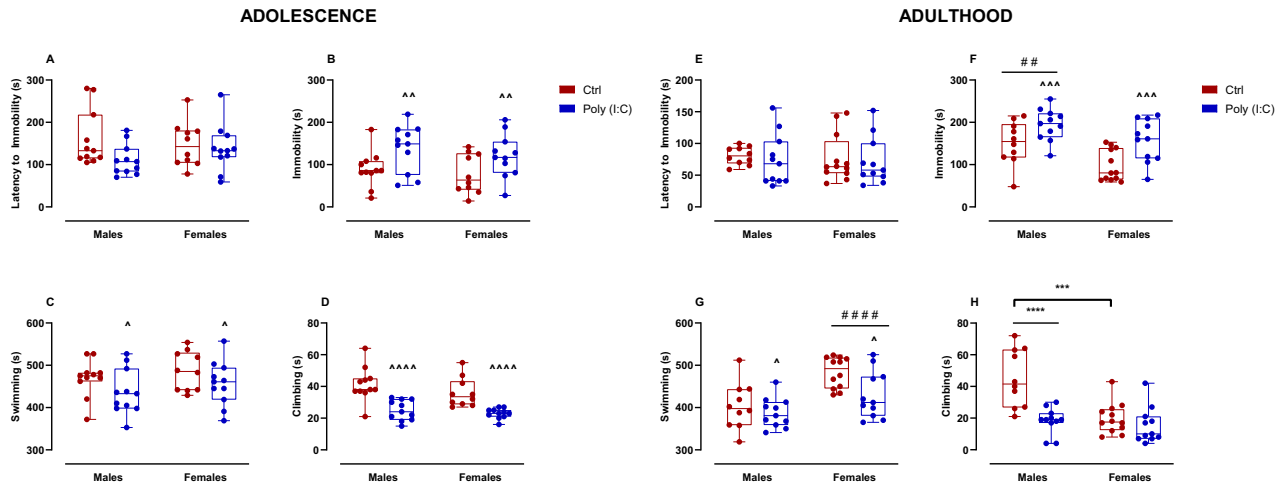

Fig. 3 Effects of MIA induced with gestational HMW poly (I:C) on coping strategies to stress in adolescent and adult male and female offspring. Graphs in (**A, E**) show the latency to the first episode of immobility (s), and graphs in (**B**. **F**) show the immobility time (s). Immobility was described by the minimum amount of movements with the anterior paws to maintain the head above the water surface. Graphs in (**C, G**) display the time (s) spent swimming. Graphs in (**D, H**) show the time of (s) spent climbing. Climbing is described by attempts to climb the wall of the cylinder. $N = 11$ rats, control and poly (I:C) adolescent male offspring, $N = 10$ control adolescent female offspring, $N = 11$ poly (I:C) female offspring. $N = 10$ control adult male offspring, $N = 11$ poly (I:C) adult male offspring, $N = 12$ control adult female offspring and $N = 11$ poly (I:C) adult female offspring. Data are expressed as means ± SEM. Main Effect of treatment: ^$p < 0.05$, ^^ $p < 0.01$, ^^^$p < 0.001$, ^^^^$p < 0.0001$. Main effect of sex: ## $p < 0.01$, #### $p < 0.0001$. Post hoc analyses: *** $p < 0.001$, ***$p < 0.0001$.

active and passive coping behaviors in response to acute stress during adulthood we used, $n = 10$ control adult male offspring, $n = 11$ poly (I:C) adult male offspring, $n = 12$ control adult female offspring and $n = 11$ poly (I:C) adult female offspring. Data analysis revealed that gestational exposure to poly (I:C) did not alter the latency to the first immobility episode in adolescent offspring (Fig. 3A) [main effect of treatment: $F_{(1,\ 39)} = 1.548$, $P = 0.220$]. However, poly (I:C) offspring exhibited a significant increase in the immobility time compared to their counterparts [main effect of treatment: $F_{(1,\ 39)} = 9.522$, $P = 0.003$], independent of sex [main effect of sex: $F(_{1,39}) = 0.949$, $P = 0.335$] (Fig. 3B). Moreover, the two-way ANOVA revealed a significant reduction in both swimming [main effect of treatment: $F(_{1,\ 39}) = 4.356$; $P = 0.043$] (Fig. 3C) and climbing behavior [main effect of treatment: $F_{(1,\ 39)} = 37.37$; $P < 0.0001$] (Fig. 3D) in both male and female MIA adolescent offspring (Supplementary table 2).

Similar to adolescent offspring, no significant differences in the latency to the first immobility episode were observed in adulthood [main effect of treatment: $F(_{1,40}) = 0.329$, $P = 0.569$] (Fig. 3E). However, maternal poly (I:C) exposure significantly increased immobility time [main effect of treatment: $F(_{1,40}) = 14.91$, $P = 0.0004$] (Fig. 3F). In contrast to the adolescent offspring, ANOVA revealed a main effect of sex in immobility time, with males showing longer immobility durations than females [$F_{(1,40)} = 11.69$, $P = 0.0015$] (Fig. 3F). Gestational HMW poly (I:C) exposure also reduced the time spent swimming, with a more pronounced effect in males than in females [main effect of treatment: $F_{(1,40)} = 6.209$, $P = 0.017$; main effect of sex: $F_{(1,40)} = 19.11$, $P < 0.0001$] (Fig. 3G). In the analyses of the climbing behaviors, a significant interaction between factors treatment x sex was observed (Fig. 3H). Post-hoc test revealed that maternal HMW poly (I:C) exposure decreased climbing time in males but not in females [treatment x sex: $F(_{1,40}) = 9.367$, $P = 0.0039$; control males vs. poly (I:C) males: $p < 0.0001$] (Fig. 3H).

Effects of gestational HMW poly (I:C) on the electrical activity of putative 5-HT DRN neurons in male and female offspring during adolescence and adulthood

Given the role of 5-HT in mood regulation and depressive disorders, we examined the electrical activity of 5-HT neurons in the DRN as a potential neural correlate of depressive-like phenotype [29]. In these experiments, we utilized $n = 6$ rats (55 cells), control adolescent males; $n = 5$ rats (42 cells), control adolescent females; $n = 5$ rats (44 cells), poly (I:C) adolescent males; $n = 5$ rats (62 cells), poly (I:C) adolescent females; $n = 6$ rats (70 cells), control adult males; $n = 6$ rats (53 cells), control adult females; $n = 5$ rats (52 cells), poly (I:C) adult males; $n = 8$ rats (66 cells), poly (I:C) adult females. Figure 4A shows the typical broad spike waveform of a putative 5-HT DRN, Fig. 4B depicts representative localization of recording sites from the DRN. Figure 4C depicts demonstrative traces of regularly and irregularly firing putative 5-HT DRN cells. Analysis of the number of cells/track (Fig. 4D), which is an index that represents the spontaneous population activity of a specific brain area, did not reveal any difference during adolescence (Fig. 4D) (Two-way ANOVA, treatment x sex $F\ (_{1,\ 17})$, $P = 0.3294$) Moreover, no main effect of treatment, sex, nor their interaction was observed on the electrical activity of putative 5-HT DRN neurons in adolescent offspring (Fig. 4E (treatment x sex ($F\ _{(1,\ 207)} = 1.785$), $P = 0.183$). No changes were observed in the number of cells/track in adulthood (Fig. 4F). However, two-way ANOVA revealed a significant interaction between treatment and sex on firing frequency in the adult offspring (Fig. 4G; treatment × sex: $F(_{1,\ 237}) = 4.867$, $P = 0.028$). Tukey's post hoc test revealed a significant increase in the firing frequency of adult poly (I:C) male offspring compared to adult control male offspring ($P = 0.023$).

Effect of HMW poly (I:C) on serum cytokine, chemokine, and growth factor levels in male and female offspring during adulthood

We evaluated the expression of cytokines, chemokines, and growth factors in the serum of adult offspring using a multiplex ELISA, which enabled the simultaneous quantification of 23 factors from each sample. All these analytes were detectable in the examined serum samples, apart from IL-18, which was undetectable in the serum of both control and MIA-exposed female offspring. Two-way ANOVA displayed a significant main effect of sex for almost all cytokines, chemokines, and growth factors of poly (I:C) offspring (Table 1). However, no significant

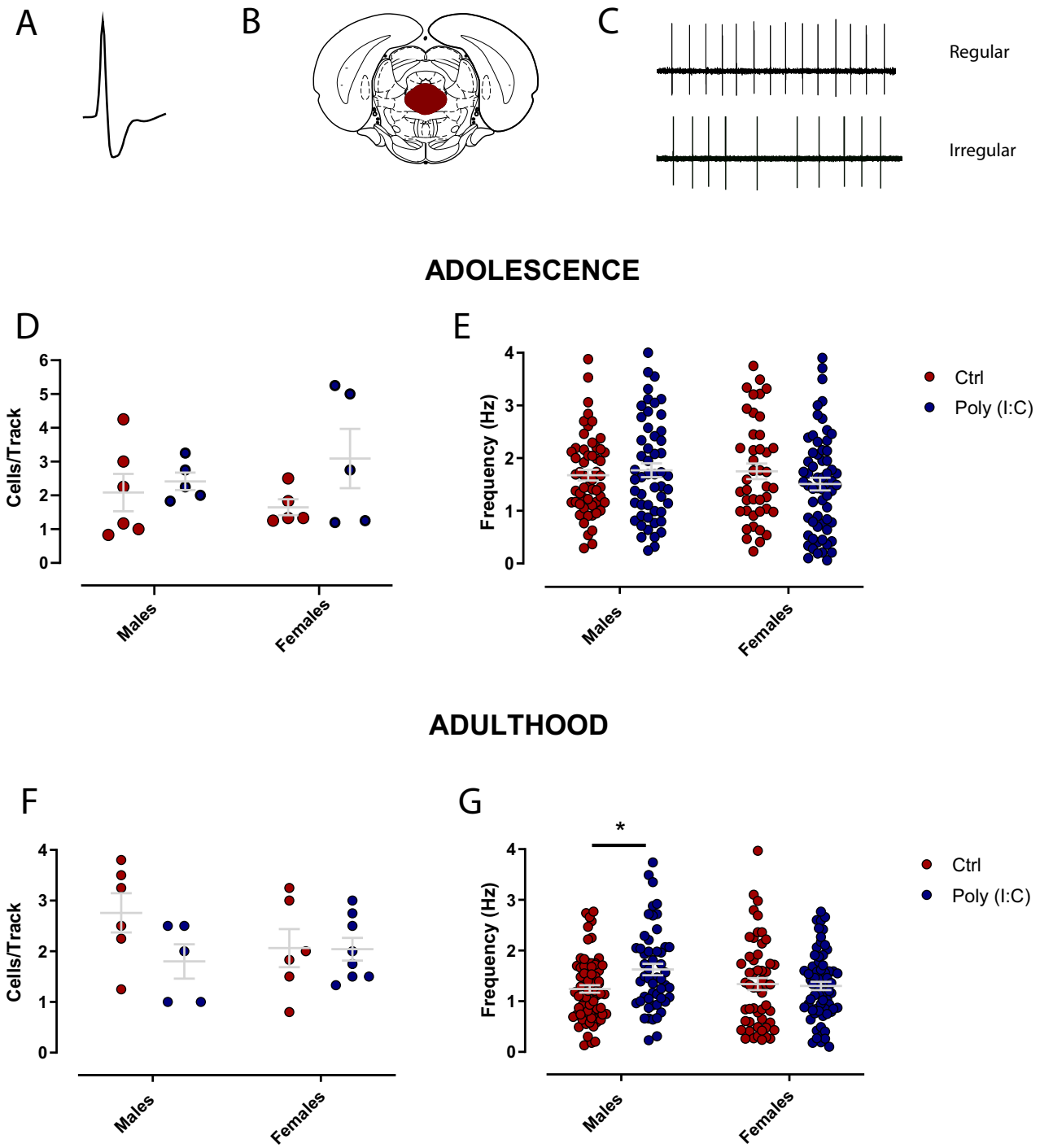

Fig. 4 Effects of MIA induced with gestational HMW poly (I:C) on putative DRN 5-HT neuron firing activity in adolescent and adult male and female offspring. (**A**) Typical spike waveform of a 5-HT neuron. (**B**) Representative localization of recording sites from the DRN (in red), as verified by histological sections. (**C**) Example of traces from regular and irregular firing pattern. (**D**) The scatter plot shows the number of spontaneously active 5-HT neurons during adolescence. (**E**) The scatter plot depicts individual and average 5-HT neuron firing rates in Ctrl and Poly (I:C), male and female adolescent offspring. (**F**) The scatter plot shows the number of spontaneously active 5-HT neurons during adulthood. (**G**) The scatter plot shows individual and average 5-HT neuron firing rates in Ctrl and Poly (I:C), male and female adult offspring. $N = 6$ rats, (55 cells) control adolescent males; $n = 5$ rats (42 cells) control adolescent females; $n = 5$ rats (44 cells) poly (I:C) adolescent males; $n = 5$ rats (62 cells) poly (I:C) adolescent females; $n = 6$ control adult males (70 cells); $n = 6$ control adult females (53 cells); $n = 5$ poly (I:C) adult males (52 cells); $n = 8$ poly (I:C) adult females (66 cells). Ctrl: control. Data are expressed as means ± SEM. *$P < 0.05$.

sex X treatment interaction was observed for any of the analytes analyzed. Specifically, the female group showed significantly lower values of all cytokines (except IL-12), chemokines (except VGF), and growth factors compared to their respective male controls and poly (I:C) offspring (see Supplementary Table 1). The main effect of poly (I:C) was significant only for IL-1α [$F(1_{,26}) = 8.2$, $P = 0.008$] and GRO/KC [$F(1,26) = 5.4$, $P = 0.029$], revealing higher levels of IL-1αin male

Table 1. Cytokine, chemokine, and growth factor concentrations in male and female serum.

	Male			Female		
	Control	**Poly (I:C)**	**↑ or ↓**	**Control**	**Poly (I:C)**	**↑ or ↓**
Pro- and anti-inflammatory cytokines (pg/ml)						
IL-1α	240.23 ± 8.72	268.37 ± 7.12	↑*	161.13 ± 3.86	170.78 ± 5.06	
IL-1β	153.89 ± 3.94	145.13 ± 5.97		93.09 ± 3.24	92.38 ± 2.17	
IL-2	4014.90 ± 161.48	3891.06 ± 171.24		3517.84 ± 122.54	3847.23 ± 202.50	
IL-4	664.24 ± 20.80	626.79 ± 28.48		234.16 ± 8.52	234.30 ± 11.14	
IL-5	908.70 ± 17.63	918.12 ± 13.92		558.82 ± 13.03	561.05 ± 12.02	
IL-6	910.58 ± 45.88	897.85 ± 56.05		522.96 ± 36.73	501.28 ± 22.15	
IL-7	205.17 ± 7.45	212.69 ± 6.50		141.24 ± 7.74	145.39 ± 8.22	
IL-10	535.89 ± 19.62	532.48 ± 26.70		294.88 ± 9.02	283.13 ± 12.70	
IL-12(p70)	528.63 ± 33.17	554.81 ± 26.46		590.95 ± 28.21	623.64 ± 25.11	
IL-13	467.95 ± 29.05	450.93 ± 30.82		194.34 ± 21.67	183.04 ± 8.91	
IL-17	143.38 ± 5.38	140.90 ± 3.45		132.71 ± 4.94	133.77 ± 7.06	
IL-18	255.18 ± 21.39	245.15 ± 12.26		N.D.	N.D.	
IFN-γ	806.12 ± 36.66	792.65 ± 33.33		457.63 ± 25.22	467.11 ± 13.99	
TNF-α	933.04 ± 62.43	853.37 ± 59.69		499.31 ± 23.18	493.58 ± 29.52	
Chemokines (pg/ml)						
MCP-1	1406.61 ± 49.23			1158.84 ± 70.95	1061.02 ± 78.50	
MIP-1α	42.75 ± 0.79	1467.61 ± 39.22		27.00 ± 1.15	26.92 ± 1.14	
MIP-3α	41.80 ± 1.92	44.58 ± 1.14		20.25 ± 0.73	18.41 ± 0.80	
RANTES	580.45 ± 58.07	42.05 ± 1.71		228.37 ± 16.21	212.31 ± 24.61	
GRO/KC	182.82 ± 5.76	572.44 ± 34.41		157.07 ± 10.60	125.95 ± 4.57	↓*
Growth factors (pg/ml)						
GM-CSF	108.79 ± 4.79	110.64 ± 3.87		88.86 ± 2.73	88.65 ± 3.27	
G-CSF	41.59 ± 1.98	40.25 ± 2.83		23.18 ± 1.43	22.55 ± 1.11	
M-CSF	33.03 ± 2.31	33.14 ± 3.89		30.80 ± 2.40	28.95 ± 1.79	
VEGF	195.95 ± 17.97	203.79 ± 16.82		260.89 ± 18.76	244.36 ± 18.58	

Data, expressed as a pg/ml, are mean ± SEM calculated from one experiment performed in duplicate ($n = 8$ ctrl males; $n = 8$ poly (I:C) males; $n = 7$ ctrl females; $n = 8$ poly (I:C) females); N.D.; not detectable: cytokine, chemokine or growth factor concentrations under the limit of detection. As two-way ANOVA does not detect a significant interaction, pairwise comparisons on the main effect were performed by using a two-tailed t-test with Bonferroni's corrected alpha values. *$p < 0.05$ compared to the control group.

serum samples ($p < 0.05$) and lower levels of GRO/KC in female serum samples ($p < 0.05$), compared to their respective controls. As shown in Table 1, the serum of male poly (I:C) offspring exhibited a significant increase in IL-1α (11% vs. Control, $p < 0.05$). Moreover, female poly (I:C) offspring showed a significant reduction in GRO/KC (20% vs. Control, $p < 0.05$). No differences in cytokine, chemokine, or growth factor levels were detected in the serum of either male or female offspring (Table 1).

DISCUSSION

Our results suggest that gestational administration of HMW poly (I:C) selectively alters depressive-like phenotypes in the offspring rather than inducing a broad depressive-like profile. While no differences in anhedonia were observed in the sucrose preference test, which evaluates key behavioral manifestations of negative valence, maternal administration of HMW poly(I:C) induced substantial alterations in stress-coping behaviors, as assessed by the forced swim test. Specifically, HMW poly(I:C) exposure during pregnancy resulted in increased immobility time in offspring of both sexes and reduced swimming behavior, with the effect being pronounced in males. The significant interaction between sex and poly (I:C) exposure in climbing behavior further suggests sex-dependent differences in adaptive behaviors to acute stress exposures. These findings corroborate previous studies and highlight how MIA may contribute to the onset of depression-related behavioral phenotypes [40, 41]. They also underscore the importance of considering both sex and the timing of exposure when investigating the behavioral consequences in offspring. Given the well-established role of the dopamine system in reward-related behaviors [44], and several previous works demonstrating MIA-induced alterations in mesolimbic dopamine functions [8, 9, 35, 45–47], we expected anhedonia-like deficits in the sucrose preference test. However, the lack of detectable changes in sucrose preference suggests a more selective impact of MIA on specific domains of the depressive-like phenotype. Previous studies employing poly (I:C) [17–19] reported concurrent elevations in forced-swim immobility and decreased sucrose preference, indicative of a more encompassing depressive-like phenotype. It should also be emphasized that these studies were performed in different species (C57BL/6 N mice vs Sprague Dawley rats) using different protocols of MIA [poly (I:C) 20 mg/kg i.p. at GD 12.5 vs HMW 4 mg/kg i.v at GD 15, and from different supplier (Sigma-Aldrich vs InvivoGen]. Indeed, several studies highlight the relevance of the poly (I:C) batch, its molecular weight (LMW versus HMW), and the vendor, all of which can critically influence behavioral readouts

[21–23, 48]. Furthermore, the behavioral alterations observed in this study were not paralleled by changes in serotonergic activity in the DRN, suggesting that these outcomes could result from mechanisms beyond classic serotonergic dysfunction. In our in vivo recordings, adult male offspring from maternal exposure to HMW poly (I:C) displayed an increased spontaneous firing rate of DRN 5-HT neurons compared with controls. This result might appear in contrast with earlier work indicating that reduced DRN activity is typically associated with depressive-like behaviors [49].

Furthermore, Csatlosova and colleagues [50] found a decreased firing rate of 5-HT neurons in the DRN, whereas we detected a stimulatory effect of prenatal HMW poly (I:C) on 5-HT neurons in the DRN. Accordingly, we previously showed that an anhedonic-like status induced by chronic neuropathic pain in rat, alters the electrophysiological activity of DRN 5-HT neurons particularly increasing their average firing frequency among others [38]. It is therefore plausible that functional maladaptations and/or distinct immune challenges [poly (I:C) at GD 15 versus LPS across GD 15-19] engage partially divergent mechanisms, yielding opposite effects on DRN excitability while converging on similar behavioral phenotypes. Mounting evidence underlines the link between immune dysregulation and depression [32, 51]. Indeed, we next examined the peripheral cytokine and chemokine profile in MIA-exposed offspring. To provide a comprehensive profile of serum cytokines, chemokines, and growth factors in adult MIA offspring, we used a multiplex immunoassay, which maximizes the simultaneous detection of multiple analytes in a single sample. At this developmental stage, the levels of almost all cytokines and chemokines were unaltered in both male and female serum. Of note, IL-1α, which was increased in the maternal serum 24 h after poly (I:C) injection (see [13]), is the only cytokine we found significantly higher in the offspring during adulthood. IL-1α, a member of the interleukin-1 family, is a key mediator of systemic inflammation, brain inflammation and injury [52]. Despite its role in inflammation, most studies have focused almost entirely on IL-1β and not on IL-1α. To our knowledge, only Garay and colleagues [53] analyzed IL-1α serum levels in MIA male mice offspring, reporting no difference compared to those of respective controls at PND 60. Among the chemokines assessed, only GRO/KC (CXCL1) levels were significantly decreased in the serum of MIA female offspring at adulthood. This chemokine acts as a chemoattractant for several immune cells and other non-hematopoietic cells at the site of injury or infection, playing a crucial role in modulating immune and inflammatory responses. To date, there is a lack of data regarding the role of GRO/KC in the context of MIA. However, it is important to emphasize that the absence of overt peripheral immune activation in adulthood does not rule out the presence of ongoing or latent neuroinflammatory processes. Indeed, neuroinflammation can occur independently of systemic cytokine elevations and may be confined to discrete brain regions involved in mood regulation and depressive-like behaviors [54]. A more detailed characterization of neuroinflammatory markers within regions involved in depression, such as the mesocorticolimbic system, hippocampus, and DRN, would therefore be instrumental in elucidating the central immune mechanisms underlying the observed behavioral alterations. Our previous work demonstrated that MIA male offspring exhibit increased expression of cyclooxygenase-2 (COX-2) and ionized calcium-binding adaptor molecule 1 (IBA-1) in the whole brain [8]. These markers are associated with latent inflammatory states and sustained microglial activation and may reflect long-lasting neuroimmune vulnerability to the emergence of latent depression-related phenotypes. In the context of established depressive-like models, our findings further highlight that different paradigms may display distinct strengths and weaknesses [55]. Classical chronic stress models, such as chronic mild stress, social defeat or unpredictable stress, and environmentally induced models of depression such as prenatal stress, maternal separation, and maternal immune activation, often yield inconsistent long-lasting behavioral alterations, likely due to methodological variability across laboratories. One mechanism commonly shared across these models, is the regulation of the hypothalamus-pituitary-adrenal (HPA) axis, along with changes in serotonergic, noradrenergic and dopaminergic systems. Accordingly, we previously showed that MIA exposures are associated with alterations in dopaminergic activity in rats [8, 9]. Moreover, it is increasingly recognized that individual variability in stress and maternal immune activation responsivity, and the emergence of resilient vs. susceptible phenotypes may shape behavioral outcomes across these models [56]. Importantly, our study was not designed to cluster offspring into resilient and susceptible subgroups, as we did not analyze entire litters with clustering-based approaches. Furthermore, several additional limitations should be acknowledged. First, although we detected alterations in stress-coping behaviors, we did not observe anhedonia-like phenotypes in the sucrose preference test, which limits our HMW-poly (I:C) model as a robust depressive-like model. Second, as we did not evaluate resilience versus susceptibility, individual variability in behavioral responses may have been overlooked. Third, our mechanistic investigation was restricted to DRN serotonergic neuronal activity and peripheral cytokine profiles in adulthood. Future studies are needed to characterize other systems potentially involved in the depressive-like phenotype, including HPA axis dysregulation, region-specific neuroinflammatory processes, and epigenetic mechanisms that may mediate long-lasting transcriptional and neuroimmune alterations induced by MIA. Finally, implementing a double-hit model would help determine whether a subsequent stressor might unmask a comprehensive depressive-like phenotype. The lack of changes in sucrose preference in our study might be plausibly explained by two methodological factors: (i) the use of the Sprague-Dawley rat, and (ii) the specific dose, molecular weight, gestational day, and route of administration of poly (I:C) employed. Depressive-like traits in MIA models are highly species/strain-dependent, with some strains showing robust anhedonia while others do not (Babri et al., 2014). Second, the poly (I:C) protocol used in our study differs substantially from those that reproducibly induce anhedonia-like behavior in mice. Studies reporting reduced sucrose preference used different species, doses, gestational time points and different poly (I:C) formulations [18, 19, 57]. Thus, both species characteristics and immune-challenge might partially explain why sucrose preference did not change in our animals. Accordingly, as recently suggested by Tillmann and colleagues [23], the effects of purified LMW poly (I:C) in the maternal immune activation model depend on the laboratory environment. In this context, the aim of our work is not to claim a comprehensive depressive-like phenotype induced by HMW poly (I:C), but rather to rigorously document the outcomes obtained under our controlled laboratory conditions. Taken together, our results underscore the importance of considering species, immune challenge characteristics, and sex as critical variables shaping the behavioral and neurobiological outcomes of the MIA model. Future studies should further aim to characterize the spatial and temporal dynamics of neuroinflammation across brain regions, thereby refining our understanding of how MIA contributes to selective behavioral manifestations relevant to depression.

DATA AVAILABILITY

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

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AUTHOR CONTRIBUTIONS

MS designed the study, performed and supervised the experiments, wrote the initial draft of the manuscript, performed data analysis and interpretation, reviewed and edited the manuscript. AM performed experiments, data analysis, reviewed and edited the manuscript; LC performed experiments, data analysis, reviewed and edited the manuscript; RM performed experiments, data analysis, reviewed and edited the manuscript; AH performed experiments, data analysis, reviewed and edited the manuscript; CS supervised experiments, data analysis, reviewed and edited the manuscript; MPC supervised experiments, data analysis, reviewed and edited the manuscript; RF supervised experiments, data analysis, reviewed and edited the manuscript; ALM supervised experiments, data analysis, reviewed and edited the manuscript; MP supervised the experiments, data analysis and interpretation, reviewed and edited the manuscript. All authors reviewed the manuscript prior to submission.

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COMPETING INTERESTS

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.

ADDITIONAL INFORMATION

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