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Prenatal immune activation in rats and adult exposure to inescapable shocks reveal sex-dependent effects on fear conditioning that might be relevant for schizophrenia

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ABSTRACT

Prenatal infection is considered a relevant factor for neurodevelopmental alterations and psychiatric diseases. Administration of bacterial and viral components during pregnancy in rodents results in maternal immune activation (MIA), leading to schizophrenia-like neurochemical and behavioral changes. Despite some evidence for abnormal fear conditioning in schizophrenia, only a few animal studies have focused on this issue. Therefore, we addressed the impact of the administration of the viral mimetic polyI:C to pregnant Long-Evans rats on the adult offspring response to inescapable shocks (IS) and contextual fear conditioning. In males, polyI:C induced a greater endocrine (plasma ACTH) response to IS and both polyI:C and IS enhanced fear conditioning and generalization to a completely different novel environment (hole-board), with no additive effects, probably due to a ceiling effect. In contrast, a modest impact of polyI:C and a lower impact of IS on contextual fear conditioning and generalization was observed in females. Thus, the present results demonstrate that polyI:C dramatically affected fear response to IS in adult males and support the hypothesis that males are more sensitive than females to this treatment. This model might allow to explore neurobiological mechanisms underlying abnormal responsiveness to fear conditioning and stressors in schizophrenia.

1. Introduction

Epidemiological evidence suggests that infections during pregnancy can alter neurodevelopment, and favor the appearance of psychiatric disorders, including schizophrenia (Brown and Meyer, 2018). In animals, the impact of infection during pregnancy has been mimicked by the administration of bacterial endotoxin (lipopolysaccharide, LPS) or the viral mimetic double-stranded RNA polyriboinosic:polycytilidic acid (polyI:C) to pregnant mothers. These two models of maternal immune activation (MIA) have been found to induce behavioral changes in the offspring that are observed in adulthood and are consistent with those expected in putative animal models of schizophrenia (Meyer et al., 2007; Estes and McAllister, 2016; Gumusoglu and Stevens, 2019). In this regard, particular attention has been paid to characterize the impact of polyI:C on prepulse inhibition, which has been found to be altered in schizophrenic patients (San-Martin et al., 2020). However, maternal infection is likely to alter a wide range of brain functions and, therefore, affects different behavioral domains. Thus, abnormal fear conditioning in schizophrenic patients has been reported (e.g., Jensen et al., 2008; Holt et al., 2009, 2012; Tuominen et al., 2021, 2022). The hippocampus and the amygdala are critical in the processing of emotions, stress and contextual fear conditioning (Ulrich-Lai and Herman, 2009; Lopresto et al., 2016) and there is the extensive evidence for structural and molecular abnormalities in the hippocampus and the amygdala of schizophrenic patients (Ho et al., 2019; Bobilev et al., 2020; Wegrzyn et al., 2022). MIA-induced morphological and biochemical changes have been

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described in the hippocampus and, to a much lower extent, in the amygdala (Gumusoglu and Stevens, 2019; Gillespie et al., 2024). Then, abnormal fear conditioning in schizophrenia and animal models are expected.

The impact of MIA on fear conditioning has been poorly explored, and results are controversial. Thus, no evidence for altered cue or contextual fear conditioning was found in adult offspring of LPS-treated rats (Petiddant et al., 2018), whereas in other studies, impaired fear extinction was observed (Okano et al., 2023; Weber-Stadlbauer et al., 2017). After polyI:C administration in C57 mice, no impact or enhanced tone fear conditioning have been reported (Meyer et al., 2006a, 2006b, Schwendener et al., 2009; Stollenwerk and Hillard, 2021). In rats, negative results of polyI:C on acquisition and extinction predominate (Yee et al., 2012; Vorhees et al., 2012; Sangha et al., 2014), with some evidence for impaired extinction recall restricted to the context after a tone fear conditioning procedure (Sangha et al., 2014). Of note, most of the above studies were done with mixed sex groups, with little evidence for sex differences when they were specifically assessed.

The above data reveal subtle, if any, alterations in fear conditioning in animals exposed to MIA. However, considering the role of hippocampus and amygdala in stress and contextual fear conditioning in animals, it is possible that more relevant differences can be found because of prior exposure to acute stress at adulthood. This interaction between MIA and adult exposure to a severe stressor is under the umbrella of the two-hits hypothesis of schizophrenia and the triggering of psychotic symptoms after acute stress (Jones and Fernyhough, 2007; Howes et al., 2017). An interesting model in this regard is the stress-enhanced fear learning (SEFL) phenomenon described and extensively characterized in Fanselow`s laboratory. They have demonstrated that prior exposure to inescapable footshocks (IS) can induce a long-lasting (days to weeks) enhancement of tone and contextual fear conditioning that reflects some kind of sensitization process (Rau et al., 2005; Rau and Fanselow, 2009; Perusini et al., 2016). These results are clearly relevant, as stress and sensitization are likely to play a significant role in schizophrenia (Walker and Diforio, 1997; van Winkel et al., 2008; Howes et al., 2017).

Thus, the aim of the present work was to study, for the first time, the impact of prenatal administration of polyI:C on contextual fear conditioning in rats previously exposed to IS during adulthood. Since acute exposure to severe stressors can induce sensitization of the hypothalamic-pituitary-adrenal axis (HPA) (Belda et al., 2015, 2016), resting and stress levels of peripheral HPA hormones (ACTH and corticosterone) were also evaluated. Since prior MIA studies have not systematically compared the two sexes and the interaction between MIA and IS has not been previously addressed, in the present study both male and female rats were included. We expected a greater impact in males in line with the greater vulnerability of males to schizophrenia.

2. Materials and methods

2.1. Animals and general procedures

The animals were maintained under standard conditions of temperature (22 ± 1 °C) and in a 12 h light-dark cycle (lights on at 8:00am). Food and water were available ad libitum, and no specific environmental enrichment was used. The experimental protocol was approved by the Ethics Committee at the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, and it was carried out in accordance with the European Council Directive (2010/63/UE) and Spanish legislation (RD 53/2013). All experimental procedures were conducted during the light period.

Progenitors were 20 females and 20 males Long-Evans rats (7 and 8 weeks old) purchased from Janvier Labs (France). Upon arrival, they were maintained 2 per cage (same sex) for 5 days and then individually housed for 3 days before mating. During this period animals were daily handled and habituated to part of the procedures associated to caudal vein i.v. administration, except i.v. administration itself. Then one female and one male were paired together for mating until pregnancy was detected by the vaginal plug (GD0), males being removed to left females alone until delivery. On GD15 and GD17, 14 females were given cadual vein i.v. administration of 5 mg/kg polyI:C in saline (1 ml/kg) and 5 females were given vehicle saline (vehicle and polyI:C groups, respectively). Dose and days of administration were based on previous studies (Oh-Nishi et al., 2010; Arsenault et al., 2014). A greater number of pregnant rats were assigned to the polyI:C group as in a parallel study we wanted to explore individual differences in the response to the mimetic. For injection, rats were gentled restrained with a cloth. Delivery took place on GD21–22 and pups were culled to 10 with an approximate ratio of 5 females and 5 males (PN1). Sex was again checked at PN8. Food intake and body weight gain of pregnant rats were evaluated from GD15 to GD17 to demonstrate a physiological impact of polyI:C without causing any additional disturbance to the animals. The number of pups and total weight per sex was measured at PN1. On PN21, each individual pup was weighed and the pups were separated from the mothers and maintained in non-sibling groups of 3–5 of the same sex and prenatal treatment per cage until PN55 when they were housed two per cage, following the same criteria as above. Rats were randomly assigned to the above-mentioned parallel study as well as to the present study. In the present study, 42 males (18 vehicle, 24 polyI:C) and 34 females (10 vehicle and 24 polyI:C) offsprings were available. The adult procedure started when the rats were about 3 months old and can be seen in Fig. 1. Blood samples were obtained in the morning under resting conditions in a subset of rats 2 days before starting adult treatments.

For adult procedures, outlined in Fig. 1, one half of the rats were randomly assigned to controls (non-IS) and the others to the shock (IS) groups. Only one rat of each sex per mother was randomly assigned to each final experimental group, except for males belonging to the vehicle groups (veh-control and veh-IS), in which case an additional animal per litter was included in 4 of the 5 mothers. The final number of rats per groups can be seen in Table 1.

2.2. Specific procedures and apparatuses

2.2.1. Physiological response to polyI:C administration

The amount of food in the feeders and the body weight of each pregnant rat were measured on GD15 before vehicle or polyI:C administration and again at the same hour on the next two days. Body weight of pregnant rats was also measure on GD18 and again after delivery on PN1. On PN1 pups were sexed, counted and all pups of the same sex belonging to the same mother were weighed together to reduce disturbances of the pups. On PN21 all pups were individually weighed.

2.2.2. Behavioral measures

Behavior of rats were video recorded when appropriate to be further measured manually by experimenters blind of each particular experimental group. It was usually measured distance travelled, frequency of rearings and time spent freezing, except when otherwise stated. Distance travelled was assessed by video tracking analysis using the center of gravity of the animal (Smart, Panlab S.L.U., Barcelona, Spain). Rearing was registered when the animals had the two front legs off the floor. Freezing was considered as the absence of all movements except for respiration. Cleaning of the apparatuses was always done before the first animal and between animals.

2.2.3. Inescapable footshocks

The IS procedure was chosen as stressor prior to fear conditioning because this SEFL model has been extensively characterized in Fanselow's lab using also Long-Evan rats (see Introduction). IS rats were exposed to shocks (context A) in Skinner boxes (Panlab, S.L.U., Barcelona, Spain) of 25 x 25 x 25 cm, with black ceiling and black wall, two gray side walls, a transparent front wall and a floor with 19 regularly spaced stainless-steel rods. The Skinner boxes were placed inside sound attenuating boxes (67 x 53 x 55 cm) provided with a fan to mitigate

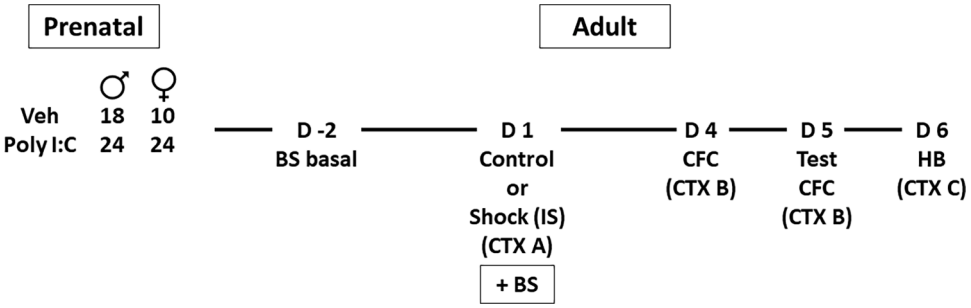

Fig. 1. Timeline of experimental procedures: pregnant rats were given vehicle or polyI:C at gestational days 15 and 17 and their offspring were left undisturbed until adulthood (about 3 months). Experimental procedures during adulthood are indicated, the day of exposure to inescapable footshocks (IS) being D1. IS exposure took place in context A, contextual fear conditioning (CFC) training and testing in context B and fear generalization in a hole-board (HB) that it is considered as context C. BS = blood sampling by tail-nick procedure.

Table 1
number of animals per experimental group.

	Veh-control	Veh-IS	Poly-control	Poly-IS
Males	9	9	12	12
Females	5	5	12	12

environmental sounds. Rats were transported to the room in small white plastic boxes (29 x 27 x 14 cm) with no bedding. Control rats were exposed to the box without being shocked for 11 min, whereas IS rats were exposed to the chambers for 3 min and then 8 shocks (1 mA, 2 s, each minute) were given. Immediately after the treatment all rats were blood sampled. Context A was cleaned with 10% ethanol.

2.2.4. Fear conditioning training

Three days after IS, control and IS rats were trained for contextual fear conditioning in a different context B. Context B was a clear Plexiglas box (57 x 41 x 70 cm) with a removable grid floor with 44 stainless steel rods (Panlab S.L.U., Barcelona, Spain). This context B is larger than those typically used for fear conditioning in rats to allow animals to better express behaviors other than freezing. The apparatus was placed in a room with black walls and fluorescent light. Two rats were simultaneously transported to the experimental room in opaque plastic cylinders containing lemon odor. After 3 min of habituation to the new chamber, rats received only one shock (1 mA, 2 s) and remained for an additional min in the chamber. Context B was cleaned with soap containing lemon odorant. Behavior of non-IS rats in this large context B allows us to evaluate whether or not polyI:C administration per se could have altered the normal behavioral pattern of rats in a novel environment.

2.2.5. Fear conditioning testing

On the day after fear conditioning, rats were tested for contextual fear conditioning learning by exposing them to context B for 8 min without shocks. Animals were transported in their home cages. The apparatus was cleaned with soap. Cleaning was done as during acquisition.

2.2.6. Fear generalization

Two days after fear conditioning rats were exposed for 8 min to a new context C (hole-board, HB). Dim illumination was provided by a white 40 W bulb placed 1.20 m above. The HB consisted of a rectangular wooden box (62 x 53 × 28 cm) with a floor containing four equidistant empty holes (diameter 4.5 cm). Each animal was placed in the apparatus initially facing the wall. Behaviors measured were: ambulation (number of areas crossed), frequency of rearing, frequency of head-dips (number of times both ears disappear into the holes) and freezing. The rats were transported in their home cages and the apparatus was cleaned with soap.

2.3. Blood sampling and analysis

Animals were blood sampled by the tail-nick procedure, which allows obtaining true resting levels of hormones, as described previously (Belda et al., 2004). Cage-mates were always sampled simultaneously. Blood (300 μl) was collected within 2 min into ice-cold EDTA capillary tubes (Microvette®, SARSTEDT) and centrifuged at 4 °C, the plasma being frozen and stored at −20 °C until analysis. Plasma ACTH and corticosterone levels were determined by well-stablished double-antibody radioimmunoassay (RIA) as described previously (Muñoz-Abellán et al., 2011).

2.4. Statistical analysis

Data were analyzed by the Statistical Package for Social Sciences (SPSS. IBM Corp., USA) version 24 for Windows. The analysis of changes in food intake or body weight in pregnant rats after polyI:C or the changes in the number of pups or pup weight were analyzed by *t*-test with Welch corrections for inequality of variances. The initial analysis of pup behavior included three between-subject factors: sex, prenatal treatment (vehicle or polyI:C) termed poly in the analysis and adult stress (control or IS exposure), termed IS in the analysis. Generalized linear model (GzLM; McCulloch and Searle, 2001) was carried out. These models do not require homogeneity of variances or normal distribution. If statistically significant interactions between factors was found that made further analysis complex, we further segregated data on the basis of sex. If an interaction between poly and IS was still found, we decomposed such interaction. The criterion for significance was set at $p < 0.05$.

Table 2
Physiological effects of prenatal polyI:C on mothers and pups.

Maternal treatment	Food intake changes	Body weight gain	Total number pups	Number male pups	Number female pups
Vehicle (5)	**2.0 ± 1.9**	**14.2 ± 4.5**	**12.2 ± 0.8**	**7.0 ± 0.7**	**5.2 ± 1.3**
PolyI:C (14)	**0.7 ± 1.7** *	**14.5 ± 3.1**	**12.0 ± 1.8**	**5.6 ± 1.4** *	**6.4 ± 1.7**

Means ± SD (number of mothers in parentheses) of food intake changes (g/rat/day) and body weight gain (g/48 h) in pregnant rats from GD15 to GD17, and number of pups on PND1. * $p < 0.05$ vs vehicle group.

3. Results

3.1. Physiological impact of polyI:C on mothers and pups

The impact of the first polyI:C administration on food intake and body weight gain of pregnant rats over the next 48 h can be seen in Table 2. Food intake was significantly reduced after polyI:C compared with vehicle ($t(14) = 2.18$, $p = 0.047$), but body weight gain was not. PolyI:C did not change neither the total number nor the total weight of pups born (Table 2), although the number of male pups was lower than in vehicle rats ($t(17) = 2.16$, $p = 0.045$). No differences were observed between groups regarding the weight of pups on PN21 (not shown).

3.2. Changes in the HPA axis

To evaluate putative long-lasting effects of poly on basal HPA activity, morning resting levels of ACTH and corticosterone were studied in a subset of animals before IS exposure (Table 3). One male (vehicle-control) and one female (polyI:C) were not included in the analysis because of extremely high hormone values. The GzLM analysis of ACTH that included the factors sex and poly showed significant effect of sex ($\chi^2(1) = 30.6$, $p < 0.001$), reflecting the higher levels in females than males, and the interaction sex x poly ($\chi^2(1) = 5.9$, $p = 0.015$). Decomposition of the interaction showed no effect of poly in males, but a significant effect in females to increase ACTH levels ($p = 0.033$). The analysis of corticosterone revealed only a significant sex effect ($\chi^2(1) = 96.0$, $p < 0.001$), with higher levels in females.

The analysis of plasma ACTH and corticosterone response to acute adult stress (Fig. 2) included the factors sex, poly and IS. The analysis of ACTH levels revealed significant effects of sex ($\chi^2(1) = 20.8$, $p < 0.001$), poly ($\chi^2(1) = 5.8$, $p = 0.016$), IS ($\chi^2(1) = 176.8$, $p < 0.001$), and the interactions sex x IS ($\chi^2(1) = 10.2$, $p < 0.001$) and poly x IS ($\chi^2(1) = 4.7$, $p = 0.031$). When segregating by sex, both poly and IS significantly enhanced ACTH response in males ($\chi^2(1) = 6.9$, $p = 0.009$ and $\chi^2(1) = 61.5$, $p < 0.001$, respectively), whereas only IS enhanced the response in females ($\chi^2(1) = 117.4$, $p < 0.001$), being poly ineffective. The analysis of plasma corticosterone response revealed significant effects of sex ($\chi^2(1) = 625.0$, $p < 0.001$), IS ($\chi^2(1) = 22.0$, $p < 0.001$), and the interaction sex x IS ($\chi^2(1) = 22.0$, $p < 0.001$). No impact of poly was found. Corticosterone levels were always lower in males than in females and the interaction reflects that IS did not increase corticosterone levels beyond those observed after exposure to the chamber in males, whereas an increase was found in females ($p < 0.001$).

3.3. Behavioral response to the fear conditioning training context B before shock exposure (Fig. 3)

The GzLM analysis of freezing revealed significant effects of sex ($\chi^2(1) = 7.3$, $p = 0.007$), IS ($\chi^2(1) = 152.9$, $p < 0.001$) and the interaction sex x IS ($\chi^2(1) = 12.3$, $p < 0.001$), whereas the interaction sex x poly x IS was marginally significant ($\chi^2(1) = 3.6$, $p = 0.057$). Levels of freezing were negligible in all control (non-IS) rats, but were considerable in IS exposed rats ($p < 0.001$ in both males and females), with overall higher levels in males than females ($p < 0.001$). Poly potentiated IS-induced freezing in females, but differences did not reach significance.

Table 3
Morning resting levels of plasma ACTH and corticosterone in adult male and female offspring of mothers treated with vehicle or polyI:C during pregnancy.

Sex-Maternal Treatment	ACTH (pg/ml)	Corticosterone (ng/ml)
Males vehicle	85 ± 23 (8)	22 ± 33 (8)
Males polyI:C	66 ± 25 (11)	13 ± 13 (11)
Females vehicle	133 ± 57 (6)	176 ± 117 (6)
Females polyI:C	170 ± 33 (12)*	223 ± 111 (11)

Means ± SD (number of rats in parentheses) are shown. Note that basal levels were assessed prior to IS and only a subset of the animals were blood sampled. Overall higher levels of both hormones were found in females as compared with males, regardless of polyI:C treatment during pregnancy. * $p = 0.015$ vs vehicle females.

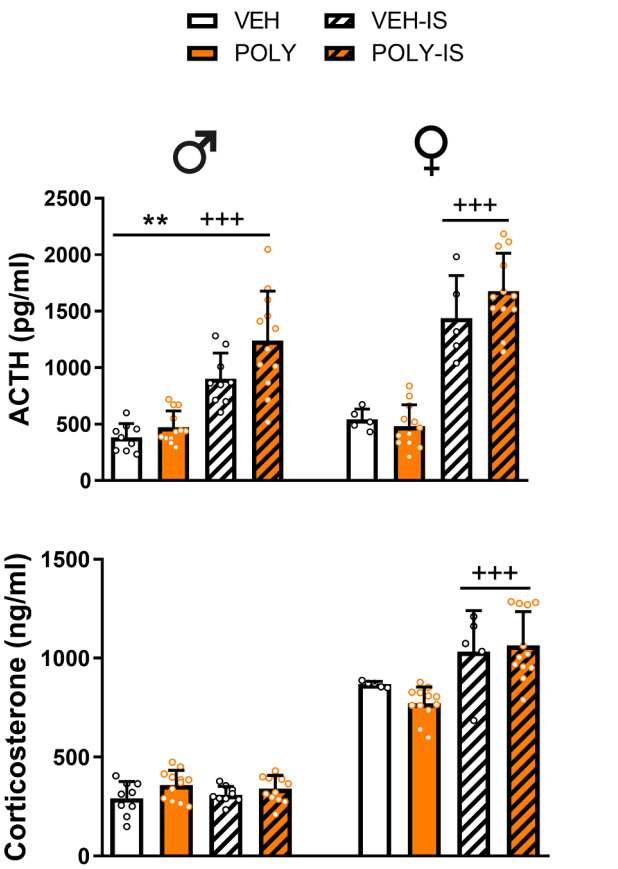

Fig. 2. Prenatal polyI:C administration effects on adult endocrine response to footshocks in context A. Means and SD of plasma levels of ACTH and corticosterone are represented. Basal levels can be seen in Table 1. Significant effect of sex was found (not indicated): For each sex: * significant poly effect, + significant IS effect within the indicated groups. Two symbols $p < 0.01$: three symbols $p < 0.001$.

The analysis of the distance travelled in the conditioning context revealed significant effects of sex ($\chi^2(1) = 23.0$, $p < 0.001$), poly ($\chi^2(1) = 4.8$, $p = 0.028$), IS ($\chi^2(1) = 197.0$, $p < 0.001$) and the interactions sex x poly ($\chi^2(1) = 5.7$, $p = 0.017$), sex x IS ($\chi^2(1) = 3.9$, $p = 0.047$), poly x IS ($\chi^2(1) = 4.2$, $p = 0.041$) and sex x poly x IS ($\chi^2(1) = 4.4$, $p = 0.035$). Overall levels of horizontal activity were modestly lower in males than females. When segregating by sex, only an effect of prior IS was found in males, dramatically decreasing horizontal activity, whereas in females, we observed significant effects of poly ($\chi^2(1) = 6.6$, $p = 0.011$), IS ($\chi^2(1) = 45.9$, $p < 0.001$) and the interaction poly x IS ($\chi^2(1) = 5.4$, $p = 0.021$). Decomposition of the interaction revealed that IS decreased activity both in veh ($p = 0.007$) and poly ($p < 0.001$) females, with greater effect in poly females ($p < 0.001$).

The analysis of rearing frequency in the conditioning context revealed significant effects of sex ($\chi^2(1) = 22.3$, $p < 0.001$) and IS ($\chi^2(1) = 222.1$, $p < 0.001$), whereas the interaction sex x poly x IS was marginally significant ($\chi^2(1) = 3.3$, $p = 0.067$). Overall levels of rearing were modestly lower in males than females. When segregating by sex, only an effect of prior IS was found in males ($\chi^2(1) = 191.2$, $p < 0.001$), markedly decreasing rearing, whereas in females, we observed significant effects of IS ($\chi^2(1) = 65.9$, $p < 0.001$) and the interaction poly x IS ($\chi^2(1) = 4.3$, $p = 0.038$). Decomposition of the interaction revealed that IS decreased rearing both in veh ($p < 0.001$) and poly ($p < 0.001$)

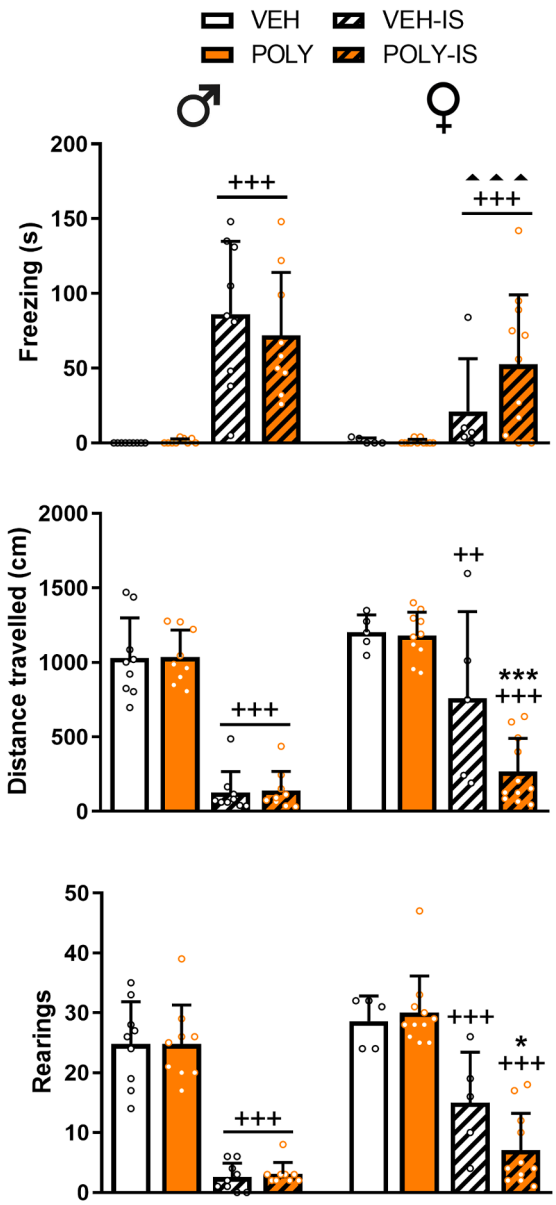

Fig. 3. Behavioral response to a new context B is affected by prior polyI:C and IS treatments. Means and SD of freezing time, distance travelled and frequency of rearings in a new context B are represented. Adult male and female offspring of pregnant mothers receiving vehicle (Veh) or polyI:C (poly) were exposed to the shock chamber (context A) without shocks (control) or were given eight shocks (IS). Three days later they were exposed to a new context B and behavior measured before being exposed to a single shock for fear conditioning. significant sex effect vs corresponding male groups: * significant poly effect vs the corresponding veh same sex group; + significant IS effect vs corresponding same sex control groups. One symbol $p < 0.05$; two symbols $p < 0.01$: three symbols $p < 0.001$.

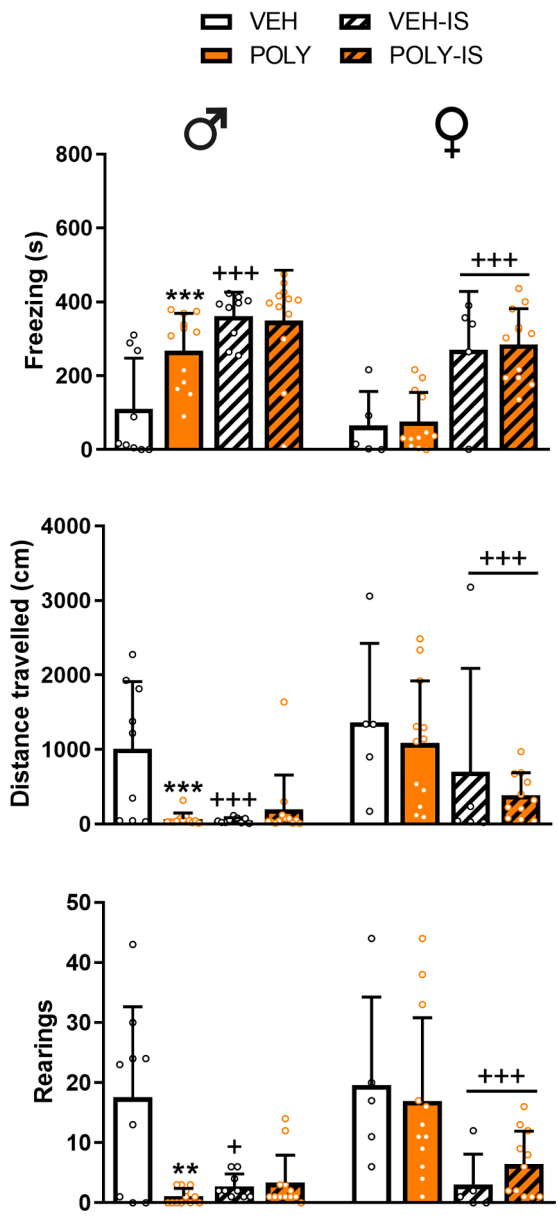

Fig. 4. Fear conditioning to context B is potentiated by prior polyI:C and IS treatments. Means and SD of freezing time, distance travelled and frequency of rearings in the conditioned context B are represented. Adult male and female offspring of pregnant mothers receiving vehicle (veh) or polyI:C (poly) were exposed to the shock chamber (context A) without shocks (control) or were given eight shocks (IS). Three days later were exposed to a new context B to induce contextual fear conditioning and 24 h later evaluated in the same context. Overall, females showed lower levels of freezing and more active behavior than males (not indicated). * Significant poly effect vs the corresponding veh same sex group; + significant IS effect vs corresponding same sex control groups. One symbol $p < 0.05$; two symbols $p < 0.01$: three symbols $p < 0.001$.

females, with greater effect in the latter group ($p = 0.013$).

3.4. *Contextual fear conditioned response (Fig. 4)*

The GzLM analysis of freezing in the conditioning context revealed significant effects of sex ($\chi^2(1) = 7.4$, $p = 0.006$), poly ($\chi^2(1) = 8.1$, $p = 0.004$), IS ($\chi^2(1) = 28.5$, $p < 0.001$) and the interactions poly x IS ($\chi^2(1) = 4.1$, $p = 0.043$) and sex x poly x IS ($\chi^2(1) = 4.1$, $p = 0.044$). Freezing was overall higher in males than females. When segregating by sex, we observed in males significant effects of poly ($\chi^2(1) = 7.5$, $p = 0.006$), IS ($\chi^2(1) = 13.9$, $p < 0.001$) and the interaction poly x IS ($\chi^2(1) = 12.2$, $p <$ 0.001). Decomposition of the interaction revealed that both prior poly and prior IS dramatically increased freezing ($p < 0.001$), with no additive effects. In females, only significant effects of IS was found, resulting in higher freezing levels ($\chi^2(1) = 14.0$, $p < 0.001$).

The analysis of distance travelled in the conditioning context revealed significant effects of sex ($\chi^2(1) = 27.6$, $p < 0.001$), IS ($\chi^2(1) = 19.7$, $p < 0.001$) and the interactions sex x poly ($\chi^2(1) = 3.9$, $p = 0.05$) and poly x IS ($\chi^2(1) = 9.7$, $p = 0.002$). Activity was overall higher in females. When segregating by sex, we observed in males significant

effects of poly ($\chi^2(1) = 5.6$, $p = 0.018$), IS ($\chi^2(1) = 7.8$, $p = 0.005$) and the interaction poly x IS ($\chi^2(1) = 11.0$, $p = 0.001$). Decomposition of the interaction revealed that both prior poly and prior IS decreased activity ($p < 0.001$), with no additive effects. In females, only a significant effect of IS was found ($\chi^2(1) = 12.1$, $p = 0.001$), resulting in lower activity.

The analysis of rearing in the conditioning context revealed significant effects of sex ($\chi^2(1) = 11.7$, $p = 0.001$), IS ($\chi^2(1) = 15.5$, $p < 0.001$) and the interactions sex x poly ($\chi^2(1) = 7.9$, $p = 0.005$), sex x IS ($\chi^2(1) = 7.8$, $p = 0.005$) and poly x IS ($\chi^2(1) = 10.2$, $p = 0.001$). Rearing was overall higher in females. When segregating by sex, we observed in males significant effects of poly ($\chi^2(1) = 10.6$, $p = 0.001$) and the interaction poly x IS ($\chi^2(1) = 8.3$, $p = 0.004$). Decomposition of the interaction revealed that both prior poly and prior IS decreased rearing ($p = 0.004$ and $p = 0.013$, respectively), with no additive effects. In females, it was only found significant effect of IS ($\chi^2(1) = 20.5$, $p < 0.001$), decreasing rearing.

3.5. Behavioral response to a novel context (HB) (Fig. 5)

The GzLM analysis of freezing in the HB revealed effects of sex ($\chi^2(1) = 12.9$, $p < 0.001$), poly ($\chi^2(1) = 4.5$, $p = 0.034$) and IS ($\chi^2(1) = 9.0$, $p = 0.003$). Males showed overall higher levels of freezing than females. When segregating by sex, we observed in males significant effects of poly ($\chi^2(1) = 4.7$, $p = 0.031$) and IS ($\chi^2(1) = 7.2$, $p = 0.007$), both treatments increasing freezing. No statistically significant differences were found in females.

Ambulation showed significant effects of sex ($\chi^2(1) = 46.9$, $p < 0.001$), IS ($\chi^2(1) = 4.6$, $p = 0.033$) and the interaction sex x poly x IS ($\chi^2(1) = 7.0$, $p = 0.008$). Males showed overall lower levels of ambulation than females. When segregating by sex, we observed in males significant effects of poly ($\chi^2(1) = 4.5$, $p = 0.033$) and a marginally significant interaction poly x IS ($\chi^2(1) = 3.2$, $p = 0.075$). Decomposition of the interaction revealed that both prior poly and prior IS decreased ambulation in males ($p = 0.006$ and $p = 0.039$, respectively), with no additive effects. In females, the interaction poly x IS was marginally significant ($\chi^2(1) = 4.5$, $p = 0.055$), the decomposition of the interaction revealing significantly lower levels in poly females after IS exposure ($p = 0.001$).

Rearing were affected by sex ($\chi^2(1) = 22.9$, $p < 0.001$), poly ($\chi^2(1) = 4.5$, $p = 0.034$), IS ($\chi^2(1) = 11.2$, $p = 0.001$) and the interaction sex x poly x IS ($\chi^2(1) = 5.4$, $p = 0.020$). Males showed overall lower levels of rearing than females. When segregating by sex, we observed in males significant effects of poly ($\chi^2(1) = 7.0$, $p = 0.008$) and marginally significant effects of IS ($\chi^2(1) = 3.0$, $p = 0.084$) and the interactions poly x IS ($\chi^2(1) = 3.7$, $p = 0.054$). Decomposition of the interaction revealed that both prior poly and prior IS both decreased rearing ($p = 0.001$ and $p = 0.016$, respectively), with no additive effects. In females, only significant effect of IS was found ($\chi^2(1) = 8.7$, $p = 0.003$), decreasing rearing.

Head-dips were affected by sex ($\chi^2(1) = 7.1$, $p = 0.008$) and poly ($\chi^2(1) = 4.6$, $p = 0.032$), with a marginally significant effect of poly x IS interaction ($\chi^2(1) = 2.8$, $p = 0.092$). Males showed overall lower levels of head-dips than females. When segregating by sex, we observed in males significant effects of poly ($\chi^2(1) = 4.2$, $p = 0.041$), IS ($\chi^2(1) = 3.9$, $p = 0.048$) and the interaction poly x IS ($\chi^2(1) = 8.2$, $p = 0.004$). Decomposition of the interaction revealed that both prior poly and prior IS decreased head-dips ($p = 0.001$ in both cases), with no additive effects. In females, any significant effect was found.

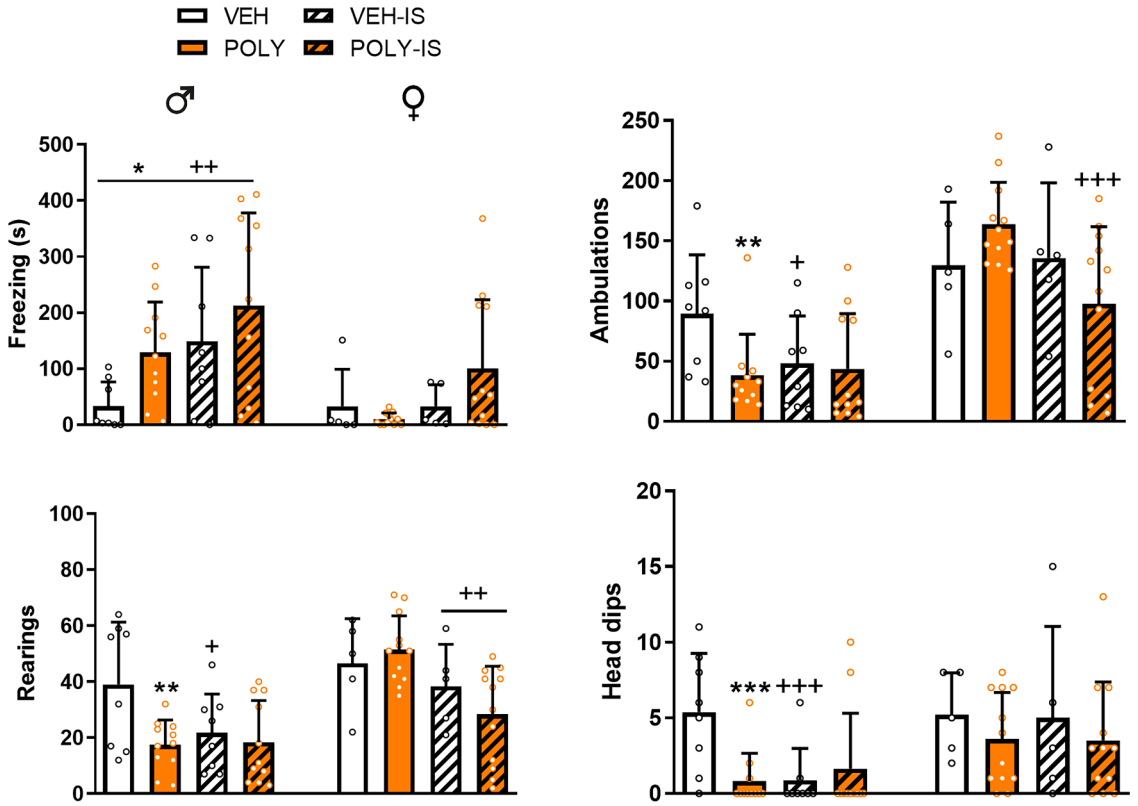

Fig. 5. Interaction of prenatal polyI:C , IS and fear conditioning on the behavior in a markedly different novel environment (holeboard, HB, context C). Means and SD of freezing time, ambulation and frequency of rearing and head-dips in the HB are represented. Adult male and female offspring of pregnant mothers receiving vehicle (Veh) or polyI:C (poly) were exposed to the shock chamber (context A) without shocks (control) or were given eight shocks (IS); Three days later all rats were exposed to context fear conditioning in a new context B and behavior in the HB was evaluated 2 days later. Overall females showed lower levels of freezing and more active behavior than males (not indicated). *Overall significant poly effect or differences vs the corresponding same sex vehicle group; + overall significant IS effect or vs corresponding same sex control group. One symbol, $p < 0.05$; two symbols $p < 0.01$: three symbols $p < 0.001$.

4. Discussion

The present results showed that polyI:C administration during pregnancy caused endocrine and behavioral alterations in the response of the offspring to IS and/or contextual fear conditioning. Some effects were more evident when combining IS exposure with contextual fear conditioning. The impact of MIA was more marked in males than females and are consonant with alterations in fear conditioning observed in schizophrenic patients.

4.1. Hypothalamic-pituitary-adrenal axis

Regardless of prenatal treatment, males showed modestly lower levels of resting ACTH and clearly lower corticosterone levels than females. In response to stress caused by exposure to the IS context (with no shocks) or to shocks, again males showed lower ACTH levels and much lower corticosterone levels than females. All these data are in accordance with our previous results after shock exposure (Daviu et al., 2014; Fuentes et al., 2018). Most published data in rats reveal higher ACTH response to stress in females and much greater differences in corticosterone (Goel et al., 2014).

Prenatal polyI:C did not alter resting hormone levels in males and modestly increased ACTH levels in females, with no effect on corticosterone. However, the ACTH response to the IS chamber and to IS was overall greater in polyI:C than vehicle males, whereas no influence of polyI:C was observed in females. Regarding corticosterone response, no impact of polyI:C was found, probably because corticosterone poorly reflects ACTH release under conditions of nearly maximum adrenocortical secretion (Armario, 2006).

The above results suggest that prenatal polyI:C administration does not modify resting activity of the HPA axis in males but enhanced the response to stress exclusively in males. Only a few studies have assessed activity of the HPA axis in pups from dams receiving polyI:C prenatally and only plasma corticosterone has been measured. Most of them reported no effects or minor effects in adult mice (Abazyan et al., 2010; Pacheco-Lopez et al., 2013; Buschert et al., 2016; Morais et al., 2018) or rats (Yee et al., 2011). In a more recent paper, plasma corticosterone was evaluated in both male and female C57 mice 90 min after a 10 min social interaction test and higher levels were found in males but not females (Zhao et al., 2021), suggesting a greater residual influence of stress associated to the test. Thus, our results, together with available data, tentatively suggest that prenatal polyI:C administration might increase HPA responsiveness to stressors only in males.

4.2. PolyI:C effects on the impact of prior IS exposure and contextual fear conditioning

The context used for contextual fear conditioning (context B) was much larger than those typically used with this procedure to allow the possibility of evaluating the behavioral impact of polyI:C per in those males or females not exposed when adults to IS. Neither horizontal nor vertical activity was found to be altered before being exposed to the conditioning shock, suggesting no effect of the prenatal treatment on general activity in a novel environment. However, prior experience with IS in a different context A notably enhanced freezing and decreased activity (ambulations and rearings) in context B, suggesting fear generalization despite major differences in the room and the apparatus (illumination, color of the walls, size and odor), which nevertheless had in common the grid floor. In studies about generalization, after changing some elements of the original context, the grid floor and the odor have been found to be the most critical elements (Gonzalez et al., 2003; Huckleberry et al., 2016). In fact, no or minor differences were observed between the original shock context and another context differing in some elements but having in common the grid (Luyten et al., 2016). The degree of generalization observed after IS exposure was greater than that observed in studies from Fanselow's lab that used similar procedures and rat strain, although the authors modified the disposition of grid rods (e.g. Rau et al., 2005). It is unclear whether the configuration of the rods is critical or if other factors can be considered.

Although IS-induced context generalization was observed in both sexes, this was overall higher in males than females. Moreover, an impact of polyI:C was only found in females, with a potentiation of IS-induced hipoactivity. Freezing followed the opposite trend but did not reach statistical significance. Although the results are suggestive of a greater impact of polyI:C in females than males, it is plausible that the lack of poly I:C effect in males was due to the already high levels of freezing in vehicle (non-poly) subjects (ceiling effect), thus behavioral inhibition reaching a ceiling effect. In any case, the greater impact of prior IS in polyI:C females suggests that polyI:C could affect fear generalization. The issue of fear generalization will be further discussed.

In the present study, conditioned freezing was overall lower in females than males. Most studies in rats have reported similar findings (e.g., Maren et al., 1994; Agnostaras et al., 1998; Brunzell et al., 2002, Yavas et al., 2021), whereas results regarding sex differences in cued fear conditioning are in general negative (Gresack et al., 2009; Milad et al., 2009) and the particular rat strain has to be considered (Pryce et al., 1999). Importantly, the present results demonstrated that polyI:C administration per se, in absence of prior IS exposure, potentiated contextual fear conditioning in males but not females, revealing a dramatic sex difference. Although only a few previous studies have focused on the impact of polyI:C on fear conditioning in males, most results are negative regarding tone fear conditioning in both rats (Vorhees et al., 2012; Yee et al., 2012; Sangha et al., 2014) and C57 mice (Meyer et al., 2006; Schwendener et al., 2009; Stollenweek and Hillard, 2021). However, either enhanced or reduced tone fear conditioning has also been found (Weber-Stadlbauer et al., 2017; Vuillermot et al., 2017, respectively). In the only study specifically addressing contextual fear conditioning, no impact of polyI:C was found in mixed sex C57 mice (Labouesse et al., 2015). Whether the results observed in the present study are due to a particular sensitivity of Long-Evans rats to polyI:C is unclear, but we have observed higher levels of contextual fear conditioning in Long-Evans as compared with Sprague-Dawley rats (unpublished).

In full agreement with prior data from Fanselow`s lab (Rau et al., 2005; 2009; Poulos et al., 2015; Perusini et al., 2016), prior exposure to IS potentiated contextual fear conditioning in both males and females of the vehicle (non-poly) groups, with overall higher levels of freezing in males. In polyI:C animals, prior IS exposure enhanced contextual fear conditioning in females, but not in males, probably due to the already high levels of freezing in absence of prior IS. Since enhanced fear conditioning after IS has been observed after the extinction of fear in the IS context, it has been suggested that it represents a sensitization phenomenon (Rau et al., 2005). The SEFL phenomenon has mainly been studied using IS, but there is sporadic evidence in rats that other stressors such as restraint can also potentiate contextual fear conditioning (e.g., Cordero et al., 2003).

4.3. How polyI:C and prior IS exposure affects behavioral inhibition in novel environments

It has been demonstrated that exposure to severe footshocks, but also to a classical brief protocol of shocks for contextual fear conditioning, can induce hypoactivity of animals in novel environments that completely differ from the shock context (van Dijken et al., 1992a, 1992b; Radulovic et al., 1998; Daviu et al., 2010, 2014). Importantly, this generalization was not observed in absence of contextual fear conditioning learning (Radulovic et al., 1998; Daviu et al., 2010), suggesting a major contribution of associative learning rather than of shock-induced sensitization. Since it is unlikely that rats cannot distinguish in the present and other studies between the shock contexts (and associated odors) and other novel environment such as a circular open-field or a conditioned place preference apparatus, we have termed

this phenomenon cognitive fear generalization (Daviu et al., 2014; Fuentes et al., 2018) to distinguish this type of generalization from that caused by partial similarities between the novel contexts and the shock context that involves deficits in configurational acquisition (O'Reilly and Rudy, 2001; Fanselow, 2010). Thus, to further study this type of generalization, all rats were assessed after contextual fear conditioning in a novel environment (context C) completely different from the IS and the fear conditioning contexts, a HB apparatus. The HB is used to evaluate for activity and exploration and has a distinctive floor provided with holes.

It is of note that in the present study, before exposure to the HB, all rats had experienced shocks (control rats in the conditioning context and IS rats both during IS and in the conditioning context) before being exposed to the HB, allowing us the opportunity to study the impact of single (contextual fear conditioning) and double (IS and contextual fear conditioning) experience of shocks. In the HB, not only hypoactivity, but also freezing, was very often observed, whereas no freezing at all is typically found in shock-naive Long-Evans rats (unpublished data), remarking the high degree of cognitive fear generalization found in the present study. In control (non-IS) males, polyI:C markedly enhanced fear generalization in the HB as evaluated by freezing and all parameters of activity, whereas no effects were found in females, indicating again that males were much more sensitive to the impact of polyI:C than females. Results in IS animals are more difficult to interpret due to the double exposure to shocks, but prior IS in non-poly rats increased freezing and reduced activity in males but not females. The double exposure to shocks in polyI:C rats did not further inhibit behavior in males, probably due to a ceiling effect, and only exerted a minor, if any, effect in females, the small effect in the latter was not explained by a prior effect of polyI:C.

The greater behavioral inhibition in the HB of males exposed to either polyI:C or IS, or both, roughly paralleled the higher levels of contextual fear conditioning. This parallelism was less obvious in females, particularly in non-poly rats exposed to IS, in which a clearly potentiated contextual fear conditioning was observed, without altered behavior in the HB. It is unclear whether females could be less sensitive to cognitive generalization than males under similar levels of contextual fear conditioning. Studies of the effects of IS on behavior in different apparatuses used to evaluate anxiety-like behavior suggest a lower impact in females (Steenbergen et al., 1990, 1991; Diehl et al., 2007). However, studies specifically addressing sex differences in the generalization of fear in rodents are scarce. Although preliminary conclusions appear to favor the hypothesis of greater generalization in females (Tronson and Keiser, 2019; Bauer, 2023), our previous results (Daviu et al., 2014, Fuentes et al., 2018) and the present data suggest that at least cognitive fear generalization is greater in males than females.

It is unclear whether possible cognitive alterations caused by polyI:C, particularly those related to hippocampal function, could have influenced contextual fear conditioning and fear generalization in the present study. However, results regarding the impact of prenatal polyI:C administration on spatial memory in the Morris water maze are in general negative (e.g., Zuckerman and Weiner, 2005; Vorhees et al., 2012).

In sum, the present results offer compelling evidence for an impact of polyI:C on fear conditioning and cognitive fear generalization that was much more evident in males than females. These data are of relevance considering that studies of fear conditioning in schizophrenia have highlighted the abnormalities of those subjects in fear generalization and the capability to distinguish between aversive and safety cues (Hofer et al., 2001; Jensen et al., 2008; Romaniuk et al., 2010; Holt et al., 2012¸Tuominen et al., 2022). The fact that we found a potentiation of cognitive fear generalization in our polyI:C model, suggests the interesting possibility that alterations in this domain could also be observed in schizoprenic patients. The study of the consequences of IS and fear conditioning after polyI:C or other animal models of schizophrenia may be useful to reveal brain abnormalities in these patients.

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Ethical statements

The experimental protocol was approved by the Ethics Committee at the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, and it was carried out in accordance with the European Council Directive (2010/63/UE) and Spanish legislation (RD 53/2013).

CRediT authorship contribution statement

Antonio Armario: Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Roser Nadal:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Silvia Fuentes:** Writing – review & editing, Validation, Methodology, Investigation. **Joan Visa:** Investigation. **Xavier Belda:** Investigation. **Sara Serrano:** Writing – review & editing, Visualization, Formal analysis. **Javier Labad:** Writing – review & editing, Project administration, Funding acquisition, Data curation, 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.

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