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Maternal immune activation transgenerationally modulates maternal care and offspring depression-like behavior

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ABSTRACT

Gestational infection is increasingly being recognized for its involvement as causative mechanism in severe developmental brain abnormalities and its contribution to the pathogenesis of psychopathologies later in life. First observations in the widely accepted maternal immune activation (MIA) model based upon the systemic administration of the viral mimetic Polyinosinic:polycytidylic acid (poly(I:C)) have recently suggested a transmission of behavioral and transcriptional traits across generations. Although maternal care behavior (MCB) is known as essential mediator of the transgenerational effects of environmental challenges on offspring brain function and behavior, the possible propagation of alterations of MCB resulting from MIA to following generations has not yet been examined. Here we show that poly (I:C) stimulation at embryonic day 12.5 (E12.5) leads to aberrant MCB and that this effect is transmitted to the female F1 offspring. The transgenerational effects on MCB are paralleled by enhanced depression-like behavior in the second generation F2 offspring with contributions of both maternal and paternal heritages. Examination of offspring hippocampal expression of genes known as targets of MCB and relevant for ensuing non-genetic transmission of altered brain function and behavior revealed transgenerationally conserved and modified expressional patterns in the F1 and F2 generation.

Collectively these data firstly demonstrate the transgenerational transmission of the impact of gestational immune activation on the reproductive care behavior of the mother. Behavioral and molecular characteristics of first and second generation offspring suggest transgenerationally imprinted consequences of gestational infection on psychopathological traits related to mood disorders which remain to be examined in future cross-fostering experiments.

1. Introduction

Early life adversity including exposure to intrauterine immune challenges resulting of infection during pregnancy is a known risk factor for the development of various psychopathologies later in life (Cowan et al., 2016; Groger et al., 1996; McEwen, 2003; Meyer et al., 2008). Epidemiological studies demonstrate a link between maternal immune activation (MIA) during pregnancy and the development of severe psychiatric disorders, including autism, personality and affective disorders (Knuesel and et al., 2014; Reisinger et al., 2015; Ronovsky et al., 2015). Excellent animal models of MIA have been generated as tools for the study of the causal relationship between MIA and offspring behavioral and emotional disturbances and to examine the relevant neurobiological mechanisms involved (Meyer et al., 2009). Among those is administration of the viral mimetic Polyinosinic:polycytidylic acid (poly(I:C)) to pregnant rodents, which causes activation of the maternal innate host defense mechanism against viruses via the toll-like receptor 3 (TLR3) pathway (Reisinger et al., 2015). Major advances in the understanding of the pathological events underlying fetal brain damage and the ensuing behavioral consequences have been made using the poly(I:C) MIA model (Labouesse et al., 2015; Meyer, 2014; Meyer and Feldon, 2012, 2009; Meyer et al., 2007). Among these, accumulating evidences for long-lasting effects of MIA are demonstrated by reports from our laboratory linking poly(I:C)-induced MIA at embryonic day (E) 12.5 with a depression-like phenotype in adult offspring (Khan and et al., 2014; Reisinger et al., 2016) at the behavioral, morphological and electrophysiological levels.

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Interestingly, a seminal study recently proposed transgenerational, non-genetic effects of MIA on offspring behavior extending beyond the first generation (F1) to the second (F2) and even third generation, without repetition of immune stimulation. Unique and shared patterns of gene expression changes have been identified in the offspring F1 and F2 amygdala in this experimental setting (Weber-Stadlbauer et al., 2016).

A prime candidate for mediating behavioral and transcriptional alterations across generations is maternal care behavior (MCB) (Champagne and Curley, 2009; Gudsnuk and Champagne, 2012, 2011; Monk et al., 2012) and MCB has been shown to be sensitive to early life adversities (Meek et al., 2001). Specifically, deficits in MCB have been suggested to promote a modulatory impact on hypothalamic-pituitaryadrenal (HPA) axis function. Glucocorticoid receptors, the high-affinity mineralocorticoid receptor and the low-affinity glucocorticoid receptor play a central role in the long-term, compensatory processes resulting from chronic stress exposure with relevance for the regulation of emotional, cognitive and neuroendocrine responses to stress and the susceptibility to the development of severe psychopathologies, including depression (de Kloet and et al., 2016; Farrell and O'Keane, 2016). The effects of MCB on the HPA axis can be propagated via epigenetic mechanisms (Meaney, 2001) - mediators of changes in gene expression without alterations of DNA sequences - to subsequent generations.

However, the particular involvement of MCB in the transgenerational effects of MIA on offspring depression-like behavior has not been examined so far. Here we employed a standard protocol for poly(I:C)-based MIA to investigate the effects of immune activation during pregnancy on MCB, its potential transmission to F1 generation females and depression-like behavior in F2 female offspring along the maternal and paternal lineages. Expressional changes of genes known as candidates for epigenetic modifications by MCB and relevant for the pathophysiology of depression were interrogated as potential molecular correlates in the F1 and F2 generations.

2. Materials and methods

2.1. Animals

C57Bl6/N mice were used for all experiments. For initial breeding animals were purchased from Charles River (Sulzfeld, Germany) at 6–8 weeks of age. All animals were housed under standard conditions in a temperature controlled colony room (22 ± 1 °C) with a 12 h light/dark cycle and food and water *ad libitum* unless otherwise stated. The light intensity was 5–10 lux inside the cages. All animal experiments were carried out in accordance with the ARRIVE guidelines and the U.K. Animals (Scientific Procedures Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments). Animal experiments described in this study were approved by the national ethical committee on animal care and use (BMWF-66.009/0200-WF/V/3b/2016; Bundesministerium für Wissenschaft und Forschung).

2.2. Timed mating

For breeding of the F1 and F2 generations, a timed mating procedure was employed as previously described (Khan and et al., 2014). Briefly, custom-made cages with a central division by a plexiglas wall allowing for odor exchange were used in order to induce the "Whitten Effect" (stimulation of the estrus in females by male pheromones) prior to mating. 3–4 females were put together on one side of the cage wall and one male on the other side for 60 h. Animals were mated overnight starting from 7 pm by placing one female into a male cage. The following day (9 am), the presence of vaginal plugs (considered as E 0.5) together with the body weight was recorded and males were transferred into a new cage.

2.3. MIA and breeding scheme

Poly(I:C) (Sigma, Vienna, Austria) was dissolved in 0.9% NaCl at a final concentration of 2 mg/ml (calculated based upon the weight of poly(I:C) in the mixture itself). Pregnant females were randomly divided into two groups, for MIA and control treatment, respectively. On E12.5, MIA dams received poly(I:C) (20 mg/kg, intraperitoneal (i.p.)) and controls were applied 0.9% NaCl (i.p.), both at 10 ml/ kg injection volume. The dosage regime and time point were chosen based upon previous studies demonstrating enhanced depression-like behavior in adult offspring after MIA on E12.5 (Khan and et al., 2014; Reisinger et al., 2016). MCB was recorded in P0 dams from postnatal day (PD) 1–6. All pups (F1 offspring) were weaned on postnatal day 21, separated by sex and group-housed until adulthood (8 weeks of age). Adult F1 offspring were separated in two cohorts which were used for hippocampal gene expression or breeding of F2 offspring respectively. The mating scheme for the generation of F1 and F2 offspring is illustrated in Fig. 1. At the age of 8 weeks mice were considered adult and were used in a timed mating procedure or for behavioral analyses. Behaviorally naïve mice were always used for breeding and gene expression analyses in order to exclude the possibility of effects of testing history and their interaction with the experimental manipulations on maternal behavior and/or transcriptional changes. In order to control for potential litter-specific effects, experimental groups using F1 and F2 offspring were always composed by selecting corresponding numbers of representative animals from different litters. The numbers of animals/samples used in each experiment is summarized in Supplementary Table 1.

2.4. Behavioral analysis

2.4.1. Maternal care behavior

P0 dams and F1 dams were assessed for MCB after birth of the F1 and F2 generations, respectively. The analysis of MCB followed a published protocol with the day of birth considered as PD 0 (Franks and B., 2011). From PD 1 to PD 6 maternal behavior was recorded daily from 11 am to 1 pm and from 3 pm to 5 pm with commercially available webcams (Logitech C525 HD Webcam, Microsoft LifeCam HD-3000, Trust Widescreen HD-Webcam, Creative LIVE! Cam Chat HD USB-Webcam). Behaviors were video-scored every 3 min by an experimenter blind to the experimental conditions. Parameters evaluated consisted of pup licking/grooming, nest-building, nursing and non-pup relevant parameters (eating/sleeping, self-grooming).

2.4.2. Sucrose preference test (SPT)

Sucrose preference in the SPT was tested in female F2 offspring. The SPT was conducted as previously described (Savalli and et al., 2015). Briefly, mice were deprived of food and water 18 h before the test where they were given the choice to consume liquid from two equal bottles, one containing a 2% sucrose solution and one filled with regular drinking water. The volumes of sucrose solution and drinking water consumed during the SPT were determined by weighing the bottles before and after the 3 h testing period and used for the calculation of the percentage of sucrose preference.

2.4.3. Forced swim test (FST)

Time spent immobile in the FST was assessed in female F2 offspring. The FST was carried out as described earlier (Monje and

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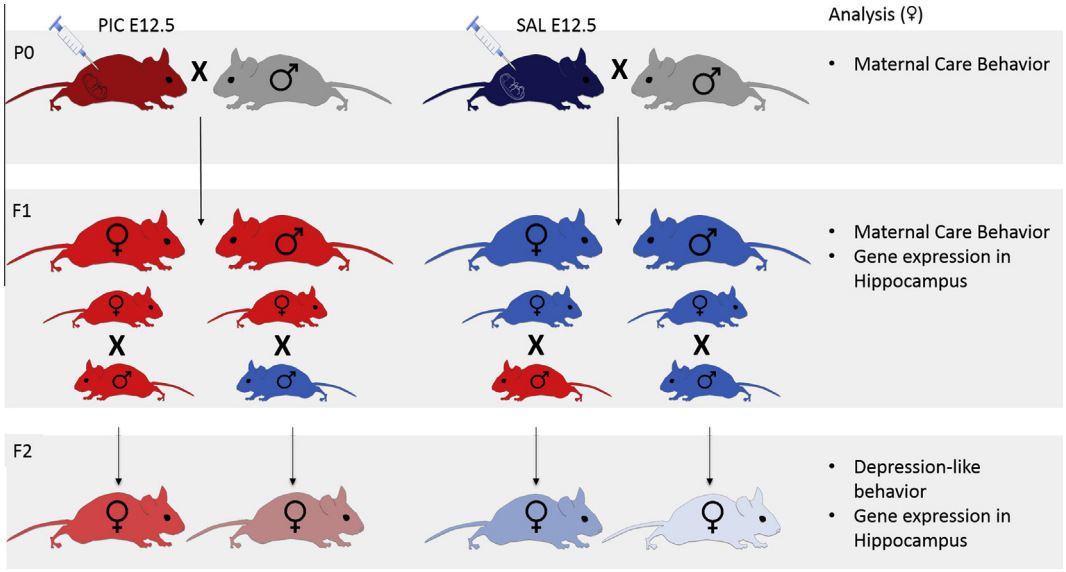

Fig. 1. Study design. Study design employed for the examination of the transgenerational effects of poly(I:C)-induced maternal immune activation at embryonic day 12.5 on maternal care, offspring depression-like behavior and hippocampal gene expression.

et al., 2011). Mice were placed in glass beakers 19 cm in diameter and 23 cm deep filled with water at 22°-24 °C. The time mice spent immobile was automatically recorded and analyzed using a commercial data acquisition and analysis software (VIDEOTRACK (PORSOLT), Viewpoint, Champagne au mont d'Or, France). The percentage of time spent immobile was calculated for each minute of the six minutes testing period.

2.4.4. Open field (OF)

The OF was used for the evaluation of exploratory, locomotor activity and anxiety-like behavior in female F2 offspring. Mice were tracked using 4 horizontal infrared beams on opposing walls, 3 cms and 10 cms (respectively) and 2 horizontal beams, 3 cms from the bottom of the open field boxes. These infrared beams were coupled to a computational tracking system (Activity Monitor, MedAssociates, St Albans, VT, USA) in an arena (27.5 cm × 27.5 cm; with 21 cm high walls). Testing time was held constant for all animals at 60 min. Standard parameters for locomotor activity and exploratory behavior were automatically recorded using the software. For analyzing anxiety-like behavior, a virtual center was user-defined in the software, equaling twenty-five percent of the total area (Bailey et al., 2009). Time spent in center zone was automatically recorded using the software.

2.4.5. Rota rod (RR)

Motor coordination was tested by evaluating the latency to fall off the RR in female F2 offspring. The automated set-up (USB Rota Rod "SOF-ENV-57X", MedAssociates Inc., St. Albans USA) comprises of a rotating drum which was accelerated from 4 to 40 RPM over the course of 5 min. Motor coordination of an animal was evaluated by the automated recording of the length of time it could stay on the rotating drum while it accelerated. When an animal could balance on the rotating drum for the entire duration of 5 min of test time at 40 RPM, the animal was removed and returned to its home cage. The RR test was performed three times for 5 min and mice received a 15 min break between trials (Rogers and et al., 1999).

2.5. Molecular analysis

2.5.1. Brain dissection

Mice were rapidly sacrificed by neck dislocation and brains were immediately extracted over ice. Hippocampal tissue was dissected and stored in RNA later buffer (Ambion, Vienna, Austria) for 24 h at 4 °C and then at −20 °C until later usage.

2.5.2. RNA extraction and qRT-PCR

Total RNA was extracted from hippocampal tissue using a commercial kit (miRNeasy Mini Kit (Qiagen, CA, USA) and transcribed into cDNA by DyNAmo cDNA Synthesis Kit (Thermo Fisher Scientific Inc.). 0.2 μL of cDNA were diluted into 7 μL of RNase-free H2O, the final volume of 7.2 μL cDNA was mixed with 7.8 μL of the SYBR Green Mastermix, consisting of 7.5 μL SYBR Green Mix and 0.15 μL from forward and reverse primer (20 nM) respectively. For primer sequences see Supplementary Table 2. The thermal cycling profiles were 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each cDNA sample was analyzed in duplicates and dCt (delta cycle threshold) values were calculated by normalizing Ct values of the gene of interest to Ct values of the housekeeping gene β-actin. Data were plotted using the formula: $2^{-\Delta dCT}$ (ddCT), where ddCT is the difference between the mean dCT of each group and the mean dCT of the control group.

2.6. Statistical analysis

All data were tested for normality using the Kolmogorov-Smirnov test prior to further statistical evaluation and outlier exclusion. Significant outliers were identified based upon the extreme studentized deviate methods using the Grubbs' test (Supplementary Table 3). Statistical analysis of MCB in P0 generation and gene expression in F1 MIA offspring was based upon using unpaired two-tailed Student's *t* test. Two-way analysis of variance (ANOVA) (mother × father) was employed for statistical evaluation of the SPT, MCB in F1 MIA offspring and gene expression in F2 MIA offspring. A mixed model design ANOVA was applied as statistical instrument for the evaluation of immobility in the FST (within-subject variables: minute 1–6, between subject-variables: mother × father). For evaluation of between-group differences in

F1 maternal behavior, F2 depression-like behavior and F2 gene expression, Scheffé pair-wise comparisons were calculated.

A linear regression model was applied for correlational analysis between maternal behavior, offspring behavior and gene expression. All data were analyzed using SPSS (IBM, SPSS 18.0) statistical software with the alpha level set at 0.05 at all instances.

3. Results

3.1. Poly(I:C) treatment at E12.5 alters maternal care behavior (MCB)

We first set-out to examine potential effects of MIA on MCB by determining the amount of time poly(I:C) stimulated and control mothers engaged in relevant behaviors from PD 1 to PD 6. The parameters evaluated focused on pup licking/grooming (LG), nest-building, nursing and non-pup relevant (eating/sleeping, self-grooming) behavior which were assessed for 4 h per day (Franks and B., 2011). Statistical analysis revealed a significant reduction in the time poly(I:C) treated mothers spent in LG behavior ($p < 0.001$) while they were more engaged in nest-building behavior ($p < 0.05$) (Fig. 2a-b). No differences in nursing and non-pup relevant behaviors examined were observed (Fig. 2c-d).

3.2. Maternal care behavior is altered in F1 MIA offspring

In order to test the hypothesis that altered MCB in poly(I:C) stimulated dams constitutes a behavioral phenotype resulting from MIA which can be transgenerationally propagated, we examined MCB in female offspring of poly(I:C) stimulated and control mothers (F1 mothers). To this end, F1 MIA and control females were mated with F1 MIA and control males in a 2 × 2 design (Fig. 1) allowing for the differential evaluation of the potential propagation of the effects of MIA on MCB through the maternal and paternal side and their possible interaction.

2-way ANOVA analysis determined a significant main effect of the father ($F_{(3,19)} = 6.1$, $p < 0.05$) and a significant mother × father interaction ($F_{(3,19)} = 17.7$, $p < 0.001$) on the time females spent in LG, with F1 dams with both mother and father originating from a background of MIA (♀PICx♂PIC) showing highest LG behavior, while MIA exclusively along the maternal, but not the paternal side (♀PICx♂SAL), was associated with lowest amount of time spent in LG in F1 dams (Fig. 3a). In contrast to the F1 generation, no apparent modulation of the parental MIA heritage on the time mothers engaged in nest-building behavior was observed (Fig. 3b). However, a significant main effect of the mother ($F_{(3,19)} = 12.9$, $p < 0.01$) and a significant mother × father interaction ($F_{(3,19)} = 6.1$, $p < 0.05$) for the time dams spent nursing the pups were revealed, as ♀PICx♂PIC F1 mothers displayed the least engagement in nursing (Fig. 3c). For the combined time investment in non-pup relevant behaviors, a significant main effect of the mother ($F_{(3,19)} = 10.0$, $p < 0.01$) was observed with ♀PICx♂PIC dams presenting with the highest levels.

3.3. Depression-like behavior in F2 generation offspring with history of MIA along the maternal and paternal lineages

Using the herein employed poly(I:C) stimulation protocol, an effect of MIA on depression-like behavior in adult F1 offspring has been described previously (Khan and et al., 2014; Reisinger et al., 2016). To investigate whether this augmented depression-like behavior of F1 MIA offspring can be transmitted to the next generation we evaluated behavioral despair using the FST and anhedonic behavior using the SPT in adult female F2 offspring

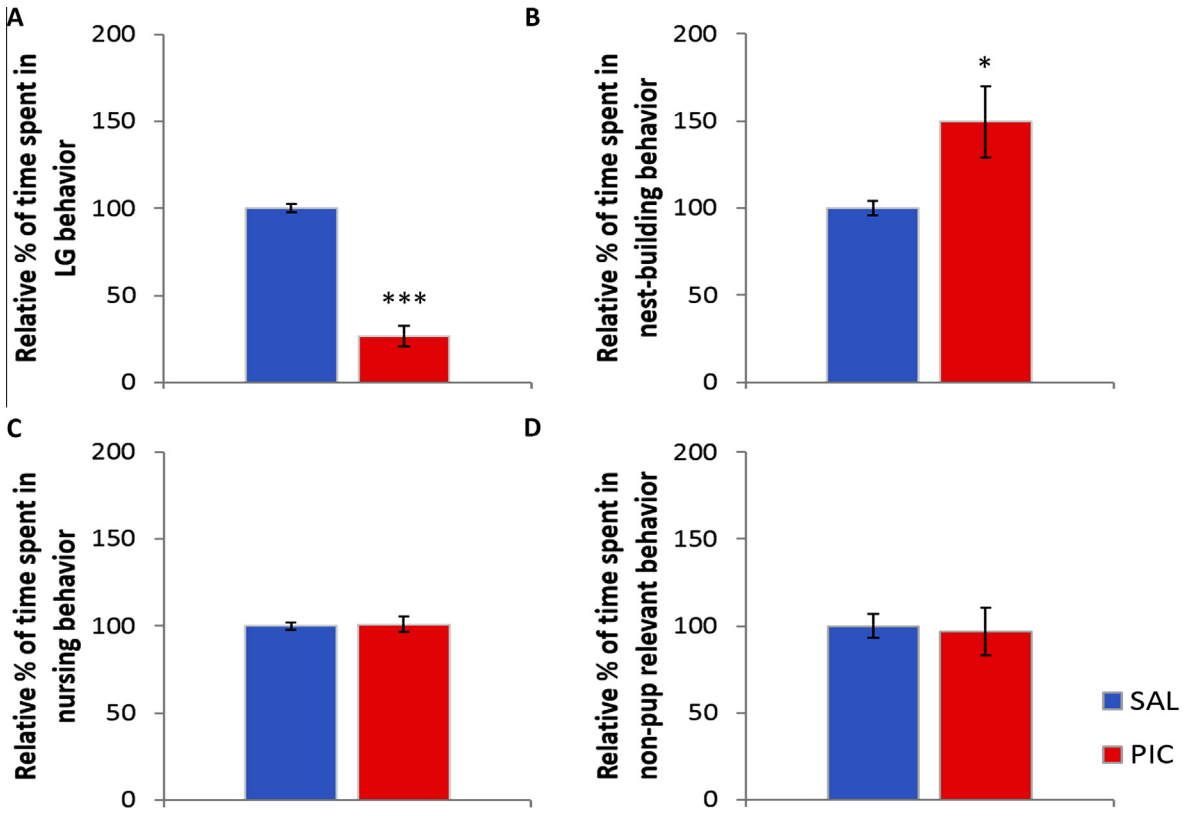

Fig. 2. Maternal care behavior in P0 dams after MIA induced by poly(I:C) stimulation at E12.5. Engagement in a.) licking/grooming, b.) nest-building, c.) nursing, d.) non-pup relevant behavior (sum of self-grooming, sleeping and eating/drinking) of poly(I:C) stimulated (PIC) relative to the percentage of total time spent by saline control (SAL) mothers. Data are depicted as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$; n = 6–10 per group.

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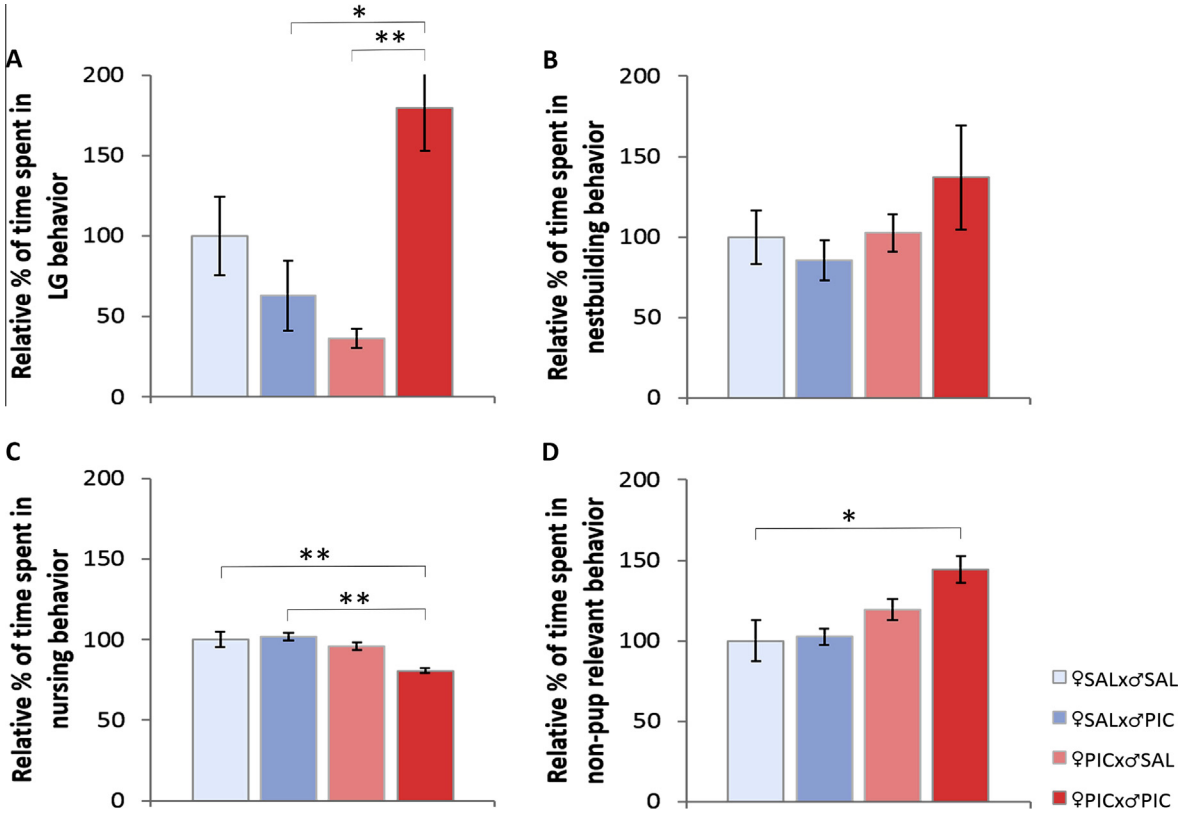

Fig. 3. Maternal care behavior in F1 dams. Engagement in a.) licking/grooming, b.) nest-building, c.) nursing, d.) non-pup relevant behavior (sum of self-grooming, sleeping and eating/drinking) of PIC or SAL mothers mated with male offspring of PIC or SAL mothers relative to the percentage of total time spent by SAL controls (mother (♀) SAL and father (♂) SAL) (n = 5–7 per group). Data are depicted as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ indicates results of post hoc pairwise comparisons using the Scheffé procedure.

derived from MIA stimulation along the maternal and paternal lineages and control animals (Fig. 1). In agreement with a recently published study (Weber-Stadlbauer et al., 2016) we observed enhanced behavioral despair in the FST in F2 offspring with history of MIA in the grandparental generation with highest immobility displayed by ♀PICx♂PIC animals with both mother and father carrying ancestral poly(I:C) stimulation. ♀PICx♂SAL F2 offspring of maternal but not paternal MIA lineages were found to present with the lowest immobility (significant main effect of mother: $F_{(3302)} = 9.46$, $p < 0.01$; significant mother × father: $F_{(3302)} = 4.43$, $p < 0.05$) (Fig. 4a). Consistent with this observation, a significant reduction in sucrose preference, indicative of increased anhedonic behavior, was noted specifically in ♀PICx♂PIC F2 offspring (significant main effect of mother: $F_{(3,47)} = 34.04$, $p < 0.001$; significant main effect of father: $F_{(3,47)} = 29.12$, $p < 0.001$, significant main effect mother × father: $F_{(3,47)} = 10.08$, $p < 0.01$) (Fig. 4b). F2 offspring depression-like behavior correlated with their mothers' display of maternal behavior. Specifically, immobility in the FST in F2 females was significantly correlated with F1 dams' nursing behavior ($R^2 = 0.61$, $p < 0.01$) (Fig. 4c) while sucrose preference in the SPT correlated with the amount of LG behavior displayed by F1 mothers ($R^2 = 0.64$, $p < 0.01$) (Fig. 4d). To rule out possible confounds on FST and SPT by possible effects of MIA on F2 offspring general behavioral functions the OF and RR tests were carried out. No significant differences between F2 offspring groups were detected in any of the parameters evaluated (Supplementary Fig. 1).

3.4. Differential hippocampal mRNA expression of mineralocorticoid receptor (MCR) and glucocorticoid receptor (GCR) in F1 MIA offspring

In search for potential neurobiological correlates accompanying the transgenerational effects of MIA on depression-like behavior in the offspring brain, we turned towards the examination of molecular elements known to be modulated as targets of altered MCB and relevant for the pathogenesis of depression. We first focused on the analysis of the F1 generation by examining possible transcriptional changes in the hippocampus of MIA as compared to control offspring. The hippocampus was selected as region of interest due to its prominent role in the neural circuitry associated with depression (Campbell and Macqueen, 2004; MacQueen and Frodl, 2011; Sheline et al., 2002). Hippocampal MCR and GCR mRNA levels were analyzed by qRT-PCR in behaviorally naïve adult female F1 MIA and control offspring. MCR and GCR are pivotal constituents of the central stress response system along the HPA- axis through their binding of adrenal steroids (de Kloet et al., 1990; Funder and Sheppard, 1987; Sutanto and de Kloet, 1991). mRNA expression of MCR and GCR, both of which have been implicated in the pathophysiology of depression (de Kloet and et al., 2016; Farrell and O'Keane, 2016), was significantly increased in the hippocampus of F1 MIA as compared to controls (MCR $p < 0.001$; GCR $p < 0.05$; Fig. 5a-b). MCR and GCR hippocampal mRNA expression in the F1 generation was significantly correlated with the amount of LG behavior displayed by their P0 mothers behavior ($R^2 = 0.8$, $p < 0.05$ and $R^2 = 0.8$, $p < 0.05$ (Fig. 4c-d). No expressional changes in other genes previously described to be regulated by MCB (Bridges, 2015; Champagne, 2011; Curley et al., 2012; Zimmermann-Peruzatto et al., 2015) and related to depression (Feldman et al., 2016; Neumann and Landgraf, 2012; Walf and Frye, 2006) (i.e. Oxytocin Receptor (OXTR), G-Protein Coupled Estrogen Receptor (GPER), Estrogen Receptor Alpha (ERa), Estrogen Receptor Beta (ERb), Vasopressin Receptor 1a (V1aR) and Vasopressin Receptor 1b (V1bR)) were found in hippocampal tissue of F1 MIA offspring (Supplementary Fig. 2).

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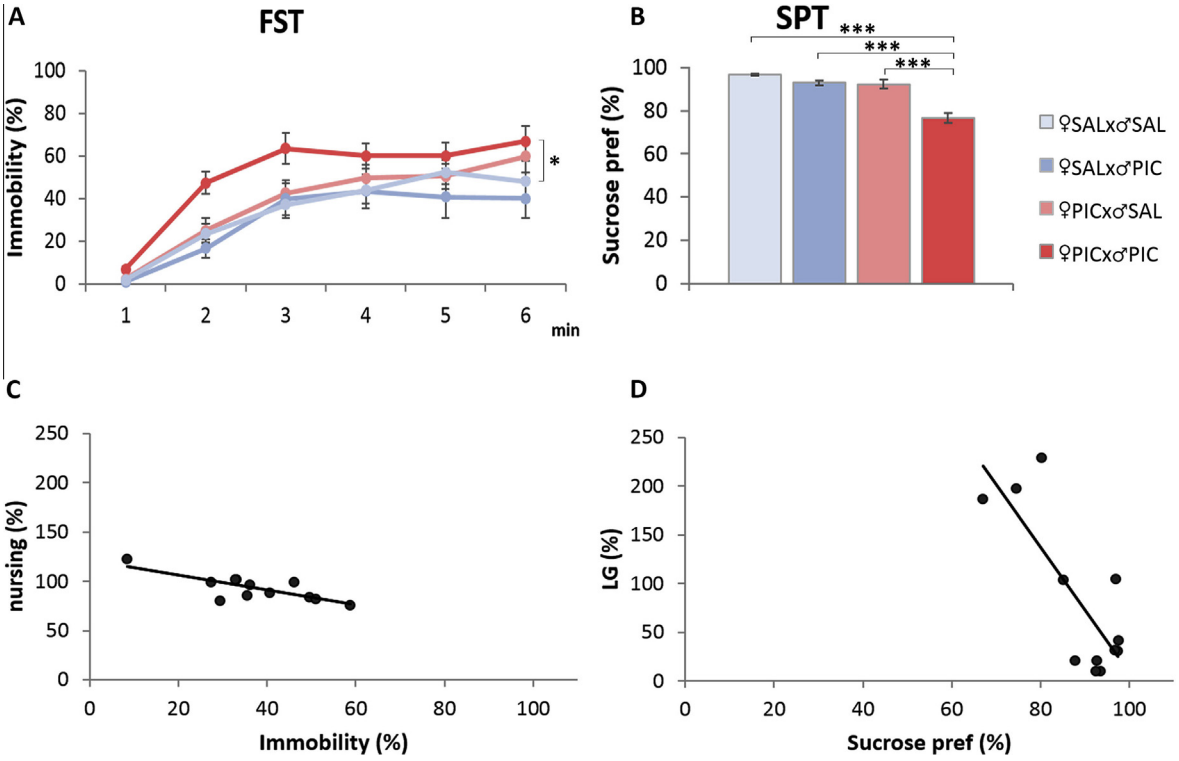

Fig. 4. Behavioral phenotypes of F2 MIA offspring. a) Percentage (%) of time spent immobile from minute 1–6 in the FST and b.) % of Sucrose preference relative to total liquid consumption in the SPT as indicator of depression-like behavior in F2 offspring (n = 8–13 per group) with heritage of poly(I:C) stimulation along the maternal (♀), paternal (♂) or both (♀ × ♂) lineages. Data are displayed as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ indicates results of post hoc pairwise comparisons using the Scheffé procedure. c.) Correlation between F1 dam nursing behavior and F2 offspring immobility in the FST d.) Correlation between F1 dam licking/grooming (LG) behavior and F2 offspring sucrose preference in the SPT.

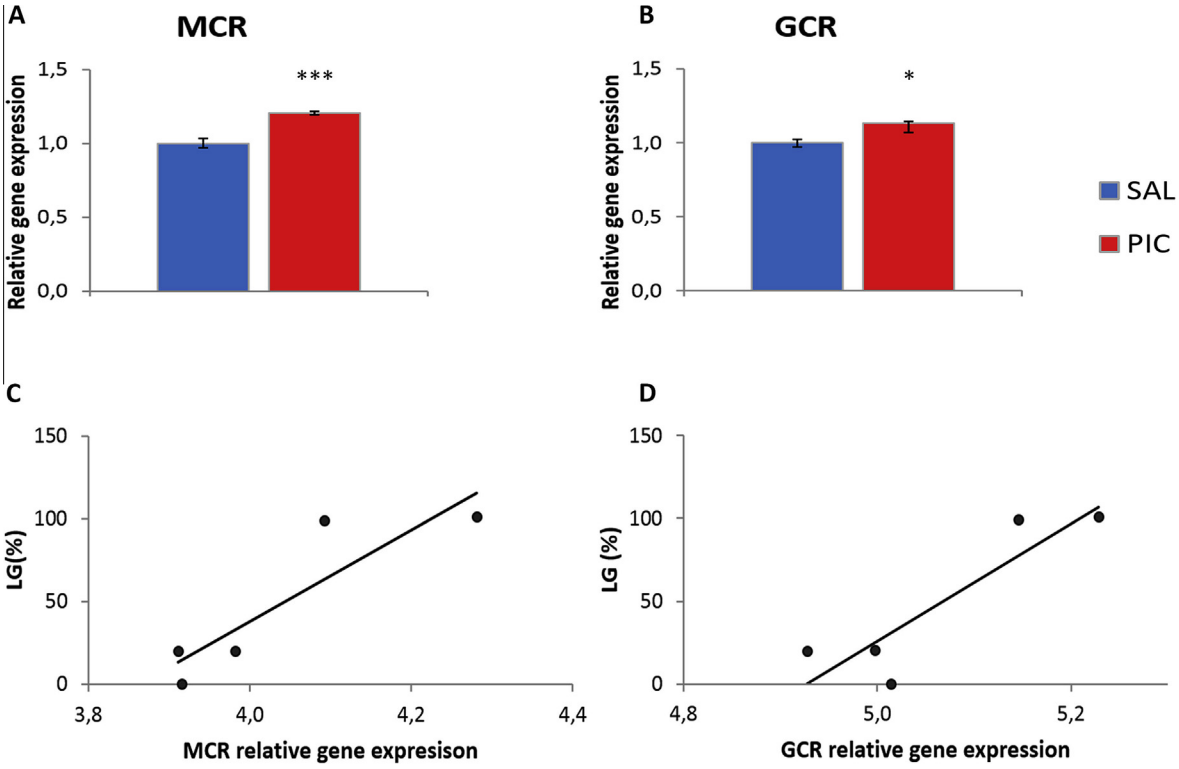

Fig. 5. Hippocampal gene expression in F1 offspring. Relative gene expression of a.) Mineralocorticoid Receptor (MCR), b.) Glucocorticoid Receptor (GCR) determined by qRT-PCR in hippocampal tissue of F1 MIA (PIC) and control (SAL) offspring (n = 7–8 per group) Results were normalized to β-actin as house-keeping gene and plotted relative to the mean of controls (SAL). Data are displayed as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$. c.) Correlation between P0 dam licking/grooming (LG) behavior and F1 offspring relative MCR expression d.) Correlation between P0 dam licking/grooming (LG) behavior and F1 offspring relative GCR expression.

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3.5. Altered hippocampal gene expression in F2 generation offspring with history of MIA along the maternal and paternal lineages

We further proceeded to examine whether the observed changes in hippocampal expression of MCR and GCR in the F1 generation were also detectable in F2 MIA offspring. To this end, qRT-PCR analysis probing MCR and GCR mRNA levles in of hippocampal tissue of behaviorally naïve adult female F2 offspring with ancestral exposure to viral-like immune stimulation during gestation along either maternal, paternal or both lineages was carried out in order to examine MCR and GCR mRNA levels. Statistical analysis revealed a significantly enhanced expression only of MCR (Fig. 6a) but not of GCR (Fig. 6b) exclusively in the hippocampus of F2 ♀PICx♂PIC mice with heritage of MIA contributed by both, the

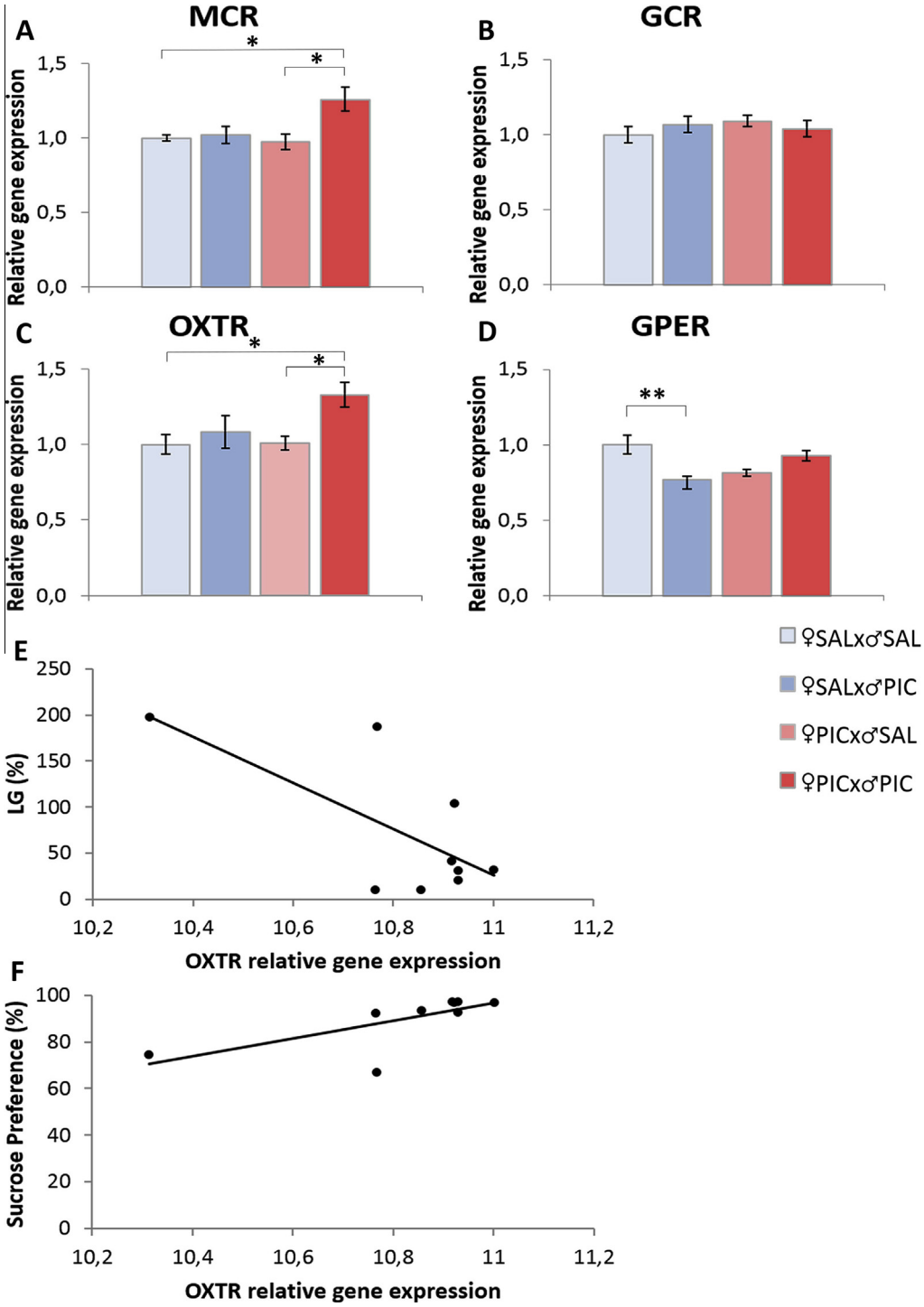

Fig. 6. Hippocampal gene expression in F2 offspring. Relative gene expression of a.) Mineralocorticoid Receptor (MCR), b.) Glucocorticoid Receptor (GCR) c.) Oxytocin Receptor (OXTR) d.) G-Protein Coupled Estrogen Receptor (GPER) determined by qRT-PCR in hippocampal tissue of F2 offspring (n = 7–8 per group) with heritage of poly(I:C) stimulation along the maternal (♀), paternal (♂) or both (♀x♂) lineages. Results were normalized to β-actin as reference gene and plotted relative to the mean of the controls (mother (♀) saline and father (♂) saline). Data are displayed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ indicates results of post hoc pairwise comparisons using the Scheffé procedure. e.) Correlation between F1 dams licking/grooming (LG) behavior and F2 offspring relative OXTR expression. f.) Correlation between F2 offspring sucrose preference in the SPT and F2 offspring relative OXTR expression.

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maternal and paternal lineages (significant main effect of mother × father $F_{(3,28)} = 8.8$, $p < 0.01$). In contrary to the F1 generation, additional specific expressional changes in two other hormonal receptors known to be directly regulated by MCB were observed in the F2 offspring hippocampus: MIA in the paternal lineage increased levels of OXTR (significant main effect of father: $F_{(3,28)} = 7.3$, $p < 0.05$) in ♀SALx♂PIC F2 and ♀PICx♂PIC F2 offspring (Fig. 6c); a significant interaction between the effects of maternal and paternal history of prenatal immune activation was found for GPER (mother × father $F_{(3,28)} = 14.4$, $p < 0.001$) with ♀SALx♂PIC F2 animals presenting with lowest levels (Fig. 6d). No differences in the expression of the other genes examined were observed between the different F2 groups (Supplementary Fig. 3). OXTR expression in F2 offspring was significantly correlated with both, the maternal care behavior in the form of LG of their mothers and their own depression-like behavior reflected in sucrose preference in the SPT test ($R^2 = 0.49$, $p < 0.05$ and $R^2 = 0.48$, $p < 0.05$) (Fig. 6e-f). No other significant correlations with regards to gene expression, F1 maternal care and F2 behavior were observed (data not shown).

4. Discussion

The currently briskly spreading outbreak of Zika virus infections in the Americas (www.cdc.gov/zika/geo/active-countries.html) and the confirmed causal relationship between gestational Zika virus infection and severe offspring brain anomalies including microcephaly (Rasmussen et al., 2016) has put the risks of maternal infection during pregnancy in the spotlight of public interest worldwide. However, long before congenital Zika virus-induced microcephaly has made global headlines, the detrimental effects of MIA, on offspring brain development and behavior have been repeatedly demonstrated (Reisinger et al., 2015; Ronovsky et al., 2015; Meyer and Feldon, 2012, 2009; Boksa, 2010; Meyer et al., 2006; Ruthschilling and et al., 2012). Along these lines, the association between gestational infection and the development of severe mental illnesses, including autism spectrum disorders, depressive disorders and schizophrenia later in life is becoming widely accepted, largely based upon strong evidences from preclinical MIA models (Meyer and Feldon, 2009; Khan and et al., 2014; Reisinger et al., 2016; Meyer et al., 2006; Malkova et al., 2012; Meyer and et al., 2006; Meyer et al., 2010; Schwartzer and et al., 2013; Winter and et al., 2009). While the consequences of MIA on neural function at the molecular, cellular and systems - i.e. behavioral- levels are being an intense focus of ongoing investigations in rodent, primate and human studies (Reisinger et al., 2015; Meyer and Feldon, 2012; Bauman and et al., 2014; Brown and Patterson, 2011) they have been majorly directed against the analysis of first generation offspring. Only recently, evidence for a transgenerational transmission and modification of the pathological traits of prenatal infection has been described in an elegant study employing the poly(I:C) MIA mouse model (Weber-Stadlbauer et al., 2016). Although there are some difference between the former and the present study in terms of the regime used for poly(I:C) stimulation (i.e. dosage and timing), we here built upon these data and set out to examine the relevance of MCB in the context of the transgenerational effects of poly(I:C)-induced MIA on offspring depression-like behavior.

There is large body of evidence illustrating the critical importance of the interaction between the neonate and their mother as predictive factor for offspring emotional behavior and neuroendocrine profiles later in life (Champagne, 2011, 2010; Claessens and et al., 2011; Jensen Pena and Champagne, 2013). The LG behavior of the female, which provides the main source of tactile stimulation for the pups, is a major element shaping the development of the offspring brain through "reprogramming" mechanisms (Fish and et al., 2004; Kaffman and Meaney, 2007) with long-lasting consequences for gene expression and behavior (Ruthschilling and et al., 2012; Hellstrom et al., 2012; Pan et al., 2014; Pedersen et al., 2011). Intriguingly, the offspring neurobehavioral phenotype, associated to psychopathological traits with relevance for anxiety, stress sensitivity and depression (Kaffman and Meaney, 2007; Curley et al., 2009), as well as the quality of the MCB, which is subject to modification by exogenous and endogenous stressors, including psychosocial and immune stress (Curley et al., 2009; Bailoo et al., 2014; Coutellier et al., 2008; Schwendener et al., 2009), can be transmitted to following generations (Curley et al., 2009; Champagne, 2008; Champagne and Meaney, 2015). The underlying mechanisms, which have been intensively studies in rodents, are based upon non-genomic, epigenetically determined, enduring changes in gene expression with alterations in steroid receptors being most prominently examined (Champagne, 2008).

Results of the present study confirm a direct dampening effect of gestational poly(I:C) stimulation on maternal behavior in the mouse, specifically LG, as previously described (Schwendener et al., 2009; Lucchina et al., 2010) and firstly suggest – in a preclinical setting - that the detrimental effects of infection during pregnancy on dams' reproductive behavior towards her pups can be transmitted across generations. The paralleling demonstrations of increased depression-like behavior and modulated hippocampal corticosteroid expression patterns in the F2 generation, implies epigenetic alterations as underlying biological principle. These experiments were designed to specifically examine consequences of MIA on female F1 and F2 offspring in light of the significantly higher prevalence of depressive disorders in women (Nolen-Hoeksema, 1987; Kessler and et al., 1994) and the fact that animal experiments in female subjects are still the orphan child of biomedical research. Impending follow-up studies will compare effects between male and female MIA offspring in order to shed light on potential sex-differences and to address the precise nature of the mediation of these effects using cross-fostering approaches.

Future cross-fostering and/or in vitro fertilization or embryo transfer experiments will aid to definitely determine a potential causal involvement of maternal behavior in the transgenerational effects of MIA on offspring emotionality and shed further light onto related, herein described effects. As such, an in vitro fertilization approach may elucidate the neurobiological underpinnings of the observed contribution of the father to the modulation of LG behavior of the F1 dam. In principle, this "father-effect" could be brought about by an adaptation of the female's strategy of reproductive investment depending on the ancestral heritage of gestational infection carried by the father of her offspring and communicated directly by male cues acting upon the female during the mating process (Gowaty and et al., 2007). Alternatively, a modulation of the behavior of the pups towards her mother – resulting from epigenetic changes carried by the father's germ cells (Gapp and et al., 2014; Rodgers et al., 2013) - could indirectly lead to an adjustment of the maternal response (Curley and et al., 2010). Cross-fostering experiments will ascertain the precise prenatal and postnatal impacts which seem to differentially contribute and/or interact to determine the final behavioral phenotype of offspring with ancestral history of gestational infection. At present, the significant correlation between dam LG and F1 and F2 hippocampal gene expression indicates a possibly relevant contribution of maternal care to offspring phenotype in the present study. This finding is further supported by the significant correlation between different aspects of maternal care (i.e. LG and nursing) and individual, depression-related behavioral read-outs (i.e. anhedonia and behavioral despair). Interestingly, there seems to be both transgenerational transmission and modification of behavioral traits and transcriptional signatures as reflected in differences in the involve-

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ment in maternal care and offspring behavior/gene expression among P0, F1 and F2 mice. Altered patterns of corticosteroid receptor mRNA expression in F1 MIA offspring and F2 females with ancestral burden of gestational immune challenge along both parental lineages suggest dysfunctions in the stress response system, to be contributing to the observed enhanced depression-like behavior. Alterations in OXTR and GPER expression in the F2 mice provide evidence for a unique transcriptional signature in second generation offspring hippocampus. This observation is in agreement with a recent description of offspring behavior and a large scale screening of amygdala gene expression patterns in the F1 and F2 generation with ancestral prenatal immune stimulation (Weber-Stadlbauer et al., 2016), also reporting of both, transgenerational transmission and modification of behavioral and gene expression traits. Interestingly, this study, employing a different protocol of poly(I:C) assisted MIA, described the emergence of offspring depression-like behavior only in the F2 generation, when examining male offspring.

5. Conclusion

The demonstration of transgenerational effects of immune challenge in the prenatal period on MCB suggest the reproductive behavior of the mother towards her pups as modulatory interface at which genes and the pre- and postnatal environment may interact to induce long-lasting, presumably epigenetically inheritable, effects on offspring brain structure, function and behavior, which remain to be confirmed in future cross-fostering studies. Gene expression data and their correlation to behavioral performance point towards an intricate web of alterations in various molecular elements jointly contributing to offspring behavioral alterations. One clear limitation of the present study is the use of a relatively small number of litters, which can produce spurious findings in multiparous species such as the mouse (Lazic and Essioux, 2013).

This study invites future research efforts specifically aiming at differentiating the effects of "nature versus nurture" and the relevance of paternal versus maternal heritages using cross-fostering and/or in vitro fertilization approaches as well as an evaluation the quality of MCB in F2 females. Moreover, albeit the recent identification of epigenetic modifications in individual molecular elements in MIA offspring brain (Reisinger et al., 2016), an unbiased screening of the "epigenome" of F1 and F2 generation MIA offspring may provide additional valuable information into the pathophysiological events resulting from prenatal immune challenge and its transgenerational effects.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2016.10.016.

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