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Maternal immune activation impairs cognitive flexibility and alters transcription in frontal cortex

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A B S T R A C T

Background: Epidemiological studies suggest that the risk of neurodevelopmental disorders such as autism spectrum disorder (ASD) and schizophrenia is increased by prenatal exposure to viral or bacterial infection during pregnancy. It is still unclear how activation of the maternal immune response interacts with underlying genetic factors to influence observed ASD phenotypes.

Methods: The current study investigated how maternal immune activation (MIA) in mice impacts gene expression in the frontal cortex in adulthood, and how these molecular changes relate to deficits in cognitive flexibility and social behavior, and increases in repetitive behavior that are prevalent in ASD. Poly(I:C) (20 mg/kg) was administered to dams on E12.5 and offspring were tested for social approach behavior, repetitive grooming, and probabilistic reversal learning in adulthood (n = 8 vehicle; n = 9 Poly(I:C)). We employed next-generation high-throughput mRNA sequencing (RNA-seq) to comprehensively investigate the transcriptome profile in frontal cortex of adult offspring of Poly(I:C)-exposed dams.

Results: Exposure to poly(I:C) during gestation impaired probabilistic reversal learning and decreased social approach in MIA offspring compared to controls. We found long-term effects of MIA on expression of 24 genes, including genes involved in glutamatergic neurotransmission, mTOR signaling and potassium ion channel activity. Correlations between gene expression and specific behavioral measures provided insight into genes that may be responsible for ASD-like behavioral alterations.

Conclusions: These findings suggest that MIA can lead to impairments in cognitive flexibility in mice similar to those exhibited in ASD individuals, and that these impairments are associated with altered gene expression in frontal cortex.

1. Introduction

Acute disruption of the maternal environment during prenatal development can significantly contribute to risk for disorders such as autism spectrum disorder (ASD) and schizophrenia (Boksa, 2010; Hsiao and Patterson, 2011; Solek et al., 2018). For example, there is growing evidence that maternal infection during pregnancy is one of the most prominent environmental risk factors of neural developmental dysfunction in subsequent offspring (Basil et al., 2014; Bilbo et al., 2018; Garbett et al., 2012). Prenatal insults, including viral infections, can negatively impact neural development leading to long lasting physiological and behavioral symptoms in the offspring (Estes and McAllister, 2016; Winter et al., 2009). Indeed, there is increasing evidence that prenatal exposure to viruses and other immune-activating factors increases risk for neurodevelopmental psychiatric disorders including ASD and schizophrenia (Brown and Derkits, 2010; Patterson, 2011). These epidemiological findings are supported by preclinical studies demonstrating that prenatal exposure to immune activation, termed maternal immune activation (MIA), alters neurodevelopment and produces behaviors relevant to schizophrenia and ASD such as deficits in social approach, working memory, and sensorimotor gating (Malkova et al., 2012; Meyer, 2014; Naviaux et al., 2013; Powell, 2010). These behavioral deficits are accompanied by alterations in striatal, limbic, and cortical brain regions implicated in neurodevelopmental and neuropsychiatric disorders (Meyer and Feldon, 2009).

Individuals with ASD have deficits in social behaviors as well as increased repetitive behaviors with restricted interests. Restrictive repetitive behaviors such as insistence on sameness and resistance to

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change are core features of ASD, are difficult to treat, and can be measured across species, though they are not fully characterized in rodent models of neurodevelopment (Amodeo et al., 2014; Bishop et al., 2013; Richler et al., 2010; Whitehouse et al., 2017). There is some evidence that MIA impairs cognitive flexibility, or the ability to actively switch or shift between behaviors, in rodent models. For example, prenatal polyriboinosinic-polyribocytidilic acid (Poly(I:C)) administration impaired set shifting and reversal learning in rats (Donegan et al., 2018; Zhang et al., 2012) and set shifting (Canetta et al., 2016) and reversal learning (Meyer et al., 2006) in mice. We examined the impact of MIA on restrictive, repetitive behaviors (e.g. cognitive flexibility, repetitive grooming) and social behavior (social approach task). Individuals with ASD have specific deficits in reversal learning paradigms that use a probabilistic reinforcement schedule (D'Cruz et al., 2016, 2013, 2011). Thus, we tested mice in a spatial probabilistic reversal learning paradigm with cross-species validity that was previously shown to reveal behavioral impairment in other mouse models of ASD (Amodeo et al., 2014).

It remains unclear how MIA interacts with underlying genetic factors to influence observed ASD phenotypes. Recent evidence suggests that dysregulation of epigenetic pathways and altered gene expression could lead to the neurodevelopmental alterations observed in the offspring (Tang et al., 2013). However, in the past decade, studies of cortical gene expression in adult offspring of Poly(I:C)-treated dams reported relatively subtle alterations (Connor et al., 2012; Richetto et al., 2017; Smith et al., 2007). This is surprising given that the prenatal Poly(I:C) model elicits robust behavioral changes in adult mice (Meyer, 2014; Naviaux et al., 2013). Several studies have implicated glutamatergic (Rojas, 2014) and the mammalian target of rapamycin (mTOR) signaling pathways in ASD (Costa-Mattioli and Monteggia, 2013). mTOR complexes are hubs in the immune signaling pathway that affect synaptic transmission (Estes and McAllister, 2015; Lombardo et al., 2018). Moreover, dysregulation of translation control pathways may impact synaptic protein expression in models of ASD (Santini and Klann, 2014). Therefore, in this study we employed next-generation high-throughput mRNA sequencing to comprehensively investigate the transcriptome profile in frontal cortex of adult offspring of Poly(I:C)-exposed dams. Because disruptions in frontal cortex function lead to deficits in cognitive flexibility (Dalton et al., 2016; Del Arco et al., 2017), we hypothesized that MIA produced by prenatal Poly(I:C) would lead to deficits in probabilistic reversal learning and that these could be correlated to gene expression changes in frontal cortex.

2. Methods and materials

2.1. Subjects

Eight week old male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were maintained on *ad libitum* water and mouse chow. Animals were housed on a 12 h:12 h reversed light-dark cycle (lights off at 7:00 am) in a temperature and humidity-controlled room. Mice were housed in standard cages (28.4 × 18.4 × 12.5 cm) in an individually ventilated caging system with corncob bedding. All studies were conducted at the University of California San Diego (UCSD) in facilities accredited with the Association of Assessment and Accreditation of Laboratory Animal Care International (AAALAC) under UCSD Institutional Animal Care and Use Committee (IACUC) approved animal protocols.

2.2. Maternal immune activation (MIA)

Male C57BL/6J mice were singly housed for 3 consecutive days. On the fourth day two, 10-week old female mice were introduced to the age-matched males and left undisturbed for three days. After three days females were singly housed for the remainder of pregnancy. Dams were checked daily for the presence of a seminal plug and then recorded as gestational day (G) 0.5. On G12.5 female mice received an intraperitoneal (ip) injection of 20 mg/kg Poly(I:C) acid potassium salt (P9582, Lot number 034M4086V, Sigma-Aldrich, St. Louis, MO) in 0.9% saline. This lot of PolyI:C administered at a dose of 5 mg/kg (IP) to naive female mice increased plasma IL-6 levels (Saline = 1.08 ± 1.31 pg/mg protein; Poly(I:C) = 26.67 ± 14.31 pg/mg protein; n = 3/group), confirming immunogenicity. The 20 mg/kg dose of Poly(I:C) administered on G12.5 has previously been shown to increase immune sensitivity and produce autism-like behaviors in offspring (Onore et al., 2014; Schwartzer et al., 2013). Control mice received 0.9% saline injections. Dams (n = 11), were left undisturbed until 5 days after birth of offspring. Pups remained with mothers until weaning on postnatal day (PD24) and then were group housed by sex and prenatal group, 3–4 per cage using 1–3 mice per litter in experiments. A total of seventeen male mice (n = 9 Vehicle, n = 8 Poly(I:C)) were tested. The same mice were tested in each of the behavioral measures, and behavioral tests were separated by at least one week.

2.3. Behavioral analysis

2.3.1. Repetitive grooming behavior

On PD 52–60 mice were assessed for repetitive grooming behavior similar to previous studies (Amodeo et al., 2014; McFarlane et al., 2008). Mice were placed into a clean cage without bedding undisturbed for 20 min and allowed to freely explore the cage for the entirety of the test. The first 10 min served as a habituation period. During the second 10 min of testing, a trained observer, situated approximately 1.6 m away and blind to group status, recorded cumulative time spent grooming all body regions. The observer was blind to experimental condition. After each mouse was tested, the cage was thoroughly cleaned with a 2% ammonium chloride solution.

2.3.2. Social approach behavior

On PD 64–74 mice were tested in the three chambered social approach test (as described in (Naviaux et al., 2013). A clear Plexiglas rectangular box (60 cm long × 60 cm wide × 30 cm tall) was divided into 3 equal sized compartments with Plexiglass dividers containing a small centered opening at the bottom to allow access to all compartments. Mice received two, 10-minute testing sessions. In the first 10-minute session mice were placed in the center compartment and allowed to explore the three chambers undisturbed. In each of the outer two chambers an empty stainless steel wire cup (Galaxy Cup Inc. Streetsboro, OH) was inverted and a plastic cup was placed on top to prohibit mice from climbing on top of the wire cup. After the first 10 min, mice were removed from the chamber while the experimenter pseudorandomly placed a sex-matched novel mouse into the inverted cup in either the left or right chamber, counterbalanced across treatment. A set of three vertically stacked Legos were placed into the opposite cup. Mice were then reintroduced to the center chamber and allowed to explore all three chambers undisturbed for another 10 min. A camera placed overhead was used to record behavior throughout testing. Ethovision 3 video tracking system (Noldus, Leesburg, VA) recorded time spent and entries into each chamber and time spent around each wire cup. To increase the sensitivity of quantifying the amount of time mice spent directly attending to the stranger or Legos, a trained observer scored time spent sniffing each wire cup. Social preference was calculated as the time spent with the stranger divided by sum of the time spent with the stranger and Legos.

2.3.3. Probabilistic discrimination and reversal learning

On PD 100–130 mice were tested in a probabilistic reversal learning test similar to that described in (Amodeo et al., 2012). For probabilistic discrimination and reversal learning mice were tested in a black Plexiglas T-maze with high walls (stem arm: 81 cm long, 10 cm wide and 25 cm high; choice arms: 35 cm long, 10 cm wide and 25 cm high). Before each trial mice were restricted to the start box (8 cm long, 10 cm

wide), which was separated from the main stem arm by a horizontal sliding door (20 cm high). Horizontal sliding doors at each arm entrance were used to help direct mice back to the start box. The walls of the testing room were adorned with posters while both choice arms were adorned with distinct visuospatial cues attached to the back and side walls. At the end of each choice arm, a food well was centered and located 3 cm away from the back wall.

2.3.4. Spatial discrimination acquisition and reversal learning

Mice were first trained in the T-maze for 2–5 days before testing. Briefly, mice were trained to retrieve ½ piece of Fruity Pebbles cereal (Post Foods, St. Louis, MO) from each food well. Mice were considered trained once they successfully completed seven trials (consuming cereal pieces from both choice arms) in a 15 min session across two consecutive days. For acquisition testing, only one of the two food wells was baited with a ½ piece of cereal in each trial. One location was designated as the "correct" spatial location and contained a ½ piece of cereal on 80% of trials. On the other 20% of trials, the "incorrect" location was baited with a ½ piece of cereal. The first two trials of each test always contained a food reinforcement in the "correct" arm. Criterion was achieved when a mouse chose the "correct" location on six consecutive trials. If a mouse chose a location with cereal, it was allowed to eat the cereal; then gently directed back to the start box and the door was closed restricting it from the choice arms. If a mouse chose the arm with no cereal, it was allowed to navigate to the unbaited food well. Subsequently, the mouse was directed back to the start box. Once mice made an arm choice, the door of the opposite choice arm was closed, restricting access. Between trials, the choice area was cleaned with 5% alcohol solution to minimize the use of odor cues.

The reversal learning test was conducted the day after acquisition. Prior to reversal learning, mice first received a retention test as in previous experiments (Amodeo et al., 2014, 2012). In the retention test, a mouse was reinforced with 80% probability on trials for choosing the spatial location that was correct in acquisition. Criterion was achieved when a mouse successfully chose the "correct" spatial location (as in acquisition) on five out of six trials. Immediately after achieving retention criterion, reversal learning began. All aspects of the reversal learning test were identical to those in acquisition, except that the opposite spatial location was considered 'correct' and reinforced with 80% probability. Criterion was met when a mouse made six consecutive correct choices.

An error analysis of reversal learning was conducted as used previously in rodent models (Brown et al., 2012; Floresco et al., 2006). The first reversal learning trial was not counted as a perseverative error, but served as initial negative feedback. On subsequent trials, if a mouse chose the previously correct spatial location, the choice was recorded as a perseverative error until a mouse made three correct choices for the newly "correct" spatial location. After selecting the correct spatial location for three consecutive trials, all subsequent entries into the previously reinforced spatial location were scored as regressive errors regardless of reinforcement availability. For example, a mouse could have initially learned to choose spatial location A, and then during reversal learning had to choose spatial location B instead. The following represents an example of the spatial location chosen on three consecutive correct trials (underlined) during reversal learning: A,**A**,**A**,B,**A**,B,**A**,-B,**A**,B,B,B,*A*,B,*A*,*A*. Therefore, a mouse would have five perseverative errors (bold) and three regressive errors (italics).

2.3.5. Statistical analysis

Behavioral data are presented as Mean ± SEM. Student's *t*-tests were conducted to compare how MIA impacted spatial acquisition learning, reversal learning, repetitive grooming, and social approach behavior as well as both perseverative and regressive type errors during reversal learning. The statistical program SYSTAT (Chicago, IL) was used to analyze the behavioral data.

2.4. RNA-Seq analysis of transcriptome

2.4.1. Tissue and sample preparation

After all behavioral tests were completed, the frontal cortices of MIA offspring were harvested on PD 189 for RNA-Seq analysis (Poly (I:C) = 9 and vehicle = 8). Animals were anesthetized with isoflurane and decapitated. The brain was immediately extracted and whole frontal cortex was obtained by slicing the adult brains coronally at Bregma 1 mm, and dissecting the frontal cortical tissue, being careful to avoid contamination with olfactory or putamen regions. Finally, the frontal cortex was flash frozen on dry ice and stored at -80 °C. Total RNA was then extracted by using RNeasy Mini kit with DNAse I treatment (Qiagen 74,104). The RNA quality, RNA integrity number (RIN), was measured using the Agilent 2200 TapeStation system (Agilent Technologies, G2965AA). All samples were with RIN > 7.

2.4.2. Transcriptome analysis (mRNA-Seq)

Strand-specific cDNA libraries were constructed by selecting polyadenylated mRNA transcripts using Truseq Stranded mRNA LT kit (Illumina, RS-122-2101 and RS-122-2102). Libraries were deeply sequenced using Illumina HiSeq 2500, generating 25 million reads (100 bp, single end sequencing).

2.4.3. Transcriptome data analysis methods

The quality of RNA-seq reads was checked by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). STAR (2.5.1b) was used to map sequence reads to the mouse mm10 reference genome and RSEM (version 1.2.31) was used to quantify gene expression. Low expressed genes with fewer than 5 read counts in at least half of samples were removed from the analysis. Differential expression analysis was conducted using Deseq2 (Love et al., 2014) with false discovery rate 0.1. We performed Gene Set Enrichment Analysis using function gesGO of Bioconductor package clusterProfiler (Yu et al., 2012). Pearson correlation was used to quantify the association between gene expression and behavioral phenotype. Previous studies from our group have shown that adult gene expression is fairly stable (Lister et al., 2013; Zhang et al., 2018), thus allowing for the comparison of behavioral data to altered gene expression in the MIA model although separated by approximately 2 months. Interaction analysis was performed by regressing gene expression on behavioral phenotype and treatment with interaction term in the model. Causal mediation analysis was performed by using R package mediation (Imai et al., 2010; Tingley et al., 2014).

3. Results

3.1. Poly(I:C)-MIA impairs probabilistic reversal learning and social approach behavior

To assess the impact of Poly(I:C)-MIA on cognitive flexibility in adult offspring, we tested mice in a probabilistic reversal learning paradigm that tests the ability to update previously learned reward contingencies (Amodeo et al., 2018). Both Poly(I:C)-MIA and control mice demonstrated comparable learning of the initial spatial discrimination, with no significant main effect of treatment on the number of trials to criterion ($t(15) = -1.47$, NS) (Fig. 1A). On the following day mice were first required to demonstrate retention of the previous spatial acquisition. We found no main effect of treatment on the number of trials to retention criterion ($t(15) = -0.34$, NS), showing that both groups had comparable retention and did not differ on recall of the previous day's learning. Once performance reached retention criterion, mice immediately began reversal trials. We found that MIA mice required more trials to reach a performance criterion for reversal learning (significant main effect of treatment, $t(15) = -5.18$, $p < .001$) (Fig. 1B).

Impaired reversal learning could be caused by failure to inhibit the previous correct choice (i.e. perseverating on the previous correct

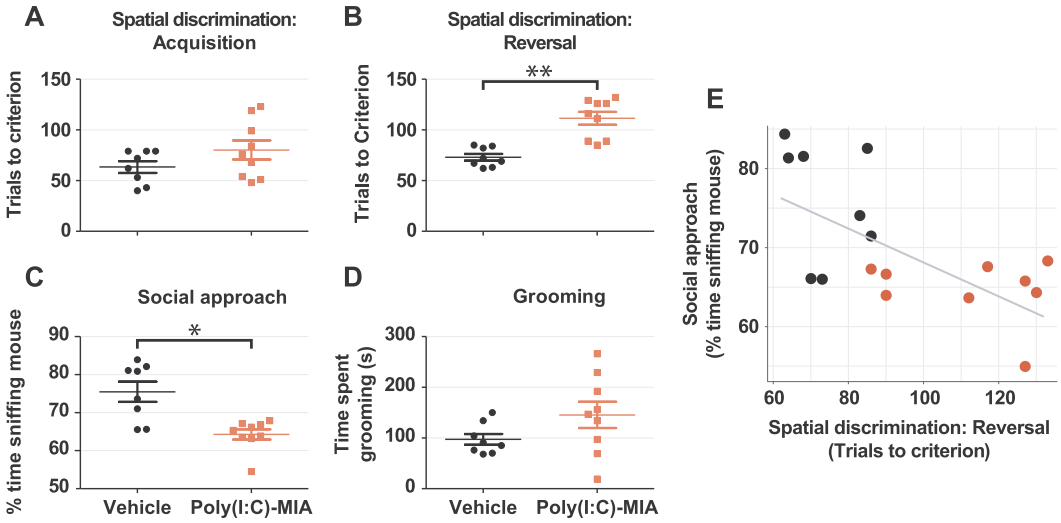

Fig. 1. Poly(I:C)-MIA impaired reversal learning of a spatial discrimination and reduced social approach. [A] Vehicle and Poly(I:C)-exposed mice did not differ on number of trials to reach criterion for acquisition of the spatial discrimination. [B]. Poly(I:C)-exposed mice required significantly more trials to reach criterion on reversal of the spatial discrimination $t(15) = -5.18, p < .001$. [C] Percent time spent sniffing the stranger mouse out of total time spent sniffing mouse or object. Poly(I:C)-exposed mice spent significantly less time sniffing the stranger mouse compared to vehicle-exposed mice ($t(15) = 3.90$, $p < .01$). [D] Poly(I:C) and Vehicle mice did not differ on time spent grooming. [E] Correlation of percent time spent sniffing the stranger mouse and trials to criterion for reversal learning of the spatial discrimination. Data are presented as Mean [± SEM], $*p < .01$, $**p < .001$ vs.vehicle ($n = 8$ Vehicle; $n = 9$ Poly(I:C)).

response), or by failure to maintain the new correct choice (i.e. choosing the new correct response but subsequently regressing to the previous correct response). To determine the nature of the reversal learning deficits in Poly(I:C)-MIA mice, we therefore analyzed perseverative errors. There was no significant difference in perseverative errors between Vehicle and Poly(I:C)-MIA mice ($t(15) = 1.56$, NS). Poly (I:C)-MIA mice committed significantly more regressive errors during reversal learning ($t(15) = 7.92$, $p < .05$). These analyses demonstrate that during reversal learning Poly(I:C)-MIA mice did not differ in initially inhibiting the previously correct choice. Instead, they were impaired in maintaining the new correct choice pattern after it was initially selected.

Impaired social behavior is a core ASD phenotype and can be altered by MIA. We found that Poly(I:C)-MIA mice spent less time sniffing an unfamiliar mouse compared to vehicle-treated mice ($t(15) = 3.90$, $p < .01$) (Fig. 1C), indicating reduced social approach behavior (Naviaux et al., 2014, 2013). We found no significant difference between the groups in time spent grooming (145 ± 26 s for Poly(I:C)-MIA vs. 95 ± 11 s for controls,$t(15) = -1.65$, $p = .12$).

3.2. Transcriptome analysis in Poly(I:C)-MIA mice indicates dysregulation of potassium ion channel activity and mTOR signaling pathway in frontal cortex

To examine the effects of immune challenge on gene expression in Poly(I:C)-MIA mice, we performed transcriptome analysis (mRNA-Seq) in frontal cortex of MIA ($n = 9$) and control offspring ($n = 8$) on PD 189. Out of 12,626 genes expressed in frontal cortex, we identified 24 differentially expressed (DE) genes in Poly(I:C)-MIA mice relative to controls (Fig. 2A; FDR < 0.1, fold-change > 1.1). The DE genes included 14 that were significantly up-regulated, and 10 down-regulated in Poly(I:C)-MIA mice (Supplementary Table 1). Among the DE genes, the top one is ribosomal protein S27 retrogene *Rps27rt* (1.49-fold-up-regulated, $p = 1.9 \times 10^{-8}$, Fig. 2B). Two other ribosomal proteins, *Rpl28-ps1* and *Rpl29*, were also up-regulated in Poly(I:C)-MIA mice. Furthermore, rapamycin-insensitive companion of TOR (*Rictor*), part of the mammalian target of rapamycin complex 2 (mTORC2, Supplementary Fig. 1A), was down-regulated in Poly(I:C)-MIA mice (1.1-fold-down-regulated, $p = 9 \times 10^{-6}$, Fig. 2B). Together with mTORC1, these genes act as the hub of immune signaling that affects synaptic transmission (Costa-Mattioli and Monteggia, 2013; Estes and McAllister, 2016). Finally, metabotropic glutamate receptor 7 (*Grm7*), a highly expressed group III mGluR in excitatory neurons, was down-regulated in Poly(I:C)-MIA mice (1.09-fold-down-regulated, $p = .0001$, Fig. 2B). Another highly expressed group I mGluR, *Grm5*, also showed a trend of alteration in Poly(I:C)-MIA offspring (1.07-fold-down-regulated, *p*-value = .0005, Supplementary Fig. 1B).

Since MIA is a neurodevelopmental disease model, we examined the dynamic trajectory of the 24 DE genes in adult MIA change during normal cortical development. We used our previously published developmental RNA-seq data (Lister et al., 2013) to identify differentially expressed genes in cortex between fetal (embryonic 14.5 days) and adult (10 weeks of age) time points. Out of 24 DE genes in adult MIA, 21 genes (87.5%) were differential expressed throughout development. Among these developmental DE genes, corticotropin releasing hormone binding protein (*Crhbp*), *Kcnk1*, tyrosine tubulin ligase like 11 (*Ttll11*), and *Grm7* were > 10 fold-up-regulated from fetal to adult (Fig. 2C). These dramatic changes indicate that these genes are critical for brain development and they were also disrupted in Poly(I:C)-MIA mice. By contrast, *Rictor* and *Rps27rt* were 1.4-fold and 3.3-fold-down-regulated during development, respectively (Fig. 2C).

To address the function of the DE genes, we performed gene set enrichment analysis (GSEA) (Subramanian et al., 2005). We found that up-regulated genes are significantly involved in monovalent transmembrane transporter activity (FDR = 0.03) and voltage-gated potassium (K+) channel activity (FDR = 0.03, Supplementary Fig. 1C). Among the genes involved in ion channel activity (Supplementary Table 2), the majority were related to K+ ion channels (Fig. 2D), especially voltage-gated and inward rectifying classes. The most significant gene in the ion channel activity gene set is K+ two pore domain channel subfamily K member 1 (*Kcnk1*) (1.11-fold-up-regulated, p-value = 5×10^{-7}, Fig. 2B).

3.3. Association of behavioral phenotypes with changes in genes expression

We further evaluated the correlation between DE genes (Fig. 3B) and reversal learning and social approach (Fig. 3C) in Poly(I:C)-MIA mice. Out of 24 DE genes, 14 genes (58%), including *Grm7*, were

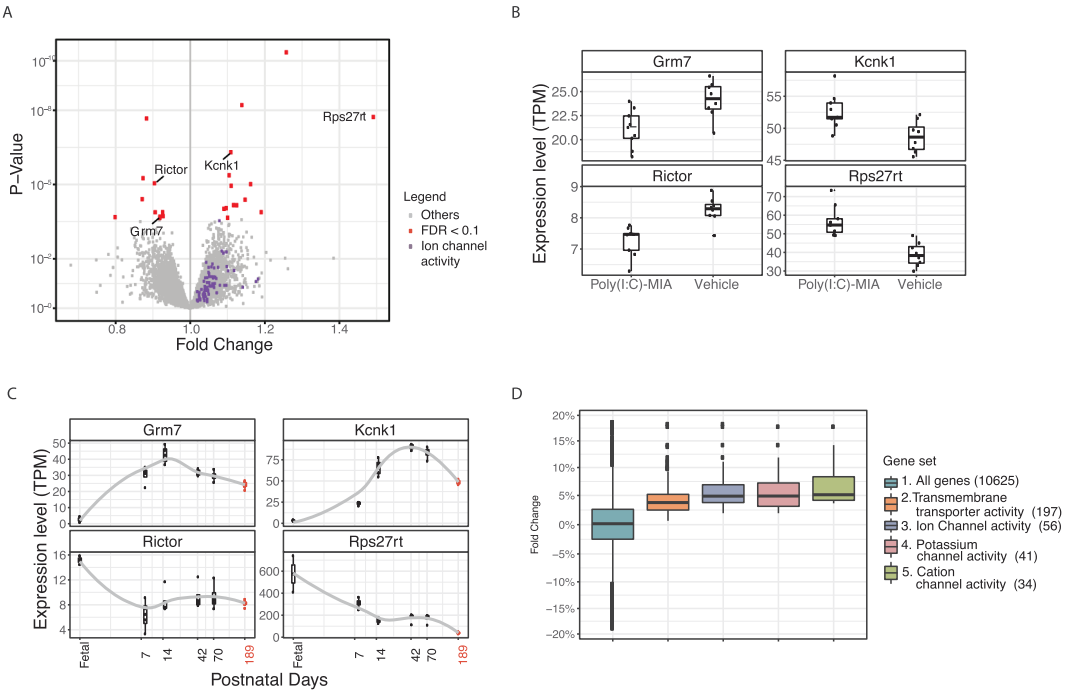

Fig. 2. Transcriptome-wide differential gene expression suggests dysregulation of ion channel activity in Poly(I:C) maternal immune activation (MIA, n = 9) compared to control, vehicle-treated mice (n = 8). (A) Volcano plot of differentially expressed genes (red dots, total of 24 genes) with FDR lower than 0.1. Gene list provided in Supplementary Table 1. The fold change of TPM values was plotted against p-value of Deseq2 analysis. A set of up-regulated genes is enriched in ion-channel activity (purple dots). All other genes that have counts > 5 in at least half of samples are shown as gray dots. (B) *Kcnk1* and *Rps27rt* are up-regulated, while *Grm7* and *Rictor* are down-regulated in Poly(I:C)-exposed offspring. (C) During brain development in control (non-Poly(I:C) treated) animals, *Grm7* and *Kcnk1* are up-regulated, while *Rictor* and Rps27rt are down-regulated (Lister et al., 2013). (D) Gene set enrichment analyses show enrichment for a set of up-regulated genes in ion-channel activity, transmembrane transport activity, potassium channel activity and cation channel activity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly correlated with reversal learning (FDR < 0.2), and 5 genes (21%) were correlated with social approach (FDR < 0.2) (Fig. 3A). Among these, *Rictor*, ribosomal protein L29 (*Rpl29*), and histone cluster 1 H2B family member C (*Hist1h2bc*) were associated with both reversal learning (Fig. 3D) and social approach (Fig. 3E).

To further investigate the interaction between maternal immune activation and gene expression on behavioral phenotype, we used two statistical models to test our hypothesis. First, we performed subgroup analysis in which we examined the correlations between gene expression and behavior within the two treatment groups separately (Supplementary Fig. 2 A-C). Overall we found little evidence for significant association between gene expression and behavioral phenotype within experimental groups. *Kcnk1* expression was positively correlated with reversal learning in the control mice ($r = 0.91$, $p < .005$; interaction (group × behavior), $p < .01$, Supplementary Fig. 2A and 2D). There was also an association between *Hist1h2bc* expression and social approach behavior in Poly(I:C)-MIA mice ($r = -0.84$, $p < .005$, interaction $p = .04$, Supplementary Fig. 2A and 2E).

Next, we used causal mediation analysis (Imai et al., 2010) to test whether the effect of treatment on behavior could be explained as an indirect consequence of the direct effect on gene expression. This analysis suggested that *Kcnk1* expression levels had a mediating effect on behavioral performance in reversal learning. This effect was blocked by Poly(I:C)-MIA (p of treatment-mediator interaction = 0.03). Taken together, the results suggest that the association between *Kcnk1* expression and behavioral phenotypes was affected by MIA.

4. Discussion

Our findings show that Poly(I:C)-MIA led to an impairment in reversal of a spatial discrimination and social approach behavior, similar to two core features of ASD. Deficits in similar 80/20 probabilistic reversal learning tasks have been observed in other rodent models relevant to ASD (Amodeo et al., 2014; Whitehouse et al., 2017). Our findings in Poly(I:C)-MIA mice are similar to those observed in individuals with ASD who exhibit reversal learning deficits only when reinforcement is probabilistic, and not when they are reliably reinforced for making a correct choice (D'Cruz et al., 2016, 2013, 2011). Previous studies have shown that individuals with ASD showed decreased activation in frontal cortex and ventral striatum during reversal learning when reward was uncertain (e.g. 80/20 reward contingency). No brain activation differences were observed when responses were certain (e.g. 100% reward contingency) (D'Cruz et al., 2016). Thus, frontal cortical alterations have been implicated in poor performance of a similar probabilistic reversal learning task in individuals with ASD. Deficits in probabilistic reversal learning observed in Poly(I:C)-MIA mice in the current study corroborate previous studies indicating cognitive flexibility deficits associated with MIA (Canetta et al., 2016; Meyer et al., 2006) and extend these findings to a translationally relevant task that has revealed deficits in ASD and a role of the frontal cortex in task performance (D'Cruz et al., 2016, 2013, 2011).

By using high throughput transcriptome sequencing to comprehensively evaluate gene expression in the brains of adult Poly(I:C)-MIA mice, the current study identified altered expression of 24 genes in the frontal cortex, albeit with small fold changes. The disruption of genes involved in glutamatergic neurotransmission (*Grm7*), K^+ ion channel activity (*Kcnk1*) as well as mTOR signaling (*Rictor*) can be long-lasting after an environmental insult during embryonic development. We hypothesized that Poly(I:C) treatment affect developmentally regulated genes. Consistent with this, 87.5% of DE genes in MIA offspring are genes that are differentially expressed during fetal to adult brain development. Furthermore, *Grm7, Rictor, Rpl29rt* and *Hist1h2bc* were associated with altered behavioral phenotypes. The causal mediation and interaction analyses indicated that expression of *Kcnk1* was altered by

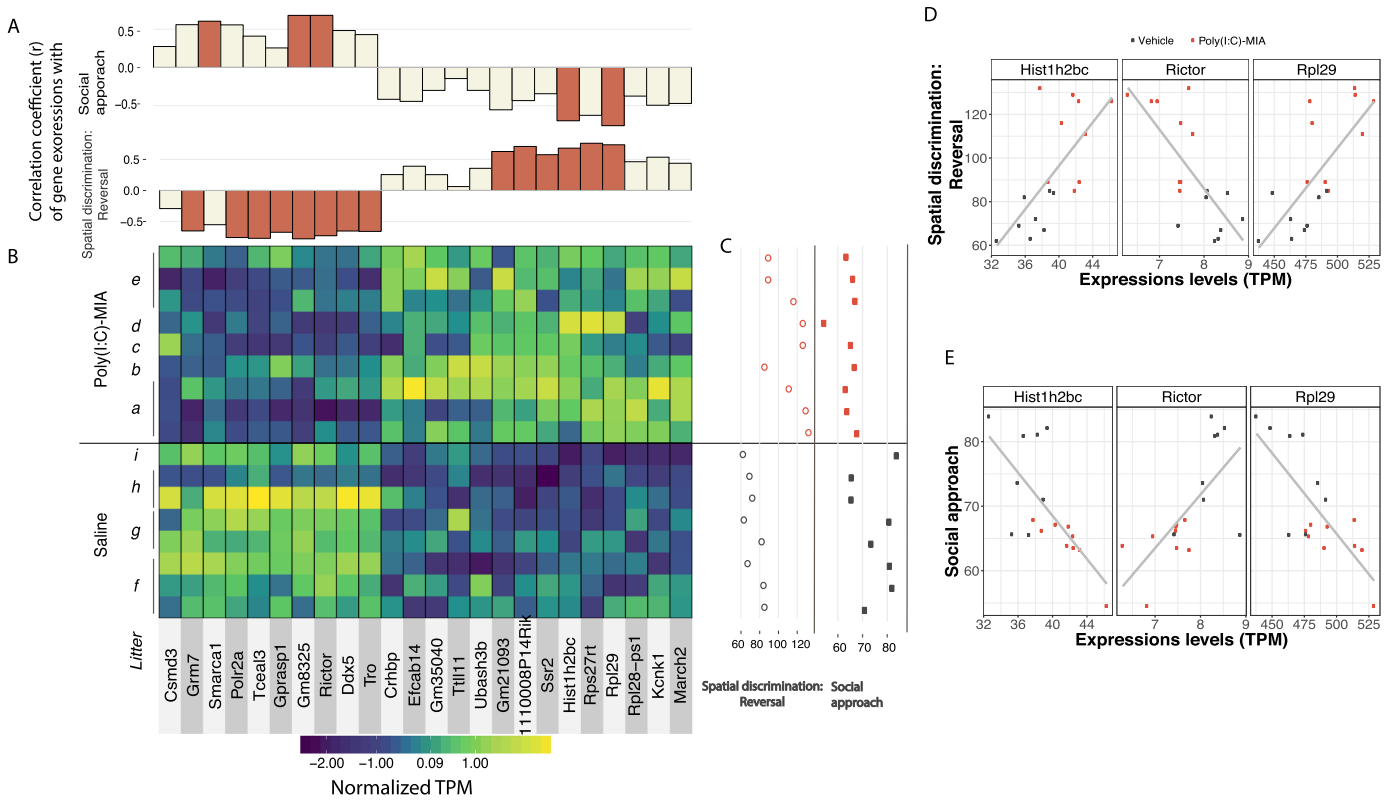

Fig. 3. Correlation between differentially expressed genes and behavior. (A) The bar plot shows Pearson correlation coefficients of individual gene expressions log10(TPM) with behavioral phenotypes in all samples ($n = 17$). The correlations with FDR < 0.2 are highlighted in red. (B) Gene expression levels. Heat map showing differences in individual gene expression (24 genes) in the cortex of Poly(I:C)-exposed offspring as compared to Vehicle-exposed offspring. The letters a-i in the row labels indicate litter IDs of mice. Each gene expression (TPM) is scaled to mean 0 and standard deviation 1 and the higher the scaling numbers, the higher the expression levels. (C) Behavioral phenotype. Each data point is the score of reversal learning (hollow circle) or social approach (filled square) in the mice with Poly (I:C) (pink dot) or vehicle(blue dot) treatment. (D) The scatter plot showing reversal learning and (E) social approach against gene expressions levels (TPM) of Hst1h2bc, Rictor and Rpl29. Their expression levels are associated with both reversal learning and social approach. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MIA and was associated with reversal learning. Finally, the GSEA analysis indicated that prenatal Poly(I:C) may disrupt ion channel activity, particularly K^+ ion channels, through a subtle increase in expression of a set of genes involved in voltage-gated and inward rectifying K+ channels.

Congruent with earlier microarray-based investigations of gene expression, we observed a subtle alteration in frontal cortex RNA expression in Poly(I:C)-MIA mice (Connor et al., 2012; Richetto et al., 2017; Smith et al., 2007). Comparing our findings with a previous study that provided a DE gene list (Connor et al., 2012; Richetto et al., 2017; Smith et al., 2007), Ribosomal protein S27, retrogene (*Rps27rt*) was the only common gene (Our data: 1.49 fold up-regulated, $p < 2 \times 10^{-8}$; Richetto et al.: fold change $= -1.2$ $p < 1 \times 10^{-3}$) (Richetto et al., 2017). However, Richetto et al. (2017) reported downregulation of *Rps27rt* in MIA mice whereas our study showed an upregulation. Furthermore, global hypoacetylation was detected in juvenile Poly(I:C)-MIA mice, but the changes were subtle in adult mice (Connor et al., 2012; Tang et al., 2013). Our finding of up-regulation in *Hist1h2bc* may related to long lasting effects of acute activation of IL-6 signaling, which has been shown to alter H3K4me3 at many transcription start site in brain tissue, including histone cluster 1 H2B family (Hist1h2b) (Connor et al., 2012).

Given the consistent findings of subtle alterations in gene expression in adult MIA offspring, the effect of Poly(I:C)-MIA on gene expression likely long precedes the manifestation of ASD-like behavioral deficits. In other words, the consistent behavioral impairments with MIA may be due to alterations in gene expression profiles at earlier time points during development which produce long lasting functional alteration as we observed in the study. Indeed, it has been shown that gene expression patterns in the developing brain are highly dynamic and reflect the underlying biological processes (Kang et al., 2011; Tebbenkamp et al., 2014). These developmentally regulated patterns of gene expression are particularly dramatic during early brain development (Lister et al., 2013; Marín, 2016). Indeed, some of the DE genes in this study, such as *Grm7* and *Kcnk1*, dramatically change their expression during the first weeks of postnatal life. Therefore, in addition to focusing on alterations in gene expression in adult offspring, it is also important to elucidate the dynamic changes during early developmental stages and understand whether these changes in expression are long-lasting and related to the behavioral deficits in MIA (Han et al., 2009).

Rictor and *Grm7* (mGluR7) were down-regulated in frontal cortex of adult Poly(I:C)-MIA offspring. *Rictor* is a crucial component defining distinct function of mTOR complex 2 (mTORC2) (Costa-Mattioli and Monteggia, 2013; Estes and McAllister, 2016). Rictor is highly expressed in the brain, notably in neurons (Huang et al., 2013), and is important for various aspects of brain development and function. For example, deletion of Rictor in neurons leads to reduced mTORC2-mediated Akt phosphorylation and results in behavioral endophenotypes that are relevant to autism or schizophrenia (Siuta et al., 2010), such as prepulse inhibition deficits (Powell et al., 2009). The mTORC2 was shown to be essential in actin dynamics–mediated late long-term potentiation and long term memory (Huang et al., 2013), which may related to impaired hippocampal long-term potentiation in MIA offspring (Khan et al., 2014; Smith et al., 2007). Disruption of mTOR signaling leads to abnormal brain development (Costa-Mattioli

and Monteggia, 2013; Takei and Nawa, 2014). Notably, it was recently reported that mTOR was down-regulated in fetal mouse brain (E15) four hours after lipopolysaccharide injections to pregnant dams (Lombardo et al., 2018). However, there were no differences in expression level of mTOR gene 24 h after LPS injection (Lombardo et al., 2018). On the other hand, mGluR7 is the most abundant and widespread metabotropic receptor in neurons (Niswender and Conn, 2010). Knockout of mGluR7 mice showed anxiolytic-like effects (Cryan et al., 2003) and abnormalities in learning tasks (Goddyn et al., 2008; Hölscher et al., 2004). Genetic studies suggesting the glutamatergic synapse pathway as involved in ASD and attention deficit hyperactivity disorder, included the dysregulation of mGluR7 (Elia et al., 2011; Luo et al., 2018). Experiments on hippocampal slices showed that rapamycin blocks mGluR-dependent long-term depression (Hou and Klann, 2004). Taken together the connection between dysregulation of mTORC2, mGluR7 and synaptic functions in MIA offspring warrants further investigation (Santini and Klann, 2014). It is important to note that the subtle changes observed in this study may result from complex patterns of gene-expression regulation across multiple neuronal cell types (Mo et al., 2015). The MIA-mediated dysregulation of glutamatergic pathways in specific neuronal types warrants further investigation.

Our results also indicate a possible dysregulation of potassium channel activity in Poly(I:C)-MIA mice, which had up-regulated expression of *Kcnk1*. This gene codes for one of the two-pore domain K^+ (K2P) channels that are major contributors to background potassium currents in cells and stabilize the resting membrane potential as well as cellular excitability (Enyedi and Czirják, 2010). Kcnk1 can mediate glutamate release from astrocytes (Hwang et al., 2014) and it contributes to the intrinsic excitability of dentate granule cells in mouse hippocampus (Yarishkin et al., 2014). Also the results of GSEA identified K+ ion-channel activity as enriched in a set of up-regulated genes, such as Kcnq3 and Kncd2. Genetic analyses of autistic individuals uncovered mutations in several types of K+ channels (Guglielmi, 2015; Schmunk and Gargus, 2013). These results have strengthened the notion that their intrinsic dysfunction may play a central etiologic role in ASD. For example, a de novo gain of function mutation in KCND2 has been reported to slow K+ channel inactivation in autism and seizures (Lee et al., 2014).

In summary, the current study characterized frontal cortex transcriptome profile of a viral-mimic of MIA effects and its correlation with behavioral phenotypes relevant to ASD. Long-term effects of MIA involved dysregulated expression of genes involved in glutamatergic pathway, mTOR signaling and potassium ion channel activity. These gene expression-phenotype correlations provide insight into genes that may underlie the ASD-like behavioral phenotype in the MIA mouse model.

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Conflict of interest

The authors declare no conflict of interest.

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