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Maternal immune activation-induced PPARγ-dependent dysfunction of microglia associated with neurogenic impairment and aberrant postnatal behaviors in offspring

Qiuying Zhao[a], Qiaozhi Wang[b], Jiutai Wang[a], Minmin Tang[a], Shugui Huang[a], Ke Peng[b], Yue Han[a], Jinqiang Zhang[a], Guangyi Liu[b], Qi Fang[c], Zili You[a,b,*]

[a] School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, Sichuan 610054, China
[b] Department of Histology and Embryology, School of Basic Medical Sciences, Southwest Medical University, Luzhou, Sichuan 646000, China
[c] Graduate Program in Neuroscience, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA

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ABSTRACT

Maternal infection during pregnancy is an important factor involved in the pathogenesis of brain disorders in the offspring. Mounting evidence from maternal immune activation (MIA) animals indicates that microglial priming may contribute to neurodevelopmental abnormalities in the offspring. Because peroxisome proliferator-activated receptor gamma (PPARγ) activation exerts neuroprotective effects by regulating neuroinflammatory response, it is a pharmacological target for treating neurogenic disorders. We investigated the effect of PPARγ-dependent microglial activation on neurogenesis and consequent behavioral outcomes in male MIA-offspring. Pregnant dams on gestation day 18 received Poly(I:C) (1, 5, or 10 mg/kg; i.p.) or the vehicle. The MIA model that received 10 mg/kg Poly(I:C) showed significantly increased inflammatory responses in the maternal serum and fetal hippocampus, followed by cognitive deficits, which were highly correlated with hippocampal neurogenesis impairment in prepubertal male offspring. The microglial population in hippocampus increased, displayed decreased processes and larger soma, and had a higher expression of the CD11b, which is indicative of the M1 phenotype (classical activation). Activation of the PPARγ pathway by pioglitazone in the MIA offspring rescued the imbalance of the microglial activation and ameliorated the MIA-induced suppressed neurogenesis and cognitive impairments and anxiety behaviors. In an *in vitro* experiment, PPARγ-induced M2 microglia (alternative activation) promoted the proliferation and differentiation of neural precursor cells. These results indicated that the MIA-induced long-term changes in microglia phenotypes were associated with hippocampal neurogenesis and neurobehavioral abnormalities in offspring. Modulation of the microglial phenotypes was associated with a PPARγ-mediated neuroprotective mechanism in the MIA offspring and may serve as a potential therapeutic approach for prenatal immune activation-induced neuropsychiatric disorders.

1. Introduction

Epidemiological data suggest that maternal infection during pregnancy increases the risk for adverse postnatal psychological outcomes, such as cognitive impairments and mental disorders (Estes and McAllister, 2016; Lowe et al., 2008). Maternal immune activation (MIA) influences fetal development in many ways in the uterus and can lead to long-term destructive effects on the offspring (Couzin-Frankel, 2013). This long-lasting impacts of MIA on the brain development are evidenced by analyses of animal models to study complex human brain disorders, such as schizophrenia, autism-spectrum disorder and major depressive disorder (Careaga et al., 2017; Jaaro-Peled et al., 2009).

Abbreviations: CNS, central nervous system; DCX, doublecortin; DG, dentate gyrus; EPM, elevated plus-maze; GCL, granular cell layer; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; i.p., intraperitoneal; LA, locomotor activity; M1, classical activation; M2, alternative activation; MCM, microglial conditioned medium; MIA, maternal immune activation; MTT, tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MWM, Morris Water Maze; NPC, neural precursor cells; PND, postnatal day; Poly(I:C), polyriboinosinic-polyribocytidilic acid; PPARγ, peroxisome proliferator-activated receptor gamma; SVZ, subventricular zone

* Corresponding author at: School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China.
E-mail address: youzili@uestc.edu.cn (Z. You).

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Animal experiments indicated that cytokine-associated inflammatory events may play a crucial role in the aberrant behavior of MIA offspring (Choi et al., 2016). However, the underlying pathophysiological mechanisms by which MIA-induced inflammation leads to neurodevelopmental and behavioral abnormality in prepubertal offspring remain poorly understood.

Recent evidence from clinical and preclinical investigations indicates that microglial dysfunction may be a critical cellular mechanism linked to brain development impairment and aberrant postnatal behaviors (Morgan et al., 2010; Wei et al., 2012). Microglia derive from primitive myeloid precursors in the embryonic yolk sac, self-renew and populate the early brain rudiment (Sousa et al., 2017). Under physiological conditions, microglia continuously survey the CNS microenvironment and respond sensitively to any potential challenge to homeostasis (Marin and Kipnis, 2017). When microglia are activated, the gene expressions and morphological characteristics altered, indicating long-lasting consequences (Graeber, 2010). Microglial priming has been considered to be a primary consequence of MIA and to underlie neuronal dysfunctions and behavioral abnormities observed in offspring (Knuesel et al., 2014). Several studies have revealed that exposing dams to Poly(I:C) across gestational windows causes persistent changes, such as the elevation of inflammatory cytokines and morphological changes, in the microglia of the offspring, indicating a long-lasting priming of the microglia (Krstic et al., 2012). Although microglial activation is necessary and is crucial for host defense, the overactivation of the microglia can lead to deleterious and neurotoxic consequences (Camara et al., 2015).

It is becoming more widely accepted that a dual role of microglia that depends on their phenotypes, *i.e.* a classical activation (M1 phenotype) and an alternative activation (M2 phenotype) (Hu et al., 2012), exists. The M1 phenotype is primarily characterized by increased pro-inflammatory mediators such as IL-1β, TNF-α and IL-6. The M2 phenotype is primarily characterized by anti-inflammatory mediators such as IL-10, IL-4 and TGF-β (Liu et al., 2013). Depending on their activation profiles, microglia show either neurotoxic or neuroprotective effects on modulating hippocampal neurogenic niches. M1 microglia release pro-inflammatory cytokines, have detrimental effects on neurogenesis and can lead to abnormal postnatal behaviors (Zhang et al., 2017). In contrast, M2 microglia release anti-inflammatory cytokines, which promote neurogenesis and repair and may be beneficial in the treatment of diseases (Zhao et al., 2014; Zhao et al., 2015).

PPARγ is a ligand-dependent transcription factor in the nuclear hormone receptor family that has been known to mediate immune inflammatory responses (Bouhlel et al., 2007). PPARγ drives monocyte activation toward the M2 alternative classification (Bouhlel et al., 2007). Inhibiting PPARγ pathway is able to induce microglial dysfunction in models of CNS diseases related to inflammation (Zhao et al., 2007). The dual roles of microglia imply a potential strategy involving modulating their polarization to treat psychiatric disorders. In the present study, we evaluated whether targeting the PPARγ pathway would be a beneficial way on neurogenic development and neurobehavioral modifying of prepubertal MIA offspring.

2. Materials and methods

2.1. Animal

All the animal experiments were approved by the institutional animal care and use committee guidelines at the University of Electronic Science and Technology of China. Adult male and female Wistar rats were obtained from Chengdu Dossy Biological Technology Co., Ltd. (China). Breeding began after one week of habituating to the new environment with standard housing conditions (12 h light-dark cycle, lights on at 7:00 a.m., temperature 23–25 °C, humidity 50–60%). Food and water were available *ad libitum*.

2.2. Maternal immune activation

Fifty-five female rats were subjected to a timed mating procedure as described previously (Zhao et al., 2014). On gestation day 18 (GD18), the pregnant rats received a single injection of the viral mimetic Poly (I:C) (polyriboinosinic-polyribocytidilic acid; potassium salt; Sigma-Aldrich) at either 1 mg/kg, 5 mg/kg, or 10 mg/kg (dissolved in 0.9% saline, intraperitoneal, i.p.) or the vehicle (three dams per group). Dams were anesthetized with pentobarbital sodium and maternal trunk blood, placenta, fetal brain were harvested at 12 h post-injection for inflammatory cytokines detection (2 pups per litter and 3 dams/litters per group). The rest of the pregnant females (forty-three dams) were randomly assigned to control or Poly(I:C) (10 mg/kg). Weight change was measured on days 1, 2, and 3 after injection. On the day of birth, the gestation duration, sex ratio, litter size, and body weight were recorded.

2.3. Drugs

After weaning on postnatal day 21 (PND 21), the prepubertal offspring from each group (n = 2 to 3 pups per dam) were housed in groups (retaining only the male pups in order to avoid possible confusions arising from sex-specific fluctuation in female offspring (Bale, 2016). The PPARγ agonist, pioglitazone (hydrochloride; Sigma-Aldrich) dissolved in 0.9% saline, was injected daily for seven consecutive days from PND 21 to PND 27 at three doses (10, 20, or 30 mg/kg, i.p.). Control (Con): n = 26; Control-Pioglitazone (Con-Piog): n = 25; Poly (I:C): n = 25; Poly(I:C)-Pioglitazone-10 mg (10 mg): n = 9; Poly(I:C)-Pioglitazone-20 mg (20 mg): n = 8; Poly(I:C)-Pioglitazone-30 mg (30 mg): n = 26.

2.4. Maternal behavior

To explore the effect of MIA on maternal care, we observed the mothers (control: n = 7, Poly(I:C): n = 9) with their offspring (n = 8 to 12 pups per group) during three 60 min periods daily (8:00, 14:00 and 20:00) for the first 7 postnatal days (See Supplementary Materials and Methods for additional information).

2.5. Locomotor activity (LA)

LA was measured using the infrared ray passive sensor system (Ye et al., 2014; Zhu et al., 2014) (Taimeng Tech Ltd., China) on the offspring rats (PND 27; Con: n = 10; Con-Piog: n = 9; Poly(I:C): n = 9; 10 mg: n = 9; 20 mg: n = 8; 30 mg: n = 10; choosing 2 to 3 pups per litter and 3 litters per group). Each rat was placed in a chamber of an autonomous movement device to accommodate to the environment for 5 min before the test. Then, a 10 min test period was recorded (total locomotion number).

2.6. Elevated plus-maze (EPM)

The EPM was performed 2 h after the LA test. The maze was made of black Plexiglas 50 cm above the floor and consisted of four arms (50 cm long and 10 cm wide): two opposing enclosed arms with walls (20 cm high) and two open arms. The rats were placed in the center of the maze facing the same closed arm and allowed to explore for 10 min. The total distance explored in each of the four arms, the time spent in the open and closed arms, and the number of entries into each arm was recorded. The percentage of time spent in the open arms and the percentage of open arm entries (time or number of open arms/time or number of open plus closed arms) were calculated and used as measures of anxiety (Grigoryan and Segal, 2013).

2.7. Morris water maze (MWM)

The MWM was performed on PND 28 (choosing 2 to 3 pups per litter and 3 to 4 litters per group). The MWM was a circular tank 1 m in diameter with a hidden 9 cm diameter platform located 2 cm below the water (20–23 °C) surface. The animals had to learn to locate the hidden platform using distal cues over four consecutive days, as previously described (Zhao et al., 2014). On day 5, a spatial probe experiment was performed on the offspring. Reversal learning of the offspring was evaluated on days 6 and 7. The escape latency and spatial probe trial time were recorded for up to 60s in each quadrant. All the MWM testing was performed between 9 a.m. and 1 p.m. to estimate the spatial learning and memory of the offspring.

2.8. Cell culture

Primary microglia were isolated from the brains of the neonatal Wistar rats using aseptic techniques. The whole brains were digested in 0.25% trypsin (Gibco), filtered by cell strainer (70 μm, Corning) and centrifuged at 1200*g*, 10 min. Microglia were collected for phenotypic identification and for preparing the microglial conditioned medium (MCM). Neural precursor cells (NPCs) were obtained from the subventricular zone (SVZ) niche of Wistar rats (PND 28) and cultured with MCM for proliferated and differentiated detection (See Supplementary Materials and Methods for additional information). To quantify the number of cells, positive cells were counted in 10 different, random fields of three coverslips per condition in each experiment, in at least three independent experiments.

2.9. RNA interference

PPARγ-specific siRNA included three siRNA products (siRNA-1: 5′-CCG CCU UAU UAU UCU GAAA dTdT-3′; siRNA-2: 5′-CGA AGA ACC AUC CGA UUGA dTdT-3′; siRNA-3: 5′-GCA AGA GAU CAC AGA GUAU dTdT-3′; RiboBio Co., China) and the control siRNA (GFP). siRNA was transfected into cells using ribo*FECT*™ CP reagent, as recommended in the manufacturer's instructions. To confirm the silencing effect of the PPARγ-specific siRNA, RNA was extracted from microglia cells after 24, 48, and 72 h at 10, 30, 50, or 100 μmol and analyzed for PPARγ expression using real time PCR (See Supplementary Materials and Methods for additional information).

2.10. Immunofluorescence

Perfusion, tissue processing, and immunohistochemical analysis were carried out on the prepubertal rats (PND 28), as previously described (Zhao et al., 2015). Offspring rats (n = 5 per group; choosing 1 to 2 pups per litter and 3 litters per group) were injected twice daily (8:00 and 16:00) with BrdU (50 mg/kg, i.p., Sigma-Aldrich) for two consecutive days on PND 27-28. Two hours after the last BrdU administration, they were anesthetized with pentobarbital sodium, perfused with 4% paraformaldehyde (PFA), and dehydrated in 30% sucrose. Coronal cryosections were cut to 35 μm using a freezing microtome (CM1900; Leica Microsystems Inc.) and stored at 4 °C. For cell slips, they were fixed in 4% PFA and stored at 4 °C. The primary antibodies were: mouse anti-BrdU (1:500; Cell Signaling Technology), goat anti-DCX (1:400; Santa Cruz), goat anti-Iba1 (1:400; Abcam), mouse anti-GFAP (1:600; Cell Signaling Technology), rabbit anti-IL-6 (1:300; Abcam), rabbit anti-Arg1 (1:100; Abcam) and rabbit anti-PPARγ (1:100; Abcam). Secondary antibodies (Jackson ImmunoResearch): DyLight 488-conjugate donkey anti-mouse (1:300), DyLight 549-conjugate donkey anti-goat (1:300), and DyLight 488-conjugate donkey anti-rabbit (1:200).

To obtain the volumes of the neurogenesis subregions, the dentate gyrus (DG) and the granular cell layer (GCL) of the hippocampus were estimated and every sixth section was stained with DAPI antibody (Roche) (Czeh et al., 2010). Total number of per animal was multiplied by six to estimate the number of cells per DG. The sections and cell slips were imaged using an Olympus BX51 microscope (40× objective) with Olympus digital camera (DP70) and processed with Olympus DP Manager software and analyzed using Image J software (version 1.45 J; National Institutes of Health).

2.11. Real time PCR

The total RNA was isolated from the hippocampus of the offspring rats (PND 28, n = 6 per group; choosing 2 pups per litter and 3 litters per group) and the primary microglia using Trizol reagent (Invitrogen Life Technologies), and 2 μg RNA was reversed to cDNA with a Synthesis Kit (TaKaRa). PCR amplification of cDNA was performed with the All-In-One RT MasterMix (Applied Biological Materials Inc.) using a PCR detection system (Bio-Rad CFX 96). The relative gene expression was calculated using the Ct method. The threshold values for the genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Primers were shown in Table S1.

2.12. ELISA

Maternal serum, placenta, fetal brain, fetal prefrontal cortex, fetal hippocampus, fetal cerebellum, hippocampus, hippocampal cytoplasm and nucleus samples of prepubertal offspring (PND 28) and primary microglial cells were harvested using protein extraction kit (Millipore) supplemented with protease inhibitor (Beyotime) (n = 5 for each condition; choosing 1 to 2 pups per litter and 3 litters per group). The homogenates were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was collected and evaluated for the concentration of IL-6 (Beijing 4A Biotech Co., Ltd., China), TNFα (Beijing 4A Biotech Co.), Arg1 (B&D Systems), and PPARγ (B&D Systems). For the hippocampal cytoplasm and nucleus samples of hippocampus (PND 28), cytoplasmic extraction reagent was added into hippocampus tissue and centrifuged at 16,000 rpm for 5 min at 4 °C, the supernatant was cytoplasmic protein; then nuclear extraction reagent was added into the pellet after removing cytoplasmic, centrifuged at 16,000 rpm for 5 min at 4 °C, the supernatant was nuclear protein. The results were expressed as the ratio of each protein to the total soluble protein content (pg/mg).

2.13. Statistical analysis

The results are presented as mean ± SEM. Statistical comparisons were assessed with a *t*-test, one-way analysis of variance, two-way or three-way repeated measures ANOVA followed by a Bonferroni's multiple comparisons *post hoc* test, when appropriate. The results were accepted as statistically significant at $p \leq 0.05$. The statistical analysis data are available in Tables S2–15.

3. Results

3.1. Poly(I:C) induced cytokine responses in maternal and fetal tissues

To test whether maternal infection during late pregnancy results in an inflammatory response and influences the development of the fetal brain, Poly(I:C) (1, 5 or 10 mg/kg) were systemically administered. Schematic diagram of the experimental design was shown in Fig. 1A. Elevations of cytokines in the maternal serum were shown by an increase in IL-6 at three dose levels of Poly(I:C) and an increase of TNFα at the 10 mg/kg Poly(I:C) treatment (Fig. 1B-C). The placental content of IL-6 and TNFα were unchanged with or without Poly(I:C) (Fig. 1B, Interaction: Group × Tissue, $F_{6,\ 45} = 7.202$, $p < 0.001$; Fig. 1C, Interaction: Group × Tissue, $F_{6,\ 45} = 18.34$, $p < 0.001$). However, their concentrations were increased in the fetal brain, especially in the fetal hippocampus, after the 10 mg/kg treatment compared with the vehicle alone or with the 1 mg/kg or 5 mg/kg treatments (Fig. 1D-E; Fig. 1D,

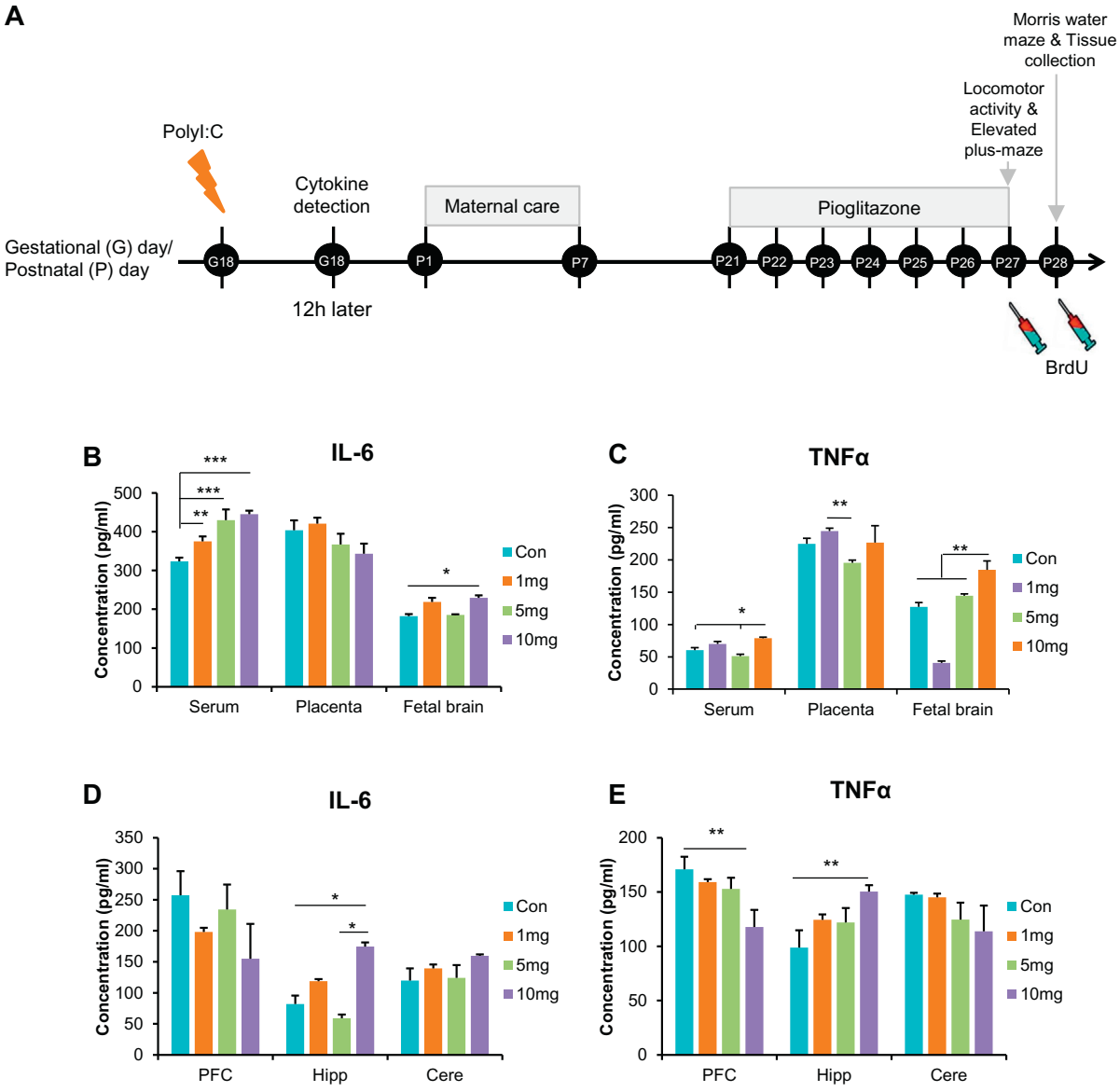

Fig. 1. Effects of MIA on the inflammatory cytokines in maternal blood, placenta and fetal brain. (A) Schematic diagram of the experimental design. (B, C) Poly(I:C) (PolyIC) administration during late gestation induced a dose-dependent elevation in the protein concentration of IL-6 and TNFα in the maternal serum (n = 3 dams/group) and the fetal brain (n = 6/group) but not in the placenta (n = 6/group), compared to control rats at 12 h post-injection. (D, E) However, higher IL-6 and TNFα were detected in the fetal hippocampus (n = 6/group) injected with 10 mg/kg relative to the 1 mg/kg and 5 mg/kg Poly(I:C) groups. PFC, Prefrontal cortex; Hipp, hippocampus; Cere, cerebellum. All data are means ± SEM. *p < 0.05, **p < 0.01.

Interaction: Group ×Tissue, $F_{6,\ 45} = 3.732$, p < 0.05; Fig. 1E, Interaction: Group ×Tissue, $F_{6,\ 45} = 5.134$, p < 0.001). Furthermore, a clear dose-dependent increase of inflammatory cytokine was detected in various tissues at 12 h post-injection. The 10 mg/kg dose of PolyI:C was chosen for the later experiments. The physiological parameters of mother and offspring (Fig. S1A-E) and the maternal behavior (Fig. S1F-G) were not affected by the Poly(I:C). These data indicate that MIA induces maternal inflammatory and fetal hippocampal inflammatory responses.

3.2. MIA-induced abnormal behaviors emerged in prepubertal offspring and were ameliorated by pioglitazone

The prepubertal MIA offspring showed impaired spatial learning compared with the control offspring on the second, third and fourth days (Fig. 2A; Day, $F_{3,\ 12} = 205.9$, p < 0.001; Group, $F_{5,\ 60} = 16.23$, p < 0.001; Interaction: Day ×Group, $F_{15,\ 60} = 2.319$, p < 0.05). The spatial probe test revealed a significantly shorter latency in the platform quadrant (Fig. 2B; $F_{5,\ 49} = 7.69$, p < 0.001) and fewer crossing times in the MIA offspring on the fifth day (Fig. S2A). In the reverse learning phase, the time to locate the platform of MIA offspring rats was longer than that of the control rats (Fig. S2B-C). Pharmacological treatment with pioglitazone (30 mg/kg) on PolyI:C offspring improved the hippocampus-dependent spatial learning and memory of the MIA offspring.

In the EPM, the MIA offspring had fewer entries (%) and less time (%) in the open arms than the control groups (Con and Con-Piog). This anxiety-like behavior was ameliorated by pioglitazone treatment (30 mg/kg; Fig. 2C; $F_{5,\ 49} = 4.141$, p = 0.003; Fig. 2D; $F_{5,\ 49} = 9.391$, p < 0.001). Importantly, these distinct behaviors observed for the different groups were not due to differences in the total ambulatory distance and locomotor activity (Fig. S2D-E).

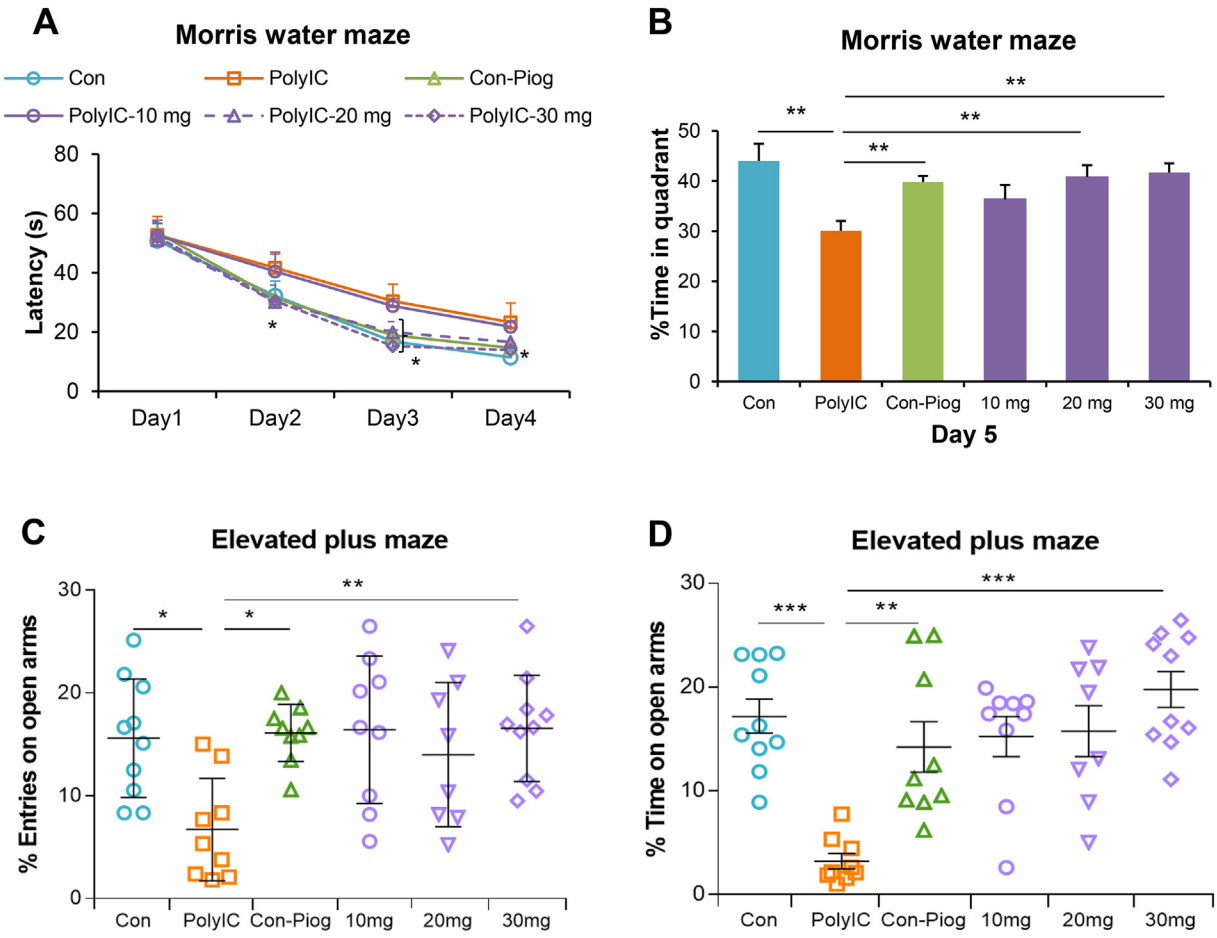

Fig. 2. Cognition-related behavioral changes of prepubertal offspring rats following MIA. (A) The latency was elevated in the offspring of Poly(I:C) and Poly(I:C) plus 10 mg/kg pioglitazone on days 2, 3, and 4 compared to the control rats in the Morris water maze test. (B) Poly(I:C)-20 mg/kg and − 30 mg/kg pioglitazone increased the percentage of time in the platform quadrant on day 5. (C-D) Percentage entries and time in open arms were evaluated by an elevated plus maze test. Control: Con; Control-Pioglitazone: Con-Piog; Poly(I:C): PolyIC; Poly(I:C)-Pioglitazone-10 mg: 10 mg; Poly(I:C)-Pioglitazone-20 m: 20 mg; Poly(I:C)-Pioglitazone-30 mg: 30 mg. n = 8–11/group. All data are means ± SEM. *p < 0.05, **p < 0.01.

3.3. Neurogenesis was increased in the hippocampal DG in the offspring with pioglitazone treatment

To examine the relationship between the MIA-induced hippocampus-related abnormal behaviors and hippocampal neurogenesis in the prepubertal offspring, we observed the BrdU-labeled proliferated cells and the DCX-represented differentiated cells in the subgranular zone (SGZ) of the hippocampus (Fig. 3A). MIA reduced the BrdU+ cell population in the offspring compared with the control group, but the generation of new cells was reversed in the pioglitazone-treated rats (Fig. 3B; Treatment, $F_{1,\ 4} = 26.65$, $p < 0.001$; Condition, $F_{1,\ 4} = 26.41$, $p < 0.001$; Interaction: Treatment ×Condition, $F_{1,\ 4} = 131.9$, $p < 0.001$; Condition is PolyI:C *versus* control. Treatment is pioglitazone *versus* control). Differentiated newborn neurons, detected by the $BrdU^+/DCX^+$ and DCX^+ cells, were also lower in the Poly(I:C)-treated offspring (Fig. 3C-D; Interaction: Treatment × Condition, $F_{1,\ 4} = 108.7$, $p < 0.001$; Interaction: Treatment ×Condition, $F_{1,\ 4} = 11.22$, $p < 0.05$). However, the volume of the DG and GCL showed no significant differences between the control, MIA, and pioglitazone-treated groups (Fig. 3E-G; Region × Treatment × Condition, $F_{1,\ 32} = 0.413$, $p > 0.05$). MIA-induced abnormal postnatal behaviors are, thus, highly associated with neurogenesis impairment.

3.4. Dynamic microglial phenotypes regulated the neurogenic microenvironment after MIA and pioglitazone treatment

To determine whether microglial phenotypes could account for the impaired neurogenesis in MIA-treated offspring, we assessed the activation states of the microglia and astrocytes in the NPC region. Representative images of Iba1 and GFAP labeling in the CA1, CA3, and DG of the hippocampus are shown in Fig. 4A and Fig. S3A. MIA induced a higher density of microglia in the DG but not the number of astrocytes (Fig. 4B and Fig. S3B). Surprisingly, pioglitazone administration reduced the microglia population but not the astrocyte number (Region × Condition × Treatment, $F_{2,\ 48} = 42.23$, $p < 0.001$). Moreover, the decrease in the number of processes and the larger soma in the microglia, which reflect the microglial activation phenotype (Fig. 4C), were reversed with pioglitazone treatment in the DG of MIA offspring (Fig. 4D-E; Interaction: Treatment × Condition, $F_{1,\ 4} = 23.34$, $p < 0.001$; Interaction: Treatment ×Condition, $F_{1,\ 4} = 0.5403$, $p > 0.05$). In addition, mRNA expression of CD11b, the microglial activation marker, was increased in the MIA offspring and returned to a lower level with pioglitazone administration (Fig. 4F; Interaction: Treatment ×Condition, $F_{1,\ 5} = 11.21$, $p < 0.05$).

To further identify the activated phenotype of the microglia, the mRNA and protein expression of the M1 and M2 markers were assessed (Kobayashi et al., 2013; Liu et al., 2013; Michelucci et al., 2009). MIA increased the M1 markers (Fig. 4G; IL-1β ~3 fold, TNF-α ~2 fold, IL-6–7 fold, CD68 ~6 fold, iNOS ~3.5 fold, IFNγ ~3 fold; M1 markers ×

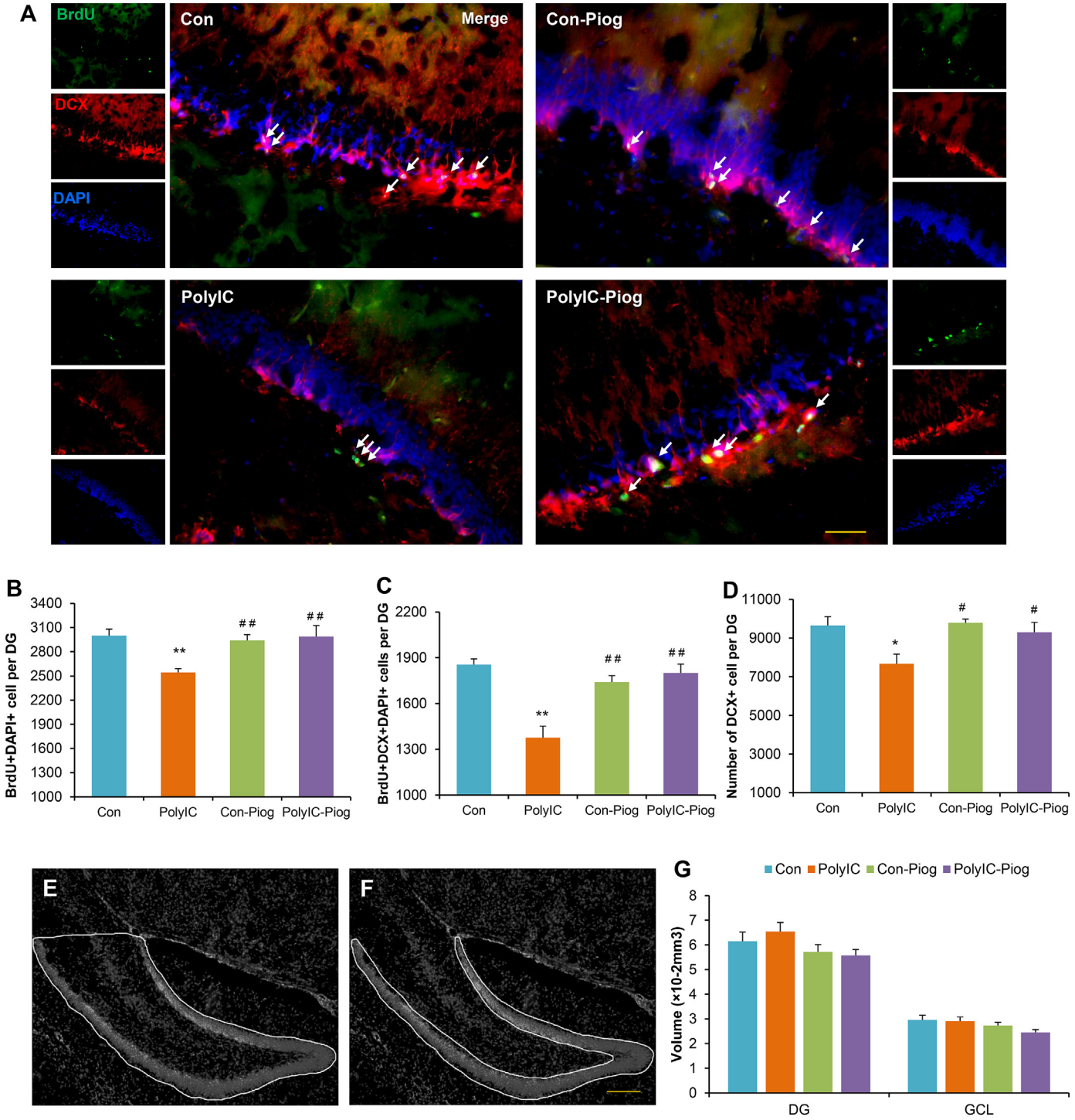

Fig. 3. Pioglitazone mitigated the neurogenesis impairment in the hippocampus of MIA offspring. (A) Representative images of neurogenesis in the DG of the hippocampus. Scale bars: 50 μm. Arrows represent BrdU + DCX + cells. The proliferative cells (B; $BrdU^+$ cells) and the differentiated cells (C-D; $BrdU^+DCX^+$ cells, DCX^+ cells) were reduced in the DG of the MIA offspring. (*E*-F) Representative images of the volume in the DG and GCL of the hippocampus. Scale bars: 200 μm. (G) No significant difference in volume was observed between groups. $n = 5$/group. All data are means ± SEM. $^{**}p < 0.01$ *vs.* Con. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* Poly (I:C).

Condition × Treatment, $F_{5,\ 120} = 5.959$, $p < 0.001$) and reduced the M2 markers (Fig. 4I; Ym1 ~0.4 fold, Arg1 ~0.5 fold, CD206 ~0.1 fold, IL-4–0.2 fold, IL-10–0.3 fold, CCR2 ~0.25 fold; Condition × Treatment, $F_{1,\ 120} = 15.55$, $p < 0.001$; M2 markers × Condition × Treatment, $F_{5,\ 120} = 0.2202$, $p > 0.05$) in the hippocampus of the offspring compared with the control group. For the protein expression assayed by ELISA, IL-6 increased and Arg1 decreased in the MIA offspring (Fig. 4H; Interaction: Treatment × Condition, $F_{1,\ 4} = 101$, $p < 0.001$; Fig. 4J; Treatment, $F_{1,\ 4} = 33.75$, $p < 0.05$; Condition, $F_{1,\ 4} = 44.58$, $p < 0.05$; Interaction: Treatment × Condition, $F_{1,\ 4} = 2.691$, $p > 0.05$). After the pioglitazone treatment, these markers returned to the normal level in the hippocampus of MIA offspring from mRNA to protein expression. In this study, we assigned the ratio of iNOS to Arg1 and TNFα to IL-10 to represent the M1-M2 dynamic alterations (Tajiki

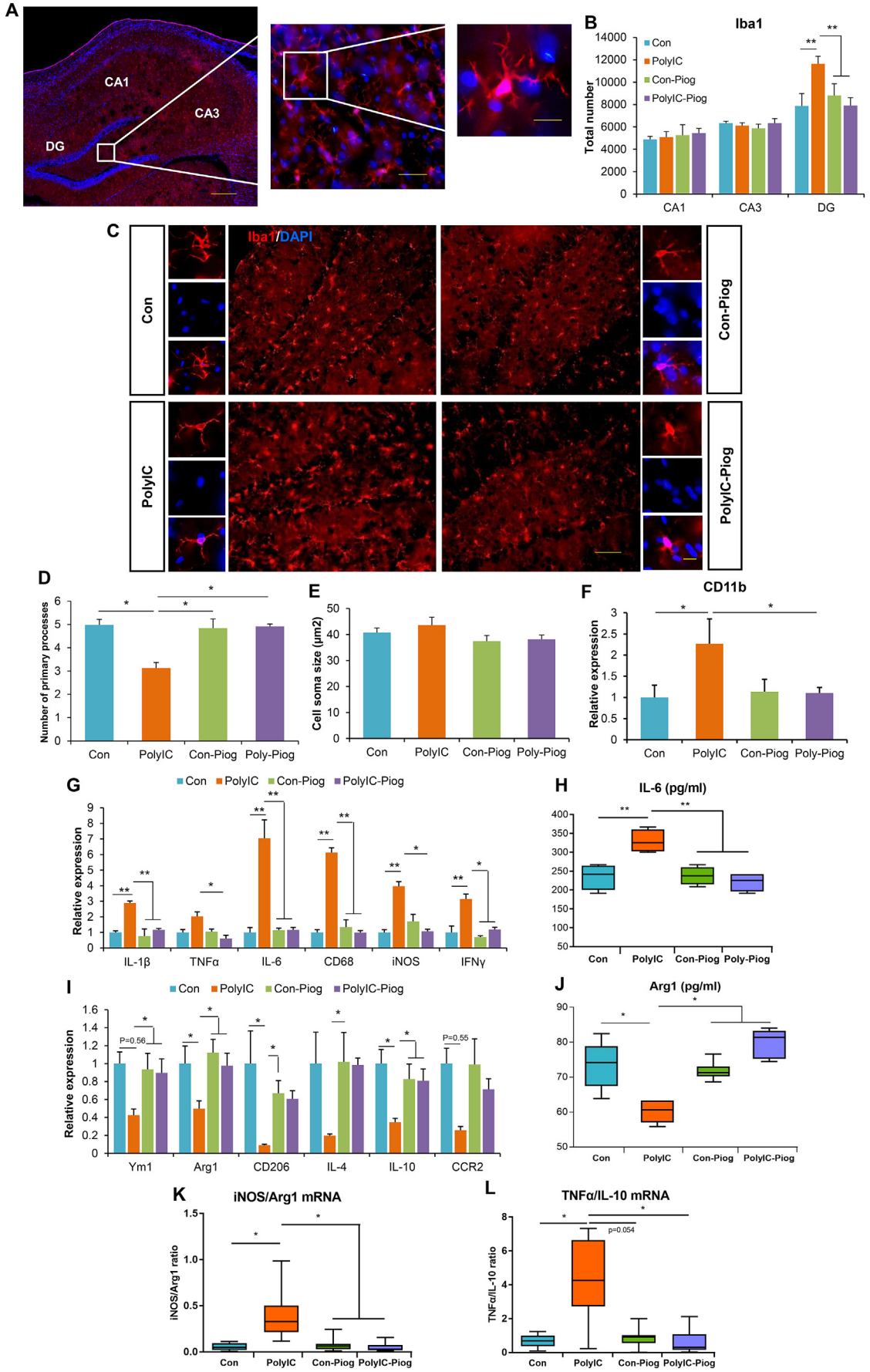

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Fig. 4. Pioglitazone switched MIA-induced microglial M1 activation to the M2 phenotype. (A) Representative fluorescent images of Iba1-labeled cells are shown. (B) Immunofluorescent quantification showed that pioglitazone decreased the MIA-induced increase of the Iba1$^+$ cells in the DG region. Scale bars: 250 μm (left panel), 50 μm (medial panel), and 20 μm (right panel). (C–F) Schematic representation (n = 5/group) and quantification of the number of microglial primary processes, cell soma size of microglia, and microglial activation marker-CD11b (n = 6/group) were showed in the hippocampus of prepubertal offspring. (G-J) Pioglitazone rescued the elevation of microglial M1 markers paralleled by the reduction of the M2 mediators after Poly(I:C) injection on the mRNA and protein levels. (K-L) A similar trend was detected in the ratio of M1/M2. IHC: n = 5/group; RT-PCR: n = 6/group; ELISA: n = 5/group. Scale bars: 100 μm. All data are means ± SEM. *$p < 0.05$, **$p < 0.01$.

et al., 1997; Yang et al., 2016). Interestingly, the data showed that iNOS/Arg1 and TNFα/IL-10 were significantly increased in the MIA offspring and reduced after pioglitazone treatment, compared with the control groups (Fig. 4K-L; Interaction: Treatment × Condition, $F_{1, 5} = 12.28$, $p < 0.05$; Interaction: Treatment × Condition, $F_{1, 5} = 10.28$, $p < 0.05$). Based on their immunofluorescence colocalization, we determined that the cellular source of IL-6 and Arg1 was microglial cells (Fig. S4). This indicated that the microglial M1 was associated with the reduced neurogenesis and abnormal behaviors.

3.5. The PPARγ pathway was required for the microglial M2 phenotype and their pro-neurogenic effects

The evidence indicated that pioglitazone plays an important role in the PPARγ-dependent microglial activation. The relative mRNA expression of PPARγ, but not PPARα or PPARβ/δ, was reduced in the hippocampus of the MIA-treated offspring and returned to the level of the control group after pioglitazone administration (Fig. 5A; PPARs × Condition × Treatment, $F_{2, 60} = 2.239$, $p > 0.05$). Concurrently, pioglitazone moderated the reduction of PPARγ protein in the MIA-offspring (Fig. 5B; Interaction: Treatment × Condition, $F_{1, 4} = 14.06$, $p < 0.05$). The protein concentration of PPARγ decreased in the cell nucleus of the hippocampus of the MIA offspring but remained unchanged in the cytoplasm (Fig. 5C; Region, $F_{1, 32} = 7106$, $p < 0.001$; Region × Condition, $F_{1, 32} = 4.671$, $p < 0.05$). Fig. 5D indicating that the PPARγ is localized in the microglia cytoplasm and nuclear region of Poly(I:C)-treated offspring. However, after the pioglitazone treatment the PPARγ was concentrated in the microglial nuclear region. This indicates that the PPARγ was activated by pioglitazone and transferred into the nuclear region where it promotes transcription of anti-inflammatory mediators.

To confirm the effect of PPARγ on the activated microglial pathway, primary microglial cells were incubated and M1 and M2 markers were detected with or without Poly(I:C), pioglitazone and GW9662

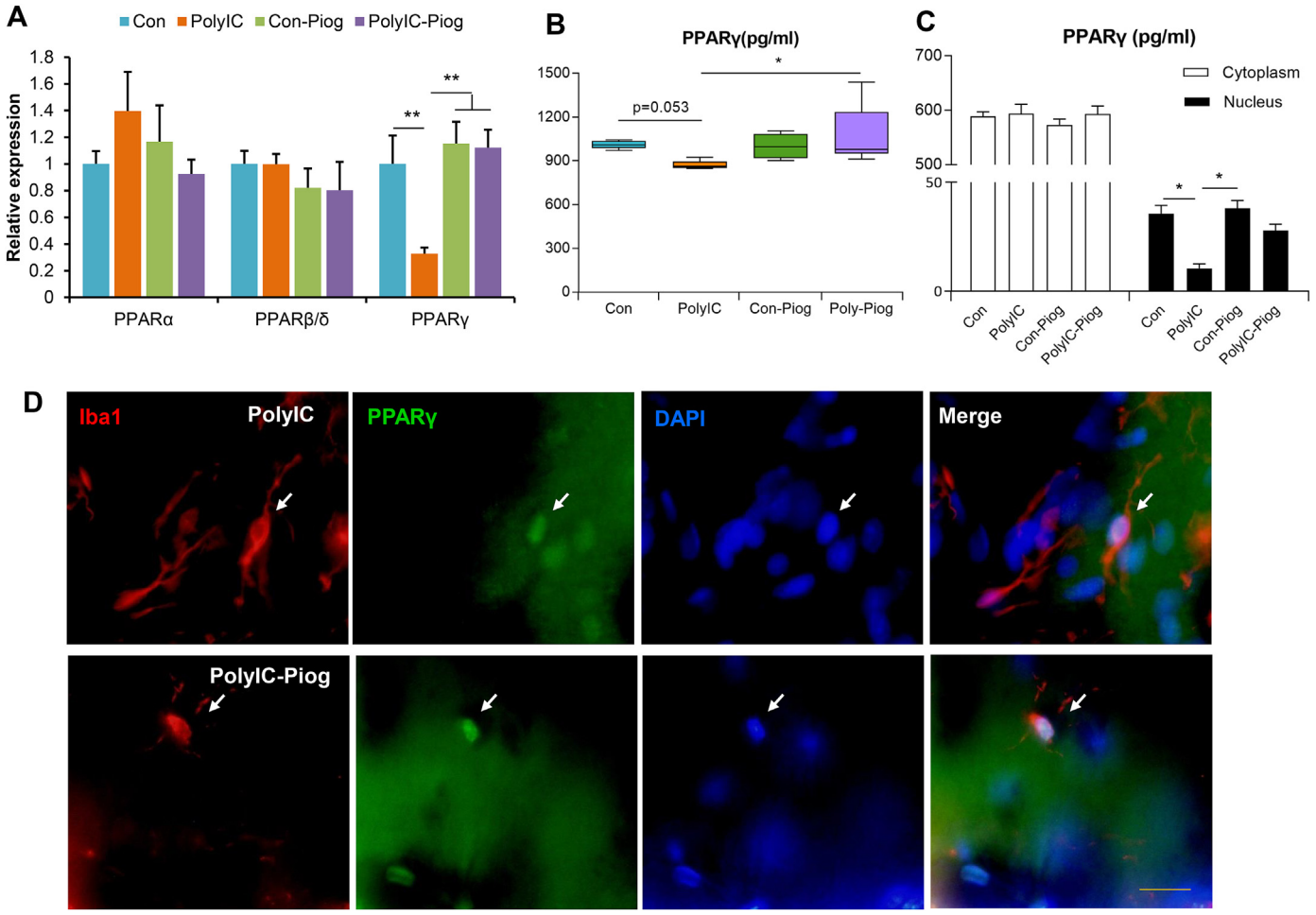

Fig. 5. Pioglitazone regulated the pathway of PPARγ in the hippocampus. (A-B) qPCR and ELISA quantified PPARγ with or without pioglitazone in the control and Poly(I:C) offspring rats. (C) The decrease of PPARγ expression was moderated by pioglitazone treatment in the nucleus of the Poly(I:C)-treated hippocampus. (D) Immunofluorescent images showed the distribution of PPARγ in a single cell. Arrows represent PPARγ positive microglial cells. RT-PCR: n = 6/group; ELISA: n = 5/group. Scale bars: 20 μm. All data are means ± SEM. *$p < 0.05$, **$p < 0.01$.

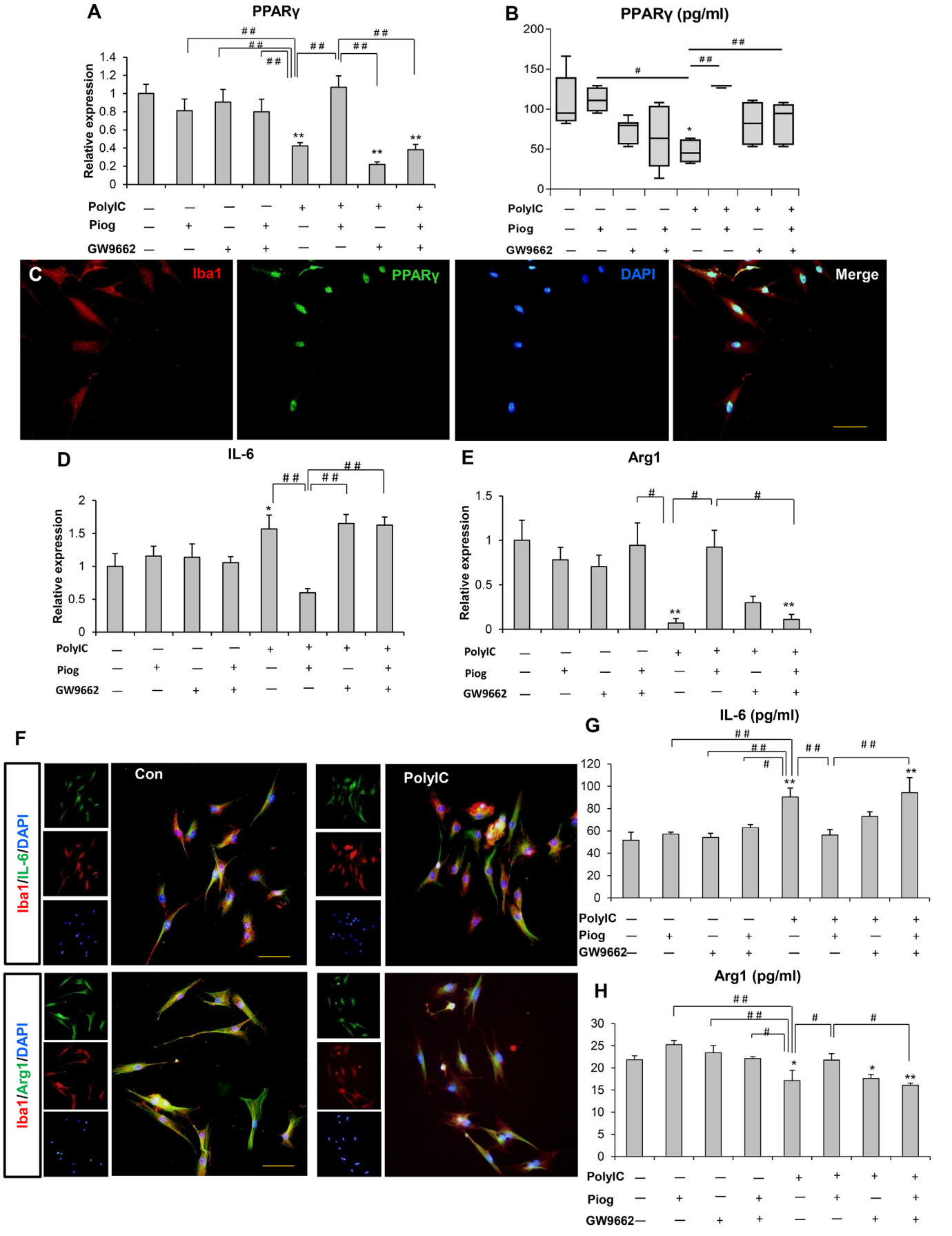

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Fig. 6. PPARγ pathway is involved in microglial phenotypic switch. PPARγ mRNA (A) and protein expression (B) were determined in primary microglia with PPARγ agonist or/and antagonist treatment. (C) Representative distribution of PPARγ positive microglia. Scale bars: 50 μm. Poly(I:C) induced the expression of M1 cytokine (D, G) and reduced the M2 marker content (E, H). The fluorescence images from the IL-6 and Arg1 immunostaining in microglia better illustrated the results shown in the quantitative histograms (F). Scale bars: 50 μm. All data are means ± SEM. *$p < 0.05$, **$p < 0.01$ *vs.* Con [Poly(I:C)(−) Piog(−) GW9662(−)]. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

treatment. The optimal treated time and the optimal concentration of pioglitazone were shown by RT-PCR in Fig. S5. The mRNA and protein expression of the PPARγ decreased with Poly(I:C) stimulation, and the expressions were mitigated by the PPARγ agonist, pioglitazone. However, the PPARγ antagonist GW9662 inhibited this mitigation (Fig. 6A-B). In Poly(I:C)-treated microglial cells, pioglitazone moderated the increase of the M1 markers: IL-6, IL-1β, TNF-α, CD68, iNOS, and IFNγ (Fig. 6D, Fig. S6) and the decrease of the M2 markers: Arg1, Ym1, CD206, IL-4, IL-10 and CCR2 (Fig. 6E, Fig. S7). The PPARγ antagonist maintained the M1 phenotype. A change in mRNA expression was accompanied by a corresponding change in protein expression (Fig. 6F-H). These results indicated that PPARγ activation switches microglial phenotypes from M1 to M2.

To evaluate whether microglial activation affects the neurogenesis of NPCs, MCM was prepared and added to the NPCs. Fig. 7A and Fig. 7B respectively represent the proliferation (BrdU staining) and differentiation (DCX and GFAP staining). Incubated with Poly(I:C) MCM, an almost 10% reduction in $BrdU^+$ proliferation cells were found in the Poly(I:C), Poly(I:C)-GW9662, and Poly(I:C)-Piog-GW9662 groups compared with the control MCM. This reduction was ameliorated in the pioglitazone-treated media (Fig. 7C; GW9662 × Condition × Pioglitazone, $F_{1,\ 16} = 19.65$, $p < 0.001$). With respect to the differentiation of NPCs, pioglitazone reversed the number of neonatal neurons (DCX positive cells), which were lower in the Poly(I:C)-incubated cultures (Fig. 7D; GW9662 × Condition × Pioglitazone, $F_{1,\ 16} = 23.16$, $p < 0.001$). However, the proportions of the astrocytes (GFAP positive cells) in the Poly(I:C)-MCM and Poly(I:C)-Piog-MCM were not significantly different from the control MCM (Fig. 7E; GW9662 × Condition × Pioglitazone, $F_{1,\ 16} = 1.121$, $p > 0.05$). With PPARγ-siRNA treatment, the effects of pioglitazone on microglial activation were eliminated in cultured primary microglia (Fig. S8). The proliferated cells decreased in the siRNA-MCM and siRNA-piog-MCM compared to the control MCM (Fig. 7F; $F_{3,\ 8} = 124.1$, $p < 0.001$). The DCX^+ cells were reduced in the siRNA- and siRNA-piog-treated MCM (Fig. 7G; $F_{3,\ 8} = 55.17$, $p < 0.001$), but siRNA did not affect the percentage of $GFAP^+$ cells (Fig. 7H; $F_{3,\ 8} = 1.9$, $p > 0.05$). The results indicated that PPARγ-mediated M2 microglia have pro-neurogenic effects.

4. Discussion

Our data showed that during development the microglia responded to MIA, resulting in an imbalance of pro−/anti-inflammatory cytokines, which associated with aberrant postnatal behaviors in the offspring. The pro-inflammatory activation of M1 microglia apparently drove the inhibition of hippocampal neurogenesis leading to behavioral impairments. Targeting the switch of microglial polarization from M1 to M2 in the PPARγ pathway had a neuroprotective effect on the MIA offspring. When PPARγ was activated by pioglitazone, it shifted microglia from the M1 to the M2 phenotype, improved neurogenesis, and ameliorated behavioral deficits in the prepubertal MIA-offspring.

MIA models suggested that using immunostimulants, such as Poly (I:C), activate the maternal immune system and trigger subsequent changes in brain development and behaviors of the offspring (Meyer et al., 2009). Epidemiological studies have indicated that the critical link in the association between MIA and increased neuropsychiatric disorders risks in offspring is a cytokine-related immunological response to infection (Gilmore and Jarskog, 1997). We showed that Poly (I:C) induced inflammatory responses in the maternal peripheral system and fetal brain, especially in the hippocampus, but not affect maternal care behavior. The maternal inflammation was administered in late gestation based on the observation that the changes in the cytokines in fetal brain after MIA may occur late in gestation but not during other gestational periods (Cai et al., 2000; Meyer et al., 2006). Maternal cytokines may be able to cross the placenta, lead to dysregulation of the microglia, and lead to an imbalance in the inflammatory cytokines in the fetal brain, eventually having an effect on abnormal postnatal behaviors (Giovanoli et al., 2013; Juckel et al., 2011). An increase in inflammatory cytokines in the maternal host and ultimately in the fetal brain may be one of the pathologies that result in developmental abnormalities with long-lasting neuropsychiatric disturbances later in life (Patterson, 2007).

The behavioral deficits in the prenatal Poly(I:C)-exposed rodents have been explained as being associated with heightened vulnerability and exaggerated inflammatory responses to environmental insults (Knuesel et al., 2014). Inflammation-mediated neurotoxicity appears to underlie the hippocampal neurogenesis impairments and behavioral abnormalities that have been observed in juvenile and adolescent offspring (Smith et al., 2007). Studies have suggested that newborn cells in the DG may be recruited in greater numbers into existing circuits and influence learning and memory processes even before they mature (Kempermann, 2002; Shors et al., 2002). Prenatal microglial programming could modulate neurogenesis, which seems to be critically involved in the precipitation of long-term cognitive and emotional consequences. Recently, we found that intragastric administration of the PPARγ agonist pioglitazone in chronic mild stress-treated mice led to a clear shift of the microglia from the M1 to the M2 phenotype, as recognized by a dynamic alteration in gene-expression profiles and phenotype markers (Zhao et al., 2016). Several studies have demonstrated that Poly(I:C) treatment during gestational windows led to persistent changes in the microglia of prepubertal offspring (Giovanoli et al., 2013; Krstic et al., 2012). Microglial priming enhanced the offspring's vulnerability to neuronal dysfunctions and behavioral aberrances. MIA is a potent microglial primer (Knuesel et al., 2014). The source of the inflammatory cytokines might be mainly from microglia, but not astrocytes in the MIA offspring.

Microglial activation exists in equilibrium between two states, characterized as M1 and M2 activations, each with different molecular profiles. The broad M1 pathway is activated following neurotoxic events and results in the release of a range of pro-inflammatory cytokines (Cherry et al., 2014). The M2-trended phenotype can be triggered by anti-inflammation cytokines (such as IL-4, IL-10 and TNFβ), some chemicals like minocycline, rapamycin, glatiramer acetate and becarotene (Cherry et al., 2014; Lan et al., 2017), and is involved in neuroprotective processes and expresses mediators with the capacity to downregulate, repair, or protect the CNS from inflammation (Cherry et al., 2014). As shown in Fig. 4, the imbalance of microglial activated phenotypes resulted in a prolonged inflammatory response in MIA offspring. The extensive microglial inflammation and continued release of over-expression of pro-inflammatory cytokines indicated that inflammatory processes influence neural progenitor cell development (Monje et al., 2002; Monje et al., 2003).

MIA-induced dysregulation of microglial polarization appears to prime the brain's responsiveness to inflammatory processes. PPARγ can initiate expression of distinct inflammatory genes after activation (Ahmadian et al., 2013). Pioglitazone, a well-established drug known to be a PPARγ agonist belonging to the class of thiozolidinodienes, is implicated in microglial polarization (Mandrekar-Colucci et al., 2012). We found PPARγ transferred into the microglial nucleus after

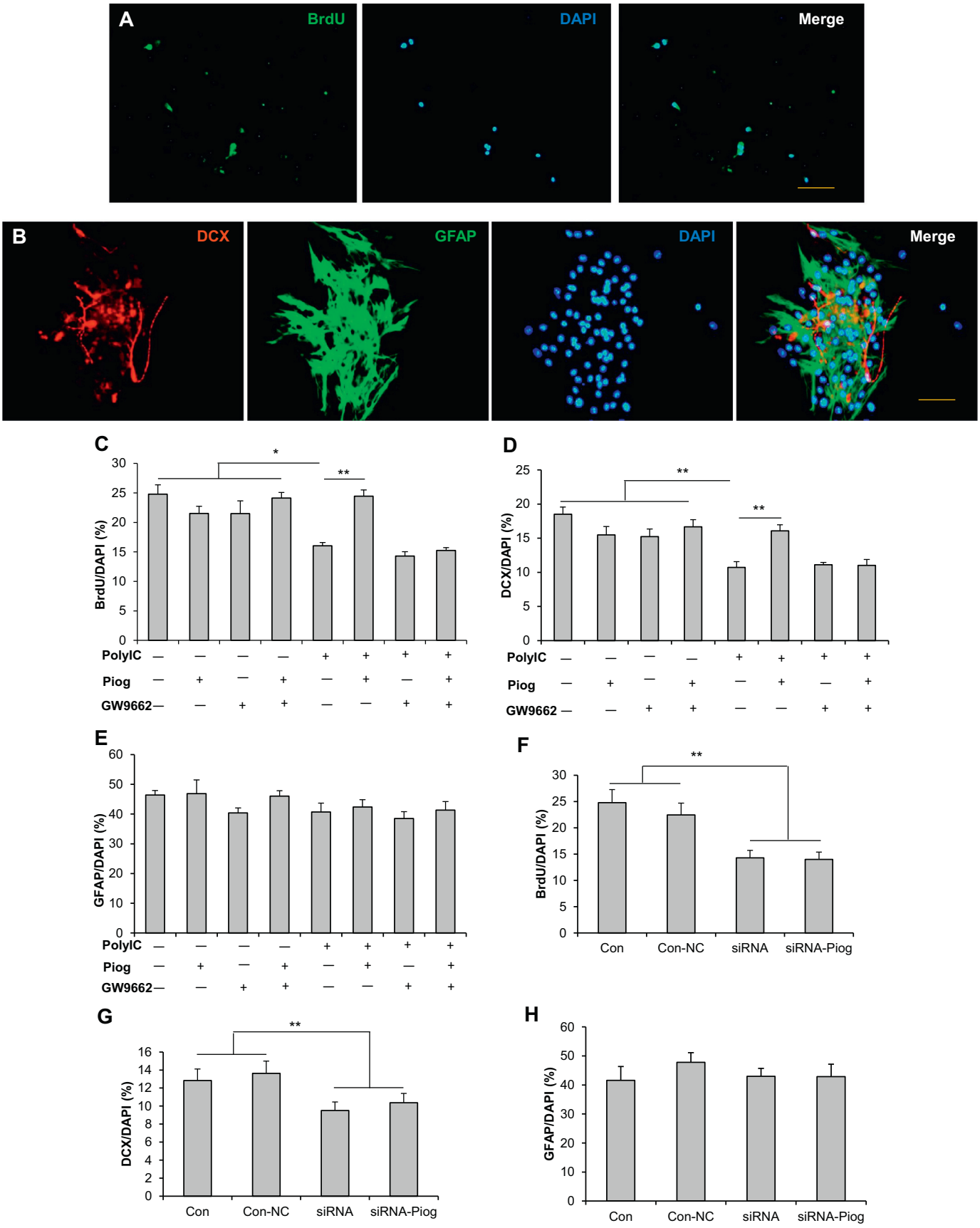

Fig. 7. Effects of PPARγ-modulated microglia on neurogenesis in MIA offspring. (A) Proliferation of NPC labeled with BrdU/DAPI immunostaining. Scale bars: 100 μm. (B) Differentiation of NPC shown with neuron-orientated DCX and astrocyte-orientated GFAP. Scale bars: 100 μm. (C-E) Poly(I:C) inhibited $BrdU^+$ and DCX^+ cells but caused no change in $GFAP^+$ cells. (F–H) PPARγ agonist increased the number of BrdU and DCX positive cells. We observed PPARγ-siRNA impaired neurogenesis but not astrocyte-orientated differentiation. All data are means ± SEM. *$p < 0.05$, **$p < 0.01$.

pioglitazone treatment and may promote transcription of the anti-inflammatory mediators. Clinical investigation showed that pioglitazone has beneficial effects on psychiatric patients with metabolic problems or diseases (Boris et al., 2007; Ghaleiha et al., 2015; Iranpour et al., 2016; Kemp et al., 2012), indicating an important translational implication for research of PPARγ mediated inflammatory signaling in mood disorders (Kirsten et al., 2018). PPARγ activation inhibited microglial proinflammatory response and had a neuroprotective effect in neuroinflammatory models (Zhao et al., 2007). Several years ago, the concept of microglial phenotypes, which has been widely accepted, was classified using the nomenclature of macrophages (Schwartz et al., 2006). However, the binary classification of the M1 and M2 functionally polarized microglia has recently been debated (Ransohoff, 2016) because microglia and macrophages have diverse characteristics in response to environmental signals. Since we aware that the description of the states as M1 and M2 is a simplified scheme, we identified the M1-M2 by a panel of expression profiles of pro- and anti-inflammatory cytokines, phenotypic markers, and morphological and functional states of the microglia.

The polarized status of microglia *in vitro* is much more straightforward than *in vivo*. Microglia polarized toward the M1 phenotype with Poly(I:C) stimulation or PPARγ-specific siRNAs. Pioglitazone enhanced and prolonged the M2 phenotype, and reduced the M1 phenotype. IL-6 is the M1 signature gene and is upregulated after microglial M1 activation; simultaneously, elevated IL-6 may be able to stimulate microglia toward M1 polarization (Qin et al., 2012). IL-6 suppresses the differentiation of NPCs into neurons *in vitro* but does not affect astrocyte-orientated differentiation (He et al., 2005; Sparkman et al., 2006). IL-6 is also known to be a key mediator of the effects of MIA on fetal brain development (Smith et al., 2007). Microglia constitute the primary immune mediators of the NPC functions (Knuesel et al., 2014). The rebalance of M1-M2 microglial phenotypes and pro−/anti-inflammatory cytokines after pioglitazone treatment was associated with the reversal of the deficient neurogenesis. Combined with *in vivo* results, this indicates that the M1-to-M2 phenotypic switch is a favorable microenvironment for ameliorating neurogenesis impairments.

5. Conclusions

MIA during pregnancy is associated with neurodevelopmental and neurobehavioral abnormalities in prepubertal offspring. The dysregulation of microglia polarization seeds the vulnerability for inflammation-induced neurotoxicity during postnatal development. By modulating the PPARγ signaling, the microglia are switched to the M2 phenotype, resulting in improved neurogenesis followed by the amelioration of behavioral abnormalities in the MIA prepubertal offspring. Our findings highlight the important role of microglial cells after maternal inflammatory exposure and the therapeutic relevance of their polarization balance. Targeting PPARγ-dependent microglial phenotypes may well shed light on the development of therapies for neurodevelopmental and psychiatric disorders.

Competing interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2019.01.005.

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