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Minocycline rescues decrease in neurogenesis, increase in microglia cytokines and deficits in sensorimotor gating in an animal model of schizophrenia

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Abstract

Adult neurogenesis in the hippocampus is impaired in schizophrenic patients and in an animal model of schizophrenia. Amongst a plethora of regulators, the immune system has been shown repeatedly to strongly modulate neurogenesis under physiological and pathological conditions. It is well accepted, that schizophrenic patients have an aberrant peripheral immune status, which is also reflected in the animal model. The microglia as the intrinsic immune competent cells of the brain have recently come into focus as possible therapeutic targets in schizophrenia.

We here used a maternal immune stimulation rodent model of schizophrenia in which polyinosinic-polycytidilic acid (Poly I:C) was injected into pregnant rats to mimic an anti-viral immune response. We identified microglia IL-1β and TNF-α increase constituting the factors correlating best with decreases in net-neurogenesis and impairment in pre-pulse inhibition of a startle response in the Poly I:C model. Treatment with the antibiotic minocycline (3mg/kg/day) normalized microglial cytokine production in the hippocampus and rescued neurogenesis and behavior. We could also show that enhanced microglial TNF-α and IL-1β production in the hippocampus was accompanied by a decrease in the pro-proliferative TNFR2 receptor expression on neuronal progenitor cells, which could be attenuated by minocycline. These findings strongly support the idea to use anti-inflammatory drugs to target microglia activation as an adjunctive therapy in schizophrenic patients.

Introduction

Schizophrenia is a devastating disorder and constitutes a social and economic burden for patients as well as families and society. During the past decades, an increasing number of studies have associated schizophrenia and inflammation (Müller et al., 2010; Fineberg et al., 2013). Concomitantly, microglia cells – the intrinsic immune competent cells of the brain - have been pinpointed in the pathophysiology of this neurodevelopmental disorder in both human patients and animal models of this disorder (Blank and Prinz, 2013; Fricker et al., 2013; Harry et al., 2012). In a subpopulation of schizophrenic patients increased microglial cellular density and activity has been found in post-mortem tissue and *in vivo* (Falkai et al., 1999; Steiner et al, 2006; van Berckel et al., 2008; Busse et al., 2012) as well as in animal models of schizophrenia with varying results depending on brain region and age investigated (Juckel et al., 2011; Garay et al., 2013; van den Eynde et al., 2014).

One way in which activated microglia contribute to pathology is through the production of pro-inflammatory cytokines. An imbalance in cytokine levels may trigger aberrant neurodevelopment in the fetus and lead to neuropathology and psychopathology in the adult offspring. Infection-induced increase of pro-inflammatory maternal cytokines may be one of the key events leading to enhanced risk for neuropsychiatric disorders in the offspring (Gilmore and Jarskog, 1997). Human studies revealed that increased maternal serum levels of the pro-inflammatory cytokine Tumor Necrosis Factor-α (TNF-α) and the chemokine Interleukin-8 (IL-8) during pregnancy are directly associated with a higher risk for schizophrenia in the progeny (Brown et al., 2004; Buka et al., 2001). In line with this finding, *Mednick* and colleagues reported that fetuses gestating during a viral epidemic are at elevated risk for developing schizophrenia (Mednick et al., 1988). Subsequent prospective studies have shown that maternal

infections of various types increase the risk for schizophrenia in the offspring three- to sevenfold (for review see Brown et al., 2010). Rodent studies have confirmed that a maternal immune response is sufficient to induce psychopathology in later life (Biscaro et al., 2012; Ozawa et al., 2006; Meyer et al., 2005; Zuckerman and Weiner, 2005; Shi et al., 2003; Abazyan et al. 2010; Zuckerman et al., 2003; Frick et al., 2013). Injection of pregnant rodents with the viral mimic polyinosinic-polycytidilic acid (Poly I:C) leads to a wide spectrum of schizophrenia-relevant behavioral deficits, such as pre-pulse inhibition of the acoustic startle response (PPI) (Gal et al., 2009; Klein et al., 2013; Kumari et al., 2008; Smith et al., 2007; Nyffeler et al., 2006; Schwarzkopf et al., 1992; for review see Yamada et al., 2000). Behavioral deficits are associated with schizophrenia-relevant neuropathological deficits including abnormalities in dopaminergic and glutamatergic neurotransmission (Winter et al., 2008; for review see Kirkpatrick, 2013), histopathological (Biscaro et al., 2012; Kühn et al., 2012) and structural changes (Piontkewitz et al., 2011a; 2012). The relevance of maternal Poly I:C -induced deficits to schizophrenia is further supported by the responsiveness of adult behavioral deficits to neuroleptic treatment (Piontkewitz et al. 2011b). Finally, prenatal Poly I:C -induced behavioral abnormalities exhibit the maturation delay of schizophrenia (Meyer et al., 2013a, b; Feldon and Weiner, 2009), enabling the elucidation of progressive mechanisms possibly underlying behavioral manifestations as well as preventive interventions. Maternal immune stimulation is thus an excellent model to study pathophysiological and therapeutic aspects relevant to schizophrenia (Meyer et al., 2012; Lipina et al. 2013; for reviews see Meyer, 2013a, b).

Schizophrenia has the most robust clinical evidence for a disease-related reduction in grey and white matter including smaller hippocampal volume assessed in chronic schizophrenic patients (Wexler et al., 2009) and animal models (Meyer et al., 2013a, b; Piontkewitz et al., 2012; Lipska

et al., 2004). Hippocampal involvement is likely to be associated with neuropsychological impairments of schizophrenia (Harrison, 2004) as well as with its psychotic symptoms (Ewing and Winter, 2013; Floresco et al., 2011).

Hippocampal structural pathology in schizophrenia might be due to aberrant neurodevelopment and abnormal neural plasticity. One particular example of cell-based brain plasticity is the generation of new neurons in the hippocampus throughout life (Altman and Das, 1965; Eriksson et al., 1998). Neurogenesis has been linked with hippocampal-dependent function (for reviews see Deng et al., 2010; Bruel-Jungerman et al., 2007). Recently, microglial activity has been shown to be important for the homeostasis of neurogenesis, predominantly through the phagocytosis of apoptotic neuronal progenitor cells (Sierra et al., 2010) and balancing apoptotic and proliferative events via TNF-α signaling (Chen and Palmer, 2013). Baseline microglial activity and cytokine levels in the hippocampus are needed to maintain baseline neurogenesis while an immune response accompanied by an increase in pro-inflammatory cytokines is thought to be detrimental for neurogenesis. Consequently, anti-inflammatory drugs have been shown to ameliorate the decrease of neurogenesis caused by pro-inflammatory cytokines (for review see Kohman and Rhodes, 2013). In schizophrenic patients *Miyaoka* and colleagues demonstrated significant and robust clinical improvements using the tetracycline minocycline - a potent inhibitor of microglial activation (Miyaoka et al., 2008; Seki et al., 2013). Minocycline has been used successfully in some clinical trials since as an adjunctive therapy to antipsychotics for schizophrenia (for review see Dean et al., 2012). How minocycline affects microglia function *in vivo* and neurogenesis is still not fully understood.

We here evaluated the effects of minocycline treatment on neurogenesis in parallel to microglia density, activation and cytokine production in the hippocampus compared to other brain regions

in the Poly I:C rat model of schizophrenia. We correlated these data with the effects of minocycline treatment on sensorimotor gating deficits - a behavioral phenotype relevant to schizophrenia.

Materials and Methods

Animals

All experimental protocols conformed to the guidelines of the European Communities Council Directive (86/609/EEC) for care of laboratory animals and were approved by the local ethic committee (Landesdirektion Dresden). Wistar Rats (Harlan laboratories) were housed in a temperature and humidity controlled vivarium with a 12-hour light-dark cycle (lights on: 6 a.m. to 6 p.m.). They had access to food and water *ad libitum*.

Poly I:C injections

Rats were mated at about an age of three months and the first day after copulation was defined as day one of pregnancy. On gestation day 15, pregnant rats received a single i.v. injection to the tail vein of either Poly I:C (4.0 mg/kg, SIGMA, Germany) dissolved in 200 µl 0.9% NaCl, or vehicle (Klein et al., 2012). On postnatal day (PND) 21, pups were weaned and housed by sex and litter and left undisturbed until behavioral phenotyping at PND 90-98. Prior to the experiments rats were handled for about ten minutes daily for three days. Handling comprised habituation to the investigator and the startle chamber. Each experimental group consisted of male subjects derived from multiple independent litters, with no more than three rats from the same litter. The following experimental groups were included into the study: NaCl H2O – animals derived from mothers injected with 0.9% NaCl during pregnancy and during treatment period supplied with drinking water. NaCl mino – animals derived from mothers injected with 0.9% NaCl during pregnancy and treated with minocycline added to drinking water. Poly I:C H2O –animals derived from mother injected with Poly I:C during pregnancy and during

treatment period supplied with drinking water. Poly I:C mino - animals derived from mothers injected with Poly I:C during pregnancy and treated with minocycline added to drinking water.

Minocycline treatment

Minocycline was added to the drinking water from PND 60 until 128, when animals were sacrificed. With an average of 5ml water intake per 250g rat per day, the animals received an average daily dosage of 3mg/kg over the course of approximately 70 days. The daily dosage was adapted from treatments in schizophrenic patients (Levkovitz et al., 2010). Water bottles were changed every second day to ensure minocycline stability.

BrdU injection

Five animals of each group received i.p. injections of 10mg/ml BrdU at 50mg/kg body weight for three consecutive days starting at PND 98 to label proliferating cells to be subsequently analyzed for levels of neurogenesis at PND 128.

Immunohistochemistry

The standard procedure for evaluation of cell proliferation and neurogenesis including analysis had been described elsewhere (Wolf et al., 2011; Wolf et al., 2010; Kempermann et al., 2008; Wiskott et al., 2006). Five animals from each group were deeply anesthetized with ketamine and perfused transcardially with cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were dissected from the skulls and postfixed overnight. Before sectioning from a dry-ice-cooled copper block on a sliding microtome (Leica, Bensheim, Germany), the hemispheres were

transferred to 30% sucrose in 0.1 M phosphate buffer, pH 7.4, until they had sunk. Brains were cut in the coronal plane in 40µm thick sections. The level of generation of new cells was determined by the *in vivo* injection of BrdU, which incorporates during the S-phase into the cell and thus labelled proliferating cells. For BrdU staining, DNA was denatured in 2N HCl for 30 minutes at 37ºC. Free-floating sections were than rinsed in 0.1 M borate buffer, pH 8.5, and thoroughly washed in tris-buffered saline (TBS), pH 7.4. To block endogenous peroxidase reactions, sections were pre-treated with 0.6% H_2O_2. The anti-BrdU antibody (Sigma) was diluted 1:500 in TBS supplemented with 0.1% TritonX-100, 0.1% Tween 20 and 3% donkey serum (TBS-plus) and the sections were incubated overnight at 4ºC. After rinsing the sections in TBS and a blocking step in TBS-plus, an incubation step with the biotinylated secondary antibody (anti-goat, Abcam) diluted 1:500 in TBS-plus followed. ABC reagent (Vectastain Elite, Vector Laboratories) was applied for one hour at a concentration of 9 µl/ml for each reagent. 3, 3'-diaminobenzidine (DAB, Sigma) was used as a chromogen at the concentration of 0.25 mg/ml in TBS with 0.01% H_2O_2 followed by rinsing with tap water and TBS. We counted the BrdU positive cells in every 6^{th} slice in the gyrus dentatus. We thus analyzed ten slices per brain containing both hemispheres. To estimate the total number of BrdU cells per brain in the gyrus dentatus, we multiplied the counted cells from ten brain sections by six. To phenotype the proliferating cells, we used triple staining for BrdU and a combination of maturation markers as applicable. A total of 50 BrdU-positive cells (five cells distributed across the whole subgranular layer of the gyrus dentatus per each brain section, total of ten sections) per animal were phenotyped. The primary antibodies were applied over night at 4°C in the following concentrations: BrdU (1:500, Sigma), anti-Doublecortin (DCX, 1:200, Santa Cruz Biotechnologies), anti-NeuN (1:100, Chemicon). Secondary antibodies were anti-goat, anti-

rabbit, anti-mouse (1:250, 2h RT, Jackson Laboratories) directly coupled to a fluorochrome for confocal analysis. Pictures were taken throughout the entire thickness of the slice containing the dentate gyrus. We used a two-way ANOVA followed by a Bonferroni post-hoc test using Prism 5 for Windows (Graphpad).

Prepulse inhibition (PPI) test

The PPI paradigm is based on the fact that a weak pre-stimulus presented 30 to 500 milliseconds (ms) before a startling stimulus gates, i.e. reduces the amplitude of the startle response. Acoustic startle reactivity (ASR) and PPI of startle reflex were assessed in a sound-attenuated chamber (41 x 41 x 41 cm) using a movement-sensitive piezoelectric measuring platform connected to a personal computer with an analogue to digital (AD) converter (Startle Response System, TSE, Bad Homburg, Germany) as described previously (Klein et al., 2013; Manahan-Vaughan et al., 2008). During test sessions, animals were placed in a wire mesh cage (22.5cm x 8cm x 8.5cm) mounted on the transducer-platform. For acoustic stimulation, two loudspeakers were mounted on both sides of the test cage at a distance of 4cm. On the days of PPI testing, the animals were transported to the experimental room with the startle-box-room and habituated for 30 minutes. The experiment consisted of a five minutes acclimatization phase and the test session. During the acclimatization phase, animals received background noise (60dB sound pressure level (SPL), white noise) followed by ten initial startle stimuli (100 dB SPL, white noise) lasting each for 20 ms. The test session consisted of seven different trial types delivered in a pseudorandom order: (i) pulse alone (100dB SPL white noise, 20 ms duration); (ii) control (no stimulus); (iii,iv) prepulse alone (72 dB or 68 dB, pure tone, 10 kHz, 20 ms duration); (v-vii) prepulse (72 dB, 68 dB, or 64 dB) each followed by a pulse with an inter-stimulus interval of 100 ms. A total of ten

presentations of each type was given with an inter-trial interval randomized between 20 and 30 s. Background noise intensity during the whole experiment was 60 dB SPL. The entire test session took about 40 minutes. PPI was calculated according to the formula 100 – 100% x (PPx/PA), in which PPx is the mean ASR of the 10 PPI trials (separate for each individual prepulse intensity) and PA is the mean ASR to the pulse alone trials. The average PPI response over the three prepulse intensities was analyzed (Klein et al., 2013) and a two-way ANOVA was performed to test for effect of phenotype (Poly I:C) and treatment (minocycline) across the whole session followed by Bonferroni post-hoc tests using Prism 5 for Windows (Graphpad).

Microglia density

Microglia were labelled using goat-anti-Iba1 antibody (1:400, Abcam) in combination with an anti-goat-biotinylated secondary antibody (1:500, Abcam), followed by a HRP conjugated antibody and DAB (Sigma) treatment. Microglia population densities (DAB and light microscopy) in the various regions were estimated using the StereoInvestigator 9 (Mbf Bioscience, 2010), following the Optical Fractioner probe by a person (LFdC) blind to the sample ID. This protocol constitutes an unbiased and efficient method to count cells by systematic-random sampling of a series of sections (40µm thick), from which further estimations can be derived. For our protocol every 12^{th} section was sampled and always in more than five sections (the recommended minimum number to use). Furthermore, the grid size for each animal was estimated for about 25 counting frames and these superimposed counting frames were defined as 111.64µm^2, consisting of two inclusion dotted lines and two exclusion lines. The counting criterion was the soma of the microglia when it came into focus, which was strictly

followed. Lastly, the estimate of precision – the Coefficient of Error (CE) – was always below 0.05, for the population size estimates derived from the counting protocol. For statistical analysis a two-way ANOVA followed by Bonferroni post-hoc tests was performed.

Microglia fluorescence intensity

Microglia were labelled using goat-anti-Iba1 antibody (1:400, Abcam) followed by the secondary antibody anti-goat Cy5 (1:250 Jackson Laboratories) described elsewhere (Vinnakota et al., 2013). For Iba1 reactivity measures (mean fluorescence intensity per area) DAPI was used as reference. The mean fluorescence intensity derived from the image J software for Iba1 were divided by the mean fluorescence intensity of DAPI from the same scan (image J) to assure technical accuracy. From hippocampus (gyrus dentatus) and cerebellum five slices of 20µm thickness per animal were scanned using a confocal microscope (Leica). Z-stacks were analysed using image J software by a person (SAW) blind to the sample identity. We did the statistical analysis using one-way ANOVA and Bonferroni post-hoc test (Prism 5, Windows).

Isolation of microglia

The remaining five animals from each group were also subjected to perfusion with 0.9% NaCl to remove the blood, followed by microglia isolation using the neuronal dissociation kit (Miltenyi, Bergisch-Gladbach) and pluriBeads™ (pluriSelect, Leipzig). Briefly, the brains were removed, hippocampi and cerebelli extracted and incubated with pre-heated enzyme mix 1 for 15 minutes at 37°C in the water bath by inverting the tube several times every five minutes. The tissue was dissociated mechanically using the Pasteur pipette by pipetting up and down ten times followed by two more incubation/dissociation steps with enzyme mix 2 at 37°C for ten minutes. The

single cell suspension was applied on a 30µm cell strainer placed on 15 ml tube and washed with 10 ml HBSS (w) followed by repeated centrifugation for ten minutes min at 300g and 4°C and washing steps. Cell pellets were resuspended in buffer and conjugated to CD11b rat pluriBeads-S™ (pluriSelect) according to the manufacturer's instructions. The suspension was sieve down a 15µm strainer - the pluriBeads™ with CD11b cells stayed on top, the unwanted cells ran through. After detaching, the CD11b cells were collected from the top of the cell strainer and frozen in lysis buffer at -80° until further use. There is a possible effect of the isolation procedure on microglial activation. Nevertheless this kind of activation is not avoidable and will occur across all sample groups. This is why we choose to show relative differences between the groups in cytokine mRNA levels.

Isolation of neuronal progenitor cells using magnetic activated cell sorting

We used the flow through after the microglia separation step to isolate the neuronal progenitor cells by the MACS technology (Miltenyi Biotec, Bergisch-Gladbach). We incubated the single cell suspension with anti-PSA-NCAM magnetic microBeads™ (Miltenyi Biotec, Bergisch-Gladbach) followed by a magnetic separation of this cell population according to the manufactures protocol. It is noteworthy that in rodent hippocampus PSA-NCAM and Doublecortin expression overlaps 100% (Nacher et al., 2002).

Cell pellets were resuspended in 70 µl MACS buffer consisting of PBS (pH 7.2), 0.5% BSA and 2 mM EDTA, well mixed and incubated for ten minutes at 4°C. 20 µl of anti-PSA-NCAM micro beads were added, well mixed and incubated for 15 minutes at 4°C. Cells were washed with 1-2 ml MACS buffer and centrifuged at 300g and 4°C for ten minutes. Supernatant was aspirated and cells resuspended in 500 µl MACS buffer. The separation column was placed into the

magnetic field of the MACS separator and rinsed with 1ml MACS buffer. The cell suspension was applied onto the column and washed three times with 500 µl MACS, only adding new buffer when the column reservoir was empty. The column was removed from the separator and placed on 5 ml tubes. 1 ml MACS buffer was used to immediately flush out the labeled cell fraction by pushing the plunger into the column. The cell suspension was transferred into a new tube, centrifuge at 300g for five minutes and immediately resuspended in lysis buffer for RNA extraction (Stratec Molecular, Berlin).

RNA preparation, RT and qPCR

Total RNA extraction from rat microglia and neuronal progenitor cells was performed with the InviTrap Spin Universal RNA Mini Kit (Stratec Molecular, Berlin) according to the manufacturer's instructions. RNA concentrations were measured with a Nanodrop 1000 (Thermo Fisher Scientific, Waltham) spectrophotometer and stored at -80 °C until further use. First strand cDNA synthesis was done with the SuperScript II reverse transcriptase (Invitrogen, Carlsbad) using oligo-dT primers$_{12\text{-}18}$ (Invitrogen, Carlsbad) according to the manufacturer's instructions. cDNA samples were stored at -20 °C until further use. Quantitative real-time PCR reactions were done in a 7500 Fast Real-Time thermocycler (Applied Biosystems, Carlsbad) using the SYBR Select Master Mix (Applied Biosystems, Carlsbad) according to the manufacturer's instructions. cDNA input ranged between 1 – 5 ng/µl of total RNA transcribed into cDNA. The primers used were: 5'-GATCGGTCCCAACAAGGAGG-3' (TNFα fwd); 5'-CAGCTGCTCCTCCGCTT-3' (TNFα rev); 5'-CTGTCTGACCCATGTGAGCTG-3' (IL-1β fwd); 5'-AGGGATTTTGTCGTTGCTTGTC-3' (IL-1β rev); 5'-CTCTTGGTGACCGGGAGAAG-3' (TNFR1 fwd); 5'-GGTTCCTTTGTGGCACTTGGT-3'

(TNFR1 rev); 5'-TAGGACTGGCGAACTGCTTC-3' (TNFR2 fwd); 5'-TCCTGGGATTTGTCATCAGGC-3' (TNFR2 re); 5'-CCTGCACCACCAACTGCTTA-3' (GAPDH fwd); 5'-AGTGATGGCATGGACTGTGG-3' (GAPDH rev). Calculation of the relative expression was done using the comparative Ct (2-ΔΔCt) method with GAPDH as reference gene. Statistical significance was tested using one-way ANOVA followed by Bonferroni post-hoc test.

Results

1. The down regulation of neurogenesis in Poly I:C offspring is normalized by minocycline treatment.

To label proliferating cells we injected BrdU i.p. four weeks prior to sacrifice. We counted the BrdU positive cells in the subgranular zone of the dentate gyrus (DG) of the hippocampus and phenotyped them using doublecortin (DXC), a marker of the late mitotic stage and NeuN, a marker of mature neurons (figure 1). In line with reports from us and others (Meyer et al., 2010; Wolf et al., 2011), we could detect a decrease in the total number of BrdU positive cells in the Poly I:C H2O offspring (2213 ± 141) vs. NaCl H2O (3006 ± 94) (figure 1A, two-way ANOVA $F_{3,20} = 20.01$, Bonferroni post-hoc test $p \leq 0.001$). The decrease in BrdU cells was mainly reflected by the drop in net-neurogenesis measured by BrdU/NeuN double positive cells (Poly I:C H2O vs. NaCl H2O: 1412 ± 210 vs. 1936 ± 91, $p \leq 0.001$). The pool of more immature neuronal progenitor cells expressing Doublecortin and BrdU was not changed. Thus the pool of mature NeuN expressing neurons was affected by the Poly I:C phenotype. We could rescue the

decrease in net-neurogenesis by giving minocycline at PND 60 onwards at a dosage of 3mg/kg/day (figure 1). Minocycline treatment significantly increased the proliferating cells labeled by BrdU (Poly I:C mino: 2852 ± 429, $p \leq 0.001$) and the net-neurogenesis measured by the total number of BrdU/NeuN double positive cells (Poly I:C mino: 1833 ± 276, $p \leq 0.01$). Representative micrographs show the co-labeling of BrdU positive cells (figure 1B) with either Doublecortin (1C) or NeuN (1D). Figure 1F and G show a representative micrograph of BrdU labeled cells for light microscopy that was used for counting the amount of BrdU cells per gyrus dentatus.

2. Pre-pulse inhibition deficits are normalized by minocycline treatment.

We next aimed to investigate the effects of minocycline on the pre-pulse inhibition of the startle response. We observed a deficit in the pre-pulse inhibition in the Poly I:C offspring (24.5% ± 8.5) compared to the NaCl offspring (40.9% ± 13.6) (figure 2, ANOVA $F_{3,18} = 4.96$, $p = 0.0067$, Bonferroni post-hoc test $p \leq 0.05$). Minocycline treatment rescued this deficit (figure 2, Poly I:C H2O vs. Poly I:C mino 24.5% ± 8.5 vs. 41.2 %± 11.59, Bonferroni post-hoc test $p \leq 0.05$).

3. Microglia density in most brain regions is not altered in Poly I:C offspring.

As a further step we investigated microglia density in different brain regions (ventral striatum (vSt), medial prefrontal cortex (mPFC), nucleus accumbens core (Nacc), cingulate cortex (Cg), gyrus dentatus (DG) and cerebellum (Ce)) since it has been used as a parameter in human post mortem and some animal studies. We could detect a significant increase in microglia density (cells per μm^2) in the NAcc of Poly I:C H2O (3.03 ± 0.4) compared to NaCl H2O (2.04 ± 0.74)

(figure 3A, two-way ANOVA $F_{4,156}$ = 1.8, Bonferroni post-hoc test $p \leq 0.01$). There was no significant difference measured in any of the other brain regions mentioned above between these two groups. Moreover there was no significant effect of minocycline treatment detectable (representative micrographs figure 3 B-I).

4. Iba1 reactivity is decreased in Poly I:C offspring but remains unaffected by minocycline treatment in hippocampus and cerebellum.

Since microglia density was not significantly changed in the gyrus dentatus, we further analyzed microglia reactivity by measuring the fluorescence intensity of Iba 1 in the gyrus dentatus and cerebellum. In the gyrus dentatus we detected a significant decrease in microglia Iba1 reactivity in the Poly I:C H2O group (8.8 ± 0.86) compared to control NaCl H2O (10.7 ± 0.83) (figure 4A,C,D, ANOVA $F_{3,20}$ = 13.52, p = 0.0001, Bonferroni post-hoc test $p \leq 0.001$). Minocycline had no effect on Iba1 reactivity in the gyrus dentatus (Poly I:C mino 8.3 ± 0.59). A similar pattern was seen in the cerebellum: there was a significant decrease in Iba1 reactivity in the Poly I:C H2O group (1.37 ± 0.06) compared to control NaCl H2O (2.77 ± 0.38) (figure 4 B, ANOVA $F_{3,20}$ = 23.22, $p \leq 0.0001$, Bonferroni post-hoc test $p \leq 0.0001$). Minocycline had no effect on Iba1 reactivity in the cerebellum (Poly I:C mino 0.94 ± 0.26). Thus neither microglia density nor microglia reactivity measured by Iba1 fluorescence intensity correlated with the pattern seen in the behavior test or neurogenesis.

5. The production of TNF-α or IL-1β is enhanced in microglia derived from Poly I:C brains in hippocampus, but not cerebellum and this enhancement is attenuated by minocycline treatment.

We measured the cytokine production of microglia by mRNA levels in the hippocampus as a more specific functional aspect of microglia activation. We compared hippocampus as a brain region relevant for schizophrenia-like behavior and harboring neurogenesis to microglial cytokine production in the cerebellum, a region not linked to sensorimotor gating performance or neurogenesis. There was no significant change in the microglia derived from the cerebellum in TNF-α or IL-1β mRNA (figure 5A).

In the contrary, in the hippocampus we found a significant increase of IL-1β mRNA in the Poly I:C H2O (1.91 ± 0.15) compared to NaCl H2O (0.84 ± 0.15) (figure 5B, ANOVA $p = 0.003$, $F_{3,16} = 8.24$, Bonferroni post-hoc test ≤ 0.05). In addition we detected a significant effect of minocycline on IL-1β mRNA levels in the Poly I:C mino group (0.93 ± 0.12) compare to Poly I:C H2O (1.91 ± 0.15, Bonferroni post-hoc test $p \leq 0.05$) in the hippocampus.

The increase in TNF-α mRNA in Poly I:C H2O in the hippocampus did not reach significance compared to NaCl H2O but was significant higher compared to NaCl mino (figure 5B, Poly I:C H2O vs. NaCl mino, 1.39 ± 0.19 vs. 0.78 ± 0.09, ANOVA $p = 0.009$, $F_{3,17} = 5.9$, Bonferroni post-hoc test $p \leq 0.05$). Similar to IL-1β, we could measure a decrease of TNF-α in the hippocampus after minocycline treatment (Poly I:C H2O vs. Poly I:C mino: 1.39 ± 0.19 vs. 0.71 ± 0.07, Bonferroni post-hoc test $p \leq 0.05$). We did not detected a significant difference between NaCl H2O and NaCl mino.

mRNA levels of both pro-inflammatory cytokines were thus down regulated towards control level specifically in the hippocampus by the treatment with minocycline (P60 onwards,

3mg/kg/day). Therefore the most reliable measure of microglia activation that could be correlated with behavior and neurogenesis in our hands was cytokine mRNA synthesis.

6. The expression level of TNF-α receptor 2 is decreased in the neuronal progenitor cells derived from hippocampus of Poly I:C offspring and rescued by minocycline treatment.

Since it is known that the expression of TNF-α receptor 1 and 2 (TNFR1 and TNFR2) in progenitor cells determines the fate of the cells (apoptosis vs. proliferation), we measured the mRNA level of both receptors in neuronal progenitor cells derived from the hippocampi of all four groups. There was no change in the level of TNFR1 detectable between the groups (figure 6A). TNFR2, however, was significantly decreased in the neuronal progenitor cells derived from Poly I:C H2O (0.49 ± 0.07) vs. NaCl H2O (1.06 ± 0.68) (figure 6 B, ANOVA $p = 0.0094$, $F_{3,18} = 5.5$, Bonferroni post-hoc test $p \leq 0.05$). This decrease of TNFR2 in Poly I:C H2O was rescued by mincocycline treatment in Poly I:C mino (figure 6 B, 1.04 ± 0.17, $p \leq 0.05$) .

Discussion

Hippocampal adult neurogenesis in the dentate gyrus contributes to brain plasticity with a pool of new neurons, which mature and integrate into functional circuits. We and others have previously shown that adult hippocampal neurogenesis is down-regulated in the offspring of *in utero* immune challenged animals (Wolf et al., 2011; Meyer et al., 2010). Studies conducted on schizophrenic patients reveal smaller hippocampal volume, which correlates with the positive symptoms of schizophrenia, and lower neurogenesis in these patients (Kühn et al., 2012; Reif et al., 2006). Decreased adult hippocampal neurogenesis leads to several cognitive deficits also in rodents (Biscaro et al., 2012). Low neurogenesis levels probably contribute to disruptions in sensorimotor gating, since the hippocampus is one of the areas implicated in the circuitry of this behavioral measure (Kühn et al., 2012; Kumari et al., 2007; Swerdlow et al., 1998). In the Poly I:C offspring, we confirm the decrease in net-neurogenesis and the altered behavioral phenotype in the PPI. Moreover we could show that minocycline treatment rescued neurogenesis and attenuated the altered behavioral phenotype when given from PND 60 onwards at a dosage of 3mg/kg/day for approximately ten weeks. The minocycline treatment itself did not affect hippocampal neurogenesis when given to control rats.

It has been shown that microglia have a role in the normal physiological guidance of adult neurogenesis in the hippocampus (Gebara et al., 2013). Moreover, microglial mediated inflammation can be detrimental for hippocampal neurogenesis (reviewed in Kohman and Rhodes, 2013 and Ekdahl et al., 2009). In addition, treatment with anti-inflammatory agents including minocycline in mouse models of sickness behavior and Alzheimer's disease, have restored hippocampal neurogenesis (Biscaro et al., 2012; Monje et al., 2003). In our present study we show similar effects of minocycline on hippocampal neurogenesis in the rat model of

schizophrenia. Taken together these findings strengthen the idea of a negative influence of activated microglia on neurogenesis and the ability of anti-inflammatory drugs to reverse this effect.

Human studies suggested an increase in the density of reactive microglia in the hippocampus of schizophrenia patients (for review see Frick et al., 2013). However, in the young adult offspring of Poly I:C exposed pregnant dams, the density of microglia was not different (Garay et al., 2013). We could confirm this observation in the hippocampus, the medial prefrontal cortex, the ventral striatum, the cingulate cortex and the cerebellum with the exception of the nucleus accumbens. Another study showed increased microglia density in aged Poly I:C offspring (PND 180) in hippocampus, corpus callosum, pons and thalamus (van den Eynde et al., 2014). We propose that the nucleus accumbens might be a region where density differences in microglia become evident in the Poly I:C model around the age of four months while the process may take longer in other brain regions. Variations in age between the animals used in different studies might explain the controversial findings regarding microglia density. It is worthwhile to note, that in our study and in the study conducted by Garay et al. Iba1 was used to label microglia, while van den Eynde used OX42 and ED-1. Moreover, we used the Stereoinvestigator tool to analyze microglial density and these technical differences may account for the different findings. Minocycline had no effect on microglia density in any of the brain regions tested. This suggests that Poly I:C exposure during pregnancy does not necessarily lead to an increased microglial density in early adulthood when behavior symptoms are already evident, while it might become important in later age. We therefore tested functional parameters of these microglial cells and focused on the hippocampus as one of the regions of adult neurogenesis.

We found that IL-1ß and TNF-α are upregulated in the Poly I:C offspring compared to either one or both of the control NaCl offspring groups indicating that microglia are chronically activated in this animal model of schizophrenia. This is in line with other studies that reported an increased level of pro-inflammatory cytokines in the brain of the adult Poly I:C offspring (Garay et al., 2013) and in the brain of fetuses after a maternal immune activation (Arrode-Brusés and Brusés, 2012; Smith et al., 2007; Giovanoli et al., 2013).

Minocycline given from PND 60 onwards attenuated the microglial pro-inflammatory cytokine production in the hippocampus paralleled by the restored deficits in sensorimotor gating and neurogenesis. This suggests that the role of microglia cells is not limited to a transient embryonic activation since features of microglial activation can also be affected in the adulthood by treating with minocycline. This is in line with findings where treatment with valporic acid suppressed glial proliferation in the Disc1-L100P mutant mice – a genetic model of schizophrenia (Lipina et al., 2012). Our data support the mild-encephalitis hypothesis where it is stated that an ongoing inflammatory state is the underlying cause of the disease (Bechter et al., 2013a, b). We could infer that a more activated microglial phenotype reflected by an increase in IL-1β and TNF-α in the Poly I:C exposed offspring might affect neurogenesis. A study by *Wu* and colleagues indicated that IL-1β has a direct and negative effect on adult hippocampal neurogenesis independent of IL-1β receptor signaling (Wu et al., 2013). A study conducted in the lab of *Palmer* (Chen and Palmer, 2013) concludes that TNF-α directly and differentially affects NPC's proliferation through activation of TNF-receptor 1 and 2 (TNFR1 and 2) thereby inducing a homeostatic regulation of adult neurogenesis. At baseline levels it promotes neurogenesis through TNFR2 and it inhibits it through TNFR1. In pathological conditions, when higher levels of TNF-α are produced the TNFR1 signaling may overrule and induce apoptosis of precursor

cells (Chen and Palmer, 2013). We have found that TNRF2 mRNA is down regulated in Poly I:C NPCs. Since signaling through TNFR2 promotes proliferation of NPCs, it is conceivable that neurogenesis is impaired in the animal model of schizophrenia at least partially due to the down regulation of this receptor. It might also be possible, that due to the down regulation of TNFR2, TNFR1 signaling may overrule and induce apoptosis similar to the in vitro situation described by Chen and colleagues.

The minocycline treatment normalized TNFR2 expression levels in the NPCs, rescued net-neurogenesis, attenuated the levels of TNF-α and IL-1β in hippocampus-derived microglia and the deficit in sensory motor gating. Since minocycline had no effect on the TNFR2 expression levels in the NPCs derived from the hippocampi of NaCl offspring per se, we propose that the mode of action of minocycline is promoted through the regulation of microglial derived TNF-α and IL-1β. In human patients minocycline had beneficial effects at a similar dosage as we have used in our study (Levkovitz et al., 2010). In a recent *in vitro* study it was shown that minocycline inhibited the production of TNF-α from IFN-γ-activated microglia (Seki et al. 2013). Our data support the use of minocycline as an add on therapy in schizophrenic patients. Moreover we provide evidence for the mode of action of minocycline on neurobiological factors such as microglia cytokine production and neuronal progenitor cell TNFR expression that contribute to a mild chronic inflammatory state in the brain and lead to lower neuronal plasticity constituting an endophenotypes in an animal model of schizophrenia.

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Figure legends

Figure 1

Neurogenesis is rescued by minocycline treatment in the Poly I:C model.

BrdU-labeled cells were counted in the subgranular zone of the dentate gyrus in the hippocampus to evaluate proliferation. Phenotyping was accomplished via the evaluation of cells double labeled for BrdU and one of the neuronal markers: doublecortin, DCX (late mitotic stage) or NeuN (mature neurons). (A) BrdU positive cells decreased in the Poly I:C H2O offspring; mainly reflected in a the drop of BrdU/NeuN double positive cells. Error bars indicate standard error means (SEM). Significance levels are denoted by ** $p<0.01$, *** $p<0.001$ based on two-way ANOVA followed by Bonferroni post-hoc test. Representative micrographs are shown from the gyrus dentatus for BrdU (B, red), Doublecortin (C, green) and NeuN (D, blue). In (E) the channels are merged and the arrowheads point to BrdU and NeuN positive cells. To count BrdU cells we used light microscopy and in (F) an overview is shown of a representative section containing the gyrus dentatus (DG). The arrowheads in (G) point to single BrdU labeled cells that were counted by hand by a person blind to the sample identity.

Figure 2

Behavioral deficits are rescued by minocycline treatment in the Poly I:C model.

The combination of inhibition of the startle response to 120db pulse for all three pre-pulses (64, 68, 72 db) revealed a deficit in the pre-pulse inhibition in the Poly I:C H2O offspring compared to controls (NaCl H2O). This effect could be rescued by minocycline-administration. Error bars indicate standard error means (SEM) and significance levels are denoted by * $p<0.05$ based on two-way ANOVA followed by Bonferroni post-hoc test.

Figure 3

Microglia density in Poly I:C model is not affected by minocycline.

Microglia density was evaluated in schizophrenia-relevant brain regions (ventral striatum (vSt), cingulate gyrus (Cg), medial prefrontal cortex (mPFC), nucleus accumbens core (NAcc), dentate gyrus of the hippocampus (DG)). We also included the cerebellum (Cereb) as a region unlikely to be affected in schizophrenia. We labeled microglia with Iba1 antibody and visualized the staining using DAB (3, 3'-diaminobenzidine). We evaluated the density using a light microscope (Zeiss) and the StereoInvestigator software. (A) Only in the NAcc of Poly I:C H2O we could detect a significant increase in microglia density compared to controls. No significant effect of minocycline treatment was detectable. Error bars indicate standard error means (SEM). Significance levels are denoted by ** $p<0.01$ based on two-way ANOVA followed by Bonferroni post-hoc test. Representative micrographs show the Iba1 staining in NaCl H2O animals in the nucleus accumbens core (B), the hippocampus (C) and the cerebellum (D). In (F, G and H) the same brain regions are shown from a brain of the Poly I:C H2O group. In (E and I) Iba1 labeled microglia are shown from the cerebellum. *LV* – lateral ventricle; *DG* – gyrus dentatus; *Cb* – 8th cerebellar lobus; *aca* – anterior commissure, ant. The dotted lines depict the areas analyzed by the StereoInvestigator software.

Figure 4

Microglial reactivity (Iba1) in Poly I:C is not affected by minocycline.

Since microglia density did not show a significant change in hippocampus we analyzed the microglial reactivity in this region using fluorescence intensity of Iba1 labeling normalized to DAPI fluorescence intensity. Cerebellum was used as a non neurogenic brain region. In the cerebellum (A) as well in the hippocampus (B) a significant decrease in Iba1 reactivity in Poly I:C H2O (black bar) was measured compared to controls (white bar). Minocycline did not have an effect in either region on Iba1 reactivity (striped bar, A and B). Significance levels are denoted by ** $p<0.01$ and *** $p<0.001$ based on one-way ANOVA followed by Bonferroni post-hoc test. In panel (C) representative micrographs show the Iba1 (red) and the DAPI (blue) staining from one 20µm Z scan of the hippocampus from a NaCl H2O animal. In panel (D) the same is shown in a section from a Poly I:C H2O animal. The dotted line depicts the area that was analyzed for mean fluorescence intensity by the image J software.

Figure 5

Hippocampus specific increase in microglial cytokines is rescued by minocycline.

Calculation of the relative expression of IL-1β and TNF-α was done using the comparative Ct ($2^{-\Delta\Delta Ct}$) method with GAPDH as reference gene. No changes in cytokine levels was seen in the cerebellum (A). A significant increase of IL-1β was measured in the hippocampus in Poly I:C H2O compared to NaCl H2O. This increase was brought back towards baseline expression in the Poly I:C mino group. A similar regulation was seen in the level of TNF-α mRNA, where minocycline had a significant effect in the Poly I:C mino group as well. The increase in TNF-α

in Poly I:C H2O is not significant compared to NaCl H2O but was significant to NaCl mino. There is no significant difference between NaCl H2O and NaCl mino.

Error bars indicate standard error means (SEM). Significance levels are denoted by * $p<0.05$ and ** $p<0.01$ based on one-way ANOVA followed by Bonferroni post-hoc test.

Figure 6

Decreased expression of TNFR2 on hippocampal neuronal progenitor cells is rescued by minocycline.

Calculation of the relative expression of TNFR1 and TNFR2 was done using the comparative Ct ($2^{-\Delta\Delta Ct}$) method with GAPDH as reference gene. No change in TNFR1 mRNA expression level was detected between the groups. TNFR2 mRNA level was significantly decreased in the Poly I:C H2O group compared to NaCl H2O. This decrease was brought back to control level by minocycline treatment (Poly I:C mino). Significance levels are denoted by * $p<0.05$ based on one-way ANOVA followed by Bonferroni post-hoc test.

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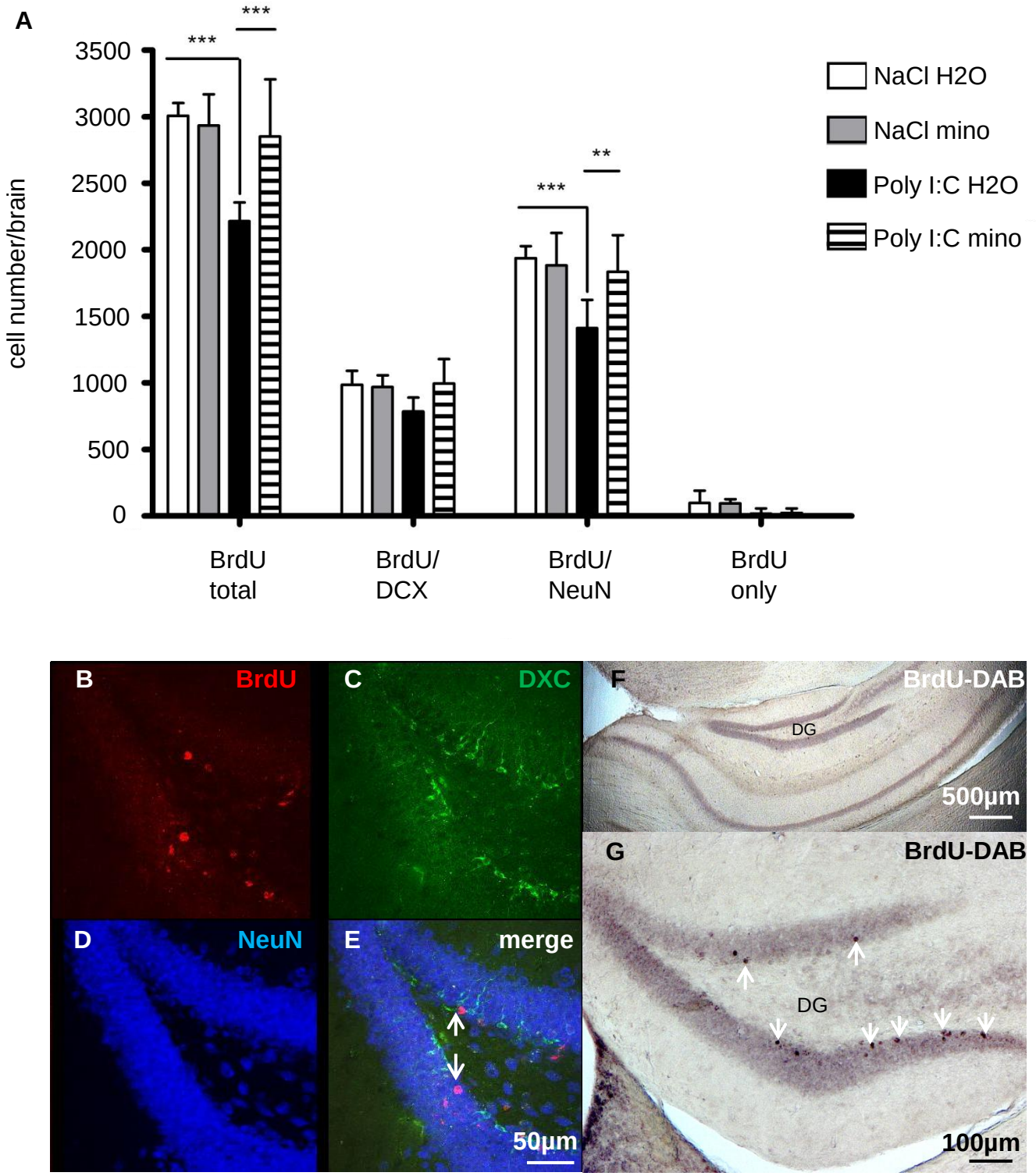
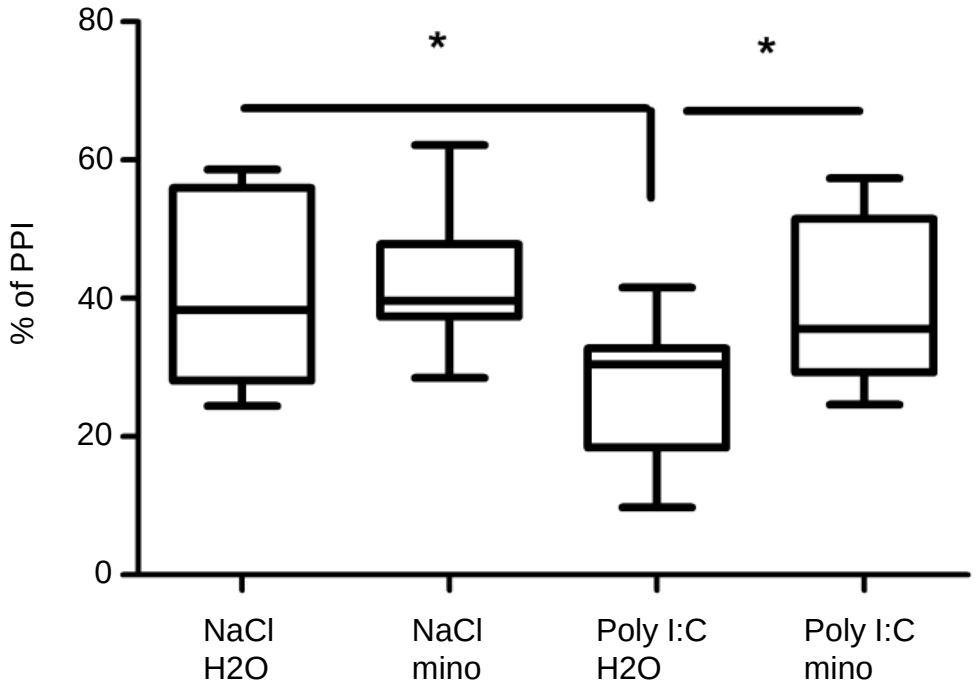
Neurogenesis is rescued by minocycline treatment in the Poly I:C model



Behavioral deficits are rescued by minocycline treatment in the Poly I:C model



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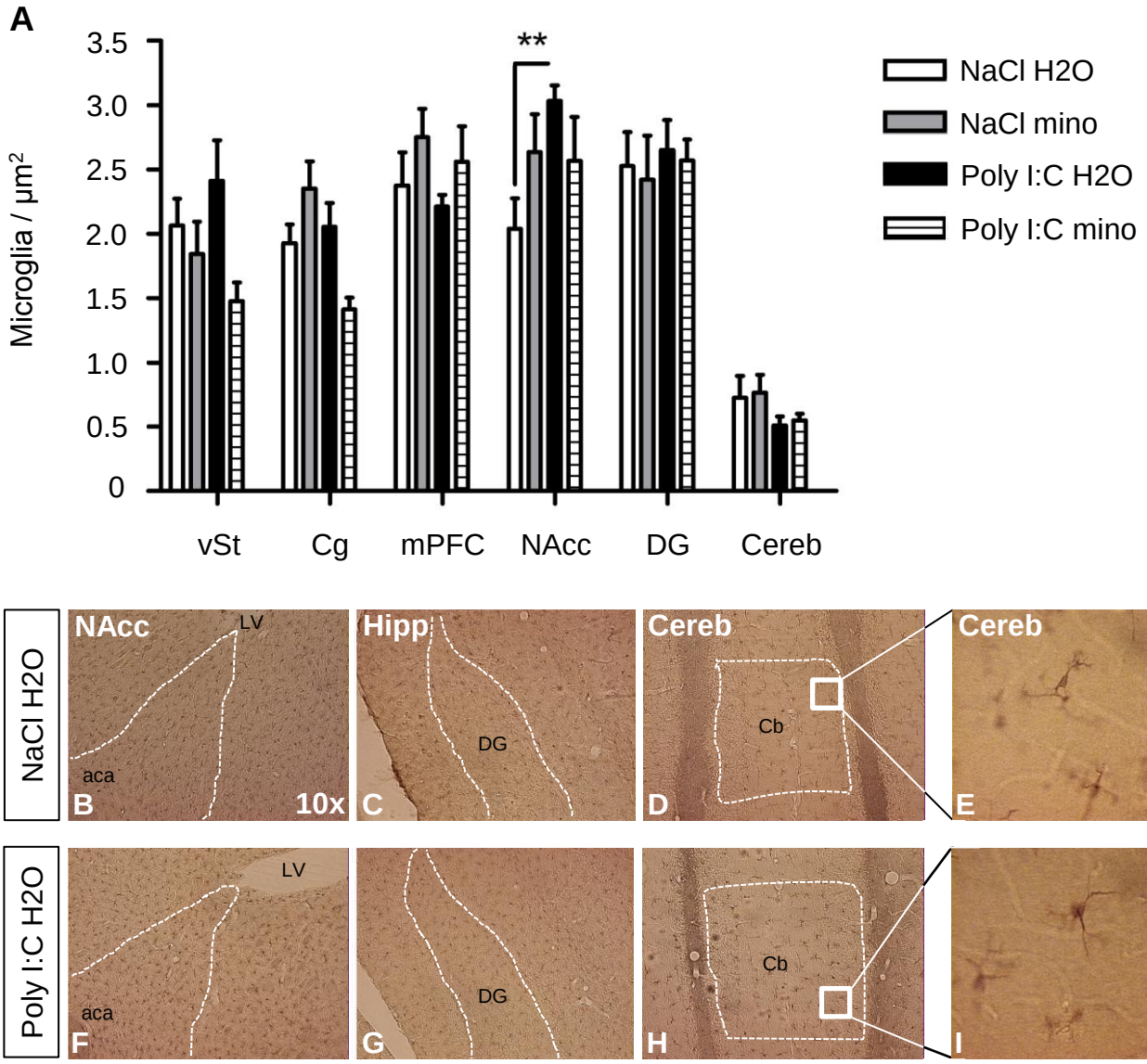

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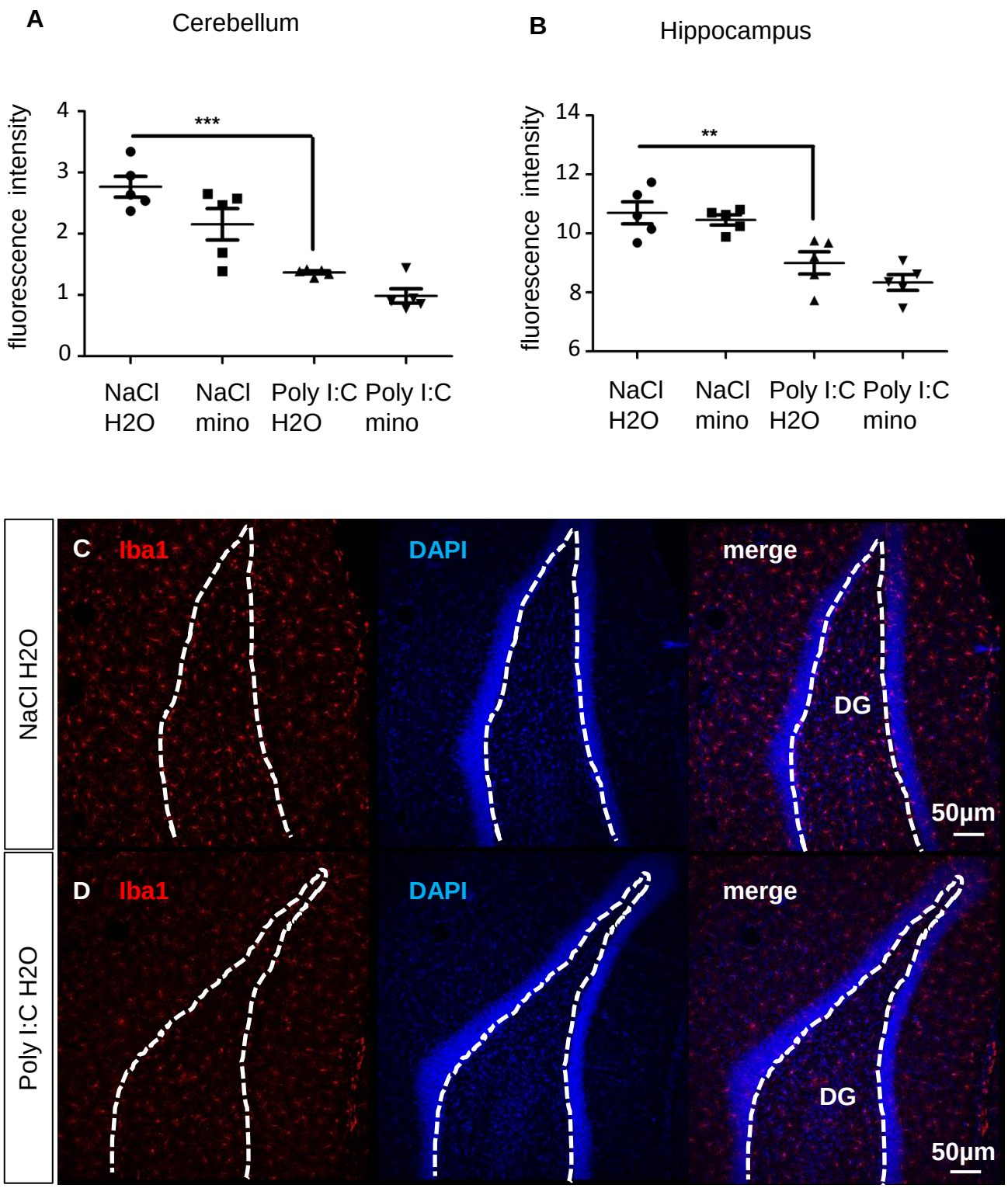
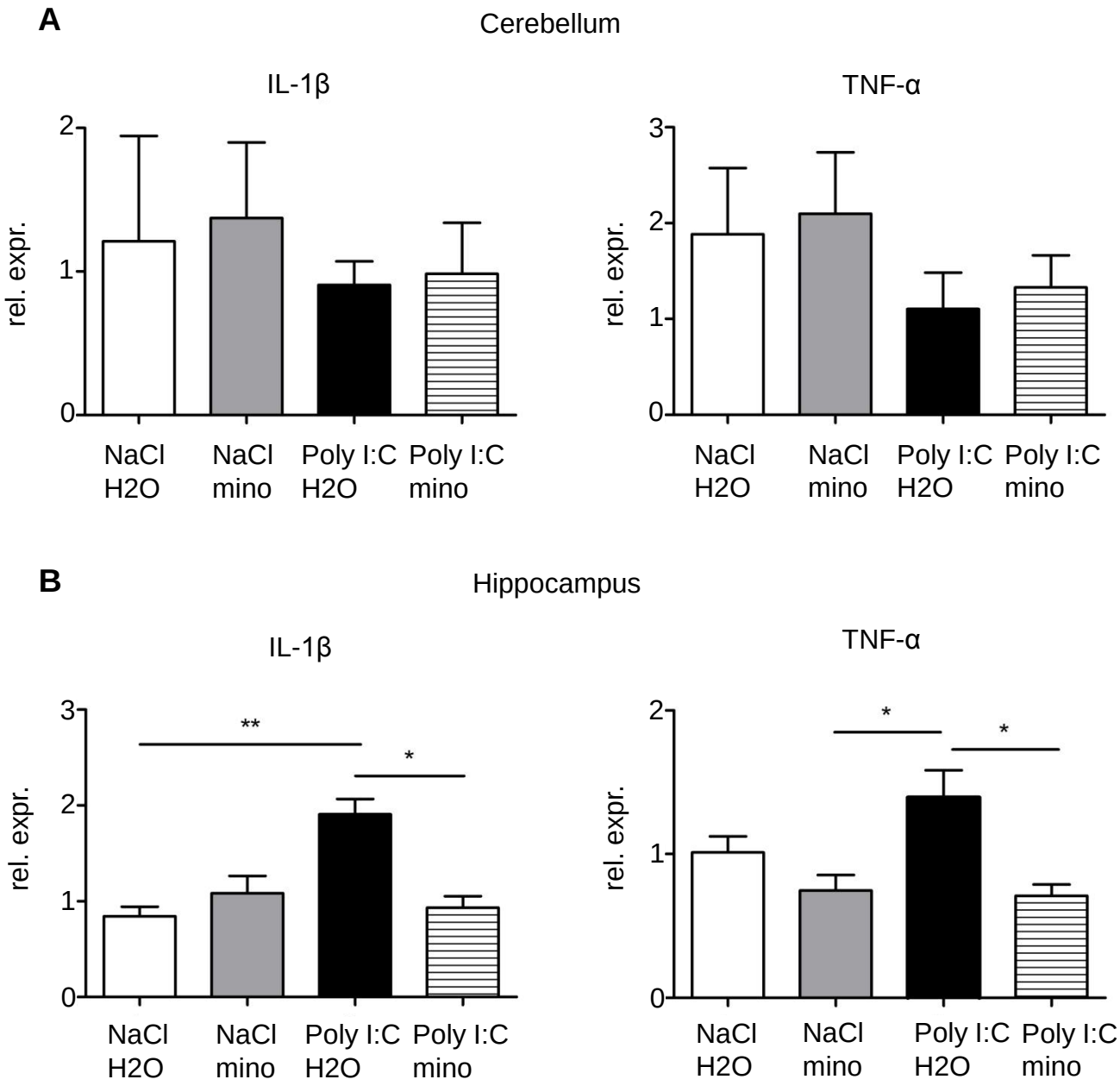


Hippocampus specific increase in microglial cytokines is rescued by minocycline



TNFR2 mRNA level is down regulated in NPCs from Poly I:C hippocampi and normalized by minocycline treatment