MURINE ADULT NEURAL PROGENITOR CELLS ALTER THEIR PROLIFERATIVE BEHAVIOR AND GENE EXPRESSION AFTER THE ACTIVATION OF TOLL-LIKE-RECEPTOR 3

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Viral infections during pregnancy significantly increase the risk for psychological pathologies like schizophrenia in the offspring. One of the main morphological hallmarks of schizophrenia is a reduced size of the hippocampus. Since new neurons are produced in this particular brain compartment throughout life, it might be possible that low neurogenesis levels triggered by a maternal viral infection contribute to developmental deficits of the hippocampus. We injected polyinosinic:polycytidylic acid (Poly I:C) in pregnant C57Bl/6 mice to stimulate an anti-viral response through TLR3 and examined gene expressions in the neuronal progenitor cells (NPCs) of the offspring at different ages. Additionally, we treated adult NPC lines with Poly I:C to investigate its direct effect. We could show for the first time that TLR3 and its downstream effector molecule IRF3 are expressed in adult NPCs. Poly I:C treatment *in vitro* and *in vivo* led to the regulation of proliferation and genes involved in antiviral response, migration, and survival. These findings indicate that NPCs of the fetus are able to react towards an *in utero* immune response, and thus, changes in the neuronal stem cell pool can contribute to the development of neurological diseases like schizophrenia.

Keywords: adult neurogenesis, TLR3, schizophrenia, neuronal progenitor cells, *in utero* viral infection

Introduction

Adult neurogenesis is a process where new neurons are produced in the brain throughout life. In mammals, such as mice, rats, guinea pigs, monkeys, and humans, the generation of new neurons occurs in the subventricular zone and the dentate gyrus, which is part of the hippocampus [1]. The hippocampus is considered to be crucially important in memory formation and consolidation. During hippocampal adult neurogenesis, neural stem cells residing in the subgranular zone produce neural progenitor cells (NPCs). These cells undergo a process of further proliferation, migration, differentiation, maturation, and integration into the neuronal circuits of the granular cell layer of the dentate gyrus [2]. Although poorly understood, adult neurogenesis in the dentate gyrus is thought to be required for cell-based plasticity at the cortico-hippocampal interface. Many extrinsic and intrinsic factors, such as neurotransmitters, growth factors, hormones, stress, physical activity, and aging can potentially regulate adult neurogenesis [3]. Furthermore, the immune system has been shown to modulate adult neurogenesis under normal physiological conditions when stimulated by immune activators [4–6].

Clinical studies have revealed that maternal bacterial or viral infection during pregnancy results in long-lasting

neurodevelopmental dysfunctions of the postnatal brain causing mental disorders like autism and schizophrenia [7, 8]. Epidemiological studies report that maternal viral infections with influenza, rubella, varicella-zoster, or poliovirus, together with genetic predisposition and stress, significantly increase the risk of developing schizophrenia pathology [9, 10]. Moreover, maternal viral infection has been cited as the ‘principal non-genetic cause of autism’ [11–13]. The neurodevelopmental hypothesis of schizophrenia describes that this psychopathological condition results from neurodevelopmental deficits caused by combination of environmental and genetic factors, which precede long before the onset of clinical symptoms [14]. Neuroimaging studies of the hippocampus in patients suffering from schizophrenia show an average hippocampal volume loss of 4–8% [15]. Additional hallmarks of schizophrenia are pathophysiological and neuropathological progress in life and impaired hippocampal structure resulting in abnormalities in associative learning and working memory [7, 16]. The strong involvement of the hippocampus in schizophrenia suggests that adult neurogenesis might contribute to the development of schizophrenic disorders when impaired. A commonly used animal model for the examination of the effects of infection during development is the maternal administration of polyinosinic:polycytidylic acid (Poly I:C). Poly I:C is a synthetic dsRNA analog and is

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a well-established agent for inducing innate antiviral response through binding to TLR3 [17].

In nature, TLR3 recognizes double-stranded RNA (dsRNA), which is an often intermediate during a viral replication cycle [18]. TLR3 mediates signals via two different pathways. The pathway through the C-terminal domain of Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule (TICAM)-1 leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). The activation of NF- κ B leads to its translocation into the nucleus, where it acts like a transcription factor resulting in the expression of pro-inflammatory cytokines, such as IL-1, IL-6, and TNF- α [17, 19–21]. The second pathway occurs through the N-terminal domain of TICAM-1, which leads to the phosphorylation of interferon regulating factor-3 (IRF-3). Phosphorylated IRF-3 homodimerizes and translocates into the nucleus, where it acts as a transcription factor for the expression of interferon- β (IFN- β) [17, 21–23]. IFN- β belongs to type I interferons, which play a major role in antiviral defense. The activation of the common type I IFN receptor (IFN α R) by IFN- β leads to the signal transduction to the nucleus through JAK-STAT pathway, which results in the expression of IFN-stimulated genes (ISGs) [24, 25]. ISGs stimulate several hundred genes some of which code for antiviral proteins.

The offspring of Poly I:C-treated mothers displays abnormal behavioral responses to prepulse inhibition and acoustic startle response, which are comparable to the human pathophysiology of schizophrenia [26, 27]. The behavioral abnormalities of the offspring result most likely from structural malformations of the neocortex and hippocampus, pyramidal cell atrophy and reduced levels of reelin immunoreactivity [28–30]. Abnormal cytokine levels of TNF- α , IL-2, IL-6, IL-8, and IL-10 following viral or Poly I:C exposure have been suggested to contribute to the symptomatology in schizophrenia [27, 29, 31, 32].

Although it is hypothesized that maternal and fetal chemokines might induce hippocampal malformation and facilitate the development of schizophrenia, little is known about the exact causes, mechanisms, and relation between a viral infection and the disease. Even less is known about the role of adult neurogenesis in schizophrenia. However, it might be possible that infection-induced defects in NPCs lead to impairments in either quality or quantity or both in the population of resulting hippocampal neurons. Although not the cause of schizophrenia, the lack of well-functioning adult neurogenesis might contribute to the disease development and symptomatology.

Therefore, we investigated the fundamental question, if murine adult NPCs from the dentate gyrus (mDG NPCs) express functional TLR3 and are able to react directly to a viral infection mimicked by Poly I:C. We examined the proliferative response and the gene expression profiles of TLR3 and IRF3 after exposure to Poly I:C. The gene expression of IFN α R served as an indicator for the regulation of the pathway that leads to the expression of interferon stimulated genes. Finally, we looked at the gene expression profiles of CXCR4 and its ligand CXCL12, which

helped to estimate possible impact on migratory behavior and survival of NPCs.

Materials and methods

Immunocytochemistry

Murine DG NPCs were fixed with 4% paraformaldehyde (PFA, Sigma) and washed twice with cold phosphate buffer saline (PBS, Biochrom AG). The samples were permeabilized with 0.25% Triton-X (Fluca) in PBS for 10 min and washed 3 $\times$ for 5 min in PBS. Next, the cells were treated with 4% fetal calf serum (FCS, Biochrom) in PBS containing 0.1% Triton-X for 30 min to block unspecific binding of the antibodies. Afterwards cells were washed 3 $\times$ for 5 min in PBS and incubated with primary antibodies against mouse nestin (1:250, Beckton–Dickinson), goat DCX (1:100, Santa Cruz) and rabbit TLR3 (1:200, Abcam) in 4% FCS in PBS at room temperature for 1 h. The cells were washed 3 $\times$ for 5 min in PBS and incubated with secondary antibodies against mouse FITC (1:250, Jackson Laboratories), anti-goat Texas Red (1:250, Jackson Laboratories), and anti-rabbit Cy5 (1:250, Jackson Laboratories) in 4% FCS in PBS with 0.1% Triton-X 1 h at room temperature. Afterwards, the cells were treated with 100 ng/ml DAPI (Sigma–Aldrich) for 3 min, mounted with mounting medium (Dako), and examined under a fluorescence microscope (Leica) with the appropriate filters for DAPI, FITC, Texas Red, and Cy5. The brains of nestin-GFP C57Bl/6 mice were fixed in 4% PFA (Sigma) overnight and afterwards transferred to 30% sucrose. The samples were cut into 40- μ m coronal slices. On day one of the procedure, the samples were washed 3 $\times$ in Tris-buffer saline (TBS, Sigma) and permeabilized with TBS-plus containing 1% Triton-X (Fluca) and 3% FCS (Biochrom) for 30 min. Next, the samples were treated with primary antibodies against goat DCX (1:100, Santa Cruz) and rabbit TLR3 (1:100, Abcam) at 4 $^{\circ}$ over night on a shaking table. On the second day, the brain slices were washed 2 $\times$ in TBS and 1 $\times$ in TBS-plus for 15 min each. In the next step, the samples were treated with the secondary antibodies anti-goat Texas Red (1:250, Jackson Laboratories) and anti-rabbit Cy5 (1:250, Jackson Laboratories) for 2 h shaking and in the dark. After washing the samples 4 $\times$ in TBS for 2 min each, they were placed on microscope slides (Thermo Scientific), followed by 30 min bath in Neoclear (Merck). In the last steps, the samples were mounted with mounting medium (Dako) and covered with cover-clips (Menzel–Gläser). The samples were examined under a confocal laser microscope (Leica) at the wavelength of 488 nm (GFP), 594 nm (Texas Red), and 633 nm (Cy5).

Cell culture

Mouse dentate gyrus neural progenitor cells (mDG NPCs) were cultured in 10-cm cell culture dishes (Falcon) at 37 $^{\circ}$ C

and 5% CO₂ using D-MEM F12 GlutaMAX™-I (Invitrogen) medium containing B27 supplement (1×, Invitrogen), epidermal growth factor (20 ng/ml, Peprotech), fibroblast growth factor (20 ng/ml, Peprotech), heparin (5 µg/ml, Sigma), and penicillin/streptomycin (100 µg/ml, Gibco). After mDG NPCs reached 70–80% confluence they were split and cultured in 6-cm cell culture dishes (TPP).

Proliferation assay

Murine DG NPCs were cultured for 24 and 48 h according without Poly I:C in case of the control and 100 and 200 µg/ml Poly I:C (Sigma) in case of the treatment. All samples received additionally 10 mM 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen) in parallel to the treatment for the detection of newly synthesized DNA. After the indicated time, the cells were harvested and EdU was detected with Click-iT Alexa Fluor 488 Flow Cytometry Assay Kit (Invitrogen) according to manufacturer's protocol. For the detection of EdU with Alexa Fluor 488 azide, FACS Canto II (BD Bioscience) with 488-nm excitation with green emission filter (530/30 nm or similar) was used. The fluorescent signal is best detected with logarithmic amplification.

Real time PCR

Murine DG NPCs were harvested after 24 and 48 h for total RNA isolation with the NucleoSpin RNA II Kit (Macherey–

Table 1. List of the genes examined by qPCR

Gapdh	Mm99999915_g1	Applied Biosystems
TLR3	Mm01207403_m1	Applied Biosystems
IRF3	Mm01203177_m1	Applied Biosystems
IFNαR	Mm00439544_m1	Applied Biosystems
CXCR4	Mm01292123_m1	Applied Biosystems
CXCL12	Mm00457276_m1	Applied Biosystems

Nagel) according to the manufacturer's protocol. Real-time PCR was performed after the reverse transcription of total RNA into cDNA with the High-Capacity cDNA RT Kit (Applied Biosystems) according to the manufacturer's protocol. The mRNA of the genes of interest was detected by specific primers designed by Applied Biosystems (Table 1). The housekeeping gene GAPDH was used as the internal reference. The mRNA detection was performed with Taqman Gene Expression assay (Applied Biosystems) according protocol on the 7500 Fast Real-Time PCR system (Applied Biosystems), and the results were examined with SDS software (Applied Biosystems) and Excel (Microsoft) using the $2^{-\Delta\Delta C_t}$ method. The gene expression of the treated cells was compared to the control, whereas the fold difference bigger than 0.5 was treated as a regulatory event.

In vivo, the hippocampi of three pups from control and three pups from Poly I:C treated mothers were collected at postnatal days (P)1, P3, P5, P10 and P15. After the dissociation of brain tissue with Neural Tissue Dissociation Kit (Miltenyi Biotec), mDG NPCs were isolated with anti-

Fig. 1. Cultured mDG NPCs were treated with antibodies against progenitor cell markers nestin and DCX as well as TLR3 and examined for the appearance and location of TLR3. (a) Negative control: HEK cells do not express TLR3. (b) Cultured nestin-positive mDG NPCs express TLR3 (fluorescence microscopy) (c) on the cell surface (arrows) and intracellular compartments (cross) (laser confocal microscopy). Scale bar: 10 µm

PSA-NCAM microbeads (Miltenyi Biotec) by MACS. The mRNA of the isolated NPCs was examined corresponding to the *in vitro* protocol.

Poly I:C treatment

Twenty-four hours after the splitting, cells were treated with 100 $\mu\text{g/ml}$ or 200 $\mu\text{g/ml}$ Poly I:C (Sigma) concentration. The controls were cultured according to the protocol without additional reagents. NPCs were harvested after 24 h and 48 h for further procedure.

In vivo, pregnant C57Bl/6 wild type or nestin-GFP transgene mice (background C57Bl/6) received an interperitoneal injection of 100 μl NaCl containing 16 mg/kg Poly I:C (Sigma) at embryonic day 15 (E15).

Animals

C57Bl/6 and C57Bl/6 Nestin-GFP (tg) mice (kind gift of Rainer Glass, University of Munich) were kept under Optimal Hygiene Conditions in individually ventilated cages at the animal facility of the Institute of Anatomy (University of Zurich) according to Swiss Federal Veterinary Office regulations. The animals had unlimited access to water and food and lived in 12-h light/dark cycle.

Statistics

We have used Student *t*-test run with EXCEL on a Macintosh System, and results were treated as significant with a *p*-value equal or below 0.05.

Results

Adult mDG NPCs express TLR3 in cell culture

First of all, we wanted to know, whether mDG NPCs express TLR3, as this information was not present in the literature. To identify and locate the receptor, we used antibodies against the well-established NPC markers nestin and doublecortin (DCX) as well as an antibody for murine TLR3 in an adult NPC culture and examined the cells under a fluorescent and confocal laser microscope. We compared the results to the TLR3 negative human embryonic kidney (HEK) cells (Fig. 1a). Our findings clearly demonstrate that adult murine NPCs express TLR3 on their cell surface as well as in intracellular compartments (Fig. 1b and c).

Murine DG NPCs alter their gene expression after treatment with a high dose of Poly I:C

Following the observation that mDG NPCs express TLR3, we went deeper and asked the question whether these cells alternate the gene expression of TLR3 and its downstream effector molecule IRF3. Furthermore, we wanted to see if mDG NPCs regulate the expression of IFN α R as a receptor for the product of the TLR3–IRF3 pathway as well as CXCR4 and its corresponding ligand CXCL12 as indicators for the migratory potential. Therefore, we treated mDG NPCs with 100 and 200 $\mu\text{g/ml}$ Poly IC for 24 and 48 h, and analyzed the corresponding mRNAs by real-time PCR (qPCR). We were able to detect the mRNA of TLR3 and IRF3, which supported our findings from microscopy analysis that mDG NPCs express the receptor and its downstream target. Additionally, we confirmed

Fig. 2. The gene expression of IRF3, CXCR4, and IFN α R alters 48 h after Poly I:C treatment, whereas the expression of TLR3 does not change at either time point. Murine DG NPCs were incubated with 100 mg/ml and 200 mg/ml Poly I:C for 24 h and 48 h. The bars represent gene expression of indicated genes in the NPCs compared to the controls and are expressed as fold difference from the baseline (indicated as dashed line). (a) No changes of TLR3, CXCR4 and IFN α /R can be observed after 24 h of exposure to Poly I:C. B. After 48 h IRF3, CXCR4, and IFN α show a tendency to be upregulated at 100 $\mu\text{g/ml}$ Poly I:C. At 200 $\mu\text{g/ml}$ Poly I:C TLR3 and CXCR4 tend to be downregulated. Bars represent the fold difference in gene expression of treated groups in comparison to the controls. The y-axis shows the fold difference. The dashed line at $y=1$ represents the normalized expression of the control. The x-axis indicates the Poly I:C concentration. $N=3/\text{group}$. Repeated three times with 100 $\mu\text{g/ml}$ Poly I:C; performed once with 200 $\mu\text{g/ml}$ Poly I:C

the findings from other studies that mDG NPCs express IFN α R and CXCR4, but not CXCL12 [33]. However, after 24 h, no regulation of the examined genes could be observed at either concentration (Fig. 2a). Nevertheless, after 48 h, the mRNA of IRF3, CXCR4 and IFN α R showed a tendency to be upregulated at 100 μ g/ml Poly I:C. At the concentration of 200 μ g/ml, TLR3 and CXCR4 showed a tendency to be downregulated (Fig. 2b). Thus, cultured NPCs express the downstream machinery of TLR3, except CXCL12, and are able to regulate them after TLR3 stimulation.

TLR3 stimulation with Poly I:C in vitro leads to increased proliferation at high Poly I:C dose

In the next step, we wanted to know how the activation of TLR3 affects proliferation as one of the most fundamental processes of NPCs. Therefore, we treated cultured mDG NPCs with Poly I:C at the concentrations of 100 and 200 μ g/ml, and measured the proliferation by flow cytometry 24 and 48 h after treatment. No effects were observed at 100 μ g/ml Poly I:C after 24 or after 48 h. However, already after 24 h, a significant increase in proliferation by 5.9% could be observed at the concentration of 200 μ g/ml Poly I:C compared to the control. The pro-proliferative impact of TLR3 stimulation was even more dramatic after 48 h, where the increase in proliferation resulted in 15.1% at the same concentration (Fig. 3). According to the gene expression results, mDG NPCs are susceptible to high dose of Poly I:C and increase their proliferation as a consequence of TLR3 stimulation.

Adult mice express TLR3 on mDG NPCs

After the described observations *in vitro*, we wanted to know how mDG NPCs behave *in vivo* regarding the TLR3-expression, proliferation, and gene expression of indicated genes. First of all, we addressed the question, whether we could detect TLR3 on mDG NPCs of adult C57Bl/6 nestin-GFP transgene animals. Therefore, we prepared slices from the brains of adult animals and treated them with antibodies against DCX and TLR3. We examined the presence of TLR3 on DCX- and nestin-positive cells of the dentate gyrus under the confocal laser microscope. Our results indicate that TLR is present on DCX as well as nestin-positive cells of adult mice (Fig. 4a and b).

TLR3 stimulation with Poly I:C leads to decreased proliferation of mDG NPCs in vivo

Next, we wanted to examine the question how mDG NPCs proliferate in adult animals after their mothers mounted an antiviral immune response during pregnancy to estimate the correlation between the model of

viral infection and maldevelopment of the microscopic organization of the dentate gyrus. To stimulate antiviral immune response through TLR3, we injected Poly I:C in pregnant mice at embryonic day 15 and examined the hippocampi of the adult offspring at postnatal day 60. Therefore, we produced histological slices and treated them with antibodies against Ki67, which is a well-known proliferation marker, and DCX. Afterwards we counted Ki67-positive and Ki67/DCX-double positive cells always in the same part of the dentate gyrus and compared the numbers to the control (Fig. 4c). The number of Ki67-positive cells was significantly reduced by 51.72% whereas the number of Ki67/DCX-double positive cells, which indicated the proliferating NPCs, was significantly decreased by 81.85% (Fig. 4d). Therefore, the treatment with Poly I:C in pregnant mice leads to an enormously significant decrease in proliferation of mDG NPCs in the adult offspring.

The genes of interest are downregulated in mDG NPCs of pups of different age

Referring to the determination of the gene expression *in vitro*, we wanted to know the expression pattern of mDG NPCs for TLR3, IRF3, IFN α R, CXCR4 and CXCL12 in pups of different age derived from Poly I:C-treated mothers. We treated pregnant C57Bl/6 mice with Poly I:C at embryonic day 15. After birth, we collected the hippocampi of pups at postnatal days (P)1, 3, 5, 10, and 70, and isolated mDG NPCs by magnetic associated cell sorting with iron-labeled polysialated neural cell adhesion molecule (PSA-NCAM) antibody, whose expression fully overlaps with the expression of DCX. Afterwards, we extracted the RNA for qPCR analysis and normalized the ratios of mRNA ex-

Fig. 3. The treatment of mDG NPCs with 200 μ g/ml Poly I:C leads to increased proliferation. Cells were treated with 100 μ g/ml and 200 μ g/ml Poly I:C for 24 h and 48 h for proliferation analysis by flow cytometry. Whereas the treatment with 100 μ g/ml Poly I:C does not show significant alteration in proliferation at indicated time points, the exposure of NPCs to 200 μ g/ml Poly I:C increases the proliferation after 24 h as well as 48 h. The bars represent the percentage of divided cells. The y-axis shows the percentage of total cells. The x-axis indicates the time of the treatments. Poly I:C concentrations are indicated in the legend and plotted on the x-axis. Ctr: control; N=3; significance level: *, $p < 0.05$

Fig. 4. Adult mDG NPCs positive for DCX and nestin-expressed TLR3 *in vivo*. Brains from adult nestin-GFP transgene mice were fixed, cut, and treated with antibodies against DCX and TLR3. The slices were placed on microscope slides and examined for TLR3 appearance and location on adult NPCs in the dentate gyrus with a confocal laser microscope. Pictures represent optical sections from a region of the dentate gyrus. (a) The arrows point to TLR3 positive signals on the cell surface and intracellular vesicles of DCX positive cells and on (b) TLR3 in intracellular vesicles of nestin as well as DCX positive cells. Scale bar: 5 μ m. The proliferation of DCX positive mDG NPCs is decreased in pups from mothers treated with Poly I:C during pregnancy. Pregnant C57Bl/6 nestin-GFP transgene mice were injected i.p. with Poly I:C at E15. The brains of the adult offspring (P60) were collected, cut, and treated with antibodies against the proliferation marker Ki67 and DCX. The hippocampi were examined for Ki67 positive as well as Ki67/DCX double-positive cells in the dentate gyrus with confocal laser microscope. The cells were counted, and the total number was calculated stereological. (c) The cells were counted always in the same region of the dentate gyrus represented by an exemplary micrograph. (d) The number of all proliferating cells is significantly decreased by 51.72%. The number of proliferation mDG NPCs is decreased by 81.85%. The y-axis represents the total number of counted cells. The x-axis shows the used markers. Bars represent the numbers of cells and the difference between the treatment and the control. Scale bar: 100 μ m. Control: $N=4$; treatment: $N=5$

pression to the control (a fold change of at least 0.5 was seen as a regulatory event). We could observe the down-regulation of TLR3 at P3 and P70 ($R_{\text{ratio}}=0.53$ and 0.49) (Fig. 5a). Also, IRF3 showed the tendency to be down-regulated at P3 ($R=0.58$) (Fig. 5b). IFN α/β R showed no significant alteration in gene expression, but was slightly downregulated at P3 ($R=0.57$) (Fig. 5c). CXCR4 was

slightly downregulated at P3 ($R=0.60$) and P5 ($R=0.75$) (Fig. 5d). CXCL12 gene expression showed a tendency to be decreased at P3 ($R=0.44$) (Fig. 5e). The data show low regulation of the examined genes, but nevertheless, the altered expression changes to the negative direction most strikingly at P3. According to the *in vitro* gene expression assays, the PSA-NCAM positive NPCs express the genes

of interest which might be regulated postnatal after the administration of the mothers to Poly I:C.

Discussion

In this study, *in vitro* and *in vivo* approaches were used to examine the presence of TLR3 and possible effects of TLR3-signaling on adult mDG NPCs. Our findings demonstrate that adult NPCs express TLR3 intracellular and on the cell surface in cell culture as well as in animals. Recently, Okun et al. showed that TLR3 is expressed in the DG and in other regions of the hippocampus. In addition, the authors claimed that nestin-positive NPCs do not express TLR3 [34]. However, in their study, except nestin-positive cells, the authors did not phenotype other cell types in the dentate gyrus. In contrast, in our study we could distinguish between DCX-, nestin-, and DCX/nestin-positive cells with corresponding markers, and observed the expression of the receptor at all these cell types. In addition, we were able to measure the mRNA of TLR3 in cultured and isolated NPCs. We could show the expression of IRF3 as the downstream effector molecule of TLR3, which suggests a functional pathway.

Our results demonstrate a pro-proliferative effect of TLR3 signaling on cultured NPCs. In one recent study on the influence of TLR3 signaling on NPC proliferation, the opposite effect has been shown. The authors observed negative regulation of NPC proliferation as a consequence of TLR3 signaling activated by Poly I:C [35]. Neverthe-

less, these findings do not contradict to our *in vitro* data, because the experimental approach differs in the choice of the model. Lathia et al. examined free floating NPC neurospheres derived from embryonic mouse brain, whereas we used an adherent NPC cell line originally derived from adult murine hippocampus. Differences in the type of progenitor cells, embryonic vs. adult, as well as the heterogeneity of cell types in a neurosphere, could explain the discrepancy between the data. However, our examination of the proliferation in adult mice derived from Poly I:C treated mothers revealed a decrease in proliferation. This defect could be the consequence of the mild inflammation causing by Poly I:C with resulting cytokine secretion or the signaling of TLR3 through the NF- κ B pathway. There is a large body of evidence that NF- κ B regulates the cell cycle through the activation of the expression of cyclin D1 and D2 [36, 37]. Cyclin D1 and D2 form a complex with cyclin dependent kinases 2, 4, or 6 and promote the G₁-to-S progression through phosphorylation and inactivation of retinoblastoma protein (pRb) members [37, 38]. Most likely, both factors contribute to the embryonic progenitor cell precursor deficits and the exact mechanisms should be examined in the future. However, our study provides the first evidence of a link between maternal and fetal immune response, and the depletion of NPC population in adult offspring. Our results are in accordance with the observation of Okun et al., where larger volumes of the DG were measured in mice lacking TLR3. The authors suggest that TLR3 contributes to the modulation of hippocampal synaptic plasticity and neurogenesis [34]. Together, our re-

Fig. 5. The application of Poly I:C into pregnant mice has little impact on the gene expression of examined genes. However, a sign for the downregulation of the genes of interest especially at P3 and P70 can be observed. Murine DG NPCs positive for PSA-NCAM from pups of the age P1, P3, P5, P10, and P70 derived from Poly I:C treated mothers were isolated and examined for the genes of interest by qPCR. (a) TLR3 is downregulated at P3 and P70. (b) IRF3 is downregulated at P3 as well as IFN α R (c) CXCR4 (d) and CXCL12 (e). The y-axis represents the fold difference whereas the x-axis shows the age of the pups. The bars represent gene expression of indicated genes in the hippocampi compared to the controls and expressed as fold difference from the baseline (indicated as dashed line). *N*=3.

sults indicate an important role of TLR3 in the regulation of adult hippocampal neurogenesis in health and disease.

Short exposure of NPCs to Poly I:C clearly did not influence the examined gene expressions. However, a prolonged exposure of NPCs to Poly I:C concentration of 100 $\mu\text{g/ml}$ led to the upregulation of IRF3 and CXCR4. In contrast, at the Poly I:C concentration of 200 $\mu\text{g/ml}$, the gene expression of TLR3 were downregulated, suggesting an adaptation to the high agonist dosage probably to prevent cytotoxic effects on NPCs. Isolated PSA-NCAM positive cells of the pups from treated mothers expressed TLR3 and IRF3 mRNA at all examined postnatal points of time. Remarkably, TLR3 as well as IRF3 were downregulated at P3 and moreover in adult mice (P70), suggesting a long-lasting effect of Poly I:C on TLR3 and its downstream signaling molecules in adult NPCs.

In addition, IFN α R mRNA was present in mDG NPCs and elevated after Poly I:C treatment, suggesting the induction of higher sensitization of NPCs to IFN- β . However, IFN α / β R mRNA was downregulated at P3 in pups. The effect of IFN- β on adult NPCs was examined in two particular studies which described the presence of IFN α R on NPCs [39, 40]. Further, Hirsch et al. observed a direct binding of IFN- β to the IFN α R, suggesting a possible function of IFN- β as a neurotropic factor. They concluded a neuroprotective role of IFN- β from the observation that IFN- β treatment of NPCs promoted their survival by decreasing apoptosis. They did not detect effects of IFN- β on differentiation or proliferation [40]. In contrast, Wellen et al. reported an inhibition of NPC proliferation by IFN- β . Moreover, they observed IFN- β -induced inhibition of neurite outgrowth and maturation of NPC-derived neurons. Nevertheless, they confirmed the findings of Hirsch et al. that IFN- β treatment does not change differentiation and does not induce apoptosis, but leads to the upregulation of the antiviral proteins Mx1 and viperin in NPCs [39]. The contradicting results may derive from the different NPC sources both studies used, which indeed are only indirectly linked to the NPCs derived from the DG. Whereas Hirsch et al. used NPCs isolated from the whole mouse brain of four-day-old pups, Wellen et al. worked with isolated NPCs from the lateral and medial ganglionic eminence of 14-day-old mouse embryos and transferred their conclusions to adult NPCs [39, 40]. Nevertheless, the proof of principle that NPCs sense and react to IFN- β with alteration in their behavior was demonstrated in these studies, indicating the importance of the IFN- β -IFN α R-ISGs signaling pathways for the regulation of NPC proliferation. Therefore, the regulation of IFN α R in NPCs after TLR3 stimulation might serve as a preparation of the cells to the level of IFN- β produced, for example, by microglia and astrocytes during an infection of the CNS [41]. Whether NPCs are able to produce IFN- β by themselves has not been investigated yet, although the expression of IRF3 as the TLR3 effector molecule provides strong evidence for IFN- β production by NPCs.

The activation of TLR3 also led to the upregulation of CXCR4 in NPCs *in vitro* after prolonged exposure but

to downregulation at high Poly I:C concentration *in vitro* as well as in isolated mDG NPCs from P3 pups. As the only known ligand of CXCR4, CXCL12 was not expressed by cultured NPCs. However, isolated NPCs did express CXCL12 at all examined ages. This discrepancy could be explained by the different stages of these two cell types. Cultured NPCs belong to an early population of progenitor cells positive for nestin and DCX, which does not express CXCL12. In contrast, in several previous studies, it has been shown that CXCR4 and CXCL12 are both expressed by PSA-NCAM positive cells and contribute to their migration and chemoattraction of other new born neurons [42, 43]. During the development of the nervous system, CXCR4 and CXCL12 are important for the morphogenesis of the hippocampus and, in particular, the DG. Thereby, CXCL12 acts as a chemotactic signal for cells expressing CXCR4. The lack of CXCR4 results in abnormal development of the DG most likely due to abnormal migration of young granule neurons, but it is also due to reduced number of dividing cells, which in addition differentiate prematurely, suggesting an important role of CXCR4/CXCL12 in mitosis and maturation [44, 45]. In the adult DG, CXCR4 is expressed by NPCs, whereas CXCL12 is expressed by young and mature granule cells in the target regions of the new generated neurons, i.e. the granular cell layer [44]. Further, CXCL12 is considered to be important for the survival of NPCs by acting as neurotropic factor. Finally, the development of new generated neurons crucially depends on CXCL12, as it modulates axon growth and synapse formation by signaling through CXCR4. This signaling leads to the regulation of GABAergic inputs to NPCs from mature granule cells, thereby supporting the maturation of young neurons [46, 47]. According to previous observations, our findings confirmed the expression of CXCR4 mRNA and the absence of CXCL12 mRNA in cultured NPCs. Although the gene expression of CXCR4 was upregulated at Poly I:C concentration of 100 $\mu\text{g/ml}$, it was downregulated at Poly I:C concentration of 200 $\mu\text{g/ml}$. These findings suggest that CXCR4 is not necessarily sensitive to TLR3 signaling, but may possess a threshold for downregulation during innate antiviral response. Unfortunately, the relationship between TLR3-signaling and CXCR4 has not been investigated yet. Solely in one study on the effect of TLR3 activation on the metastasis of nasopharyngeal carcinoma, it has been reported that TLR3 activation leads to dose-dependent downregulation of CXCR4 providing evidence for a connection between these two receptors [48]. Nevertheless, our findings indicate that a prolonged exposure to high TLR3-agonist concentration results in the regulation of CXCR4 through an unknown mechanism and thus can impair the migratory and maturation potential of adult NPCs probably resulting in insufficient guidance of NPCs to the granular zone. In fact, according to the diagram of CXCR4 expression, its mRNA level seems to rise after the lowest point at P3 to nearly normal expression at P70. Whether the reduced expression of CXCR4 during the first postnatal days has long lasting effects on hippocampal function remains to be elucidated.

Our study provides the first evidence that adult NPCs express functional TLR3 and that maternal immune challenge has the potential to modulate adult hippocampal neurogenesis. Both might be useful to understand how multifactorial psychopathological diseases develop and what can be done for prevention and treatment. A very recent paper speculated on changes in the total amount of doublecortin and Sox2 in the offspring of Poly I:C treated dams [49]. Since Forrest et al. used whole brain homogenate, no conclusion about adult neurogenesis in the dentate gyrus can be drawn.

To examine further the physiology of adult NPCs after fetal exposure to maternal infection would not only provide a better understanding of the biology behind adult neurogenesis, but could also give the possibility to interfere pharmacologically and prevent the damage of the NPC population *in utero* or even in later life. To achieve that goal, many further experiments have to be carried out, but the fast progress in the field of adult neurogenesis in the past years raises the hope that adult neural stem cells might soon become targets for successful therapies treating neurological and neurodegenerative disorders.

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