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Abstract: Prenatal exposure to infectious or inflammatory insults can increase the risk of developing neuropsychiatric disorder in later life, including schizophrenia, bipolar disorder, and autism. These brain disorders are also characterized by pre- and postsynaptic deficits. Using a well-established mouse model of maternal exposure to the viral mimetic polyriboinosinic-polyribocytidilic acid [poly(I:C)], we examined whether prenatal immune activation might cause synaptic deficits in the hippocampal formation of pubescent and adult offspring. Based on the widely appreciated role of microglia in synaptic pruning, we further explored possible associations between synaptic deficits and microglia anomalies in offspring of poly(I:C)-exposed and control mothers. We found that prenatal immune activation induced an adult onset of presynaptic hippocampal deficits (as evaluated by synaptophysin and bassoon density). The early-life insult further caused postsynaptic hippocampal deficits in pubescence (as evaluated by PSD95 and SynGAP density), some of which persisted into adulthood. In contrast, prenatal immune activation did not change microglia (or astrocyte) density, nor did it alter their activation phenotypes. The prenatal manipulation did also not cause signs of persistent systemic inflammation. Despite the absence of overt glial anomalies or systemic inflammation, adult offspring exposed to prenatal immune activation displayed increased hippocampal IL-1 levels. Taken together, our findings demonstrate that age-dependent synaptic deficits and abnormal pro-inflammatory cytokine expression can occur during postnatal brain maturation in the absence of microglial anomalies or systemic inflammation.

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Prenatal immune activation causes hippocampal synaptic deficits in the absence of overt microglia anomalies

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Abstract

Prenatal exposure to infectious or inflammatory insults can increase the risk of developing neuropsychiatric disorder in later life, including schizophrenia, bipolar disorder, and autism. These brain disorders are also characterized by pre- and postsynaptic deficits. Using a well-established mouse model of maternal exposure to the viral mimetic polyriboinosinic-polyribocytidilic acid [poly(I:C)], we examined whether prenatal immune activation might cause synaptic deficits in the hippocampal formation of pubescent and adult offspring. Based on the widely appreciated role of microglia in synaptic pruning, we further explored possible associations between synaptic deficits and microglia anomalies in offspring of poly(I:C)-exposed and control mothers. We found that prenatal immune activation induced adult onset of presynaptic hippocampal deficits (as evaluated by synaptophysin and bassoon density). The early-life insult further caused postsynaptic hippocampal deficits in pubescence (as evaluated by PSD95 and SynGAP density), some of which persisted into adulthood. In contrast, prenatal immune activation did not change microglia (or astrocyte) density, nor did it alter their activation phenotypes. The prenatal manipulation did also not cause signs of persistent systemic inflammation. Despite the absence of overt glial anomalies or systemic inflammation, adult offspring exposed to prenatal immune activation displayed increased hippocampal IL-1β levels. Taken together, our findings demonstrate that age-dependent synaptic deficits and abnormal pro-inflammatory cytokine expression can occur during postnatal brain maturation in the absence of microglial anomalies or systemic inflammation.

1. Introduction

Maternal infectious or inflammatory insults during pregnancy have been repeatedly implicated in the etiology of developmental neuropsychiatric disorders, including schizophrenia (Brown and Derkits, 2010; Canetta et al., 2014b), autism (Atladóttir et al., 2010; Brown et al., 2014), and bipolar disorder (Canetta et al., 2014a; Parboosing et al., 2013). Preclinical support for these epidemiological associations has been obtained by various translational rodent models demonstrating multiple brain and behavioral abnormalities following prenatal exposure to infection and/or immune activation (reviewed in Boksa, 2010; Harvey and Boksa, 2012; Meyer, 2014; Meyer and Feldon, 2010). Increasing evidence suggests that cytokine-associated inflammatory events, together with downstream pathophysiological effects such as oxidative stress and (temporary) macronutrient and micronutrient deficiency, are critical in mediating the adverse effects of maternal infection on the fetal system (reviewed in Meyer, 2014; Miller et al., 2013). Disruption of normal fetal development (Meyer et al., 2008; Vuillermot et al., 2010), together with subsequent changes in brain maturation (Hadar et al., 2015; Piontkewitz et al., 2011; Richetto et al., 2014), may then confer increased risk of behavioral and cognitive dysfunctions in later life.

In recent years, there has been growing interest in the potential role of microglia in normal brain development and maturation, especially in those events that relate to synaptic pruning (Bilimoria and Stevens, 2015; Kreisel et al., 2014; Paolicelli et al., 2011; Schafer et al., 2012; Schafer and Stevens, 2013). Microglia are the major immunocompetent cells residing in the brain parenchyma and can adopt different morphological characteristics and functions: In their ramified state, they constantly survey the brain

microenvironment to detect and to respond to alterations in brain homeostasis, which in turn can induce the transition of ramified into phagocytic microglia (Gomez-Nicola and Perry, 2015). In contrast to ramified microglia with small cell bodies and extensive arborization, phagocytic microglia are characterized by enlarged cell soma and less arborization. As phagocytes, they can quickly remove cellular debris and dying neurons, thereby preventing possible toxic damage to neighboring cells.

The phagocytic capacity of microglia is particularly important during early brain development and subsequent maturation, where they can contribute to the removal of excessive synapses in certain neuronal pathways (Bilimoria and Stevens, 2015; Kreisel et al., 2014; Paolicelli et al., 2011; Schafer et al., 2012). For these reasons, it has been suggested that abnormal microglia functions during critical periods of brain development and maturation may be an important etiopathological mechanism linking neuroinflammation to synaptic deficits (Rao et al., 2012). Synaptic deficits are a hallmark of various neurodevelopmental brain disorders, including schizophrenia and autism (reviewed in Eastwood, 2004; Ebrahimi-Fakhari and Sahin, 2015; Harrison, 2004). These disorders have also been associated with altered microglia functions and associated neuroinflammatory changes (Arion et al., 2007; Doorduin et al., 2009; Fillman et al., 2013; Fung et al., 2014; Morgan et al., 2010; Tetreault et al., 2012; van Berckel et al., 2008; Volk et al., 2015). It remains elusive, however, whether and to what extent such microglial abnormalities may be responsible for the development of synaptic deficits as, for example, seen in schizophrenia or autism (Catts et al., 2013; Onore et al., 2012).

Against these backgrounds, the present study aimed at exploring potential associations between impaired synaptic development and microglial abnormalities in a model of prenatal immune activation with relevance to neurodevelopmental disorders. We

used a well-established mouse model of maternal treatment with the viral mimetic polyriboinosinic-polyribocytidilic acid [poly(I:C)], which is known to capture a wide spectrum of neuronal and behavioural abnormalities relevant to psychiatric disorders, especially schizophrenia and autism (reviewed in Meyer, 2014; Reisinger et al., 2015). Prenatal poly(I:C) exposure in rats has also been shown to cause synaptic deficits (Forrest et al., 2012; Oh-Nishi et al., 2010), but their possible association with microglial abnormalities and related neuroimmune changes remain unexplored thus far.

Using the maternal poly(I:C) administration model in mice, we assessed the expression of synaptic proteins, microglia density and activation, systemic and peripheral inflammation, and selected behavioral functions. We focused our neuroanatomical investigations on the hippocampus for two main reasons. First, it is one of the brain areas strongly implicated in the neuropathology of schizophrenia and other brain disorders with neurodevelopmental components (Harrison, 2004; Tamminga et al., 2010). Second, the hippocampal formation is markedly affected by prenatal immune challenge and is of key interest to current research in this field (reviewed in: Meyer and Feldon, 2009; Piontkevitz et al., 2012a). The assessment of synaptic proteins included synaptophysin, bassoon, synaptic Ras GTPase-activating protein 1 (SynGAP), and postsynaptic density protein 95 (PSD95). Synaptophysin and bassoon are two major presynaptic proteins that are localized in or associated with presynaptic vesicles (Elferink and Scheller, 1995; Schoch and Gundelfinger, 2006; Shin, 2014; Wiedenmann and Franke, 1985). On the other hand, SynGAP and PSD95 are two major postsynaptic proteins that are enriched at postsynaptic sites of excitatory synapses, where they critically help organizing and strengthening excitatory receptor complexes and their associated signaling proteins (Kim et al., 1998; Kim and Sheng, 2004; Sheng and Hoogenraad, 2007). Microglia density was examined by

quantification of cells immunoreactive for the ionized calcium-binding adaptor molecule 1 (Iba1), whereas their activation status was assessed through the analysis of microglia morphology and cluster of differentiation 68 (CD68) immunoreactivity (Colton and Wilcock, 2010; Franco and Fernández-Suárez, 2015; Ransohoff and Perry, 2015). In addition, we measured the density of glial fibrillary acidic protein (GFAP)-positive astrocytes and the levels of cytokines in plasma and hippocampus to assess the effects of prenatal immune activation on astrogliosis and inflammatory signaling, respectively. To confirm the negative influence of prenatal immune challenge on behavior, we measured sensorimotor gating using the paradigm of prepulse inhibition (PPI) of the acoustic startle reflex. PPI deficiency is a well-replicated behavioral manifestation emerging in rats and mice exposed to prenatal immune activation (for a review, see Boksa, 2010; Meyer, 2014; Meyer and Feldon, 2010; Reisinger et al., 2015) and is frequently observed in neuropsychiatric disorders with neurodevelopmental components (Braff et al., 2001; Swerdlow et al., 2008). All analyses were conducted during puberty and adulthood in order to identify possible maturation-dependent effects of prenatal immune activation.

2. Methods

2.1. Animals

C57BL6/N mice were used throughout the study. Female and male breeders were obtained from Charles River Laboratories (Sulzfeld, Germany) at the age of 10–14 weeks. Breeding began after 2 weeks of acclimatization to the animal holding rooms, which were temperature- and humidity-controlled (21 ± 1°C, 55 ± 5%) facilities under a reversed light–dark cycle. All animals had *ad libitum* access to food (Kliba 3430, Kaiseraugst, Switzerland)

and water. All procedures described in the present study had been previously approved by the Cantonal Veterinarian's Office of Zurich. All efforts were made to minimize the number of animals used and their suffering.

2.2. Maternal immune activation during pregnancy

For the purpose of the maternal immune challenge, C57BL6/N female mice were subjected to a timed mating procedure as described previously (Meyer et al., 2005). Pregnant dams on gestation day (GD) 9 were randomly assigned to receiving either a single injection of poly(I:C) (potassium salt; Sigma-Aldrich, Buchs, St. Gallen, Switzerland) or vehicle. Poly(I:C) (5 mg/kg; calculated based on the pure form poly(I:C)) was dissolved in sterile pyrogen-free 0.9% NaCl (vehicle) solution to yield a final concentration of 1 mg/ml and was administered intravenously (i.v.) into the tail vein under mild physical constraint. The dose of poly(I:C) was selected based on previous dose-response studies (Meyer et al., 2005). A total of 16 pregnant dams were used, half of which were allocated to the poly(I:C) treatment, and the other half to the vehicle treatment.

The selected gestational window (i.e., GD 9) in mice corresponds roughly to the middle of the first trimester of human pregnancy with respect to developmental biology and percentage of gestation from mice to humans (Clancy et al., 2007). It was selected based on our previous findings showing that poly(I:C) exposure on GD 9 leads to multiple behavioral abnormalities in the adult offspring, including deficits in PPI (Meyer et al., 2005, 2006). We have also verified the effectiveness of this poly(I:C) administration protocol in mice in terms of the elicited cytokine-associated inflammatory response in maternal and fetal tissues (e.g., Meyer et al., 2006).

2.3. Allocation and testing of the offspring

All offspring were weaned and sexed on postnatal day (PND) 21. Littermates of the same sex were caged separately and maintained in groups of 3-5 animals per cage. Only male animals were included in all experiments. Poly(I:C) and control offspring stemmed from multiple independent litters (N = 8 for each prenatal treatment group) to avoid possible confounds arising from litter effects. For each maturational stage (i.e., pubescence and adulthood), 1 male offspring per litter was randomly selected for the initial PPI test and subsequent immunohistochemical evaluations of glial and synaptic markers, leading to a group size of N=8 offspring per treatment group and age. Pubescent and adult testing began with the assessment of PPI on PND 35 and PND 84, respectively. Following a resting period of 5-6 days, the animals were then sacrificed on PND 40 and PND 90, respectively, for the purpose of the immunohistochemical evaluations in pubescence and adulthood.

The remaining offspring from poly(I:C) and control litters were used for the analysis of plasma and hippocampal cytokines in pubescence (N = 10 per treatment group) and in adulthood (N = 8 per treatment group). The animals were killed on PND 40 and PND 90 to match the age of those offspring, which were assigned to the immunohistochemical evaluations of glial and synaptic markers.

The pubescent and adult stages were defined based on the gradual attainment of sexual maturity and age-specific behavioral discontinuities from younger to older animals (Spear, 2000) and based on our previous findings revealing a post-pubertal onset of PPI deficits following poly(I:C)-induced prenatal immune activation in mice (Vuillermot et al., 2010).

2.4. Prepulse inhibition of the acoustic startle reflex

Sensorimotor gating was assessed using the paradigm of prepulse inhibition (PPI) of the acoustic startle reflex. PPI of the acoustic startle reflex refers to the reduction in startle reaction in response to a startle-eliciting pulse stimulus when it is shortly preceded by a weak prepulse stimulus (Braff et al., 2001; Swerdlow et al., 2008). The apparatus consisted of four startle chambers for mice (San Diego Instruments, San Diego, CA, USA) and has been fully described elsewhere (Meyer et al., 2005). In the demonstration of PPI, the animals were presented with a series of discrete trials comprising a mixture of 4 trial types. These included pulse-alone trials, prepulse-plus-pulse trials, prepulse-alone trials, and no-stimulus trials in which no discrete stimulus other than the constant background noise was presented. The pulse and prepulse stimuli used were in the form of a sudden elevation in broadband white noise level (sustaining for 40 and 20 ms, respectively) from the background (65 dB_A), with a rise time of 0.2–1.0 ms. In all trials, three different intensities of pulse (100, 110, and 120 dB_A) and three intensities of prepulse (71, 77, and 83 dB_A, which corresponded to 6, 12, and 18 dB_A above background, respectively) were used. The stimulus-onset asynchrony of the prepulse and pulse stimuli on all prepulse-plus-pulse trials was 100 ms (onset-to-onset).

The protocol used for the PPI test was extensively validated before (e.g., Vuillermot et al., 2010). A session began with the animals being placed into the Plexiglas enclosure. They were acclimatized to the apparatus for 2 min before the first trial began. The first 6 trials consisted of 6 startle-alone trials; such trials served to habituate and stabilize the animals' startle response and were not included in the analysis. Subsequently, the animals were presented with 10 blocks of discrete test trials. Each block consisted of the following: three pulse-alone trials (100, 110, or 120 dB_A), 3 prepulse-alone trials (+6, +12, or +18 dB_A above

background), 9 possible combinations of prepulse-plus-pulse trials (3 levels of pulse × 3 levels of prepulse), and one no stimulus trial. The 16 discrete trials within each block were presented in a pseudorandom order, with a variable interval of 15 s on average (ranging from 10 to 20 s). For each of the 3 pulse intensities (100, 110, or 120 dB_A), PPI was indexed by percent inhibition of the startle response obtained in the pulse-alone trials by the following expression: 100% × [1 − (mean reactivity on prepulse-plus-pulse trials/mean reactivity on pulse-alone trials)], for each animal, and at each of the three possible prepulse intensities (+6, +12, or +18 dB_A above background). In addition to PPI, reactivity to pulse-alone trials and prepulse-alone trials were also analyzed.

2.5. Immunohistochemistry

The animals were deeply anesthetized with an overdose of Nembutal (Abbott Laboratories, North Chicago, IL, USA) and perfused transcardially with 0.9% NaCl, followed by 4% phosphate-buffered paraformaldehyde solution containing 15% picric acid. The dissected brains were postfixed in the same fixative for 6 h and processed for antigen retrieval involving overnight incubation in citric acid buffer (pH 4.5) followed by a 90 s microwave treatment at 480 W according to protocols established before (Giovanoli et al, 2013; Vuillermot et al., 2010). The brains were then cryoprotected using 30% sucrose in PBS, frozen with powdered dry ice, and stored at −80°C until further processing.

Perfused brain samples were cut coronally at 30 μm thickness from frozen blocks with a sliding microtome. Eight serial sections were prepared for each animal and, after rinsing in PBS, stored at −20°C in antifreeze solution (30% glycerol and 30% ethylene glycol in PBS at 25 mM and pH 7.4) until further processing. For immunohistochemical staining, the slices were rinsed three times for 10 min in PBS, and blocking was done in PBS, 0.3% Triton X-

100, 10% normal serum for 1 h at room temperature. The following primary antibodies were used: Rabbit anti-Iba1 (Wako, Neuss, Germany; diluted 1:2,000), rat anti-CD68 (AbD Serotec, Oxford, UK; diluted 1:5,000), rabbit anti-GFAP (Dako, Baar, Switzerland; diluted 1:5,000), rabbit anti-synaptophysin (Sigma, diluted 1:3,000), mouse anti-bassoon (Stressgen Biotechnologies, Victoria, Canada; diluted 1:5,000), mouse anti-PSD95 (Pierce Antibody Products, diluted 1:800), and rabbit anti-SynGap (Affinity BioReagents, Colorado, USA; diluted 1:400). All primary antibodies were validated before (Giovanoli et al, 2013; Nyffeler et al., 2006, 2007). They were diluted in PBS containing 0.3% Triton X-100 and 2% normal serum, and the sections were incubated free-floating overnight at room temperature. After three washes with PBS (10 min each), the sections were incubated for 1 h with the biotinylated secondary antibodies diluted 1:500 in PBS containing 2% NGS and 0.3% Triton X-100. Sections were washed again three times for 10 min in PBS and incubated with Vectastain kit (Vector Laboratories, Burlingame, CA, USA) diluted in PBS for 1 h. After three rinses in 0.1 M Tris-HCl, pH 7.4, the sections were stained with 1.25% 3,3-diaminobenzidine and 0.08% H_2O_2 for 10–15 min, rinsed again four times in PBS, dehydrated, and coverslipped with Eukitt (Kindler, Freiburg, Germany).

2.6. Unbiased stereological estimations

The numbers of Iba1-, CD68-, or GFAP-immunoreactive cells were determined by unbiased stereological estimations using the optical fractionator method (Gundersen et al., 1988). With the aid of the image analysis computer software Stereo Investigator (version 6.50.1; MicroBrightField, Williston, VT, USA), every section of a one-in-eight series was measured, resulting in an average of 4-5 sections per brain sample. The following sampling parameters were used: (1) a fixed counting frame with a width of 60 µm and a length of 60

μm; and (2) a sampling grid size of 200 × 150 μm. The counting frames were placed randomly at the intersections of the grid within the outlined structure of interest by the software. The cells were counted following the unbiased sampling rule using the 40× oil lens [numerical aperture (NA), 1.3] and included in the measurement when they came into focus within the optical dissector (Howard and Reed, 2005). All immunohistochemical preparations were quantified in the dorsal cornu amonis 1 to 3 (CA1 –CA3) regions of the hippcoampus (Bregma -1.3 to -2.7 mm), thereby including stratum oriens (so), stratum radiatum (sr), and stratum lacunosum moleculare (slm). Preliminary analyses have revealed no layer-specific effects of prenatal immune activation (data not shown), so that the data representing the entire dorsal CA region are presented. In addition, the immunohistochemical preparations were quantified in the dorsal stratum moleculare (sm) of the dentate gyrus (DG; Bregma -1.3 to -2.7 mm). In view of the dorsal-ventral dichotomy of the hippocampus, we extended parts of the stereological estimations to the ventral part of the hippocampus (Bregma -2.9 to -3.5 mm), thereby including the same ventral layers (CA: so, sr, slm; DG: sm) as for the dorsal subregion. All brain regions and layers were delineated according to the "*Mouse Brain in Stereotaxic Coordinates*" (Franklin and Paxinos, 2008). These stereological methods were previously validated to capture overt microglia anomalies under conditions of hippocampal inflammation (Giovanoli et al., 2013).

2.7. Assessment of microglia morphology

Iba1-immunoreactive microglia were visualized under the 63× oil lens [numerical aperture (NA), 1.4] using a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany). Various parameters of microglia cell morphology were assessed in the CA1 slm and DG sm according to methods described before (Giovanoli et al., 2013). In brief, a counting frame of

100 µm × 100 µm was randomly placed into three sections of a one-in-eight series. All microglia cells captured by the counting frame were included in the morphological analyses, except when microglial processes were obscured by either background labeling or other cells. 3 to 4 microglia cells in every section of a one-in-eight series (see above) were traced using the software Stereo Investigator (version 6.50.1; MicroBrightField), for which cell soma area and the number of primary and secondary processes were estimated, giving a total of 12 to 16 cells per animal, as described before (Giovanoli et al., 2013).

2.8. Optical densitometry

Quantification of synaptophysin, bassoon, PSD95, and SynGap immunoreactivity was achieved by means of optical densitometry using NIH ImageJ software as described before (Giovanoli et al, 2013; Nyffeler et al., 2007). In brief, digital images were acquired at a magnification of 5.0× using a digital camera (Axiocam MRc5; Carl Zeiss) mounted on a Zeiss Axioplan microscope. Exposure times were set so that pixel brightness was never saturated. Pixel brightness was measured in the respective areas of one randomly selected brain hemisphere. In addition, pixel brightness was measured in non-immunoreactive areas of the corpus callosum as background measurements. The background-corrected relative optical densities were averaged per brain region and animal. Four to 5 sections per animal were analyzed for each brain region of interest. All immunohistochemical preparations were quantified in the dorsal CA and DG regions (Bregma -1.3 to -2.7 mm) as described above. Synaptophysin and PSD95 immunoreactivities were also quantified in the ventral subregions of the hippocampus (Bregma -2.9 to -3.5 mm), thereby including the same layers (CA: so, sr, slm; DG: sm) as for the dorsal subregions.

2.9. Preparation of plasma samples and hipocampal homogenates

Mice were killed by decapitation and trunk blood was collected in heparinized tubes (Microvette CB 300 LH, Sarstedt, Nümbrecht, Germany). Plasma was separated by centrifugation (2000 × g, 5 min) and stored at −20°C until later analyses. The brains were extracted from the skull and placed ventral side up on an ice-chilled plate for extraction of the left and right hippocampi. The entire hippocampi were weighed and stored at −80°C until further processing. Frozen hippocampal samples were placed in 300 µl lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.6 M NaCl, 0.2% Triton X-100, 0.5% bovine serum albumin, and protease inhibitors (1 mM benzamidine, 0.1 mM benzethonium chloride and 0.1 mM phenylmethylsulfonyl fluoride). Once placed in the lysis buffer, samples were allowed to thaw and were then homogenized (TissueTearor; BioSpec Products, Bartlesville, OK, USA) for 10 s, sonicated (Vibra Cell; Sonics & Materials, Newtown, CT, USA) for 20 s at 10 mV, and centrifuged as described before (Giovanoli et al., 2013). The hippocampal supernatants were aliquoted and frozen at –80°C until the cytokine assays were performed (see below).

2.10. Cytokine assay

Cytokine levels in plasma and hippocampal supernatants were quantified using a customized Meso-Scale Discovery (MSD) V-Plex electrochemiluminescence assay for mice, which allows ultralow detection of multiple cytokines in mouse plasma and supernatants (Burguillos, 2013). V-plex plus 96-well plates coated with primary antibodies directed against interleukin (IL)-1β, IL-4, IL-6, and tumor necrosis factor (TNF)-α were used and were treated with the corresponding detecting antibodies, which were prelabeled with SULFO-TAG™ (MSD, Rockville, Maryland, USA). The plates were read using the SECTOR PR

400 (MSD) imager and analyzed using MSD's Discovery Workbench analyzer and software package. All assays were run according to the manufacturer's instructions. IL-1β, IL-6 and TNF-α were selected to cover prototypical pro-inflammatory cytokines secreted by classically activated (M1) microglia, and IL-4 was selected to probe a prototypical anti-inflammatory cytokine inducing alternative (M2) microglia activation (Cherry et al., 2014). The detection limits were 0.04 pg/ml for IL-1β, 0.06 pg/ml for IL-4, 0.5 pg/ml for IL-6, and 0.03 pg/ml for TNF-α. To express hippocampal cytokine levels, the cytokine concentrations quantified in the corresponding hippocampal lysates were normalized to the animals' hippocampal weights measured immediately after hippocampal dissection.

2.11. Statistical analyses

All data were analyzed using full-factorial parametric analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) post-hoc comparisons or restricted ANOVAs whenever appropriate. Percent PPI was analyzed using a 2 × 2 × 3 × 3 (prenatal treatment × age × prepulse level × pulse level) ANOVA, and reactivities to pulse-alone trials and prepulse-alone trials were analyzed using 2 × 2 × 3 (prenatal treatment × age × pulse level) and 2 × 2 × 3 (prenatal treatment × age × prepulse level) ANOVAs, respectively. All immunohistochemical and cytokine data were analyzed using a 2 × 2 (prenatal treatment × age) ANOVA. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the statistical software StatView software (version 5.0) implemented on a PC running the Windows XP operating system.

3. Results

3.1. Prenatal immune activation induces an adult onset of prepulse inhibition deficits

Consistent with previous reports (Lipina et al., 2013; Vuillermot et al., 2010), we found that poly(I:C)-induced prenatal immune activation induced an adult onset of PPI deficits (**Fig.1A**). ANOVA yielded a significant main effect of age [$F(1,28) = 136.75$, $p < 0.001$], reflecting the general increase in % PPI displayed by adult relative to pubescent animals, as well as a significant interaction between age and prenatal treatment [$F(1,28) = 4.80$, $p < 0.05$]. Subsequent post-hoc analyses verified that adult but not pubescent offspring of poly(I:C)-exposed mothers displayed a significant ($p < 0.05$) reduction in %PPI compared to adult control offspring (**Fig. 1A**).

Prenatal immune activation did not significantly affect the reactivity to pulse-alone trials (**Fig. 1B**) or prepulse-alone trials (**Fig. 1C**). For both measures, ANOVA only revealed a significant main effect of age [pulse: $F(1,28) = 5.25$, $p < 0.05$; prepulse: $F(1,28) = 59.80$, $p < 0.001$], reflecting the general increases in pulse- or prepulse-induced reactivity from pubescence to adulthood.

3.2. Prenatal immune activation induces an adult onset of presynaptic hippocampal deficits

As depicted in **Fig. 2A**, the relative density of the presynaptic marker synaptophysin was generally increased in the dorsal CA and DG of adult relative to pubescent mice, leading to a significant main effect of age [CA: $F(1,28) = 44.87$, $p < 0.001$; DG: $F(1,28) = 47.94$, $p < 0.001$]. Adult (but not pubescent) offspring of poly(I:C)-exposed mothers displayed a significant decrease in the relative density of synaptophysin compared to adult control offspring. This effect emerged similarly in the dorsal CA and DG (**Fig. 2A,B**) and was supported by the

significant interaction between prenatal treatment and age [CA: $F(1,28) = 8.64$, $p < 0.01$; DG: $F(1,28) = 5.95$, $p < 0.05$], and by the subsequent post-hoc comparisons confirming the significant group differences in adulthood (for both CA and DG, $p < 0.05$). Similar findings were obtained with regards to synaptophysin expression in the ventral CA and DG subregions (**Suppl. Fig. S1**).

In contrast to synaptophysin, the density of the presynaptic marker bassoon generally decreased from pubescence to adulthood (**Fig. 2C**), leading to a significant main effect of age [CA: $F(1,28) = 76.18$, $p < 0.001$; DG: $F(1,28) = 86.80$, $p < 0.001$]. Prenatal immune activation significantly reduced the relative density of bassoon in the dorsal CA and DG of adult but not pubescent offspring (**Fig. 2C,D**), as supported by the significant interaction between prenatal treatment and age [CA: $F(1,28) = 5.84$, $p < 0.05$; DG: $F(1,28) = 7.29$, $p < 0.01$]. Subsequent post-hoc comparisons verified the significant group differences in adult offspring (for both CA and DG, $p < 0.01$).

3.3. Prenatal immune activation induces a pubescent onset of postsynaptic hippocampal deficits

The relative density of the postsynaptic protein PSD95 in both the dorsal CA and DG generally decreased from pubescence to adulthood, leading to a significant main effect of age [CA: $F(1,28) = 27.87$, $p < 0.001$; DG: $F(1,28) = 36.98$, $p < 0.001$]. Offspring of poly(I:C)-exposed mothers displayed a significant reduction of PSD95 in the dorsal CA and DG. This effect was already present in pubescence and persisted into adulthood (**Fig. 3A,B**), as supported by the significant main effect of prenatal treatment [CA: $F(1,28) = 23.99$, $p < 0.001$; DG: $F(1,28) = 32.19$, $p < 0.001$]. Prenatal immune activation led to a similar reduction in PSD95 expression in the ventral CA and DG subregions, which was evident in pubescent

and adult offspring of poly(I:C)-exposed offspring relative to age-matched controls (**Suppl. Fig. S2**).

In addition to its age-independent effect on PSD95 (Fig. **3A,B**), prenatal immune activation also reduced the relative density of the postsynaptic protein SynGap. In contrast to PSD95, however, prenatal poly(I:C) treatment selectively decreased SynGap density in the dorsal CA region at pubescent but not adult age (Fig. **3C,D**). This age-dependent effect in the dorsal CA region was supported by the significant interaction between prenatal treatment and age [$F(1,28) = 6.93$, $p < 0.05$], and by the subsequent post-hoc analysis confirming the significant group difference in pubescent animals ($p < 0.01$). No significant effects were obtained with regards to SynGAP density in the dorsal DG.

3.4. Prenatal immune activation does not alter hippocampal microglia density or activation statuses

Unbiased stereological estimations of Iba1-positive microglia showed that prenatal immune activation did not alter the total number of microglia cells in the dorsal CA and DG, neither in pubescence nor in adulthood (**Fig. 4A,B**). There were also no group differences with regards to Iba1-positive microglia in the ventral CA and DG subregions (**Suppl. Fig. S3A**). The number of Iba1-positive microglia was generally lower in the dorsal CA of adult as compared to pubescent offspring, leading to a significant main effect of age [$F(1,28) = 6.06$, $p < 0.05$]. This age-dependent decrease, however, similarly emerged in poly(I:C)-exposed and control offspring (**Fig. 4A,B; Suppl. Fig. S3A**).

There were also no signs of altered microglia activation in poly(I:C)-exposed relative to control offspring. We found no group differences with respect to cell soma area and primary or secondary branches of Iba1-positive cells in the CA (**Fig. 4C**) or DG subregions

(**Suppl. Fig. S4**), suggesting that microglia morphology was not changed between poly(I:C)-exposed relative to control offspring. Furthermore, the number of microglia expressing CD68, which is typically increased in the lysosome of activated (phagocytic) cells (Colton and Wilcock, 2010), was not changed in the dorsal CA and DG subregions of poly(I:C)-treated offspring relative to controls (**Fig. 4D,E**). There were also no group differences with regards to CD68-positive cells in the ventral CA and DG subregions (**Suppl. Fig. S3B**). Consistent with the age-dependent decrease of Iba1-positive cells (**Fig. 4A**), the number of CD68-positive microglia was generally lower in the dorsal CA and DG of adult as compared to pubescent offspring (**Fig. 4D**), leading to a significant main effect of age [CA: $F(1,28) = 4.78$, $p < 0.05$; DG: $F(1,28) = 6.35$, $p < 0.05$]. Similar findings were obtained for the ventral CA and DG subregions (**Suppl. Fig. S3B**).

We further examined whether possible microglia alterations might exist in offspring of poly(I:C)-mothers at an earlier maturational stage, namely at juvenile age (PND 21). These additional analyses, however, similarly revealed no significant group differences with regards to Iba1-or CD68-positive microglia cells in the juvenile hippocampus (**Suppl. Fig. S5**). Hence, we found no evidence for microgliosis or altered microglia activation in juvenile offspring exposed to prenatal viral-like immune activation.

Consistent with previous studies (Nyffeler et al., 2006), poly(I:C)-induced prenatal immune activation did also not alter the numbers of GFAP-positive in the hippocampus at pubescent or adult ages (**Fig. 4F,G**). In the dorsal CA region, there was a general decrease in the number of astrocytes in adult relative to pubescent animals [main effect of age: $F(1,28) = 7.50$, $p < 0.05$], whereas an opposite age-dependent effect emerged in the dorsal DG region [main effect of age: $F(1,28) = 9.16$, $p < 0.01$].

3.5. Prenatal immune activation increases hippocampal IL-1β levels in adulthood

As depicted in **Fig. 5A**, we found that prenatal immune activation selectively increased hippocampal IL-1β levels in adult but not pubescent offspring. ANOVA of hippocampal IL-1β revealed a significant interaction between prenatal treatment and age [$F(1,32) = 4.90$, $p < 0.05$], and subsequent post-hoc analyses confirmed the significant group difference in hippocampal IL-1β levels at adult age ($p< 0.05$). Prenatal immune activation did not affect the hippocampal levels of the other cytokines of interest (IL-4, IL-6 and TNF-α; **Fig. 5A**). There was only a general age-dependent effect for the hippocampal levels of IL-4 [main effect of age: $F(1,32) = 5.63$, $p < 0.05$] and TNF-α [main effect of age: $F(1,32) = 4.82$, $p < 0.05$], with lower levels measured in adult as compared to pubescent offspring (**Fig. 5A**).

No group differences in plasma levels of IL-1β, IL-4, IL-6 or TNF-α were detected (**Fig. 5B**), indicating that prenatal immune activation did not induce systemic inflammation at pubescent or adult ages. Plasma levels of IL-1β and TNF-α generally decreased as a function of age (**Fig. 5B**), leading to significant main effects of age [IL-1β: $F(1,32) = 17.29$, $p < 0.001$; TNF-α: $F(1,32) = 6.72$, $p < 0.05$]. No other main effects or interactions attained significance.

4. Discussion

The present study demonstrates that viral-like prenatal immune activation in mice impairs the expression of major pre- and post-synaptic synaptic proteins in the hippocampal formation. Consistent with other findings (Oh-Nishi et al., 2010), we have previously shown that the same prenatal manipulation does not lead to neuronal loss in this brain area (Nyffeler et al., 2006). It thus follows that hippocampal synaptic deficits emerging following prenatal viral-like immune activation are not secondary to neuronal loss. Interestingly, the

nature of synaptic deficits induced by this prenatal insult was markedly influenced by the maturational stage of the offspring. Clear deficits in the expression of the presynaptic proteins synaptophysin and bassoon were evident only in adult but not pubescent offspring of immune-challenged mothers. In contrary, prenatal immune activation led to a decrease in the expression of the major postsynaptic protein PSD95 regardless of the offspring's age. In addition, it impaired hippocampal SynGap expression specifically in pubescent but not adult offspring. It thus seems that prenatal viral-like immune activation can led to an early pubescent onset of postsynaptic hippocampal deficits, whereas presynaptic abnormalities are only manifested once the offspring reach adulthood. Our study does not provide insights into the developmental processes underlying this differential temporal onset. Hence, it remains elusive whether they may be somehow interrelated, or alternatively, whether distinct developmental processes may be involved. In support of the former possibility, however, it has been shown that postsynaptic proteins can induce presynaptic assembly and drive the formation of new presynaptic contacts (Biederer et al., 2002; Scheiffele et al., 2000). Furthermore, the postsynaptic scaffolding protein PSD95, along with its interaction partners, can modulate the release probability of presynaptic transmitter vesicles in a retrograde manner (Futai et al., 2007). The early onset of postsynaptic deficiencies identified here is consistent with previous findings showing that prenatal immune activation induces early developmental changes in the expression of *N*-methyl-D-aspartate (NMDA) receptor subunits (Khalil et al., 2013), which co-localize with several PSD proteins such as PSD95 (Sheng, 2001). Therefore, it could be speculated that the presence of early (pubescent) postsynaptic deficits may facilitate or even drive the subsequent development of presynaptic deficits, which in turn would appear only with a certain delay in adulthood.

Interestingly, the delayed emergence of presynaptic hippocampal deficits coincided with the adult onset of prenatal infection-induced PPI deficits. The adult emergence of poly(I:C)-induced PPI deficit is consistent with numerous previous studies in mice (e.g., Lipina et al., 2013; Pacheco-López et al., 2013; Vuillermot et al., 2010) and rats (Hadar et al., 2015; but see also Wolff and Bilkey, 2010). Since the hippocampal formation is one of several neuronal substrates modulating sensorimotor gating (Bast and Feldon, 2003), deficient hippocampal expression of presynaptic proteins may readily contribute to the attenuation of PPI in immune-challenged offspring. Even though this hypothesis warrants further examination, it would be consistent with other environmental stress models showing an association between reduced hippocampal synaptophysin expression and emergence of PPI deficits (Varty et al., 1999). Besides post-pubertal synaptic deficits, the delayed onset of prenatal poly(I:C)-induced deficits in PPI may also involve altered functional maturation of the subcortical dopamine system (Hadar et al., 2015; Vuillermot et al., 2010), brain volumetric changes throughout adolescence (Piontkewitz et al., 2011), and altered maturation of prefrontal GABAergic systems (Richetto et al., 2014). It should also be noted that prenatal poly(I:C)-induced immune activation can lead to various behavioral and neuronal abnormalities with early juvenile or adolescent onsets, including hypersensitivity to dopamine-stimulating psychotomimetic drugs (Meyer et al., 2008,; Vuillermot et al., 2010), cognitive deficits (Richetto et al., 2013), impairments in social interaction (Aavani et al., 2015), and deficits in hippocampal neurogenesis (Meyer et al., 2006). Hence, whereas the present findings may be more important for neuropsychiatric disorders with adult onsets, prenatal immune activation models are generally also relevant for neurodevelopmental disorders that are characterized by overt symptomatology in childhood or early adolescence.

Besides their potential involvement in PPI, the synaptic deficits identified here are likely to contribute to other functional abnormalities typically associated with prenatal viral-like immune activation. In particular, the disruption of hippocampal synaptic integrity may change the electrophysiological properties of hippocampal cells, thereby altering the firing activity of hippocampal neurons. Such abnormalities have indeed been noted by numerous previous investigations using prenatal poly(I:C) models in rodents (Dickerson et al., 2010, 2014; Savanthrapadian et al., 2013; Wolff and Bilkey, 2015; Zhang and van Praag, 2015). Furthermore, the synaptic deficits may also contribute to the cognitive impairments that have frequently been observed following prenatal immune activation (reviewed in Boksa, 2010; Harvey and Boksa, 2012; Meyer and Feldon, 2010; Reisinger et al., 2015).

Based on the growing evidence suggesting an important role of microglia in forming and maintaining synaptic integrity (Bilimoria and Stevens, 2015; Kreisel et al., 2014; Paolicelli et al., 2011; Schafer et al., 2012; Schafer and Stevens, 2013), we hypothesized that the prenatal infection-induced synaptic abnormalities would be associated with altered densities and/or activation patterns of microglia. Contrary to this hypothesis, we did not find any evidence for overt microglia abnormalities in offspring exposed to poly(I:C)-induced prenatal immune activation. In fact, our data highlight that a prenatal poly(I:C) challenge, which is effective in causing marked deficits in hippocampal synaptic protein expression and behavioral abnormalities, does not necessarily lead to increased Iba1-postive microglia (or GFAP-positive astrocyte) density in the offspring's hippocampal formation. We were also unable to detect signs of altered microglia activation in poly(I:C)-exposed offspring, as evaluated by microglia morphology and CD68 immunoreactivity. We acknowledge that our study was not designed to detect possible changes in microglia functions that could occur at early fetal or neonatal developmental windows (Arsenault et

al., 2014; Pratt et al., 2013). Therefore, our study does not negate a possible role of microglia in mediating early neurodevelopmental effects of prenatal immune activation. At the same time, however, the design of our study readily allowed us to identify potential microglia abnormalities from the pubescent (PND 40) to the adult (PND 90) stage and to establish a putative association with pre- and postsynaptic hippocampal deficits. Since our study failed to establish such an association, we feel it is reasonable to conclude that the adult onset of hippocampal synaptic deficits was not primarily the result of altered synaptic pruning by microglia. Based on our additional evaluation of possible microglia abnormalities at an earlier juvenile (PND 21) stage, we believe that altered synaptic pruning by microglia similarly plays only a minor role in inducing the hippocampal synaptic deficits in the pubescent offspring of infected mothers.

Hence, even though abnormalities in microglia functions can occur following prenatal immune challenge (Borrell et al., 2002; Juckel et al., 2011; Van den Eynde et al., 2014; Zhu et al., 2014), our findings suggest that such changes are not a prerequisite for synaptic deficits to occur, at least within the dorsal and ventral hippocampus. This interpretation is consistent with the findings derived from disease models of chronic neurodegeneration, suggesting that microglia do not play an active role in either synaptic stripping or synapse degeneration in the hippocampal formation (Perry and O'Connor, 2010; Sisková et al., 2009). Instead, these models highlight that synaptic elimination and envelopment of degenerating terminals can be a neuron autonomous event. Furthermore, our interpretation is also in line with other studies using the prenatal poly(I:C) administration model showing that significant neuronal and behavioral abnormalities can occur in the absence of overt microglia abnormalities (Garay et al., 2013; Missault et al., 2914; Pineda et al., 2013; Willi et al., 2013). Additional investigations will therefore be

needed to identify the cellular and molecular processes underlying the emergence of hippocampal synaptic deficits following prenatal immune challenge. On speculative grounds, one possible mechanism may be related to impairments in postnatal hippocampal neurogenesis, which have been repeatedly found in various models of prenatal immune challenge (Cardon et al., 2010; Cui et al., 2009; Meyer et al., 2006, 2010; Piontkewitz et al., 2012b; Zhang and van Praag, 2015). An alternative (but not mutually exclusive) mechanism may relate to epigenetic modifications impacting on the transcription of genes that encode for synaptic proteins. Even though this possibility warrants direct examination, epigenetic modifications at other gene loci have recently been identified in mouse models of prenatal immune activation (Basil et al., 2014; Connor et al., 2012; Hollins et al., 2014; Tang et al., 2013). Yet another possibility would be that the emergence of hippocampal synaptic deficits following prenatal immune activation might involve (developmental) changes in the kynurenine pathway. As discussed in detail elsewhere (Schwarcz et al., 2012), this pathway is strongly influenced by inflammatory processes, so that some effects of prenatal poly(I:C)-induced immune activation might be mediated by transient and/or long-term changes in the synthesis of kynurenine and its metabolites. Circumstantial support for this hypothesis derives from studies showing that inhibition of the kynurenine pathway during gestation can lead to changes in synaptic transmission, neuronal morphology and plasticity in the rat hippocampus (Forrest et al., 2013a,b; Khalil et al., 2014). Whatever precise mechanism involved, we believe that the deficits in hippocampal synaptic proteins emerging in pubescent and/or adult offspring of infected mothers may have an early developmental origin rather than being the result of abnormal microglia functions and associated changes in adolescent synaptic pruning.

In fact, the age-dependent increase in hippocampal synaptophysin expression, which was seen in both poly(I:C)-exposed and control offspring, may even more generally question whether the mouse hippocampal formation undergoes marked synaptic pruning between pubescence and adulthood. Synaptophysin is one of the most widely used synaptic markers, which is typically taken to index synaptic integrity and presynaptic density (Elferink and Scheller, 1995; Wiedenmann and Franke, 1985). Synaptophysin is already expressed during early neuronal differentiation, where it appears to have important roles in synapse formation (Eastwood et al., 2006; Friedman et al., 2000; Zai et al., 2000). The present findings of an age-dependent increase in hippocampal synaptophysin expression are consistent with previous studies that examined its basal expression from the early neonatal to the adolescent period (Eastwood et al., 2006). Similar to our results, Eastwood et al. (2006) did not find any evidence for an adolescence-related decline in basal hippocampal synaptophysin expression, as it is seen, for example, in cortical brain areas (Glantz et al., 2007; but see also Webster et al., 2011). In as much as synaptophysin can be considered a presynaptic marker (Elferink and Scheller, 1995; Wiedenmann and Franke, 1985), our findings presented here, together with those reported by Eastwood et al. (2006), suggest that the overall presynaptic density in the hippocampus is not reduced but rather increased between pubescence and adulthood.

At the same time however, we found that the hippocampal expression of the presynaptic protein bassoon generally decreased from pubescence to adulthood. This is in stark contrast to the expression pattern of synaptophysin, which generally increased from pubescent to adulthood. We admit that we have no parsimonious explanation for this differential pattern of temporal expression. On speculative grounds, however, this difference may be explained by the distinct role of these two presynaptic proteins in

governing synaptic strength and neurotransmitter release. The vesicle-associated protein synaptophysin is involved in the regulation of synaptic strength (Han and Stevens, 2009; Schmitt et al., 2009), but it does not seem to be required for the actual release of neurotransmitters from presynaptic vesicles (McMahon et al., 1996). On the other hand, bassoon is a scaffold protein of the presynaptic active zone and critically determines presynaptic neurotransmitter release through various (but not mutually exclusive) mechanisms (Davydova et al., 2014; Matz et al., 2010; Mendoza Schulz et al., 2014; Schröder et al., 2013;), including reloading of presynaptic vesicles to release sites at excitatory synapses (Hallermann et al., 2010). Based on our data, it may be speculated that hippocampal synaptic strength generally increases from pubescence to adulthood (as indexed by the overall increase in synaptophysin expression in adult relative to pubescent animals), which is consistent with the maturation-dependent increases in synaptic strength occurring in other brain areas (Kasanetz and Manzoni, 2009). One limitation of our study is, however, that we cannot further define whether the age-dependent changes in synaptophysin expression may reflect alterations in the number of presynaptic contacts and/or variations in the number of presynaptic vesicles per synapse. Moreover, it remains to be further explored whether the age-dependent changes in hippocampal bassoon expression may reflect changes in neurotransmitter reloading and/or basal neurotransmitter release, and whether they are specific to a certain neuronal population (e.g., excitatory pyramidal cells or inhibitory interneurons). Despite these limitations, our results tentatively suggest that prenatal immune activation impairs the maturation of presynaptic cellular processes that govern synaptic strength and neurotransmitter release."

Another intriguing finding of our study is that adult offspring of poly(I:C)-exposed mothers display a significant increase in hippocampal IL-1β expression even in the absence

of overt changes in microglia density and/or activation status. Even though in line with other studies (Garay et al., 2013), this effect may seem paradoxical in view of the numerous observations suggesting that microglia, especially those acquiring a pro-inflammatory (M1) phenotype, are the major source of IL-1β (and other pro-inflammatory cytokines) in the brain parenchyma (Hanisch, 2002; Ransohoff and Perry, 2009). Although this may hold true for various neuroinflammatory conditions, in which overt microgliosis and increased pro-inflammatory microglia activity exist, it is unlikely to be the case in our model. We also did not find associations between IL-1β expression in the hippocampus and plasma IL-1β concentrations, or between hippocampal IL-1β expression and astrocyte numbers. These findings indicate that neither systemic inflammation nor astrogliosis may be responsible for the increased hippocampal IL-1β levels in adult offspring of poly(I:C)-exposed mothers. In addition to microglia and astrocytes, however, neurons are known to express cytokines, including IL-1β, as well (Acarin et al., 2000; Erta et al., 2012; Freidin et al., 1992; Liu et al., 2005). Neuronal sources of cytokines have received somewhat less attention, but still appear to be relevant under various pathological conditions such as stress exposure (Kwon et al., 2008) and gray matter damage (Acarin et al., 2000; Liu et al., 2005). Even though the underlying mechanisms and functional role of increased hippocampal IL-1β expression remains to be examined in our model, our data clearly demonstrate that abnormal pro-inflammatory cytokine expression in the brain can occur without concomitant microglia abnormalities. These findings may also have important implications for the current attempts to define "neuroinflammation" in neurodevelopmental disorders such as schizophrenia and autism, especially for those that rely on the examination of microglia only (Doorduin et al., 2009; Kenk et al., 2015; Morgan et al., 2010; Pasternak et al., 2015; Tetreault et al., 2012; van Berckel et al., 2008).

In conclusion, our study shows that maternal poly(I:C)-induced immune activation in mice causes age-dependent hippocampal deficits in the offspring. Given the validity of the prenatal poly(I:C) administration model for neuropsychiatric disorders with neurodevelopmental components (Meyer and Feldon, 2010; Meyer, 2014; Reisinger et al., 2015), our findings suggest that prenatal infection and/or inflammation may be an early environmental risk factor for the development of synaptic abnormalities in these disorders. Another important implication of our findings is that the hippocampal synaptic deficits in offspring exposed to prenatal immune activation were not paralleled by overt microglial abnormalities, suggesting that the latter is not a prerequisite for the former. Interestingly, a recent study using the same poly(I:C)-based immune activation model in mice showed that there were no significant differences in fetal microglial cell density or activation levels between offspring of immune-stimulated and control mothers (Smolders et al., 2015). Our interpretations are consistent with these findings and generally question a major involvement of microglia-driven processes in precipitating short- and long-term (hippocampal) abnormalities following prenatal immune activation. Future attempts to identify the mechanisms underlying these hippocampal deficits should thus go beyond the possible role of altered microglia functions.

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Conflict of interest

All authors declare no conflict of interest.

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Figures

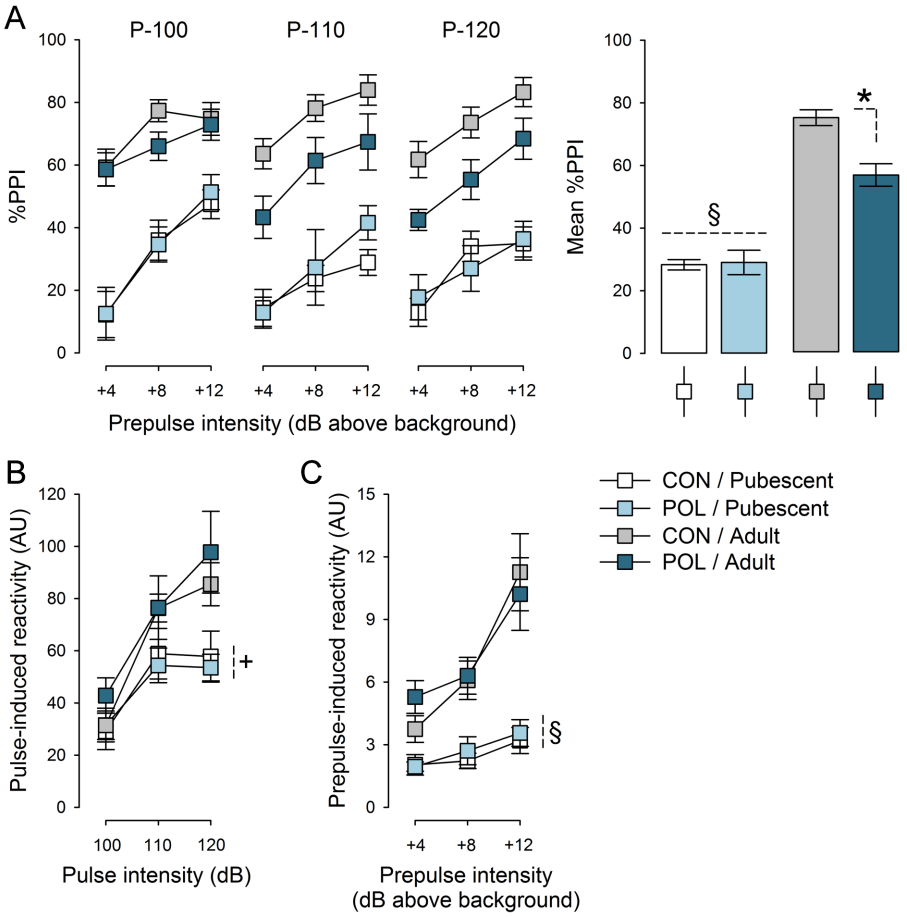


Figure 1. Effects of prenatal immune activation on prepulse inhibition of the acoustic startle reflex. **(A)** The line plot shows percent prepulse inhibition (%PPI) as a function of the 3 distinct pulse levels (P-100, P-110 and P-120, which correspond to 100, 110 and 120 dB_A) and prepulse levels (+6, +12 and +18 dB_A above background of 65 dB_A) for pubescent and adult offspring of poly(I:C)-exposed (POL) and control (CON) mothers, and the bar plot depicts the mean %PPI across all prepulse and pulse stimuli used. $^{\S}p < 0.001$, reflecting the significant difference between pubescent and adult offspring based on ANOVA; $^{*}p < 0.05$, reflecting the significant difference between adult CON and adult POL offspring based on Fisher's LSD post-hoc test. **(B)** The line plot illustrates the startle reactivity (in arbitrary units, AU) to pulse-alone trials for the 3 different pulse levels. $^{\S}p < 0.05$, reflecting the significant difference between pubescent and adult offspring based on ANOVA. **(C)** The line plot shows the prepulse-elicited reactivity (AU) for the 3 different prepulse levels (above background of 65 dB_A). $^{\S}p < 0.001$, reflecting the significant difference between pubescent and adult offspring based on ANOVA. $N = 8$ in each group; all values are means ± s.e.m.

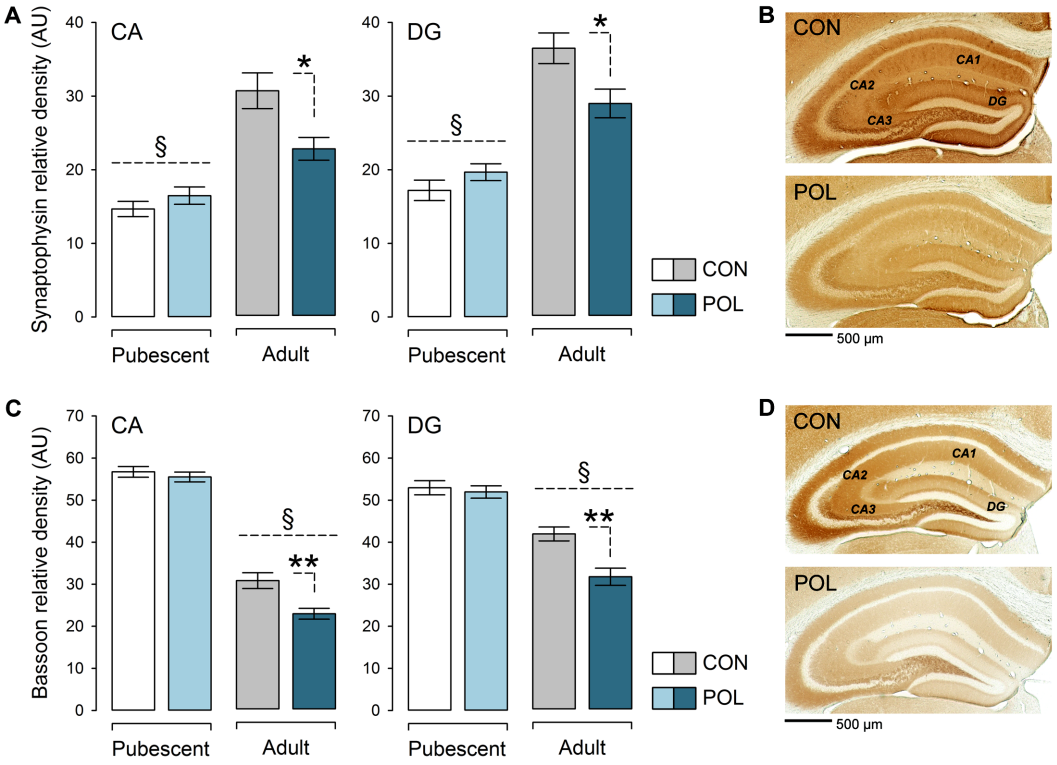


Figure 2. Effects of prenatal immune activation on presynaptic proteins in the hippocampus. **(A)** Relative optical density (in arbitrary units, AU) of synaptophysin in the dorsal cornu amonis (CA; CA1–CA3) and dentate gyrus (DG) of pubescent and adult offspring born to poly(I:C)-exposed (POL) and control (CON) mothers. §$p < 0.001$, reflecting the significant difference between pubescent and adult offspring based on ANOVA; *$p < 0.05$, reflecting the significant difference between adult CON and adult POL offspring based on Fisher's LSD post-hoc test. **(B)** The photomicrographs show representative hippocampal sections of adult CON and POL offspring stained with anti-synaptophysin antibody. **(C)** Relative optical density (AU) of bassoon in the CA and DG of pubescent and adult offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. §$p < 0.001$, reflecting the significant difference between pubescent and adult offspring based on ANOVA; **$p < 0.01$, reflecting the significant difference between adult CON and adult POL offspring based on Fisher's LSD post-hoc test. **(D)** The photomicrographs show representative hippocampal sections of adult CON and POL offspring stained with anti-bassoon antibody. $N = 8$ in each group; all values are means ± s.e.m.

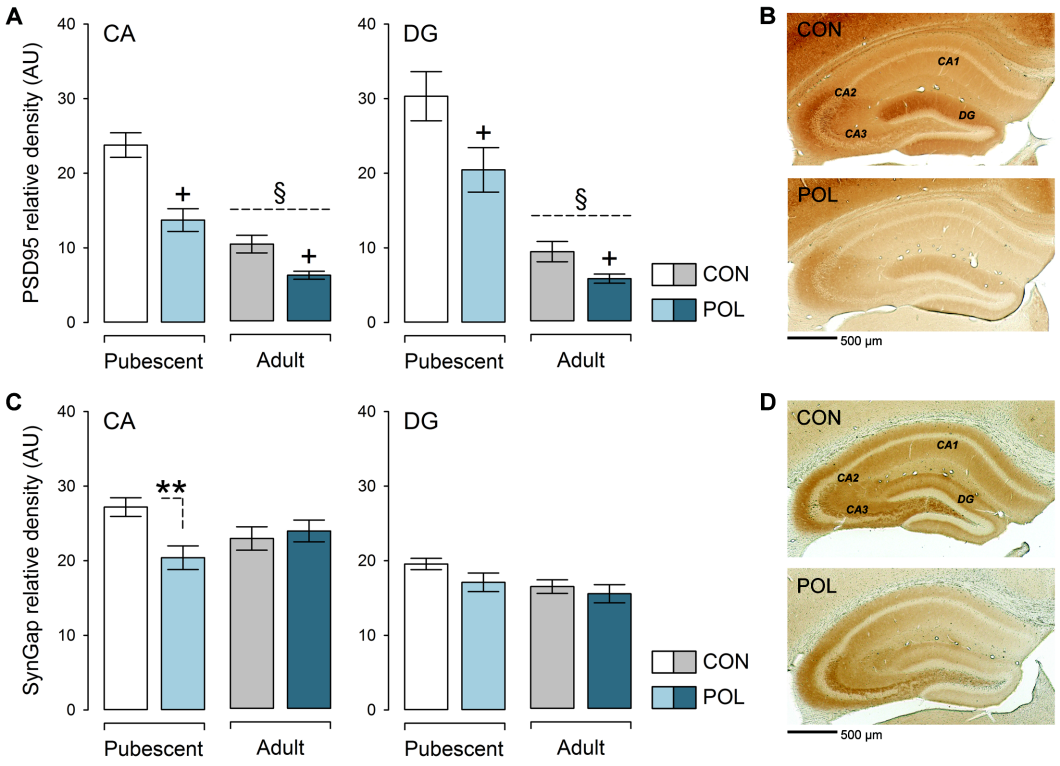


Figure 3. Effects of prenatal immune activation on postsynaptic proteins in the hippocampus. **(A)** Relative optical density (in arbitrary units, AU) of PSD95 in the dorsal cornu amonis (CA; CA1–CA3) and dentate gyrus (DG) of pubescent and adult offspring born to poly(I:C)-exposed (POL) and control (CON) mothers. $^{+}p < 0.001$, reflecting the significant difference between CON and POL offspring associated with the main effect of prenatal treatment by ANOVA; $^{\S}p < 0.001$, reflecting the significant difference between pubescent and adult offspring based on ANOVA. **(B)** The photomicrographs show representative hippocampal sections of adult CON and POL offspring stained with anti-PSD95 antibody. **(C)** Relative optical density (AU) of SynGap in the CA and DG of pubescent and adult offspring born to poly(I:C)-exposed (POL) or control (CON) mothers. $^{**}p < 0.01$, reflecting the significant difference between pubescent CON and pubescent POL offspring based on Fisher's LSD post-hoc test. **(D)** The photomicrographs show representative hippocampal sections of pubescent CON and POL offspring stained with anti-SynGap antibody. $N = 8$ in each group; all values are means ± s.e.m.

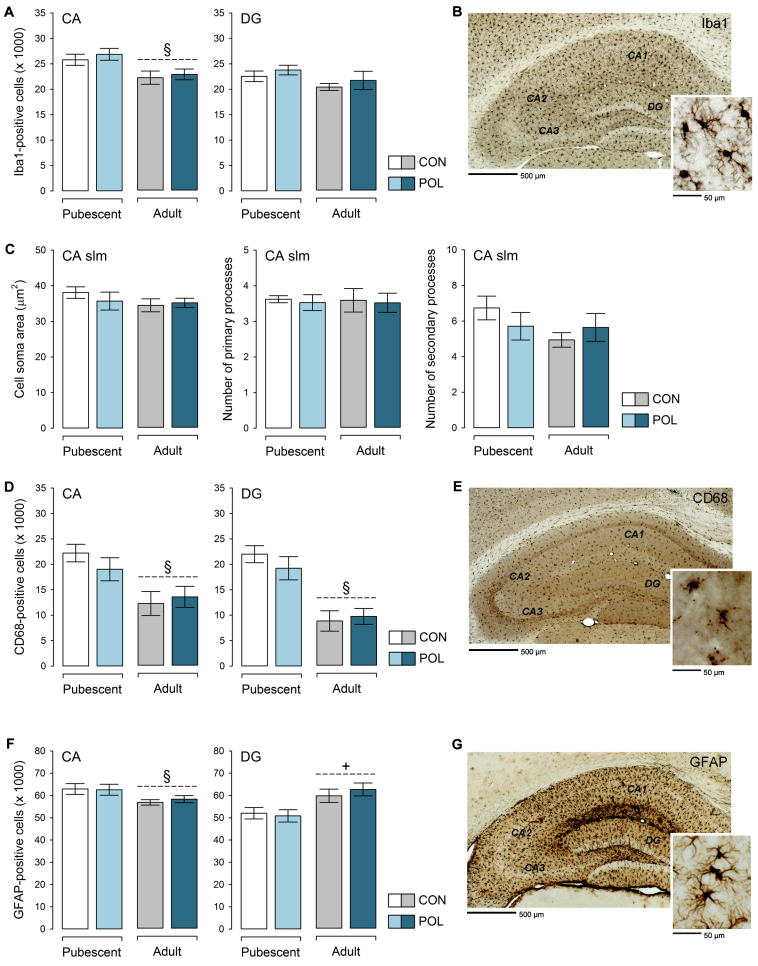


Figure 4. Effects of prenatal immune activation on microglia and astrocyte density and morphology. **(A)** Stereological estimates of Iba1-positive microglia in the dorsal cornu amonis (CA; CA1–CA3) and dentate gyrus (DG) of pubescent and adult offspring born to poly(I:C)-exposed (POL) and control (CON) mothers. §$p < 0.05$, reflecting the significant difference between pubescent and adult offspring based on ANOVA. **(B)** The photomicrograph shows a representative hippocampal section stained with anti-Iba1 antibody, and the inlet depicts Iba1-positive microglia at high magnification. **(C)** Cell soma area, number of primary processes, and number of secondary processes of Iba1-positive microglia in the CA stratum lacunosum moleculare (slm) of pubescent and adult CON and POL offspring. **(D)** Stereological estimates of CD68-positive microglia in the CA and DG of pubescent and adult CON and POL offspring. §$p < 0.05$, reflecting the significant difference between pubescent and adult offspring based on ANOVA. **(E)** The photomicrograph shows a representative hippocampal section stained with anti-CD68 antibody, and the inlet depicts CD68-positive microglia at high magnification. **(F)** Stereological estimates of GFAP-positive astrocytes in the CA and DG of pubescent and adult CON and POL offspring. §$p < 0.05$ and $^{+}p < 0.01$, reflecting the significant difference between pubescent and adult offspring based on ANOVA. **(E)** The photomicrograph shows a representative hippocampal section stained with anti-GFAP antibody, and the inlet depicts GFAP-positive astrocytes at high magnification. $N = 8$ in each group; all values are means ± s.e.m.

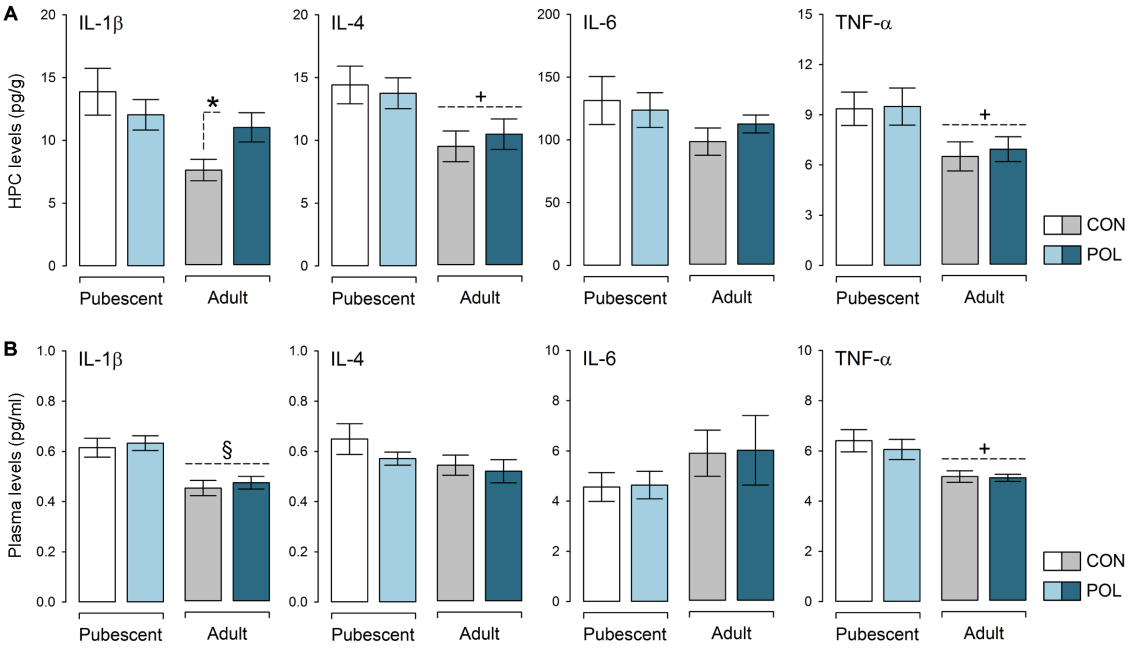
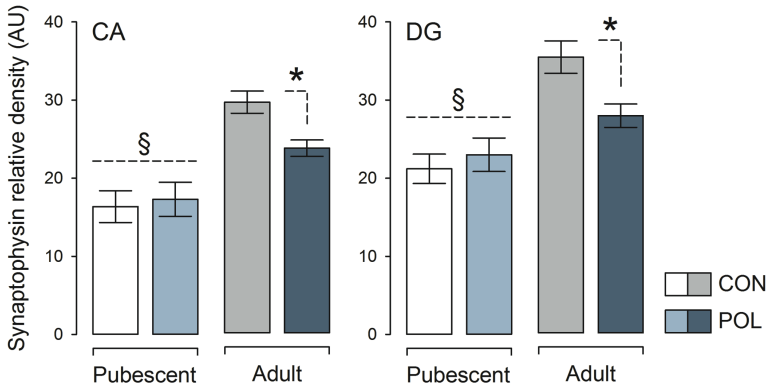
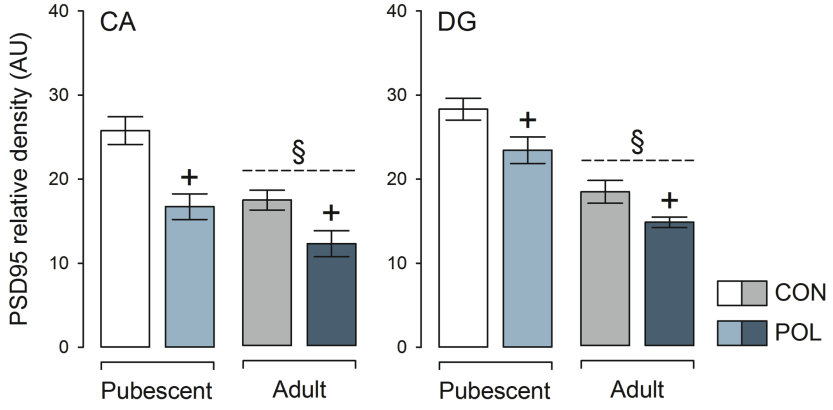


Figure 5. Effects of prenatal immune activation on cytokine levels in the hippocampus and plasma. **(A)** Cytokine levels in the hippocampus (HPC) of pubescent and adult offspring born to poly(I:C)-exposed (POL) and control (CON) mothers. $^{*}p < 0.05$, reflecting the significant difference between adult CON and adult POL offspring based on Fisher's LSD post-hoc test; $^{+}p < 0.05$, reflecting the significant difference between pubescent and adult offspring based on ANOVA. **(B)** Cytokine levels in the plasma of pubescent and adult POL and CON offspring. $^{+}p < 0.05$ and $^{\S}p < 0.001$, reflecting the significant difference between pubescent and adult offspring based on ANOVA. $N = 10$ per treatment group in pubescence, and $N = 8$ per treatment group in adulthood; all values are means ± s.e.m.

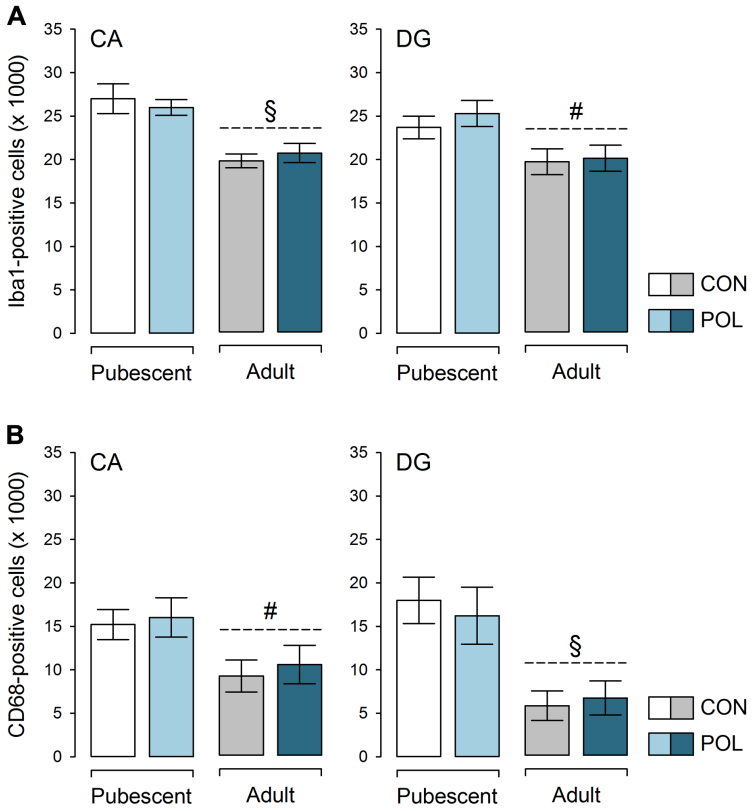
SUPPLEMENTARY DATA



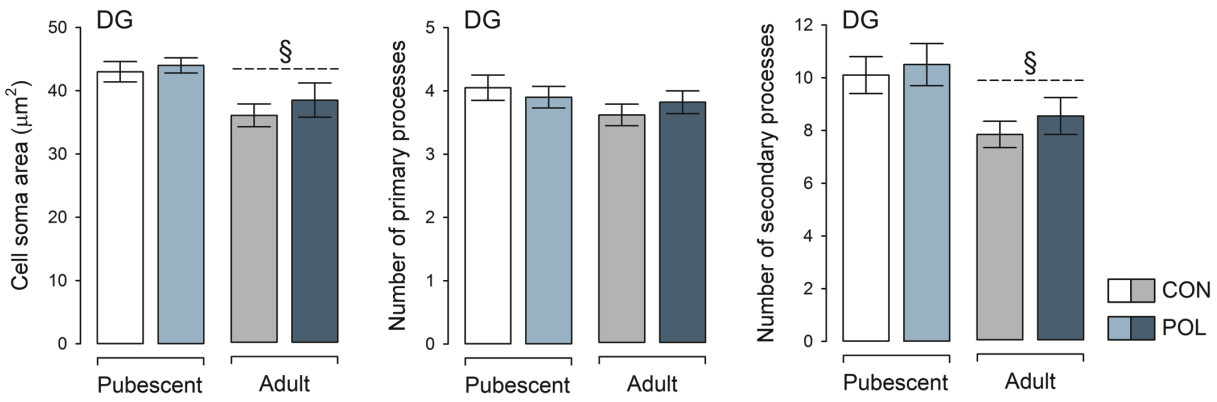
Supplementary Figure S1. Relative optical density (in arbitrary units, AU) of synaptophysin in the ventral cornu amonis (CA; CA1–CA3) and dentate gyrus (DG) of pubescent and adult offspring born to poly(I:C)-exposed (POL) and control (CON) mothers. §$p < 0.001$, reflecting the significant difference between pubescent and adult offspring based on ANOVA [CA: $F(1,28) = 36.43$; DG: $F(1,28) = 39.01$]; *$p < 0.05$, reflecting the significant difference between adult CON and adult POL offspring based on Fisher's LSD post-hoc test following the presence of a significant interaction between prenatal treatment and age [CA: $F(1,28) = 5.26$, $p < 0.05$; DG: $F(1,28) = 5.83$, $p < 0.05$]. $N = 8$ in each group; all values are means ± s.e.m.



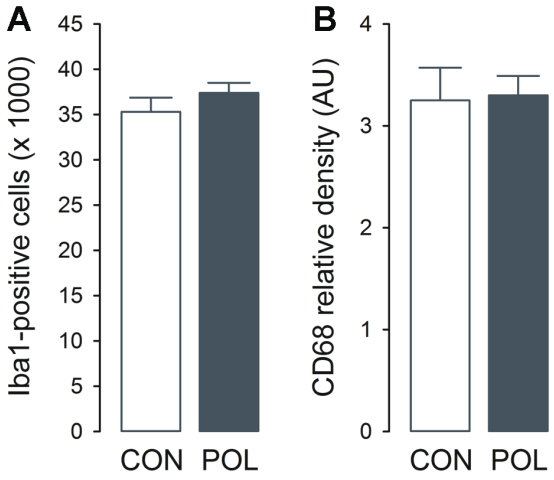
Supplementary Figure S2. Relative optical density (in arbitrary units, AU) of PSD95 in the ventral cornu amonis (CA; CA1–CA3) and dentate gyrus (DG) of pubescent and adult offspring born to poly(I:C)-exposed (POL) and control (CON) mothers. +$p < 0.001$, reflecting the significant difference between CON and POL offspring associated with the main effect of prenatal treatment [CA: $F(1,28) = 28.71$; DG: $F(1,28) = 20.36$] revealed by ANOVA; §$p < 0.001$, reflecting the significant difference between pubescent and adult offspring based on the main effect of age [CA: $F(1,28) = 26.13$; DG: $F(1,28) = 24.22$, $p < 0.001$] revealed by ANOVA. $N = 8$ in each group; all values are means ± s.e.m.



Supplementary Figure S3. (A) Stereological estimates of Iba1-positive microglia in the ventral cornu amonis (CA; CA1–CA3) and dentate gyrus (DG) of pubescent and adult offspring born to poly(I:C)-exposed (POL) and control (CON) mothers. $^{\#}p < 0.05$ and $^{\S}p < 0.01$, reflecting the significant difference between pubescent and adult offspring based on the main effect of age [CA: $F(1,28) = 9.43$, $p < 0.01$; DG: $F(1,28) = 5.92$, $p < 0.05$) revealed by ANOVA. **(B)** Stereological estimates of CD68-positive microglia in the CA and DG of pubescent and adult CON and POL offspring. $^{\#}p < 0.05$ and $^{\S}p < 0.01$, reflecting the significant difference between pubescent and adult offspring based on the main effect of age [CA: $F(1,28) = 5.08$, $p < 0.05$; DG: $F(1,28) = 9.38$, $p < 0.01$] revealed by ANOVA. $N = 8$ in each group; all values are means ± s.e.m.



Supplementary Figure S4. (A) Cell soma area, number of primary processes, and number of secondary processes of Iba1-positive microglia in the dentate gyrus (DG) of pubescent and adult CON and POL offspring. §$p < 0.01$, reflecting the significant difference between pubescent and adult offspring based on the main effect of age [cell soma area: $F(1,28) = 8.98$, $p < 0.01$; number of secondary processes: $F(1,28) = 10.36$, $p < 0.01$] revealed by ANOVA. $N = 8$ in each group; all values are means ± s.e.m.



Supplementary Figure S5. Microglia parameters in juvenile offspring of prenatally immune-challenged and control mothers. Pregnant C57BL6/N female mice were treated with the viral mimetic poly(I:C) (5mg/kg, i.v.) or control vehicle solution (sterile pyrogen-free 0.9% NaCl), and the resulting offspring were killed shortly after weaning on postnatal day 21 for immunohistochemical analyses of microglia parameters as fully described in the main text. **(A)** Stereological estimates of Iba1-positive microglia in the hippocampal formation (including CA1–CA3 and dentate gyrus region) of juvenile offspring born to poly(I:C)-exposed (POL) and control (CON) mothers. **(B)** Relative optical density (in arbitrary units, AU) of CD68 immunoreactivity in the hippocampal formation (including CA1–CA3 and dentate gyrus region) of juvenile POL and CON offspring. N(CON) = 9 and N(POL) = 10; the offspring stemmed from 12 independent litters (6 CON and 6 POL litters). All values are means ± s.e.m.