Maternal inflammation significantly impacts cortical interneuron development in a subtype-specific manner

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Abstract

Severe infections during pregnancy are one of the major risk factors for cognitive brain impairment in offspring. It has been suggested that maternal inflammation leads to dysfunction of cortical GABAergic interneurons that in turn underlies cognitive impairment of the affected offspring. However, the evidence comes largely from studies of adult or mature brain and how impairment of inhibitory circuits arises upon maternal inflammation is unknown. Here we show that maternal inflammation affects multiple steps of cortical GABAergic interneuron development, i.e. proliferation of precursor cells, migration and positioning of neuroblasts as well as neuronal maturation. Importantly, the development of distinct subtypes of cortical GABAergic interneurons was discretely impaired as a result of maternal inflammation. This translated into a reduction in cell numbers and redistribution across cortical regions and layers. Furthermore, vulnerability of GABAergic interneuron subtypes was associated with varying impact of maternal inflammation on interneuron precursor pools that depends on the stage of brain development. Thus, differential effect of maternal inflammation on GABAergic interneuron subtypes and the time of insult might be key factors contributing to etiology of cognitive impairment in maternal inflammation-affected offspring.

Introduction

Development of the fetal brain is highly influenced by the maternal environment. A multitude of factors such as maternal nutrition, stress, hormonal imbalance as well as the maternal immune status play key roles in shaping normal brain development[1]. Maternal inflammation is known to increase the risk for severe psychiatric disorders including schizophrenia, bipolar disorder, intellectual disability, anxiety, autism spectrum disorders (ASDs) and cerebral palsy[2,3] with high societal costs[4]. Although the exact mechanism of adverse neurodevelopment upon maternal inflammation is not known, data from animal experiments and clinical observations suggest that the cytokine-related pro-inflammatory response in the mother contributes to disordered development of the fetal brain and predisposes the offspring to additional stressors during postnatal maturation of the brain[5–7].

Cortical GABAergic interneurons arise during embryogenesis in the ganglionic eminences (GEs). Two of the three classes of GABAergic interneurons are generated in the medial ganglionic eminence (MGE), i.e., the parvalbumin (PV)- and somatostatin (SST)-expressing interneuron types, whereas the third class is generated in the caudal ganglionic eminence (CGE) and gives rise to a highly heterogeneous group of neurons that express serotonin receptor 3A (5HT3AR) and consists of vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), reelin-positive and a few other subtypes[8–11]. In mice, cortical GABAergic interneurons are produced during middle-late gestation (E9.5-E18.5)[12,13], and migrate tangentially from the GEs to the cortical plate, where they start migrating radially into prospective cortical layers[14,15]. Subsequently, a significant proportion of interneurons undergoes programmed cell death[16,17], while the surviving cortical interneurons mature over two to three months in mice[9].

Excitation-inhibition imbalance in the brain has been proposed as a major factor underlying the behavioral outcomes and cognitive decline associated with various neurodevelopmental disorders, including those associated with maternal inflammation[18–21]. Indeed, several studies show changes in activity and distribution of GABAergic interneurons in the cortex and hippocampus of the adult mouse brain upon maternal inflammation[22–25]. Such abnormalities are thought to occur due to

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perturbed brain development during early fetal or juvenile periods. Importantly, the development of neuronal circuits in the brain continues until late adolescence, up to 60-70 days postnatally in rodents and 20-25 years in humans[26]. Despite being acute, maternal inflammation can have long-lasting effects on several critical developmental processes such as precursor cell proliferation, neuronal migration and differentiation during embryogenesis, as well as postnatal neuronal maturation including neuronal survival, dendritic, synaptic and axonal pruning and synaptogenesis. Some studies have addressed the developmental impairment of principal cortical neurons upon maternal inflammation[6,27,28], but surprisingly little is known regarding how development and maturation of cortical GABAergic interneurons are affected, and more importantly how those defects of GABAergic interneurons that are observed in adult[22–25,29] arise during brain development. Abnormal development of cortical inhibitory circuits will have a significant impact on animal behavior leading to phenotypes resembling human psychiatric disorders as has been shown for a number of genetic mouse models[30–32]. Furthermore, as cortical GABAergic interneurons represent a diverse class of neurons with more than twenty subtypes[33,34], various subtypes of GABAergic interneurons might be differentially affected by maternal inflammation contributing to the complex behavioral abnormalities of the offspring.

To study the impairment of cortical interneuron development due to maternal inflammation, we utilized a mouse model of maternal inflammation that involved injecting polyriboinosinic–polyribocytidilic acid (poly I:C) at gestational day 9.5 (GD9.5). Poly I:C is a synthetic polynucleotide that mimics viral double stranded (ds) RNA and is known to cause an acute inflammatory response by activating pro-inflammatory cytokines including IL-6[35,36]. Following this, we undertook a detailed analysis of embryos and pups exposed to maternal inflammation. We found poly I:C to have an acute effect on GABAergic interneuron precursor proliferation with follow-up effects on the migration, positioning and maturation of GAD+ neuroblasts. The effect of maternal inflammation was interneuron subtype-specific and demonstrated differential vulnerability of interneuron subtypes to mother-derived insults.

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Materials and Methods

Animal breeding and genotyping

All animal experiments were conducted in accordance with the guidelines of the National Animal Ethic Committee of Denmark. C57BL/6J (Janvier Labs), PV[Cre] (017320, Jackson Labs), ROSA-tdTomato (007905, Jackson Labs) and GAD67-EGFP (Gad1[tm1.1Tama])[37] mice were used in this study, maintained in IVC cages and provided food and water *ad libitum*. Heterozygous GAD67-EGFP mice were bred with wildtype mice. Date of the vaginal plug was treated as GD0.5, and embryos and pups were timed accordingly. GD is used here to refer to the maternal stage of pregnancy while E is to indicate the age of the embryo.

EGFP+ animals were identified by PCR using the following primers (in 5'-3' orientation) that amplified a 345 bp region:

EGFP_F   CCTACGGCGTGCAGTGCTTCAGC

EGFP_R   CGGCGAGCTGCACGCTGCGTCCTC

Induction of Maternal Immune Activation using poly I:C

Commercially prepared poly I:C (Sigma, P9582) was purchased and dissolved in phosphate buffered-saline (PBS) to give a 1 mg/ml stock solution.

Pregnant females at E9.5 received a single tail intravenous (i.v) injection of poly I:C equivalent to 5mg/kg body weight under mild restraint. Control females received an equal volume of PBS.

For experiments involving BrdU labelling of progenitors, a final dose of 50 mg/kg of BrdU (Sigma, B5002) was injected intra-peritoneally (i.p) into dams at GD14.5. Dams were sacrificed 2 hours after labelling and embryo were dissected and their heads collected in 4% paraformaldehyde solution.

Plasma IL-6 estimation

Three hours-post poly I:C or PBS injection, blood samples were taken from the tail vein in EDTA pre-coated Eppendorf tubes. Following centrifugation (3000 rpm, 10 mins), the supernatant was

collected in a separate tube and stored at -80°C when all samples were analyzed together. A Mouse IL-6 DuoSet ELISA kit (R&D Systems) was used to measure IL-6 levels. Absorbance was measured at 450 and 560 nm using a Glomax microplate reader (Promega). For analysis, readings at 560 nm were subtracted from those at 450 nm and normalized to blank controls. Values were obtained by linear regression of a plot of concentrations of known standards versus normalized absorbance values.

Mouse behavior

PBS- and poly I:C-treated offspring (P35, male and female) were first acclimatized to the room and subsequently placed in the center of a circular open field (OF) of 1 m diameter. Movement was recorded for 10 mins using the Noldus Ethovision XT video tracking system v5 (Noldus Information Technology). Time spent in the central zone (0.7 m inner diameter) and periphery was extracted as were basic locomotor parameters such as total distance travelled, in total and in 1-min intervals and mean velocity.

Social interaction was tested immediately after the Open Field test. For this, two rectangular wire containers (6.2 cm x 7.8 cm x 12.4 cm, HxWxL) large enough to hold a mouse and allowing for social interaction were placed equidistant from the center of the open field. An unknown mouse was alternated between the two containers with the other kept empty. A region of 10 cm around the containers was treated as the social interaction zone, the remaining part of the arena as a non-social zone. Time spent by control and poly I:C mice in the social and non-social zones was extracted via Ethovision.

Acoustic startle reaction (ASR) and pre-pulse inhibition (PPI) were tested at 5 weeks of age as described[38] in two chambers (San Diego Instruments, San Diego, USA) with 70 dB(A) white background noise. A piezoelectric accelerometer transduced displacement of mouse test tubes (∅ 3.6 cm) in response to movements of the animal. Animals were acclimatized for 5 min in the tube before sessions started and ended with 5 startle trials of 40 ms 120 dB(A) bursts of white noise. In

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between, 35 trials were delivered in semi-randomized order (10 trials of 120 dB(A); 5 each of 4 pre-pulse + startle trials (pre-pulses of 72, 74, 78, and 86 dB(A)); 5 trials with only background noise). Tube movements were averaged over 100 ms following onset of the startle stimulus (AVG). The five AVGs for each pre-pulse intensity were averaged and used to calculate PPI, which was expressed as percent reduction in averaged the pre-pulse AVGs compared to the average of the 10 middle startle trials: %PPI = [1-(Pre-pulse + pulse/Pulse)] x 100.

Perfusion, Sectioning and Immunohistochemistry

All steps were performed at ambient temperature unless otherwise noted.

Postnatal mice were anaesthetized with a combination of xylazine and ketamine injected i.p. This was followed by transcardial perfusion initially with cold PBS to flush out blood and then with cold 4% paraformaldehyde (PFA). Brains were then removed and post-fixed in 4% PFA overnight at $4°C$ before being stored in PBS with 0.01% Sodium Azide.

Brains were sectioned using a vibrating microtome (Leica, VT1000S) at a thickness of 50 μm and stored in PBS with 0.01% Sodium Azide at $4°C$. Embryonic day (E) 10.5 to 14.5 embryos were fixed in 4% PFA overnight, dehydrated in 30% sucrose and sectioned at 25 μm thickness using a cryostat (Leica CM3050).

For staining, sections were blocked and permeabilized with 3% BSA (in 0.2% Triton X-100 containing PBS). Subsequently, they were incubated overnight at $4°C$ with appropriate primary antibodies. The following day, sections were washed and incubated with Alexa Fluor conjugated secondary antibodies for 2 hours at room temperature. Nuclei were counterstained with DAPI (Sigma) and coverslips were mounted on slides with FluorSave (Merck).

For BrdU and Nkx2.1 double labelling, antigen retrieval was first performed using sodium citrate solution (10mM) at $85°C$ for 15 min. Sections were then cooled and treated with 1M HCl at $37°C$ for 30 min. After quenching with 10mM Tris-HCl (pH8.5), antibody labelling was carried out as described above. A description of antibodies used in this study is given below:

ANTIBODY	MANUFACTURER	CATALOG NUMBER	CONCENTRATION
Chicken anti- GFP	Invitrogen	A10262	1:1000
Rabbit anti- Parvalbumin	Swant	PV27	1:1000
Mouse anti- SST	Santa Cruz	sc-55565	1:1000
Rabbit anti- VIP	Acris	20077	1:1000
Rabbit anti- Nkx2.1	Abcam	ab76013	1:500
Mouse anti- BrdU	Becton Dickinson	347580	1:500
Rabbit anti- Caspase3	R&D Systems	MAB835	1:1000
Mouse anti- COUP-TFII	R&D Systems	PP-H7147-00	1:100
SECONDARY ANTIBODIES			
Goat anti-chicken 488	Life Technologies		1:500
Goat anti-mouse 546	Life Technologies		1:500
Donkey anti-rabbit 594	Life Technologies		1:500
Donkey anti-mouse 647	Life Technologies		1:500
Donkey anti-rabbit 647	Life Technologies		1:500

Image acquisition and analysis

Images were acquired using a confocal microscope (Leica SP8, Leica Microsystems) and analyzed using ImageJ (NIH) and Imaris (Bitplane AG). After correcting for brightness and contrast, figures were prepared using Adobe Illustrator (Adobe Inc). Graph preparation and statistical analysis were carried out using Prism 7.0 (GraphPad).

Distribution of GABAergic interneurons across the cortex was analyzed in the region of interest (ROI) spanning from the upper edge of the corpus callosum or the subventricular zone (SVZ) to the pia at P1-60 old mice or E14.5-E17.5 old mice, respectively, and having 200-400 μm in width

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(medial-lateral) depending on age. The whole length of ROI was subdivided in 10 equal bins for E14.5-P9 ages, and in 6 layers at P15-60 when cortical layering could be distinguished based on DAPI staining.

The angle of neuroblast migration at E17.5 was measured in the intermediate zone and cortical plate using ImageJ relative to the vertical axis stretching from the ventricle to the pia.

Electrophysiology

Acute brain slices were prepared from P53-57 old mice. To this end, mice were deeply anesthetized with Isoflurane (3%) and the brains quickly removed and dissected in ice-cold sucrose-containing ACSF (212 mM sucrose, 0.02 mM $CaCl_2$, 7mM $MgCl_2$, 3 mM KCl, 1.25mM NaH_2PO_4, 26mM $NaHCO_3$, 10mM d-glucose oxygenated with carbogen). 250µm thick coronal brain slices were cut with the help of a tissue slicer (Leica VT1200S, Wetzlar, Germany) in ACSF (25 mM d-glucose, 125 mM NaCL, 1.25mM NaH_2PO_4, 26mM $NaHCO_3$, 2.5 m M KCl, 2 mM $CaCl_2$, 1mM $MgCl_2$ oxygenated with carbogen).

Brain slices were visualized under an upright Olympus BX51WI microscope fitted with a 40x water-immersion objective (LUMPlan FI/IR, NA 0.8w; Olympus, Japan) with infrared optics. Whole-cell recordings were obtained from single neurons in layer 2/3 of the somatosensory cortex under visual guidance. On average, 2-3 neurons per brain slice were used. Borosilicate glass pipettes (3-5MΩ) were pulled with a micropipette puller (Sutter Instruments, Novato, CA, USA) and filled with intracellular solution (130 mM K-gluconate, 10 mM HEPES, 10 mM phosphocreatinine-Na, 10 mM Na-gluconate, 4 mM ATP-Mg, 0.3 mM GTP, 4 mM NaCl; pH 7.2). Intrinsic membrane properties and firing patterns were recorded in current clamp mode with 50 pA current steps ranging from -50 pA to 900 pA. Intrinsic membrane properties and firing properties were analyzed with IGOR Pro (WaveMetrix, USA) and Microsoft Excel (Microsoft, USA).

Statistical analysis

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Normality of data distribution was analyzed by D'Agostino and Shapiro-Wilk's tests. Normally distributed data was analyzed using the Student's t-test (for 2 groups) or by 1-way ANOVA test (for more than 2 groups). Welch's correction was applied when standard deviations of the two groups differed from each other. For simultaneous comparison of 2 parameters between 2 and more groups, we used 2-way-ANOVA and the Bonferroni post-hoc test. Equality of variances was analyzed using Dunnett's test. Statistical analysis of the data was performed with Prism (GraphPad software, USA).

Results

Maternal inflammation model in transgenic mice that labels all cortical GABAergic interneurons

In order to reveal how the development of cortical GABAergic interneurons is affected by maternal inflammation, we utilized the GAD67-EGFP knock-in transgenic mouse line that labels all GABAergic interneurons in the cortex by EGFP[37]. Maternal inflammation was induced by injecting poly I:C in pregnant dams at GD9.5 corresponding to the early first trimester of pregnancy in humans[39]; the period of highest risk of induction of neurodevelopmental disorders caused by maternal infections[40]. This period also marks the onset of cortical GABAergic interneuron production in mice which starts in the MGE at E9.5[41]. We confirmed the induction of inflammation by an increase in maternal plasma IL-6 levels three hours after injection of poly I:C (Figure 1a). To validate that maternal inflammation in our model had a functional impact on the offspring, we performed behavioral experiments on wild-type mice testing for social interaction and memory, open field and startle (including pre-pulse inhibition) behavior. Control mice on average spent significantly more time interacting with the unfamiliar mouse in comparison to the inanimate object (the empty container), while offspring affected by maternal inflammation did not have such a preference (Figure 1b). Control offspring also interacted with the unfamiliar mouse for longer durations than those affected by maternal inflammation (Figure 1c). In addition, offspring affected by maternal inflammation displayed signs of hyperactivity observed as an increase in the total distance travelled and in running speed in the open field test (Figure 1d,e). Startle behavior was similar in control and maternally inflamed offspring for the basal startle, i.e. the response to the 120 dB noise bursts (data not shown). However, there was a significant effect of prenatal exposure on prepulse inhibition. Separate analysis of each prepulse (72, 74, 78 and 86 dB) showed that this owed to differences at the 72 dB prepulse level, as offspring from Poly I:C mothers had lower inhibition compared to control offspring (Figure 1f, Suppl Figure 1). Thus, maternal inflammation triggered by poly I:C injection led to impaired social behavior, increased anxiety and sensorimotor gating deficits in wild-type mice. These phenotypes were similar to those observed in previous studies of poly I:C injected mice mice[5,6,22,42–46].

Maternal inflammation impacts early stages of cortical GABAergic interneuron development

While the effect of maternal inflammation on offspring behavior has been well documented, there is little information about how maternal inflammation affects the development of inhibitory circuits in the cortex that might underlie abnormal animal behavior. We therefore investigated the effect of poly I:C (injected at GD9.5) on the early stages of interneuron development. Cortical GABAergic interneurons originate between E9.5 and 18.5 in the GEs[12,13] from where they migrate tangentially into the developing cortex. They enter the cortical plate at two principal sites – a superficial stream along the marginal zone just below the pia, and a deeper stream at the intermediate zone/ sub-ventricular zone (IZ/SVZ)[47,48]. Once in the developing cortical plate, GABAergic interneurons switch to a radial mode of migration and invade the cortical plate (Figure 2a). Strikingly, we observed a clear effect of maternal inflammation on cortical GABAergic interneurons early during their development, since already at E14.5 fewer EGFP+ neuroblasts were observed in the IZ of poly I:C exposed embryos at the regions of the developing cortex corresponding to the motor and somatosensory cortices (Figure 2b,c) (note that in Figure 2 and further, motor and somatosensory regions in the developing cortex are those that will become motor and somatosensory cortices in the adult brain). To study differences in migratory patterns of GABAergic interneurons, we chose E17.5 time-point when the majority of interneurons reach the developing cortex and start to migrate radially within the cortex. We divided the cortex into 10 equally sized bins (see materials and methods) and quantified the number of EGFP+ neuroblasts in each. Remarkably, maternal inflammation resulted in large-scale redistribution of GABAergic interneurons with an evident reduction in density of EGFP+ neuroblasts in the regions corresponding to the motor and somatosensory cortices of the developing cortex at E17.5 (Figure 2d,d'). Quantification of densities per bin showed that pups exposed to maternal inflammation had fewer EGFP+ neuroblasts in the superficial layers of the cortical plate (Figure 2e, bins 3-4 for M1 and bin 4 for S1), which also resulted in a decrease in total neuroblast density across the cortical plate and intermediate zone (Figure 2f). However, while the developing somatosensory cortex showed an additional increase in

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percentage of EGFP+ neuroblasts to stack in the middle layers, a similar effect was not seen in the motor cortex (Figure 2d,d',e, bin 5), indicating a differential effect of maternal inflammation on different cortical areas.

To account for the effect on distribution of GAD+ neuroblasts caused by acute maternal inflammation we analyzed the directionality of neuroblast migration in the IZ and cortical plate at E17.5. While GAD+ neuroblasts in the cortex of control offspring predominantly migrated towards the pia, their direction in inflammation-affected cortices was significantly different with a greater fraction migrating laterally (Figure 2f).

Maternal inflammation impairs positioning of GABAergic interneurons in the developing cortex

As interneuron migration continues until the second postnatal week, we chose to analyze whether these initially observed differences continue to affect the positioning of interneurons in the postnatal cortex. We hence studied several postnatal stages from P3 to P30 to understand how maternal inflammation can lead to a disordered arrangement of GABAergic interneurons in the mature cortex.

At P3 and P6, the difference in GAD+ cell density had amplified to cover the entire length of the cortical plate with the exception of the lowermost bins (bins 9-10, Figure 3a,b). Furthermore, the magnitude of the difference was consistently greater in the prospective somatosensory cortex than the motor cortex. Interestingly, the relative distribution of GAD+ neuroblasts across bins was similar in pups exposed to PBS or poly I:C (Suppl Figure 2a,b). This suggests that the decrease in GAD+ neuroblasts is equally distributed across the whole length of the cortex. By P9, while the reduction in density persisted in the somatosensory cortex, much lower difference could be observed in the motor cortex (Figure 3c) indicating a differential effect of maternal inflammation on neuroblasts destined for distinct cortical regions.

Cortical GABAergic interneurons are overproduced during embryogenesis, and half of them subsequently undergo apoptosis within the second postnatal week[16,17]. We therefore investigated whether an increase in programmed cell death of cortical interneurons due to maternal

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inflammation could explain in part the reduction of their number in the cortex of maternal inflammation-affected mice. To this end, we counted activated caspase-3 and EGFP-expressing cells in several sections covering the motor and somatosensory cortices. At both P3 and P6, we observed a similar number of activated caspase-3+ (Casp3+) as well as EGFP+ Casp3+ cells in control pups and pups exposed to maternal inflammation (Figure 3d,e). Thus, activation of the maternal immune system during early gestation did not augment apoptosis in the developing cortices of pups exposed to maternal inflammation.

To follow up the effect of maternal inflammation during postnatal brain maturation, we further studied the density and positioning of interneurons at P15. Similar to P9, the density of GAD+ interneurons in S1 was severely reduced (Figure 3f,g). Analysis of the layer-wise distribution of interneurons showed an equal effect across the whole cortical length in the somatosensory cortex (Figure 3h). In contrast, in the motor cortex, despite a small decrease in the density of GAD+ interneurons in the layer 2/3, no statistically significant difference was observed (Suppl. Figure 2c). We hence show that an acute induction of inflammation in pregnant dams impairs migration and final positioning of GAD+ neuroblasts with the effect being more pronounced in the somatosensory than in the motor cortex.

Differential effect of maternal inflammation on distinct subtypes of cortical GABAergic interneurons
While functional impairment of cortical GABAergic interneurons has been proposed to be one of the major factor contributing to imbalance between excitation and inhibition in patients with schizophrenia and other psychiatric disorders, most of the attention has been directed towards dysfunction of PV+ GABAergic interneurons[25,49,50]. However, cortical GABAergic interneurons are highly heterogeneous, and PV+ interneurons represent only one class of interneurons[8]. Therefore, the differential impact of maternal immune activation on development and maturation of various interneuron subtypes remains to be elucidated.

To this end, we studied the effect of maternal inflammation on the organization of the three largest interneuron populations, i.e. those that express PV, SST and VIP (Figure 4a,b, Suppl. Figure 2d,e).

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In the somatosensory cortex, the previously observed decrease in the density of GAD+ interneurons stemmed from a decrease in both PV+ and SST+ interneurons (Figure 4a-d). Interestingly, there was a clear layer-specific effect of maternal inflammation in the distribution of PV+ and SST+ interneurons. Thus, the decrease in number of PV+ interneurons was restricted to layer 4 at P15 (Figure 4c), whereas the reduction of SST+ interneurons was more pronounced across all layers, albeit statistically significant only in layers 2-4 at P15 (Figure 4d). There was no effect of maternal inflammation on the distribution of CGE derived VIP+ interneurons at P15 (Suppl. Figure 2d,e). At P60, the density of PV and SST was not statistically significant between maternal inflammation-affected and control animals even though an appreciable difference could be seen in the case of L4 SST+ neurons (Figure 4e,f). This is likely caused by the non-uniform expansion of cortical areas during synaptogenesis and gliogenesis[51]. In the motor cortex, there was no significant effect on distribution of interneuron subtypes at P15 and P60 (data not shown).

In addition to the reduced interneuron density, a reduction in PV expression in the cortex and hippocampus has been described in several studies for adult mice[21]. While we confirmed that maternal poly I:C exposure led to a similar reduction in PV expression in both M1 and S1 cortical regions at P60 (Figure 4i,j), surprisingly, PV+ interneurons in both regions had an excess of PV expression at P15 (Figure 4g,h). As parvalbumin is an important calcium binding protein in neurons[52], and its expression positively correlates with neuronal activity in the developing cortex[53], the developmental impairment in calcium buffering capacity in PV-expressing neurons might contribute to their functional impairment. Previous studies have shown maternal immune activation to be detrimental to PV+ interneurons of the medial prefrontal cortex (mPFC) leading to decreased GABAergic transmission onto pyramidal neurons[22]. We thus assessed the morphology and functional properties of PV+ interneurons in the superficial layers of the somatosensory cortex in control and animals prenatally exposed to maternal inflammation at P53-57. PV+ interneurons were labelled by Cre-recombinase dependent tdTomato expression (*PV^Cre^; ROSA-tdTomato*) and were assessed for intrinsic electrophysiological properties using whole-cell patch-clamp recordings of fluorescent neurons in acute brain slices (Figure 4k-n). The resting membrane potential in poly

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I:C-exposed animals was found to be significantly hyperpolarized as compared to controls (Figure 4k,l). In addition, the firing frequency of PV+ interneurons was reduced in poly I:C-exposed animals (Figure 4n) reflecting an overall slower spiking rate due to maternal immune activation.

To investigate if the changes in electrophysiological properties are accompanied by anatomical changes, we analyzed the morphology of biocytin-filled PV+ interneurons (Figure 4o). There was no significant effect of maternal inflammation on the length of dendritic filaments (Figure 4p) and branch depth (Figure 4q). However, Sholl analysis of the reconstructed interneurons showed a significant reduction in overall dendritic tree complexity of the maternal inflammation-affected PV+ interneurons (Figure 4r). These results indicate that acute maternal immune response affects morphology and electrophysiology of GABAergic interneurons.

Timing of maternal inflammation affects discreet pools of interneuron progenitors

One of the most remarkable effects of maternal inflammation on development of the GABAergic interneurons was the decrease in the density of EGFP+ neuroblasts as early as E14.5 when maternal immune activation was induced at GD9.5 (Figure 2b,c). As cortical GABAergic interneurons are generated by precursor cells mainly in the MGE and CGE between E9.5 and 18.5[12,13], acute maternal inflammation might affect the generation of GABAergic interneurons. To investigate this, GAD67-EGFP pregnant mice were injected with poly I:C at GD9.5 and proliferating cells were labelled using a 2-hour pulse of the thymidine analog, 5-bromo-2'-deoxyuridine (BrdU), at GD10.5 or GD14.5. By co-labeling interneuron precursors for BrdU along with Nkx2-1 (MGE-specific marker[41,54]) or COUP-TFII (CGE-specific marker[55]), we sought to ascertain differences in proliferation of interneuron precursors due to maternal inflammation. At E10.5, we found a ~25% decrease in the percentage of Nkx2.1+ precursors that co-labelled with BrdU in embryos exposed to maternal inflammation in comparison to unexposed mice (Figure 5a,b). In contrast, both groups showed equal rates of Nkx2.1+ progenitor cycling five days post poly I:C treatment, i.e. at E14.5 (Figure 5c). This indicates an acute effect of maternal inflammation on the cycling of interneuron progenitors. In keeping with the acute response of progenitors, maternal inflammation exhibited no

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effect on the proliferation of COUP-TFII+ progenitors over the same period (Figure 5d-e), which correlated with a lack of an effect on VIP+ interneurons in the mature cortex, thus indicating a subtype-specific effect of maternal inflammation on interneuron progenitors. Since the majority of CGE-derived interneurons are born between E12.5-18.5[12], we injected dams with poly I:C at GD12.5 to investigate whether a later induction would impair the proliferation of CGE progenitors. As with the previous analysis, a 2-hour pulse of BrdU was given before collecting the embryonic brains for analysis at E14.5. We observed a ~20% decrease in COUP-TFII+ progenitors that co-labelled with BrdU, similar to the effect on Nkx2.1+ progenitors at E9.5-E10.5 (Figure 5f). Thus, progenitors of GABAergic interneurons exhibit differential vulnerability that depends on stage of embryonic brain development.

Discussion

Despite significant evidence showing that maternal immune activation during pregnancy leads to cognitive dysfunction in the offspring that might be triggered by excitation-inhibition imbalance, little is known how the development of inhibitory system in the brain is affected. Here we showed that maternal immune activation results in multiple "hits" on development of GABAergic neurons, thus affecting proliferation of precursors, migration and positioning of neuroblasts as well as their maturation.

Genetic and environmental factors that affect cortical development provide important insights into the cellular and molecular basis of neurodevelopmental disorders. Available epidemiological and clinical findings[56] point towards a link between maternal infections during gestation and increased risk of developing a mental disorder due to impaired cortical development. While the precise mechanism of action is elusive, an increase in maternal cytokine levels is thought to lead to the transmission of maternal inflammation to the fetal brain[3]. However, the developmental changes occurring upon maternal inflammation in the fetal brain and during postnatal brain maturation have been understudied owing to ethical and technical challenges in humans. Thus, rodent models of maternal inflammation provide an excellent alternative to study the effects of an acute inflammatory insult on brain development. Accordingly, poly I:C and lipopolysaccharide (LPS) have emerged as two popular molecules that mimic viral and bacterial infections respectively and robustly activate the maternal immune system. In this study, we mimicked maternal viral infection by injecting poly I:C at GD9.5 and followed the development of cortical GABAergic neurons in the offspring brains from E10.5. Strikingly, in spite of an acute immune response, we revealed that the effect was both immediate, i.e. affecting proliferation of precursors of interneurons, and long-lasting, i.e. affecting migration of neuroblasts and maturation of cortical GABAergic neurons during late embryonic or early postnatal brain development.

One of our major findings is that the effect of maternal inflammation has a differential effect on GABAergic interneuron subtypes, highlighting subtype-specific vulnerability of neurons to maternally-derived stimuli. Hitherto, studies have revealed a convergent effect of genetic and

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environmental schizophrenia-related insults on PV+ interneurons[21,25]. However, while PV+ interneurons are crucial in synchronizing spike timing via gamma-oscillations, suppression of their activity alone has been found insufficient to reproduce schizophrenia-like phenotype in genetic or environmental mouse models of schizophrenia[57]. We show here a differential impact of maternal inflammation on interneuron subtypes and cortical regions that goes beyond PV+ interneurons. GABAergic interneurons in the somatosensory cortex were more affected in comparison to their counterparts in the motor cortex. Furthermore, the impact on PV+ interneurons was localized specifically to layer 4 and could be traced as early as P15, suggesting a specific developmental impairment. Importantly, we found a significant impact of maternal inflammation on SST+ interneurons, which were affected not only in layer 4 as PV+ interneurons but also in layer 2/3 despite both subtypes being derived from MGE-derived Nkx2.1+ progenitors. The overlap in layer 4 is of significance as SST+ interneurons in this layer target mainly PV+ fast-spiking interneurons unlike in layer 2/3 where pyramidal neurons are the primary targets[58]. Selective ablation of SST+ interneurons during development has been shown to arrest the maturation of PV+ interneurons in layer 5/6 suggesting an early role for SST+ interneurons in PV+ interneuron maturation[59].

The effect of maternal inflammation at GD9.5 on MGE-derived PV+ and SST+ interneurons in the offspring can be observed as early as E10.5, due to decreased proliferation of MGE-derived Nkx2.1+ progenitors. However, this effect was acute with proliferation returning to wild type levels by E14.5. This immediate and short-lived effect of maternal inflammation is in line with previous reports on cortical progenitors in an LPS model[28,60]. Contrary to the effect on MGE-derived subtypes, we found no change in CGE-derived VIP+ interneurons in animals exposed to maternal inflammation at E9.5, and neither CGE-derived COUP-TFII+ progenitors nor VIP+ interneurons in the mature cortex were affected. This is ostensibly due to a later 'birthdate' of CGE-derived interneurons between E12.5-18.5[12]. However, the effect on CGE-derived COUP-TFII+ progenitors could be discerned by inducing inflammation at a later developmental stage, GD12.5. Thus, our results show a developmental time-dependent impact of maternal inflammation on distinct interneuron precursor pools.

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It can hence be appreciated that the time of insult coupled with cellular or genetic vulnerabilities can produce varying outcomes on interneuron subtypes. Timing of the developmental insult will determine those developmental processes in the cortex that are affected, leading to variation in the cognitive phenotypes and severity of the affected offspring. Indeed, it has been shown that a difference in the timing of maternal inflammation leads to distinct behavioral dysfunctions[24,61] (see also[62] for review). Over the course of cortex development, maternal inflammation could interfere with: (1) interneuron precursor proliferation, (2) neuronal migration and positioning between cortical areas and within cortical layers, (3) neuronal maturation and circuit connectivity. Our data shows that acute maternal inflammation affects multiple stages of interneuron development. Thus, in addition to the impact on precursor proliferation, described above, we show that migration of interneurons into the dorsal cortex was impaired as early as E14.5. The effect of maternal inflammation on the distribution of cortical GABAergic interneurons was maintained at E17.5 but could only be seen in the superficial cortical bins. This could be due to a greater proportion of GAD+ neuroblasts in maternal inflammation-exposed embryos migrating laterally and further work using time-lapse microscopy will be important to clarify the mechanism of this impairment. Likewise, while analysis of early postnatal stages showed a decreased density of GAD+ neuroblasts in regions of the developing cortex that correspond to both motor and somatosensory cortices, by P15 the effect persisted only in the somatosensory cortex. This suggests a differential regional impairment of cortical interneurons. Despite this, perinatal reduction in the number of GAD+ neuroblasts in the motor cortex might still affect the maturation of early cortical circuits and have functional outcomes that could not be measured in this study.

In addition to the differences in proliferation and migration, the decrease in interneuron numbers can also be attributed to programmed cell death. Previous studies have shown that GAD+ neuroblasts undergo apoptosis during the first two postnatal weeks with a peak between P6-P9[16,17]. However, at both P3 and P6, we observed comparable numbers of GAD+ neuroblasts expressing activated caspase-3 suggesting that maternal inflammation does not affect interneuron cell death.

Functionally, PV+ interneurons in our model showed a decreased maximum firing frequency in the absence of changes in input resistance. The maximum firing frequency in PV+ interneurons has been described to increase during postnatal development[29,63], thus suggesting a maturational delay of PV+ interneurons in our study due to maternal inflammation. This maturational delay is also corroborated by decrease in dendritic complexity of maternal inflammation-affected PV+ interneurons. Alterations in cellular properties of GABAergic interneurons can alter the perisomatic inhibition of pyramidal neurons thus altering the excitation-inhibition balance in the neocortex[29]. Furthermore, human studies show widespread changes in neonatal cortical connectivity due to increase in the level of maternal proinflammatory cytokines[64,65]. Importantly, GABAergic interneurons are necessary for maturation of early cortical circuits that provide the foundation for adult cortical connectivity[66,67]. Thus, maturational delay of GABAergic interneurons due to maternal inflammation can have broad effect on cognitive properties of the affected offspring.

The poly I:C model of maternal inflammation presents high construct validity and parallels the effects seen in humans[3,62]. Based on translation of brain development milestones, the early time-point of induction (GD9.5) overlaps with the first trimester of human pregnancy[39] as well as with the onset of neurogenesis of cortical GABAergic interneurons[68]. Given the significant evidence for impairment of cortical GABAergic interneurons in human postmortem brain studies of patients with mental illness[69–71], the dysfunction of developmental programs for GABAergic interneurons might have causative effect on cognitive phenotype of the patients. Further studies of GABAergic circuitry development in environmental and genetic mouse models mimicking human developmental insults will reveal commonalities and differences in the affected subtypes of GABAergic interneurons across all developmental processes. Such data will provide mechanistic insight into etiology of human neurodevelopmental disorders and will identify therapeutical window during brain development when impairment GABAergic circuitry can be corrected.

Acknowledgements

Author Contributions

This study was conceived by MPN, UP, NAV and KK. NAV and MPN performed the poly I:C injections and downstream experiments including design, collection, analysis and interpretation of data. JG and DW collected and analyzed specific developmental stages. MKM and JvE performed and analyzed electrophysiology. KSH contributed to the design, execution and analysis of behavioral experiments. NAV and KK jointly wrote the paper. All authors read and approved the manuscript.

Conflict of Interest Statement

The authors declare that they have no potential conflicts of interest

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Figure Legends

Figure 1: Poly I:C induces rapid upregulation of IL-6 in maternal plasma and behavioral impairments in animals prenatally exposed to maternal inflammation

(a) Upregulation of IL-6 protein in maternal plasma 3 hours after i.v. injection of poly I:C (n=5 each)

(b,c) Prenatal exposure to maternal inflammation results in impaired social-zone preference (b) (n=12 each) as well as a decrease in time spent in social zone (c) (F=3.23).

(d,e) Mice exposed to maternal inflammation during gestation present signs of hyperactivity as seen by total distance travelled (d) (n=12 each) and speed of running over a 10 minute period (e) (n=12 each)

(f) Maternal inflammation during early gestation induces schizotypal effects in offspring that can be gauged by a decreased pre-pulse inhibition (at 72dB) (n=15 and 9, for PBS and poly I:C, respectively)

Comparison of means by Student's t-test in (a,d,f), t-test with Holm-Sidak correction for multiple comparison in (b) and two-way ANOVA in (c,e) (mean±SEM are shown, p-values denoted as asterisks with <0.05 shown as *, <0.005 shown as ** and <0.0005 shown as ***)

Figure 2: Early migratory deficits of GAD-EGFP+ neuroblasts due to maternal inflammation

(a) A schematic overview of the migratory routes taken by GAD+ neuroblasts during embryonic development[47,48]

(b,c) Reduction in the density of EGFP+ neuroblasts migrating into the cortical intermediate zone at E14.5 as a result of maternal inflammation. Panel (b) shows somatosensory region of the developing cortex. Dotted box denotes the region of counting. Quantifications from the dotted boxes are shown in (c) (motor cortex: n=8 each; somatosensory cortex: n=8 each)

(d,e) Superficial regions of the cortical column show a reduction in density of EGFP+ neuroblasts due to maternal inflammation. Cortical columns from developing motor (d) and

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somatosensory (d') cortical regions were divided into 10 equal-sized bins and the number of EGFP+ cells counted in each (with the exception of bin 1). Bin 10 represents the upper margin of the IZ. Densities in the graphs (e) show a reduction in bins 3-4 (motor bin 3: p=0.004, bin 4 p=0.004; somatosensory bin 4: p=0.002).

(f) Embryos exposed to maternal inflammation showed a decreased total density of migratory neuroblasts at E17.5. EGFP+ cells were counted in the cortical plate and intermediate zone across bins 2-10 (n=19 each).

(g) Radar plot showing that a greater percentage of neuroblasts in the cortical plate migrate laterally due to maternal inflammation as assessed by the angle of leading process. Angles were grouped into 30° bins and relative percentages were plotted. Each spoke represents 10%.

Comparison of means by Student's t-test in (c,f), t-test with Holm-Sidak correction for multiple comparison in (e) (mean±SEM are shown, p-values denoted as asterisks with <0.05 shown as *, <0.005 shown as ** and <0.0005 shown as ***)

Scale bars: 50 µm

Figure 3: Maternal inflammation causes large-scale impairment in the distribution of GAD-EGFP+ neuroblasts and mature interneurons in the cortex

(a-c) Graphs showing a decrease in density of EGFP+ cells measured by counting EGFP+ cells in 10 equally sized bins in the cortical plate of the presumptive motor and somatosensory cortices. Dotted lines represent the margins of IZ, CP and MZ respectively (P3 motor: F=21.85, P3 somatosensory: F=24.1; P6 motor: F=21.72, P6 somatosensory: F=19.59; P9 motor: F=12.22, P9 somatosensory: F=13.77)

(d,e) Equal number of total apoptotic cells (Casp3+) and apoptotic interneurons (Casp3+ EGFP+) in PBS and maternal inflammation-exposed pups at P3 and P6. Graphs show total number of cells counted in several sections spanning the motor and somatosensory regions of the cortex (n=7 each).

(f-h) Significant reduction in the density of interneurons in the somatosensory cortex at P15. EGFP+ cells were quantified in the total cortical thickness as well as in each layer of the somatosensory. Panels in (f) show representative images of regions used for quantification. Graphs show the total (g) (n=8 and 7, for PBS and poly I:C, respectively) as well as the layer-wise (h) (p-value for each comparison< 0.005) reduction in EGFP+ cell density.

Comparison of means by Two-way ANOVA in (a-c), Student's t-test in (g) and t-test with Holm-Sidak correction for multiple comparison in (h) (mean±SEM are shown, p-values denoted as asterisks with <0.05 shown as *, <0.005 shown as ** and <0.0005 shown as ***)

Scale bar: 20 µm (d) and 50 µm (f)

Figure 4: Interneuron subtype-specific and development stage-dependent effects of maternal inflammation

(a-f) Maternal inflammation resulted in a decreased density of PV and SST expressing interneurons at P15 but not at P60. Panels show representative images from P15 (a) and P60 (b). A significant decrease in PV+ interneurons could be seen in L4, while the difference was more pronounced in L2-4 for SST+ interneurons (n=8 and 7, for PBS and poly I:C, respectively). An appreciable but not statistically significant decrease could be seen at P60 (f).

(g-j) Reciprocal changes in PV expression between P15 and P60. At P15, PV+ interneurons in both motor and somatosensory cortical regions in maternal inflammation-exposed animals showed an increased expression of PV (h) (n=8 and 7, for PBS and poly I:C, respectively) measured by the signal intensity in immunolabeled images (g). By P60 (i), this effect had reversed and instead a decreased expression was observed in both motor and somatosensory cortical regions (j) (n=6 each).

(k-n) Maternal inflammation resulted in altered intrinsic physiological properties of PV+ fast-spiking interneurons. Representative traces are shown in (k) and cells showed a hyperpolarized RMP (l) (PBS: -65.13mV±1.14, Poly I:C: -68.42mV±0.75, n=25 cells each) despite no change in

the input resistance (m). Additionally, a decrease in firing frequency could also be observed (n) (PBS: 145.1Hz±7.61, Poly I:C: 123.1Hz±4.36, n=25 cells each).

(o-r) Altered morphological complexity of PV+ fast-spiking interneurons was also observed by biocytin back-filling and reconstruction (o). Differences in filament length and branch depth (p,q) did not reach statistical significance, while Sholl analysis showed a decreased complexity (r) (F=179.7).

Comparison of means by t-test with Holm-Sidak correction for multiple comparison in (c-f and h,j,r) (mean±SEM are shown, p-values denoted as asterisks with <0.05 shown as *, <0.005 shown as ** and <0.0005 shown as ***)

Scale bar: 50 μm (a,b) and 30 μm (o)

Figure 5: Acute and developmental stage-dependent impairment of precursor proliferation due to maternal inflammation

(a-c) Maternal inflammation affected the immediate proliferation of MGE-derived Nkx2.1+ precursors. Dams were injected with poly I:C at GD9.5 and embryos assessed at E10.5 after a 2-hour BrdU pulse-chase (a-c). Quantification of proliferating Nkx2.1+ BrdU+ cells showed that maternal inflammation resulted in an immediate reduction in Nkx2.1+ proliferating cells (b) (n= 7 and 6, for PBS and poly I:C, respectively) that did not sustain until E14.5 (c).

(d-f) CGE-derived COUP-TFII precursors showed a similar immediate decrease after maternal inflammation at E12.5 (d,f) (n=7 and 6, for PBS and poly I:C, respectively) that coincided with the later neurogenic window of CGE-derived precursors. Similar to Nkx2.1+ cells, COUP-TFII+ precursors remained unaffected when studied at E14.5 after poly I:C injection at GD9.5

Comparison of means by Student's t-test in (b,c,e,f) (mean±SEM are shown, p-values denoted as asterisks with <0.05 shown as *, <0.005 shown as ** and <0.0005 shown as ***)

Scale bar: 50 μm (a,d)

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Suppl. Figure 1: Auditory evoked pre-pulse inhibition (PPI) across several frequencies of pre-pulse

PPI was found to be significant only at 72dB but not at the other pre-pulse frequencies

Comparison of means by two-way ANOVA to analyze prepulse inhibition, with prepulse as the repeated measure and t-test with Holm-Sidak correction for multiple comparison (mean±SEM are shown, p-values denoted as asterisks with <0.05 shown as *)

Suppl. Figure 2: Relative distribution of EGFP+ neuroblasts at P3 and P6 and VIP+ interneurons at P15
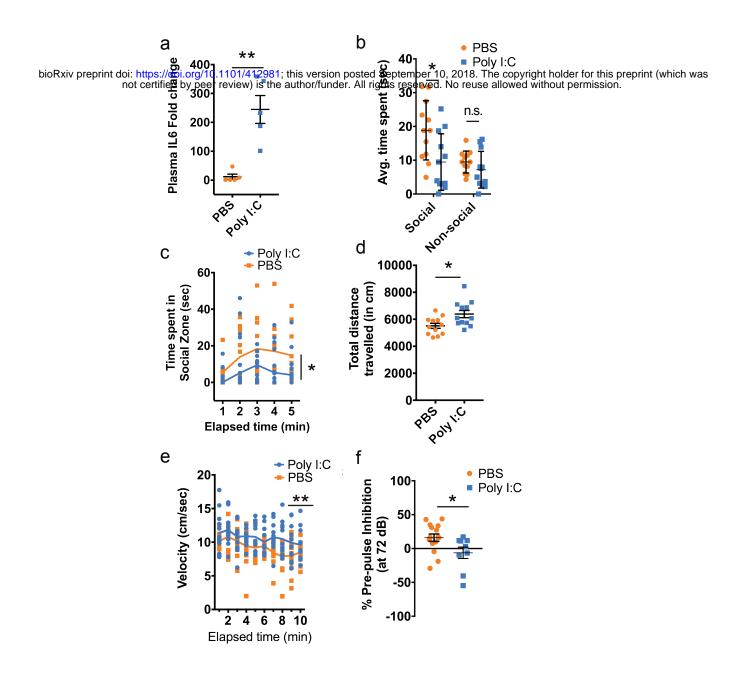
(a,b) Graphs showing comparable proportions of GAD-EGFP+ neuroblasts in PBS- and maternal inflammation-exposed offspring across the cortex at P3 (a) and P6 (b)
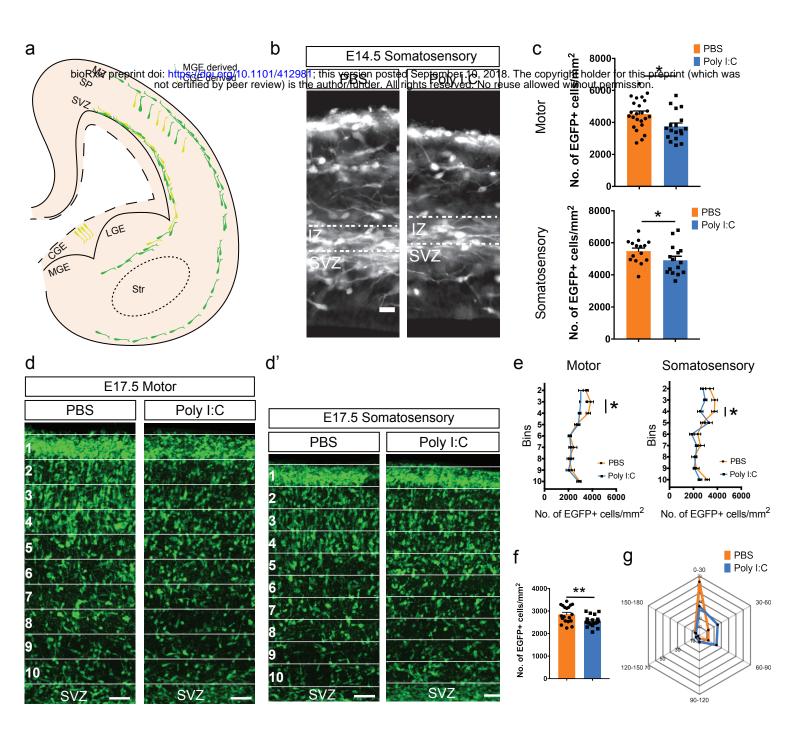
(c) Layer-wise density of EGFP+ interneurons does not show any change in the motor cortex at P15
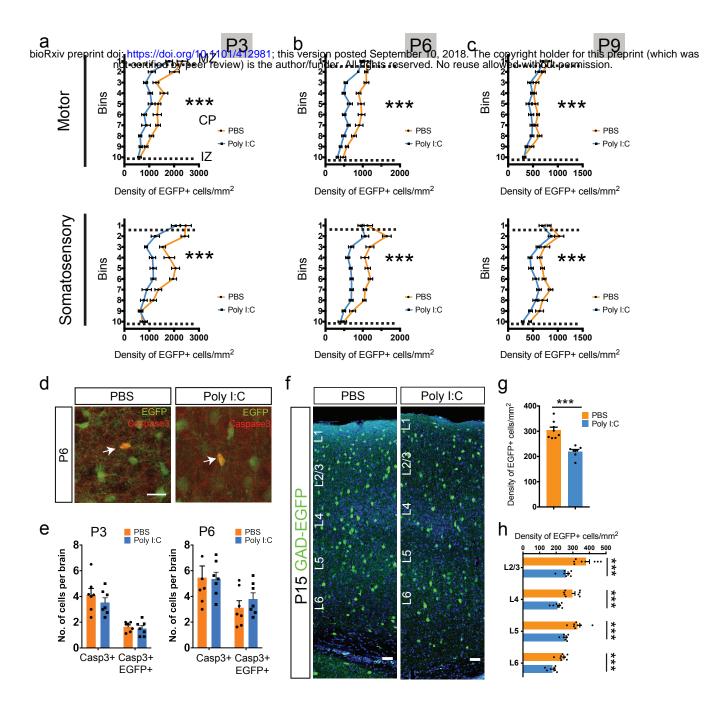
(d,e) Maternal inflammation at GD9.5 does not affect the distribution of the CGE-derived VIP+ interneuron subtype at P15
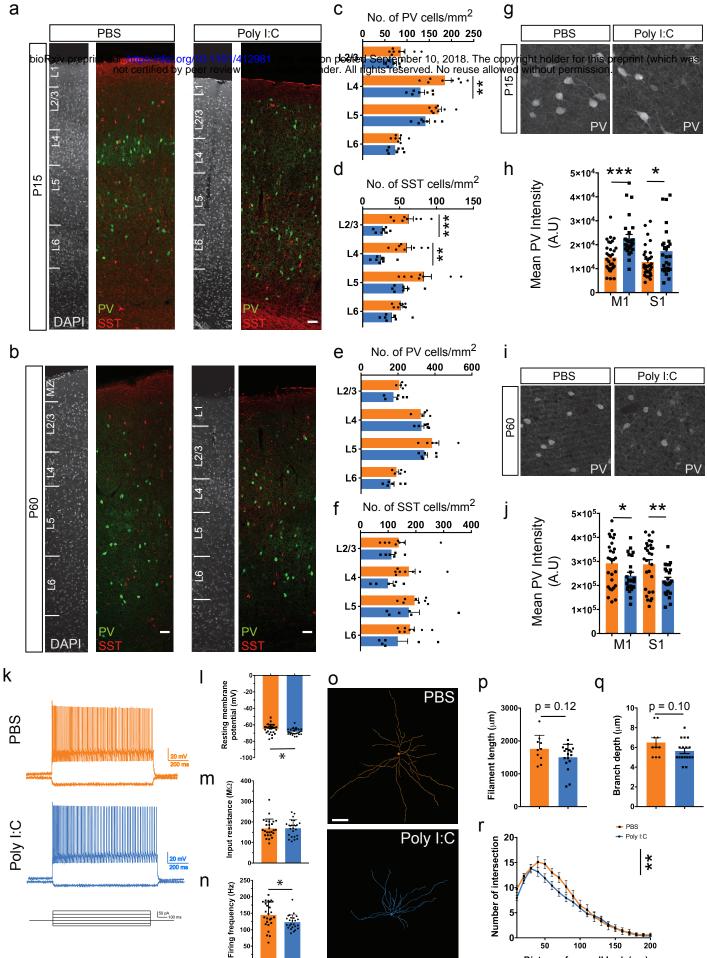
Comparison of means by Student's t-test with Holm-Sidak correction for multiple comparisons in (a-c,e) (mean±SEM are shown, p-values<0.05 denoted as asterisks with 0.05 shown as *, 0.005 shown as ** and 0.0005 shown as ***)

Scale bars: 50 µm

a

b — E14.5 Somatosensory — PBS — Poly I:C

c — Motor — Somatosensory

d — E17.5 Motor — PBS — Poly I:C

d' — E17.5 Somatosensory — PBS — Poly I:C

e — Motor — Somatosensory

f

g