Poly I:C-induced maternal immune challenge reduces perineuronal net area and raises spontaneous network activity of hippocampal neurons in vitro

David Wegrzyn1 | Marie-Pierre Manitz3 | Michael Kostka1 | Nadja Freund3 | Georg Juckel2 | Andreas Faissner1

Abstract
Activation of the maternal immune system (MIA) during gestation is linked to neuropsychiatric diseases like schizophrenia. While many studies address behavioural aspects, less is known about underlying cellular mechanisms. In the following study, BALB/c mice received intraperitoneal injections of polyinosinic-polycytidylic acid (Poly I:C) (20 µg/ml) or saline (0.9%) at gestation day (GD) 9.5 before hippocampal neurons were isolated and cultured from embryonic mice for further analysis. Interestingly, strongest effects were observed when the perineuronal net (PNN) wearing subpopulation of neurons was analysed. Here, a significant reduction of aggrecan staining intensity, area and soma size could be detected. Alterations of PNNs are often linked to neuropsychiatric diseases, changes in synaptic plasticity and in electrophysiology. Utilizing multielectrode array analysis (MEA), we observed a remarkable increase of the spontaneous network activity in neuronal networks after 21 days in vitro (DIV) when mother mice suffered a prenatal immune challenge. As PNNs are associated with GABAergic interneurons, our data indicate that this neuronal subtype might be stronger affected by a prenatal MIA. Degradation or damage of this subtype might cause the hyperexcitability observed in the whole network. In addition, embryonic neurons of the Poly I:C condition developed significantly shorter axons after five days in culture, while dendritic parameters and apoptosis rate remained unchanged. Structural analysis of synapse numbers revealed an increase of postsynaptic density 95 (PSD-95) puncta after 14 DIV and an increase of presynaptic vesicular glutamate transporter (vGlut) puncta after 21 DIV, while inhibitory synaptic proteins were not altered.

KEYWORDS
extracellular matrix, multielectrode array, neuronal networks, perineuronal nets, schizophrenia
1  |  INTRODUCTION

Schizophrenia is a severe neuropsychiatric disorder with a frequent onset at the transition of late adolescence to early adulthood. Studies of the last decades unravelled a strong genetic component for the manifestation of this disorder (Cardno & Gottesman, 2000; Fischer, 1973; Henrikson, Nordgaard, & Jansson, 2017). Nevertheless, there is a growing evidence that environmental factors play an important role for the development of schizophrenia. Specifically, infections during pregnancy and the upregulation of immune factors seem to have a strong influence on the development of the embryonic central nervous system (CNS) and are increasing the risk for schizophrenia in the offspring (Allswede & Cannon, 2018; Blomstrom et al., 2016; Labouesse, Langhans, & Meyer, 2015; Meyer, 2013). Interestingly, there is much evidence for this association including infections with pathogens like influenza (Brown et al., 2004; Limosin, Rouillon, Payan, Cohen, & Strub, 2003; McGrath, Pemberton, Wetham, & Murray, 1994; Mednick, Machon, Huttunen, & Bonett, 1988), rubella (Brown et al., 2001), toxoplasma gondii (Brown et al., 2005; Mortensen et al., 2007), polio (Suvisaari, Haukka, Tanskanen, Hovi, & Lonnqvist, 1999) and herpes simplex type 2 (Buka, Cannon, Torrey, & Yolken, 2008; Buka et al., 2001). As most pathogens are unable to pass the placental barrier, it is proposed that maternal immune activation (MIA) and the associated increase of pro-inflammatory cytokines in the serum affect the development of the foetal central nervous system in an indirect way (Ashdown et al., 2006; Gilmore & Jaruskog, 1997; Patterson, 2009).

In order to reconstruct and to analyse the impact of a maternal immune activation on foetal CNS development, the Poly I:C model has been established and revealed significant alterations in the offspring of treated rodents. Injections with Poly I:C in the early and mid-gestation period reduced significantly behavioural parameters like prepulse inhibition (PPI) and latent inhibition (LI) in the offspring of rodents and primates (Bauman et al., 2014; Drazanova et al., 2018; Esslinger et al., 2016; Hui et al., 2018; Meyer & Feldon, 2012; Wolff & Bilkey, 2008). Deficits in the PPI and LI are well characterized in schizophrenia patients. Interestingly, a knockout of interleukin-6 could reduce the behavioural abnormalities induced by a prenatal immune activation via Poly I:C administration (Smith, Li, Garbett, Mirkics, & Patterson, 2007). Beside behavioural changes, an elevated number of activated microglia could be observed in the hippocampus and striatum in the offspring of Poly I:C-treated mice at postnatal day 30 (PND30) (Juckel et al., 2011; Manitz et al., 2013). In addition, the sensitivity to dopamine receptor (DR) and NMDA receptor (NMDAR) agonists was increased in the offspring of Poly I:C-treated mice and rats (Wolff & Bilkey, 2008; Zuckerman, Rehavi, Nachman, & Weiner, 2003; Zuckerman & Weiner, 2005). Concomitant to the higher sensitivity of dopamine and NMDA receptors, multiple changes in the basal neurotransmitter levels could be observed (Winter et al., 2009). Another interesting study analysed the effects of a prenatal immune activation via Poly I:C administration at GD E15.5 and documented a reduced hippocampal volume, a reduced number of parvalbumin-positive interneurons and a reduced synaptic inhibition in mature dentate gyrus (DG) neurons (Zhang & van Praag, 2015).

There is a growing evidence of extracellular matrix (ECM) abnormalities in different animal models of neuropsychiatric diseases. A current study revealed significantly decreased PNN areas in the medial prefrontal cortex in the offspring of a Poly I:C rat model (Paylor et al., 2016). Furthermore, PNN alterations were also observed in a ketamine animal model of schizophrenia and in an early life stress animal model (Castillo-Gomez et al., 2017; Matuszko, Curreli, Kaushik, Becker, & Dityatev, 2017). PNNs are specialized structures of the ECM and were first described by Camillo Golgi (Celio, Sprefacico, De Biasi, & Vitellaro-Zuccarello, 1998). They form net-like structures around the neuronal soma including proximal dendrites and are often associated with fast-spiking parvalbumin-positive interneurons (Bruckner et al., 1994; Carulli et al., 2010; Dzyubenko, Gottschling, & Faisser, 2016; Hartig, Brauer, Bigl, & Bruckner, 1994; Wintergerst et al., 1996; Yamada, Ohgomori, & Jinno, 2015). There is growing evidence that alterations of PNNs are involved in the pathophysiology of schizophrenia (Berretta, 2012; Bitanhirwe & Woo, 2014; Wen, Binder, Ethell, & Razak, 2018). Post-mortem studies of schizophrenia patients revealed alterations in PNNs in the amygdala, olfactory epithelium, entorhinal cortex, superior temporal cortex and prefrontal cortex (Bitanhirwe & Woo, 2014; Mauney et al., 2013; Pantazopoulos et al., 2013; Pantazopoulos, Woo, Lim, Lange, & Berretta, 2010; Pietersen et al., 2014).

In the light of these observations, we wanted to determine if a prenatal immune challenge exercises influence on the development of hippocampal neurons and on the formation of PNNs in vitro. Therefore, we utilized the Poly I:C mouse model for the induction of a MIA at GD 9.5 and prepared neuronal cultures from embryonic hippocampi at embryonic stage E15.5. Here, we focused on embryonic hippocampal neurons, as several studies revealed structural as well as electrophysiological abnormalities of the hippocampus in different MIA models (Ito, Smith, Hsiao, & Patterson, 2010; Lowe, Luheshi, & Williams, 2008; Zhang & van Praag, 2015). Furthermore, the hippocampus plays a special role in the early development of schizophrenia (Lieberman et al., 2018). In order to determine cell-specific effects of a MIA, we concentrate exclusively on neuronal characteristics and analysed axonal as well dendritic parameters after a short cultivation time of three and five DIV. Additionally, we determined structural synapse numbers after a longer cultivation time of 14 and 21 DIV. For this purpose, native cortical astrocytes
were added in an indirect contact with embryonic neurons derived from Poly I:C and saline-treated mouse dams. Using this co-culture combination, we exclusively focused on neuron-specific changes induced by a MIA. Beyond structural analysis, electrophysiological MEA recordings of cultured neurons derived from Poly I:C- and saline-treated animals were performed. Last, we focused on PNNs and used a triple staining for the visualization of glutamatergic synapses on PNN-wearing neurons in culture.

2 | MATERIAL AND METHODS

2.1 | Animal housing and ethical standards

The following study was performed in accordance with the Council Directive of the European Parliament and the Council of 22 September 2010 (2010/63/EU) on the protection of animals used for scientific purposes. Experiments were approved by the animal care committee of North Rhine-Westphalia, Germany, based on the LANUV (Landesamt für Umweltschutz, Naturschutz und Verbraucherschutz, North Rhine-Westphalia, D-45659 Recklinghausen, Germany; approval: 84–02.04.2016.A017). The study was supervised by the animal welfare commissioner of Ruhr-University Bochum. Male and female BALB/c and SV129 mice (Charles River, Sulzfeld, Germany) were housed with a constant 12 hr light–dark cycle, temperature (21°C) and humidity (60%–70%), as well as food and water access ad libitum. Animals were kept in a regular animal facility with conventional housing. When a vaginal plug was observed after a 1:1 pairing, female mice were considered pregnant and separated. Embryonic and postnatal mice of both sexes were used for all experimental procedures. The total number of pregnant dams and embryonic mice used for the experiments are listed in the following Table 1. All efforts were undertaken to reduce the number of animals used for this study.

2.2 | Initiation of sickness behaviour

Polyinosinic-polycytidylic acid sodium (Sigma-Aldrich; Cat. No.: P1530) was prepared for injection by re-suspension in sterile NaCl (0.9%; Baxter Deutschland GmbH) at a concentration of 2.5 mg/ml and was stored at −20°C until use. On day nine of gestation, mice were randomly divided into two groups (N = 6/group) and were injected intraperitoneally (i.p.) with either Poly I:C (20 mg/kg) or sterile NaCl (controls). The usage

| Experiment                                | Total number of used pregnant dams per condition | Total number of embryonic mice per condition |
|-------------------------------------------|--------------------------------------------------|---------------------------------------------|
| Burrowing behaviour                       | 6                                                |                                             |
| Apoptosis DIV3                            | 5                                                | 25                                          |
| Apoptosis DIV5                            | 5                                                | 25                                          |
| Axo-dendritic DIV3                        | 4                                                | 20                                          |
| Axo-dendritic DIV5                        | 4                                                | 20                                          |
| Excitatory synapse numbers DIV14          | 5                                                | 25                                          |
| Excitatory synapse numbers DIV21          | 5                                                | 25                                          |
| Inhibitory synapse numbers DIV14          | 3                                                | 15                                          |
| Inhibitory synapse numbers DIV21          | 3                                                | 15                                          |
| Perineuronal net properties DIV14         | 5                                                | 25                                          |
| Perineuronal net properties DIV21         | 5                                                | 25                                          |
| MEA analysis DIV14                        | 7\(^*\)                                          | 35                                          |
| MEA analysis DIV21                        | 5                                                | 25                                          |
| Synapses on PNN(+) neurons DIV14          | 5                                                | 25                                          |
| Synapses on PNN(+) neurons DIV21          | 5                                                | 25                                          |

\(^*\)An additional dam was included without testing burrowing behaviour.
of the BALB/c mouse strain and the definition of the Poly I:C dose are based on a previously published study (Shi, Fatemi, Sidwell, & Patterson, 2003). Here, Shi and colleagues observed a significant impairment of prepulse inhibition in the offspring when pregnant BALB/c mice were previously treated with Poly I:C (20 mg/kg) at gestation day 9.5.

2.3 | Burrowing test

We used the burrowing test (adapted and modified (Deacon, 2006)) to assess sickness behaviour in mice challenged peripherally with Poly I:C. The test was performed in two phases: baseline and testing. To acquire an individual baseline, mice were placed in individual cages, each containing one burrowing tube (plastic cylinder, 20 cm long, 5.7 cm diameter, sealed at one end, standing on two feet at a height of 1.5 cm above the ground, and filled with 30 g of sunflower seeds). After two hours, the tubes were removed, and the amount of remaining seeds was measured. Then, the tubes were returned to the cages. A second overnight measure was obtained the next morning. On the same day, mice were injected with either Poly I:C or NaCl. Five hours after the injections, tubes were placed in the cages. After additional two hours, the weight of the remaining sunflower seeds was determined. The burrowing test was performed between 5–7 hr after Poly I:C challenge, because this time point demarcates the nadir of burrowing activity (Cunningham, Campion, Teeling, Felton, & Perry, 2007) and overnight. The results are expressed as the amount of sunflower seeds displaced from the tubes.

2.4 | Cell culture

2.4.1 | Primary astrocytic culture

Native cortical astrocytes from postnatal (P)1–3 SV129 mice were used as a feeder cell layer for neurons and prepared as described previously with minor modifications (McCarthy & de Vellis, 1980).

After decapitation, brains of postnatal SV129 mice were removed and transferred to HBSS −/− (Thermo Fisher Scientific Inc.; Cat. No.: 14170088). Next, cortices were isolated, freed from meninges and subsequently digested in a solution consisting of 30 U/ml Papain (Worthington; Cat. No.: LS003126), 80 µg/ml (w/v) DNase I (Worthington; Cat. No.: LS002007), 0.96 µg/ml L-Cysteine (Sigma-Aldrich; Cat. No.: C2529) in DMEM (Thermo Fisher Scientific Inc.; Cat. No.: 41960029). Here, three brains (≈ 6 cortices) were used per approach and incubated for 1h at 37°C. The digestion was stopped by adding freshly prepared astrocyte medium (DMEM, 10% (v/v) horse serum (Sigma-Aldrich; Cat. No.: S9135), 0.1% (v/v) gentamycin (Sigma-Aldrich; Cat. No.: S9135), and the tissue was carefully triturated. Afterwards, the suspension was centrifuged for 5 min at 1,000 rpm. The supernatant was aspirated and discarded, and the pellet was resuspended in astrocyte medium. Next, the cells were plated out on a Poly-D-Lysine (PDL) (10 µg/ml; Sigma-Aldrich; Cat. No.: P0899) coated T-75 flask (Sarstedt). Therefore, 1 ml cell suspension was added to the previously prepared 9 ml pre-warmed astrocyte medium in the flask. The mixed glial culture was incubated at 37°C and 6% (v/v) CO₂ in the incubator, and the medium was exchanged every third day.

After 7–10 DIV, astrocytes formed a dense monolayer with microglia and oligodendrocyte precursor cells (OPCs) on their surface. Therefore, the culture flasks were placed on an orbital shaker for 1 hr at 250 rpm and 37°C. Then a medium exchange was performed, and the flasks were set again in the incubator for 1 hr. Afterwards, the cultures were shaken overnight at 250 rpm and 37°C. On the next day, a medium exchange was done and 20 µM cytosine-1-β-D arabinofuranoside (Ara-C) (Sigma-Aldrich; Cat. No.: C1768) was added to the cultures for 48–72 hr in order to remove the remaining microglial cells and OPCs.

For the indirect co-cultivation of cortical astrocytes with hippocampal neurons derived from embryonic mice of Poly I:C- and saline-treated BALB/c dams, astrocytes were plated in cell culture transwell inserts (Falcon by Thermo Fisher Scientific Inc.; Cat. No. 08–770) with a permeable membrane (pore size: 0.4 µm) (Gottschling, Dzyubenko, Geissler, & Faissner, 2016; Pyka et al., 2011). Therefore, astrocytes were washed once with PBS and incubated with 0.05% (v/v) Trypsin-EDTA (T/E) (Gibco; Cat. No.: 25300054) for 7–10 min at 37°C and 6% CO₂ in the incubator. Afterwards, the astrocytes detached from the bottom of the T-75 flask and were collected in astrocyte medium. After a centrifugation step (5 min, 1,000 rpm), the supernatant was aspirated, and the cell pellet was resuspended in 1 ml astrocyte medium. In a final step, 25,000 astrocytes were plated out per transwell insert (pre-coated with 10 µg/ml (w/v) PDL). Finally, the inserts were placed in a 24-well plate (Nunc by Thermo Fisher Scientific Inc.) and 500 µl astrocyte medium was added around every insert. The plate with the inserts was incubated at 37°C and 6% (v/v) CO₂. After the astrocytes have grown to confluence at the insert membrane, the preparation of neurons was performed. The native cortical astrocytes derived from postnatal SV129 mice purely served the purpose of supplying the neurons and enable a cultivation time up to 21 days in completely defined medium.

2.4.2 | Primary neuronal culture

Hippocampal neurons were isolated from BALB/c embryonic mice of the developmental stage E15.5 derived from
Poly I:C- and saline-treated mouse dams. The neurons were cultured in an indirect co-culture setup with native cortical astrocytes (Gottschling et al., 2016). Six days after the treatment with Poly I:C or saline pregnant, animals were killed, and embryonic mice (E15.5) were isolated. The preparation of Poly I:C and saline-treated animals was performed simultaneously per experimental repetition, and five embryonic brains (♂ 10 hippocampi) of each group were isolated and used for the experiments. The brains were transferred to preparation medium (HBSS (Gibco; Cat. No.: 14170088), 0.6% (w/v) glucose (SERVA Electrophoresis GmbH; Cat. No.: 22,700), 10 mM HEPES (Gibco; Cat. No.: 15630080)) and the dissected hippocampi were also collected in 1 ml of the preparation medium. The digestion was performed in 1 ml MEM (Gibco; Cat. No.: 31095029) containing 30 U/ml papain, 0.96 µg/ml (w/v) L-cysteine and 80 µg/ml DNase I for 15 min at 37°C. Afterwards, the digestion was stopped by the replacement of the digestion solution by hippocampus medium (MEM, 10 mM sodium pyruvate (Sigma-Aldrich; Cat. No.: S8636), 2% (v/v) B27 (Gibco Cat. No.: 17504044), 1 mg/ml (w/v) ovalbumin (Sigma-Aldrich; Cat. No.: A7641), 0.05 mg/ml (w/v) gentamicin). The tissue was washed three times with hippocampus medium and carefully triturated to a single cell suspension.

For the analysis of axo-dendritic parameters after three and five DIV 30,000 neurons were plated out in 500 µl hippocampus medium on poly-L-ornithine (PLO) (15 µg/ml; Sigma-Aldrich; Cat. No.: P3655) coated coverslips without astrocyte inserts. For the analysis of synaptic puncta 35,000 neurons were cultured in 500 µl medium on PLO-coated coverslips in an indirect contact with astrocytes. Therefore, the astrocyte medium in the cell culture transwell inserts with confluent grown astrocytes was removed and replaced by hippocampus medium. After the neurons have settled down on the coverslips, the culture inserts with astrocytes were hung in special 24-well plates (Falcon; Product No.: 353504).

## 2.5 Immunocytochemistry

### 2.5.1 Visualization of dendrites, axons and apoptotic nuclei

For the analysis of axo-dendritic parameters as well as apoptosis, neurons were fixed for ten minutes with pre-warmed paraformaldehyde (PFA) (4% (w/v); Carl Roth GmbH & Co. KG; Cat. No.:4235.1) after three and five DIV. Next, neurons were washed three times with PBS and incubated with the primary antibody solution in a humid chamber. The antibodies (see Table 2) were diluted in PBT1 (PBS with 0.1% (v/v) Triton X-100 (AppliChem GmbH; Cat. No.: A49750500) and 1% (w/v) BSA (AppliChem)). Afterwards, coverslips were washed three times with PBS/A (PBS containing 0.1% (w/v) BSA (AppliChem)) and incubated with the secondary antibody solution for 2 hr at room temperature. The secondary antibodies (see Table 2) were diluted in PBS/A. In addition, bisbenzimide (Hoechst 33258, 1:100.000; Sigma-Aldrich) was used for the staining of nuclei. After the incubation with secondary antibodies, three washing steps with PBS and one washing step with MilliQ water was done. Finally, coverslips were mounted on microscope slides with ImmuMount (Thermo Fisher Scientific Inc.; Cat. No.: 9990402).

### 2.5.2 Staining of synaptic proteins

Neurons were fixed after 14 and 21 days in culture for 10 min with 4% PFA (w/v) at room temperature. Then, coverslips

| Antibody Target | Host | Clonality | Ig-type | Dilution | Company | RRID |
|----------------|------|-----------|---------|----------|---------|------|
| Aggrecan       | Rabbit | Polyclonal | —       | 1:500    | Merck KGaA | AB_90460 |
| βIII-Tubulin   | Mouse | Monoclonal | IgG2b   | 1:300    | Sigma-Aldrich | AB_477590 |
| Caspase 3      | Rabbit | Polyclonal | —       | 1:300    | Sigma-Aldrich | AB_476884 |
| Gephyrin       | Mouse | Monoclonal | IgG1    | 1:500    | Synaptic Systems GmbH | AB_887717 |
| Map2           | Rabbit | Polyclonal | —       | 1:300    | Sigma-Aldrich | AB_1840999 |
| PSD−95         | Mouse | Monoclonal | IgG2a   | 1:500    | Merck KGaA | AB_1121285 |
| Tau            | Chicken | Polyclonal | —       | 1:300    | Abcam | AB_1310734 |
| vGAT           | Guinea pig | Polyclonal | —       | 1:500    | Synaptic Systems GmbH | AB_887878 |
| vGlut          | Guinea pig | Polyclonal | —       | 1:1,000  | Synaptic Systems GmbH | AB_887873 |
| Biotin-conjugated WFAa | — | — | — | 1:100 | Sigma-Aldrich | AB_2620171 |

*Used as primary antibody in immunocytochemistry.
were washed three times with washing solution containing 10% (v/v) foetal bovine serum (FBS) (Sigma-Aldrich; Cat. No.: F7524), 0.1 mM glycine (VWR International GmbH; Cat. No.: 101196X) and 0.1% (v/v) Triton X-100 (AppliChem; Cat. No.: A49750500) in PBS. Afterwards, primary antibodies (see Table 2) were diluted in washing solution and incubated with coverslips for 1 hr at room temperature. Thereafter, coverslips were washed three times with wash solution and incubated with the secondary antibody solution (see Table 3) for 1 hr at room temperature. Last, coverslips were washed three times with PBS, once with MilliQ water and mounted on microscope slides with ImmuMount.

For the immunocytochemical staining of inhibitory synaptic proteins, a protocol by Dobie and colleagues was used, with minor modifications (Dobie & Craig, 2011). Hippocampal neurons were fixed with 4% (w/v) PFA for 10 min at room temperature. Afterwards, coverslips were rinsed three times with PBST (0.25% (v/v) Triton X-100 in PBS) and subsequently blocked with 10% (w/v) BSA (Carl Roth GmbH & Co. KG; Cat. No.: 8076.2) in PBS for 30 min at 37°C. Next, neurons were incubated with primary antibody solution in a humid chamber for 2 hr at 37°C. Primary antibodies (see Table 2) were diluted in blocking solution (3% (w/v) BSA in PBS). After incubating the neurons with the primary antibody solution, coverslips were washed three times with blocking solution. After that, secondary antibodies (see Table 3) were diluted in 3% (w/v) BSA in PBS and incubated with neurons for 1 hr at 37°C. Finally, the coverslips were washed three times with PBS, once with MilliQ water and mounted on microscope slides with ImmuMount.

2.6 | Enzymatic digestion with chondroitinase ABC

To prove the specificity and localization of the aggrecan signal (see Supplementary Figures 1 and 2), native hippocampal neurons were treated every third day with 50 mU/ml chondroitinase ABC (ChABC, Sigma-Aldrich, Cat. No.: C3667). Control neurons were treated with the same volume of BSA (0.01% (w/v) in H2O, Sigma-Aldrich, Cat. No.: A9418). After cultivation time of 14 and 21 days, in vitro neurons were fixed and stained immunocytochemically as previously described (see 2.5.1).

2.7 | Electrophysiology

2.7.1 | Cultivation of primary hippocampal neurons on multielectrode arrays

The spontaneous network activity of hippocampal neurons derived from embryos of Poly I:C- and saline-treated mouse dams was determined via MEA analysis. First, electrode fields of standard MEA plates (60MEA200/30iR-Ti-gr; Multichannel systems; Item No.: 890103) were coated with 0.05% (v/v) poly-ethylenimine (PEI, Sigma-Aldrich; Cat. No.: P3143) for 1 hr at room temperature. Afterwards, the PEI was aspirated and MEA plates were washed 4–5 times with MilliQ water. Then, the plates were air-dried and left for at least 30 min under ultraviolet light. Next, the electrode fields of MEAs were coated with laminin in a concentration of 5 µg/ml for 30 min in the incubator at 37°C and 6% CO2. After the second coating, the laminin solution was removed and directly replaced by the neuronal cell suspension. A total number of 30.000 neurons in 30 µl hippocampus medium (≈1 × 10⁶ cells/ml) were plated out on the midpoint of the electrode field. After an incubation time of ten minutes, the cell density was controlled under the microscope and supplemented if necessary. In a next step, the MEAs were flooded with 1 ml of hippocampus medium and incubated for 1 hr at 37°C and 6% CO2. After the neurons have settled, cell culture transwell inserts with cortical astrocytes were placed in specially manufactured racks after the astrocyte medium had been replaced by hippocampus medium and added to the neurons. These co-cultures were then placed in plastic boxes.
with a fluorinated ethylene propylene membrane (Teflon®, ALA MEA Sheets from ALA Scientific Instruments Inc. by Multi Channel Systems MCS GmbH) in the lid, which allows for gas exchange and limits evaporation (Geissler & Faissner, 2012).

The neuron-astrocyte co-cultures on the MEAs were incubated in boxes at 37°C and 6% CO2, until the recordings were performed.

**2.7.2 | MEA recordings**

Recordings of the spontaneous network activity of cultured neurons were performed after a cultivation time of 14 and 21 DIV. At first, the astrocyte containing inserts were replaced by an airtight lid to prevent contaminations. The transport of the MEA plates from the incubator to the recording chamber may cause an overactive network. Therefore, MEA plates were initially placed for ten minutes on a 35°C pre-heated pre-amplifier. After normalization of the network activity, recordings for 10 min were performed with a sample rate of 20 kHz, considering all 60 electrodes. For the recordings, the program MC_Rack (Version 3.9.0, Multichannel Systems) was used. Additionally, an adjusted frequency of 200 Hz was set for the high-pass filter that eliminated field potentials from the raw data. A spike detector was used, which recorded single amplitudes 4.5-fold higher than the standard deviation. For the detection of bursts, following settings were determined: maximal interval initiating a burst: 10 ms, maximal interval terminating a burst: 100 ms, maximal interval between two bursts: 210 ms, minimal duration of a burst: 50 ms, minimal number of spikes per burst: 5.

After the final recordings, the medium was replaced by 1% tergazyme (w/v) (Alconox) and incubated overnight at
**FIGURE 2** Early development of embryonic hippocampal neurons derived from Poly I:C- and saline-treated mouse dams. (a-d) Pregnant mice received i.p. injections either with Poly I:C (20 mg/kg) or saline (0.9%) at GD 9.5. Afterwards, hippocampal neurons were isolated at embryonic day 15.5 and cultured for 3 and 5 DIV for further analysis. (e-h) The percentage of apoptotic neurons in the cultures was determined via immunocytochemical staining against the activated form of the protease caspase 3 (red), while nuclei were visualized with bisbenzimide (blue). (i-l) Axons and dendrites were visualized immunocytochemically with antibodies against the microtubule-associated proteins MAP2 (red) and Tau (green). (m) No differences concerning the apoptosis rate could be observed in the neuronal cultures of both groups. (n,o) Hippocampal neurons isolated from the embryos of Poly I:C-treated mother mice formed significantly shorter axons after five DIV compared to the saline control group \( (p = .0031) \). (p,q) While the quantification of the dendritic number did not show significant differences, a significant reduction in the dendritic length in the Poly I:C group was observed after three DIV \( (p = .0381) \). However, this difference vanished after 5 DIV. Statistics: Five independent experimental repetitions (\( N = 5 \)) have been performed for the caspase 3 analysis, and the data of 25–30 recordings (\( n = 25–30 \)) have been quantified. In total, 7,500–10,000 cells were counted per condition. Data are shown as mean ± SD (Mann–Whitney U test for the data at DIV3, \( p \leq .05 \); t test for the data at DIV5 \( p \leq .05 \)). Scale bar: 100 µm. Four independent experiments (\( N = 4 \)) were done for the investigation of axonal and dendritic parameters. Per experimental repetition 30–40 cells (\( n = 30–40 \)) were analysed for the axonal parameters and 28–30 (\( n = 28–30 \)) cells for the dendritic parameters. Data are shown as mean ± SEM (Mann–Whitney U test, \( p \leq .05 \)). Scale bar: 50 µm.
room temperature. The next day, the tergazyme solution with degraded cells and debris was aspirated, and the MEA plates were subsequently washed three times with MilliQ water. After the washing steps, MEA plates were stored at 4°C for further use.

2.8 | Microscopy

For the documentation of active-caspase 3 as well as MAP2 and Tau immunostained neurons, the AxioPlan2 fluorescence microscope (Zeiss) was used. Images were recorded with an affiliated AxioCam MRm digital camera (Zeiss). The data documentation was done with the associated AxioVision 4.5 software (Zeiss).

Images of excitatory and inhibitory synaptic puncta were recorded using the confocal laser scanning-microscope LSM 510 Meta (Zeiss). Here, 5–7 z-stacks (with a width of 0.25 µm) were recorded dependent on the size and depth of the neuron. After the recording, the single stacks were overlaid to a so-called maximum intensity projection. Values for the gain and threshold were kept constant for all recordings.

2.9 | Data quantification

Apoptotic neurons were quantified after three and five DIV via active-caspase 3 staining using the Multi-point tool in ImageJ (version 1.52h). Here, the number of caspase 3-positive cells per image was set in relation to the total number of nuclei per image, stained with bisbenzimide.

The number of axonal and dendritic branches was also analysed manually with the Multi-point tool in ImageJ. For the analysis of axonal and dendritic lengths, the Freehand line tool in ImageJ was used after defining a certain distance, based on the corresponding scale bar.

The analysis of excitatory and inhibitory synaptic puncta was performed with the ImageJ Plugin “Puncta Analyzer” from Barry Wark (licensed under http://www.gnu.org/copyleft/gpl.html). The following settings were adjusted for the detection of puncta: rolling ball radius = 50 pixel, size (pixel²) = 2-infinity and circularity = 0.00–1.00. The percentage increase/decrease of synaptic puncta on PNN carrying neurons was calculated using the following formula:

\[
\frac{\text{value} - \text{saline mean value}}{\text{saline mean value}} \times 100.
\]

A detailed description of the MEA recordings as well as spike and burst quantification can be found in 2.6.2.

The areas of PNNs and somata were quantified with the “Freehand Selections” tool in ImageJ after the definition of a distance, depending on the scale bar. For the determination of the PNN fluorescence intensity, the corrected total cell fluorescence (CTCF) was calculated, as previously described (McCloy et al., 2014).

2.9.1 | Statistical analysis

The statistical analysis of the data was performed with Prism (GraphPad, version 8.2.1). First, the data sets were tested regarding their distribution using the Kolmogorov–Smirnov test. For normally distributed values, the unpaired t test was used and for not normally distributed, the Mann–Whitney U test was applied. A detailed number of independent experiments and the corresponding level of significance were specified in the figure legends. The significance level was set at p ≤ .05. Data are given in the result text part as mean ± SEM and in figures as mean value with standard deviation (±SD). Additional information is given at the end of the figure legends.

3 | RESULTS

3.1 | Control of the sickness behaviour via burrowing test

First, the induction of the sickness behaviour was controlled via the burrowing test, as previously described (Deacon, 2006) (Figure 1a–c). The measurement of the displaced sunflower seeds revealed no significant differences between both groups after 2 and 12 hr in the baseline recordings (Figure 1b). Next, the burrowing behaviour was analysed after injections with Poly I:C or saline (Figure 1c). Animals which received a saline injection displaced 14.35 ± 6.40 g sunflower seed 7 hr after the injection. In contrast, animals with a Poly I:C injection displaced only 0.049 ± 0.034 g and showed therefore a significantly (t = 2.234, df = 10; p = .0495) reduced burrowing behaviour and a reduced general activity. After 19 hr, Poly I:C-treated animals returned to their normal behaviour and did not show significant differences to the animals of the saline group (t = 0.1715, df = 10; p = .867).

3.2 | Prenatal immune challenge induced by Poly I:C administration worsens axonal elongation of hippocampal neurons in vitro

In order to investigate the impact of a prenatal immune activation induced by the administration of Poly I:C at GD 9.5, the apoptosis rate of embryonic hippocampal neurons in vitro was first analysed (Figure 2a–h). Therefore, embryonic mice of Poly I:C- and saline-treated mouse dams were isolated at developmental stage E15.5 and hippocampal neurons were cultured for 3 and 5 DIV. After fixation, neurons were immunostained with an antibody against the active form of caspase 3 and the apoptosis rate was determined by cell counting (Figure 2h). The quantification revealed similar values for the apoptosis rate of both groups at
both analysed time points (Figure 2h). After three DIV, an apoptosis rate of 17.56 ± 0.91% was observed in neuronal cultures derived from embryos of Poly I:C-treated mouse dams and 17.98 ± 0.69% in neuronal cultures derived from embryos of saline-treated control mice (Mann–Whitney U test = 10,594; p = .383). After five DIV, an apoptosis rate of 21.27 ± 0.98% was observed in neuronal cultures of the Poly I:C-treated group and 20.78 ± 1.12% in the saline control group (t = 0.3285, df = 275; p = .743). In addition, axonal and dendritic parameters were analysed using immunocytochemistry with antibodies against the microtubule-associated proteins MAP2 and Tau (Figure 2i-l). While MAP2 can be strongly detected in the dendrites and the soma of neurons (Caceres, Banker, Steward, Binder, & Payne, 1984), Tau exclusively accumulates in axons (Binder, Frankfurter, & Rehuhn, 1985). This allows for a differentiated analysis of axonal and dendritic characteristics.

Neurons of both groups did not show significant differences concerning the axonal lengths and branches after three days in culture (Figure 2n,e). At this time point, neurons isolated from embryos of Poly I:C-treated mice displayed an average axon length of 110.16 ± 3.61 µm with 3.88 ± 0.23 branches. In comparison, neurons of the control group showed an average axon length of 108.62 ± 3.43 µm (Mann–Whitney U test = 11,992; p = .979) with 4.12 ± 0.30 branches (Mann–Whitney U test = 9,344; p = .871). Interestingly, a significant reduction of axonal lengths could be observed in the Poly I:C-treated group after 5 DIV. While neurons of the control condition formed axons with a length of 167.20 ± 5.48 µm, a significantly reduced average axon length of 148.61 ± 4.51 µm could be observed in the Poly I:C-treated group (Mann–Whitney U test = 8,414; p = .003). Contrarily, the number of axonal branches did not differ between the Poly I:C and the saline condition after 5 days in culture (Poly I:C: 6.73 ± 0.35; saline: 8.35 ± 0.54; Mann–Whitney U test = 7,225; p = .102).

After analysing the axonal compartment, dendritic parameters were quantified with a focus on the number of dendrites per neuron and the average length of the longest dendrite (Figure 2p,q). Hippocampal neurons prepared from embryonic mice of Poly I:C-treated dams formed 5.62 ± 0.16 dendrites after three DIV and 7.00 ± 0.19 dendrites after five DIV. No statistical significance could be observed to neurons of the control condition, which formed 5.55 ± 0.18 dendrites after three DIV (Mann–Whitney U test = 6,264; p = .483) and 6.89 ± 0.19 dendrites after five DIV (Mann–Whitney U test = 7,024; p = .741). A mild, but significant difference was observed for the dendritic length after three days in vitro (Poly I:C: 37.92 ± 0.99 µm; saline: 35.01 ± 0.10 µm; Mann–Whitney U test = 6,084; p = .038). However, this difference vanished after five DIV, where neurons of the Poly I:C condition showed an average dendritic length of 46.22 ± 1.37 µm in comparison to the saline control neurons with an average dendritic length of 47.61 ± 1.43 µm (Mann–Whitney U test = 6,812; p = .483). In summary, the analysis of the early in vitro development of hippocampal neurons derived from embryonic mice of Poly I:C- and saline-treated mouse dams revealed no differences for the apoptosis rate and only minor aberrations concerning the axo-dendritic parameters. The strongest difference could be observed regarding the average axonal lengths of embryonic neurons derived animals with an immune challenge.

### 3.3 | Treatment of mouse dams with Poly I:C at gestation day 9 alters the amount of excitatory synaptic puncta of embryonic hippocampal neurons in vitro

For the analysis of structural synapse numbers, hippocampal neurons derived from embryonic mice of Poly I:C- and saline-treated mouse dams were cultured in an indirect co-culture setup with native cortical astrocytes for 14 and 21 days (Gottschling et al., 2016). After this cultivation time, neurons formed a complex and highly branched network (Figure 3a-d). Excitatory synaptic proteins were visualized via immunostaining with antibodies against vGlut and against PSD-95 (Figure 3e-H). For the visualization of inhibitory synaptic proteins, antibodies against vGAT and against gephyrin were used (Figure 3i-l). A colocalization of both fluorescence signals resulted in yellow puncta which were determined as structural glutamatergic or GABAergic synapses (Gottschling, Wegrzyn, Denecke, & Faissner, 2019) (Figure 3m). The number of synaptic puncta per recorded neuron was quantified and included somatic as well as dendritic synapses. We additionally used aggrecan as a PNN marker and excluded aggrecan-positive neurons for this quantification.

After 14 DIV, a significantly increased number of PSD-95 and colocalized puncta could be observed in cultured neuronal networks of the Poly I:C condition (Figure 3n). While neurons of the control condition formed 6,169.54 ± 153.74 PSD-95 and 2,777.47 ± 87.70 colocalized puncta, neurons derived from animals with a prenatal immune challenge formed 6,757.89 ± 136.38 PSD-95 (t = 2.863, df = 198; p = .0047) and 3,042.98 colocalized puncta (t = 2.284, df = 198; p = .023). The number of vGlut puncta did not differ significantly between neurons of both groups (Poly I:C: 3,605.17 ± 85.71; saline: 3,457.28 ± 116.26; Mann–Whitney U test = 4,297; p = .0047) and 3,042.98 colocalized puncta, neurons derived from animals with a prenatal immune challenge formed 6,757.89 ± 136.38 PSD-95 (t = 2.863, df = 198; p = .0047) and 3,042.98 colocalized puncta (t = 2.284, df = 198; p = .023). The number of vGlut puncta did not differ significantly between neurons of both groups (Poly I:C: 3,605.17 ± 85.71; saline: 3,457.28 ± 116.26; Mann–Whitney U test = 4,297; p = .086).

After 21 DIV, neurons isolated from the embryonic hippocampus of Poly I:C-treated mouse dams formed significantly more vGlut puncta (3,622.27 ± 75.64; t = 2.091; df = 198; p = .038) in comparison to the control condition (3,381 ± 86.95). The previously observed increase of PSD-95 and colocalized puncta at DIV 14 in the Poly I:C-treated group was no longer observable after 21 DIV. Here, neurons from the Poly I:C-treated condition formed 6,542.36 ± 200.59 PSD-95 (t = 0.6338, df = 198;
FIGURE 3  Analysis of the glutamatergic and GABAergic synapse formation of cultured neuronal networks after 14 and 21 DIV. (a-d) Hippocampal neurons derived from embryonic mice of Poly I:C- (20 mg/kg) and saline(0.9%)-treated mouse dams were cultured for 14 and 21 days in an indirect co-culture setup with native cortical astrocytes. Neurons of both groups formed a dense and highly branched network. (e-h) Excitatory synaptic proteins were visualized with antibodies against vGlut, (red) and against PSD-95 (green) and recorded via confocal laser scanning microscopy. A colocalization of both signals yielded in yellow puncta, which were determined as structural glutamatergic synapses (m, left). (i-l) Inhibitory synaptic proteins were visualized immunocytochemically with antibodies against vGAT (green) and the postsynaptic scaffolding protein gephyrin (red). The colocalization of the signals resulted in yellow puncta, which were quantified as structural GABAergic synapses (m, right). White boxes beside each image show magnified regions on dendrites. (n) Quantification of excitatory puncta revealed significantly increased numbers of PSD-95 ($p = .0047$) and colocalized puncta ($p = .0234$) after 14 DIV and significantly increased numbers of vGlut puncta ($0.0378$) after 21 DIV in the Poly I:C group. (o) Comparison of inhibitory puncta did not show significant differences between both groups after 14 and 21 DIV. Synaptic puncta of the whole recorded cell, including soma and dendrites, were quantified. Statistics: Five independent experiments ($N = 5$) have been performed for the analysis of structural glutamatergic synapse numbers and 20 cells ($n = 20$) were quantified per experiment. Data are shown as mean ± SEM ($t$ test was used for all conditions except vGlut DIV14, where the Mann–Whitney U test was used, $p ≤ .05$). Scale bar: 50 µm. Three independent experimental repetitions ($N = 3$) were done concerning the structural inhibitory synapse staining. Here, also 20 cells ($n = 20$) were analysed per experimental repetition. Data are shown as mean ± SD (Mann–Whitney U test, $p ≤ .05$). Scale bar: 50 µm.
The quantification of inhibitory synaptic puncta revealed no significant changes between both groups at all analysed time points (Figure 3o). After 14 DIV 973.88 ± 78.44 puncta for vGAT, 3,246.93 ± 199.15 puncta for gephyrin and 474.23 colocalized puncta were determined for neurons of the control group. In comparison, 914.07 ± 89.98 puncta for vGAT (Mann–Whitney U test = 1587; \( p = .264 \)), 3,424.92 ± 202.42 puncta for gephyrin (Mann–Whitney U test = 1702; \( p = .607 \)) and 440.56 ± 32.00 ( \( t = 0.7518, df = 117; p = .388 \)) colocalized puncta were observed for neurons of the Poly I:C-treated condition. After 21 DIV 790.97 ± 80.11 vGAT puncta, 3,409.87 ± 177.17 gephyrin puncta and 471.43 ± 40.50 colocalized puncta were observed in neuronal control cultures. Similar values were detected in the Poly I:C-treated condition (vGAT: 802.68 ± 74.38; Mann–Whitney U test = 1,660; \( p = .463 \)); gephyrin: 3,288.23 ± 205.59; Mann–Whitney U test = 1662; \( p = .469 \) colocalization: 431.37 ± 41.07; Mann–Whitney U test = 1626; \( p = .363 \)).

Summarized, it could be observed that the prenatal activation of the immune system via intraperitoneal injections of Poly I:C increased the presence of postsynaptic PSD-95 and colocalized puncta in neuronal cultures after 14 DIV. However, this enhanced number of postsynaptic and colocalized puncta disappeared after 21 DIV and was interestingly replaced by a significantly increased number of presynaptic vGlut puncta. In addition, it is worth mentioning that the inhibitory synaptic proteins vGAT and gephyrin did not show any significant alterations in this study.

### 3.4 Prenatal activation of the maternal immune system strongly raises the spontaneous network activity of hippocampal neurons after 21 DIV

After investigating the structural synapse properties of cultured neuronal networks, functional analysis was performed. Therefore, embryonic mice of Poly I:C- and saline-treated mouse dams were used for the cultivation of hippocampal neurons on electrode fields of multielectrode arrays (Figure 4a,b). As before, neurons of both groups were cultured indirectly with native cortical astrocytes for 14 and 21 DIV (Geissler & Faisstner, 2012). An increasing spontaneous activity could be observed in both groups from the measurement after 14 DIV to the measurement after 21 DIV (Figure 4c-f).

The quantification of the recordings revealed interesting and significant differences in neuronal networks derived from the Poly I:C-treated group. First, spontaneous occurring action potentials per electrode, so-called spikes, were analysed (Figure 4g). After 14 DIV, recordings revealed a significant reduction in the number of spikes. Here, 2,788.76 ± 158.69 spikes were observed in the control condition and 2,112.11 ± 90.62 spikes in Poly I:C condition (Mann–Whitney U test = 79.939; \( p = .019 \)). Interestingly, a strong and significant increase in the number of spikes could be detected in networks derived from the Poly I:C-treated group after 21 DIV. At this time point, 5,318.89 ± 289.47 spikes could be detected in neurons isolated from embryonic mice of Poly I:C-treated mouse dams in comparison to 3,218.02 ± 221.16 spikes in the control condition (Mann–Whitney U test = 31.778; \( p < .001 \)).

Next, bursts were analysed which are defined as sequence of synchronously occurring action potentials with specific characteristics listed in section 2.6.2 (Figure 4h) (Canepari, Bove, Maeda, Cappello, & Kawana, 1997; Segev, Shapira, Benveniste, & Ben-Jacob, 2001). After 14 DIV, no significant changes could be observed concerning the number of bursts. Neurons of the control condition exhibited 53.09 ± 2.43 bursts per measurement in comparison to 45.57 ± 2.57 bursts in the network from the Poly I:C-treated condition (Mann–Whitney U test = 82.290; \( p = .093 \)). MEA recordings of hippocampal neurons derived from Poly I:C-treated mother mice revealed 136.47 ± 7.52 bursts and were significantly (Mann–Whitney U test = 33.118; \( p < .001 \)) higher in comparison to neurons derived from embryonic mice of saline-treated dams (97.16 ± 6.41). The analysis of the burst duration revealed no significant differences between both groups after 14 (Poly I:C: 364.72 ± 12.04; saline: 357.14 ± 11.11; Mann–Whitney U test = 70.333; \( p = .409 \)) and 21 DIV (Poly I:C: 396.38 ± 27.64; saline: 347.58 ± 17.84; Mann–Whitney U test = 33.426; \( p = .346 \)). Last, the percentage of spikes occurring in bursts was determined. After 14 DIV, no significant changes were observed between the Poly I:C and saline condition. However, a significantly increased number of spikes occurring in bursts were detected in cultures derived from embryonic mice of Poly I:C-treated dams after 21 DIV. Here, 64.09 ± 1.64% of spikes in bursts could be observed in the cultures created from the Poly I:C mice and 50.71 ± 2.11% of spikes in bursts created from control mice (Mann–Whitney U test = 27.671; \( p < .001 \)).

In summary, the analysis of the spontaneous network activity uncovered an interesting link between the prenatal treatment with Poly I:C and an elevated level of activity. Especially, the number of spikes and bursts was highly increased after a cultivation time of 21 DIV. This increase correlated strongly with the higher number of presynaptic vGlut puncta observed in the previous experiment (Figure 3n).

### 3.5 Analysis of perineuronal net wearing neurons derived from embryonic mice of mouse dams with maternal immune activation

For the analysis of PNNs, neuronal cultures were prepared as described previously from embryonic mice isolated
from Poly I:C- and saline-treated mouse dams and cultured in an indirect contact with native cortical astrocytes. The visualization of the PNNs was done by immunostaining with an antibody against aggrecan, a main component of PNNs in combination with antibodies against excitatory synaptic proteins after 14 and 21 DIV (Figure 5a-g)

**FIGURE 4** Functional analysis of neuronal network properties via multielectrode array (MEA) recordings. (a,b) After the i.p. treatment of pregnant mice at GD 9.5 with Poly I:C (20 mg/kg) or saline (0.9%), embryonic mice (E15.5) were used for the cultivation of hippocampal neurons in an indirect contact with native cortical astrocytes on MEAs for 14 and 21 DIV. (c-f) Representative recordings of the spontaneously arising network activity of neuronal networks cultured on MEAs for 14 and 21 DIV. (g) Quantification of spontaneously occurring action potentials, so-called spikes, revealed first a significant reduction after 14 DIV, followed by a strong and significant increase in the Poly I:C group after 21 DIV ($p < .001$). (h) The organized network firings, called bursts, were also significantly increased in neuronal cultures derived from embryonic mice of Poly I:C-treated mouse dams after 21 DIV. (i) Analysis of the average burst duration unravelled no significant changes between both groups. (j) The percentual proportion of spikes occurring in bursts was significantly increased in networks derived from animals with an immune challenge after 21 DIV ($p < .001$). Statistics: Seven experiments ($N = 7$) have been performed for the analysis of spikes and bursts after 14 DIV and five experiments ($N = 5$) for the analysis after 21 DIV with the data of 60 analysed electrodes ($n = 60$) per experiment. For the analysis of the burst duration and the percentage of spikes in bursts, seven experiments ($N = 7$) were done for the point in time DIV14 and five experiments ($N = 5$) for the point in time DIV21. Here, the data of 50–58 ($n = 50–58$) electrodes were used for the statistics. Data are shown as mean ± SD (Mann–Whitney U test, $p \leq .05$)
FIGURE 5  Immunocytochemical analysis of perineuronal nets in cultured hippocampal neurons isolated from embryonic mice of Poly I:C- and saline-treated mouse dams. (a-d) PNNs were formed by a subtype of neurons and were detected immunocytochemically after 14 and 21 DIV with an antibody against the core component aggrecan (blue). (e-g) A triple staining against vGlut (red), PSD-95 (green) and aggrecan (blue) was used in order to visualize structural glutamatergic synapses on the somata of PNN-wearing neurons. (h) The amount of PNN-wearing neurons did not differ between both conditions after 14 and 21 DIV. (i) The quantification of PNN areas revealed interestingly a significant reduction after 21 DIV in the Poly I:C group ($p < .001$). (j) Analysing the corrected total cell fluorescence (CTCF), a significantly attenuated intensity of the aggrecan-positive PNNs was observed in the Poly I:C condition after 21 DIV ($p = .0014$). (k) The average soma areas of PNN-wearing neurons in the Poly I:C group were significantly smaller after 14 DIV ($p = .0169$) and 21 DIV ($p = .0034$) in comparison to the saline control condition. (l) Soma areas of PNN (−) neurons in the Poly I:C condition show a significantly increased size after 14 DIV ($p = .0020$). (m) Quantification of excitatory synaptic puncta on the PNN covered soma region unravelled a significant reduction of PSD-95 puncta in the Poly I:C condition after 14 DIV ($p = .0124$). After 21 DIV, vGlut ($p = .0017$), PSD-95 ($p = .0005$) and colocalized signals ($p = .0489$) were all significantly decreased in the prenatal MIA group. Statistics: In order to determine the percentage of PNN (+) neurons, five independent experiments ($N = 5$) were performed and 50–54 fluorescence images ($n = 50–54$) were quantified per experiment. In total, 1850–1950 neurons were counted per condition. Data are shown as mean ± SD (Mann–Whitney U test, $p \leq .05$). For the analysis of PNN parameters and the number of glutamatergic synapses on PNN (+) neurons five, independent experiments ($N = 5$) were done and 20 neurons ($n = 20$) per experiment quantified. Data are shown as mean ± SD except subfigure 5 m which represents mean ± SEM (Mann–Whitney U test, $p \leq .05$). Scale bar: 50 µm.
(Gottschling et al., 2019). The specificity of the aggrecan signal and its extracellular localization was proven by a chronic chondroitinase ABC (ChABC) treatment of native neurons (see Figure S1 and S2). When neurons were treated every third day with ChABC, aggrecan could not be detected anymore by immunocytochemistry, proving an extracellular localization. Similar observations have been reported when cultured cortical neurons were treated with ChABC after fixation. In that study, the authors demonstrated that the degradation of GAG side chains of CSPGs and of hyaluronan strongly disrupted the structure of PNNs. Consequently, components like aggrecan or tenascin-R that do not possess an own transmembrane domain diffused away from the PNN assembly. In conclusion, PNN constituents are integrated via CS- and hyaluronan-dependent interactions of ECM components (Giamanco, Morawski, & Matthews, 2010). Hence, we propose that a chronic addition of ChABC to the culture medium caused a removal of aggrecan core proteins from the PNNs, as revealed by a loss of immunocytochemical staining.

First, the percentual amount of PNN-wearing neurons was determined and revealed no significant differences between both groups after 14 (Poly I:C: 12.26 ± 1.14%; saline: 12.68 ± 0.82%; Mann–Whitney U test = 1,235; \( p = .456 \)) and 21 DIV (Poly I:C: 11.38 ± 0.90%; saline: 13.68 ± 0.98%; Mann–Whitney U test = 1,327; \( p = .196 \)) (Figure 5h). Regarding the area of PNNs, a significant reduction could be observed after 21 DIV (Figure 5i). Here, embryonic neurons derived from saline-treated mouse dams formed PNNs with an average area of 1,212.97 ± 53.86 \( \mu m^2 \) while neurons of the Poly I:C condition formed PNNs with a significantly smaller average area of 835.36 ± 42.38 \( \mu m^2 \) (Mann–Whitney U test = 2,909; \( p < .001 \)). After 14 DIV, a small but not significant reduction of the PNN area could be already detected (Poly I:C: 1,188.18 ± 42.16 \( \mu m^2 \); saline: 1,281.90 ± 46.36 \( \mu m^2 \); \( t = 1.485; df = 198; p = .139 \)). In addition to the analysis of the PNN area size, the fluorescence intensity was determined via the calculation of the corrected total cell fluorescence (CTCF) (Figure 5j). After 14 DIV, PNNs of the control group showed a fluorescence intensity of 174,054.38 ± 7,913.38 in comparison to the Poly I:C-treated group with 157,073.50 ± 8,828.07 (Mann–Whitney U test = 4,284; \( p = .80 \)). After 21 DIV, a fluorescence intensity of 165,352.47 ± 8,990.99 was observed in neuronal cultures of the saline-treated condition and a significantly downregulated fluorescence intensity of 128,305.58 ± 8,450.73 in neuronal cultures derived from the Poly I:C-treated group (Mann–Whitney U test = 3,690; \( p = .0014 \)).

Last, the number of excitatory synaptic puncta on the soma regions of PNN-positive neurons was determined (Figure 5f,g,m). PNNs mostly cover neuronal somata as well as the proximal dendrites. As we see differences in soma size and perineuronal net area, we focused on this explicit region. Interestingly, neuronal cultures derived from embryonic mice of Poly I:C-treated mouse dams showed a significantly reduced number of PSD-95 puncta by \(-18.43 ± 3.37\% \) (Mann–Whitney U test = 3,976; \( p = .0124 \)) and of the vGlut and colocalized puncta, no significant changes were observed between both groups after 14 DIV (vGlut: \(-14.12 ± 4.15\% \); Mann–Whitney U test = 4,206; \( p = .022 \)). After a cultivation time of 21 DIV, stronger effects could be observed. The number of chimeric puncta \((18.74 ± 4.45\% \); Mann–Whitney U test = 4,021; \( p = .017 \)) and colocalized puncta \((-23.43 ± 4.45\% \); Mann–Whitney U test = 3,569; \( p = .0005 \)) were significantly reduced in neuronal cultures of the Poly I:C-treated condition. In summary, the analysis of PNN-wearing neurons revealed a significant reduction of PNN areas and PNN fluorescence intensities after 21 DIV. Interestingly, the number of structural glutamatergic synapses was also decreased on PNN-positive neurons of the Poly I:C-treated condition after 21 DIV. Finally, we could also observe a significant reduction of the soma area in neuronal cultures derived from Poly I:C-treated mice after 21 DIV.
4 | DISCUSSION

4.1 | Summary

In our study, we analysed the impact of a prenatal immune challenge via intraperitoneal Poly I:C injections on the development of embryonic hippocampal neurons in vitro. Analysis of the early neuronal development in vitro revealed only minor effects. Here, the apoptosis rate and dendritic parameters remained unchanged at both measured time points. However, a significantly reduced axonal complexity could be observed after five days in culture. The strongest effects were observed concerning the spontaneous network activity. This was first significantly reduced after 14 DIV and then increased in neuronal networks derived from embryonic mice of the maternal immune challenge group after 21 DIV. The elevated activity was accompanied by an increased number of presynaptic vGlut puncta on PNN-negative neurons. Additionally, significantly decreased PNN areas and reduced PNN staining intensities could be observed at this time point.

4.2 | Induction of the systemic inflammatory challenge and effects on early neuronal development

First, we utilized the burrowing test to confirm sickness behaviour of mice after Poly I:C challenge. Therefore, mice were tested after seven hours postimmune challenge. We could observe that mice treated with Poly I:C displayed reduced burrowing activity compared to controls (Figure 1c). However, the next day, Poly I:C-treated mice have recovered their burrowing behaviour. These results are congruent with those from other studies (Cunningham et al., 2007; Konat, Borysiewicz, Fil, & James, 2009) and indicate that the mice effectively suffered from a systemic immune response. In this way, we ascertain that the embryonic mice used for the cell culture experiments were exposed to inflammatory mediators. Different studies have proven a link between prenatal Poly I:C injections and behavioural changes (e.g. prepulse inhibition or latent inhibition) in the offspring (Meyer, Feldon, Schedlowski, & Yee, 2005; Smith et al., 2007; Wolff & Bilkey, 2008). A previous study dealing with the Poly I:C mouse model revealed a strong increase of the cytokines IL-6 and TNF-α in unstimulated heparinized blood samples 2 hr after intraperitoneal Poly I:C injection (Esslinger et al., 2016). Both detected cytokines share properties that allow them to pass the placental barrier (Zaretsky, Alexander, Byrd, & Bawdon, 2004). Additionally, it has been proven that especially IL-6 can pass the placental barrier with a higher rate in the mid-gestation period than in the late gestation period and induce foetal injury or stimulation of foetal stress hormones (Allswede & Cannon, 2018; Dahlgren, Samuelsson, Jansson, & Holmang, 2006). Interestingly, analysis of foetal brains after induction of a MIA via Poly I:C administration at GD 9.5 revealed significantly increased protein levels of IL-6 after three and six hours, while IL-6 mRNA levels remained unchanged (Meyer et al., 2006). Knockout studies of IL-6 demonstrated a prevention of inflammatory responses in placental–foetal axis and a reduction of behavioural abnormalities in MIA-induced mouse models (Smith et al., 2007; Wu, Hsiao, Yan, Mazmanian, & Patterson, 2017). These data indicate that the increased levels of cytokines like IL-6 and TNF-α can reach the embryonic central nervous system and affect the neuronal development, which was analysed in our study.

Neuronal apoptosis in vitro was determined via immunocytochemical active-caspase 3 staining after three and five days, without significant differences between the groups (Figure 2m). An interesting study focused on the analysis of neuronal survival after the administration of increasing concentrations of IL-1β, IL-6 and TNF-α and revealed that only high concentrations of these cytokines decreased the viability of cultured cortical neurons (Gilmore, Fredrik Jarskog, Vadlamudi, & Lauder, 2004). In vivo analysis of active-caspase 3-positive neurons in the offspring hippocampus also did not show significant changes when Poly I:C was administered at GD 9.5 (Meyer et al., 2006). Therefore, we hypothesize that the concentrations of released immune factors induced by the administration of Poly I:C were possibly not high enough to induce neuronal cell death in our cultures. Another possible explanation might be that neuronal cell death occurred earlier, before the removal and preparation of embryonic hippocampi. The same work by Gilmore et al. show a clear reduction of dendritic nodes when cytokines were added to the medium. In our study, we only observed minor and transient effects regarding dendritic arborization after 3 days in vitro (Figure 2p,q). The mild phenotype might be explained by the fact that the maternal immune activation does not influence all embryonic mice in the same severe way. Recently published studies discuss the sex of the animals as an important protection from MIA effects (Chavez-Valdez et al., 2019; Haida et al., 2019). As we used the hippocampi of five embryonic mice per culture, it is plausible that our cultures also include neurons from animals which were lesser affected by the MIA. Although the biological significance appears to be low for our cultures, it might be greater for individual animals. This aspect includes also differences in the axonal compartment. However, further analysis of dendrites after longer cultivation time points would be useful, because the dendritic complexity rises after 5 days in culture. Additionally, the analysis of dendritic spines in this model would be of great interest. Interestingly, the morphological analysis of the axonal complexity revealed a significant reduction of the axonal length and an impaired but not significant axonal branch number (Figure 2n,o). In contrast...
to the mild effects on dendrite parameters, the axonal development seems to be more affected by Poly I:C treatment. The impaired axonal development is interesting because of the dysconnectivity hypothesis in schizophrenia (Friston, 1998; Zalesky et al., 2011). However, less is known about the effects of a prenatal immune activation on the axonal guidance or axonal complexity in prenatal MIA animal models. A study by Makinodan and colleagues could show a delay in axonal development and a significantly decreased axonal diameter in the hippocampus of Poly I:C-treated mice at postnatal day 14, supporting our observations (Makinodan et al., 2008). As already indicated in the introduction, our study focused on neuron-specific changes and excluded the impact of astrocytes. As astrocytes contribute to the formation of the tetrapartite synapse (Chelini, Pantazopoulos, Durning, & Berretta, 2018) and also react to inflammation or injury (Sofroniew, 2015), it would be of great interest to switch the combination of the co-culture and analyze the influence of astrocytes derived from newborn mice of Poly I:C-treated dams with native embryonic neurons. Interestingly, some previous studies already unravelled a link between a prenatal MIA and alterations in GFAP expression and S100b levels (Samuelsson, Jennische, Hansson, & Holmang, 2006; de Souza et al., 2015). In addition, it is known that astrocytes express aggrecan, which is a main component of PNNs. Therefore, one could assume that a combination of neurons and astrocytes derived from Poly I:C animals might show more severe effects.

4.3 Perineuronal net formation and hyperexcitability of the neuronal networks

The most pronounced effect of the Poly I:C treatment in our mouse model was visible with regard to the altered network activity on multielectrode arrays (Figure 4g-j). While the number of spontaneous occurring action potentials was significantly lowered after 14 DIV, a strong and significant increase was observed after 21 DIV. Based on our analysis of early neuronal development, we assume that the impaired axonal outgrowth and branching might be a possible explanation for the reduced number of spikes after 14 DIV. However, changes in basal neurotransmitter levels (Winter et al., 2009) as well as long-lasting epigenetic changes (Basil et al., 2014) might be other possible explanations. Additionally, we observed a significantly increased number of PSD-95 puncta at that point in time, while presynaptic terminals were not altered. We suppose that the increase is caused by greater soma areas of PNN-negative neurons (see Figure 5i). Cytoskeletal alterations induced by the prenatal immune activation could in turn explain the increased cell surface of hippocampal neurons in our cultures. An interesting proteome analysis revealed changes in neurite outgrowth and cytoskeletal proteins in a prenatal LPS rat model (Gyorffy et al., 2016).

However, less is known about the neuronal cell volume or cytoskeletal abnormalities in MIA models. Here, most studies focus on the cell numbers or volumes of brain regions. Interestingly, previous studies focused on neurotransmission in the hippocampus in Poly I:C or lipopolysaccharide (LPS)-induced MIA models unravelled differences in network properties in offspring animals. Lowe and colleagues analysed neuronal transmission in hippocampal slices of rats descending from LPS-treated dams and observed on the one hand smaller evoked field excitatory postsynaptic potentials (fEPSPs), but on the other hand a heightened intrinsic excitability of CA1 pyramidal neurons (Lowe et al., 2008). This increased excitability of CA1 neurons fits in to our electrophysiological MEA data after 21 DIV. Another study demonstrated a reduced frequency and increased amplitudes of miniature excitatory postsynaptic currents (mEPSPs) in the CA1 region of animals derived from Poly I:C-treated mouse dams (Ito et al., 2010). The increased spontaneous activity in our cultures after 21 days went along with a reduced area and staining intensity of PNNs as well as reduced soma areas of PNN-wearing neurons. In agreement with our observations, experiments with a Poly I:C rat model exhibited reduced areas of PNNs in the medial prefrontal cortex after an induced maternal immune challenge at mid-gestation day 15 (Paylor et al., 2016). Another in vivo study dealing with the effects of a prenatal immune activation via Poly I:C administration at a later time point of gestation (G15) in mice revealed dominant effects on hippocampal properties including a reduced hippocampal volume, decreased number of parvalbumin-positive interneurons and a lowered synaptic inhibition in mature DG neurons (Zhang & van Praag, 2015). This partially matches with our data, because Zhang et al. observed in their study a reduced number of action potentials in the hippocampus in the offspring. However, we could detect a strong increase of neuronal activity after longer cultivation period. Nevertheless, the different time point of Poly I:C administration and the in vivo situation could be reasons for this divergence. We assume that the increased number of action potentials after 21 DIV in our study might be explained by a stronger damage to parvalbumin interneurons, reminiscent of observations reported by Zhang et al. in their study. Parvalbumin-positive interneurons are often associated with PNNs (Bruckner et al., 2010; Carulli et al., 2010; Hartig et al., 1994; Wintergerst et al., 1996; Yamada et al., 2015) and displayed significantly reduced soma areas after 14 and 21 days in our neuronal cultures (Figure 5i,j). In addition, a reduction of structural synapse numbers could be observed on the soma of PNN-positive neurons (Figure 5m). This could be an indication for a damage or degradation of this neuronal subtype. Parvalbumin-positive interneurons are crucial for inhibitory gamma wave oscillations and the synchronization of the neuronal network (Bartos et al., 2002). Lodge and colleagues could show that a loss of parvalbumin-positive
interneurons is associated with diminished oscillatory activity in an animal model of schizophrenia and discuss this as a possible explanation for the origin of hippocampal hyperactivity (Lodge, Behrens, & Grace, 2009). As a matter of fact, it has already been shown that parvalbumin-positive interneurons are more sensible to environmental factors or oxidative stress (Inan, Petros, & Anderson, 2013; Steullet et al., 2017). In addition, alterations of PNNs have effects on the balance of excitatory and inhibitory synapse numbers as well as receptor motility in the neuronal membrane (Bakalo, Schachner, & Dityatev, 2001; Frischknecht et al., 2009; Geissler et al., 2013; Gottschling et al., 2019). An altered synaptic transmission is also an important characteristic of schizophrenia, as it has been nicely described for GABA (Stan & Lewis, 2012) and dopamine transmission (Brisch et al., 2014). The increased network activity in our MEA cultures was accompanied by elevated levels of presynaptic vGlut which might also contribute to the hyperactivity. However, in literature little is known about the expression of vGlut1 in prenatal MIA models. A study by Pendyala et al. revealed significantly decreased levels of vGlut1 in mixed cerebellar cultures (Pendyala et al., 2017). Nevertheless, the development of the cerebellum differs strongly from the development of the hippocampus. Here, we can just carefully suspect that the increased levels of presynaptic vGlut1 might be caused by an aberrant axonal branching and possible excess of presynaptic terminals.

Summarized, the observations of our study support the growing number of hints for a correlation between a prenatal immune activation and alterations in electrophysiological properties of hippocampal neurons in the offspring. Here, especially a damage of PNN-wearing GABAergic interneurons might be an important link for the imbalance in neurotransmission. Given that, so far, our results were only found in embryonic hippocampal cell cultures, future studies may focus on the embryonic in vivo situation and analyse the effects of a MIA on the development and differentiation of interneurons.

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CONFLICT OF INTEREST
The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS
A.F. and G.J. designed and supervised the study. D.W., M.P.M. and M.K. performed the experiments, analysed and interpreted the data. G.J., N.F. and M.P.M provided the Poly I:C mouse model and affiliated materials. A.F., G.J and N.F. interpreted the data. D.W. drafted the manuscript and A.F., G.J and N.F. revised the paper. All authors discussed the results and commented the manuscript.

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ORCID
Andreas Faissner https://orcid.org/0000-0002-2211-8259

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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