Changes in Neuronal Excitability by Activated Microglia: Differential Na+ Current Upregulation in Pyramid-Shaped and Bipolar Neurons by TNF-α and IL-18

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Microglia are activated during pathological events in the brain and are capable of releasing various types of inflammatory cytokines. Here, we demonstrate that the addition of 5% microglia activated by 1 μg/ml lipopolysaccharides (LPS) to hippocampal cultures upregulates Na+ current densities (I_{NavD}) of bipolar as well as pyramid-shaped neurons, thereby increasing their excitability. Deactivation of microglia by the addition of 10 ng/ml transforming growth factor-β (TGF-β) decreases I_{NavD} below control levels suggesting that the residual activated microglial cells influence neuronal excitability in control cultures. Preincubation of hippocampal cultures with 10 ng/ml tumor necrosis factor-α (TNF-α), a major cytokine released by activated microglia, upregulated I_{NavD} significantly by ~30% in bipolar cells, whereas in pyramid-shaped cells, the upregulation only reached an increase of ~14%. Incubation of the cultures with antibodies against either TNF-receptor 1 or 2 blocked the upregulation of I_{NavD} in bipolar cells, whereas in pyramid-shaped cells, increases in I_{NavD} were exclusively blocked by antibodies against TNF-receptor 2, suggesting that both cell types respond differently to TNF-α exposure. Since additional cytokines, such as interleukin-18 (IL-18), are released from activated microglia, we tested potential effects of IL-18 on I_{NavD} in both cell types. Exposure to 5–10 ng/ml IL-18 for 4 days increased I_{NavD} in both pyramid-shaped as well as bipolar neurons, albeit the dose–response curves were shifted to lower concentrations in bipolar cells. Our results suggest that by secretion of cytokines, microglial cells upregulate Na+ current densities in bipolar and pyramid-shaped neurons to some extent differentially. Depending on the exact cytokine composition and concentration released, this could change the balance between the activity of inhibitory bipolar and excitatory pyramid-shaped cells. Since bipolar cells show a larger upregulation of I_{NavD} in response to TNF-α as well as respond to smaller concentrations of IL-18, our results offer an explanation for the finding, that in certain conditions of brain inflammations periods of dizziness are followed by epileptic seizures.

Keywords: voltage-activated sodium currents, microglia, hippocampal neurons, interleukin-18, tumor necrosis factor-α, lipopolysaccharide, transforming growth factor-β, glial cells

INTRODUCTION

Glial cells influence neuronal functions in various ways. Apart from preventing mutual excitation of adjacent neurons by K^+ buffering and glutamate uptake (1), they can also modulate neuronal excitability. For instance, thyroid hormone triiodo-L-thyronine (T3) upregulates Na^+ currents and thereby increases excitability of postnatal rat hippocampal and cortical neurons (2, 3) via secretion of proteins [most prominently basic fibroblast growth factor (FGF-2)] from satellite cells (4).

Microglia are the resident macrophage-like population in the central nervous system (CNS) (5–7). They are continually active in their resting state, palpating and surveying their microenvironment with extremely motile processes (8). Pathological events lead to a rapid change of the microglial morphology (9) and the expression of cell surface proteins (10) such as antigens (11). A well-established method of activating microglia in culture is their stimulation via lipopolysaccharides (LPS) (12), the major surface membrane components of Gram-negative bacteria. The LPS stimulus on microglia is mediated by Toll-like receptors 1–9 (TLR), especially TLR2 (13). TLRs are a major family of pattern recognition receptors that mediate innate immunity but also link with the adaptive immune response, therefore providing a mechanism by which microglia are able to sense both pathogen- and host-derived ligands within the CNS (14, 15). In this activated state, microglia secrete a variety of cytokines (16), including proinflammatory factors like the IL-1/IL-18 family members and TNF-α.

Some growth factors and cytokines do not only regulate cell survival, differentiation, and proliferation but are also able to modulate the expression of ion channels. Long-term effects on the expression of Na^+ channels by nerve growth factor (NGF) have already been shown in DRG neurons (17, 18) and nociceptive primary afferent neurons (19). Likewise, FGF-2 upregulates Na^+ current density in PC12 cells (20) as well as hippocampal neurons (4). Recently, TNF-α has been shown to contribute to the upregulation of Nav1.3, Nav1.8 (21), and Nav1.7 (22) in dorsal root ganglion (DRG) neurons (21) and to enhance Na^+ currents in injured DRG neurons (18). Since the voltage-gated sodium channels are essential for the generation and conduction of electrical impulses in excitable cells, an upregulation of the Na^+ current density (I_{NavD}) especially of Nav1.7 and Nav1.8 has been suggested to be involved in the enhancement of excitability in inflammation in the peripheral nervous system (23–27) leading to hyperalgesia, inflammatory, and neuropathic pain.

In the CNS, inflammations are accompanied by conditions ranging from dizziness to epileptic seizures (28). In infectious diseases, transient epileptic episodes have been observed (29, 30), which have been suggested to be caused by excessive cytokine release from activated microglia (31, 32). Epileptiform neuronal excitability can be induced by changes in the intrinsic neuronal excitability governed by the membrane density of voltage-activated sodium channels (Nav1.1–Nav1.3 and Nav1.6) leading to a stronger increase of excitation compared with inhibition in the neuronal network. We have recently obtained the first evidence that in hippocampal cultures, the addition of 5% microglia stimulated by 1 μg/ml LPS as well as an incubation of the cultures with 100 ng/ml TNF-α leads to an upregulation of I_{NavD} (33). As in the conditions of neuroinflammation in the CNS periods of dizziness may be followed by epileptic events (34) and TNF-α can suppress cortical excitability (35), it is intriguing to approach the question of whether activated microglia could regulate I_{NavD} differentially in pyramid-shaped (excitatory) and bipolar (inhibitory) neurons. The main aim of this study was to investigate whether both types of neurons regulate I_{NavD} differently in the presence of activated microglia, whether different TNF-α receptor subtypes are involved in the response of bipolar and pyramid-shaped cells, and whether additional cytokines, which are released from microglia, such as interleukin-18 (36), could participate in the regulation of I_{NavD} in both cell types. In order to obtain a first evidence whether already the presence of residual microglia in control cultures influences I_{NavD}, we additionally investigated whether an inhibition of microglia by transforming growth factor β (TGF-β) (37) influences I_{NavD}.

MATERIALS AND METHODS

Cell Cultures

Experiments were performed on cell cultures obtained from hippocampi dissected from brains of 2–4 days postnatal Wistar–Hannover rats. The hippocampi were collected in ice-cold modified phosphate buffered saline (MPBS) [0.89 mM KH_2PO_4, 2.7 mM KCl, 5 mM Na_2HPO_4, 137 mM NaCl, 10 mM glucose, 10 mM HEPES, 1 mM pyruvate, 1 mM glutamine, 1 mg/ml bovine serum albumin (BSA), 25 U/ml penicillin, and 25 μg/ml streptomycin (P/S from PAA, Germany)]. After addition of 10 μg/ml desoxyribonuclease I and 5 μl/ml of a 2.5% trypsin solution and gentle dissociation, the tissue was incubated under agitation for 7 min at 37°C. The digestion was stopped with 50 μl/ml fetal calf serum (FCS, Invitrogen, Karlsruhe, Germany) and afterward triturated 15 times with a 1-ml Eppendorf pipette tip. The dissociated cells were collected in a 10-ml tube and centrifuged at 1,000 rpm at 4°C for 10 min. The pellet was resuspended in RPMI (Invitrogen, Karlsruhe, Germany), supplemented with 10% FCS, P/S, and glutamine. The cells were preplated at 37°C and 5% CO_2 in a humidified atmosphere for 1 h in a B 5060 incubator (Heraeus, Hanau, Germany). After preplating, the neuron-enriched supernatant was collected and centrifuged at 1,000 rpm at room temperature for 10 min. The pellet was resuspended in supplemented RPMI medium. The 3.5-cm plastic petri dishes were coated with poly-D-lysine (5 μg/ml in sterile water, for 1 h) and 300,000 cells of the suspension were transferred into 1-cm diameter glass rings in the center of the dishes. In some experiments, ~15,000 microglia were added directly to the cells in the central glass ring. To expose neurons to microglia-secreted factors in the absence of direct membrane contact 60,000 microglia were transferred into cell culture inserts (DIM 23/34, 0.4 MY) (Fisher scientific, Schwerte, Germany). Since the inserts had a diameter of 2.4 cm (four times the surface of the glass rings) four times as many microglia were seeded to achieve a comparable density of microglia directly above the neurons.

After culturing for 1 day, the medium was exchanged with Neurobasal medium (NB) supplemented with B18 prepared according to the composition described in Ref. (38) without the thyroid hormone triiodo-L-thyronine (T3) (to minimize potential influences of astroglia-secreted factors on Na$^+$ current densities) and treated for 24 h with 4 µM Cytosin-β-D-Arabinofuranoside (AraC) to prevent further proliferation of glial cells and subsequent overgrowth of the cultures. After the third day in culture, the NB-medium was renewed and supplemented with the factors to be investigated, 10 ng/ml TGF-β (Sigma Aldrich, Munich, Germany), 10–100 ng/ml TNF-α (Peprotech, Hamburg, Germany), 1 µg/ml LPS, 5, 8 or 10 ng/ml IL-18 (Invivogen, CA, USA), and/or 10 µg/ml anti-TNFR 1/2 (R&D-Systems, MN, USA) (as indicated in the figures).

Isolation of Microglia

Microglial cells were obtained from whole brain mixed glial cell cultures by a shaking procedure following the protocol published by McCarthy et al. (39) and modified as described by Kleinsimlinghaus et al. (40). Cortices were dissected from 0 to 3 days postnatal Wistar–Hannover rats and dissociated by passing through 125- and 36-nm nylon meshes. The cells were centrifuged at room temperature at 900 rpm for 10 min and resuspended in 5 ml glial mixed medium (GMM) composed of DMEM:Ham's F12 (1:1) supplemented with 10% FCS, P/S, and glutamine. The cells harvested from 1.5 brains were transferred to uncoated T-75 flasks (Sarstedt, Nümbrecht, Germany) and precultured for 10–12 days in GMM at 37°C, 5% CO$_2$, and a humidified atmosphere in an incubator (B 5060, Heraues, Hanau, Germany) with medium changes every 3–4 days.

Following the preculture period, microglia were isolated from the underlying layers of stronger adhering astrocytes and oligodendrocyte precursor cells by shaking the flasks for 3 h on an orbital shaker (ES-W, Kühner AG, Birsfelden, Switzerland) in the incubator. The supernatant containing more than 90% microglia was centrifuged for 5 min at 1,000 rpm at room temperature and added to the neuron-enriched cultures.

Patch-Clamp Recordings

Na$^+$ currents were quantified using whole cell patch clamp recordings. All measurements were performed at room temperature using a Patch-Clamp L/M-EPC7 amplifier (List Medical, Darmstadt, Germany). The maximal measurement period for one cell culture dish was limited to 60 min since the number of successful recordings with small leakage currents decreased due to deteriorating cells. Signal filtering was performed using the EPC7 10-kHz lowpass filter and data obtained were digitized using PClamp 10 software (Molecular Devices, Sunnyvale, CA, USA) at a sampling rate of 20 kHz. Data were digitized with a Digidata 1440A board (Molecular Devices) and stored on a personal computer. Patch pipettes were fabricated from borosilicate glass capillaries (GB-150TF-8P, Science Products, Hofheim, Germany) using a PP-830 puller (Narishige Europe, London, UK) and had resistances of 6–10 MΩ. The pipette solution contained in mM: CaCl$_2$ 0.1, EGTA 1.1, MgCl$_2$ 5, NaCl 5, HEPES 10, and CsF 100; The bath solution contained in mM: CdCl$_2$ 0.5, CaCl$_2$ 1, MgCl$_2$ 1, 4-Aminopyridine 4, glucose 10, HEPES 10, TEA-Cl 10, and NaCl 100. The osmolarity of bath and pipette solution was adjusted to the osmolarity of the NB medium (40).

Na$^+$ currents were recorded using a series of step depolarizations, starting from a holding potential of −77 mV (after a liquid junction potential correction of −7 mV) in increments of 5 mV. Maximal peak Na$^+$ currents were determined as peak currents at a test potential of −12 mV, which corresponds to the maximum of the current–voltage relationship.

Voltage-dependent inactivation of the Na$^+$ currents was determined by applying a series of prepulse steps of 200 ms duration in 5 mV increments starting at an initial hyperpolarization to −82 mV, which were followed by a depolarization step to a test potential of −17 mV to evoke maximal Na$^+$ currents. Peak Na$^+$ currents versus prepulse potential were fitted to a modified Boltzmann equation [$I/I_0 = 1/(1 + \exp([V_m − V_{In1/2}]/S))$] $V_{In1/2}$ = prepulse potential at which half of the channels are inactivated; I_0 = current elicited from the most negative prepulse potential; S = slope factor].

Leakage and capacitive artifacts were subtracted using a P/4 protocol. By normalizing the peak Na$^+$ current to the cell capacitance (calculated from the integral of the charging curve), Na$^+$ current densities were calculated. Cells were only included in the statistical evaluation if their leak currents did not surpass 100 pA; their series resistances did not surpass 20 MΩ and Na$^+$ currents displayed an activation voltage range of at least 20 mV in the I/V curve to minimize errors of poor membrane voltage control. A liquid junction potential of −7 mV with respect to the bath solution was corrected manually. For statistical comparison, analysis of variance (ANOVA) followed by Tuckey's *Post hoc* test was used (OriginPro 9.0G, Origin Lab Corporation, Northampton, MA, USA), as it represents a conservative approach when sample sizes are not equal, and we expect homoscedasticity, normal distribution, and independence of our data.

RESULTS

Regulation of Na$^+$ Current Density by Microglial Activation and Deactivation

We first investigated, whether a cocultivation of hippocampal neurons with a surplus of either activated or deactivated microglia for 7 days influences their Na$^+$ current density. To this aim, control Na$^+$ currents were measured in hippocampal cultures obtained from 2- to 4-day-old postnatal rats cocultivated with ~15,000 (5%) microglia. Currents recorded from control neurons after 7 days in culture were compared with those recorded in sister cultures in which microglia had been activated by addition of 1 µg/ml LPS after the first 3 days in culture. In a third series of dishes, the influence of microglia on neurons was abolished as follows: to suppress direct membrane interactions between microglia and neurons, instead of adding microglial cells directly, the same density of microglia was separated from the neurons by seeding into inserts. To inactivate protein secretion from microglia 10 ng/ml TGF-β was added in every medium exchange throughout the whole culture period
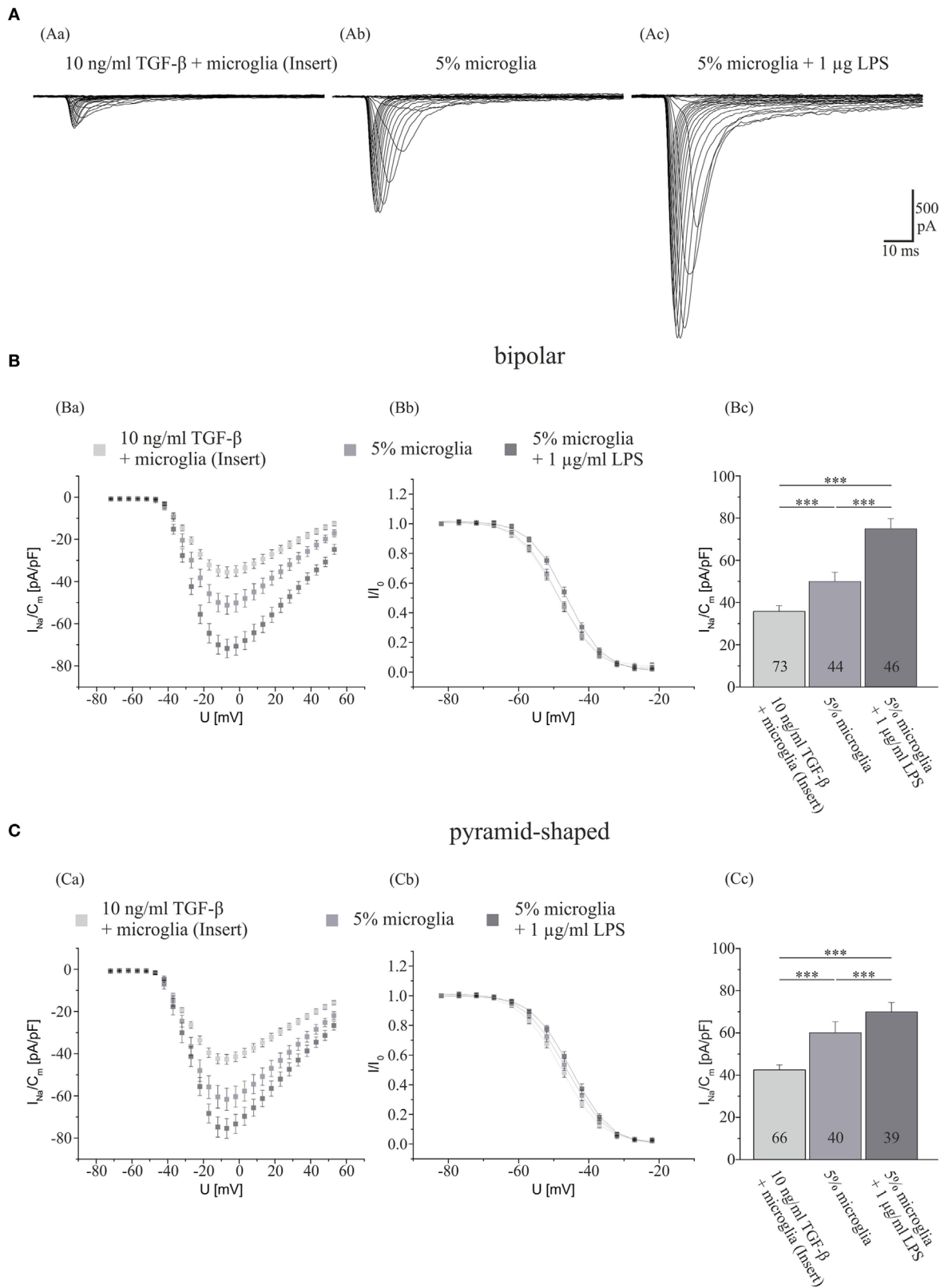
FIGURE 1 | Influences of LPS exposure for 4 days and TGF-β exposure for 7 days in the presence of microglia on Na+ current density of cultured hippocampal neurons.

(Continued)

FIGURE 1 | Continued

(A) Series of original Na$^+$ current recordings from bipolar neurons elicited by step depolarizations in 5 mV increments starting from a holding potential of −77 mV after the different treatments. Analysis of the whole data set for **(B)** bipolar and **(C)** pyramid-shaped cells. **(Ba,Ca)** Average current–voltage relationships for Na$^+$ currents normalized to capacitance recorded from neurons cultured in the presence of microglia and either 4 days of exposure to 1 μg LPS or 7 days of exposure to TGF-β. **(Bb,Cb)** Influence of TGF-β and LPS on steady-state inactivation of Na$^+$ currents. Solid lines represent fits to the modified Boltzman equation as detailed in Section "Materials and Methods." **(Bc,Cc)** Peak Na$^+$ current densities of neurons cultured in the presence of microglia and either 4 days of exposure to 1 μg/ml LPS or 7 days of exposure to TGF-β determined at test potentials of −12 mV starting from holding potentials of −77 mV. All recordings performed at day 7 in culture. Numbers in bar charts indicate numbers of cells recorded from. Database for every column derived from 7 to 10 different preparations. Error bars represent means ± SE, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$.

of 7 days. As shown in **Figure 1**, activation of microglia via LPS treatment resulted in a significant increase in Na$^+$ current density in both bipolar and pyramid-shaped cells increasing I_{NaVD} in bipolar cells from 49.9 ± 4.4 pA/pF (mean ± SE) to 69.9 ± 4.5 pA/pF in LPS-stimulated cultures ($p < 0.005$). Likewise, the Na$^+$ current density increased from 60.2 ± 5.2 to 74.8 ± 4.8 pA/pF in pyramid-shaped neurons ($p < 0.005$). The suppression of microglial activation by 10 ng/ml TGF-β led to a significant decrease in Na$^+$ current density to 34.9 ± 2.5 pA/pF (bipolar, $p < 0.005$) and 42.4 ± 2.4 pA/pF (pyramid-shaped, $p < 0.005$). This suggests that the degree of microglia activation in the vicinity of neurons considerably influences neuronal Na$^+$ current density. Additionally, the Na$^+$ current densities of bipolar and pyramid-shaped neurons were significantly different in cultures incubated with 10 ng/ml TGF-β ($p < 0.01$). As shown in **Figures 1Bb,Cb**, no changes in voltage dependence of activation and inactivation were observed.

Effects of Neuroactive Factors Derived from Activated Microglia
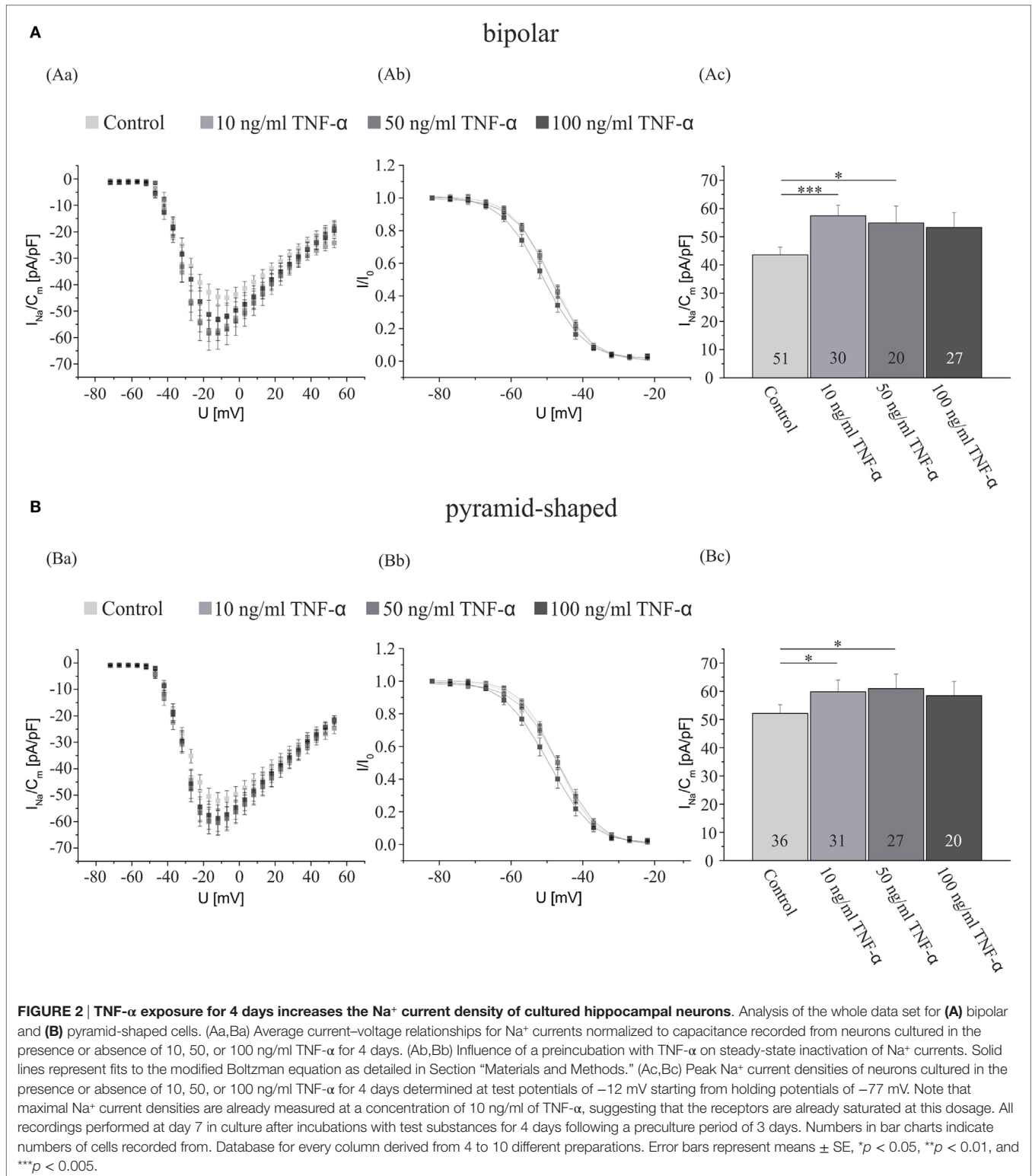### Effect of TNF-α on the Na$^+$ Current Density

After showing that activated microglia exert a considerable effect on the Na$^+$ current density of hippocampal neurons, we now focused on identifying responsible neuroactive factors released from activated microglia. Since TNF-α regulates the Na$^+$ current density in central (33) and peripheral DRG neurons (18, 21), we first recorded a dose–response curve of TNF-α effects on hippocampal neurons (**Figure 2**). Hippocampal neuron-enriched cultures obtained from 2- to 4-day-old postnatal rats were preincubated for 3 days and exposed to NB medium supplemented with or without (control) either 10, 50, or 100 ng/ml TNF-α. As the records in **Figure 1** show for TGF-β treated cultures, in control cultures containing no surplus of microglial cells, current densities in bipolar neurons were significantly smaller than in pyramid-shaped neurons (43.6 ± 2.7 pA/pF in bipolar cells versus 52.2 ± 3.1 pA/pF in pyramidal cells, $p < 0.05$). A preincubation with all tested concentrations of TNF-α induced an increase in Na$^+$ current density. A preincubation with 10 ng/ml significantly increased I_{NaVD} in both neuron types, while 50 ng/ml led to a significant increase only in bipolar neurons. We observed a maximal increase in bipolar neurons by ~30% (from 43.6 ± 2.7 pA/pF, $n = 51$) and in pyramid-shaped neurons by ~15% (from 52.2 ± 3.1 pA/pF, $n = 36$). This suggests that the effect is already maximal at a concentration of 10 ng/ml.

Role of TNF-Receptor 1 and 2 in the Na$^+$ Current Upregulation

To investigate whether TNF-α is involved in the upregulation of Na$^+$ current density by activated microglia and which receptors mediate the effect, we now performed experiments using antibodies against TNF-receptors (TNFR 1 and 2). Cultures obtained from 2- to 4-day postnatal rats were cocultivated with ~15,000 microglia (5%). After 3 days of preincubation, the cultures were exposed to NB medium supplemented with either 1 μg/ml LPS, 1 μg/ml LPS plus 10 μg/ml antibody against TNFR 1, 1 μg/ml LPS plus 10 μg/ml antibody against TNFR 2, or without supplements (control). As shown in **Figure 3**, we observed significant differences in the Na$^+$ current regulation in bipolar and pyramid-shaped neurons. In bipolar neurons, a blockage of both TNFR 1 and 2 led to a decrease of the Na$^+$ current density from 69.9 ± 4.5 pA/pF back to control level (in the presence of 5% microglia, all values between 50 and 55 pA/pF). In pyramid-shaped neurons, only a blockage of TNFR 2 led to a decrease of the Na$^+$ current density back to control level (5% microglia) of ~60 pA/pF, whereas in the presence of antibodies against TNFR 1, the same elevated Na$^+$ current density as in LPS-activated microglia of ~75 pA/pF was observed.
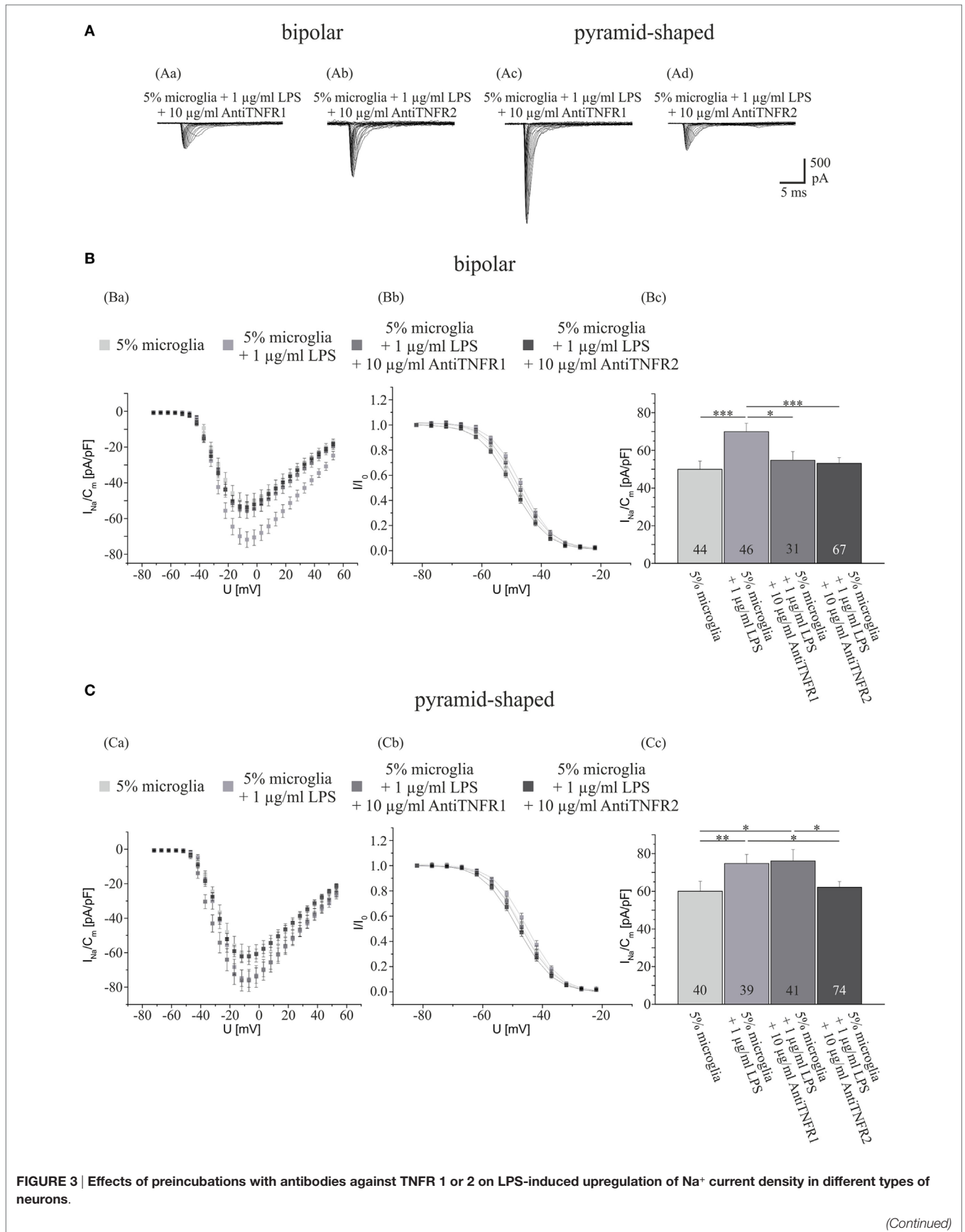
Regulatory Effect of IL-18 on the Na$^+$ Current Density

The increases in Na$^+$ current density induced by an incubation with TNF-α were smaller than the increase observed after cocultivation with LPS-activated microglia (compare **Figure 1** with **Figure 2**). Furthermore, the blockage of the TNF-α receptors by antibodies did not reduce the Na$^+$ currents to the levels obtained in the presence of TGF-β if the microglia were added in cell culture inserts (compare **Figures 1Bc,Cc** with **Figures 3Bc,Cc**). We thus considered that other factors released from microglia might additionally contribute to the regulation of I_{NaVD}. Potential candidates are proinflammatory cytokines of the IL1/18-family. Preliminary data yielded no significant change in the Na$^+$ current density in hippocampal neurons incubated with 0.01 ng/ml IL-1α and 0.02 ng/ml IL-6 (data not shown). Since we were not aware of any investigations of IL-18 effects on voltage-activated ion currents we now investigated, whether a preincubation with this interleukin for 4 days affects I_{NaVD} in bipolar and hippocampal neurons. Cultures obtained from 2- to 4-day-old postnatal rats were exposed to NB medium supplemented with or without (control) 5, 8, or 10 ng/ml IL-18 after 3 days of preincubation. As shown in **Figures 4Ac,Bc**, a significant increase in the Na$^+$ current

FIGURE 2 | TNF-α exposure for 4 days increases the Na$^+$ current density of cultured hippocampal neurons. Analysis of the whole data set for **(A)** bipolar and **(B)** pyramid-shaped cells. (Aa,Ba) Average current–voltage relationships for Na$^+$ currents normalized to capacitance recorded from neurons cultured in the presence or absence of 10, 50, or 100 ng/ml TNF-α for 4 days. (Ab,Bb) Influence of a preincubation with TNF-α on steady-state inactivation of Na$^+$ currents. Solid lines represent fits to the modified Boltzman equation as detailed in Section "Materials and Methods." (Ac,Bc) Peak Na$^+$ current densities of neurons cultured in the presence or absence of 10, 50, or 100 ng/ml TNF-α for 4 days determined at test potentials of −12 mV starting from holding potentials of −77 mV. Note that maximal Na$^+$ current densities are already measured at a concentration of 10 ng/ml of TNF-α, suggesting that the receptors are already saturated at this dosage. All recordings performed at day 7 in culture after incubations with test substances for 4 days following a preculture period of 3 days. Numbers in bar charts indicate numbers of cells recorded from. Database for every column derived from 4 to 10 different preparations. Error bars represent means ± SE, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$.

density was observed in both, bipolar and pyramid-shaped neurons. While bipolar neurons reached a saturation level at 5 ng/ml IL-18 with an increase of I_{NaVD} by 40%, maximal values were observed for pyramid-shaped neurons only at higher concentrations of 8–10 ng/ml with average increases of I_{NaVD} by 40%. These observations suggest cell-type specific different dose–response curves for the regulation of the Na$^+$ current density by IL-18.

FIGURE 3 | Effects of preincubations with antibodies against TNFR 1 or 2 on LPS-induced upregulation of Na+ current density in different types of neurons.

(Continued)

(A) Series of original current recordings from bipolar neurons elicited by step depolarizations in 5 mV increments starting from a holding potential of −77 mV. Analysis of the whole data set for **(B)** bipolar and **(C)** pyramid-shaped cells. (Ba,Ca) Average current–voltage relationships for Na+ currents normalized to capacitance recorded from neurons cultured in the presence of microglia and 4 days of exposure to 1 µg LPS and 4 days of either antibody against TNFR 1 or 2. (Bb,Cb) Influence of TNFR 1 and 2 blockage on steady-state inactivation of Na+ currents. Solid lines represent fits to the modified Boltzman equation as detailed in Section "Materials and Methods." (Bc,Cc) Peak Na+ current densities of neurons cultured in the presence of microglia and 4 days of exposure to 1 µg LPS and 4 days of either antibody against TNFR 1 or 2 determined at test potentials of 12 mV starting from holding potentials of −77 mV. Note that the Na+ current density of bipolar neurons is decreased by both antibodies against TNFR 1 and 2, whereas the Na+ current density of pyramid-shaped neurons is only decreased by antibodies against TNFR 2 to control levels. All recordings performed at day 7 in culture after incubations with test substances for 4 days following a preculture of 3 days. Numbers in bar charts indicate numbers of cells recorded from. Database for every column derived from 7 to 15 different preparations. Error bars represent means ± SE, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$.
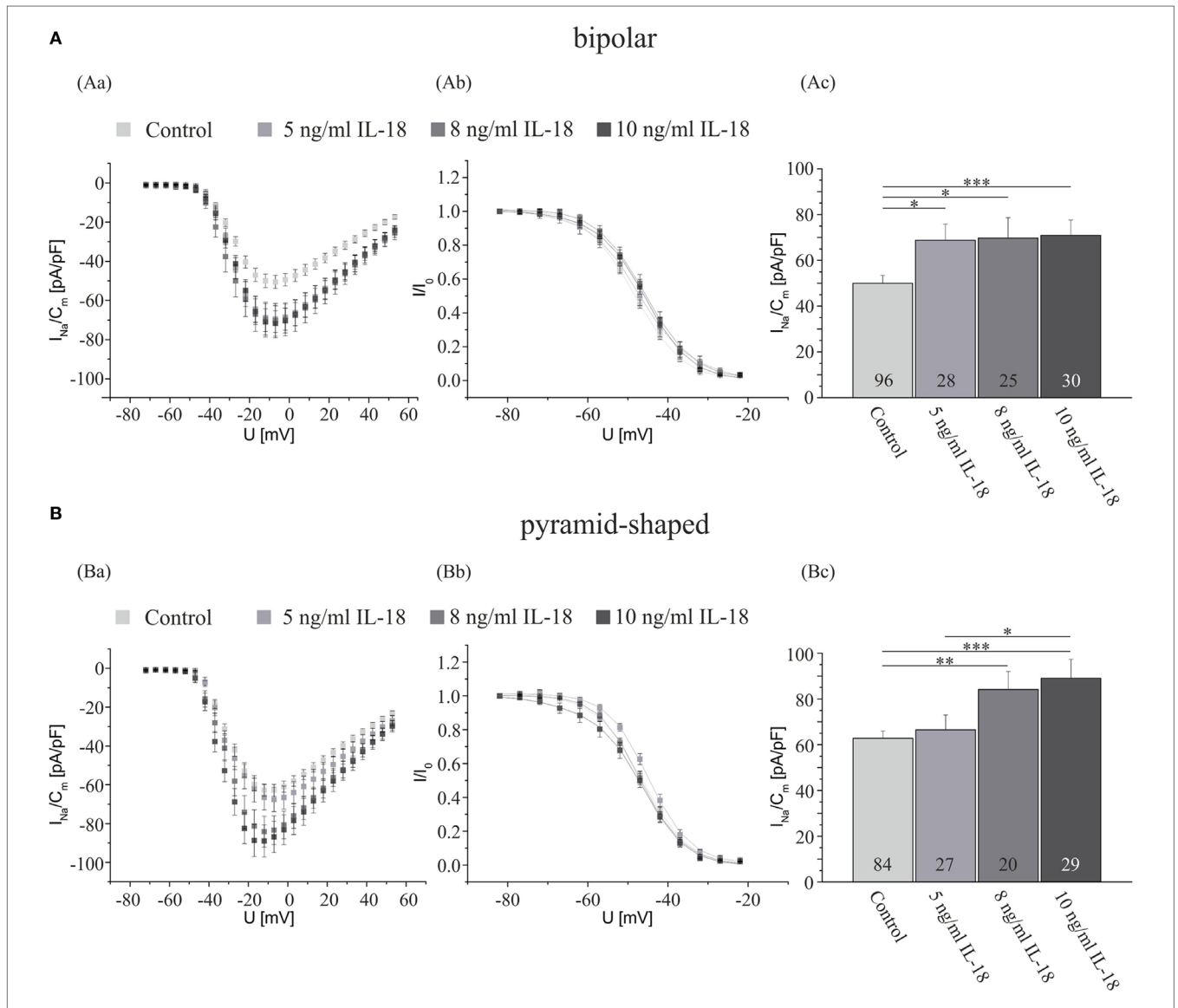
FIGURE 4 | IL-18 exposure for 4 days increases the Na+ current density of cultured hippocampal neurons. Analysis of the whole data set for **(A)** bipolar and **(B)** pyramid-shaped cells. (Aa,Ba) Average current–voltage relationships for Na+ currents normalized to capacitance recorded from neurons cultured in the presence or absence of 5, 8, or 10 ng/ml IL-18 for 4 days. (Ab,Bb) Influence of IL-18 on steady-state inactivation of Na+ currents. Solid lines represent fits to the modified Boltzman equation as detailed in Section "Materials and Methods." (Ac,Bc) Peak Na+ current densities of neurons cultured in the presence or absence of 5, 8, or 10 ng/ml IL-18 for 4 days determined at test potentials of −12 mV starting from holding potentials of −77 mV. Note that the Na+ current density increases in a dose-dependent manner. The dose–response curve for pyramid-shaped neurons is shifted toward higher concentrations. All recordings performed at day 7 in culture after incubations with test substances for 4 days following a preculture period of 3 days. Numbers in bar charts indicate numbers of cells recorded from. Database for every column derived from 7 to 24 different preparations. Since not all experiments were performed in sister cultures incubated under all four conditions the data for the control cultures in this series of experiments have been pooled. Error bars represent means ± SE, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$.

DISCUSSION

As Na$^+$ currents are essential for the initiation and propagation of neuronal firing, changes in the current density can lead to abnormal neuronal activity. Na$^+$ current density can be modulated by small molecular weight neurotransmitters, such as acetylcholine and dopamine by the activation of phosphorylation cascades in a form of short-term plasticity (41). Na$^+$ channels may be regulated acutely via the activation of G-proteins (41–43) and over larger time spans by an increase in channel synthesis [see e.g. Ref. (44)].

Apart from release of neurotransmitters out of neurons, astrocytes and microglia are sources of additional neuroactive factors acting at a longer time scale in the CNS [see e.g. Ref. (45)]. For instance, by release of FGF-2 from thyroid hormone-stimulated satellite cells the Na$^+$ current density of neurons and thus their excitability can be increased over the course of several days in postnatal, maturing neurons (33). In addition, our research group had previously observed, that LPS-stimulated microglia can increase Na$^+$ current density, and that this effect can be mimicked by 100 ng/ml TNF-α (33). Here, we extend these observations showing that the presence of activated microglia in hippocampal cultures effects Na$^+$ current densities to some extent differently in bipolar and pyramid-shaped neurons, potentially changing the balance between excitation and inhibition. Thus conditions of neuronal hyper- as well as hypoexcitability under inflammatory conditions could be explained by the release of neuroactive factors from microglia.

Role of Microglia

Microglia are part of the innate immune system in the CNS and play an important role in inducing and propagating inflammatory signals in response to activation of the peripheral immune system (46) or during brain lesion. TGF-β is a known deactivator of microglia. Kim et al. (47) suggest TGF-β as Phosphatidylinositol 3-Kinase (PI3K) inhibitor, which in turn is linked to the TLR4-mediated cytokine release (48). TGF-β is a 24-kDa protein produced by various types of cells, not only from T and B lymphocytes (49, 50), activated macrophages (51, 52) but also from resident glial cells of the CNS such as astrocytes and microglia (53–55). The effects of TGF-β on cells of the monocyte lineage are multipotential with diverse effects, such as inducing chemotaxis on monocytes and IL-1 production (56). Interestingly, IL-1 and IL-1α induce TGF-β expression in glial cells, which suggests a positive autocrine feedback loop (57). In contrast, TGF-β blocks Interferon-γ (IFN-γ)-induced macrophage activation (58, 59), including the induction of class II major histocompatibility complexes (MHC) antigen (60) and the reduction of macrophagic inhibition of intracellular replication of certain parasites (61). Furthermore, it suppresses proliferation and LPS-induced activation of microglia (62, 63), regulates cell survival (47), and decreases cytokine release, including IL-1, IL-1α, IL-6, TNF-α, and nitric oxide (NO) (37, 62).

Transforming growth factor-β regulates ion channels in microglia. It upregulates delayed rectifier (DR) Kv1.3 K$^+$ channel though it shows no effect on inward rectifier (IR) K$^+$ channels

(64, 65), which could lead to an inhibition of vesicle release by hyperpolarization.

In accordance with previous observations of an inhibitory role of TGF-β on microglia, we here observed (see **Figure 1**) that the incubation of microglia-enriched cultures with 10 ng/ml TGF-β leads to a decrease of the Na$^+$ current density in both, bipolar and pyramid-shaped neurons to values smaller than those observed in control cultures. Although further experiments are needed to further corroborate this hypothesis, our observation suggests that in untreated control cultures, the residual 1–3% microglia release neuroactive factors which influence the Na$^+$ current of neurons in their vicinity. LPS stimulates the release of various neuroactive factors from microglia (12). As shown in **Figures 1** and **3**, we extend our previous findings (33) that the presence of LPS-treated microglia leads to an upregulation of I_{NavD}, and show here that a larger increase in the Na$^+$ current density can be observed in bipolar compared with pyramid-shaped neurons. Guo et al. (66) linked conditions of chronic pain to the innate immune system of the CNS by the activation of TLRs on microglia, which are activated among others, by LPS. Our findings of the increase of the Na$^+$ current density of neurons by LPS-activated microglia support the idea of microglia as a link to hyperalgesia and hyperexcitability.

Effects of Neuroactive Factors Released from Microglia

Tumor necrosis factor-α is the most prominent cytokine released from microglia. In the brain, it has mostly been associated with leukocyte recruitment through the regulation of adhesion processes and is involved in the activation of glial cells in the process of gliotic scar formation (67). TNF-α belongs to the superfamily of type II transmembrane proteins with intracellular N-terminus (68). It originates in a 26-kDa homotrimeric pro-molecule, which is converted by the metalloprotease TNF-α converting enzyme (TACE) to a diffusible peptide, which forms a non-covalently bound trimer (69) and is secreted from macrophages and monocytes [see e.g. Ref. 70, 71]. The source of TNF-α in the CNS are activated microglia (72). To our knowledge, effects of TNF-α on Na$^+$ current densities in central neurons have only been investigated in one study (33). Here, we extend these previous results showing that already an incubation of cultures for 4 days with a concentration of 10 ng/ml elicits maximal effects and that the upregulation of the I_{NavD} is slightly larger in bipolar compared with pyramid-shaped cells. In our previous publication (33), we had observed a significant upregulation after treatment with 100 ng/ml TNF-α in the order of magnitude of 20% using a larger database of about 70 cells recorded under each condition. In the present series of experiments, we recorded from a smaller number of cells under each concentration such that the treatment with 100 ng/ml TNF-α did not yet reach significance. The decline seen with increasing concentrations in the present series of experiments might result from the deleterious effects of higher dosages of TNF-α, which made it increasingly difficult to obtain stable patch clamp recordings from neurons.

Tumor necrosis factor-α binds exclusively to two receptors, TNFR 1 and 2. TNFR 1 is regarded as the primary signaling receptor for systemic TNF-α inflammatory responses (73, 74). TNFR 2 may mediate TNF-α effects in a paracrine or autocrine manner because it is strongly activated by membrane-bound TNF-α (75). Distinct effects of TNFR activation on the TTX-resistant Nav1.8 currents of DRG neurons (76) could be observed.

Our present findings suggest that bipolar neurons are regulated by both TNFR subtypes, while pyramid-shaped neurons are mainly regulated by TNFR 2, suggesting that I$_{NavD}$ in both types of neurons can be regulated differentially by TNF-α.

The observation that the blockage of TNF-α receptors reduced I$_{NavD}$ to a smaller extent than by the inactivation of microglia by TGF-β suggests, that additional cytokines, released by microglia could contribute to I$_{NavD}$-regulation by microglial activation.

Apart from TNF-α, microglia have been shown to release cytokines from the IL-1 family (77). IL-18 is a member of the IL-1 family of proinflammatory cytokines. It is synthesized as an inactive 24-kDa precursor protein that is subsequently cleaved by caspase-1 into its active form (78). The IL-18 receptor (IL-18R) is expressed on a variety of cells, including hypothalamic neurons and murine glia (79, 80).

The effect of cytokines of the IL-1/IL-18 family on Na$^+$ currents of hippocampal neurons has to our knowledge not been investigated. Interestingly, both interleukins and TNF-α are implicated in sleep regulation (81). Both enhance non-rapid eye movement sleep (NREMS). While the exact mechanism is currently unknown, it might be possible that an alteration of neuronal excitability might be involved in the enhancement of the NREMS phase. Interestingly though, a variety of cytokines has been linked to the increase of NREM sleep during inflammation, including IL-1, IL-18, and TNF-α (81–83).

Our present results suggest that IL-18 may additionally regulate I$_{NavD}$ in central neurons. Differences in the dose–response curves observed in bipolar and pyramid-shaped neurons suggest that bipolar cells might already respond to lower activations of microglial cells releasing less IL-18. Our findings that bipolar inhibitory neurons are regulated more strongly by IL-18 and TNF-α and subsequently lead to an overall hypoexcitability of the neural network and may explain symptoms such as dizziness or an increase in NREM sleep during infectious diseases.

Tumor necrosis factor-α not only exerts long-term effects but is also capable of an acute p38-mediated modulation of Tetrodotoxin-resistant (TTX-R) Na$^+$ channels in mouse sensory neurons (84). During phosphorylation, changes in the voltage-dependence of inactivation have been observed by Chizhmakov et al. and Franceschetti et al. (85, 86). For both cytokines tested here, we observed no significant changes in the voltage-dependence of the inactivation of the Na$^+$ currents. This indicates that the increased Na$^+$ current densities might rather be caused by increased ion channel densities instead via short-term regulatory mechanisms such as phosphorylation (87).

Taken together, this study presents evidence that two major cytokines, namely TNF-α and IL-18, released from microglia activated by pathological events such as CNS injury or inflammation (6, 14, 15, 28, 36, 88), not only effect, e.g., cell survival (89) and death (90), DNA-synthesis (91), cell proliferation (92, 93), cell differentiation (89, 94), and neuronal cell fate in embryonic neural progenitor cells (NPC) (95) but also in addition upregulate Na$^+$ current density and thus excitability in hippocampal neurons. The differential regulation of the Na$^+$ current densities in bipolar and pyramid-shaped neurons can lead to an imbalance between inhibition and excitation in the neural network. At the network level, this could lead to an overall neuronal hypoexcitability, which might be an explanation for the symptom of dizziness during neuroinflammation (96). These findings are in line with the results of Richter et al. that TNF-α reduces the amplitude of cortical spreading depression (CSD) by the activation of TNFR 2 in cortical inhibitory neurons (35). The differentiation by the TNFR subtypes enables an asymmetric increase of Na$^+$ currents by the same concentration of TNF-α and might be an additional explanation that different concentrations of TNF-α can lead to bidirectional effects on cortical network activity (97). Another mechanism of differential Na$^+$ current density regulation is the dose-dependent effect of IL-18. At low concentrations, it mainly affects the Na$^+$ current density of inhibitory interneurons, while at higher concentrations (8–10 ng) also pyramid-shaped neurons are affected. This finding could contribute to the origin of a general inhibition of cortical networks followed by a general excitation with a further increase of the neuroinflammation. Such a mechanism, leading with a delay of several days after the onset of the infection first to dizziness and then to epileptic seizures might also explain the neurological symptoms, which occurred during immune reactions in 20% of the patients over the course of the recent outbreak of a Shiga-toxin producing *E. coli* infection (98).

ETHICS STATEMENT

AUTHOR CONTRIBUTIONS

ID designed the experiments and participated in data analysis, interpretation, and writing of the manuscript. LK acquired and analyzed most of the data, participated in the conception of the experiments, prepared the figures, and drafted the article. BI contributed to the conception of the experiments, some of the data and tutoring of LK during his Master's thesis, which contains parts identical to the present publication. All authors approved the version to be published.

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