Quantification of the Functional Expression of the Ca\(^{2+}\) -Activated K\(^{+}\) Channel KCa3.1 on Microglia from Adult Human Neocortical Tissue

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The KCa3.1 channel (KCNN4) is an important modulator of microglia responses in rodents, but no information exists on functional expression on microglia from human adults. We isolated and cultured microglia (max 1% astrocytes, no neurons or oligodendrocytes) from neocortex surgically removed from epilepsy patients and employed electrophysiological whole-cell measurements and selective pharmacological tools to elucidate functional expression of KCa3.1. The channel expression was demonstrated as a significant increase in the voltage-independent current by NS309, a KCa3.1/KCa2 activator, followed by full inhibition upon co-application with NS6180, a highly selective KCa3.1 inhibitor. A major fraction (79%) of unstimulated human microglia expressed KCa3.1, and the difference in current between full activation and inhibition (ΔKCa3.1) was estimated at 292 ± 48 pA at −40 mV (n = 75), which equals at least 585 channels per cell. Serial KCa3.1 activation/inhibition significantly hyperpolarized/depolarized the membrane potential. The isolated human microglia were potently activated by lipopolysaccharide (LPS) shown as a prominent increase in TNF-α production. However, incubation with LPS neither changed the KCa3.1 current nor the fraction of KCa3.1 expressing cells. In contrast, the anti-inflammatory cytokine IL-4 slightly increased the KCa3.1 current per cell, but as the membrane area also increased, there was no significant change in channel density. A large fraction of the microglia also expressed a voltage-dependent current sensitive to the KCa1.1 modulators NS1619 and Paxilline and an inward-rectifying current with the characteristics of a Kir channel. The high functional expression of KCa3.1 in microglia from epilepsy patients accentuates the need for further investigations of its role in neuropathological processes.

Key words: neuroinflammation, potassium channel, patch clamp, glial cell, IK channel, BK channel

Introduction

Microglia are the resident immune cells of the human brain that serve an important role of continuously surveying the central nervous system (CNS) tissue and conduct maintenance work which for instance includes removing dead cells and tissue debris. However, microglia can, directly or indirectly, become excessively activated as characterized by release of a range of cytokines and growth factors that can be neurotoxic and cause tissue destruction, for example, in diseases like Alzheimer’s disease and multiple sclerosis. Microglia are, therefore, considered important target cells for therapeutic intervention in a number of neurodegenerative diseases (for recent reviews, see Kettenmann et al., 2011 and Michell-Robinson et al., 2015).

Ion channels expressed on microglia cells may contribute to both restorative and degenerative processes, in particular, via their influence on the microglia membrane potential, regulation of intracellular Ca\(^{2+}\) signaling, migration, and volume regulation. The intermediate-conductance Ca\(^{2+}\) -activated K\(^{+}\) channel, KCa3.1 (Ishii et al., 1997), which is a voltage-independent potassium channel encoded by KCNN4, is an important player in the regulation of immune responses.
mediated by T cells (Ghanshani et al., 2000), B cells (Wulff et al., 2004), and peripheral macrophages (Kang et al., 2014). KCa3.1 has also been reported to be expressed by rodent microglia (Ferreira et al., 2014; Kaushal et al., 2007; Khanna et al., 2001; Wong and Schlichter, 2014) and implicated in a number of experimental animal models involving pathology in the CNS, e.g., ischemic stroke (Chen et al., 2011), traumatic brain injury (Mauler et al., 2004; Urbahns et al., 2003), spinal cord injury (Bouhy et al., 2011), and experimental autoimmune encephalitis (Reich et al., 2005). In rodent microglia in vitro cultures, KCa3.1 was originally shown to act in a proinflammatory manner participating in the respiratory burst process (Khanna et al., 2001) and pharmacological blocking of KCa3.1 by the selective KCa3.1 inhibitor TRAM-34 gave reduced nitric oxide (NO) production following activation by lipopolysaccharide (LPS) (Kaushal et al., 2007) or amyloid-β oligomers (AβO) (Maewa et al., 2011). These effects could explain the in vivo neuroprotection of TRAM-34. However, in vitro experiments have also shown that, in addition to its classical proinflammatory role, KCa3.1 can also play a role in interleukin-4 (IL-4)-mediated activation of rat microglia (Ferreira et al., 2014), as significantly larger KCa3.1 currents could be measured after treatment with IL-4, which resulted in a TRAM-34-sensitive increased migratory potential (Ferreira et al., 2014).

Despite the solid evidence from rodent microglia studies, functional expression and physiological/pathophysiological importance of KCa3.1 in adult human microglia has not yet been reported. Hitherto, the few functional studies using human microglia of fetal origin (abortions) or microglia isolated from removed neuronal tissue from epilepsy or cancer patients have failed to reveal the presence of KCa3.1 channels (Bordey and Spencer, 2003; McLarnon et al., 1997; Nordenberg et al., 1994). However, based on immunohistochemistry of postmortem tissue, Chen et al. (2015) recently showed a clear expression of KCa3.1 protein in the infarcted area in stroke patients.

As prominent differences in expression profiles and responses occur between rodent and human microglia (Smith and Dragunow, 2014), the verification of functional expression of KCa3.1 in human microglia from adults is an important piece of missing evidence in the translational validation of KCa3.1 as a target for modulation of neuroinflammatory diseases. The access to neural tissue removed within a short timeframe allows production of primary cultures of microglia cells with high purity and relevant cellular physiological properties. In this study, we conducted experiments on immunohistochemically characterized human microglia isolated from neocortex of adults undergoing therapeutic surgery due to medically intractable mesial temporal lobe epilepsy. We determined—by use of selective pharmacological tools combined with electrophysiology—the fraction of isolated human microglia cells expressing functional KCa3.1, as well as the current density and the overall impact of the channel on the membrane potential in resting human microglia. We also investigated whether activation of these cells in vitro by IL-4 or LPS would change the functional expression of KCa3.1.

We conclude that KCa3.1 constitute a major K+ conductance in isolated human neocortical microglia under all conditions investigated. We also found functional expression of the BK channel (KCa1.1) as well as an unidentified inward-rectifying K+ channel (Kir).

Materials and Methods
Preparation of Human Microglia Cultures
The temporal neocortex was obtained from eight epilepsy patients undergoing anterior temporal lobectomies (ATL). During the operation, part of the temporal neocortex was removed before performing an amygdalohippocpectomy. In all subjects, the amygdala and hippocampus were determined to be the epileptogenic zone during the epilepsy surgery evaluation. Histopathological preparations of neocortex were normal in all subjects. Written informed consent was obtained from all patients before the surgery. All studies were in accordance with the protocol approved by the Rigshospitalet’s Ethical Committee in the Capital Region of Denmark (H-2-2011-104).

The neocortex was immediately placed in high-glucose Dulbecco’s Modified Eagle Medium (DMEM) containing 1 mM pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine on ice. The CNS resection was carefully cleaned from any blood residues and meninges. The tissue was cut into smaller pieces with a pair of scissors. Following a centrifugation step for 10 min at 400g, the tissue was incubated with 0.05% trypsin–EDTA (Gibco) containing 0.15 KU/mL DNase (Sigma) for 15 min in a water bath at 37°C. The tissue was further dissociated using a Potter-Elvehjem homogenizer. Fetal bovine serum (FBS) was added to stop the trypsinization and the cell lysate was centrifuged for 10 min at 400g. The tissue was incubated with 0.05% trypsin–EDTA (Gibco) containing 0.15 KU/mL DNase (Sigma) for 15 min in a water bath at 37°C. The tissue was further dissociated using a Potter-Elvehjem homogenizer. Fetal bovine serum (FBS) was added to stop the trypsinization and the cell lysate was centrifuged for 10 min at 400g to remove the trypsin solution. The pellet was resuspended in DMEM medium with aforementioned supplements as well as 5% FBS. The cell suspension was seeded at 2 × 10^5 cells/mL in either four-chamber LabTek glass slides (Fisher Scientific) for cell culture analysis, 24-well plates for molecular characterization, or 35 mm petri dishes containing round glass coverslips (Ø 3.5mm) for electrophysiological recordings. The cells were left to attach for 1–2 h after

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**Abbreviations**

| Code | Description |
|------|-------------|
| IL-4 | interleukin-4 |
| IV | current-voltage relation |
| KCa1.1 | large-conductance Ca^{2+}-activated K+ channel (a.k.a. BK channel) |
| KCa2 | small-conductance Ca^{2+}-activated K+ channel (a.k.a. SK channel) |
| KCa3.1 | intermediate-conductance Ca^{2+}-activated K+ channel (a.k.a. IK channel) |
| Kir | inward-rectifying K+ channel |
| Kv | outward-rectifying potassium channel |
| LPS | lipopolysaccharide |
| TNF-α | tumor necrosis factor alpha |
which debris was removed by washing the bound cells twice with fresh cell culture medium. The cells were allowed to recover for ≥5 days with the medium changed every 3–4 days.

**Immunocytochemistry and Analysis of Cell Culture Purity**

On day 6 postisolation, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. Following 2 × 5 min of washing in PBS, the cells were incubated with PBS containing 0.3% Triton X-100 for 20 min. This was followed by incubation in a blocking buffer consisting of 5% goat serum (Dako) in PBS for 20 min. The following primary antibodies were used to identify cells in the culture: rabbit anti-ionized calcium-binding adaptor molecule 1 (iba1; microglia; 1:1000; Wako Pure Chemical Industries) in combination with mouse anti-microtubule-associated protein 2 (MAP2; neurons; 1:1000; Sigma) and rabbit anti-glial fibrillary acidic protein (GFAP; astrocytes; 1:1000; Abcam) in combination with mouse anti-CC-1 (oligodendrocytes; 1:500; Abcam). A negative control was included where the primary antibody incubation step was omitted.

The cells were incubated with the primary antibodies diluted in PBS included where the primary antibody incubation step was omitted. Anti-CC-1 (oligodendrocytes; 1:500; Abcam). A negative control was included where the primary antibody incubation step was omitted. Following 2 min of washing in PBS, the cells were incubated with PBS containing 5% goat serum for 2 h at room temperature. The cells were washed extensively for 3 × 5 min in PBS after which a mix of the following secondary antibodies was added: Alexa Fluor 555-conjugated anti-rabbit (1:400, Life Technologies) and Alexa Fluor 488-conjugated anti-mouse (1:400; Life Technologies) for 45 min at room temperature. The cells were again washed 3 × 5 min in PBS and the slides were finally mounted with Slow-Fade® Gold mounting medium containing DAPI nuclear dye (Life Technologies). Gray-scale photographs of each staining were captured using an Axio Imager fluorescent microscope (Zeiss). Ten frames per marker and isolation session were captured for subsequent image analysis using the ImageJ software. Altogether, the purity analysis was done on 3 isolation session were captured for subsequent image analysis using the ImProm-II™ Reverse Transcriptase (Promega). For qPCR experiments, a reaction mix of 20 μL per sample containing 10 μL SYBR Green master mix (Bio-Rad Laboratories), 4.7 μL RNase-free water, 0.15 μL of each primer, and 15 μL cDNA template was prepared. The following primers were used (Nhu et al., 2010): TNF-α Fw: 5’-CCC AGG GAC CTC TCT CTA ATC A-3’ Re: 5’-GGT TGA GGG TTT GCT ACA AGA TG-3’, IL-1β Fw: 5’-AAA TAC CTT TGG CCT TGG GC-3’ Re: 5’-TTT GGG ATC TAC ACT CTC CAG CT-3’, IL-6 Fw: 5’-GTA GCC GCC CCA CAC AGA-3’ Re: 5’-CAT GTC TCC TTT TTT CTC AGG GCT G-3’, GAPDH Fw: 5’-GCA TGA TGT CCA GAG CTC-3’ Re: 5’-GGT ATC GTG GAA GGA-3’. The following PCR cycling conditions were used: 94°C for 10 min followed by 40 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 90 s. The concluding melt curve (50–94°C) analysis showed a single PCR product which was also confirmed using gel electrophoresis. Relative gene expression was calculated using the 2^(-ΔΔCt) method. All samples were run in duplicates and were normalized against the expression levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Microglia Patch Clamp Electrophysiology**

To induce activation in microglia, they were treated for 2–4 days with either 20 ng/mL interleukin-4 (IL-4; PeproTech) or 100 ng/mL LPS (Sigma) diluted in the regular cell culture medium. Age-matched unstimulated microglia were treated exactly the same way, only no stimulating reagent was added to the regular cell culture medium. Coverslips from the three different dishes were transferred to a chamber mounted on the stage of an inverted microscope. The cells were superfused with extracellular saline (in mM: 126 Na-gluconate, 14 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); pH adjusted to 7.4 with NaOH) and CaCl₂, 1 MgCl₂, 10 HEPES; pH adjusted to 7.4 with NaOH) at a rate of 1–2 mL/min using a gravity-driven system. The pipette solution (in mM: 110 K-gluconate, 15 KCl, 4.5 CaCl₂, 1 MgCl₂, 5 ethylene glycol tetraacetic acid (EGTA), 15.6 KOH, 2 MgATP, 10 HEPES; pH 7.2) was adapted from Ferreira et al. (2014) and contained ~1 μM free Ca²⁺ as calculated by the Maxchelator program (http://maxchelator.stanford.edu/webmaxc/webmaxcE.htm). The pipettes were created from borosilicate glass (Vitrex) using a DMZ-Universal puller (Zeitz-Instruments GmbH) and had a resistance of 3–5 MΩ. Upon establishment of the whole-cell configuration, a voltage ramp protocol (−120 to 30 mV in 150 ms) was applied every 5 s from a holding potential of −90 mV. Series resistance compensation and fast and slow capacitance cancellations were updated at every application of the protocol to track the stability of the recording. The C胞 parameter (pF/cell) measured immediately after whole-cell establishment was used to estimate the cell-to-cell variability in sizes, based on the value of specific capacitance of 1 μF/cm² membrane area.

For the current-clamp experiments, an extracellular saline with high chloride concentration was used (in mM: 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES; pH adjusted to 7.4 with NaOH) and the pipette solution contained in mM: 109 KCl, 5.2 CaCl₂, 1.4 MgCl₂, 10 EGTA, 31 KOH, 10 HEPES; pH 7.2 giving a calculated free [Ca²⁺] of 0.2 μM according to Maxchelator, see above). All recordings were conducted at room temperature using an HEKA EPC-9 amplifier and the PULSE software (HEKA Elektronik).
Pharmacological Tool Compounds
The KCa3.1/KCa2 activator NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime), the KCa3.1 inhibitor NS6180 (4-[[3-(trifluoromethyl)phenyl]-methyl]-2H-1,4-benzothiazin-3(4H)-one), and the KCa3.1 activator NS1619 (1-(2’-hydroxy-5’-trifluoromethylphenyl)-5-trifluoromethyl-2(3H)benzimidazolone) were synthesized according to previous descriptions (Olesen and Waetjen 1993; Olesen et al, 1994; Strobaek et al., 2004, 2013). The BK channel inhibitor Paxilline (a tremorgenic alkaloid, Knaus et al., 1994) was purchased from Sigma-Aldrich. All the compounds were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM, aliquoted and stored at −20°C. The compounds were diluted in the extracellular saline to a stock concentration of 10 mM, aliquoted and stored at −20°C. On the day of experiment, the compounds were diluted in the extracellular saline to the final concentrations. The highest concentration of DMSO used in our experiments was 0.1%. Control experiments showed that the microglia currents were unaffected by this concentration of DMSO (data not shown).

Statistical Analysis
All data are given as means ± SEM unless indicated otherwise. For TNF-α release experiments, the number of observations (n) means the number of replicates in one experiment; for the patch clamp experiments (n) means the number of individual cells measured. Data sets were analyzed for statistically significant differences (P < 0.05) between treatments, time points, or concentrations using a t-test or a one-way ANOVA with a Newman–Keuls post-hoc test, unpaired or paired test as appropriate in each situation. For changes in proportion between groups, a chi-square (χ²) test was used.

Results
Isolation and Culturing of Human Microglia
To evaluate the expression of KCa3.1 channels in adult human microglia, we first needed to establish an isolation procedure to obtain high purity in vitro cultures. Purity and viability of the cells 6 days following the isolation procedure were investigated using immunocytochemistry (Fig. 1A,B). We found that the cultures contained 92% pure microglia (Iba1), 1% astrocytes (GFAP), and no oligodendrocytes (CC-1) or neurons (MAP2) (Fig. 1C). Seven percent of the DAPI+ cells remained unstained. As seen from the figure, the Iba1+ microglia are large cells (notably bigger than isolated rat microglia) and exhibit various morphologies but typically had a larger cell body and shorter/thicker processes compared to the few astrocytes that survived the isolation. These characteristics were used in the selection of cells to patch in the electrophysiological experiments.

We next established that the cells remained functional in culture. Different concentrations of LPS (0, 0.3, 1.0, and 10 ng/mL) were added to the microglia culture medium, which was then analyzed for released amounts of the proinflammatory cytokine TNF-α 4 h later. No detectable levels of TNF-α could be measured in the absence of LPS indicating that the microglia, although originating from an adult human brain and despite the isolation procedure, remained in an inactivated state with respect to TNF-α production. Addition of 0.3 ng/mL, LPS caused a significant increase of TNF-α (P < 0.01) to a final concentration of 400 pg/mL. Higher concentrations of LPS, 1.0 and 10 ng/mL, did not significantly result in additional elevated levels of TNF-α compared to the lower LPS concentration (Fig. 1D). Furthermore, treatment of the microglia cells in vitro with 0.3 ng/mL LPS resulted in a significant increase in the gene expression of the proinflammatory cytokines TNF-α and IL-1β (P < 0.05) and a trend toward an increase in IL-6 expression (P = 0.06) (Fig. 1E). These results made us confident that the isolation procedure yielded a pure and functional microglia culture to be used in the time span of 5–9 days in the current experimental series. We, furthermore, noted that the human microglia did not exhibit noteworthy proliferation in this period.

Human Microglia Express Several Types of Ca2+-Activated K+ Channels
The aforementioned morphological characteristics guided the selection of cells for whole-cell patch clamp experiments and the visually observed large size of the cells were confirmed by membrane capacitance measurements (23.5 ± 7.6 pF; n = 19). We next addressed whether the human microglia cells express functional KCa3.1 channels. For these experiments, a set of intracellular (pipette) and extracellular salines with reduced Cl− concentrations were used to minimize possible currents through Cl− channels and electrogenic anion transporters, and with a relatively high free intracellular Ca2+ concentration (~1 μM) to enhance the chances of detecting Ca2+-activated K+ currents. Whole-cell currents were elicited by applying voltage ramps of 150 ms duration starting at −120 mV and ending at 30 mV. Since KCa3.1 are K+ selective channels that are not gated by voltage, they mediate currents that are essentially linearly dependent on voltage, they mediate currents that are essentially linearly dependent on voltage in the range of −90 (E0) to 0 mV. Figure 2A shows the current–voltage (IV) relationships recorded from a microglia cell before and after application of the highly selective and potent (IC50 = 9 nM) KCa3.1 inhibitor NS6180 at a concentration of 1 μM (Jenkins et al., 2013; Jorgensen et al., 2013; Strobaek et al., 2013) (see also Supporting Information, Table 1). The complete inhibition of KCa3.1 (>99% as calculated from the Hill equation) strongly reduced the slope of the IV curve, and caused a distinct depolarizing shift in the reversal potential from ~74 to ~13 mV consistent with a decreased total K+ conductance. The difference IV curve conforms well to the predicted KCa3.1 IV relationship with a reversal potential close to EK (~89 mV). This experiment shows that the linear current in this cell is mainly mediated by KCa3.1 without significant contribution from KCa2 channels which are insensitive to NS6180 (for quantification of KCa3.1, see later). As shown in Fig. 2B (different cell), the control IV relationship
in some cells crossed the voltage axis at a more depolarized level (in this case at \(-55\) mV). This is attributable to the combination of a relatively low \(K_{Ca3.1}\) expression/activation possibly combined with some contribution of background cation and/or residual \(Cl^-\) conductances with equilibrium potentials close to zero or above. Upon application of increasing concentrations of the \(K_{Ca3.1}/K_{Ca2}\) activator NS309, however, a clear and concentration dependent increase in the linear current component occurred and the zero current potential gradually shifted towards \(E_K\), reflecting an increasingly dominating K\(^+\) conductance in this cell. Figure 2C shows the current from this cell analyzed at \(-40\) mV as a function of time with the periods and concentrations of NS309 indicated by the bars above. The average equilibrium responses vs concentrations (\(n = 7\); Fig. 2D) were fitted to a Hill equation and gave an \(EC_{50}\) value of 44 nM, a value close to the observed \(EC_{50}\) value for NS309 activation of human \(K_{Ca3.1}\) channels expressed in HEK-293 cells recorded under
identical conditions (20 nM, see Supporting Information, Fig. 1). The concentration–response plot also shows that a near maximal response to NS309 is obtained at a concentration of 500 nM, which we continued to use for quantification throughout the rest of the study.

The KCa3.1 current was sometimes recorded in combination with a putative K+ current with outward-rectifying characteristics (Kor) seen as an increased slope of the IV curve at positive potentials, or an inward-rectifying current (Kir) causing an abrupt conductance increase at potentials negative toEK (for a summary of IV phenotypes of the microglia population, see Supporting Information, Fig. 2). Figure 3 shows the representative recording from a microglia cell expressing both KCa3.1 and Kor as evident from the IV relationships in panel A. The analysis was in this case performed at two different voltages: −40 mV (KCa3.1) and +30 mV (sum of KCa3.1 and Kor). Following the establishment of a baseline (with a small but stable current run down at +30 mV), 500 nM NS309 was applied and a clear increase in current amplitude could be seen at both −40 mV and +30 mV (Fig. 3A,C). Upon application of NS6180, the current at −40 mV was strongly reduced, proving that the current also in this cell was carried by KCa3.1 (NS6180 exerts no effect on KCa2a at this concentration, see Strobaek et al., 2013). After having removed the KCa3.1 current by NS6180, the outward-rectifying K+ current now became clear (Fig. 3B). In rat microglia, the voltage-dependent outward-rectifying K+ current has been described as being
mediated by various K⁺ channels including Kv1.3 (Jou et al. 1998) (antibody staining only); (Fordyce et al. 2005; Khanna et al. 2001). We were curious to reveal what mediates this current in human microglia, especially since a large proportion of cells (80%) actually exhibit a discernible K⁺ current. We exploited the fact that NS6180 does not readily wash off from the KCa3.1 channel at a concentration of 1 μM and this particular experiment was, therefore, continued by changing directly into a saline containing the KCa1.1 selective activator NS1619 (10 μM). This resulted in a prominent current increase at +30 mV, whereas the current at −40 mV remained negligible (Fig. 3B,C). Following co-addition of the selective KCa1.1 inhibitor Paxilline, the current at +30 mV was fully inhibited. Since Kᵣ was sensitive to NS1619 and completely inhibited by Paxilline in 9 experiments, we conclude that the predominant voltage-dependent outward current in human microglia is mediated by KCa1.1. Note that this finding is different from our identical experiments with microglia isolated from rat pups, which clearly express a Kv-type K⁺ current activating at negative membrane potentials, see Supporting Information Fig. 3. In contrast, for the Kᵣ component also faintly co-expressed with KCa3.1 in some cells, we have no clue to the molecular identity and we did not characterize it further in any detail. We did, however, ascertain that NS309 and NS6180 did not change this component (see Supporting Information, Fig. 4).

Quantification of Functional KCa3.1 Channels in Unstimulated Microglia Cells

To quantify the amplitude of the maximally attainable KCa3.1 current, we systematically measured the responses to sequential application of NS309 and NS6180 (as shown in Fig. 3) in a large number of cells and we defined the ΔKCₐ₃.1 current as the difference in current size between full activation (NS309) and full inhibition (NS6180). A cell with a ΔKCₐ₃.1 current difference of ≥20 pA was considered to express functional KCa3.1 channels, whereas cells with a current change < 20 pA were considered a nonexpresser (see below and Discussion for further elaboration). Typical examples of unresponsive vs responsive cells can be seen in Fig.
FIGURE 4: KCa3.1 currents responsive to NS309 and/or NS6180 were recorded in 79% of the human microglia population. Microglia cells were considered as responsive to NS309 (500 nM) and/or NS6180 (1 μM) if the current amplitude at −40 mV changed by ≥20 pA in whole-cell experiments as those described in Fig. 2 and 3. (A) Time course obtained from a microglia cell that did not respond to NS309 (500 nM) alone nor in combination with NS6180 (1 μM) and which was therefore considered as a nonresponder. (B) The bars show the number of cells that responded (n = 75 equivalent to 79%) and the number of unresponsive cells (n = 20 equivalent to 21%) as defined above. C, time course showing a representative experiment from a microglia cell that did respond to NS309 (500 nM) and where the current was inhibited by NS6180 (1 μM). C, current amplitudes from the 42 microglia cells where both NS309 and NS6180 were applied. The current amplitudes pre- and postapplication of NS309 (500 nM) and after application of NS309 in combination with NS6180 (1 μM) were plotted with currents from individual cells connected by lines. D, representative current-clamp experiment showing a pronounced hyperpolarization upon application of NS309 (500 nM) and a depolarization upon co-application of NS6180 (1 μM). The compounds were present in the extracellular saline during the periods indicated by the bars. D, the membrane potential recorded before (Control) and after NS309 (NS309) as well as after co-application of NS309 and NS6180 (NS309 + NS6180) was plotted and data from individual cells (n = 7) connected by lines. The average membrane potential before application of NS309 was −24 ± 6 mV, after NS309 it was −67 ± 5 mV and after co-application of NS6180 −25 ± 5 mV. Paired one-way ANOVA * P < 0.05, *** P < 0.001.
A typical example of an IV profile of a microglia cell treated with either IL-4 or LPS and with a dominant KCa3.1 current can be seen in Fig. 5A; P > 0.05. We did also not observe any time-dependent changes in current amplitudes for either IL-4 or LPS (Supporting Information, Fig. 5B,C) over the 4 days in which the cells were stimulated (P > 0.05). This is also in line with previous studies from rats, where the KCa3.1 current amplitude did not change following treatment with IL-4 over 6 treatment days in vitro (Ferreira et al. 2014). Hence, the results shown and discussed in the following are exclusively due to the treatment effects, and not influenced by a time overlay.

A typical example of a membrane potential vs time recording. Upon stabilization the membrane potential fluctuated around a value of −16 mV. Application of 500 nM NS309 immediately hyperpolarized the cell to −77 mV and the fluctuations were much reduced as expected from the increased KCa3.1 open state probability in the presence of NS309 combined with the much reduced driving force on the K-ion. Co-application with NS6180 resulted in a depolarization to −21 mV. The quantification and summary statistics are illustrated in Fig. 4Di (n = 7). In conclusion, the degree of activation of the KCa3.1 channel expressed in human microglia cells exerts a profound effect on the membrane potential and is, therefore, concluded to represent a significant hyperpolarizing reserve in human microglia. In contrast, application of NS1619 at 10 μM elicited no hyperpolarization, in accordance with the strong and fast deactivation of the KCa1.1 channel at negative membrane potentials (results not shown).

Quantification of KCa3.1 Functional Expression in LPS- and IL-4-Stimulated Microglia

All experiments conducted so far were performed on the unstimulated and supposedly “resting” human microglia, but we were also interested in investigating whether the expression and/or function of KCa3.1 would change upon in vitro activation by standard proinflammatory (LPS) and anti-inflammatory (IL-4) treatments as has been demonstrated for rodents. The microglia cells were stimulated for 2–4 days with either 100 ng/ml LPS or 20 ng/ml IL-4 after which the electrophysiological measurements were performed. As the treatments were conducted over a few days in vitro, we tested whether the baseline current, the current following activation with NS309, as well as the ΔKCa3.1 current remained stable over days in vitro. Indeed, in unstimulated cells, no changes in current pre- or post-NS309 application or ΔKCa3.1 could be seen between day 5 (the earliest day the cells were used postisolation) up until day 9 (Supporting Information, Fig. 5A; P > 0.05). We did also not observe any time-dependent changes in current amplitudes for either IL-4 or LPS (Supporting Information, Fig. 5B,C) over the 4 days in which the cells were stimulated (P > 0.05). This is also in line with previous studies from rats, where the KCa3.1 current amplitude did not change following treatment with IL-4 over 6 treatment days in vitro (Ferreira et al. 2014). Hence, the results shown and discussed in the following are exclusively due to the treatment effects, and not influenced by a time overlay.

It is worth noting that our criterion for a KCa3.1 expressing cell is a ΔKCa3.1 >20 pA, which is equivalent to 400 pS. Microglia cells expressing <40 KCa3.1 channels have therefore been categorized as nonexpressers in this study.
after the application of NS6180 was conducted and plotted as individual data points for the respective treatments (Fig. 5Aii,Bii). Figure 5Aiii,Biii shows the average current for each treatment group. In both the microglia population activated by IL-4 and the population activated by LPS, a clear and significant increase in the current was observed in response to NS309 ($P < 0.001$). The addition of NS6180 substantially inhibited the current in both cases ($P < 0.001$) even further below the baseline current ($P < 0.05$). In summary, this means that activated cells, similar to unstimulated microglia, respond significantly to KCa3.1 pharmacology.

As described above (Fig. 4B), 79% of our unstimulated microglia population expressed functional KCa3.1 channels. This proportion did not change significantly when the cells were stimulated with either IL-4 or LPS where 86% ($n = 38$) and 73% ($n = 42$) of the recorded cells expressed functional KCa3.1 channels, respectively (Fig. 6A; $P = 0.35$). Based on the IV phenotyping, the proportion of cells expressing $K_u$ and $K_m$ currents also remained constant, (see Supporting Information Fig 2B,C). Further investigation of the current amplitude differences between unstimulated and stimulated microglia revealed that IL-4-treated cells had a significantly greater $\Delta K_{C_{a3.1}}$ window compared to unstimulated cells (Fig. 6B; $P < 0.01$). The difference between full activation and full block in these cells was measured to be $607 \pm 88$ pA compared to a $\Delta K_{C_{a3.1}}$ current of $292 \pm 48$ pA in unstimulated cells and $478 \pm 102$ pA in LPS-treated cells. However, it was also noted that microglia exposed to IL-4 had a significantly greater cell membrane surface area (measured as cell capacitance) compared to both unstimulated cells ($P < 0.001$) as well as LPS-treated cells ($P < 0.01$). There was no difference in membrane capacitance between unstimulated and LPS-

FIGURE 5: Quantification of the KCa3.1 current in microglia activated by IL-4 or LPS. (A) Current traces obtained from voltage-clamp experiment as described in Fig. 2 but from a microglia cell treated with IL-4 (20 ng/mL) for 4 days in vitro. The traces were obtained before (Control) and after application of NS309 (500 nM) and after NS309 in combination with NS6180 (1 $\mu$M) as indicated at the traces. (Aii) Current amplitude at $-40$ mV of individual microglia cells pre- and postapplication of NS309 and NS309 in combination with NS6180. (Aiii) Averages of the currents from panel Aii. Panel B–Biii same as A–Aiii but after treatment with LPS (100 ng/mL). The bars in Aii and Biii represent the mean $\pm$ SEM; paired one-way ANOVA * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 

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treated microglia (Fig. 6C). However, when the current for each individual cell was normalized against its cell size, the current difference between unstimulated microglia and microglia exposed to IL-4 disappeared (Fig. 6D; $P > 0.05$). This indicates that IL-4 treated cells have the same number of KCa3.1 channels per membrane area and that the larger $\Delta \text{KCa3.1}$ current is due to the greater cell size rather than having additional channels present per membrane area.

**Discussion**

We have established methods for isolation, cultivation, and in vitro maintenance of highly purified human microglia cultures essentially devoid of neurons and other glial cells, by using adult brain tissue surgically resected from patients with treatment-resistant epilepsy. The microglial source was neocortical tissue removed in the surgery to get access to the epileptic foci in the hippocampus. This tissue showed no discernable histopathology and was, therefore, considered relatively unaffected by the patient’s epilepsy and potentially a reference for future studies with foci-derived microglia or microglia from established neuroinflammatory diseases. Experimental support for considering these microglia as resting cells were lack of basic TNF-$\alpha$ release and negligible gene expression levels of TNF-$\alpha$, IL-6, and IL-1$\beta$ combined with a massive upregulation and secretion upon challenge with the standard proinflammatory molecule LPS, a response in accordance with the expression of toll-like receptors (TLRs) on prenatal human microglia (Jack et al. 2005). As $K^+$ channels, in particular, the KCa3.1 channel, are important in rodent microglia and often upregulated during activation, a primary goal of this study was to make a quantitative exploration of the basic functional expression of KCa3.1 in these adult human microglia cells compared to similar measurements reported in the literature using rodent microglia. Furthermore, we also aimed at exploring whether standard pro- and anti-inflammatory stimulations in vitro might influence the expression level.

**Unstimulated Human Microglia Cells**

We found that a large fraction (79%) of the microglia cells expressed KCa3.1, usually as the predominant $K^+$ conductance in the physiological membrane potential range. We quantified this functional expression by calculating the difference in current between full pharmacological activation by NS309 and full inhibition by NS6180. This $\Delta \text{KCa3.1}$ value was found on average to amount to 292 pA at $-40 \, \text{mV}$, which we—provided certain reasonable assumptions, see the Results section—estimated to be equivalent to a minimum average number of 585 functional KCa3.1 channels per cell.

The functional impact of KCa3.1 activation/inhibition was directly confirmed by current clamp measurements: From an average resting membrane potential of $-24 \, \text{mV}$, all cells responded with a distinct hyperpolarization upon NS309 application and with an ensuing depolarization by adding NS6180. Thus, activation/inhibition of KCa3.1 strongly
influences the membrane potential of unstimulated human microglia cells. This is considered a significant finding, as many downstream functions of \( K_{Ca3.1} \) activation (e.g., regulation of transmembrane \( Ca^{2+} \)-flux) are mediated via changes in the membrane potential.

**IL-4 and LPS Stimulation**

With \( K_{Ca3.1} \) established as a major \( K^+ \) channel in the untreated microglia, we focused on possible changes in functional expression level upon proinflammatory (LPS), respectively, anti-inflammatory (IL-4) stimulations. There was no significant change in the fraction of \( K_{Ca3.1} \) expressing cells compared to the resting condition (79%) by either treatment (84% for IL-4 and 73% for LPS). The average \( \Delta K_{Ca3.1} \) current in the \( K_{Ca3.1} \) expressing fraction of the IL-4 treated cells were significantly increased to 207% of the unstimulated current level, which is equivalent to an increase in average channel numbers from 584 to 1209 channels per microglia cell. In contrast, no significant change was observed in the LPS-treated cells. Interestingly, a closer analysis revealed that the IL-4 stimulated cells had significantly increased input capacitance (30 pF) compared to unstimulated cells (20 pF), reflecting an increase in the cell membrane area as a result of the stimulation, whereas the recorded capacitance (22 pF) of LPS-stimulated cells was not significantly different from that of the resting cells. Using the standard value for specific cell membrane capacitance (1 \( \mu \)F/cm\(^2\)), the channel density per unit membrane area was thus calculated at 0.14 channels/\( \mu \)m\(^2\) (unstimulated cells), 0.22 channels/\( \mu \)m\(^2\) (IL-4 stimulated cells), and 0.20 channels/\( \mu \)m\(^2\) (LPS), respectively. As these numbers are not significantly different, we conclude that in vitro treatment with neither IL-4 nor LPS changed the density of \( K_{Ca3.1} \) channels on the human microglia cells.

**Comparison to Other Studies**

As previously mentioned, there are relatively few reports on the functional expression of \( K^+ \) channels from human microglia cells, in particular, from adults. Bordey and Spencer (2003) performed patch clamp studies on putative microglia directly in slices from hippocampi isolated from epileptic or brain tumor patients, and only found cells with very low or even lack of functional activity of any ion channels at negative membrane potentials (extremely low total input conductance, 0.28 nS, put into perspective by comparison with the specific \( K_{Ca3.1} \)-mediated conductance of 5.84 nS found in our study). At positive potentials, a voltage and \( Ca^{2+} \)-activated \( K^+ \) channel with high unitary conductance was identified, which clearly was due to \( K_{Ca1.1} \). Norenberg et al. (1994) also used an adult microglia preparation, and observed currents consistent with \( K^+ \) channels followed by an outward-rectifying conductance depending on intracellular \( K^+ \) and with the general characteristics of a \( K^+ \) channel. No \( K^+ \) conductance with \( K_{Ca3.1} \) properties was identified. McLarnon et al. (1997) investigated microglia from human fetuses, and found evidence of A-type \( K^+ \) currents in unstimulated cells, and, upon stimulation with the proinflammatory cytokine IFN-\( \gamma \), another outward-rectifying \( K^+ \) conductance with all biophysical characteristics consistent with \( K_{Ca1.1} \). Again, however, no indication of currents attributable to \( K_{Ca3.1} \) was noted. Recently, Chen et al. (2015) reported, as part of their studies on microglia in stroke, expression of \( K^+ \) channels by immunohistochemistry on postmortem human tissue, and found evidence for presence of \( K_{Ca3.1} \) and \( K_{Ca1.3} \) protein on microglial macrophages in the infarcted area of patients, whereas no protein was detected in noninfarcted areas. No functional studies were performed.

A quite extensive body of literature exists on the basic expression of \( K_{Ca3.1} \) as well as other \( K^+ \) channels in primary or acutely isolated rodent microglia under different stimulation paradigms in vitro. A consistent finding across studies suggest expression of \( K_{Ca3.1} \), \( K_{Ca2.1} \), and \( K_{Ca2.3} \) (Chen et al., 2015; Ferreira et al., 2014, 2015; Kaushal et al., 2007) with some reports also being consistent with functional expression of \( K_{Ca2.3} \) (Schlichter et al., 2010; Siddiqui et al., 2014). While our study clearly confirmed functional expression of \( K_{Ca3.1} \) in the human microglia and also a \( K_{Ca} \) current (possibly attributed to \( K_{Ca2.1} \)), it is noteworthy that we found no evidence for functional expression of \( K_{Ca1.3} \) (or any other \( K_{Ca} \) channel), since the measured \( K_{Ca} \) currents were sensitive to both NS1619 and Paxilline and therefore attributable to \( K_{Ca1.1} \). It may be argued that the ramp protocol used is not optimal for detection of an inactivating channel like \( K_{Ca1.3} \), and that we therefore have underestimated or even missed it. However, control experiments using the same voltage-clamp ramp protocol with rat microglia clearly showed the \( K_{Ca1.3} \) current. We also found no evidence for a major contribution of \( K_{Ca2.3} \) channels, since NS6180 (no effect on \( K_{Ca2.3} \) at 1 \( \mu \)M) essentially eliminated the NS309 augmented voltage-insensitive \( K^+ \)-current.

Focusing specifically on the functional expression level of \( K_{Ca3.1} \) in microglia from rat pups, Ferreira et al. (2014) reported a basic NS309/TRAM-34 defined \( K_{Ca3.1} \) conductance (using a protocol resembling the \( \Delta K_{Ca3.1} \) method used here) of 1.7 pA/pF in the unstimulated cell and 39 pA/pF (current measured at +80 mV) upon stimulation with IL-4. For comparison, a recalculation of our corresponding data from the human microglia yields ~49 pA/pF for the unstimulated cells and ~74 pA/pF for the IL-4-stimulated cells. Using acutely isolated microglia from mice, Chen et al. (2015) also reported a TRAM-34 defined \( K_{Ca3.1} \) conductance of 20 pS/pF from acutely isolated and unstimulated cells, as well as values of 72 pS/pF from microglia isolated from a MCAO induced infarct, and 84 pS/pF from microglia...
isolated upon intracerebroventricular LPS injection. In comparison, our values expressed in the same units are 285 pS/pF for unstimulated cells and 436 pS/pF respectively 410 pS/pF for IL-4 and LPS stimulated cells. The unstimulated human microglia cells from this study thus expressed substantially higher KCa3.1 currents than both unstimulated primary rat microglia and acutely isolated mouse microglia (10–30 times), and were furthermore on par with the expression levels obtained with the various in vitro/in vivo stimulation procedures applied with the rodent microglia (one to three times).

In conclusion, we have found a very substantial functional expression of KCa3.1 channels in microglia from adult epilepsy patients. It is quite remarkable that the level is as high—or even higher—than observed in LPS or otherwise experimentally activated rodent microglia. It is also noteworthy that the KCa3.1 expression could not be increased further by standard pro- or anti-inflammatory treatments as has repeatedly been demonstrated in the literature for rodent microglia. We suggest that these human microglia cells are already maximally primed with respect to KCa3.1 expression. These findings may raise several important questions for ion and functional activity reflects the normal situation in causing a microglia phenotype with a high KCa3.1 level.

In conclusion, we have found a very substantial functional expression of KCa3.1 channels in microglia from adult epilepsy patients. It is quite remarkable that the level is as high—or even higher—than observed in LPS or otherwise experimentally activated rodent microglia. It is also noteworthy that the KCa3.1 expression could not be increased further by standard pro- or anti-inflammatory treatments as has repeatedly been demonstrated in the literature for rodent microglia. We suggest that these human microglia cells are already maximally primed with respect to KCa3.1 expression. These findings may raise several important questions for future studies; for example whether this high KCa3.1 expression and functional activity reflects the normal situation in healthy resting adult microglia or whether the nonpathological neocortical tissue is somehow influenced by the epileptic condition of the patients (or by their medication history), thereby causing a microglia phenotype with a high KCa3.1 level.

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Author contributions
LVB, DS, CH, and PC designed research; LVB, DS, CH, and JK performed research; LVB, DS, and PC analyzed the data; LVB, DS, LP, JDM, and PC wrote the paper.

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