Quantification of the functional expression of the Ca2+ -activated K+ channel KCa 3.1 on microglia from adult human neocortical tissue.

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Title:

Quantification of the Functional Expression of the Ca^{2+}-Activated K^+ Channel $K_{Ca}3.1$ on Microglia from Adult Human Neocortical Tissue

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Main points:

K^+ currents in primary human microglia are mainly due to Ca^{2+}-activated K^+ channels

Electrophysiology and pharmacology revealed functional $K_{Ca}3.1$ and $K_{Ca}1.1$ in a majority of microglia

IL-4 or LPS treatment did not change the $K_{Ca}3.1$ current density

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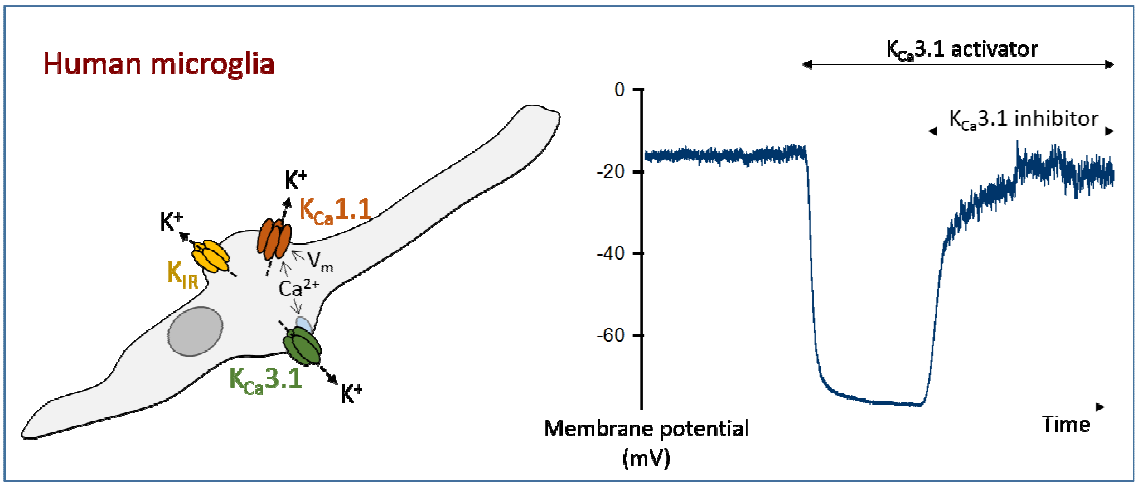

Key words:

Neuroinflammation, potassium channel, patch clamp, glial cell, IK channel, BK channel

Abbreviations:

IL-4, interleukin-4; IV, current-voltage relation; $K_{Ca}1.1$, large-conductance Ca^{2+}-activated K^+ channel (a.k.a. BK channel); $K_{Ca}2$, small-conductance Ca^{2+}-activated K^+ channel (a.k.a. SK channel); $K_{Ca}3.1$, intermediate-conductance Ca^{2+}-activated K^+ channel (a.k.a. IK channel); K_{ir}, inward-rectifying K^+ channel; K_{or}, outward-rectifying potassium channel; K_v, voltage-dependent K^+ channel; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor alpha.

Abstract

The $K_{Ca}3.1$ channel (*KCNN4*) is an important modulator of microglia responses in rodents, but no information exists on functional expression in 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 $K_{Ca}3.1$. The channel expression was demonstrated as a significant increase in the voltage-independent current by NS309, a $K_{Ca}3.1/K_{Ca}2$ activator, followed by full inhibition upon co-application with NS6180, a highly selective $K_{Ca}3.1$ inhibitor. A major fraction (79 %) of unstimulated human microglia expressed $K_{Ca}3.1$, and the difference in current between full activation and inhibition ($\Delta K_{Ca}3.1$) was estimated at 292±48 pA at -40 mV (n=75), which equals at least 585 channels/cell. Serial $K_{Ca}3.1$ activation/inhibition significantly hyperpolarized/depolarized the membrane potential. The isolated human microglia were potently activated by LPS shown as a prominent increase in TNF-α production. However, incubation with LPS neither changed the $K_{Ca}3.1$ current nor the fraction of $K_{Ca}3.1$ expressing cells. In contrast, the anti-inflammatory cytokine IL-4 slightly increased the $K_{Ca}3.1$ current per cell, but since 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 $K_{Ca}1.1$ modulators NS1619 and Paxilline and an inward-rectifying current with the characteristics of a K_{ir} channel. The high functional expression of $K_{Ca}3.1$ in microglia from epilepsy patients accentuate the need for further investigations of its role in neuropathological processes.

Introduction

Microglia are the resident immune cell 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, $K_{Ca}3.1$ (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). $K_{Ca}3.1$ 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, $K_{Ca}3.1$ was originally shown to act in a pro-inflammatory manner participating in the respiratory burst process (Khanna et al. 2001) and pharmacological blocking of $K_{Ca}3.1$ by the selective $K_{Ca}3.1$ inhibitor TRAM-34 gave reduced nitric oxide (NO) production following activation by

lipopolysaccharide (LPS) (Kaushal et al. 2007) or amyloid-β oligomers (AβO) (Maezawa 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 pro-inflammatory role, $K_{Ca}3.1$ can also play a role in interleukin-4 (IL-4)-mediated activation of rat microglia (Ferreira et al. 2014), since significantly larger $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 $K_{Ca}3.1$ channels (Bordey and Spencer 2003; McLarnon et al. 1997; Norenberg et al. 1994). However, based on immunohistology of *post mortem* tissue, Chen et al. (2015) recently showed a clear expression of $K_{Ca}3.1$ protein in the infarcted area in stroke patients.

Since prominent differences in expression profiles and responses occur between rodent and human microglia (Smith and Dragunow 2014) the verification of functional expression of $K_{Ca}3.1$ in human microglia from adults is an important piece of missing evidence in the translational validation of $K_{Ca}3.1$ as a target for modulation of neuroinflammatory diseases. The access to neural tissue removed within a short timeframe allow production of primary cultures of microglial cells with high purity and relevant cellular physiological properties. In the present 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 $K_{Ca}3.1$, 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 $K_{Ca}3.1$. We conclude that $K_{Ca}3.1$ constitute a major K^+ conductance in isolated human neocortical microglia under all conditions investigated. We also found functional expression of the BK channel ($K_{Ca}1.1$) as well as an unidentified inward-rectifying K^+ channel (K_{ir}).

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 amygdalohippocampectomy. In all subjects the amygdala and hippocampus were determined to be the epileptogenic zone during the epilepsy surgery evaluation. Histopathological examinations 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 400 x g 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^{o}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 400 x g to remove the trypsin solution. The pellet was re-suspended in DMEM medium with above mentioned supplements as well as 5% FBS. The cell suspension was seeded at 2 x 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 hours after 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 six post-isolation the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. Following 2 x 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 containing 5% goat serum for 2 hours at room temperature. The cells were washed extensively for 3 x 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 x 5 min in PBS and the slides were finally mounted with Slow-Fade® Gold mounting medium containing DAPI nuclear dye (Life Technologies). Grayscale 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 procedures (i.e. 30 frames per marker

altogether). The number of cells stained with each specific marker in each frame was counted and expressed as a percentage of the number of $DAPI^+$ nuclei in that same frame.

Quantification of pro-inflammatory cytokine expression following LPS stimulation

On day six post-isolation the microglia were exposed to 0, 0.3, 1 or 10 ng/ml lipopolysaccharide (LPS) for four hours. Two replicates were included per concentration. After the stimulation the cell culture medium was collected and stored at -80^{o}C until the ELISA analysis could be performed. The amount of TNF-α released by microglia was analyzed using a Human TNF-α ELISA Ready-SET-GO!® (eBiosciences) according to the manufacturer's instructions.

The gene expression of the pro-inflammatory cytokines interleukin-6 (IL-6), interleukin-1 beta (IL-1β) and TNF-α was quantified using quantitative real-time PCR (qPCR). Following exposure to 0.3 ng/ml LPS for four hours the stimulated and unstimulated control cells were washed in PBS and lysed in RLT buffer (Qiagen). The total ribonucleic acid (RNA) was subsequently extracted using the RNeasy Mini Kit (Qiagen) as per manufacturer's instructions. The optional DNase treatment step was included in the procedure. The extracted RNA was reverse transcribed into cDNA using the ImProm-II^{TM} Reverse Transcriptase (Promega). For qPCR experiments a reaction mix of 20 µl per sample containing 10 µl IQ 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′-GCT TGA GGG TTT GCT ACA ACA TG-3′, IL-1β Fw: 5′-AAA TAC CTG 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 CTC AGG GCT G-3′, GAPDH Fw: 5′-GGA TGA TGT TCT

GGA GAG C-3′ Re: 5′-GGT ATC GTG GAA GGA CTC-3′. The following PCR cycling conditions were used: 94^{o}C for 10 min followed by 40 cycles of 94^{o}C for 30s, 60^{o}C for 45s and 72^{o}C for 90s. The concluding melt curve (50^{o}C to 94^{o}C) analysis showed a single PCR product which was also confirmed using gel electrophoresis. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ 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 $CaCl_2$, 1 $MgCl_2$, 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, 4.3 $CaCl_2$, 1 $MgCl_2$, 5 EGTA, 15.6 KOH, 2 MgATP, 10 HEPES; pH 7.2) was adapted from Ferreira et al. (2014) and contained ~1 μM free Ca^{2+} 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 in order to track the stability

of the recording. The C_{slow} 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/cm^2 membrane area.

For the current-clamp experiments an extracellular saline with high chloride concentration was used (in mM: 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES; pH adjusted to 7.4 with NaOH) and the pipette solution contained in mM: 109 KCl, 5.2 $CaCl_2$, 1.4 $MgCl_2$, 10 EGTA, 31 KOH, 10 HEPES; pH 7.2 giving a calculated free [Ca^{2+}] of 0.2 μM according to Maxchelator, see above). All recordings were conducted at room temperature using a HEKA EPC-9 amplifier and the PULSE software (HEKA Elektronik).

Pharmacological tool compounds

The $K_{Ca}3.1$/$K_{Ca}2$ activator NS309 (6,7dichloro-1*H*indole-2,3-dione 3-oxime), the $K_{Ca}3.1$ inhibitor NS6180 (4-[[3-(trifluoromethyl)phenyl]methyl]-2H-1,4–benzothiazin-3(4H)-one) and the $K_{Ca}1.1$ activator NS1619 (1-(2′-hydroxy-5′-trifluoromethlphenyl)-5-trifluoromethyl-2(3H)benzimidazolone) were synthesized according to previous descriptions (Olesen and Waetjen 1993; Strobaek et al. 2013; Strobaek et al. 2004). 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^{o}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 (χ^2) test was used.

Results

Isolation and culturing of human microglia

In order to evaluate the expression of $K_{Ca}3.1$ 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 six 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 pro-inflammatory cytokine TNF-α four hours 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 respectively, 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 pro-inflammatory cytokines TNF-α and IL-1β ($p<0.05$) and a trend towards 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 Ca^{2+}-activated K^+ channels

The above mentioned 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 $K_{Ca}3.1$ channels. For these experiments a set of intracellular (pipette) and extracellular salines with reduced Cl^- concentrations were used in order to minimize possible currents through Cl^- channels and electrogenic anion transporters, and with a relatively high free intracellular Ca^{2+} concentration (~1 μM) in order to enhance the chances of detecting Ca^{2+}-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 $K_{Ca}3.1$ are K^+ selective channels that are not gated by voltage they mediate currents that are essentially linearly dependent on voltage in the range of -90 (= E_K) to 0 mV. Fig. 2A shows the current-voltage (IV) relationships recorded from a microglia cell before and after application of the highly selective and potent (IC_{50} = 9 nM) $K_{Ca}3.1$ inhibitor NS6180 at a concentration of 1 μM (Jenkins et al. 2013; Jorgensen et al. 2013; Strobaek et al. 2013) (see also Supplementary Table 1). The complete inhibition of $K_{Ca}3.1$ (> 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 mV to -13 mV consistent with a decreased total K^+ conductance. The difference IV curve conforms well to the predicted

$K_{Ca}3.1$ IV relationship with a reversal potential close to E_K (-89 mV). This experiment shows that the linear current in this cell is mainly mediated by $K_{Ca}3.1$ without significant contribution from $K_{Ca}2$ channels which are insensitive to NS6180 (for quantification of $K_{Ca}3.1$, 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 $K_{Ca}3.1$ 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 $K_{Ca}3.1/K_{Ca}2$ 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. Fig. 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 $K_{Ca}3.1$ channels expressed in HEK-293 cells recorded under identical conditions (20 nM, see Supplementary 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 $K_{Ca}3.1$ current was sometimes recorded in combination with a putative K^+ current with outward-rectifying characteristics (K_{or}) seen as an increased slope of the IV curve at positive potentials, or an inward-rectifying current (K_{ir}) causing an abrupt conductance increase at potentials negative to E_K (for a summary of IV phenotypes of the microglia population, see Supplementary Fig. 2). Fig. 3 shows a representative recording from a microglia cell expressing both $K_{Ca}3.1$ and K_{or} as evident from the IV relationships in panel A. The analysis

was in this case performed at two different voltages: -40 mV ($K_{Ca}3.1$) and +30 mV (sum of $K_{Ca}3.1$ and K_{or}). 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 size 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 $K_{Ca}3.1$ (NS6180 exerts no effect on $K_{Ca}2$ at this concentration, see Strobaek et al. (2013)). After having removed the $K_{Ca}3.1$ current by NS6180 the outward rectification of the current remaining positive to 0 mV now became more clear (Fig. 3B). In rat microglia the voltage-dependent outward-rectifying K^+ current has been described as being mediated by various K_v channels including $K_v1.3$ (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_{or}. We exploited the fact that NS6180 does not readily wash off from the $K_{Ca}3.1$ channel at a concentration of 1 μM and this particular experiment was therefore continued by changing directly into a saline containing the $K_{Ca}1.1$ 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 $K_{Ca}1.1$ inhibitor Paxilline the current at +30 mV was fully inhibited. Since K_{or} was sensitive to NS1619 and completely inhibited by Paxilline in nine experiments, we conclude that the predominant voltage-dependent outward current in human microglia is mediated by $K_{Ca}1.1$. Note that this finding is different from our identical experiments with microglia isolated from rat pups, which clearly express a K_v-type K^+ current activating at negative membrane potentials, see Supplementary Fig. 3. In contrast, for the K_{ir} component also faintly co-expressed with $K_{Ca}3.1$ 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 Supplementary Fig. 4).

Quantification of functional $K_{Ca}3.1$ channels in unstimulated microglia cells

In order to quantify the amplitude of the maximally attainable $K_{Ca}3.1$ 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 $\Delta K_{Ca}3.1$ current as the difference in current size between full activation (NS309) and full block (NS6180). A cell with a $\Delta K_{Ca}3.1$ current difference of $\geq$ 20 pA was considered to express functional $K_{Ca}3.1$ channels, whereas cells with a current change < 20 pA were considered a non-expresser (see below and Discussion for further elaboration). Typical examples of unresponsive vs responsive cells can be seen in Fig. 4A and Fig. $4C_i$. A total of 95 human microglia cells were tested out of which 75 cells (79%) turned out to be responsive and 20 cells (21%) unresponsive to NS309 and NS6180 (Fig. 4B). A quantitative assessment was conducted of the cells responsive to $K_{Ca}3.1$ pharmacology: On average this population exhibited a highly significant increase in the $K_{Ca}3.1$ current following application of NS309 ($p<0.001$) (Fig. $4C_i$, C_{ii}). Similarly there was a highly significant decrease of the $K_{Ca}3.1$ current following co-application of NS309 and NS6180 ($p<0.001$), reaching a value of -13 ± 8 pA, which was furthermore also significantly lower than the initial baseline current ($p<0.05$). We never observed cells where NS309-facilitated currents could not be inhibited by NS6180 which rules out the possibility that $K_{Ca}2$ channels could have contributed significantly to the total voltage-independent current in these cells. On average the human microglia cells displayed a $\Delta K_{Ca}3.1$ current of 292 ± 48 pA (n = 42), which can be converted to total $K_{Ca}3.1$ single cell chord conductance, G:

$$G = \Delta K_{Ca}3.1/(V_m - E_K) = 292 * 10^{-12}\ A/0.05\ V = 5.84\ nS$$

Assuming a single channel chord conductance, g = 10 pS at room temperature and near physiological intracellular and extracellular K^+ concentrations (Christophersen 1991; Sauve et al. 1986) *and* that full activation by NS309 results in an open state probability (P_o) of 1, this conductance can be converted to an absolute number of expressed functional channels (N) per microglia cell:

$$N = G/g = 5840\ pS\ per\ cell/10\ pS\ per\ channel = 585\ channels/cell^1$$

Note, however, that since the effect of NS309 has not been investigated in single channel recordings, the $P_o(max) = 1$ is a theoretical limiting assumption, and the calculated channel number per cell is therefore an average minimum estimate.

We wanted to elucidate the impact of $K_{Ca}3.1$ activation and inhibition on the microglia membrane potential, which in many cases is the immediate mediator of down-stream effects of $K_{Ca}3.1$ activation under physiological/pathophysiological conditions. Thus, we performed current-clamp experiments (no applied current) using basically the same experimental paradigm as above. For these experiments, however, we used extracellular and intracellular salines with near physiological [Cl^-] concentrations, and a pipette solution which was buffered closer to the expected level of intracellular "resting" free Ca^{2+}. Fig. $4D_i$ shows 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 $K_{Ca}3.1$ 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. $4D_{ii}$ (n = 7). In conclusion, the degree of activation of the $K_{Ca}3.1$ 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 $K_{Ca}1.1$ channel at negative membrane potentials (results not shown).

Quantification of $K_{Ca}3.1$ 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 $K_{Ca}3.1$ would change upon *in vitro* activation by standard pro-inflammatory (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. Since 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 $\Delta K_{Ca}3.1$ current remained stable over days *in vitro*. Indeed, in unstimulated cells no changes in current pre- or post-NS309 application or $\Delta K_{Ca}3.1$ could be seen between day 5 (the earliest day the cells were used post-isolation) up until day 9 (Supplementary. Fig. 5A; $p>0.05$). We did also not observe any time dependent changes in current amplitudes for either IL-4 or LPS (Supplementary Figs 5B and 5C) 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 $K_{Ca}3.1$ 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 an IV profile of a microglia cell treated with either IL-4 or LPS and with a dominant $K_{Ca}3.1$ current can be seen in Figs. $5A_i$ and $5B_i$ respectively. Quantitative analysis of the currents pre- and post-NS309 as well as after the application of NS6180 was conducted and plotted as individual data points for the respective treatments (Figs. $5A_{ii}$ and $5B_{ii}$). Figs. $5A_{iii}$ and $5B_{iii}$ show 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 $K_{Ca}3.1$ pharmacology.

As described above (Fig. 4B), 79% of our unstimulated microglia population expressed functional $K_{Ca}3.1$ 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 $K_{Ca}3.1$ channels, respectively (Fig. 6A; $p=0.35$). Based on the IV phenotyping the proportion of cells expressing K_{ir} and K_{or} currents also remained constant, (see Supplementary Figs 2B and 2C). Further investigation of the current amplitude differences between unstimulated and stimulated microglia revealed that IL-4 treated cells had a significantly greater $\Delta K_{Ca}3.1$ 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 ± 88 pA compared to a $\Delta K_{Ca}3.1$ current of 292 ± 48 pA in unstimulated cells and 478 ± 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 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 $K_{Ca}3.1$ channels per membrane area and that the larger $\Delta K_{Ca}3.1$ 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-α release and negligible gene expression levels of TNF-α and IL-1β combined with a massive upregulation and secretion upon challenge with the standard pro-inflammatory molecule LPS, a response in accordance with the expression of toll-like receptors (TLRs) on prenatal human microglia (Jack et al. 2005). Since K^+ channels, in particular the $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 $K_{Ca}3.1$, 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 $\Delta K_{Ca}3.1$ value

allowed clear distinction from the expression of other major ion conductances, such as a K_{ir} channel (unknown molecular background) and the K_{or}, which were identified as $K_{Ca}1.1$ by its voltage-dependence and its pharmacology. The $\Delta K_{Ca}3.1$ value was found on average to amount to 292 pA at -40 mV, which we - provided certain reasonable assumptions, see the Results section - estimated to be equivalent to a minimum average number of 585 functional $K_{Ca}3.1$ channels per cell.

The functional impact of $K_{Ca}3.1$ activation/inhibition was directly confirmed by current clamp measurements: From an average resting membrane potential of -24 mV all cells responded with a distinct hyperpolarization upon NS309 application and with an ensuing depolarization by adding NS6180. Thus, activation/inhibition of $K_{Ca}3.1$ strongly influences the membrane potential of unstimulated human microglia cells. This is considered a significant finding, since many down-stream functions of $K_{Ca}3.1$ activation (e.g. regulation of transmembrane Ca^{2+}-flux) are mediated via changes in the membrane potential.

IL-4 and LPS stimulation

With $K_{Ca}3.1$ established as a major K^+ channel in the untreated microglia, we focused on possible changes in functional expression level upon pro-inflammatory (LPS) respectively anti-inflammatory (IL-4) stimulations: There was no significant change in the fraction of $K_{Ca}3.1$ expressing cells compared to the resting condition (79 %) by either treatment (84% for IL-4, 73 % for LPS). The average $\Delta K_{Ca}3.1$ current in the $K_{Ca}3.1$ 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/μm^2 (unstimulated cells), 0.22 channels/μm^2 (IL-4 stimulated cells), and 0.20 channels/μm^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 $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 $K_{Ca}1.1$. Norenberg et al. (1994) also used an adult microglia preparation, and observed currents consistent with Na_v channels followed by an outward-rectifying conductance depending on intracellular K^+ and with the general characteristics of a K_v channel. No K^+ conductance with $K_{Ca}3.1$ properties was identified. McLarnon et al. (1997) investigated microglia from human foetuses, and found evidence of A-type K_v currents in unstimulated cells, and, upon stimulation with the pro-inflammatory cytokine IFN-ɤ, another outward-rectifying K^+ conductance with all biophysical characteristics consistent with

$K_{Ca}1.1$. Again, however, no indication of currents attributable to $K_{Ca}3.1$ was noted. Recently, Chen et al. (2015) reported, as part of their studies on microglia in stroke, expression of K^+ channels by immunohistochemistry on *post mortem* human tissue, and found evidence for presence of $K_{Ca}3.1$ and $K_v1.3$ protein on microglia/macrophages in the infarcted area of patients, whereas no protein was detected in non-infarcted areas. No functional studies were performed.

A quite extensive body of literature exists on the basic expression of $K_{Ca}3.1$ 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 $K_{Ca}3.1$, $K_{ir}2.1$, and $K_v1.3$ (Chen et al. 2015; Ferreira et al. 2014; Ferreira et al. 2015; Kaushal et al. 2007) with some reports also being consistent with functional expression of $K_{Ca}2.3$ (Schlichter et al. 2010; Siddiqui et al. 2014). While our study clearly confirmed functional expression of $K_{Ca}3.1$ in the human microglia and also a K_{ir} current (possibly attributed to $K_{ir}2.1$), it is noteworthy that we found no evidence for functional expression of $K_v1.3$ (or any other K_v channel), since the measured K_{or} currents were sensitive to both NS1619 and Paxilline and therefore attributable to $K_{Ca}1.1$. It may be argued that the ramp protocol used is not optimal for detection of an inactivating channel like $K_v1.3$, 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 $K_v1.3$ current. We also found no evidence for a major contribution of $K_{Ca}2$ channels, since NS6180 (no effect on $K_{Ca}2.3$ at 1 μM) essentially eliminated the NS309 augmented voltage-insensitive K^+-current.

Focusing specifically on the functional expression level of $K_{Ca}3.1$ in microglia from rat pups, (Ferreira et al. 2014) reported a basic NS309/TRAM-34 defined $K_{Ca}3.1$ conductance (using a protocol resembling the $\Delta K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 (1-3 times).

In conclusion, we have found a very substantial functional expression of $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 $K_{Ca}3.1$ expression. These findings may raise several important questions for future studies; for example whether this high $K_{Ca}3.1$ expression and functional activity reflects the normal situation in healthy resting adult microglia *or* whether the non-pathological 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 $K_{Ca}3.1$ 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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LVB, DS, CH, JK and PC were all employees at Saniona A/S at the time the work was conducted.

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Footnotes

[1] It is worth noting that our criterion for a $K_{Ca}3.1$ expressing cell is a $\Delta K_{Ca}3.1 > 20$ pA, which is equivalent to 400 pS. Microglia cells expressing less than 40 $K_{Ca}3.1$ channels have therefore been categorized as non-expressers in this study.

Tables

Supplementary Table 1. The name, structure and main action of the compounds used in this study.

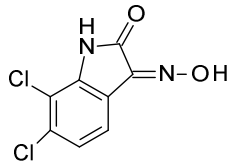
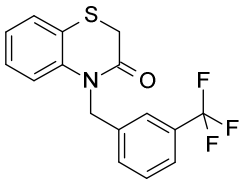
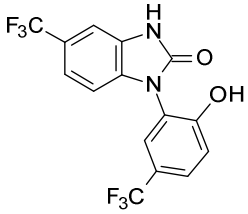
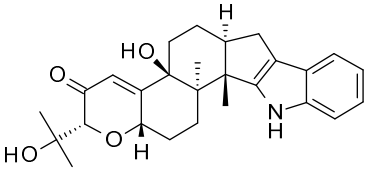
Compound	Structure	Type	References
NS309		$K_{Ca}3.1$ activator $K_{Ca}2$ activator	Strobaek et al. 2004
NS6180		$K_{Ca}3.1$ inhibitor	Jorgensen et al. 2013 Strobaek et al. 2013 Jenkins et al. 2013
NS1619		$K_{Ca}1.1$ activator	Olesen et al. 1994 Gribkoff et al. 1996
Paxilline		$K_{Ca}1.1$ inhibitor	Strobaek et al. 1996 Gribkoff et al. 1996

Figure legends

Figure 1. The human microglia culture is pure and responsive to stimulation with LPS. Photomicrographs of the cell culture with (A) human microglia stained for Iba1 in red and (B) astrocytes stained for GFAP in green 6 days post-isolation. The boxes in the bottom right corners show a microglia and an astrocyte, respectively, at higher magnification. Counterstaining of $DAPI^+$ nuclei can be seen in blue. A-B (including insert): Scale bars: 50 μm. (C) Quantitative purity analysis of the human microglia cultures (n = 3) at 6 days post-isolation. In addition to Iba1 and GFAP the cultures were stained for neurons (MAP2) and oligodendrocytes (CC-1) and stained cells were expressed as a percentage of the total amount of $DAPI^+$ nuclei. (D) Quantification of TNF-α release following incubation with LPS (0.3, 1, and 10 ng/ml) for four hours shows a significant response at all LPS concentrations tested. C-D: Bars represent mean ± SEM; D: unpaired one-way ANOVA ** $p<0.01$, *** $p<0.001$. (E) Gene expression analysis of pro-inflammatory cytokines following stimulation with LPS for four hours. Bars represent mean ± SEM; unpaired t-test * $p<0.05$, ** $p<0.01$.

Figure 2. Human microglia express $K_{Ca}3.1$ channels sensitive to the inhibitor NS6180 and the activator NS309. (A) Current-voltage (IV) plot from a human microglia cell obtained during a whole-cell recording in voltage-clamp mode. The currents were elicited by applying voltage ramps (from -120 mV to 30 mV in 150 ms) every 5 seconds from a holding potential of -90 mV. The current traces appear voltage-independent (linear) and were obtained before (Control) and after the application of the $K_{Ca}3.1$ selective inhibitor NS6180 (1 μM). The $K_{Ca}3.1$ IV curve (red trace) was obtained by subtracting the NS6180 trace from the Control trace. E_K indicates the calculated equilibrium potential for K^+ (-90 mV) with the salines used (see method section). (B) IV plot showing the effect of increasing concentrations of the

$K_{Ca}3.1$ activator NS309. The individual current traces were obtained before or after application of NS309 in the concentrations indicated to the right of the traces. (C) Time course of the experiment in (B) plotted as the current amplitude measured at -40 mV as a function of time. NS309 was present in the extracellular saline as indicated by the bars. (D) Averaged concentration-response plot for NS309. For each cell (n=7) the responses to NS309 were fitted to a Hill equation (EC_{50} values in the individual cells ranged from 16 to 85 nM) and normalized to the fitted current maxima. These normalized data points were averaged and plotted in (D) where each point represent the mean ± SD from 4-5 cells and the solid line represents the result of a Hill fit to these averaged data yielding an EC_{50} value of 44 nM (n_H = 1.1).

Figure 3. In addition to $K_{Ca}3.1$ human microglia also have an outward-rectifying K^+ current mediated by $K_{Ca}1.1$. (A) Current traces obtained from a whole-cell experiment performed as described in Figure 2. The traces were obtained before (1) and after application of NS309 (500 nM; 2), and after subsequent co-application of NS309 (500 nM) and NS6180 (1 μM) (3). (B) Current traces from the same cell as shown in (A) and with identical trace 3. Trace 4 was obtained upon subsequent application of the specific $K_{Ca}1.1$ activator NS1619 (10 μM) and trace 5 following co-application of NS1619 (10 μM) and Paxilline (1 μM). The time course of the experiment shown in (A) and (B) was analyzed at -40 mV (■, $K_{Ca}3.1$) as well as at +30 mV (○, $K_{Ca}3.1$ and $K_{Ca}1.1$) and plotted as a function of time in (C). The bars above the analyses indicate the periods where the different compounds were present in the extracellular saline and the numbers shows where the traces in panel (A) and (B) were obtained. The experiment is representative of n=9.

Figure 4. $K_{Ca}3.1$ 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 Figure 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 non-responder. (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_i) 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_{ii}) Current amplitudes from the 42 microglia cells where both NS309 and NS6180 were applied. The current amplitudes pre- and post-application 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_i) 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_{ii}) 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 * $p<0.05$, *** $p<0.001$.

Figure 5. Quantification of the $K_{Ca}3.1$ current in microglia activated by IL-4 or LPS. (A_i) Current traces obtained from voltage-clamp experiment as described in Figure 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 μM) as indicated at the traces. (A_{ii}) Current amplitude at -40 mV of individual microglia cells pre- and post-application of NS309 and NS309 in combination with NS6180. (A_{iii}) Averages of the currents from panel A_{ii}. Panel B_i - B_{iii} Same as A_i-A_{iii} but after treatment with LPS (100 ng/ml). The bars in A_{iii} and B_{iii} represent the mean ± SEM; paired one-way ANOVA * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 6. IL-4 or LPS treatment do not alter $K_{Ca}3.1$ current densities. (A) The bars represent the percentage of cells in the microglia population that express $K_{Ca}3.1$ channels based on the 20 pA criterion described in Figure 4. Microglia cultures incubated with IL-4 (20 ng/ml) or LPS (ng/ml) for 2-4 days *in vitro* do not have an altered proportion of cells expressing functional $K_{Ca}3.1$ channels compared to untreated cultures (χ^2 test; $p > 0.05$). (B) The $\Delta K_{Ca}3.1$ current defined as the difference in current at -40 mV between full activation by 500 nM NS309 and full block by 1 μM NS6180 was calculated and plotted for unstimulated and IL-4 as well as LPS treated cells. IL-4 treated cells show a significant increase in the $\Delta K_{Ca}3.1$ current compared to unstimulated cells. (C) The membrane capacitance (pF) was measured and plotted for unstimulated and IL-4 as well as LPS treated cells. IL-4 treated cells have a larger membrane area (capacitance) compared to both unstimulated and LPS treated cells. (D) The $\Delta K_{Ca}3.1$ current from individual cells was divided by the membrane capacitance of the cell and plotted as a function of treatment. This measure of $K_{Ca}3.1$ current densities showed no significant differences between untreated and IL-4 or LPS-treated cells. Lines in B-D represent the mean ± SEM; unpaired one-way ANOVA ** $p<0.01$, *** $p<0.001$.

Supplementary Figure 1. (A) IV plot showing the effect of increasing concentrations of the $K_{Ca}3.1$ activator NS309 in a HEK-293 cell stably expressing human $K_{Ca}3.1$ channels (Jensen et al. 1998). The traces were obtained upon application of voltage ramps (from -120 mV to 30 mV in 150 ms) every 5 seconds from a holding potential of -90 mV and NS309 was present as indicated to the right. E_K indicates the calculated reversal potential for K^+ (-90 mV) with the salines used. (B) Time course of the experiment in (A) showing the current measured at -40 mV as a function of time. NS309 was present in the extracellular solution as indicated by the bars. (C) Concentration-response plot from where an EC_{50} was estimated to 20 nM (n_H = 1.1) (n = 6) as described in legend to Fig. 2D. The data points represent the mean ± SD. The experiments were performed using the same experimental conditions, voltage-ramp protocol and the same saline solutions (~1 μM free Ca^{2+}) as for the microglia experiments.

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Supplementary Figure 3. Electrophysiological whole-cell recording of a rat microglia cell using identical electrophysiology protocol and salines as for the human microglia experiments. Mixed glia cell cultures were generated from rat pup brains according to

standard procedures and were allowed to recover in culture for 14 days. The microglia were subsequently isolated from the confluent astrocyte layer by shaking followed by plating in petri dishes containing glass coverslips for electrophysiological recordings. The trace was obtained upon application of voltage ramps (from -120 mV to 30 mV in 150 ms) every 5 seconds from a holding potential of -90 mV. E_K indicates the calculated reversal potential for K^+ (-90 mV) with the salines used.

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Supplementary Figure 5. The $K_{Ca}3.1$ current amplitude in human microglia does not change over the time in culture. (A) Baseline, NS309-induced and $\Delta K_{Ca}3.1$ current amplitude in unstimulated cells from day 5 (earliest experimental day) to day 9 (last experimental day) post-isolation. (B-C) Baseline, NS309 and $\Delta K_{Ca}3.1$ current amplitude in IL-4 (20 ng/ml, B) and LPS (100 ng/ml, C) treated cells after 2, 3 or 4 days of stimulation. The incubation with IL-4 and LPS was started after 5 days in culture. No difference in the $K_{Ca}3.1$ current amplitude was observed over the length of the stimulation. Lines represent the mean ± SEM; unpaired one-way ANOVA $p>0.05$.

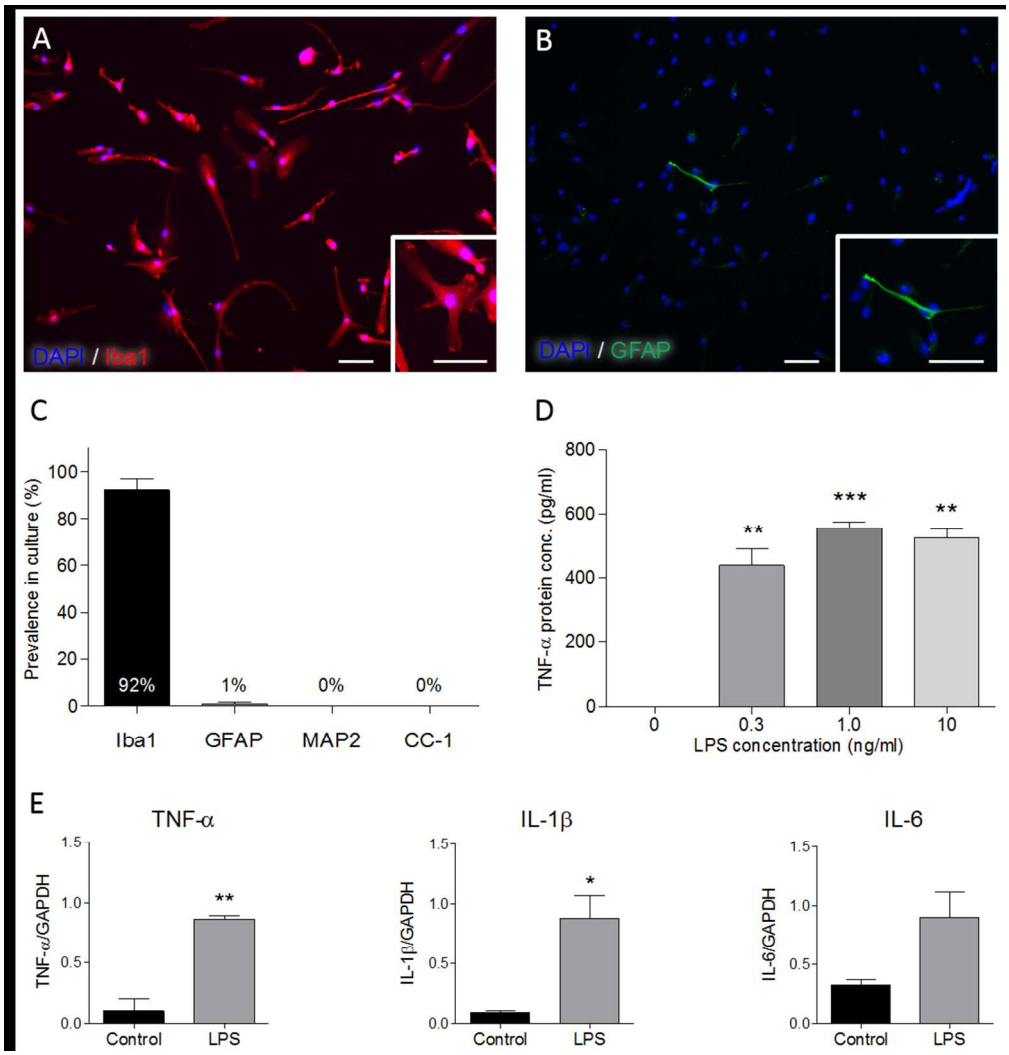

Figure 1. The human microglia culture is pure and responsive to stimulation with LPS. Photomicrographs of the cell culture with (A) human microglia stained for Iba1 in red and (B) astrocytes stained for GFAP in green 6 days post-isolation. The boxes in the bottom right corners show a microglia and an astrocyte, respectively, at higher magnification. Counterstaining of $DAPI^+$ nuclei can be seen in blue. A-B (including insert): Scale bars: 50 µm. (C) Quantitative purity analysis of the human microglia cultures (n = 3) at 6 days post-isolation. In addition to Iba1 and GFAP the cultures were stained for neurons (MAP2) and oligodendrocytes (CC-1) and stained cells were expressed as a percentage of the total amount of $DAPI^+$ nuclei. (D) Quantification of TNF-α release following incubation with LPS (0.3, 1, and 10 ng/ml) for four hours shows a significant response at all LPS concentrations tested. C-D: Bars represent mean ± SEM; D: unpaired one-way ANOVA ** $p<0.01$, *** $p<0.001$. (E) Gene expression analysis of pro-inflammatory cytokines following stimulation with LPS for four hours. Bars represent mean ± SEM; unpaired t-test * $p<0.05$, ** $p<0.01$.

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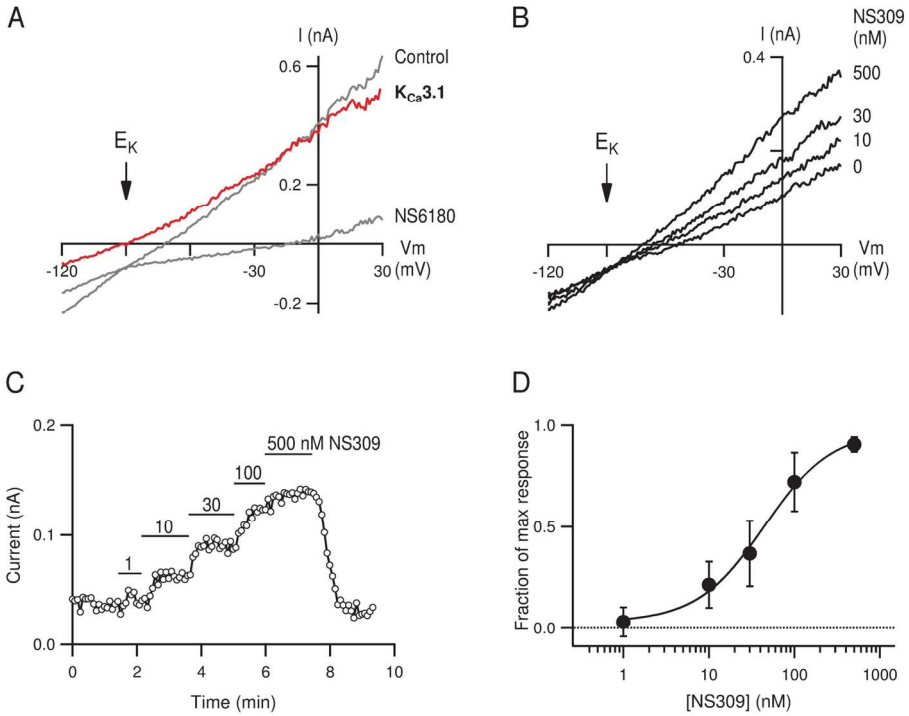

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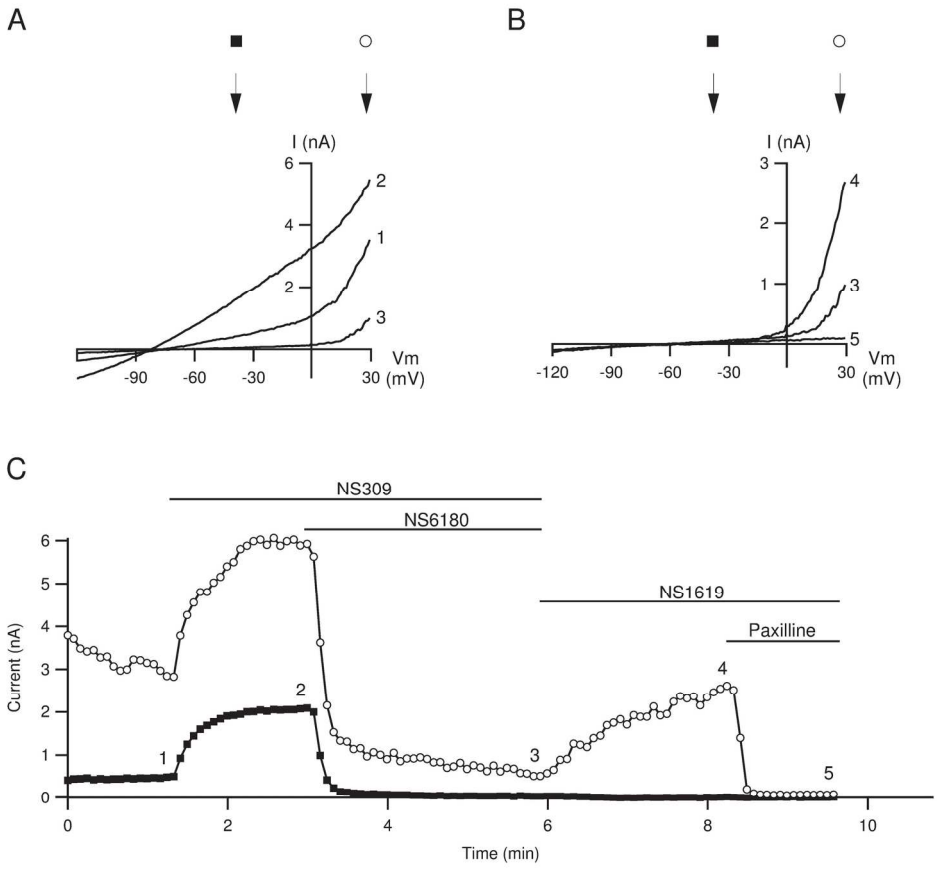

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Fig. 3
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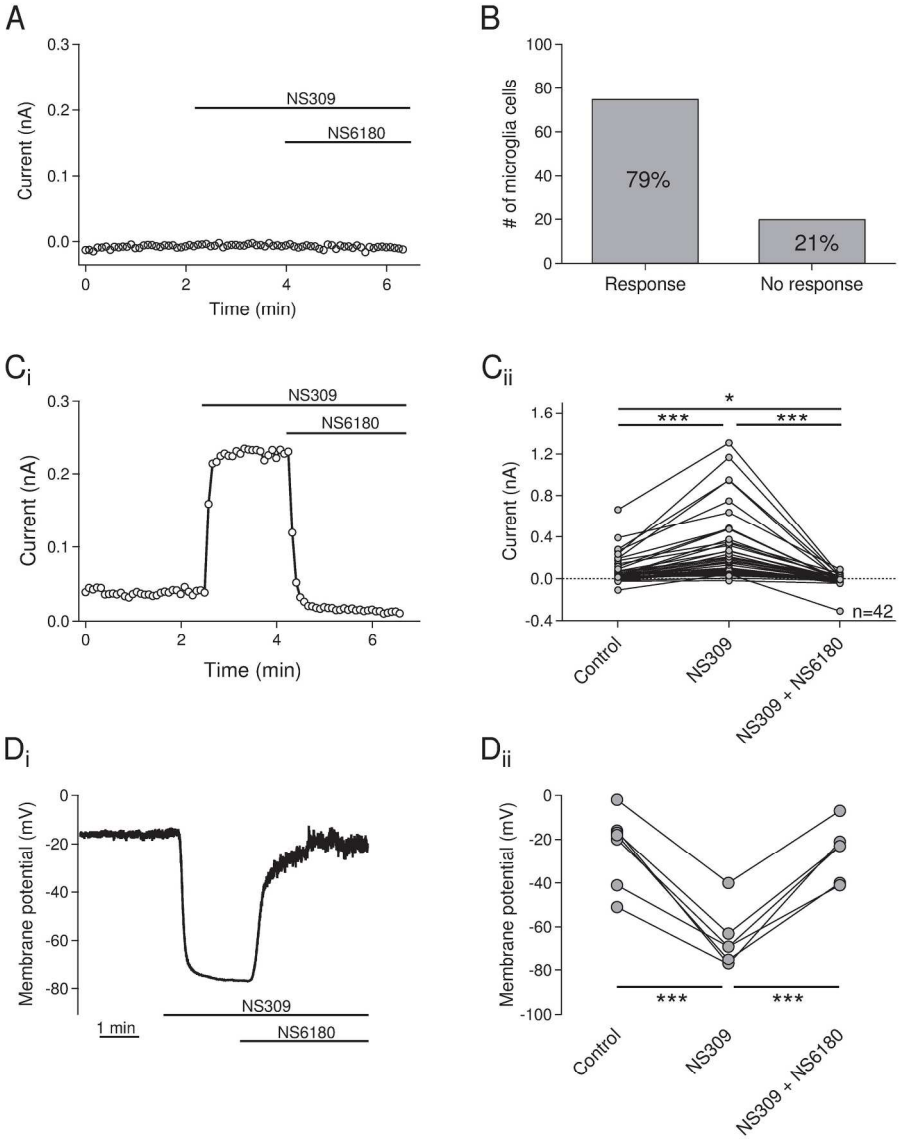

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Fig. 4

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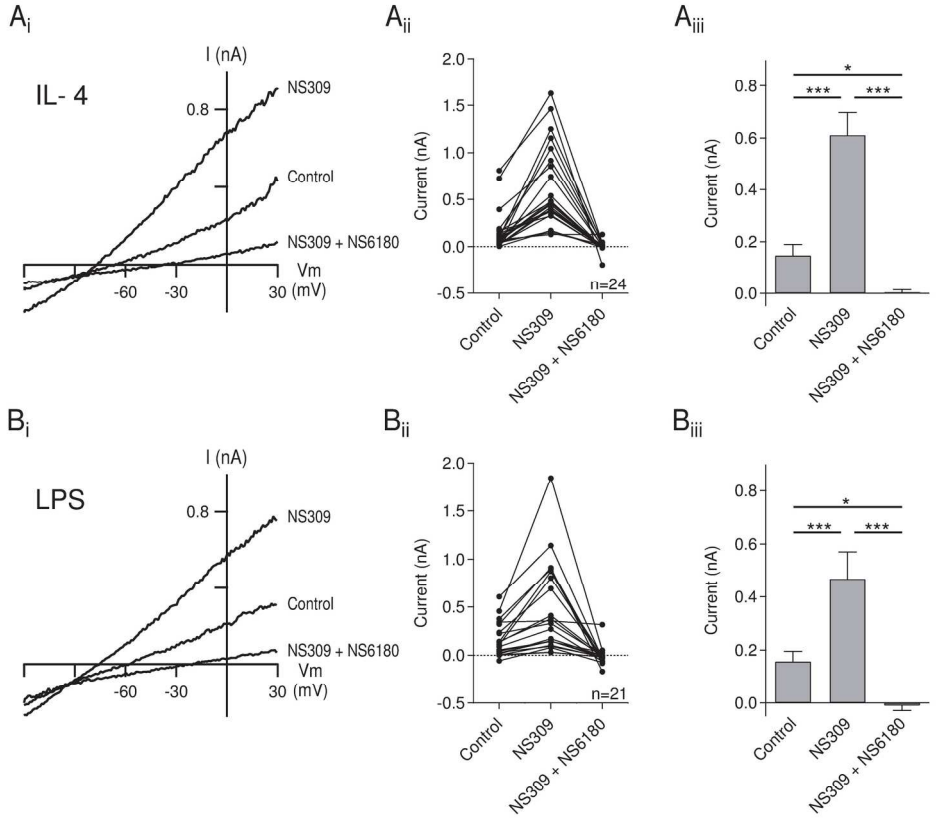

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Fig. 5
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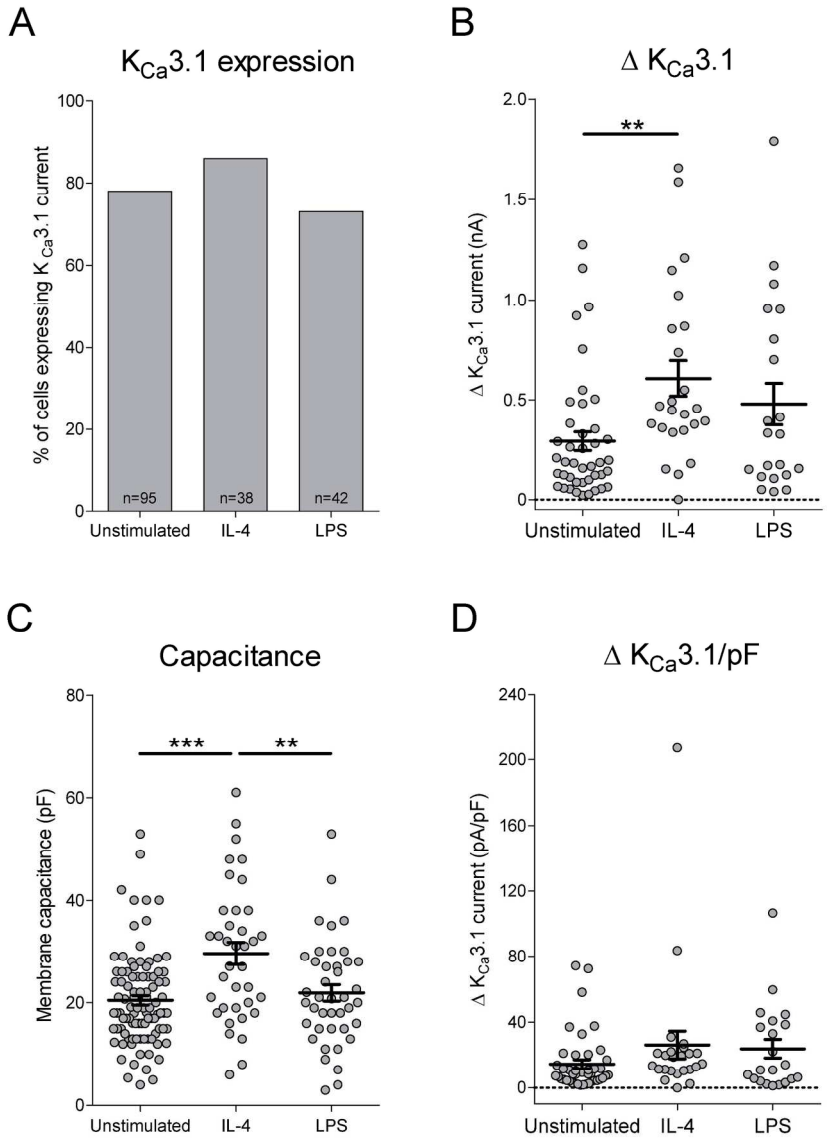

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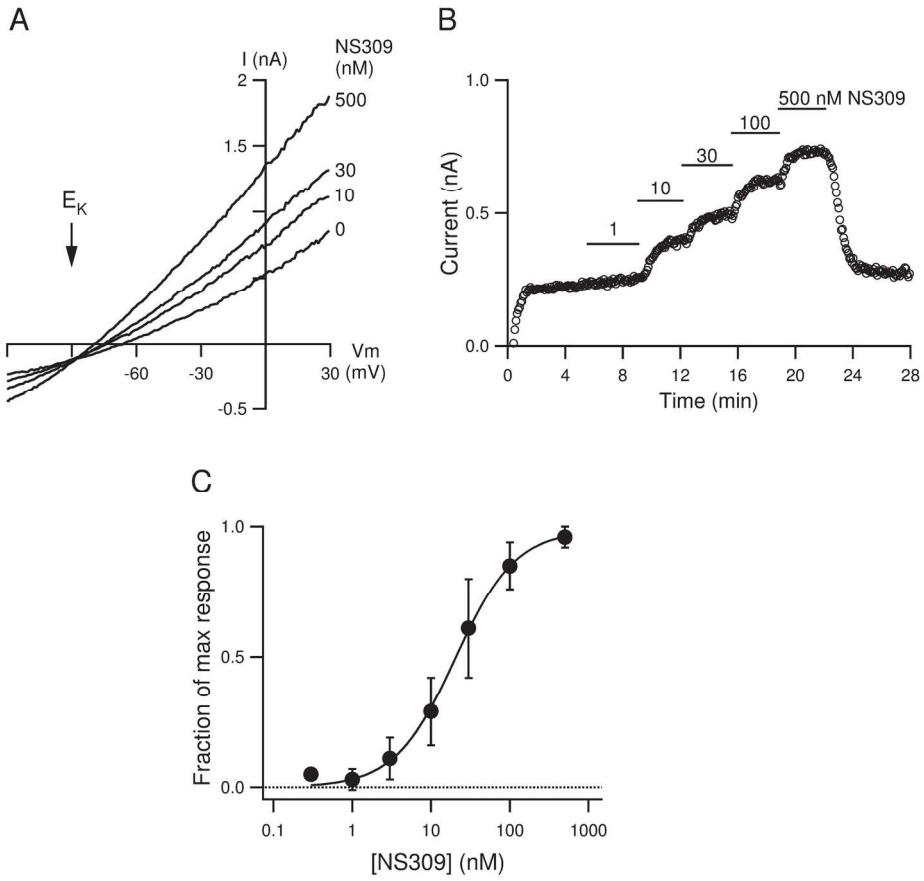

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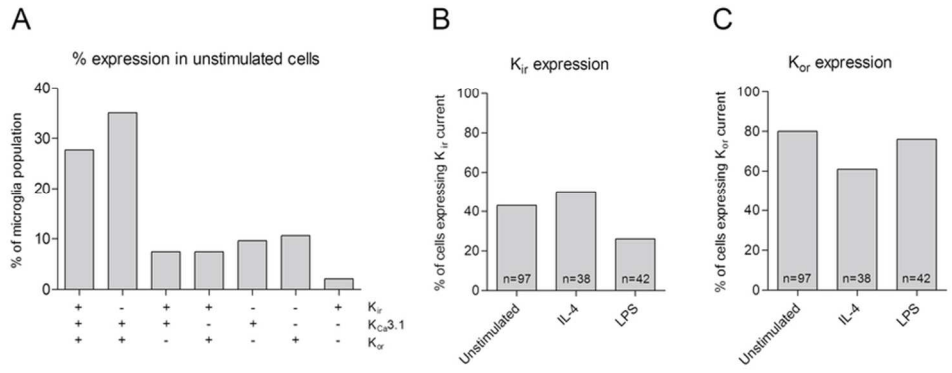

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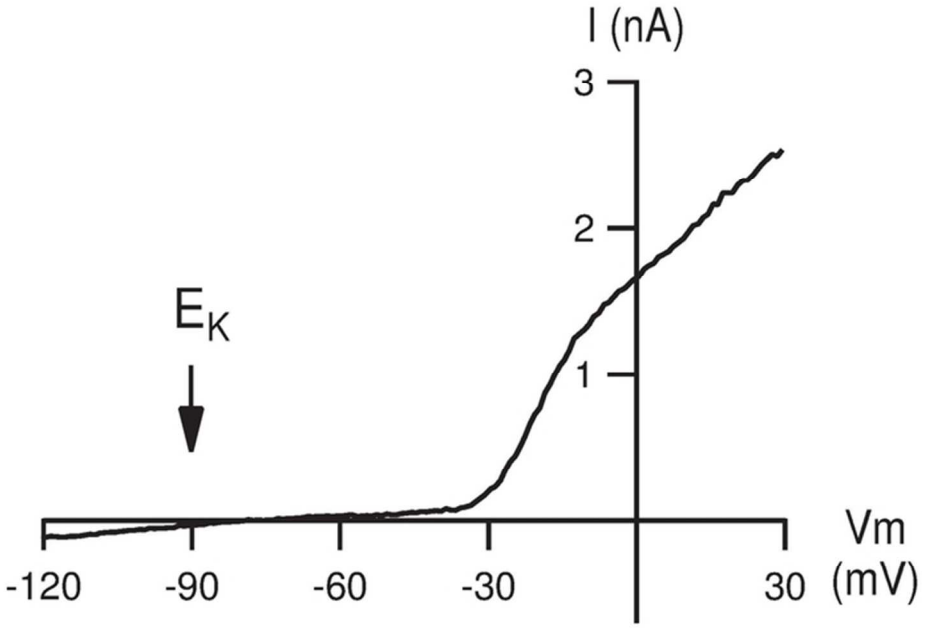

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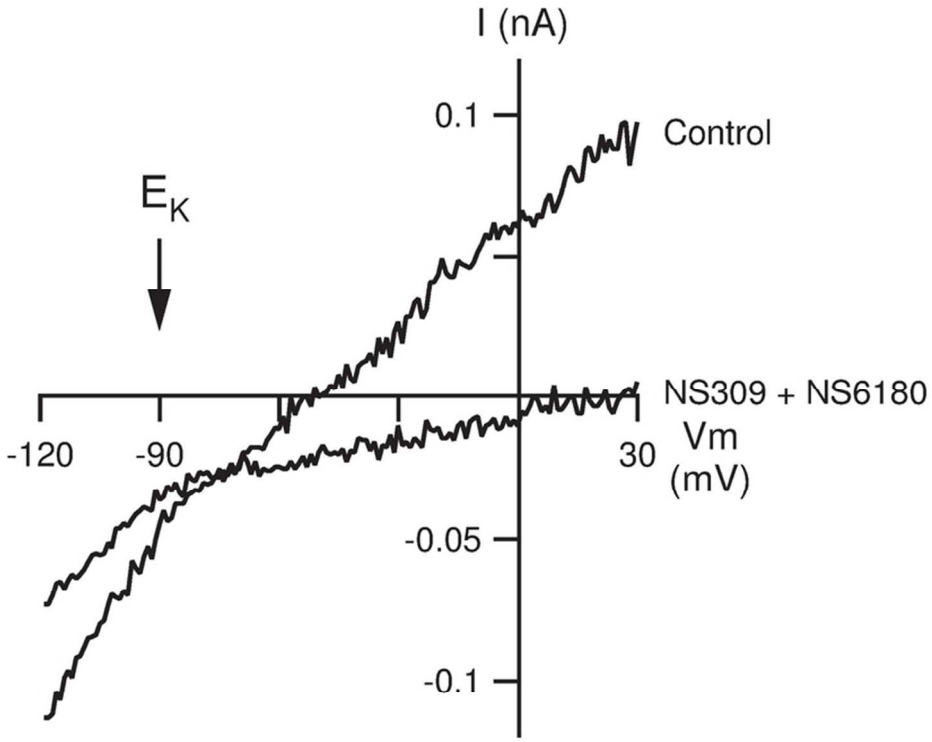

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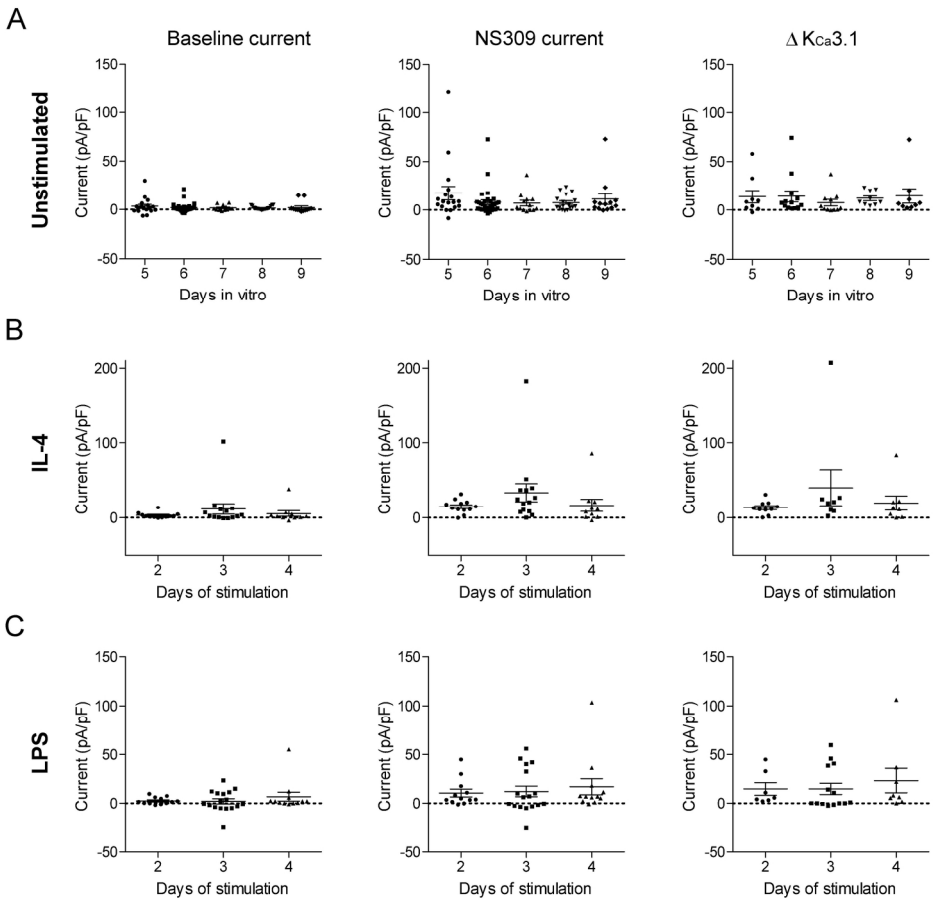

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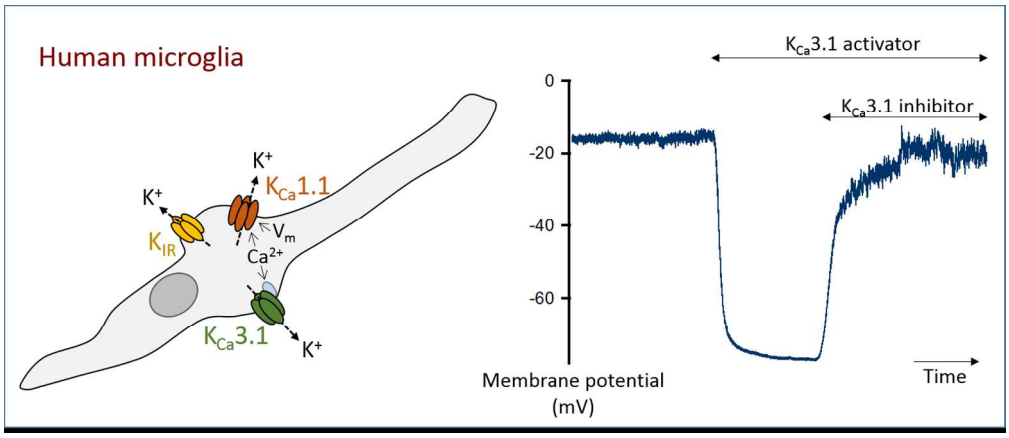

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