The Role of Microglia Membrane Potential in Chemotaxis

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Short report

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Abstract

Microglia react to danger signals by rapid and targeted extension of cellular processes towards the source of the signal. This positive chemotactic response is accompanied by a hyperpolarization of the microglia membrane. Here we show that optogenetic depolarization of microglia has little effect on baseline motility, but significantly slows down the chemotactic response. Reducing the extracellular Ca\(^{2+}\) concentration mimics the effect of optogenetic depolarization. As the membrane potential sets the driving force for Ca\(^{2+}\) entry, hyperpolarization is an integral part of rapid stimulus-response coupling in microglia. Compared to typical excitable cells such as neurons, the sign of the activating response is inverted in microglia, leading to inhibition by depolarizing channelrhodopsins.

Introduction

Microglia – the only resident immune cells in the brain – are the first responders to external and internal threats in the central nervous system. They account for 5–10% of total brain cells and constantly surveil the brain parenchyma. Under physiological conditions microglia are highly ramified, have a small cell body and span approximately 50 µm in diameter. With their thin processes, microglia detect changes in the extracellular space, such as dying neurons, danger signals (e.g. adenine nucleotides), invading organisms, or alterations in neuronal activity (Nimmerjahn, 2005).

Under physiological conditions, the tandem-pore domain halothane-inhibited K\(^+\) channel 1 (THIK-1) and possibly transient receptor potential (TRP) and chloride-conducting channels contribute to the resting membrane potential in microglia, which sits around −40 mV (Madry et al., 2018; Newell and Schlichter, 2005; Schilling and Eder, 2015). Deviations from this potential in both directions have been linked to microglia activation. Under pathological conditions, microglia exhibit two modes on how they change their membrane potential, (i) sustained hyperpolarization in chronically activated microglia and (ii) rapid, transient hyperpolarization induced by extracellular ATP in the vicinity of tissue damage. Sustained pathological hyperpolarization in activated and in aged microglia is correlated with an increased expression of Kir2.1 and Kv1.3 channels (Rangaraju et al., 2015; Schilling and Eder, 2015). This additional hyperpolarization is thought to be a precondition for the initiation of microglia functions, such as phagocytosis, chemotaxis and cytokine release, which are potentially promoted by the larger driving force for Ca\(^{2+}\) influx (Ince et al., 1988; Kouri et al., 1980; Paolicelli et al., 2011). While sustained hyperpolarization is accompanied by process retraction leading to an ameboid microglia morphology, transient hyperpolarization due to local tissue damage induces rapid process extension towards the source of extracellular ATP and Ca\(^{2+}\) transients in the processes directed towards the damage site (Eder, 2005; Lee et al., 2008; Nimmerjahn, 2005; Pozner et al., 2015). Detection of extracellular ATP and chemotaxis are largely mediated by the purinergic Gi-protein coupled receptor P2Y12 and ion channels receptors P2 \(\times\) 4 and P2 \(\times\) 7 (Davalos et al., 2005; Haynes et al., 2006; Ohsawa et al., 2007; Swiatkowski et al., 2016).
Although originally classified as non-excitable cells, microglia exhibit strong electrophysiological stimulus-response features. While correlations between microglia activation and membrane potential are numerous, they have only been described phenomenologically, and it is not well understood whether changes in membrane potential are cause or consequence of microglia activation. Selective investigation of microglia in their native environment is hampered by the fact that identical molecular pathways exist in other types of glial cells and in neurons (Kouri et al., 1980; Rangaraju et al., 2015; Schilling and Eder, 2015; Wendt et al., 2017). Therefore, pharmacological manipulations are extremely difficult to interpret unless microglia are cultured in isolation. In isolation, however, microglia assume an activated phenotype with retracted processes, making it impossible to investigate the activation process from the resting state. Genetic manipulations have the required cell-type specificity, but knockdown or knockout approaches change the system in a chronic fashion and may trigger the activation of compensatory mechanisms.

Here we adopt an optogenetic approach to investigate context-dependent behavior of microglia. Using a specific Cre-driver line, we express the light-gated cation channel ChETA specifically in microglia. Two-photon time-lapse imaging allowed us to quantify chemotactic responses while manipulating the membrane potential with blue light pulses. We demonstrate that ATP-mediated hyperpolarization is not an epiphenomenon, but causally related to the chemotactic response.

Results And Discussion

ChETA effectively depolarizes microglia in response to blue light illumination

We achieved visualization of microglia and expression of the light-gated ion channel ChETA by crossing the tamoxifen-inducible microglia-specific Cre-driver line Cx3cr1CreERT2 with the conditional R26-CAG-LSL-ChETA mouse line. To visualize microglia in control experiments, we used the conditional reporter line R26-CAG-LSL-tdTomato (Fig. 1A, B). From those mice and littermate controls lacking ChETA, we prepared organotypic hippocampal slice cultures, a three-dimensional system in which the cellular architecture is well preserved. Application of (Z)-4-hydroxytamoxifen in the culture medium (24 h) activated cre/loxP-system, leading to selective construct expression in the entire microglia population (Fig. 1C). Although microglia become activated during the cutting process and assume a morphology similar to pathologically-activated microglia shortly after preparation, they regained their highly ramified resting morphology over 9–12 days in vitro (DIV) (Fig. 1D-G). Sholl analysis showed that at 29 DIV, their morphology was indistinguishable from microglia we imaged through a cranial window (green markers, Fig. 1H). The fraction of microglia in stratum radiatum of organotypic cultures fluctuated between 5–10% of all cells, which is not different from the density we measured in vivo (Fig. 1I). Furthermore, microglia membrane properties recorded in mature organotypic slice cultures (membrane resistance, capacitance, resting potential; Fig. 2A, B) were very similar to those reported in acute brain slices (Boucsein et al., 2000; Pagani et al., 2015).
The channelrhodopsin-2 variant ChETA (ChR2/E123T/H134R) was originally developed for fast response kinetics at the expense of smaller photocurrents (Gunaydin et al., 2010). Therefore, relatively high light intensities and expression levels are required to spike neurons (Berndt et al., 2011). Microglia, however, exhibit an almost 10-times higher input resistance compared to neurons, so small photocurrents generate large changes in microglia membrane potential (Schilling and Eder, 2015). Using increasing blue light intensities, we achieved a maximal depolarization of $\Delta V_m = 36 \text{ mV}$ from microglia resting potential (Fig. 2B, D). During high intensity light pulses, depolarization reached a plateau at 0 mV, the reversal potential of ChR2 (Berndt et al., 2011). Even prolonged repetitive depolarization using light flashes at 1 Hz frequency did not lead to desensitization, but produced photocurrents of constant amplitude (Fig. 2E, F). In summary, our genetic approach guarantees specific expression of the channelrhodopsin variant ChETA exclusively in microglia and allows rapid light-controlled depolarization in a dose-dependent and cell-type specific fashion.

**Optogenetic depolarization of microglial membrane potential mildly affects microglia morphology and baseline surveillance**

Resting microglia constantly monitor their surrounding parenchyma and the surveyed area greatly depends on their morphology. In the resting state, microglia scan larger areas due to the higher number and greater length of processes compared to pathologically activated microglia (Raivich et al., 1999; Streit et al., 1999). The morphological change during pathological activation is often accompanied by strong changes in microglia membrane potential (Eder et al., 1995; Izquierdo et al., 2019; Nguyen et al., 2017a; Visentin et al., 2001). Thus, a direct link between morphology and membrane potential is plausible. Consistent with this, Madry et al. found that pharmacological block of the constitutively open outward-rectifying THIK-1 potassium channel, or genetic knock-out thereof, leads to a more depolarized membrane potential in microglia and a more activated morphological phenotype with reduced surveillance (Madry et al., 2018). To investigate whether microglia depolarization by itself causes motility changes, we optically modulated the membrane potential (480 nm light flashes for 20 min) while tracking microglia motility over 50 minutes with two-photon microscopy. Optogenetic depolarization did not induce any morphological changes and had only minor, transient effects on baseline motility compared to microglia in control slices that did not express ChETA (Supplemental Fig. 1). Taken together, our data show that strong short-term depolarization of microglia membrane potential does not induce any lasting changes in morphology or surveillance behavior.

**Optogenetic microglia depolarization decelerates chemotactic response kinetics**

Microglia maintain a negative resting potential around $-40 \text{ mV}$ and rapidly hyperpolarize further when sensing extracellular ATP or other nucleotides. This instantaneous hyperpolarization is mediated via the activation of P2Y12 receptors, which results in opening of THIK-1 channels and occurs simultaneously with the onset of microglia process movement towards the source of ATP (Madry et al., 2018;
Swiatkowski et al., 2016). Hyperpolarization could be part of the trigger mechanism for the rapid chemotactic response, or just an epiphenomenon. To find out, we altered microglia membrane potential during the chemotactic response using optogenetic depolarization.

After recording microglia baseline motility for 15 minutes with two-photon microscopy, we induced spatially precise tissue damage in a region surrounded by three or four microglia by parking the IR-laser in the center of the field of view for 1.5 seconds. The distance between microglia process tips and laser damage was 35–50 µm (Fig. 3A, B). Microglia rapidly responded to the laser damage by extending their processes and within a few minutes, the site of damage was completely surrounded by pseudopodia.

To investigate how effectively optogenetic depolarization reduced ATP-mediated hyperpolarizing currents, we performed whole-cell patch clamp recordings from microglia during laser damage (Fig. 3C, D). Using 480 nm illumination before and during tissue damage, we were able to substantially reduce the amount of hyperpolarization in microglia (Fig. 3D). In addition to light stimulation during laser damage, we illuminated the field of view with 1 Hz flashes at 480 nm between the acquired stacks to depolarize microglia throughout the chemotactic response (Fig. 3F, insert).

After determining the light intensities and frequencies required to effectively depolarize microglia, the actual chemotactic experiments were then performed without patch-clamp electrodes. We included two types of control experiments: Microglia expressing only the fluorescent protein (no ChETA) and microglia that expressed ChETA, but were not illuminated. These control groups showed similar response kinetics in response to laser damage, demonstrating that neither ChETA expression nor light application alone had an effect on microglia responsiveness (T½ ctrl = 7.7 ± 0.7 min, free area ctrl = 36% ± 8%; T½ ChETA_nolight = 7.3 ± 0.7 min, free area ChETA_nolight = 37% ± 6%; Fig. 3G, H). In contrast, microglia expressing ChETA exhibited significantly slower response kinetics when illuminated compared to both control microglia with light and ChETA expressing microglia without light stimulation (T½ ChETA+light = 10.7 ± 0.6 min, free area ChETA+light = 61% ± 3%, Fig. 3G, H). Under all conditions, response kinetics did not correlate with either microglia distance from laser damage or temperature, which was tightly controlled during the experiments (Supplemental Fig. 2).

In microglia, an increased frequency of Ca²⁺ transients is correlated with pathophysiological activation and other response properties such as chemotaxis. Processes moving towards a source of extracellular ATP exhibit significantly more Ca²⁺ transients compared to resting microglia or processes that are located away from the tissue damage (Pozner et al., 2015). We hypothesize that microglia response kinetics are partially guided by Ca²⁺ influx into the processes located towards the ATP source. Thus, it is conceivable that optogenetic depolarization slowed down chemotaxis by reducing the driving force for Ca²⁺ influx. To investigate whether response kinetics are dependent on Ca²⁺ entry, we performed a series of chemotactic experiments in nominally Ca²⁺-free extracellular solution. In Ca²⁺-free extracellular solution, process extension towards the damaged area was significantly slowed down (ΔT½ Ca-free = 3.1 min; Supplemental Fig. 3), with kinetics similar to the responses we recorded during optogenetic depolarization (ΔT½ ChETA + light = 3 min).
Together, these data demonstrate that microglia hyperpolarization is an important component of the rapid chemotactic response and suggest that the coupling of membrane potential to process elongation might be mediated by enhanced influx of Ca\textsuperscript{2+} ions. However, the onset of chemotactic responses was not affected, neither in optogenetic depolarization nor in Ca\textsuperscript{2+}-free extracellular solution, indicating that this important cellular function is controlled by multiple pathways that act in parallel, partially redundant fashion. Some of them, e.g. G-protein coupled P2Y\textsubscript{12} receptors are not at all expected to depend on membrane potential or calcium influx. Thus, controlling the membrane potential of microglia can significantly slow down, but not completely switch off the injury response.

**Microglia hyperpolarization is not an epiphenomenon**

Microglia are the only resident immune cells in the brain and although they maintain a negative resting potential and rapidly respond to an external stimulus (ATP) with strong transient hyperpolarization, they are considered non-excitable cells. The classical definition refers to the ability of neurons being able to generate action potentials in response to electrical stimulation. However, many neuronal subtypes never fire action potentials, but respond with graded de- or hyperpolarizing changes in membrane potential (e.g. retinal bipolar cells, photoreceptor cells, most insect neurons), demonstrating that electrical stimulus-response coupling does not require generation of spikes (Barlow, 1953; Baylor and Fuortes, 1970; Werblin and Dowling, 1969). Microglia exhibit reliable changes in membrane potential upon external stimulation, which in turn affect crucial microglia functions such as chemotaxis or phagocytosis (Wu et al., 2007; Zhao et al., 2018). Here we provide evidence that membrane hyperpolarization is not just a byproduct, but part of the signaling pathway for rapid stimulus-response coupling. As the definition of electrical excitability should be consistent across cell types, we suggest classifying microglia as excitable cells.

Previous studies investigating the depolarizing effect of pharmacological block of K\textsuperscript{+} channels on chemotactic response kinetics yielded conflicting results: While K\textsuperscript{+} channel block with quinine reportedly abolishes chemotaxis, K\textsuperscript{+} channel block with tetrapentylammonium (TPA) did not seem to affect response kinetics (Madry et al., 2018; Wu et al., 2007). It is difficult to determine whether the stimulation strength was comparable in both studies, as chemotaxis was induced by pressure application of ATP at different concentrations and evaluated with different metrics. In both studies, chemotaxis assays were performed in acute brain slices which obviously had sustained severe tissue damage during the slicing process. The state of microglia activation at baseline might therefore depend on subtle details of the slice-making and incubation procedure, leading to poor reproducibility across labs. Microglia in mature organotypic cultures may provide a more stable baseline for chemotaxis and other assays. In addition, our optogenetic approach reduces the risk of unspecific side effects that is the bane of pharmacology. Madry et al. also generated THIK-1 knock-out mice in which microglia are chronically depolarized and show no ATP-induced currents. The chemotactic response was not tested in these animals, but THIK-1-KO microglia had fewer and less motile processes at baseline, suggesting microglia activation. As we show here, acute optogenetic depolarization does not change the morphology and hardly affects surveillance
activity of microglia. Thus, it is likely that chronic depolarization triggers changes in expression profile, e.g. downregulation of P2Y12 and upregulation of A2A receptor expression (Orr et al., 2009). While P2Y12 receptors are responsible for the chemotactic response, A2A receptors mediate process retraction from ATP in activated microglia (Madry et al., 2018; Orr et al., 2009).

Hyperpolarization increases the driving force for calcium ions

Ca\(^{2+}\) signaling is known to play a key role in microglia activation. The main entry route is not via voltage-gated Ca\(^{2+}\) channels, which are only expressed under pathological conditions (Eder, 1998; Espinosa-Parrilla et al., 2015; Saegusa and Tanabe, 2014), but through ligand-gated receptors with high Ca\(^{2+}\) permeability. Thus, in contrast to neurons, depolarization reduces Ca\(^{2+}\) influx in microglia. A direct link between hyperpolarization and Ca\(^{2+}\) signaling has been demonstrated in many types of immune cells and is strengthened by the finding that under resting conditions, microglia exhibit almost no Ca\(^{2+}\) transients (4% of resting microglia show a spontaneous Ca\(^{2+}\) transient during a 20 min recording session (Pozner et al., 2015)). Pathological hyperpolarization strongly affects microglia function and is accompanied by an increased number and intensity of Ca\(^{2+}\) transients (Brawek et al., 2017; Eichhoff et al., 2011). In particular, potassium channel expression plays an important role in the regulation of the hyperpolarized membrane potential (for review see (Madry and Attwell, 2015; Nguyen et al., 2017b; Thei et al., 2018)). In addition, it was shown that chemotactic responses are accompanied by local Ca\(^{2+}\) transients in processes approaching the source of ATP. Thus, optogenetic depolarization might effectively decrease Ca\(^{2+}\) currents through ligand-gated receptors, thereby decelerating chemotactic responses. Corroborating evidence comes from our chemotaxis experiments in Ca\(^{2+}\)-free saline which phenocopied the effect of optogenetic depolarization. Actin polymerization, one of the major driving forces for pseudopod extension during chemotaxis, is highly regulated by Ca\(^{2+}\)-activated enzymes. In our optogenetic depolarization experiments, we were only able to slow down process extension and not completely prevent all movements, which might be due to our pulsed light protocol in combination with a fast closing opsin. However, as the reversal potential for Ca\(^{2+}\) is far above the reversal potential of ChETA (-5 mV), even constant illumination would not prevent Ca\(^{2+}\) influx completely. The remaining slowed down chemotactic response may therefore be driven by residual calcium influx or by parallel, Ca\(^{2+}\)-independent signaling pathways.

Conclusions

We have shown that ATP-mediated hyperpolarization is not just an epiphenomenon of microglia activation, but rather that it is essential for rapid microglia process extension towards the site of injury. Microglia could therefore be considered excitable cells, but they react in an inverted fashion when compared to neurons: Depolarization dampens their reactivity while hyperpolarization is part of their active response to extracellular danger signals. Beyond chemotaxis, optogenetic control of microglia
membrane potential opens the possibility to investigate other physiological processes that may depend on membrane potential, such as cytokine release via exosomes, without affecting other cell types.

Materials And Methods

Mouse slice culture

Mice carrying a tamoxifen-inducible Cre-recombinase in microglia and the floxed fluorescent marker tdTomato (B6.129; B6.129 - Cx3cr1tm2.1(cre/ERT2)Jung; JAX 020940; JAX 007909) were crossed with mice heterozygously carrying the Gt(ROSA26)Sortm1(CAG−COP4*E123T*H134R,−tdTomato)Gfng (JAX 017455) allele to generate mice expressing ChETA and tdTomato in microglia and littermate controls without ChETA expression in microglia. Hippocampal slice cultures from sex-matched ChETA-expressing mice and littermate controls of were prepared at postnatal day 4–7 as described (Gee et al., 2017). Briefly, mice were anesthetized with 80% CO₂ and decapitated. Hippocampi were dissected in cold slice culture dissection medium containing (in mM): 248 sucrose, 26 NaHCO₃, 10 glucose, 4 KCl, 5 MgCl₂, 1 CaCl₂, 2 kynurenic acid and 0.001% phenol red. pH was 7.4, osmolarity 310–320 mOsm kg⁻¹, and solution was saturated with 95% O₂, 5% CO₂. Tissue was cut into 410 µM thick sections on a tissue chopper and cultured at the medium/air interface on membranes (Millipore PICMORG50) at 37°C in 5% CO₂. For the first 24 h of incubation, 1 µM (Z)-4-hydroxytamoxifen (Sigma H7904) was added to the slice culture medium to induce Cre-activation. No antibiotics were added, and slice culture medium was partially exchanged (60–70%) twice per week and contained (for 500 ml): 394 ml Minimal Essential Medium (Sigma M7278), 100 ml heat inactivated donor horse serum (Sigma H1138), 1 mM L-glutamine (Gibco 25030-024), 0.01 mg ml⁻¹ insulin (Sigma I6634), 1.45 ml 5M NaCl (Sigma S5150), 2 mM MgSO₄ (Fluka 63126), 1.44 mM CaCl₂ (Fluka 21114), 0.00125% ascorbic acid (Fluka 11140), 13 mM D-glucose (Fluka 49152).

Slice cultures were used for experiments between 12 and 28 days in vitro. Mice were housed and bred at the University Medical Center Hamburg-Eppendorf. All procedures were performed in compliance with German law and the guidelines of Directive 2010/63/EU. The study was approved by the local authorities (Amt für Verbraucherschutz, Lebensmittelsicherheit und Veterinärwesen, Hamburg; permission # 42/17).

Mouse genotyping

Tail biopsies were taken from mice at postnatal day 3–4 and lysed using 75 µl alkali buffer containing (in mM): 25 NaOH, 0.2 Na₂-EDTA*2H₂O for 60 min at 95 °C and then neutralized using 75 µl 40 mM Tris-HCl. PCR-based genotyping of the Rosa26 locus was performed using the primer combinations 5’-AAGGGAGCTGCAGTGGAATCTCTGGGAAGTC-3’ and 5’-CCGAAATCTGCTGGAAAGTC-3’ for WT and 5’-GGCATTAAGCGAGCTATCC-3’ and 5’-CTGGTCTGTTACGCGATGG-3’ for knock-in alleles.

Perfusion of animals and preparation of slices
Mice were transcardially perfused with cold phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in PBS under ketamine (130 mg/kg, WDT) and xylazine (10 mg/kg, WDT) anesthesia. Brains were extracted and post-fixed for 24 h in 4% PFA in 4% paraformaldehyde for 24 h at 4°C. Before slicing, the tissue was changed to 1x PBS for 20 minutes, then slices of 40 µm were sectioned using Leica vibratome, collected and stored in PBS.

**Immunohistochemistry**

Organotypic slice cultures were fixed in 4% paraformaldehyde in PBS for 45 min at room temperature and stored in PBS at 4 ºC until further use. Fixed cultures or brain slices from perfused animals were blocked for 2 hours at room temperature using goat-serum based blocking solution containing (in %): 10 Goat serum (Capricorn), 0.2 Bovine Serum Albumin (Sigma-Aldrich A2153-10G), 0.5 TritonX-100 (Sigma Aldrich T-9284). Primary antibodies were directed against IBA1 (rabbit-anti-iba1 WAKO chemicals 019-19741 1:1000) and tdTomato (mouse-anti-dsRed Takara 632392 1:1000). Secondary antibodies were goat anti-rabbit or anti-mouse conjugated with Alexa dyes 488 and 568 (1:1000), respectively (Life Technologies A11008 and A11004). Antibodies were prepared in carrier solution containing (in %): 1 Goat serum (Capricorn), 0.2 Bovine Serum Albumin (Sigma-Aldrich A2153-10G), 0.3 TritonX-100 (Sigma Aldrich T-9284). Primary antibodies were applied overnight at 4 ºC; before application of secondary antibodies, slices were washed three times 10 min with PBS and then incubated with secondary antibodies for 2 hours at room temperature. Slices were washed for three times with PBS, incubated with DAPI (Molecular Probes, Invitrogen) for 5 min and then mounted (Thermo Scientific 9990402). Slices were imaged using an Olympus FV-1000 confocal microscope and microglia morphology and cell count was analyzed using the Imaris Software (Bitplane).

**Two photon imaging and microglia patch clamp**

Organotypic hippocampal slice cultures were placed in the recording chamber of a custom-built two-photon laser scanning microscope based on an Olympus BX51WI microscope with a pE-4000 LED light source for epifluorescence and activation of ChETA. The LED light source was controlled using a TTL trigger input. Power density of the 470 nm light was measured using a digital optical power and energy meter console with an attached photodiode power sensor (Thorlabs).

The microscope was equipped with a WPlan-Apochromat 40 × 1.0 NA (Zeiss) or HC Fluotar L 25 × 0.95 NA (Leica) objective and controlled by ScanImage 2017b (Vidrio). A pulsed Ti:Sapphire laser (Chameleon Ultra, Coherent) controlled by an electro-optic modulator (350 – 80, Conoptics) was used to image microglia with 1040 nm. Z-stacks were acquired with step intervals between 2 and 3 µm.

The slices were continuously perfused with either an artificial cerebrospinal fluid (ACSF) saturated with 95% O₂ and 5% CO₂ consisting of (in mM): 119 NaCl, 26.2 NaHCO₃, 11 D-glucose, 1 NaH₂PO₄, 2.5 KCl, 4 CaCl₂, 4 MgCl₂. (pH 7.4, 308 mOsm) at 27–29° C or with a HEPES-buffered solution (in mM): 135 NaCl, 2.5 KCl, 10 Na-HEPES, 12.5 D-glucose, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂ (pH 7.4). Nominally Ca²⁺-free
extracellular solution consisted of (in mM): 135 NaCl, 2.5 KCl, 10 Na-HEPES, 12.5 D-glucose, 1.25 NaH$_2$PO$_4$, 3 MgCl$_2$ (pH 7.4).

Whole-cell recordings from microglia in stratum radiatum were made with patch pipettes (6–8 MΩ) filled with (in mM): 135 K-gluconate, 4 MgCl$_2$, 4 Na$_2$-ATP, 0.4 Na-GTP, 10 Na$_2$-phosphocreatine, 3 sodium-L-ascorbate, and 10 HEPES (pH 7.2, 295 mOsm kg$^{-1}$).

Fluorescence guided microglia whole-cell patch clamp recordings were done using a Multiclamp 700B amplifier (Molecular Devices) under the control of Ephus software written in Matlab (The MathWorks) (Suter et al., 2010). Series resistance was below 20 MΩ. Z-stacks of patch-clamped microglia were acquired to document their morphology.

The tissue damage was performed in a standardized fashion in a region between three to four microglia at a distance of approximately 35–50 µm by parking the IR-laser in the center of the imaged region.

**Image analysis of microglia baseline surveillance and chemotaxis**

**Microglial baseline surveillance.** We combined several analysis steps into a semi-automated workflow. Image data were background subtracted (rolling ball radius = 30 pixels) and median filtered (radius = 1 pixel) with ImageJ. In order to correct for small positional shifts that occurred during acquisition, we registered all images from a time-lapse recording to the first image of the series (Efficient subpixel image registration by cross-correlation, version 1.1, MATLAB Central File Exchange). Images were then binarized using Otsu's method (threshold set to 50% calculated value). Based on a maximum projection of both images and binary images, a mask was drawn to define the region considered for motility analysis. Within the masked region we counted the number of pixels that remained stable, that were lost, and that were gained throughout the time series. From the covered areas and perimeters, we calculated ramification and surveillance indices.

**Microglial response to laser-induced tissue damage.** We created a semi-automated workflow (Matlab R2016b, MathWorks®, code available on GitHub) to analyze microglial responses to laser-induced tissue damage. Individual images from a time-lapse series (2D, Number, time step) were median filtered (3 × 3 kernel size), converted to binary form (Otsu's method), polished using dilation, erosion steps and filling of holes. A spot (eccentricity < 0.5, size > 50 pixel) near the image center was defined as the site at which tissue damage was initiated. The image from the tissue damage time point with detected spot coordinates was presented to the user for visual inspection; the location of the spot was corrected if necessary. For further processing, images prior to the damage time point were averaged (median pixel values) to obtain a background image from which an intensity threshold was calculated (locally adaptive threshold, specificity = 0.5). All images were then background subtracted and binarized using the intensity threshold. The region around the damage center (spot dilated with a 9 × 9 disc) was masked and objects smaller than 5 pixels were removed. Following polishing using a dilation/erosion (5 × 5 kernel), step
object areas, distances, and positions relative to the damage center sectioned into 36 radial slices were extracted.

**Abbreviations**

**ACSF:** Artificial cerebrospinal fluid

**CAG:** strong synthetic promoter

**ChETA:** Channelrhodopsin E to T, accelerated

**CX3CR1:** CX3C chemokine receptor 1

**IBA1:** ionized calcium-binding adapter molecule 1

**Kir2.1:** voltage-gated potassium channel, inward rectifier

**Kv1.3:** voltage-gated potassium channel, delayed rectifier

**LSL:** Lox-STOP-lox cassette

**P2X4:** purinergic receptor, ligand-gated ion channel

**P2X7:** purinergic receptor, ligand-gated ion channel

**P2Y12:** purinergic Gᵢ-protein coupled receptor

**PBS:** phosphate-buffered saline

**R26:** ROSA26 locus for ubiquitous gene expression

**tdTomato:** dimeric red fluorescent protein

**THIK-1:** tandem-pore domain halothane-inhibited K⁺ channel 1

**TRP:** transient receptor potential channel

**Declarations**

**Ethics approval and consent to participate**

Animal experiments were approved by the local authorities (Amt für Verbraucherschutz, Lebensmittelsicherheit und Veterinärwesen, Hamburg, Germany; Permission # 42/17).

**Consent for publication**
Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Author’s contributions

LL and TGO designed the experiments and prepared the manuscript. LL performed microglia patch clamp recordings, microglia chemotaxis and motility experiments, immunohistochemical experiments and all data analysis. MLB performed microglia motility experiments and immunohistochemical experiments. CS wrote software to analyze microglia motility and chemotaxis. All authors read and approved the final manuscript.

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References

1. Barlow, H.B. (1953). Summation and inhibition in the frog's retina. J. Physiol. 119, 69–88.
2. Baylor, D.A., and Fuortes, M.G.F. (1970). Electrical responses of single cones in the retina of the turtle. J. Physiol. 207, 77–92.
3. Berndt, A., Schoenenberger, P., Mattis, J., Tye, K.M., Deisseroth, K., Hegemann, P., and Oertner, T.G. (2011). High-efficiency channelrhodopsins for fast neuronal stimulation at low light levels. Proc.
4. Boucsein, C., Kettenmann, H., and Nolte, C. (2000). Electrophysiological properties of microglial cells in normal and pathologic rat brain slices. Eur. J. Neurosci. 12, 2049–2058.

5. Brawek, B., Liang, Y., Savitska, D., Li, K., Fomin-Thunemann, N., Kovalchuk, Y., Zirdum, E., Jakobsson, J., and Garaschuk, O. (2017). A new approach for ratiometric in vivo calcium imaging of microglia. Sci. Rep. 7, 6030.

6. Davalos, D., Grutzendler, J., Yang, G., Kim, J.V., Zuo, Y., Jung, S., Littman, D.R., Dustin, M.L., and Gan, W.-B. (2005). ATP mediates rapid microglial response to local brain injury in vivo. Nat. Neurosci. 8, 752–758.

7. Eder, C. (1998). Ion channels in microglia (brain macrophages). Am. J. Physiol. 275, C327–C342.

8. Eder, C. (2005). Regulation of microglial behavior by ion channel activity. J. Neurosci. Res. 81, 314–321.

9. Eder, C., Fischer, H.G., Hadding, U., and Heinemann, U. (1995). Properties of voltage-gated potassium currents of microglia differentiated with granulocyte/macrophage colony-stimulating factor. J. Membr. Biol. 147, 137–146.

10. Eichhoff, G., Brawek, B., and Garaschuk, O. (2011). Microglial calcium signal acts as a rapid sensor of single neuron damage in vivo. Biochimica et Biophysica Acta - Molecular Cell Research 1813, 1014–1024.

11. Espinosa-Parrilla, J.F., Martínez-Moreno, M., Gasull, X., Mahy, N., and Rodríguez, M.J. (2015). The L-type voltage-gated calcium channel modulates microglial pro-inflammatory activity. Mol. Cell. Neurosci. 64, 104–115.

12. Gee, C.E., Ohmert, I., Wiegert, J.S., and Oertner, T.G. (2017). Preparation of Slice Cultures from Rodent Hippocampus. Cold Spring Harb. Protoc. 2017.

13. Gunaydin, L.A., Yizhar, O., Berndt, A., Sohal, V.S., Deisseroth, K., and Hegemann, P. (2010). Ultrafast optogenetic control. Nat. Neurosci. 13, 387–392.

14. Haynes, S.E., Hollopeter, G., Yang, G., Kurpius, D., Dailey, M.E., Gan, W.-B., and Julius, D. (2006). The P2Y12 receptor regulates microglial activation by extracellular nucleotides. Nat. Neurosci. 9, 1512–1519.

15. Ince, C., Coremans &Ypey, J.M.C.C.D.L., Leijh, P.C.J., Verveen, A.A., and Van Furth, R. (1988). Phagocytosis by human macrophages is accompanied by changes in ionic channel currents. J. Cell Biol. 106, 1873–1878.

16. Izquierdo, P., Attwell, D., and Madry, C. (2019). Ion Channels and Receptors as Determinants of Microglial Function. Trends Neurosci. 42, 278–292.

17. Kouri, J., Noa, M., Diaz, B., and Niubo, E. (1980). Hyperpolarisation of rat peritoneal macrophages phagocytosing latex particles. Nature 283, 868–869.

18. Lee, J.E., Liang, K.J., Fariss, R.N., and Wong, W.T. (2008). Ex Vivo Dynamic Imaging of Retinal Microglia Using Time-Lapse Confocal Microscopy. Invest. Ophthalmol. Vis. Sci. 49, 4169–4176.
19. Madry, C., and Attwell, D. (2015). Receptors, Ion Channels, and Signaling Mechanisms Underlying Microglial Dynamics. J. Biol. Chem. 290, 12443–12450.

20. Madry, C., Kyrargyri, V., Arancibia-Cárcamo, I.L., Jolivet, R., Kohsaka, S., Bryan, R.M., and Attwell, D. (2018). Microglial Ramification, Surveillance, and Interleukin-1β Release Are Regulated by the Two-Pore Domain K+ Channel THIK-1. Neuron 97, 299–312.e6.

21. Newell, E.W., and Schlichter, L.C. (2005). Integration of K+ and Cl-currents regulate steady-state and dynamic membrane potentials in cultured rat microglia. J. Physiol. 567, 869–890.

22. Nguyen, H.M., Grössinger, E.M., Horiuchi, M., Davis, K.W., Jin, L.-W., Maezawa, I., and Wulff, H. (2017a). Differential Kv1.3, KCa3.1, and Kir2.1 expression in "classically" and "alternatively" activated microglia. Glia 65, 106–121.

23. Nguyen, H.M., Blomster, L.V., Christophersen, P., and Wulff, H. (2017b). Potassium channel expression and function in microglia: Plasticity and possible species variations. Channels 11, 305–315.

24. Nimmerjahn, A. (2005). Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma in Vivo. Science 308, 1314–1318.

25. Ohsawa, K., Irino, Y., Nakamura, Y., Akazawa, C., Inoue, K., and Kohsaka, S. (2007). Involvement of P2X4 and P2Y12 receptors in ATP-induced microglial chemotaxis. Glia 55, 604–616.

26. Orr, A.G., Orr, A.L., Li, X.J., Gross, R.E., and Traynelis, S.F. (2009). Adenosine A2A receptor mediates microglial process retraction. Nat. Neurosci. 12, 872–878.

27. Pagani, F., Paolicelli, R.C., Murana, E., Cortese, B., Angelantoni, S.D., Zurolo, E., Guiducci, E., Ferreira, T.A., Garofalo, S., Catalano, M., et al. (2015). Defective microglial development in the hippocampus of Cx3cr1 deficient mice. Front. Cell. Neurosci. 09, 111.

28. Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T.A., Guiducci, E., Dumas, L., et al. (2011). Synaptic pruning by microglia is necessary for normal brain development. Science 333, 1456–1458.

29. Pozner, A., Xu, B., Palumbos, S., Gee, J.M., Tvrzik, P., and Capecchi, M.R. (2015). Intracellular calcium dynamics in cortical microglia responding to focal laser injury in the PC::G5-tdT reporter mouse. Front. Mol. Neurosci. 8, 12.

30. Raivich, G., Bohatschek, M., Kloss, C.U.A., Werner, A., Jones, L.L., and Kreutzberg, G.W. (1999). Neuroglial activation repertoire in the injured brain: Graded response, molecular mechanisms and cues to physiological function. Brain Research Reviews 30, 77–105.

31. Rangaraju, S., Gearing, M., Jin, L.-W., and Levey, A. (2015). Potassium channel Kv1.3 is highly expressed by microglia in human Alzheimer's disease. J. Alzheimers. Dis. 44, 797–808.

32. Saegusa, H., and Tanabe, T. (2014). N-type voltage-dependent Ca2+ channel in non-excitatory microglial cells in mice is involved in the pathophysiology of neuropathic pain. Biochem. Biophys. Res. Commun. 450, 142–147.

33. Schilling, T., and Eder, C. (2015). Microglial K+channel expression in young adult and aged mice. Glia 63, 664–672.
34. Streit, W.J., Walter, S.A., and Pennell, N.A. (1999). Reactive microgliosis. Progress in Neurobiology 57, 563–581.
35. Suter, B.A., O’Connor, T., Iyer, V., Petreanu, L.T., Hooks, B.M., Kiritani, T., Svoboda, K., and Shepherd, G.M.G. (2010). Ephus: multipurpose data acquisition software for neuroscience experiments. Front. Neural Circuits 4, 100.
36. Swiatkowski, P., Murugan, M., Eyo, U.B., Wang, Y., Rangaraju, S., Oh, S.B., and Wu, L.-J. (2016). Activation of microglial P2Y12 receptor is required for outward potassium currents in response to neuronal injury. Neuroscience 318, 22–33.
37. Thei, L., Imm, J., Kaisis, E., Dallas, M.L., and Kerrigan, T.L. (2018). Microglia in Alzheimer’s Disease: A Role for Ion Channels. Front. Neurosci. 12.
38. Visentin, S., Renzi, M., and Levi, G. (2001). Altered outward-rectifying K+ current reveals microglial activation induced by HIV-1 Tat protein. Glia 33, 181–190.
39. Wendt, S., Maricos, M., Vana, N., Meyer, N., Guneykaya, D., Semtner, M., and Kettenmann, H. (2017). Changes in phagocytosis and potassium channel activity in microglia of 5xFAD mice indicate alterations in purinergic signaling in a mouse model of Alzheimer’s disease. Neurobiol. Aging 58, 41–53.
40. Werblin, F.S., and Dowling, J.E. (1969). Organization of the retina of the mudpuppy, Necturus maculosus. II. Intracellular recording. J. Neurophysiol. 32, 339–355.
41. Wu, L.-J., Vadakkan, K.I., and Zhuo, M. (2007). ATP-induced chemotaxis of microglial processes requires P2Y receptor-activated initiation of outward potassium currents. Glia 55, 810–821.
42. Zhao, Y., Wu, X., Li, X., Jiang, L.-L., Gui, X., Liu, Y., Sun, Y., Zhu, B., Piña-Crespo, J.C., Zhang, M., et al. (2018). TREM2 Is a Receptor for β-Amyloid that Mediates Microglial Function. Neuron 97, 1023–1031.e7.