DETECTION OF PRO-INFLAMMATORY ION CHANNEL ACTIVITY IN HUMAN MICROGLIA, THE BRAIN MACROPHAGES

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A research group at Saniona A/S has established a method for studying functional ion channel expression and activity in human microglia, the macrophages of the brain, isolated from epilepsy patients. Various ion channels on microglia cells are thought to promote or dampen inflammation in the brain, and inhibiting or facilitating these ion channels by pharmacological means could serve as a novel handle to treat inflammatory diseases. Through an extensive collaboration between Saniona A/S, the Neurobiology Research Unit at the Copenhagen University Hospital (Rigshospitalet) and the EU consortium INMiND (HEALTH-F2-2011-278850), we have been able to study the functional expression of several ion channels in microglia derived from adult human CNS tissue. The new findings that these channels are in fact functionally expressed in human microglia is of crucial importance in order to enhance successful translation of previous findings in animal experiments to humans.

Microglia are the macrophages of the central nervous system

Microglia are the macrophages of the central nervous system (CNS), i.e. the brain and the spinal cord. They share a similar heritage to peripheral macrophages, however, during embryonic development the microglia migrate into the CNS where they remain after the blood-brain-barrier has sealed itself, and where the microglia subsequently survive through local self-proliferation. Microglia carry out many of the same functions as the peripheral macrophages do. They scavenge the CNS tissue to look for potential invading pathogens and/or damaged tissue. They can respond to pro-inflammatory stimuli and become activated, a process that is characterised by migration towards the stimuli, proliferation, secretion of pro-inflammatory molecules and phagocytosis of pathogens, cell debris and dead cells. They are also thought to play a role in facilitating the generation of neurons from progenitor cells in the brain. In other words, microglia cells are vital in maintaining homeostasis in the CNS.

Despite these many important functions, during pathology, microglia can easily become over-activated and harmful in their nature by the abnormal changes that occur in the brain. Plaque formation and excess levels of the peptide amyloid-beta in Alzheimer's disease, a massive neuronal cell death in traumatic injuries (e.g. traumatic brain injury and stroke) and T cell infiltration in multiple sclerosis are all examples of pathological features in the CNS that trigger microglia to become over-activated (1,2).

Finding a treatment for inflammation in the brain

Activated microglia can greatly damage the healthy neuronal tissue. Since the CNS cannot easily regenerate itself these harmful aspects of the microglia cells are usually detrimental and irreversible. Existing anti-inflammatory treatments (e.g. glucocorticoids) can reduce the activation profile of microglia but have both severe side effects as well as a limited treatment time span. However, finding an anti-inflammatory and neuroprotective treatment that does not undermine the natural healing ability of microglia and that does not prevent the microglia from their ability to detect and remove pathogens whilst at the same time dampening the harmful aspect of microglia over-activation remains a challenge.

An example of a putatively important ion channel target is the calcium-activated potassium channel, $K_{Ca}3.1$ (encoded by the *KCNN4* gene), which is an ion channel present on rodent immune cells such as macrophages, T cells, B cells, neutrophils, mast cells and dendritic cells in the periphery and microglia in the CNS(1,3). It is thought to play an inflammatory role in these cells, by analogy to T cells, by facilitating an increase of intracellular Ca^{2+} levels, a typical sign and mediator of activation in these cells (Figure 1). In addition, $K_{Ca}3.1$ seems to participate in pro-inflammatory activities of rodent microglia such as the production of reactive oxygen species and other pro-inflammatory molecules, factors that are directly implicated in neurotoxicity. Many of these pro-inflammatory events can be directly prevented in both *in vitro* and *in vivo* models of neuroinflammation by blocking the $K_{Ca}3.1$ channel with e.g. the selective small molecule TRAM-34 (4). Most importantly, blocking $K_{Ca}3.1$ does not result in an impaired clearance of viral

particles suggesting that using $K_{Ca}3.1$ as an anti-inflammatory target could be safer than current treatment options (5).

Human microglia in a Petri dish

Although we know quite a lot about the role of ion channels in rodent microglia, very little is known about their presence and role in human microglia. Studying human microglia has been greatly hampered by the fact that it is very difficult to get access to human CNS tissue. There are three potential sources of human microglia that have been previously used in the literature; microglia isolated from 1) foetal tissue, 2) postmortem tissue and 3) microglia isolated from epilepsy patients undergoing surgical treatment or from brain cancer patients. There is no doubt that all three methods have both *pro et contra* aspects to take into consideration but the choice is in the end usually driven by availability.

The CNS tissue used for our study came from the supposedly non-pathological cortex of epilepsy patients. In order for the surgeon to gain better access to the hippocampus (located closer to the middle of the brain) which is the pathological area in this group of epilepsy patients, a small part of the cortex (the outer layer of the neuronal tissue) has to be surgically removed. The cortex in these patients is considered healthy, consequently the microglia in this tissue should, in theory, be in a resting state. However, one has to keep in mind that it does come from patients that have severe epilepsy and most likely have received various different types of medication prior to surgery. All these things could ultimately affect both the neurons and the microglia in the tissue, which has to be taken into consideration when interpreting the results from the experiments using this method.

We isolate and generate pure human microglia cultures from the freshly dissected CNS tissue (>92% microglia cells). This is done through both enzymatic and mechanical homogenisation steps where bonds between tissue and cells are being dissolved. A cell suspension of dissociated microglia is subsequently plated in small petri dishes for electrophysiological recordings. The cell culture purity can be determined by immunofluorescent staining (Figure 2). In culture, human microglia cells would typically display a large cell body with a few long processes of various lengths

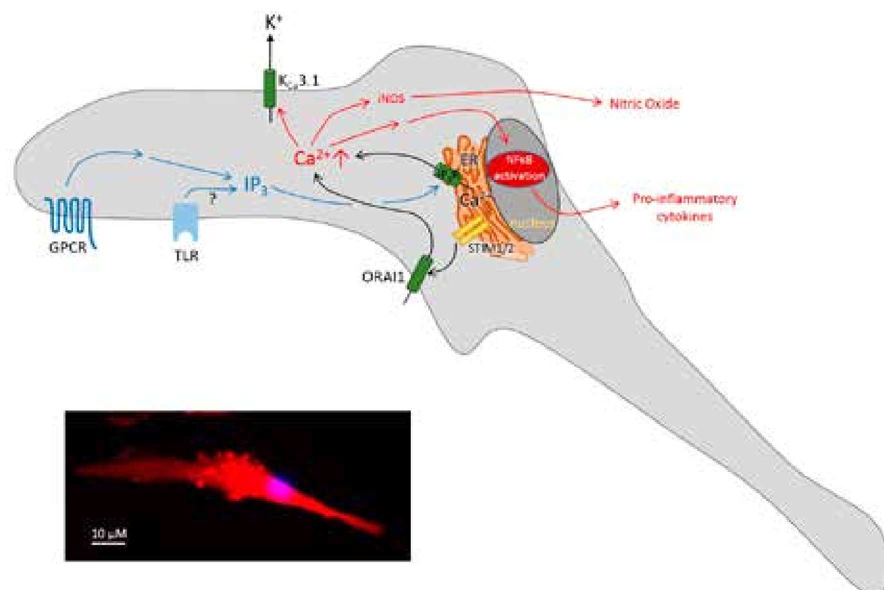

Figure 1. *Schematic illustration of the hypothesised mechanism of microglia activation. Ligand binding to pro-inflammatory receptors e.g. certain G-protein coupled receptors (GPCR) and toll-like-receptors (TLR) activates a cascade of intracellular signalling pathways ultimately leading to the release of Ca^{2+} from the endoplasmic reticulum (ER) through the inositol triphosphate receptor (IP_3R). Reduced Ca^{2+} levels in the ER initiates STIM1/2 to couple with ORAI1 allowing for a direct flow of Ca^{2+} into the cell. Elevated intracellular levels of free Ca^{2+} ($[Ca^{2+}]_i$) activates $K_{Ca}3.1$ to mediate K^+ flow out of the cell, thereby causing a hyperpolarization of the microglia membrane and an acceleration of the Ca^{2+}-influx which facilitate the activation process further. Increases in $[Ca^{2+}]_i$ additionally triggers downstream signalling pathways including the enzyme iNOS and the transcription factor NFkappaB ultimately leading to the generation and release of pro-inflammatory molecules (cytokines) and nitric oxide (NO). Local net KCl and H_20 loss via $K_{Ca}3.1$, various chloride channels, and aquaporins, are also important for microglia migration (not illustrated). The photomicrograph below the illustration shows a real microglia cell in a petri dish. The photograph has been acquired from Figure 2.*

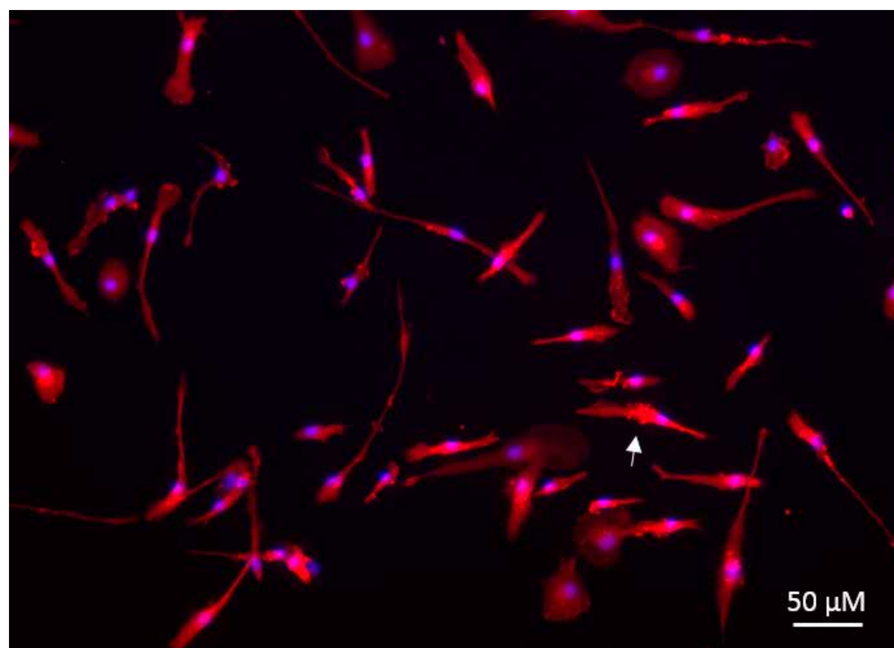

Figure 2. *Human microglia cells in vitro 6 days after they were isolated. The cells have been antibody-labelled with a red fluorescent dye and counterstained with a blue nuclear dye. The arrow indicates the microglia cell that can be seen at higher magnification in Figure 1.*

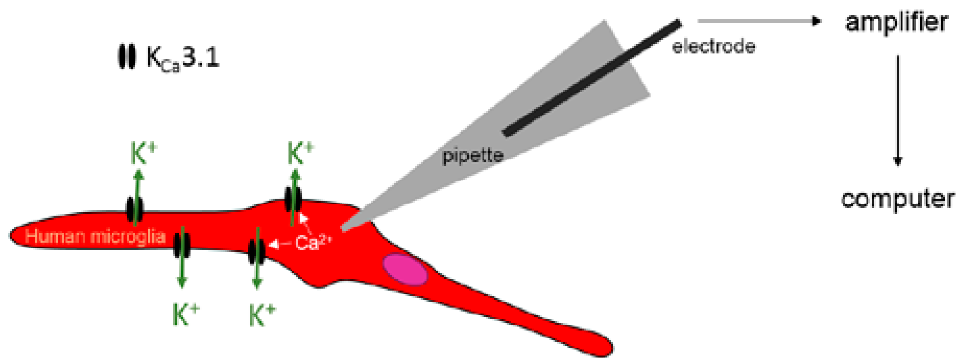

Figure 3. *Schematic representation of a human microglia cells undergoing electrophysiological whole-cell recording. The electrode in the pipette picks up flow of ions across the cell membrane. This information is sent to the computer and can subsequently be analysed after the conclusion of the experiment. The illustration focuses on a particular functional type of ion channel selective for K^+ (and thus mediates outward current) and is activated by a rise in intracellular Ca^{2+}, such as is the case for the previously mentioned $K_{Ca}3.1$ channel.*

stretching away from the cell body. Upon stimulation of these cells with the pro-inflammatory bacterial molecule lipopolysaccharide (LPS) we saw a strong pro-inflammatory cytokine response generated by these cells. There was no response in the cells that had not received LPS indicating that the cells were not already activated before the experiment started.

To test whether human microglia express specific ion channels we use an electrophysiological approach. Using this technique we can measure the electrical current across the plasma membrane that is generated by a flow of ions through a specific ion channel protein. By attaching a glass pipette to the cell membrane (Figure 3) and thereafter creating an approximately 1 µm hole in the membrane by applying gentle suction, the total amount of current across the microglia membrane can be measured (whole-cell current) in that particular cell. For isolation of the current components through specific ion channels we employ a variety of strategies, such as using special salt solutions on the extracellular and intracellular side of the membrane, specific activation protocols aimed at eliciting currents through specific channel types only (e.g. steps in membrane potentials for voltage-dependent ion channels, extracellular application of neurotransmitters for ligand activated ion channels, or intracellular application of Ca^{2+} for stimulation of Ca^{2+}-activated ion channels), as well as pharmacological tool compounds developed for high selectivity for certain ion channels.

Human vs. rodent microglia ion channel recordings are in for surprises

An important conclusion of our studies is that some ion channel types identified are the "expected ones" as compared to rodent microglia, whereas others found are usually not considered to play a significant role in rodents. Another very important conclusion is that some channels (such as the previously mentioned $K_{Ca}3.1$) are expressed in much higher densities on the cell membrane of our adult human microglia preparation than on rodent microglia even after these have been stimulated with various pro- or anti-inflammatory agents, thus hinting to an accentuated physiological role for human microglia *in situ*. Even though further studies are needed in order to find out whether the observed differences are rodent/human-, young/old-, or health/disease-based variations, the results hitherto obtained clearly point to the need for much more human microglia-based information on expression and function, preferably from neuroinflammatory diseases, in order to validate specific ion channels for therapeutic use. We think that the established procedure for microglia isolation and ion channel recording represents a good foundation for such studies.

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