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Electrophysiological properties and intracellular calcium recordings of microglial cells from the adult brain

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Für meine Familie

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List of abbreviations

5HT	Serotonin (5-Hydroxytryptamine)
5HT7	Serotonin receptor 7
ASCF	Artificial spinal cord fluid
ADP	Adenosine diphosphate
AM	Acetoxymethyl ester
ATP	Adenosine-5'-triphosphate
BBB	Blood brain barrier
BrdU	Bromdesoxyuridin
BSA	Bovine serum albumin
CMV	*Cytomegalovirus*
CX3CR1	fractalkine receptor 1
diF	Integrated fluorescence
DNA	Deoxyribonucleic acid
dt	Duration in s
DMEM	Dulbecco's Modified Eagle Medium
E.coli	*Escherichia coli*
ER	Endoplasmatic reticulum
ELISA	Enzyme linked immunosorbent assay
ET-A	Endothelin receptor A
ET-B	Endothelin receptor B
F	Fluorescence
F/F0	Baseline fluorescence intensity
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
G	Specific conductance in nS/pF
GCaMP2	eGFP based single fluophore calcium sensor protein
eGFP	Enhanced green fluorescence protein
GFP	Green fluorescence protein

HBSS	Hanks Balanced Solution
I	Current in A (ampere)
Iba-1	Ionized calcium binding adaptor molecule 1
IL	Interleukin
INF	Interferon
Kd	Dissociation constant
Kir	Potassium inward rectifier channel
Kv	Potassium outward rectifier channel
LB medium	*Lysogeny broth* culture medium
LPS	Lipopolysaccharide from *E.coli*
LTR	Long terminal repeat
max rFI	maximal relative fluorescence intensity
MCAO	Middle cerebral artery occlusion
mRNA	Messenger ribonuclein acid
NO	Nitric oxide
NG2	Proteoglycan neuro-glia2
NGS	Normal goat serum
Nr	Number
P_2	Purinergic receptor typ 2
P_2X	Purinergic receptor typ 2 X
P_2Y	Purinergic receptor typ 2 Y
P	Postnatal day
p	p-value (statistics)
PBS	Phosphate buffered saline
Plat-E	Platinum E
rFI	Relative fluorescence intensity
RT	Room temperature
SD	Standard deviation
TNF	Tumor necrosis factor
UTP	Uridine-5'-triphosphate

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1. Summary

1.1 English summary

Microglial cells are the resident immune cells of the brain and are important for fight against microorganisms, clearance of damaged tissue and scar formation. If the brain gets injured microglial cells transform from a resting to an activated stage. The physiological changes that accompany with microglia activation are not fully understood in the adult brain, because so far adequate models to study the cells are missing. Microglia were investigated mainly in cell culture prepared from neonatal rodends. A few recent studies on microglia from adult animals advert that these cells differ in their physiological properties compared to cultured microglia from neonatals. Therefore I investigated the physiology of microglia cells of the adult brain in the present work.

First I characterized cultured microglia cells from the adult brain by patch clamp technique and compared them to microglial cells from neonatal mice. Following properties could be identifyed:

(1) Adult cultured microglia cells exhibit in general less inward and outward currents then cultured microglial cells from neonatal brains.

(2) The current amplitudes were increasing culturing time.

(3) The amplitude and conductance of inward currents in unstimmulated microglia was age dependent, whereas outward currents did not differ.

(4) Adult microglia from distant brain regions, namely cerebral cortex and corpus callosum did not differ in their current profiles.

(5) Upon mimicing bacterial infection by stimulation with LPS, adult microglia were able to induce outward currents. Unexpectedly microglia from juvenilles where not able to develop outward currents upon LPS stimulation.

In vitro studies show that microglia express a variety of neurotransmitter and neuropeptide receptors which are linked to Ca^{2+} dependent pathways. These signaling molecules were found to modulate important microglia functions. In the second part of this work I introduce a new approach which allowed me to extent calcium measurements to microglia in the brain tissue environment of adult mice. A retrovirus encoding for eGFP or a calcium sensor protein (GCaMP2) was injected into the cortex of mice two days after microglial proliferation was stimulated by a stab wound. 3, 6, 21 and 42 days after the stab wound injury acute brain slices were prepared. Characterization of the eGFP positive cells in the side of injury by patch clamp experiments and by antibody staining identified them as highly activated microglial cells at 3 and 6 days. EGFP positive cells at day 42 showed properties of resting microglia. Upon GCaMP2 expression transient intracellular Ca^{2+} increase in response to of ATP, endothelin-1, substance P, histamine and serotonin application were recorded. The fluorescence amplitude to ATP was higher at day 6 in comparison to all other time points. The responses to all other ligands did not differ significantly between the time points. About half of the microglial cells that responded to ATP also responded to endothelin-1, serotonin and histamine. Only substance P at day 6 showed a complete overlap with the ATP responding microglial population, while at day 42 this population was reduced to 55 %. Adult microglia in the brain slice showed an increased responsiveness to the tested ligands in comparison to cultured microglia from neonatal mice.

My studies support the hypothesis that adult microglia differ in their physiology from microglia of neonatal mice. The present results are contributing to the better understanding of brain injury of adults.

1.2 Deutsche Zusammenfassung

Mikrogliazellen sind die Immunzellen des Gehirns und sind für die Verteidigung gegen Mikroorganismen, die Säuberung von zerstörtem Gewebe und für Narbenbildung von Bedeutung. Im Falle einer Gehirnverletzung wechseln Mikroglia von einem ruhenden in einen aktivierten Zustand. Die physiologischen Veränderungen im Gehirn eines Erwachsenen, die mit Mikrogliaaktivierung einhergehen nicht vollständig verstanden, weil geeignete Modelle zum Studium der Zellen fehlen. Mikroglia wurden bisher hauptsächlich in Zellkultur untersucht, welche aus neugeborenen Nagetieren gewonnen wurden. Einige wenige kürzlich angefertigte Untersuchungen an Mikroglia von erwachsenen Tieren weisen darauf hin, dass sich diese Zellen in ihren physiologischen Eigenschaften von kutivierten Mikrogliazellen aus neugebornen Tieren stammend unterscheiden. In der vorliegenden Arbeit habe ich daher die Physiologie von Mikroglia des Gehirns von erwachsenen Mäusen untersucht.

Zunächst habe ich kultivierte Zellen des erwachsenen Gehirns mittels Patch Clamp-Technik untersucht und diese mit Mikrogliazellen von neugebornen Mäusen verglichen. Folgende Eigenschaften könnten dabei identifiziert werden:

(1) Erwachsene kultivierte Mikrogliazellen zeigen generell weniger Einwärts- und Auswärtsströme als kultivierte Mikroglia von Gehirnen Neugeborener.
(2) Die Stomamplitude war abhängig von der Kultivierungszeit.
(3) Die Amplitude und Leitfähigkeit der Einwärtsströme von unstimmulierten Mikrogliazellen war altersabhängig, wärend sich die Auswärtsströme nicht unterschieden.
(4) Erwachsene Mikroglia von verschiedenen Gehirnregionen, namentlich cerebraler Kortex und Corpus callsoum unterschieden sich nicht in ihren Stromprofilen.
(5) Nach Imitation einer bakteriellen Infektion mittels LPS, waren adulte Mikroglia in der Lage Auswärtströme induzieren. Unerwarteterweise konnten Mikroglia von jugendlichen Mäusen keine Auswärtsströme entwickeln.

In vitro-Studien zeigen, dass Mikroglia eine Vielzahl an Neurotransmitter- und Neuropeptid-Rezeptoren exprimieren, welche mit Kalzium abhangigen Signalwegen verbunden sind. Im zweiten Teil dieser Arbeit stelle ich einen neuen Ansatz vor, welcher mir erlaubt Kalziummessungen an Mikrogliazellen auf das Gehirngewebe von erwachsenen Mäusen auszuweiten. Ein Retrovirus, welcher entweder für eGFP oder das Calciumsensorprotein GCaMP2 codiert, wurde in das Gehirn von Mäusen injeziert, zwei Tage nach dem Mikrogliazellen mittels Stichwundenverletzung zur Proliferation angeregt wurden. 3, 6, 21 und 42 Tage nach Stichwundenverletzung wurden akute Hirnschnitte vorbereitet. Die Charakterisierung der eGFP positiven Zellen nahe der Verletzung mittels Patch Clamp-Experimenten und Antikörperfärbung ergab, dass es sich haupsächlich um aktivierte Mikrogliazellen an Tag 3 und 6 handelte. EGFP positive Zellen an Tag 42 zeigten Eigenschaften von ruhenden Mikrogliazellen. Nachdem GCaMP2 in den Zellen exprimiert wurde konnte ich vorrübergehende intrazelluläre Kalziumerhöhungen als Antwort auf die Applikation von ATP, Endothelin-1, Substanz P, Histamin und Serotonin messen. Die Fluoreszenzamplitude war an Tag 6 im Gegensatz zu allen anderen Zeitpunkten erhöht. Die Antworten der anderen Liganden unterschieden sich nicht zwischen den Zeitpunkten. Ungefähr die Hälfte der Zellen, die auf ATP reagierten, antworteten auch auf Endothelin-1, Serotonin und Histamin. Nur Substanz P zeigte eine komplette Übereinstimmung mit der auf ATP reagierenden Zellpopulation an Tag 6, wärend an Tag 42 nur noch 55% der Zellen reagierten. Erwachsene Mikrogliazellen im Gehirnschnitt zeigten ein erhöhtes Antwortverhalten gegenüber den getesten Liganden im Vergleich zu kultivierten Mikrogliazellen von neugeborenen Mäusen.

Meine Untersuchungen unterstützen die Hypothese, dass sich erwachsene Mikroglia in ihrer Physiologie von Mikrogliazellen neugeborner Mäuse unterscheiden. Die vorliegenden Ergebnisse werden dazu beitragen Gehirnverletzungen von Erwachsenen besser zu verstehen.

2. Introduction

2.1 Microglia – immune defense of the brain

The immune system of the body is classified into innate and adaptive immune system. Microglial cells are displaying the only cell type of the innate immunity in the healthy brain parenchyma and are believed to be brain macrophages. Cells of the innate immunity are immediately available to combat a wide range of pathogens without requiring prior exposure (Janeway, 2001). Microglia cells as well as other tissue macrophages originate from hematopoietic stem cells in the bone marrow, which develop into myloid precursor cells and diffentiate into monocytes. Monocytes circulate in the blood and mature into tissue macrophages. Microglia cells invade during embryonal and early postnatal development into the brain parenchyma (Perry & Gordon, 1991; Theele & Streit, 1993; Cuadros & Navascués, 2001). In the healty adult brain microglia turnover is low (Priller et al., 2001a). It was suggested that the cells survive a whole life long. Microglial cells appear in a ramified morphology, exhibiting a small cell body with numerous long thin branched processes. Every cell is occupying a micro territory in the CNS parenchyma, which is non-overlapping with other microglia cells (Del Rio-Hortega, 1932). Microglia processes are highly versatile, and serve for scanning permanently their environment (Nimmerjahn *et al*., 2005; Davalos *et al*., 2005). In the non-pathological brain microglial cells regularly pinocytose molecules, endocytose debris and apoptotic cellular material (Ward *et al*., 1991). Therefore the cells form phagosomes at the end of their processes (Peri & Nüsslein-Volhard, 2008), which often appear as bulbous protrusions. Microglia were found to scan synaptic activity with their processes (Wake *et al*., 2009). Nevertheless studies on adult resting microglia cells are rare, since the cells react to every damage of the brain.

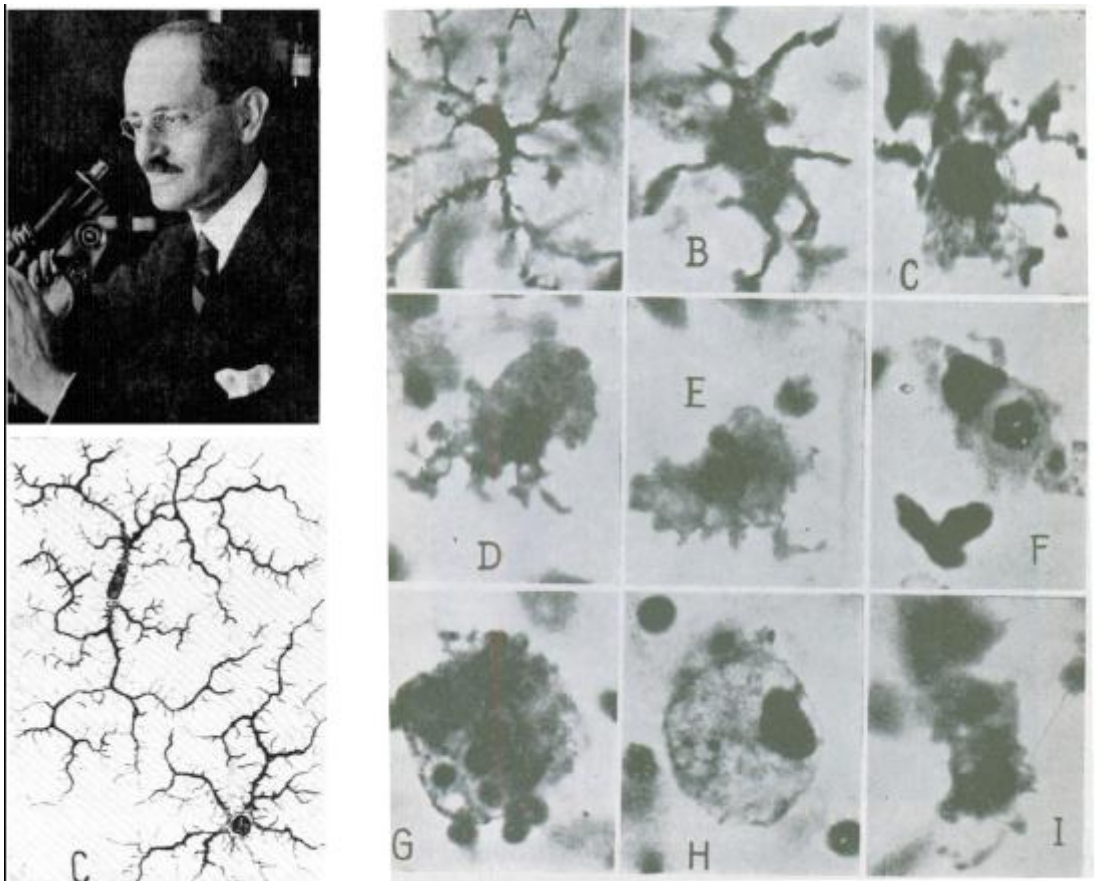


Figure 2. 1 The work of Pio del Rio Hotega on microglia. Left side, upper picture: Photo of del Rio Hortega who described microglia first. Left side lower picture: Drawing of resting microglia from del Rio Hortega. Right side: Series of pictures showing microglia stained with silver carbonate impregnation method during transformation from a resting to an activated stage. Every picture shows a distinct time points after entorhinal cortex lesion. Pictures were published in Del Rio-Hortega, 1932.

Microglia cells express diverse receptor types. They use their receptors to sense signaling molecules and changes in ion homeostasis induced for instance by acute injury. After a focal lesion microglial cells polarize and focus their processes within minutes toward the lesion site (Davalos *et al*., 2005; Haynes *et al*., 2006). Nucleotides such as adenosine triphosphate (ATP) are released by injured neuronal cells and attract microglia via activation of purinoreceptors. Microglia cells change their phenotype in response to injury, which is in general termed as microglia activation (Figure 2.1 right side). The intensity of activation depends to the grade of

the insult and the position of the cell to the injury. Microglia activation is accompanied by changes in morphology and physiological properties. Activated microglia cells can migrate to the lesion side. Within days the cells proliferate in the vicinity of the lesion, a process which is named reactive microgliosis (Fujita & Kitamura, 1975; Streit, 2000). Microglial cells communicate via inflammatory mediators like cytokines and chemokines, grow factors but also proteins and peptides may play a role in the regulation of microglial functions. Microglia induce actively inflammation by the release of cytotoxic molecules like NO and reactive oxygen species to kill pathogens (Paakkari & Lindsberg, 1995). At the lesion side the cells become phagocytotic active to clear the lesion site from bacteria, injured or dead cells and debris (Hanisch & Kettenmann, 2007). Microglia are found to be activated under several pathological conditions. Acute inflammation is the term used to describe early and transient episodes, whereas chronic inflammation occurs when the activation of immune cells persists for example in autoimmune diseases. In acute injury, for example including traumatic brain injury, infection or stroke, microglia are involved in mediating neuroinflammation. The inflammatory response is necessary for recruitment of cells from the blood system. Monocytes and cells of the adaptive immune system are attracted to support the innate immune answer. After clearance of the lesion side microglia cells are involved in scar formation by stimulating astrogliosis and tissue generation, which is necessary for wound healing (Schilling *et al*., 2009; Zhang *et al*., 2010). In neurodegenerative pathologies like in the cases of Morbus Alzheimer or in Chorea Huntington microglia cells are permanently activated which might lead to chronic inflammation.

2.2 Cellular responses to stab wound injury

Stab wound injury is a kind of open traumatic brain injury. Mechanical force leads thereby to destructions of neuronal tissue and blood brain barrier breakdown (Persson, 1976). Neural cell death not only occurs as a consequence of the mechanical force, but also by secondary damage, which includes leakage of blood components into the brain parenchyma and changes in homeostasis. As a consequence neurons, astrocytes and oligodendrocytes get damaged or dye.

The first glia cell reaction to stab wound injury of the brain was described by Huntington & Terry, 1966. Activation of microglia cells demonstrate the first response mechanism to the acute injury, followed by astro- and/or oligodendrocytic precursors (Hampton *et al*., 2004). The number of reactive microglia cells next to the side of injury is maximal at 3 days after stab wound injury. Afterwards the number of cells decline until they reach density levels of non-injured cortical tissue (Fujita *et al*., 1998). The increase in number originates in parts from migration from juxtaposed regions of the injury (Rappert *et al*., 2004), recruitment of myeloid cells from the blood (Wirenfeldt *et al*., 2005) and proliferation (Amat *et al*., 1996; Fujita *et al*., 1998; Norton, 1999). Glia cells proliferate in defined time windows. Microglia are the first cells proliferating after stab wound injury, mainly after 2-3 days, followed by NG2 precursor cells that are able to differentiate into astrocytes and oligodendrocytes. Astrocytes start to proliferate later (Hampton *et al*., 2004; Alonso, 2005) from day 3 on.

During brain injury macrophages from the blood invade the lesion side. Microglia and macrophages share many properties and are not distinguishable by morphology nor by cellular markers (Kreutzberg, 1996). Invading macrophages as well as microglial cells express the antigens Iba-1, CX3CR1 and tomato lectin, specific markers relevant for this work (Acarin *et al*., 1994; Ito *et al*., 1998; Rappert *et al*., 2004). Using eGFP transgenic bone marrow chimeric mice, researchers were able to distinguish between these two cell types. Nevertheless the number of invading macrophages remains low. After MCAO only 10-15% of the Iba-1 positive cells were eGFP labeled at day 2. In the model of perforate pathway lesion 5% of the cells were eGFP labeled after 7 days. Macrophages start to invade at day 2-3 after MCAO into the lesion side (Schilling *et al*., 2003, 2009). Invaded macrophages are able to proliferate (Norton, 1999; Wirenfeldt *et al*., 2007).

2.4 Potassium channels in microglia

Cultured microglia from early postnatal animals are electrophysiologically best characterized. These cells express constantly prominent inwardly, but no outwardly rectifying potassium channels (Kir) (Kettenmann *et al*., 1990). Predominant current is

a Kir2.1-like inward rectifier (Nörenberg et al., 1994a; Visentin et al., 1995; Schlichter et al., 1996a). Kir currents are characterized by a time-dependent inactivation. The same current profile was found in invading amoeboid microglia cells during brain development (Brockhaus *et al*., 1993). Inward currents might be involved in the regulation of the membrane potential and in regulation of calcium entry (Franchini *et al*., 2004).

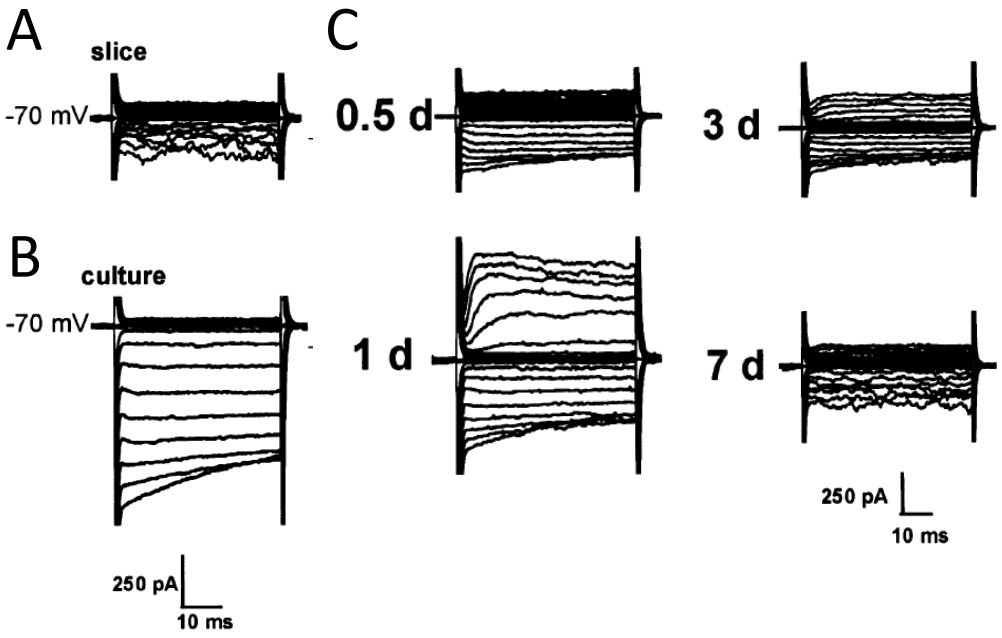


Figure 2. 2 Comparison of microglial currents. Current profiles in response to de- and hyperpolarising voltage steps of a resting microglia from acute brain slices (A), cultured microglia from neonatal (P0-3) animals (B) and activated microglial cells from acute brain slices 0.5, 1, 3 and 7 days after facial nerve axotomy. Modified from Boucsein *et al*., 2000.

Cultured microglia develop outward potassium currents when treated with inflammatory mediators like bacterial endotoxins or proinflammatory cytokines like INF-gamma (Nörenberg *et al*., 1992; Prinz *et al*., 1999). The outward currents are of shaker potassium channel type, most likely of Kv1.3 and Kv1.5 (Kotecha & Schlichter, 1999; Schilling *et al*., 2000; Pannasch *et al*., 2006), but also Kv 1.1 (Wu *et al*., 2009) and Kv 1.2 (Li *et al*., 2008) are described to be expressed. Results of *in vitro* electrophysiological experiments suggest that the ion channel expression

pattern of activated monocyte-derived macrophages does not differ from that of activated microglial cells (Eder, 1998).

However, the most experiments were performed using primary cultured microglia from neonatal or early postnatal rodents. From the rare electrophysiological studies available on microglia *in situ* (Kotecha & Schlichter, 1999; Boucsein *et al*., 2000; Schilling & Eder, 2007) I learned that the *in situ* microglia from the adult brain differs in their potassium channel properties when compared to that of primary cultured microglia (Figure 2.2). Electrophysiological characterization of cultured adult microglia are missing. Patch clamp experiments of acute brain slices from adult mice revealed that microglia under resting condition do express little, if any potassium channels. During activation the cells developed a Kir current within the first 12 h after injury. At 24 h an additional potassium outward current was found, which could be recorded within the next 3 days (Boucsein *et al*., 2000; Lyons *et al*., 2000). Afterwards first the outward current declines, followed by the inward current back to levels comparable with resting microglia.

Outwardly rectifying potassium channels are found to regulate proliferation (Kotecha & Schlichter, 1999; Pannasch *et al*., 2006) production of reactive oxygen and nitrogen species (Khanna et al., 2001a; Kaushal et al., 2007), release of cytokines (Caggiano & Kraig, 1998; Franciosi *et al*., 2006; Stock *et al*., 2006) , production of reactive oxygen species (Fischer *et al*., 1995; Eder, 2005) and migration (Schilling *et al*., 2004). Thus microglial potassium currents can be used as a marker of microglia activation.

2.5 Neuropeptid and –transmitter receptors

Microglial cells are able to sense a bunch of different molecules with specific surface receptors. Many neurotransmitter and –peptides bind to G-protein coupled receptors. Activation of G_q-protein evokes an increase in intracellular calcium (Färber & Kettenmann, 2005). In microglia cells two main pathways lead to increase of cytosolic calcium. (1) Agonist binding to G-protein coupled receptors leads to calcium release from intracellular stores and (2) opening of calcium permeable ion channels in the plasma membrane.

The most studies on neuropeptide and neurotransmitter receptors were accomplished on isolated microglia in cell culture prepared from brains of embryonic or neonatal pups of rodents. In acute brain slices amoeboid invading microglia of young mice were investigated by calcium imaging and patch clamp experiments. A few studies on acute brain slices from adult animals are existent (Boucsein *et al*., 2003). Only one recent study describes calcium signals of microglia *in vivo* (Eichhoff *et al*., 2010). Microglial cells express a huge repertoire of purinergic receptors, which are activated by purines and pyrimidines, for example ATP, UTP and ADP (Ralevic & Burnstock, 1998; Abbracchio & Burnstock, 1998). ATP can be released form nerve terminals as conventional transmitter or as co transmitter from synaptic vesicles. Also other cell types release ATP including for example endothelium, astrocytes and oligodendrocytes. The presence of ATP-sensitive P_2X and P_2Y purinoceptors has been shown in cultured microglial cells (Nörenberg et al., 1994b; Langosch et al., 1994) Microglia *in vivo* respond with an increase in intracellular calcium to ATP application and patch clamp experiments on resting microglia in acute brain slices revealed a current response (Boucsein *et al*., 2003; Eichhoff *et al*., 2010). ATP is the signaling molecule most responsible for activation of microglia during acute injury. Upon acute damage of neural tissue microglia arms were attracted dependent on P_2Y receptors (Davalos *et al*., 2005; Dibaj *et al*., 2010). ATP binding to P2Y receptors activates G_q and the enzyme phospholipase C with the subsequent release of inositol-1,4,5-phosphate, which results then in the release of calcium from intracellular stores like the ER. Depletion of the Ca^{2+} pool from the ER can initiate a capacitative entry of Ca^{2+} from the extracellular space by opening of store operated channels in the plasma membrane. Microglia express more than one P2Y subtype (Boucsein *et al*., 2003). During acute injury microglia sense extracellular ATP, possibly released from astrocytes or dying cells (Verderio & Matteoli, 2001). Astrocytes release ATP for instance in response to mechanical stimulation (Shiga *et al*., 2001). In culture it was shown that astrocytic ATP can cause a Ca^{2+} response and a K^+ conductance in microglia via P_2X_7 receptors (Verderio & Matteoli, 2001; Schipke *et al*., 2002). P_2X receptors are activated only at higher ATP concentrations and couple to a non-selective cationic membrane channel allowing influx of Na^+ and Ca^{2+} and efflux of K^+, which leads to a transient cell depolarization (Walz *et al*., 1993;

Ferrari *et al*., 1996; Boucsein *et al*., 2003). P_2X receptors are up regulated after spinal cord injury (Tsuda *et al*., 2008; Ulmann *et al*., 2008), ischemia (Franke *et al*., 2004) and in Alzheimer`s disease (McLarnon *et al*., 2006). Controversially, calcium signals by P_2 receptor activation were reduced in cultured microglia treated with LPS (Möller *et al*., 2000).

Neuropeptides and neurotransmitter like endothelin-1, serotonin, histamine and substance P are able to regulate the blood brain barrier in the in non-injured brain. After injury these substances are up regulated in spinal cord fluid and in the blood. Microglia cells express receptors for all substances. Therefore they might play a role during stab wound injury.

Endothelin-1 is a 21 amino acid hormone peptide. Translation from the endothelin gene results in the polypeptide preproendothelin- 1, which is converted into big endothelin-1 by endopeptidases. Big endothelin-1 is cleaved to the active molecule endothelin-1 by endothelin converting enzyme-1, a metalloprotease, and can be released by several cell types including astrocytes and endothelia cells. It acts via two receptors (ET-A and ET-B) and requires intracellular calcium for its action. Microglial ET-A and B receptor mRNA expression was found in cultured microglial cells. Also calcium signals could be recorded in 13% of the cells (Möller *et al*., 1997). Expression studies showed that Endothelin-1 mRNA was up regulated in LPS stimulated cultured microglia, but not *in vivo* after intracerebroventricular LPS injection (Lund *et al*., 2006). Recent studies confirmed that endothelin receptors as well as endothelin expression *in vivo* was upregulated after MCAO (Li *et al*., 2010). Endothelins were found to be released from amoeboid microglia (Wu *et al*., 2009). Endothelin-1 has extremely potent and long-lasting contractile effects on cerebral arteries (Hall & Brain, 1994; Lehmberg *et al*., 2003) and might be therefore involved in the regulation of the blood brain barrier. Studies in gerbils treated with endothelin receptor antagonist showed reduced postischemic neurological deficits. Furthermore the authors found that endothelins plays a role in mediating leukocyte-endothelium interaction after global cerebral ischemia (Lehmberg *et al*., 2003). Endothelins lead to recruitment of monocytes and lymphocytes (Hickey 1999; Lossinsky and Shivers 2004; Taupin 2008). Astrogliosis and scar formation was stimulated by endothelins (Tsang *et al*., 2001). Endothelin-1 concentration in cerebrospinal fluid is elevated in

stroke patients, as well as in patients with subarachnoid hemorrhage (Kessler *et al*., 2005). Similarly, ischemia or trauma in experimental animals results in an elevation of the endothelin-1 level in the central nervous system (Petrov *et al*., 2002). Endothelin-1 from resident microglia might lead to the relaxation of the blood brain barrier and is therefore be involved in leukocyte recruitment in the healthy brain (Hickey *et al*., 1999; Lossinsky & Shivers, 2004)

Substance P is a neuropeptide of the nerves innervating the vasculature of the cerebral arteries. Strong increase in its concentration was found after subarachnoid hemorrhage in the cerebral spinal cord fluid (Tran Dinh *et al*., 1994). In Parkinson patients decreased levels of substance P were found (Cramer *et al*., 1991). Many pathological processes (ischemia, infections, hypertensive insult, etc.) alter the integrity of this barrier.

The effects of sensory neuropeptides are particularly prominent at the level of the vasculature where they cause vasodilation of arterioles, plasma protein extravasation in post-capillary venules and leukocyte adhesion to endothelial cells (Geppetti *et al*., 1995).

Histamine receptors are described to be G-protein coupled. Histamine was found to increase calcium response in 30% of cultured microglia increase originated from inositol-1,4,5-phosphate-induced Ca^{2+} release from the ER (Bader *et al*., 1994). Histamine is a neurotransmitter which leads to opening of the blood brain barrier. Histamine is involved in neutrophil and lymphocyte recruitment after stroke (Hiraga *et al*., 2007). Increased levels of blood plasma histamine were found in Parkinson patients (Coelho *et al*., 1991). Since histamine is able to modulate the BBB by itself (Boertje *et al*., 1989, 1992; Easton *et al*., 1997) it is very likely that it crosses the BBB and defuse in the brain parenchyma during neuronal disease. In the healthy brain Histamine is not able to cross the BBB (Reilly & Schayer, 1970). Microglia can produce histamine by themselves (Katoh *et al*., 2001). Histaminergic neurons from the hypothalamus send out axons contacting neurons innervating astrocytes, and most of the microvessels of the brain (Takagi *et al*., 1986; Wada *et al*., 1991), suggesting that histamine may play an important role in cerebrovascular modulation (Watanabe *et al*., 1984).

Serotonin receptors on microglia cells are not well investigated. 5HT7-

receptors were detected in a microglia cell line. Application of serotonin caused increased permeability of microvessels. It can be released from circulating platelets, but also from mast cells, endothelium, and serotoninergic vascular nerves.

2.6 The calcium sensor GCaMP2

GCaMP2 is a calcium sensor based on a circularly permutated eGFP coupled to a fragment of a myosin kinase M13 and a modified calmodulin protein. GCaMP2 has a low basic fluorescence. Upon calcium binding to M13 and the calcium binding domain of calmodulin a conformational change in the circularly permutated eGFP molecule occurs, which leads to an increase in fluorescence (Figure 2.3). GCaMP2 has a very low Kd value of 146 nM and a Hill coefficient of 3.8. The sensor was designed for reduced magnesium sensitivity. Maximal excitation wavelength is 488 nm and maximal emission was measured at 508 nm (Tallini *et al*., 2006).

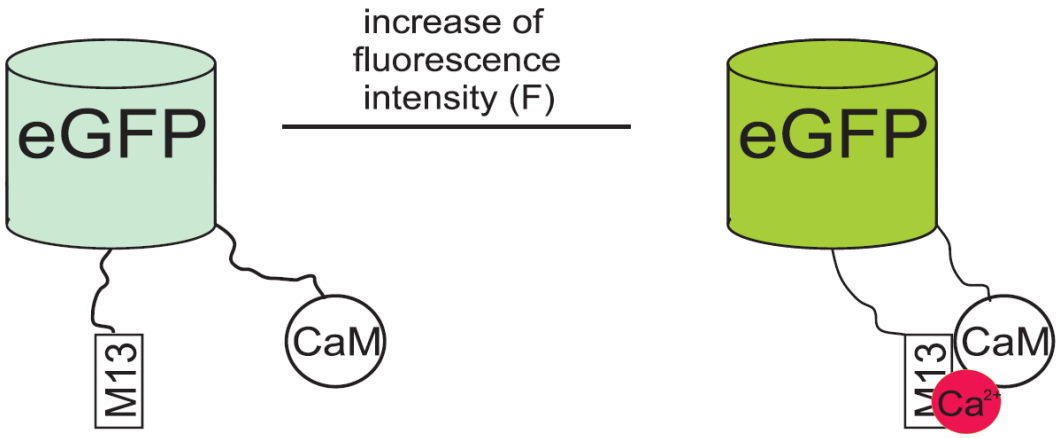


Figure 2. 3 Schematic representation of the calcium sensor GCaMP2. The sensor was constructed based on a circularly permutated eGFP connected to M13-fragment of myosin kinase and modified calmodulin. Upon calcium binding the basic fluorescence increases. Picture modified from Nakai et al., 2001.

3. Aim

Physiological properties, like membrane currents and intracellular calcium of microglial cells were mainly studied in primary cell cultures prepared from neonatal rodents. A few studies on microglia from brain slices of adult mice revealed that the adult cells show a distinct phenotype and differ in their physiological properties. Microglia from neonatal animals might therefore reflect the developing brain. For studying microglia from adult animals, new techniques need to be developed to access the cells. New isolation and culturing methods allowed me to study cultured microglia from adult mice.

The aim of this study is (1) to characterize the electrophysiological properties of adult microglia in cell culture and compare them to microglia preparations of neonatal animals *in vitro* and microglia of adult animals *in situ*. The hypothesis is that microglia reflect the properties of the adult brain and therefore differ from neonatal microglia after isolation.

Signaling molecules like neurotransmitters/-peptides, chemokines and blood components can modulate microglia properties *in vitro*. Neurotransmitter/-peptide receptors were identified in microglial cells *in vitro* and many bind to G-protein coupled receptors which lead to an increase in intracellular calcium. The molecular imaging of calcium ions in response to ligand application in adult microglia was only investigated in one recent study (Eichhoff *et al*., 2010).

The aim of this work is (2) to express a genetically encoded calciumsensor in adult microglial cells *in vivo*. This technique will allow the identification of endogenous ligands of microglia in the context of their surrounding brain tissue. My hypothesis is that ligand induced calcium signals change dependent on the microglial activation status after injury. Furthermore I suggest, that calcium signaling in response to neurotransmitter and neuropeptides of adult microglia in brain slices differ compared to cultured microglial cells prepared from neonatal animals.

Following steps need to be realized to answer this hypothesis:

1. Recloning of the calcium sensor GCaMP2 insert into a retroviral vector

2. Retroviral transduction and expression tests of eGFP and calcium sensor GCaMP2 in microglial cells *in vitro* and *in situ*
3. Characterization of eGFP expressing microglia in the stab wound injury
4. Identification of endogenous ligands in GCaMP2 expressing microglia with calcium imaging technique in acute brain slices
5. Application of endogenous ligands at different time points after stab wound injury and comparission of the calcium signals to microglia in cell culture

4. Material and methods

4.1 Material

4.1.1 Drugs and chemicals

Material	Company
Ampicillin	Sigma-Aldrich, Munich, Germany
Aqua-Poly/Mount	Polysciences Europe GmbH, Eppelheim, Germany
Blasticidin	Sigma-Aldrich, Munich, Germany
Bovine serum albumin (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
70µm cell strainer	BD Bioscience, Heidelberg, Germany
Chlodronate	Calbiochem, Darmstadt Germany
Dulbecco's Modified Eagle Medium (DMEM)	GIBCO®Media, Invitrogen, Darmstadt, Germany
Fetal calf serum (FCS)	GIBCO®Serum, Invitrogen, Darmstadt, Germany
Glucose	Merck, Damstadt, Germany
Hanks Balanced Solution (HBSS)	GIBCO®Products, Invitrogen, Darmstadt, Germany
HEPES for cell culture	GIBCO®Products, Invitrogen, Darmstadt, Germany
Ketanest	Bela-Pharm, Vechta, Germany
LPS (Lipopolysaccharide from *E.coli*)	AXXORA DEUTSCHLAND GmbH, Lörrach, Gemany
Normal goat serum (NGS)	GIBCO®Products, Invitrogen, Darmstadt, Germany
Percolll	GE Healthcare, Munich, Germany

PBS (Phosphate buffered saline)	GIBCO®Products, Invitrogen, Darmstadt, Germany
Pyromycin	Sigma-Aldrich, Munich, Germany
Rhomputin	Bayer Vital, Mannheim, Germany
Trypsin	GIBCO®Products, Invitrogen, Darmstadt, Germany
Trition X-100	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
homogenizer (glass potter)	Sigma-Aldrich, Munich, Germany
Glass capillaries with filaments	Hilgenberg, Malsfeld, Germany
Glue (Sekundenkleber)	UHU GmbH & Co. KG Bühl/Baden, Germany

Table 4. 1 List of drugs and chemicals

4.1.2 Media and buffers

Name	Composition
Artificial spinal cord fluid (ASCF)	NaCl 134.0 mM, KCl 2.5 mM, MgCl2 1.3 mM, CaCl2 2.0 mM, K2HPO4 1.2 mM, Glucose 10.0 mM, NaHCO3 26.0 mM, adjusted with NaOH to pH 7.4
Dulbecco's Modified Eagle Medium (DMEM)	GIBCO®Media, supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin)
Griess-Reagenz	Solution A: 300 mg Naphthylethylene in 300ml Aqua dest. Solution B: 3g Sulfanilamid, 18ml H3PO4 (85%ig)
HEPES buffer	NaCl 150.0 mM, KCl 5.4 mM, MgCl2 1.0 mM, CaCl2 2.0 mM, HEPES 10.0 mM, Glucose 10.0 mM, adjusted with NaOH to pH 7.4
Intracellular pipette solution	KCl 130 mM, MgCl2 2 mM, CaCl2 0.5 mM, EGTA 5.0 mM, and HEPES 10 mM, adjusted with NaOH to pH 7.3, 285 mmol
L929 conditioned medium	L929-Fibroblasts were grown to 80% confluence in a T75 flask, 30ml fresh normal medium was added, after 2 days medium was harvested, medium was steril filtered and mixed with 2/3 DMEM
LB medium	1.0% Tryptone, 0.5% Yeast extract, 1.0% NaCl, pH 7.0
Transfer buffer	140mM NaCl, 5mM KCl, 1.1mM Na_2HPO_4 x $2H_2O$, 1% Hepes pH 6.75-6.76
0.9 % saline	0.9% NaCl, Aqua dest. sterile

Table 4. 2 List of media and buffer

4.1.3 Fluorescent probes, enzymes and antibodies

Probe	Company
Alexa® 594 goat anti rabit	Invitrogen, Karlruhe, Germany
Alexa Fluor®594 -conjugated tomato lectin	0.47 mg/ml Tomatolectin from *Lycopersicon esculentum* (Vector Laboratories, Burlingame, USA) 26.8µg/ml Alexa® 594, Succinimidester (Invitrogen, Karlruhe, Germany) 5 mM Phosphate 15 mM NaCl 0.1 mM CaCl2 Linkage by BioTez GmbH, Berlin, Germany
Bgl II	New England Biolabs GmbH, Frankfurt am Main, Germany
EcoR I	New England Biolabs GmbH, Frankfurt am Main, Germany
Fluo-4 AM, cell permeant	Invitrogen, Karlruhe, Germany
Not I	New England Biolabs GmbH, Frankfurt am Main, Germany
Pst I	New England Biolabs GmbH, Frankfurt am Main, Germany
Secondary antibodies conjugated to fluorescein isothiocyanate (FITC), rhodamine red or Cy5	Jackson ImmunoResearch Europe Ltd., Suffolk, UK
Iba-1 antibody	Wako Pure Chemical Industries, Ltd., Japan

Table 4. 3 List of fluorescent probes, enzymes and antibodies

4.1.4 Kits

Kits	Company
BCA total protein assay	Pierce Biotechnology, Rockford, USA
BrdU labeling and detection kit III	Roche, Mannheim, Germany
Easypure Kit	Biozym. Hess-Oldendorf, Germany
Enzyme linked immunosorbent assay (ELISA) for IL-1beta, IL-6 and TNFalpha	R&D Systems, Wiesbaden, Germany
EndoFree® Plasmid Maxi Kit	Qiagen, Hilden, Germany
Invisorb®SpinPlasmid Mini	Invitek, Berlin, Germany
Klenow Fill-In Kit	Stratagene GmbH, Heidelberg, Germany
LigaFastTM Rapid DNA Ligation System	Promega, Mannheim, Germany
RNeasy micro kit	Invitrogen, Karlruhe, Germany

Table 4. 4 List of kits

4.1.5 Cells

Cells	Company
Competent *E.coli* cells JM 109	Promega, Mannheim, Germany
L929 fibroblast cell line	Promega, Mannheim, Germany
Microglia cells	Isolation from C57/Bl6 mice
Platinum E (Plat-E)	Cell Biolabs, San Diego, USA

Table 4. 5 List of cells

4.1.6 Devices

Devices	Company
Axiovert FS microscope	Zeiss, Oberkochen, Germany
Leica LSM laser scanning confocal microscope	Leica, Wetzlar, Germany
Microplate plate reader Infinite M200	Tecan, Crailsheim, Germany
Monochromator Polychrome II	Till Photonics, Martinsried, Germany
Patch clamp amplifier EPC10	HEKA electronics, Lambrecht, Germany
Stereotactic alignment system	David Kopf Instruments, Tujunga, USA
Vibratome VT 1000 S	Leica, Heidelberg, Germany

Table 4. 6 List of devices

4.1.7 Software

Software	Company
Adobe Illustrator 11.0	Adobe Systems, San Jose, CA, USA
Adobe Photoshop CS 8.0	Adobe Systems, San Jose, CA, USA
ICE software	Own development
Image J 1.43	http://rsbweb.nih.gov/ij/index.html
Microsoft Office 2003/ 2007/ 2010	Microsoft Deutschland, Berlin, Germany
Origin 7.0	OriginLab, Northhampton, USA
SPSS foe Windows 11.5.1	SPSS/ IBM, NY, USA
TIDA	HEKA electronics, Lambrecht/Pfalz, Germany

Table 4. 7 List of software

4.2 Methods

4.2.1 Microglia preparations

4.2.1.1 Microglia cultures from neonatal mouse brain

Primary microglia cultures were prepared from cerebral cortex of neonatal C57/Bl6 mice (P 0-P3) as described previously (Prinz *et al*., 1999). In brief, cortical tissue was freed of blood vessels and meninges in Hank's Balanced Salt Solution (HBBS) and trypsinized in 1 % trypsin and 0.05 % deoxyribonuclease for 5 min at RT. Digested tissue was dissociated with a fire-polished pipette, and washed twice in HBBS. Dissociated cells were plated on T75 flasks coated with poly-L-lysine and cultured in standard cell culture medium (DMEM). After 9 – 12 days with medium change every three days, microglia were isolated from the whole brain culture by gentle shaking and collected at 800 rpm for 10 min at RT. The cells were then seeded on glass coverslips or 96-well plates at a density of 1-1.5 × 10^5 cells/coverslip or 5× 10^5 cells/well, respectively. Cultures usually contained >95% microglia detected by isolectin B4 (*Griffonia simplicifolia*). Experiments were performed within 1 to 3 days after plating.

4.2.1.2 Microglial cultures from adult mouse brain - Percoll isolation

Isolation of microglia from adult mice was adapted from de Haas *et al*. 2007. 8 weeks old C57BL/6 mice were transcardially perfused with 0.9 % saline to clear the intravascular compartment of blood cells. Extracted brains were stored in ice-cold Hank's balanced salt solution containing 15mM HEPES and 0.5 % Glucose throughout the whole isolation procedure. Tissue was mechanically grinded in a tissue homogenizer and subsequently filtered through a 70µm cell strainer. Single cell suspension was pelleted at 300 g for 10 min at 4°C with low brake and resuspended in ice-cold 75 % Percolll obtained by mixing nine volumes Percoll and one volume 10x HBSS followed by dilution of this stock Percoll solution with PBS. This was gently overlayed with ice-cold 25 % Percoll solution followed by one layer with PBS. This density gradient was centrifuged at 800 g for 25 min at 4°C with low acceleration and no brake. After removing the myelin layer at the 0/25 interface with

a Pasteur pipette, the 25/75 interface containing mononuclear cells was collected using a fresh Pasteur pipette. For centrifugation cell-Percolll suspension was filled maximally with ice-cold PBS. Centrifugation at 300 g for 20 min at 4°C with low brake resulted in a cell pellet which was subsequently used for RNA isolation using the RNeasy micro kit according to the manufacturer's instructions.

4.2.1.3 Microglial cultures from juvenile and adult mouse brain - isolation from whole brain cultures

For preparation of whole brain cultures tissue from the brains of newborn C57/Bl6 mice (P 0-P3) was freed from blood vessels and meninges in Hank's Balanced Salt Solution (HBBS) and trypsinized in 1 % trypsin and 0.05 % deoxyribonuclease for 5 min at RT. Digested tissue was dissociated with a fire-polished pipette, and washed twice in HBBS. Dissociated cells were plated on T75 flasks with poly-L-lysine and cultured in DMEM. Cells were allowed to grow for 7 days until 100 % confluency was reached. 200 µg/ml chlodronate was added and cells were incubated over night at 37°C, 100rpm on a shaker and washed twice in HBSS. Next day brains of mice (P 22 and P 49-56) were freed of blood vessels and meninges in HBBS and trypsinized in 1 % trypsin and 0.05 % deoxyribonuclease for 5 min at RT. Digested tissue was dissociated with a fire-polished pipette, and washed twice in HBBS. Dissociated cells were plated on top of the whole brain cultures. Medium was changed next day. After 5 days medium was exchanged to 33 % L929 conditioned medium.

4.2.1.4 Acute brain slice preparation

Mice were decapitated. Skin and skull was opened and whole brain was removed and washed in ice cold ACSF. The brain was cut transversally in two parts. Forebrain was fixed with glue to a slicing chamber. Ice cold ACSF solution was added. 130 µm horizontal brain slices were prepared by vibratome VT 1000 S and stored in room temperature ACSF until recording.

4.2.2 Cell culture assays

4.2.2.1 Proliferation assay

Microglia were stimulated for 48 h in 96-well plates with standard cell culture medium (DMEM), DMEM mixed with 50 % virus conditioned medium (virus), DMEM mixed with 33 % L929 conditioned medium (L929) or DMEM mixed with 33 % L929 conditioned medium and 50 % conditioned medium (L929+virus). To quantify cell proliferation, 100 µl BrdU labeling solution/ml normal medium was added to cells according to manufacturer's instructions of the BrdU labeling and detection kit III. The amount of BrdU incorporation by the cells was taken as a measure of proliferation. In brief, cells were first fixed at -20°C for 30 min followed by partial digestion of cellular DNA with nuclease solution for another 30 min at 37°C. Digested samples were further incubated with anti-BrdU-POD solution for 30 min at 37°C under 5 % CO2. Finally, peroxidase solution with enhancer was added for 30 min at RT. Cleavage of ABTS produced a colored reaction product which was then quantified at 405 nm against 490 nm using a microplate reader (Infinite M200 Tecan). Basal and LPS-induced BrdU incorporation (i.e. proliferation) was normalized to respective controls and to the protein amount per well determined with BCA total protein assay (Pierce Biotechnology).

4.2.2.2 Cytokine assay

To determine cytokine and chemokine release *in vitro*, cells plated in 96-well plates were treated for 48 h with test substances diluted in normal medium. Medium was collected after 48 h treatment and measured for the released amount of IL-6, IL-1beta and TNF-alpha, using ELISA according to the manufacturer's instructions (R&D Systems). The resulting colorimetric reaction product was measured at 450 nm against 540 nm using a microplate reader (Infinite M200 Tecan). Standard curves ranging from 125 to 2000 pg/ml were used. BCA protein assay was performed following instructions of the manufacturer. Cytokine release was normalized to protein amount for each well.

4.2.2.3 NO assay

To determine the relative amount of NO release, Griess reagent was used. Cultured microglia plated in 96-well plates were stimulated for 48 h with DMEM, virus, L929, L929 and virus or with LPS (100 ng/ml, positive control). 50µl of cell culture supernatant from each well were mixed with 50 µl Griess reagent. The resulting colorimetric reaction product was measured at 540 nm by microplate reader (Infinite M200 Tecan).

4.2.3 Retroviral gene transfer

The task of this work was to express the recombinant proteins eGFP and GCaMP2 in microglial cells. Retroviral gene transfer is a technique for efficiently introducing stable, heritable genetic material into the genome of any dividing cell type (Ausubel *et al*., 1995; Coffin *et al*., 1996). The production of retrovirus is described in the following section in detail. Virus was produced by transiently transfecting a retroviral expression vector into an ecotropic packaging cell line.

4.2.3.1 Retroviral vectors

The retroviral vector pMP71-GCre encodes for eGFP (Engels *et al*., 2003). The eGFP fragment was restricted by EcoRI and NotI and exchanged by the 1369bp insert GCaMP2 fragment, which was obtained from pN1-GCaMP2 (Tallini *et al*., 2006) expression vector restricted with BglII and NotI. The fragments were separated by gel electrophoresis and isolated with Easypure Kit. Klenow Fill-In Kit was used to create blunt ends. The GCaMP2 fragment was ligated to a pMP71 backbone by *E.coli* using LigaFastTM Rapid DNA Ligation System. Competent *E.coli* cells were used to amplify the ligated vector. The cells were plated on 50 µg/ml ampicillin supplemented agar plates. Colonies were isolated and plasmid DNA was purified by Invisorb®SpinPlasmid Mini. Correct cloning was examined in a test restriction with PstI. An adequate probe was selected and used for retransformation into competent *E.coli* to amplify the retroviral expression vector. Cells were grown in 50 µg/ml ampicillin supplemented LB medium overnight. The retroviral expression vector was

isolated by EndoFree® Plasmid Maxi Kit. In Table 4.8 the expression vectors used in the present work are listed.

Expression vector	Promotor	Protein expression
pMP71-GCre	retroviral LTR	eGFP
pN1-GCaMP2	CMV	eGFP based calcium sensor GCaMP2
pMP71-GcaMP2	retroviral LTR	eGFP based calcium sensor GCaMP2

Table 4. 8 List of expression vectors, promotor sequences and recombinant proteins (for detailed vector maps see appendix)

4.2.3.2 Production of retroviral vector particles

For production of the virus particles the retrovirus packing cell line PlatE was used (Morita *et al*., 2000). This cells lines package recombinant retroviral RNAs into infectious, replication-incompetent particles. The viral gag, pol, and ecotropic env genes, necessary for particle formation and replication, are stably integrated into the genome. The vector particles were produced by transiently transfecting PlatE cells with pMP71-GCre or pMP71-GCaMP2 vector with the following protocol:

Cells were seeded to $7.5x10^5$/3.5 cm well. 24 h later the cells were transfected by calcium phosphate transfection. 15 µl calcium phosphate (2.5 M) and 18 µg retroviral expression plasmid DNA (pMP71-GCre, pMP71-GCaMP2) were diluted in 135 µl water and mixed. During mixing 150 µl transfer buffer was added drop wise. The solution was incubated 15-20 min at room temperature and mixed again. The entire 300 µl solution was then added to one well of the PlatE. The cells were incubated for 6 h at 37°C, 5 % CO_2, 90 % humidity and medium was changed to standard cell culture medium. Virus conditioned medium was filtered through a 0.45 µm pore size filter 36-40 h after transfection and used for transduction *in vitro* or *in vivo*.

4.2.3.3 *In vitro* transduction

Cultured microglial cells were prestimulated with L929 conditioned medium (33%) to trigger proliferation (Sawada *et al*., 1990). After 18 h medium was exchanged by virus conditioned medium (50 %) and mixed with fresh standard cell culture medium (22 %) and L929 conditioned medium (33 %). Cultured microglia plated in 96-well plates or in coverslips were stimulated for 24 or 48 h at 37°C, 5 % CO_2, 90 % humidity.

4.2.3.4 Induction of the stab wound and transduction *in vivo*

All mice used for this study were kept under standard housing conditions with a 12 h/12 h dark-light cycle and with food and water *ad libitum*. All experiments were performed according to the guidelines of the German law for animal protection.

In order to trigger microglia proliferation, a stab wound was induced by inserting a needle into the cortex. After two days, 1 µl retroviral conditioned medium was injected into the same location leading either to eGFP or GCaMP2 expression in dividing cells *in vivo:* Adult C57BL/6 mice (8-12 weeks) were anesthetized by i.p. injection with Rhomputin (20 mg/ml, 9.2 mg/kg KG, Bayer Vital) and Ketanest (100 mg/ml, 131mg/kg KG, Bela-Pharm). During surgery, animals were restrained using a stereotactic alignment system (David Kopf Instruments). A 1 cm-transsection was cut into the scalp. Scull and meninges were drilled through by a cannula (18 ga). A 1µl Hamilton syringe (22 ga, Ø 0.7 mm, blunt tip) was inserted into the right frontal cortex (1.5 mm deep, Bregma coordinates: +2 mm lateral, +2 mm frontal) for 3 min. After removing the needle the skin was closed by saturation. Two days after induction of the stab wound, animals were anesthetized by i.p. injection with Rhomputin and Ketanest as described above. The animals were restrained in the stereotactic alignment system. The incision was opened and the Hamilton syringe was inserted in the side of the stab wound followed by injection of 1 µl retrovirus suspension within 5 min. 200 nl virus was injected per min into the cortex by withdrawing the needle stepwise out of the tissue. Survival times of the mice were 3, 6 21 or 42 days. Brains were used to prepare brain slices (Figure 4.1).

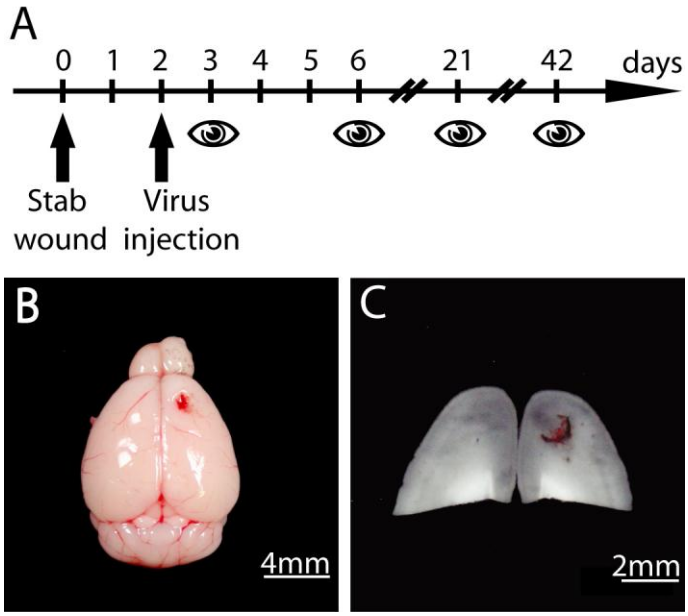


Figure 4. 1 *In vivo* transduction and stab wounded brain. A, Timeline of stab wound injury, virus injection and observation time points at 3, 6 21 and 42 days. B, Stab wounded mouse brain 6 days after stab wound injury. The injury was induced into the right frontal cortex. C, Horizontal acute brain slice (130 µm) with stab wound in the right frontal cortex.

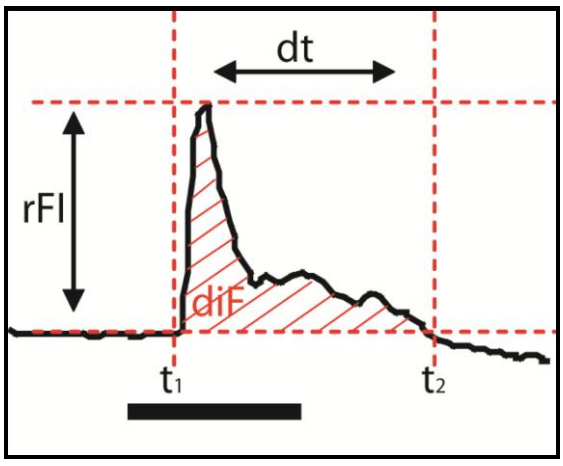
4.2.4 Physiological methods

4.2.4.1 Patch clamp experiments

Acute brain slices were prepared as described above. Slices or coverslips were placed in a chamber on the stage of a microscope (Axiovert FS, Zeiss, Oberkochen). Acute brain slices were superfused with artificial spinal cord fluid. For cell culture studies, coverslips with cells were placed in the recording chamber and superfused with HEPES buffer at pH 7.4, 3–5 ml/min. Cells were approached using microelectrodes with a resistance of 5–8 MΩ pulled from borosilicate capillaries with filaments (Hilgenberg, Malsfeld). All recordings were performed at room temperature. Whole cell uncompensated currents were measured with conventional electronics (EPC9/10-amplifier, HEKA electronics, Lambrecht/Pfalz).

4.2.4.2 Calcium imaging

Acute brain slices were prepared as described above. The slices were incubated in ACSF supplemented with 1 % Alexa Fluor®594-conjugated tomato lectin (short tomato lectin) for 25 min. After staining, the slices were superfused with ACSF. For cell culture experiments, coverslips were superfused with HEPES buffer. Buffer flow was adjusted to 3–5 ml/min. Fluorescence pictures were taken every 3 s at 488 nm excitation wavelength produced by a Polychrome II monochromator (Till Photonics, Martinsried). High pass emission filter was used (535DF35, Omega optics, maximal emission at 535 nm) to detect eGFP fluorescence. A perfusion system was installed to change the solution within seconds. Microglia were identified at excitation wavelength 560nm (emission filter at 630 nm) to detect fluorescence of tomato lectin. Data were recorded with an LCD camera and TIDA (HEKA electronics, Lambrecht/Pfalz) and ICE software (own development). Relative fluorescence intensity (rFI) was measured as the ratio of fluorescence intensity during the experiment over the average baseline fluorescence intensity (F/F0). Calcium signals were only considered as signals, if rFI was ≥ 4 times of SD of F/F0. Duration (dt) and the maximal change of relative fluorescence intensity (max rFI) of every cell was determined. Integrated fluorescence (diF) was determined by the following equation:



$$diF = \int_{t1}^{t2} rFI\ dt$$

Figure 4. 2 Qualification of the fluorescence signal by definition of relative fluorescence intensity (rFI), duration (dt) and integrated fluorescence (diF). t_1 start of the fluorescence signal, t_2 end of the fluorescence signal.

4.2.5 Immunohistochemistry

130 µm slices from patch clamp or imaging studies were fixed for 1 h in ice cold 4 % paraformaldehyde. After fixation slices were stored in 0.1 M PB until staining. Slices were incubated with 2 % Trition X-100, 2 % BSA and 10 % normal goat serum. Primary antibody Iba-1 (Wako Pure Chemical Industries, Ltd.) was diluted in 0.1 M PBS, 0.5% Triton X-100, 10 % normal goat serum and incubated for 48 h at 4°C. Appropriate specific secondary antibodies conjugated to FITC, rhodamine red or Cy5 were diluted to 1:200 and applied for primary antibody detection. Slices were washed 3 times and mounted on glass slides by Aqua-Poly/Mount.The specimens were observed by using a Leica LSM laser-scanning confocal microscope. Sections were optically sliced in the z-plane by using 0.5 µm intervals, and cells were rotated in orthogonal planes to verify double labeling.

4.2.6 Statistics

Data were tested for normality with the Shapiro-Wilk test. Electrophysiological data and calcium imaging sets were mostly not normal distributed, therefore Kruskall-Wallis test (nonparametric) was used. For pair wise testing Mann-Whitney-U-Test with Bonferroni-posthoc correction was performed. Data are graphically depicted as box plots with median (black bar); 75^{th} percentile (upper top of the box), 25^{th} percentile (upper top of the box) and whiskers indicating the minimum and maximum of the data set within one group. In the text data are written as median, 75^{th} percentile, 25^{th} percentile. For comparison of the percentage of responding cells between the time points Pearson Chi-square test was used. Statistical analysis was accomplished by SPSS software. P-values were considered as significant, if * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Cell culture assay are displayed as averaged values of 3 repetitions á 8 experiments ± SD. Student's two-tailed t-test was performed with Microsoft Office Exel software. P-values were considered as significant, if * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

5. Results

5.1 Electrophysiological characterization of adult microglia

5.1.1 Microglial cells from different preparations

Cultured microglial cells from neonatal pups are the most common microglia model investigated by whole cell patch clamp technique, whereas adult microglia have been only characterized in brain slices. Here microglial cells from adult mice were isolated and patched after different culturing times. The current profiles were compared to non-isolated adult microglia from brain slices (P 49-56) and cultured microglia from neonatal pups (P 0-3). Cells were clamped to a holding potential of -70 mV. Current amplitudes to 50 ms depolarizing and hyperpolarizing voltage steps between 50 mV and -160 mV with 10 mV increments were recorded. The current of the -150 mV step (inward current) and of the 0 mV step (outward current) was averaged over 50 ms.

Microglia from acute brain slices of the frontal cortex showed small inward currents with a median of -46.050 pA; -62.676; -33.861 and small outward currents of 24.364 pA; 19.864; 28.406 (Figure 5.1 A, n=10). Controversially cultured microglia derived from neonatal pups showed huge inward (-424.438 pA; -564.107; -232.042, $p< 0.001$) and outward currents (77.941 pA ; 53.321; 112.741, $p< 0.001$) (Figure 5.1 E, n=22). I wanted to know which kind of currents appeared in cultured microglia from the adult brain. Microglia were isolated by Percoll gradient from the cortex of adult mice and seeded on coverslips. When microglial cells were cultured for 3-8 h (Figure 5.1 B; n= 15) I found similar inward currents (-69.590 nA; -129.004; -43.090; $p=0.11$) but reduced outward currents (10.050 pA; 8.265; 13.966; $p<0.001$) in comparison to non-isolated microglia from brain slices. The Percoll isolated adult microglia were kept in cell culture overnight (Figure 5.1 C; n= 63). When microglial currents were investigated between 22-36 h in culture increased inward currents (-111.714 pA; -189.570; -67.390; $p<0.001$) were found, but outward currents (9.040 pA; 6.050; 13.050; $p<0.001$) were still reduced in comparison to microglia from brain slices. 73 % of the microglia kept for 22-36 h in cell culture showed increased inward currents at -150 mV compared to microglia 3-8 h in culture. The inward rectifying currents inactivated time dependent (τ=34.48 ms ± SD 24.59), when the cells were clamped to

hyperpolarizing voltage potentials lower than -120 mV. 50.7 % of the cells showed inactivating inward currents when cultured for 22-36 h (n=65).

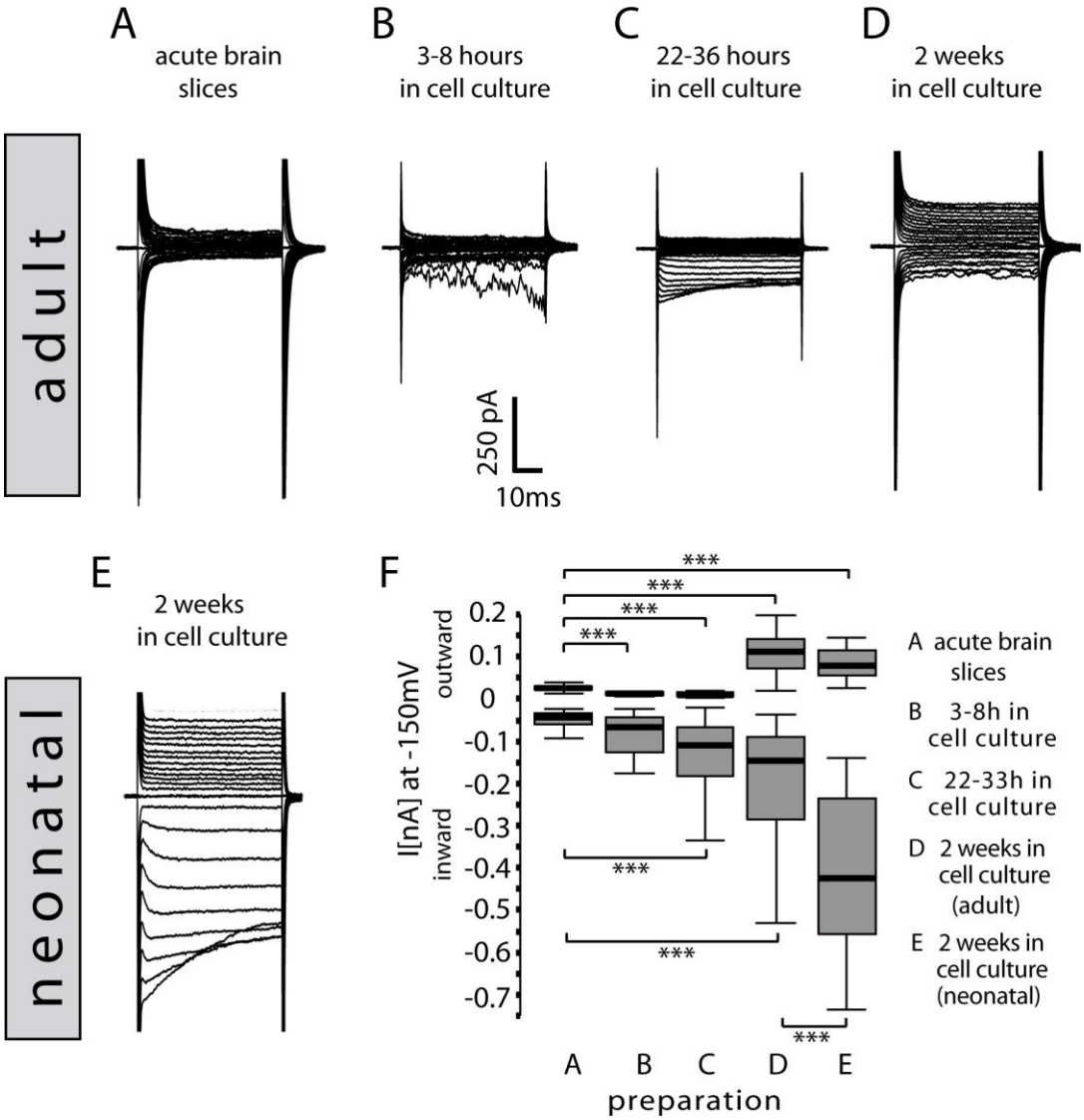


Figure 5. 1 Whole-cell patch clamp recordings from adult microglia cells of different preparations. Current profiles in response to a 10 mV voltage step protocol from a holding potential of -70 mV. Depolarizing voltage steps up to 50 mV and hyperpolarizing voltage step up to -160 mV were applied for 50 ms. Adult microglial cells of acute brain slices (A, n=10), adult microglia in cell culture for 3-6 h (B, n=15) or for 22-36 h (C, n=65) after Percoll isolation, adult microglia (P 49-56) grown on whole cell culture for 2 weeks (D, n=17), neonatal microglia (P 0-3) grown on whole cell culture for 2 weeks (E, n=22). F, Comparison of median current amplitudes (black bar) at a potential of -150 mV (inward current, lower box plots) and at a potential of 0 mV (outward current, upper box plots). Mann-Whitney U-test, *** $p<0.001$.

A new preparation technique developed by Jörg Scheffel in the laboratory of Prof. Uwe Karsten Harnisch (Institute of Neuropathology, University of Göttingen, Germany, not published) allowed me to characterize cultured adult microglia after 2 weeks in culture for the first time. In this method adult microglia were grown on top of neonatal whole cell brain cultures. The cells can be isolated 2 weeks after preparation. This preparation method is comparable with the preparation of neonatal microglia, which are also grown on whole brain cultures for 2 weeks and then isolated and seeded on coverslips. To characterize microglia earned from this preparation I performed patch clamp recordings (Figure 5.1 D). These adult microglia revealed less rectifying inward currents (-148.280 pA; -288.458; -90.956; $p<0.001$) compared to neonatal microglia, while outward currents (111.200 pA; 65.406; 142.237; $p=0.087$) were comparable. Adult microglia from brain slices showed tiny inward ($p<0.001$) as well as tiny outward currents ($p<0.001$) in contrast to microglia cultured for 2 weeks. 46 % of the adult microglia cultured for 2 weeks showed inactivation of inward currents ($\tau=23.69$ ms ± 34.78, $n=13$) when the cells were clamped to more hyperpolarizing potentials then -120 mV.

Taken together isolated microglia from the adult brain showed small current amplitudes like microglia from brain slices shortly after isolation. The current amplitudes increased during the culturing time. After longer culturing period (2 weeks) inward as well as outward currents were highly increased in comparison to microglia cells from brain slices. Nevertheless all microglia from the adult brain (isolated as well as non-isolated) show smaller inward current amplitudes than neonatal microglia. Inactivation of inward currents was found in adult as well as in neonatal microglia.

5.1.2 Adult microglia isolated by Percoll gradient

Microglia cells kept in culture for 22-36 h showed increased inward currents (Figure 5.1 C) in comparison to adult microglia from brain slices (Figure 5.1 A) when clamped to -150mV. On the other hand statistical analysis revealed no significant difference in the current amplitude at -150 mV between isolated microglia kept for 3-8 h and microglia 22-36 h in cell culture (see above), even when 73% of the cells showed

increased inward currents. Microglia cells showed strong inactivation of their inward current at -150 mV (Figure 5.2 A). Since inactivating currents were absent at -120 mV I analyzed additionally the current amplitudes of this step. Microglia in slices were ramified whereas isolated microglia in culture were roundish or had only a few processes. Since current amplitudes can be dependent on the morphology I calculated the cell specific conductance G [pS/pF] for every single cell for better comparison of microglia from acute brain slices and isolated microglia in culture. I calculated the mean membrane capacitance and the conductance for in- and outward currents. The outward conductance was estimated from the current difference between 0 and -20 mV and the inward conductance from -100 mV and -120 mV. The cell specific conductance G [pS/pF] was then determined by dividing the outward and inward conductance respectively by the mean membrane capacitance. For better overview I summarized the values for microglial current amplitudes and specific conductances in Table 5.1.

Preparation	Value	I [pA] inward	I [pA] outward	G [nS/pF] inward	G [nS/pF] outward
Slice	median	-3.117	2.277	-0.037	0.022
	SD	1.623	1.259	0.034	0.014
	75th percentile	-2.616	2.963	-0.030	0.040
	25th percentile	-4.138	1.787	-0.069	0.020
3-8h	median	-0.835	1.005	-0.009	0.011
	SD	2.103	0.747	0.022	0.008
	75th percentile	-0.646	1.397	-0.007	0.015
	25th percentile	-2.759	0.827	-0.029	0.009
22-36h	median	-2.252	0.904	-0.024	0.010
	SD	4.057	0.514	0.043	0.005
	75th percentile	-1.227	1.305	-0.013	0.014
	25th percentile	-6.332	0.605	-0.067	0.007

Table 5. 1 Electrophysiological data of non isolated and isolated microglia from cerebral cortex.

Analysis of the current amplitudes showed in principle the same results then analysis of the conductance. Isolated adult microglia patched within 3-8 h in culture revealed decreased inward ($p=0.007$) and outward conductance ($p=0.004$) in comparison to adult microglia cells from brain slices (Figure 5.2 C). The outward conductance was still reduced after a longer culturing period of 22-36 h ($p<0.001$) when compared to adult microglia from brain slices and did not differ from adult microglia cultured for 3-8 h. On the other hand inward conductance of adult microglia 22-36h in culture were significantly increased ($p=0.014$) compared to adult microglia cultured for 3-8 h and reached similar values to adult microglia from brain slices ($p=0.144$).

Detailed analysis of the conductance confirmed that Percoll isolated adult microglia loose inward and outward conductance during the isolation process (Figure 5.2 C), which was not expected by analyzing the currents at -150mV which showed inactivation, but could be uncovered by analyzing the currents at -120mV. Microglia cells from cerebral cortex were able to compensate for the lost currents after a culturing time of 22-36 h.

So far I only investigated microglia cells from the cerebral cortex. Percoll isolation of adult microglia is a suitable method to investigate also microglia from other brain regions, since microglia properties might differ between brain regions. Therefore non-isolated and cultured microglia of corpus callosum were investigated by patch clamp experiments (Figure 5.2 D and E). Like for microglia from cerebral cortex I determined currents and conductances as described above. Microglia patched in the corpus callosum and in cerebral cortex in brain slices showed no difference in inward conductance ($p=0.912$) nor in outward conductance ($p=0.579$) when compared to each other. I summarized the values for median current amplitudes and specific conductances for microglia from the corpus callosum in Table 5.2.

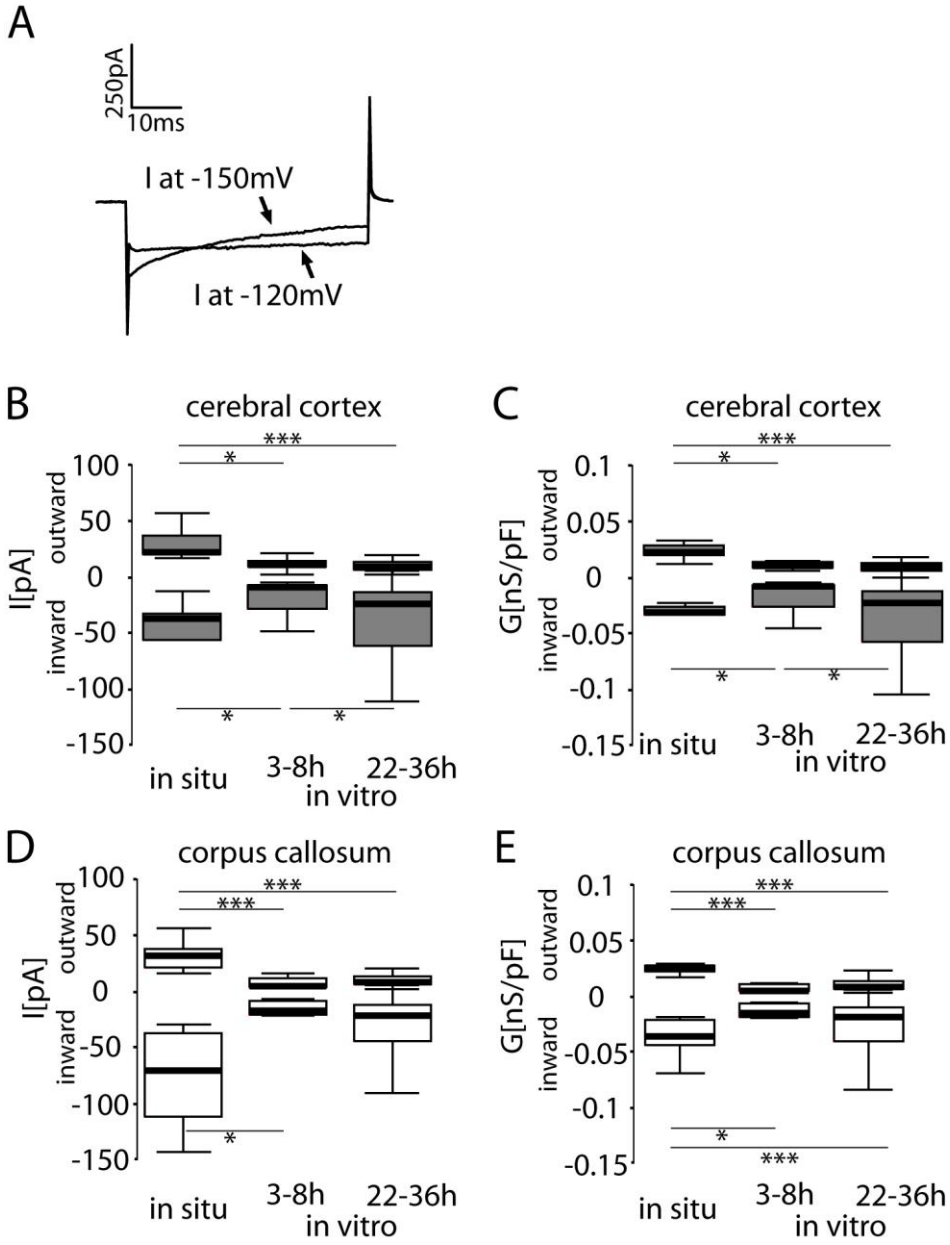


Figure 5. 2 Adult microglia isolated from cortex and corpus callosum. A, Inward currents (I) of a microglia cell cultured for 26 h clamped to -120 mV and -150 mV. Microglia cell shows strong inactivation of the current at -150 mV but not at -120 mV. B, Quantification of averaged current amplitudes (I [pA]) at 0 mV and -120 mV of microglia cells from brain slices (n=10), isolated microglial cells cultured for 3-8 h (n=17) and for 22-36 h (n=63) of the cerebral cortex. C, Quantification of specific conductance (G [nS/pF]) of the same cells like in B. D, Quantification of averaged current amplitudes (I) at 0 mV and -120 mV of microglia cells from brain slices (n=10), isolated microglial cells patched at 3-8 h (n=8) and 22-36 h (n=68) from corpus callosum. E, Quantification of specific conductance (G) of the same cells like in D. Mann-Whitney U-test, * $p<0.05$, *** $p<0.001$.

Preparation	Value	I [pA] inward	I [pA] outward	G [nS/pF] inward	G [nS/pF] outward
slice	median	-3.620	2.567	-0.069	0.033
	SD	1.851	0.606	0.043	0.012
	75th percentile	-3.523	3.972	-0.021	0.028
	25th percentile	-11.401	2.139	-0.050	0.022
3-8 h	median	-1.482	0.546	-0.016	0.006
	SD	2.143	0.343	0.023	0.005
	75th percentile	-0.683	1.318	-0.006	0.011
	25th percentile	-1.983	0.447	-0.019	0.005
22-36 h	median	-1.861	0.876	-0.020	0.009
	SD	2.459	0.676	0.026	0.007
	75th percentile	-1.049	1.453	-0.010	0.014
	25th percentile	-4.308	0.720	-0.040	0.007

Table 5. 2 Electrophysiological data of non isolated and isolated microglia from corpus callosum.

Microglia isolated from the corpus callosum showed reduced inward conductance ($p=0.01$) and outward conductance ($p<0.001$) when cultured for 3-8 h (Figure 5.2 E) in comparison to microglia from brain slices. The same results I found for microglia from cerebral cortex. After 22-36 h in culture the cells showed still reduced outward conductance ($p<0.001$) in comparison to non isolated microglia. Isolated cells from the corpus callosum did not show a significant increase inward conductance ($p=0.275$) as I found for microglia of the cerebral cortex after 22-36 h in culture (see above) compared to microglia 3-8 h in culture. Nevertheless direct comparison between isolated cultured microglia from corpus callosum and cerebral cortex did not show a difference in current amplitudes nor in conductances (all $p>0.05$). Summarized, microglia from cerebral cortex and from corpus callosum showed less inward and outward currents as well as reduced inward and outward conductance 3-8h after isolation from the brain tissue. Microglia from cerebral cortex and corpus

callosum do not differ in their current profiles, when directly compared. Nevertheless a minor difference in inward current activation after longer culturing periods of microglia was uncovered. Microglia from cerebral cortex were able to compensate for the loss of inward conductance, whereas microglia from corpus callosum showed still reduced inward conductance after 22-36h.

5.1.3 Adult microglia from whole brain cultures

A new preparation technique developed by Jörg Scheffel in the laboratory of Prof. Uwe Karsten Hanisch (Institute of Neuropathology, University of Göttingen, Germany, not published) allowed me to investigate not only cultured adult microglia, but also microglia from other age of mice. Here I ask the question how microglial cells from different age respond to an inflammatory stimulus. I chose LPS, which leads to microglia activation by binding to the microglial Toll-like receptor 4 (Qi & Shelhamer, 2005). LPS is a cell wall component of gram negative bacteria and induces an inflammatory response in microglial cells.

Microglial cells from neonatal (P 0-3), juvenile (P 22) and adult (P 49-56) animals were grown on whole brain cultures. Microglial cells were isolated after 2 weeks in culture. Isolated microglia were seeded on coverslips. Neonatal, juvenile and adult microglia were stimulated with 100 ng LPS overnight. Representative current profiles of control and LPS treated microglia of different age are shown in Figure 5.3. Amplitudes of currents were determined at -150 mV (inward) and 0 mV (outward). Specific conductance was calculated as described above.

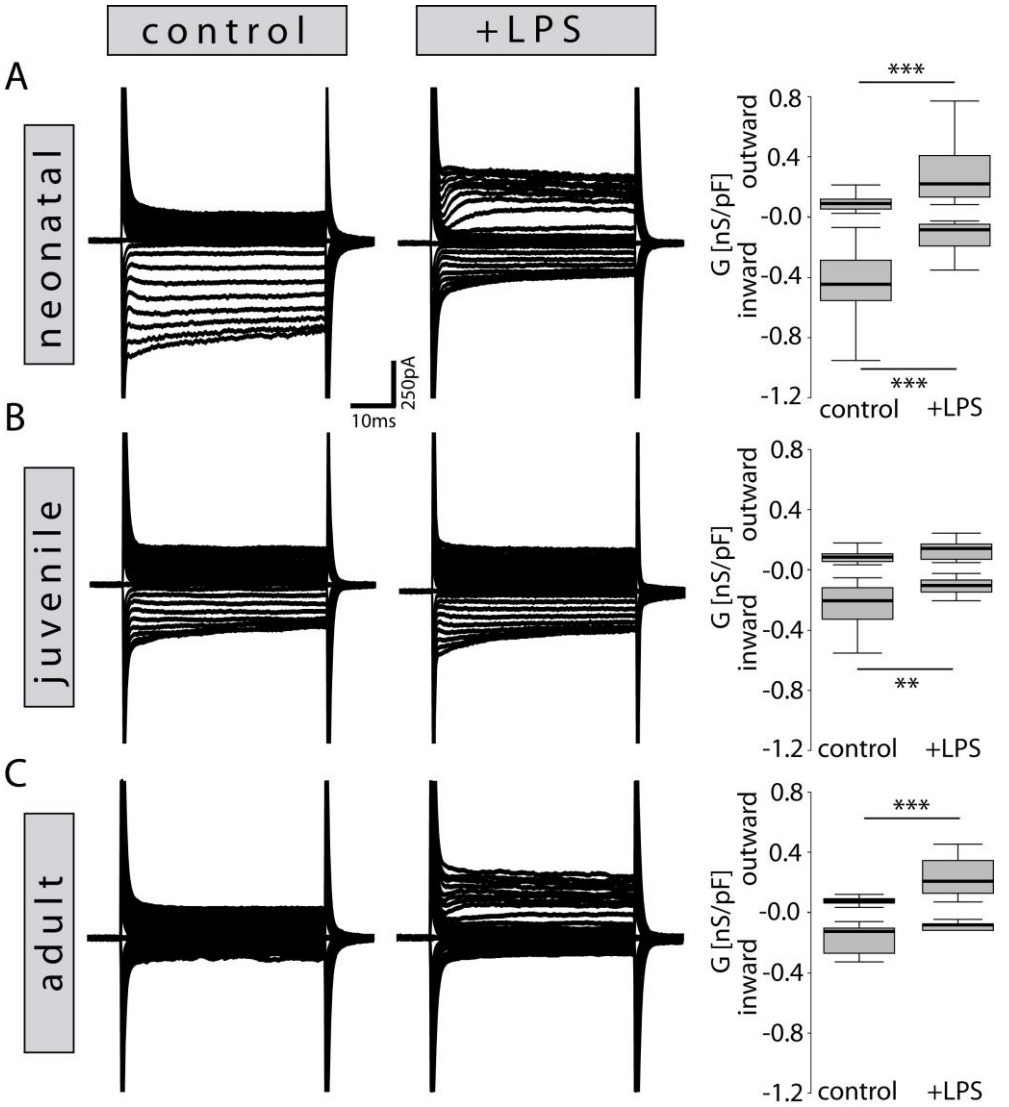


Figure 5. 3 Current profiles of cultured microglia prepared from neonatal, juvenile and adult mice. A-C, Control microglia are shown on the left side, whereas microglia stimulated with LPS (100 ng/ml) are in the middle. On the right side box plots of specific conductance G [nS/pF] of control and LPS treated microglia are displayed. A, Cultured microglia from early neonatal pups (P 0-3). B, Cultured microglia from juvenile mice (P 22). C, Cultured microglia from adult mice (P 49-54). Mann-Whitney U-test, * p<0.05, ** p< 0.01, *** p<0.001.

Patch clamp recordings from microglial cells of neonatal animals revealed inward rectifying currents and outward rectifying currents as described above. Upon stimulation with LPS the current profile changed. Inward currents decreased, whereas outward currents increased (Figure 5.3 A, n= 16). LPS-stimulated microglia

showed significant smaller inward conductance (p<0.001) and higher outward conductance (p<0.001) then non-stimulated microglia. Non-stimulated microglia from juveniles showed smaller inward conductance (p=0.01), than microglial cells from neonatal animals, but comparable outward conductance (p=0.296) (Figure 5.3 B, n=14). When microglia were treated with LPS (n= 16) surprisingly the microglia current profile did not change significantly for inward currents (p=0.173) and outward currents (p=0.544), whereas the inward conductance changed significantly (p=0.009) but the outward conductance not (p=0.109). Non-stimulated microglia (Figure 5.3 C, n= 17) cells from adult animals revealed also smaller inward currents (p=0.01) but similar outward currents (p=0.255) compared to microglia of neonatal animals. Upon LPS stimulation (n=14) the outward conductance increased (p<0.001) and inward conductance did not chance significantly (p=0.118) compared to non-stimulated microglia from adult animals. All median current amplitudes were summarized in Table 5.3., all median conductances in Table 5.4.

		without LPS		**with LPS**	
Group	**Value**	**I [pA] inward**	**I [pA] outward**	**I [pA] inward**	**I [pA] outward**
neonatal	median	-42.444	7.794	-10.753	18.110
	SD	18.050	4.388	16.585	15.044
	75th percentile	-23.204	11.274	-6.749	32.004
	25th percentile	-56.411	5.321	-17.984	10.259
juvenile	median	-16.508	10.089	-7.240	10.711
	SD	21.961	5.389	7.544	8.382
	75th percentile	-4.527	17.065	-5.401	22.965
	25th percentile	-31.453	7.097	-13.461	6.721
adult	median	-14.828	9.439	-7.968	18.683
	SD	16.382	13.206	7.207	15.291
	75th percentile	-9.096	14.237	-3.932	23.725
	25th percentile	-28.846	5.962	-13.470	12.788

Table 5. 3 Current amplitudes of cultured microglia from cerebral cortex of neonatal, juvenile and adult mice with and without LPS stimulation.

Taken together these results show that inward currents and inward conductances of control microglia in cell culture declines with age, but outward currents were equal in all 3 ages. Upon stimulation with an inflammatory stimulus, microglia from neonatal and juvenile animals change their current profile by reducing their inward currents and inward conductance. Microglia from neonatal and adult animals showed increased outward currents and conductances when treated with LPS, whereas the outward current and conductance in microglia from juvenile animals was not affected.

		without LPS		with LPS	
Group	**Value**	**G [nS/pF] inward**	**G [nS/pF] outward**	**G [nS/pF] inward**	**G [nS/pF] outward**
neonatal	median	-0.445	0.092	-0.085	0.219
	SD	0.206	0.066	0.185	0.197
	75th percentile	-0.285	0.121	-0.043	0.412
	25th percentile	-0.561	0.052	-0.212	0.122
juvenile	median	-0.202	0.090	-0.101	0.150
	SD	0.150	0.039	0.086	0.065
	75th percentile	-0.114	0.117	-0.063	0.189
	25th percentile	-0.343	0.060	-0.158	0.077
adult	median	-0.121	0.082	-0.080	0.214
	SD	0.131	0.030	0.154	0.118
	75th percentile	-0.092	0.106	-0.066	0.353
	25th percentile	-0.277	0.066	-0.143	0.129

Table 5. 4 Conductances of cultured microglia from cerebral cortex of neonatal, juvenile and adult mice with and without LPS stimulation.

5.2 Retroviral gene transfer – a tool to study microglial cells

5.2.1 Expression of a calcium sensor *in vitro*

Virus are naturally evolved vehicles which efficiently transfer genes into host cells. This ability makes them desirable for engineering virus vector systems for the delivery of therapeutic genes and gives the possibility to transfer genetic tools into target cells. In contrast to many other transfection methods, retroviral transduction can be used for *in vivo* gene transfer. I created a gamma-retroviral vector pMP71-GCaMP2 by molecular cloning, which carries the transgene GCaMP2. The GCaMP2 sequence codes for a single fluophore eGFP-based calcium sensor protein (Tallini et al., 2006; Nakai et al., 2001). The protein is expressed in the cytosol of the cell and upon calcium binding the basic fluorescence intensity of the protein changes. First I tested the pMP71-GCaMP2 construct in microglia cell culture for expression and functionality. Cell division, which is accompanied with breakdown of the nucleus, is necessary for expression of the transgene in the gamma-retroviral infected cells. Primary microglial cells divide little in culture, therefore microglial proliferation was triggered by treatment with conditioned medium of the fibroblast cell line L929 (Sawada *et al*., 1990).

Microglial cells were treated with standard cell culture medium (Figure 5.4 A) with virus conditioned medium only (Figure 5.4 B), with L929 conditioned (Figure 5.4 C) or with L929 and virus conditioned medium (Figure 5.4 D). To determine expression efficiency, I fixed the cells after 24 and 48 h and stained GCaMP2 expressing microglia with antibody directed against eGFP (which also recognizes GCaMP2) and DAPI. In contrast to control medium, L929 treated microglial cells showed a higher number of nuclei, which were mostly distributed pair wise, indicating that L929 triggers microglial proliferation. Neither microglial cells treated with virus conditioned medium only nor microglia treated with L929 conditioned medium only did express GCaMP2. Microglia expressed GCaMP2 only when treated with both L929 and virus conditioned medium. After incubation with L929 and virus conditioned medium 32 % (± 6 % SD) of the cells expressed GCaMP2 at day 2, at day 3 the percentage of GCaMP2 expressing cells was 37 % (± 2 % SD).

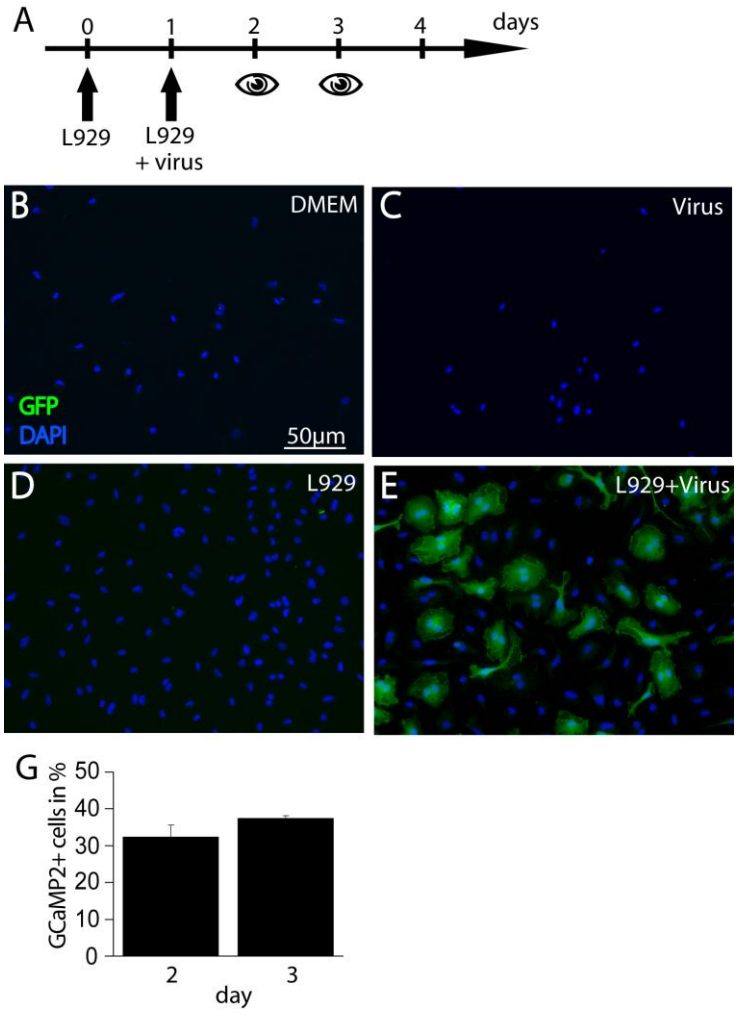


Figure 5. 4 *In vitro* expression of GCaMP2 in primary microglia. Timeline of microglial transduction in culture. Proliferation was triggered with L929 conditioned medium (L929), retroviral transduction was induced by adding L929 and virus conditioned medium (L929+virus). Primary microglia were observed at day 2 and day 3. Primary microglia treated with standard cell culture medium (B) or virus only (C) or L929 conditioned medium only (D) or treated with L929 conditioned medium and GCaMP2 virus (E). Cells were fixed and stained with GFP antibody (green) and DAPI (blue) after 48 h of retroviral incubation (day 3). G, Quantification of GCaMP2 expressing cells after stimulation with L929 conditioned medium and GCaMP2 retrovirus at day 2 and day 3.

To test the functionality of the GCaMP2 calcium sensor, the fluorescence intensity was recorded by taking serial images before (Figure 5.5 A), during (Figure 5.5 B) and after application of 500 µM ATP for 60 s. Sampling frequency was 0.3 Hz. The

fluorescence of GCaMP2 significantly increased during ATP application (Figure 5.5 C). At day 2 84 % (±3 % SD, 306 cells/ 4 coverslips) of the GCaMP2 positive primary microglia (set to 100 %) responded with an increase in cytosolic calcium to ATP application, 87 % (±1 % SD; 310 cells/ 4 coverslips) responded at day 3 (Figure 5.5 D). These data indicate that retroviral transfer of GCaMP2 and its expression can be used to study calcium in microglia.

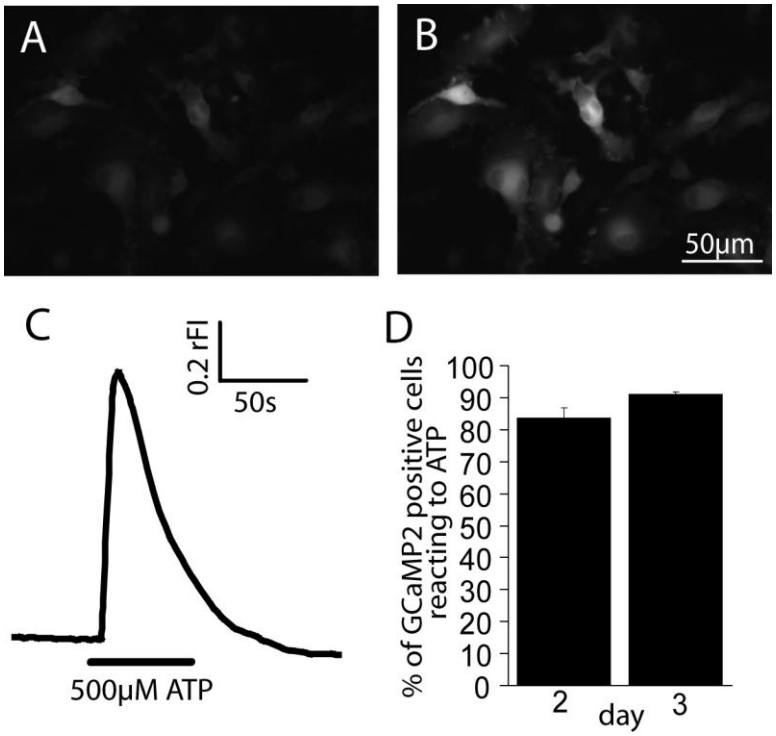


Figure 5. 5 Functionality of GCaMP2 in primary microglia. Pictures of a recording from a calcium imaging experiment before application (A) and during application (B) of 500 µM ATP (60 s) *in vitro*. Primary microglia were excited at 488 nm, 0.3 Hz. Emission was recorded at 510 nm. C, Averaged fluorescence recording of GCaMP2-expressing cultured microglial cells (n=20) in A and B. D, Percentage of GCaMP2 positive cells responding to ATP at day 2 and day 3.

5.2.2 Microglia properties are not changed by the retrovirus

To test whether the transfer of GCaMP2 affects microglial properties, I transduced primary microglia cells *in vitro*. Microglia were stimulated for 48 h with standard cell culture medium (DMEM), virus conditioned medium (virus), L929 conditioned

medium (L929) or L929 conditioned medium and virus conditioned medium (L929 + Virus) as described above.

Proliferation of primary microglial cell culture was determined by measuring the BrdU incorporation after 48 h (Figure 5.6). No increase in BrdU positive cells was observed by addition of the virus containing medium (1.1 ± 0.18 SD) compared to standard cell culture medium (1.0 ± 0.22 SD; p=0.057). I also could not find a difference of L929 conditioned medium (1.61 ± 0.21 SD) versus L929 and virus conditioned medium (1.53 ± 0.25 SD; Figure 5.6 A; p=0.19). L929 conditioned medium induced microglia proliferation around 1.6 fold compared to DMEM treated microglia (p< 0.001).

48 h after transduction, I measured the release of the pro-inflammatory cytokines TNF-alpha, IL-1beta and IL-6 in the supernatant. To correct for possible effects of proliferation or cell death, total protein was measured with the BCA total protein assay (Pierce Biotechnology). The cytokine concentrations were normalized to the amount of protein for each well. INF-gamma/LPS treatment (100 ng/100 U/ml) served as positive control for TNF-alpha and IL-6 release. 100 ng/ml LPS was used as a positive control for stimulating the release of IL-1beta. TNF-alpha release was not affected by the virus (Figure 5.6 B). DMEM treated primary microglia released 0.562 pg/µg ± SD 0.588 TNF-alpha in comparison to virus treated cells, which released 1.063 pg/µg ± SD 0.915 TNF-alpha (p=0.2). Cells treated with L929 conditioned medium released 0.699 pg/µg ± SD 0.697 TNF-alpha. When cells were treated with L929 and virus conditioned medium 0.8 pg/µg ± SD 0.718 TNF-alpha were measured. L929 treated cells were not different in comparison to cells treated with L929 and virus (p=0.77). INF-gamma/LPS treated cells released 2.504 pg/µg ± SD 0.484 TNF-alpha (p<0.001 vs DMEM). Also IL-1beta release was not changed by the virus (Figure 5.6 C). I found that upon DMEM treatment primary microglia released 5.114 pg/µg ± SD 0.667 IL-1beta, upon virus treatment 4.211 pg/µg ± SD 0.736, which was not significant (p=0.073). L929 treatment evoked 4.185 pg/µg ± SD 0.982 IL-1beta release, whereas L929 and virus treatment leads to 3.917 pg/µg ± SD 0.964 IL-1beta release, which was not significant (p=0.58). The positive control was LPS which leads to the release of 11.521 pg/µg ± SD 4.21 IL-1beta (p<0.001 vs DMEM). IL-6 release was not affected by the virus since microglia treated with virus

conditioned medium released 0.232 pg/µg ± SD 0.125 IL-6 in comparison to DMEM treated released 0.106 pg/µg ± SD 0.068 IL-6 (p=0.06, Figure 5.6 D). L929 conditioned medium evoked release of 0.1,371 pg/µg ± SD 2,007 IL-6 compared to L929+virus induced 0,183 pg/µg ± SD 0,071 (p=0.37). As positive control I used INF-gamma/LPS treatment which resulted in a higher release of 12.978 pg/µg ± SD 1.92 IL-6 (p<0.001 vs DMEM).

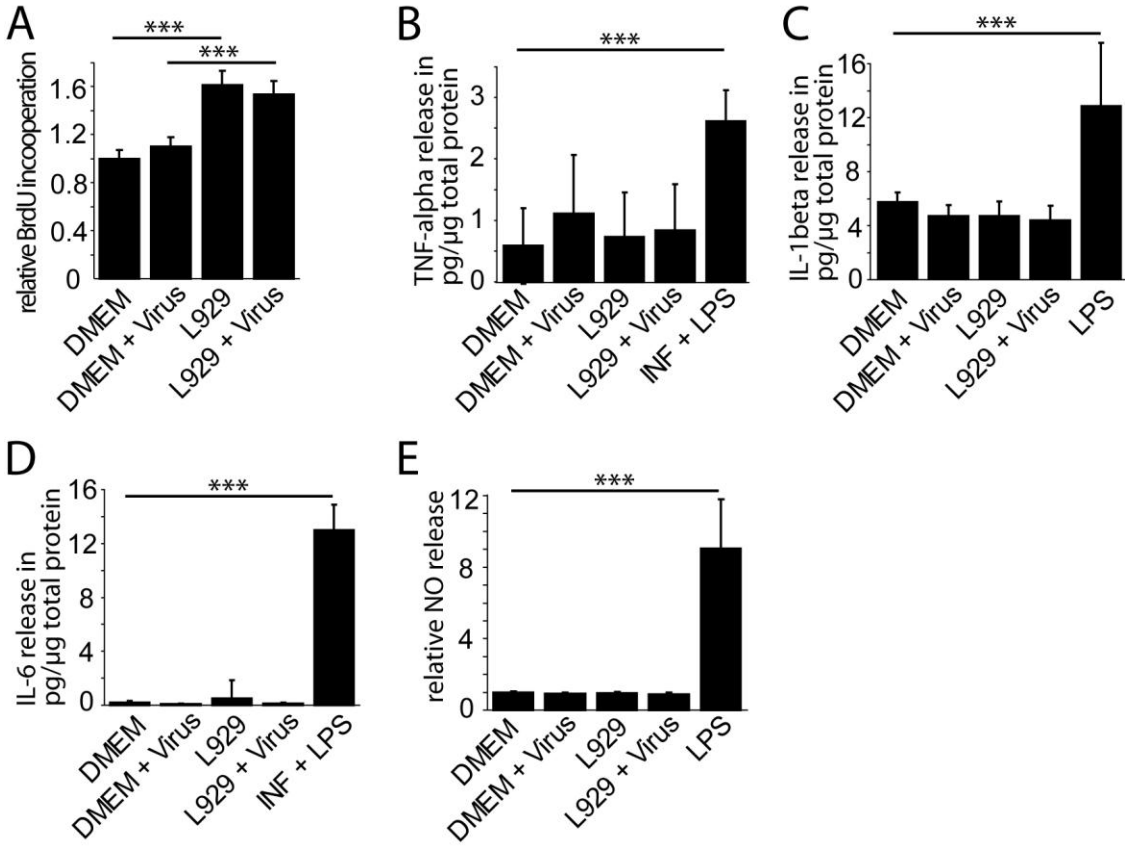


Figure 5. 6 Microglial proliferation, release of pro inflammatory cytokines and NO release are not affected by retroviral transduction with GCaMP2. Primary mouse microglia were treated with standard cell culture medium (DMEM), virus conditioned medium (virus), L929 conditioned medium (L929) or L929 conditioned medium and virus conditioned medium (L929+virus) for 48h. Primary microglia were tested for proliferation by BrdU incorporation (A), release of the cytokines TNF-alpha (B), IL-1beta (C) and IL-6 (D) as well as NO release (E) was measured. As positive controls the cells were treated with LPS or LPS and INF-gamma (INF), respectively. Student's two-tailed t-test *** p<0.001. Data are mean of 3 repeats a 8 experiments ± SD.

Furthermore NO release was tested (Figure 5.6 E). NO release was also not altered when the cells were incubated with the virus conditioned medium (1.12 ± 0.18 SD) compared to standard cell culture medium which was normalized to 1 (± 0.23 SD; p= 0.67) as control. NO release of microglia treated with L929 conditioned medium was 1.14 (± 0.13 SD) and did not differ from microglia treated with L929 conditioned medium and virus (1.26 ± 0.12 SD; p=0.15). LPS served as positive control and resulted in a 9.02 fold (± 2.76 SD) NO release (p<0.001 vs DMEM).

5.3 Expression of eGFP *in vivo*

5.3.1 Identification and characterization of virus transduced microglial cells by patch clamp technique in acute brain slices

Since the retroviral construct is only expressed by proliferating cells, proliferation was triggered by placing a stab wound in the right frontal cortex. Microglia proliferation started 1 day after stab wound injury and proliferation peaks at day 2-3 (Amat *et al*., 1996; Hampton *et al*., 2004). Accordingly, eGFP virus was injected two days after stab wound injury into the same location. eGFP positive cells were then studied 3, 6, 21 and 42 days after stab wound injury by preparing 130µm thick acute brain slices.

eGFP positive cells were only found in the vicinity of the stab wound. To characterize the membrane currents of virus-infected cells at defined times after stab wound injury, I used the patch clamp technique in the whole cell configuration. eGFP expression was found preferentially into microglia, identified by their specific current profiles. The membrane current pattern of microglial cells depends on their activation state. Microglial cells in acute slices from normal brain are characterized by a low membrane conductance. At a pathologic site, microglial cells express an inward rectifying conductance early after injury, and after a delay, an additional outward rectifying conductance (Boucsein *et al*., 2000). Membrane currents were recorded from eGFP positive cells from a holding potential of -70 mV. Current responses to depolarizing and hyperpolarizing voltage steps between 50 mV and -160 mV were recorded for 50ms with 10 mV increment. The membrane conductance was calculated based on voltage steps between 0 and -20 mV and between -100 mV and -120 mV as described above. To verify that the patched cell was eGFP-positive, the

cell was filled with AlexaFluo594 via the patch pipette and the two labels were compared. For controls, I used non-injured heterozygous CX3CR1-GFP mice, which express GFP under the microglia/macrophage specific CX3CR1 promoter (Jung *et al*., 2000). As previously described, the control microglia show small inward and outward currents (Figure 5.7 A and F black line, n=10) as well as small inward and outward conductances (Figure 5.7 G). Cells from mice 3 days after stab wound injury exhibited increased inward and outward currents (Figure 5.7 B and F red line). Membrane conductance was significantly increased at de- and hyperpolarizing voltages in comparison to control cells (Figure 5.7 G, n=14, in- and outward $p<0.001$). At day 6 outward conductance ($p=0.015$), as well as inward conductance ($p=0.005$) was increased in comparison to controls (Figure 5.7 C and G, n=14).

Cells at day 21 showed only increased inward conductance (Figure 5.7 D and G, n=13, $p=0.02$) in comparison to controls, but there was no significant difference in the outward conductance ($p=0.08$) as compared to controls. In animals 42 days after stab wound injury, the inward and outward currents were very similar to controls (Figure 5.7 E and F gray line, n=10) The inward conductance was not significantly different as compared to control cells (Figure 5.7 G, $p=0.87$). Also the outward conductance equates with the control ($p=1.285$).

Since current profiles display different activation stages of microglial cells they can be used as marker for activation. Summarized, microglia cells at day 3 and day 6 are highly activated, since they express inward and outward currents. Microglia at 21 reflect an intermediate stage of activation, because the express only inward currents. Since microglia cells at 42 day after injury did not differ significantly from controls they might be considered as resting phenotype. The number of experiments and the values for conductance are summarized in Table 5.5. I also injected the retrovirus at the same time as the stab wound was placed. I prepared slices the following 3, 6, 21 and 42 days and found no transduced cells at the location of the stab wound (data not shown). The delay in the injection of the virus was therefore an essential prerequisite to obtain significant expression of GCaMP2.

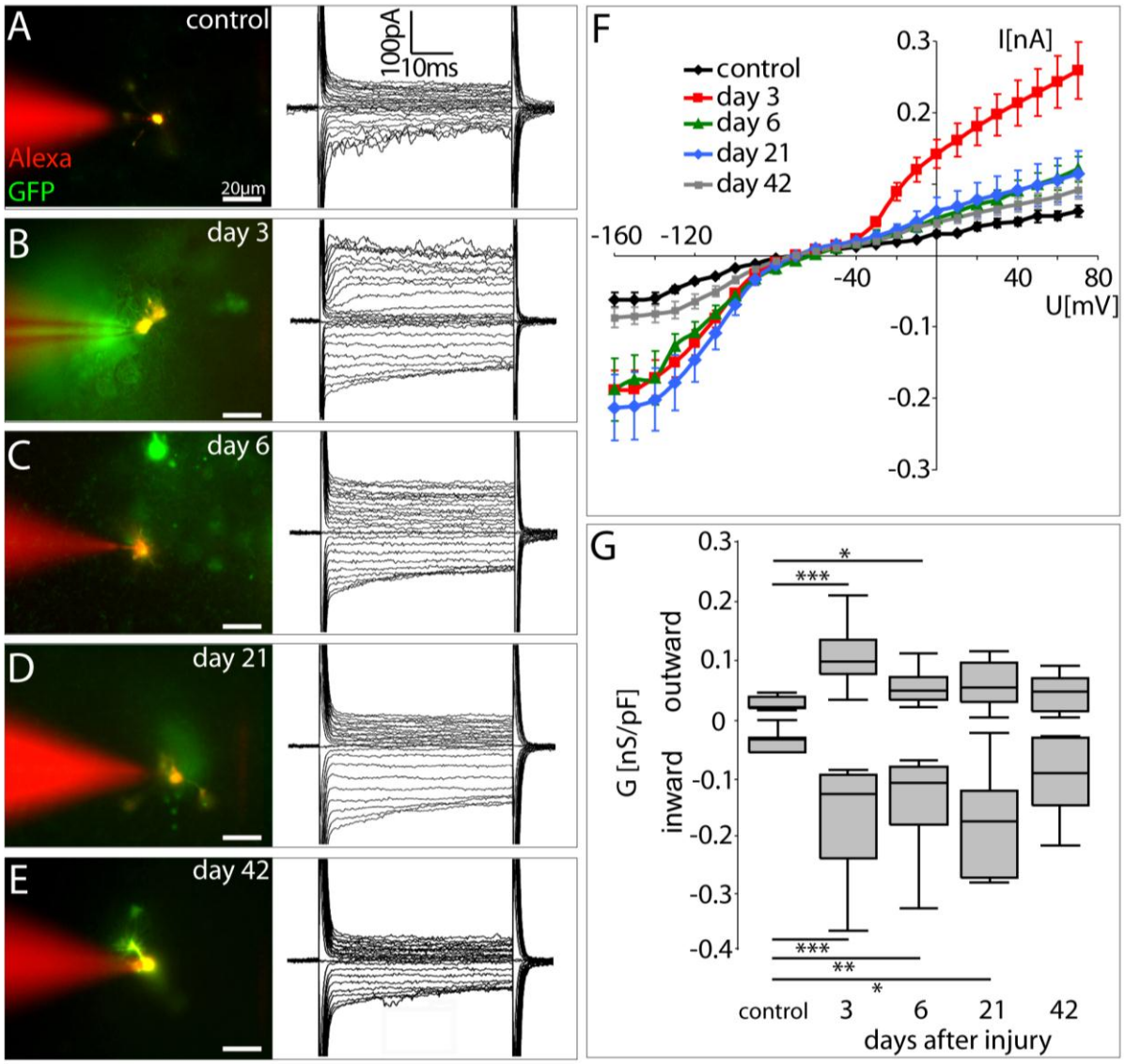


Figure 5. 7 Electrophysiological characterization of microglial cells in the stab wound lesion. Virus transduced eGFP positive microglia (green) were investigated via whole cell patch clamp technique. During recording the cells were filled with AlexaFluo594 via the patch pipette. A-E left, Images of eGFP-positive and AlexFluo594-filled microglial cells in slices obtained at different time points after stab wound injury. The corresponding current profiles are shown on the right side. Cells were clamped from a holding potential of -70 mV to a series of 50 ms voltage steps ranging from 70 mV to -160 mV with 10 mV increments. F, Average current (I) to voltage (U) from all recordings obtained at the different time points after injury. G, Boxplots of specific median conductance (G) was calculated from currents recorded at -20 mV and 0 mV (outward) and conductance (inward) at -100 mV and -120 mV. Mann-Whitney U-test, * $p<0.05$, *** $p<0.001$.

Days after injury	Nr of cells / animals	Value	G [nS/pF] inward	G [nS/pF] outward	Significance inward	Significance outward
Control	10/3	median	-0.035	0.022	*** vs day 3	
		SD	-0.036	-0.012	* vs day 6	*** vs day 3
		75th percentile	-0.030	0.041	* vs day 21	* vs day 6
		25th percentile	-0.069	0.020		
3	14/3	median	-0.129	0.098		*** vs control
		SD	-0.121	-0.069	*** vs control	* vs day 6
		75th percentile	-0.095	0.148	* vs day 42	* vs day 21
		25th percentile	-0.258	0.073		* vs day 42
6	14/3	median	-0.109	0.049		
		SD	-0.083	-0.038	* vs control	* vs control
		75th percentile	-0.081	0.082		* vs day 3
		25th percentile	-0.200	0.033		
21	13/3	median	-0.176	0.055		
		SD	-0.169	-0.061	* vs control	* vs day 3
		75th percentile	-0.103	0.101		
		25th percentile	-0.278	0.030		
42	10/3	median	-0.092	0.048		
		SD	-0.070	-0.050	* vs day 3	* vs day 3
		75th percentile	-0.031	0.075		
		25th percentile	-0.162	0.014		

Table 5. 5 Electrophysiological properties of microglia after stab wound injury. Mann-Whitney U-test *** p< 0.001, * p< 0.05.

5.3.2 Identification and quantification of microglial cells by immunohistochemistry

To characterize the population of virus-infected microglia in detail, I analyzed slices which were prepared as described for patch clamp recording. Slices were fixed and stained with Iba-1 antibody to label microglia (Figure 5.8 A). I quantified the number of eGFP/Iba-1 double positive cells in the slice (Figure 5.8 B). GFP positive cells were exclusively found in the area of the stab wound lesion of the 130 µm thick brain slice.

Since not all eGFP positive cells were positive for Iba-1, I determined the percentage of Iba-1 positive cells of all eGFP expressing cells (Figure 5.8 C). 3 days after injection I found 62 (± 24 SD) eGFP/ Iba-1 positive cells per slice (average of 8 slices/4 animals). 86 % (± 21 % SD) of all eGFP-positive cells were labeled by Iba-1. These cells had an amoeboid morphology and lacked processes. In comparison to control tissue, microglial density was higher around the injection site (observation, not quantified). 6 days after stab wound I found in average 75 (± 21 SD) eGFP/ Iba-1 positive cells per slice with amoeboid morphology (N= 8 slices/ 5 animals). 69 % (± 26 % SD) of all eGFP positive cells were co-labeled with Iba-1 staining. In comparison to slices of the control and day 3, the density of Iba-1 positive cells at the injury site was even higher (observation, not quantified). At day 21 I found in average only 19 (± 5 SD) eGFP/Iba-1 positive cells per slice (N=5 slices/5 animals). These cells had some processes (observation, not quantified). 66 % (± 30 % SD) of eGFP expressing cells were positive for Iba-1. The density of Iba-1 positive cells was still higher than in control slices but clearly reduced as compared to slices of day 6 (observation, not quantified). In average only 11 (± 5 SD) eGFP/Iba-1 positive cells per slice were found at day 42 (4 slices of 4 animals counted). 73 % (± 36 % SD) of all eGFP positive cells were Iba-1 positive. The eGFP/Iba-1 positive cells had several processes closer resembling ramified, resting microglia. The overall density of Iba-1 positive cells was similar as at day 21 (observation, not quantified).

Summarized eGFP/Iba-1 positive cells were mainly found in animals 3 and 6 days after stab wound injury, whereas the number of eGFP/Iba-1 cells was low at day 21 and 42. During wound healing the cells changed their phenotype from an amoeboid to ramified.

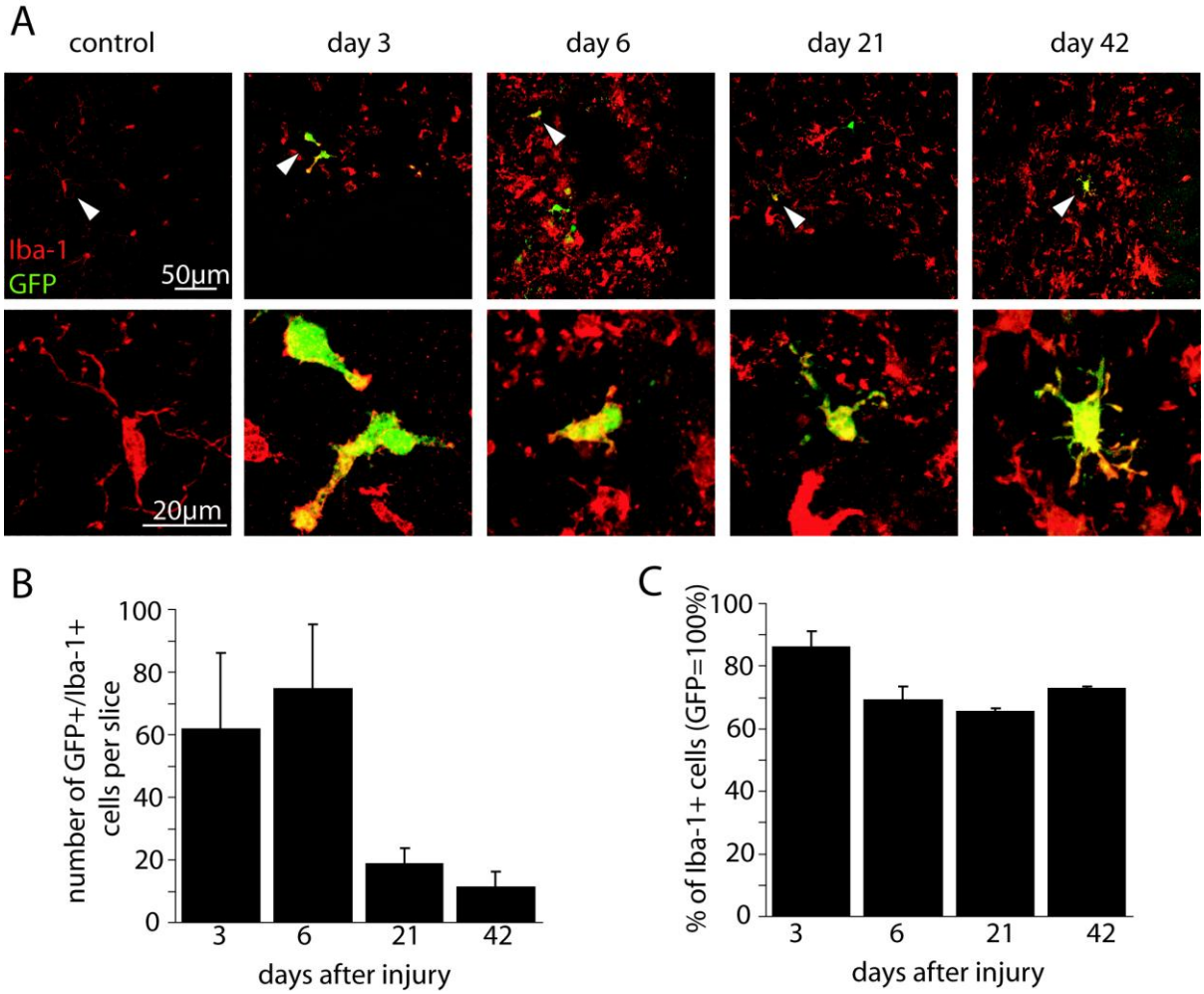


Figure 5. 8 Phenotype and quantification of virus-infected microglia in brain slices. A, Superimposed confocal images (1 µm z-stacks of 9 images) in the vicinity of the stab wound area (upper panels) at 3, 6, 21 and 42 days after stab wound injury. Representative microglia cells in higher magnification (lower panels) B, Quantification of the total number of eGFP/Iba-1 positive cells per slice. C, Percentage of Iba-1 positive cells within the population of eGFP positive cells.

5.4 Expression of a calcium sensor *in vivo*

5.4.1 Intracellular calcium increase in response to ATP in acute brain slices

Instead of eGFP virus, as in the patch clamp experiments described above, I injected GCaMP2 virus into the frontal cortex of mice. GCaMP2 was expressed to measure calcium signals from microglia cells in acute brain slices. GCaMP2 positive cells were studied 3, 6, 21 and 42 days after stab wound injury in 130 µm thick acute brain slices. Figure 5.9 A-F shows a recording of GCaMP2 fluorescence in response to ATP application (1 mM, 60 s) in a slices obtained 6 days after stab wound injury. From the immuno histology experiments I concluded that some Iba-1 negative cells also become transducted by the retrovirus. For identification of microglia in the brain slices I stained with Alexa594-conjugated tomato lectin before recording (Acarin *et al*., 1994). GCaMP2 fluorescence overlaid with the tomato lectin staining allowed me to identify responding microglia. The traces of fluorescence intensity in response to ATP of every tomato lectin/GCaMP2 positive cell of the example recording is shown in Figure 5.9 F.

Responses to ATP were also recorded in slices obtained 3, 21 and 42 days after stab wound injury (Figure 5.9 G). To quantify the responses, the maximal increase of relative fluorescence intensity (max rFI), the duration of the signal (dt) and the integrated fluorescence change (diF) were determined. The values for all time points are summarized in Table 5.6. Median values for max rFI were significantly higher at day 6 as compared to values obtained 3, 21 and 42 days after injury ($p<0.001$, $p=0.001$; $p=0.007$). In comparison to day 3 dt was significant longer at 6 and 42 days ($p=0.001$; $p=0.024$). At day 3 diF was significant smaller compared 6 days ($p<0.001$), 21 days ($p=0.002$) and 42 days ($p=0.001$). All p-values were summarized in Table 5.7.

Taken together I found intracellular calcium increase in microglia cells of acute brain slices in response to ATP. ATP calcium transients were measured at 3, 6 21 and 42 days after stab wound injury. Quantification revealed that at day 3 I found the smallest calcium signals. These signals were increased in microglia cells at day 6 after stab wound injury. In later time points, namely day 21 and 42 the signals were declined.

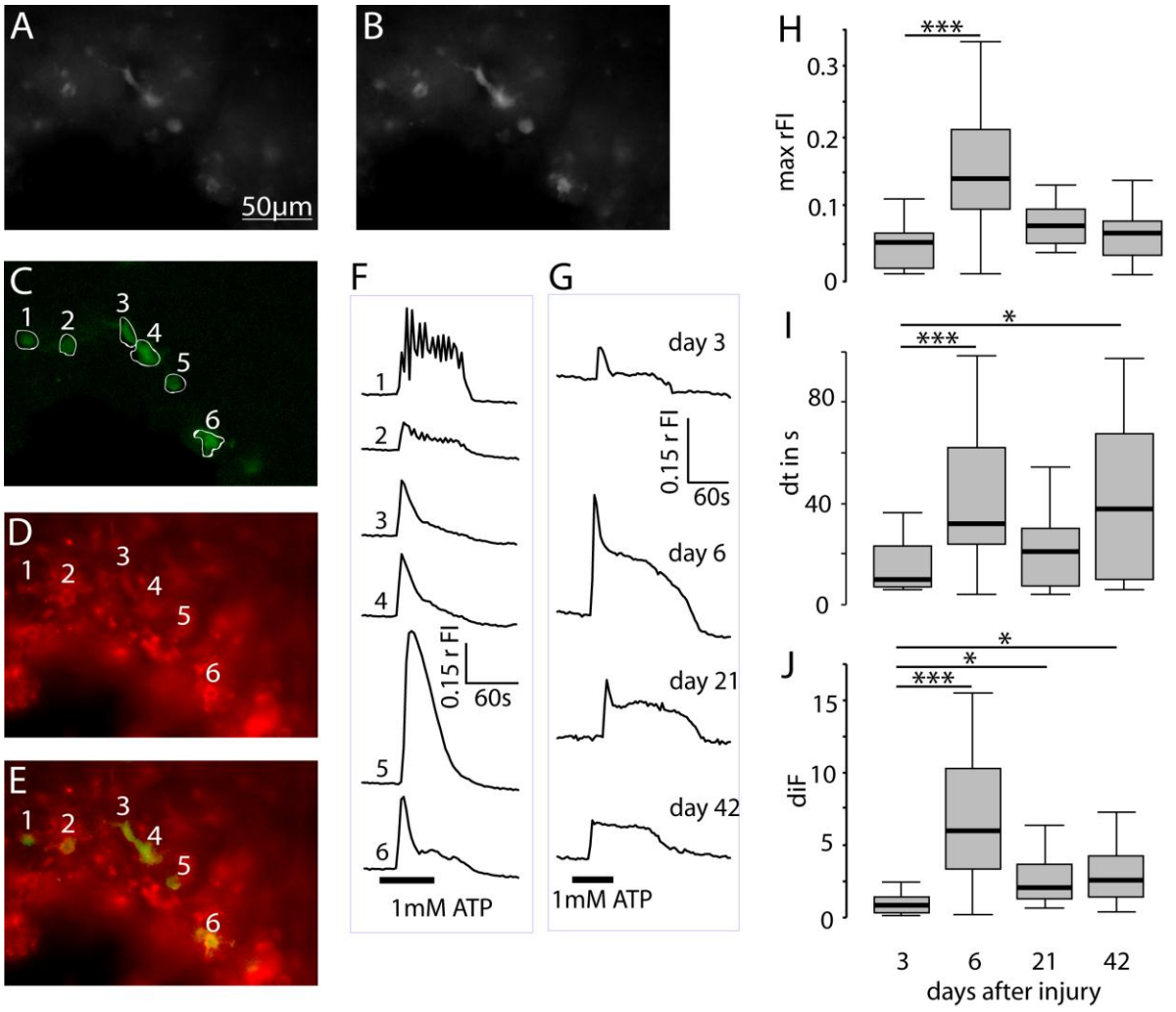


Figure 5. 9 Microglia *in situ* respond to ATP application with an increase in intracellular calcium. A and B, Fluorescence picture (excitation 488nm, emission 510nm) before (A) and during application of 1 mM ATP for 60s (B). C, White outlines with numbers indicate the region of interest in an acute brain slice in which changes in relative fluorescence intensity (rFI) were recorded. D, Tomato lectin staining was used to identify microglial cells. E, Overlay of C and D. F, Changes of fluorescence intensity over time from the regions of interest denoted in C, black bar is indicating application of 1 mM ATP for 60 s. G, ATP application at different time points after stab wound injury at 3, 6, 21 and 42 days. Relative fluorescence (rFI) changes from single microglial cells in response to application of 1 mM ATP for 60 s (black bar). H, Quantification of maximal relative fluorescence intensity (max rFI). I, Quantification of the duration (dt, middle graph) of the signal. J, Quantification of the integrated fluorescence (diF, lower graph). Mann-Whitney U-test, * $p<0.05$, *** $p<0.001$.

Days after stab wound injury	Number of cells / experiments/animals	Value	Max rFI	dt in s	diF
3	19/9/3	median	0.054	10.000	0.844
		75th percentile	0.068	24.391	1.450
		25th percentile	0.017	6.000	0.301
6	33/8/4	median	0.142	31.860	6.003
		75th percentile	0.212	62.805	10.576
		25th percentile	0.099	23.962	3.148
21	15/7/3	median	0.078	21.047	2.080
		75th percentile	0.101	30.078	3.771
		25th percentile	0.049	6.000	1.273
42	17/7/3	median	0.067	37.844	2.603
		75th percentile	0.112	73.711	4.726
		25th percentile	0.031	9.961	1.148

Table 5. 6 Quantification of microglial calcium signaling in response to ATP.

Groups	Max rFI	dt in s	diF
day 3 vs day 6	0.000	0.001	0.000
day 3 vs day 21	0.002	0.347	0.033
day 3 vs day 42	0.001	0.024	0.288
day 6 vs day 21	0.001	0.034	0.000
day 6 vs day 42	0.007	0.910	0.001
day 21 vs day 42	0.637	0.146	0.485

Table 5. 7 Summary of all p-values of microglial calcium signaling in response to ATP.
Mann-Whitney U-test *** p< 0.001, **p<0.01 * p< 0.05

5.4.2 Calcium transients in response to substance P

To test for the reactivity of tomato lectin positive cells to the neuropeptide substance P, I applied the ligand (4 µM, 60 s) to acute brain slices. 4.5 minutes later ATP was applied as control (1 mM, 60 s). Only cells which responded to ATP were evaluated.

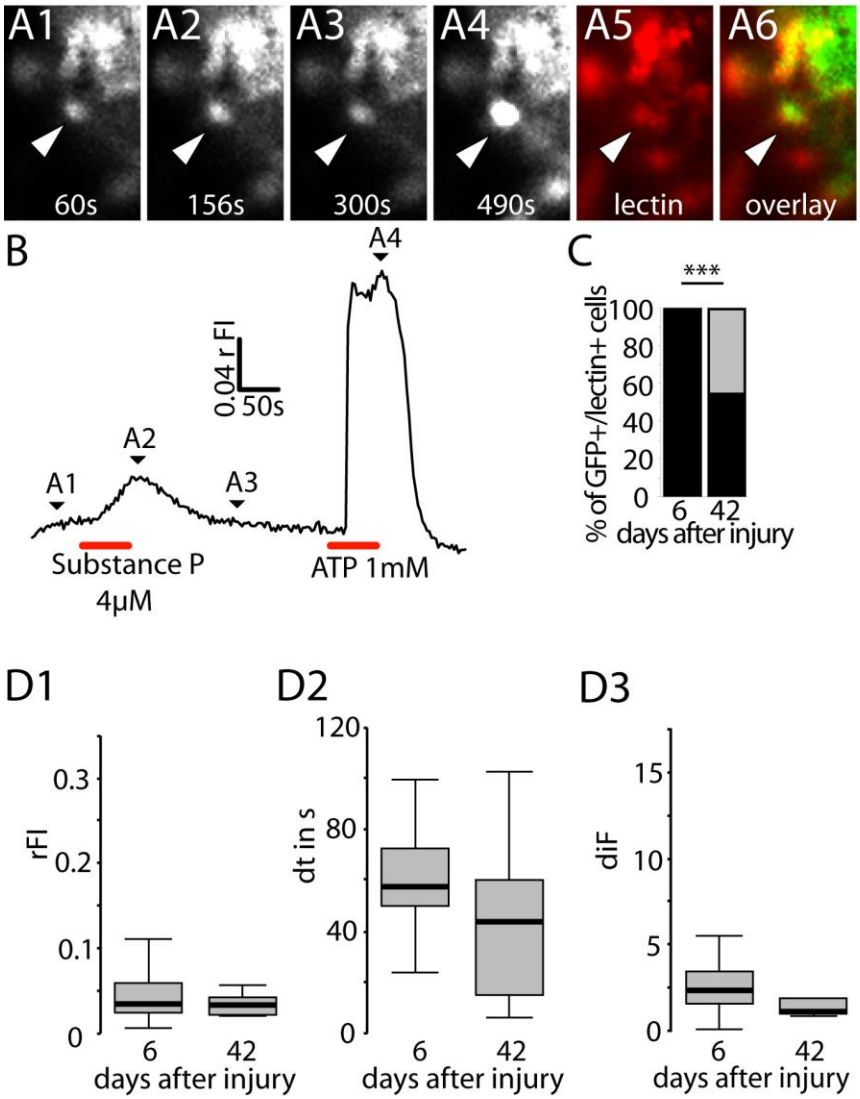


Figure 5. 10 Microglia *in situ* respond to substance P with an increase in intracellular calcium. A, Fluorescence images before (A1) and during (A2) application of 4 µM substance P and before (A3) and during (A4) application of 1 mM ATP. A5, Tomato lectin staining. A6, Overlay of the fluorescence of the calcium sensor during application of substance P (A2) and tomato lectin staining (A5). B, Trace on the left shows changes in the relative fluorescence intensity (rIF) obtained from a microglial cell at day 6 after stab wound injury, indicated by a white arrowhead in A. Bars indicate the application of substance P and ATP for 60 s. Triangles indicate when images A1-4 were recorded. Graph on the right shows the percentage of cells that responded to substance P (black bar) normalized to the ATP responsive population (=100 % gray bar) at day 6 and day 42. Pearson Chi-square test; *** $p<0.001$. D, Quantification of the microglial calcium in response to substance P. Analysis of rFI (D1), dt (D2) and diF (D3). Mann-Whitney U-test.

Figure 5.10 A shows fluorescence pictures of a recording of a representative microglial cell responding to Substance P. Figure 5.10 B shows the corresponding fluorescence trace. I quantified the number of microglia responding to Substance P application and found that 98 % of cells also responded at day 6 (Figure 5.10 C). The population of responsive cells was significantly reduced to 55 % at 42 days ($p<0.001$) after stab wound injury. I determined max rFI, dt and diF. None of the parameters was differed comparing the time points 6 and 42 days. Table 5.8 summarizes all values.

Days after stab wound injury	Nr of cells/ experiments/ animals	Value	Max rFI[a]	dt in s[a]	diF[a]	% of ATP responders[b]
6	64/8/3	median	0.034	57.453	2.386	98.4 ***
		75th percentile	0.061	72.610	3.505	
		25th percentile	0.024	48.500	1.569	
42	11/6/4	median	0.034	43.782	1.127	54.5
		75th percentile	0.046	70.965	2.303	
		25th percentile	0.022	12.761	0.981	

Table 5. 8 Quantification of microglial calcium signaling in response to Substance P
[a]Mann-Whitney U-test. [b]Pearson-Chi-Square test *** $p< 0.001$.

5.4.3 Calcium transients in response to endothelin-1

I tested the reactivity in terms of calcium transients of tomato lectin positive cells to endothelin-1 at the time points 3, 6, 21 and 42 days after stab wound injury. Endothelin-1 was applied at a concentration of 400 nM for 60 s, 4.5 min later ATP was applied at a concentration of 1mM for 60 s. Figure 5.11 A und B show the recording of a representative microglia responding to Endothelin-1 at 6 days after stab wound and its subsequent trace. Endothelin-1 triggered a response in 33 % of the cells at day 3, in 42 % of the cells at day 6, in 27 % of the cells at day 21 and in 56 % of the cells at day 42 (Figure 5.11 C). The number of responding cells was different between day 21 and 42 ($p=0.008$). All over the number of cells responding

to Endothelin-1 was increasing. I quantified max rFI, dt and diF of the calcium signals to Endothelin-1, but could not find any difference comparing the time points. Table 5.9 summarizes all values.

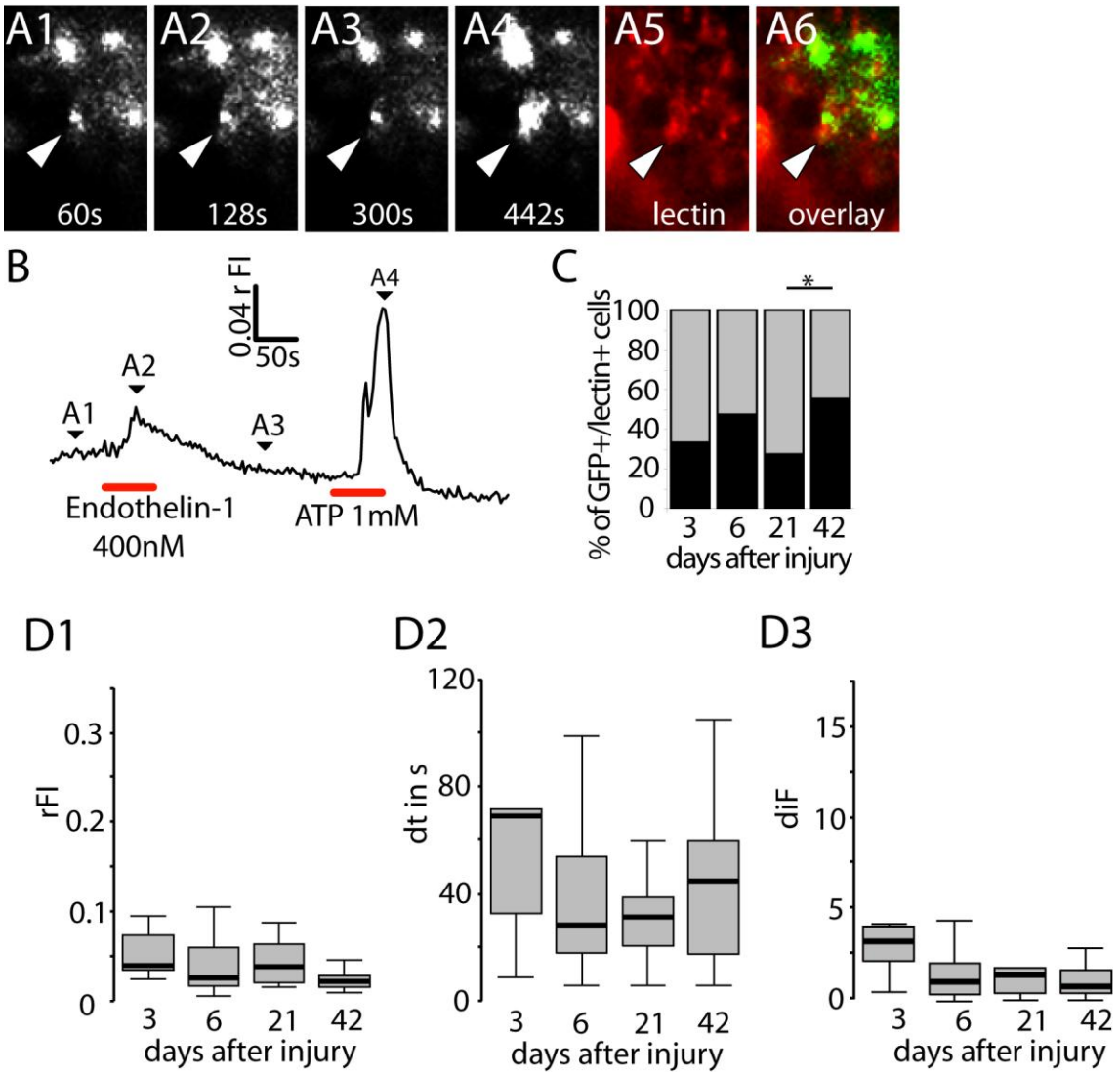


Figure 5. 11 Microglia *in situ* respond to endothelin-1 with an increase in intracellular calcium. A, Fluorescence images before (A1) and during (A2) application of 400 nM endothelin-1 and before (A3) and during (A4) application of 1 mM ATP. A5, Tomato lectin staining. A6, Overlay of the fluorescence of the calcium sensor during application of endothelin-1 (A2) and tomato lectin staining (A5). B, Trace on the left shows changes in the relative fluorescence intensity (rIF) obtained from a microglial cell at day 6 after stab wound injury, indicated by a white arrowhead in A. Bars indicate the application of endothelin-1 and ATP for 60 s. Triangles indicate when images A1-4 were recorded. Graph on the right shows the percentage of cells that responded to Endothelin-1 (black bar) normalized to the ATP

responsive population (=100 % gray bar) at day 6 and day 42. Pearson Chi-square test; * p<0.05. D, Quantification of the microglial calcium in response to endothelin-1. Analysis of rFI (D1), dt (D2) and diF (D3). Mann-Whitney U-test.

Days after stab wound injury	Nr of cells/ experiments/ animals	Value	Max rFI[a]	dt in s[a]	diF[a]	% of ATP responders[b]
3	27/7/3	median	0.039	69.469	3.339	33
		75th percentile	0.082	102.484	4.263	
		25th percentile	0.030	27.211	2.265	
6	133/28/8	median	0.025	28.695	1.121	42
		75th percentile	0.059	54.477	2.140	
		25th percentile	0.015	18.281	0.365	
21	51/19/5	median	0.037	31.743	1.496	27
		75th percentile	0.068	43.024	2.705	
		25th percentile	0.018	18.047	0.450	
42	81/40/12	median	0.020	45.094	0.826	55*** vs day 21
		75th percentile	0.027	61.876	1.788	
		25th percentile	0.014	16.524	0.449	

Table 5. 9 Quantification of microglial calcium signaling in response to Endothelin-1.
[a]Mann-Whitney U-test. [b]Pearson-Chi-Square test *** p< 0.001

5.4.4 Calcium transients in response to histamin

I tested the reactivity of tomato lectin positive cells in response to histamine at the time points 6 and 42 days after stab wound injury. Histamin was applied at a concentration of 1 mM for 60 s, followed by ATP application 4.5 min later at a concentration of 1 mM for 60 s. Figure 5.12 A displays a responding microglia cell and the corresponding trace is show in 5.12 B. Histamine evoked a calcium response in 64 % at day 6 and 53 % at day 42 of the ATP reacting microglia population (Figure 5. 12 C). The number of responding cells to histamine was not significantly different

between day 6 and day 42. When I compared max rFI, dt and diF of the fluorescence transients, I did not find a significant difference of the responses to histamine recorded at day 6 and day 42. Table 5.10 is summarizing all values.

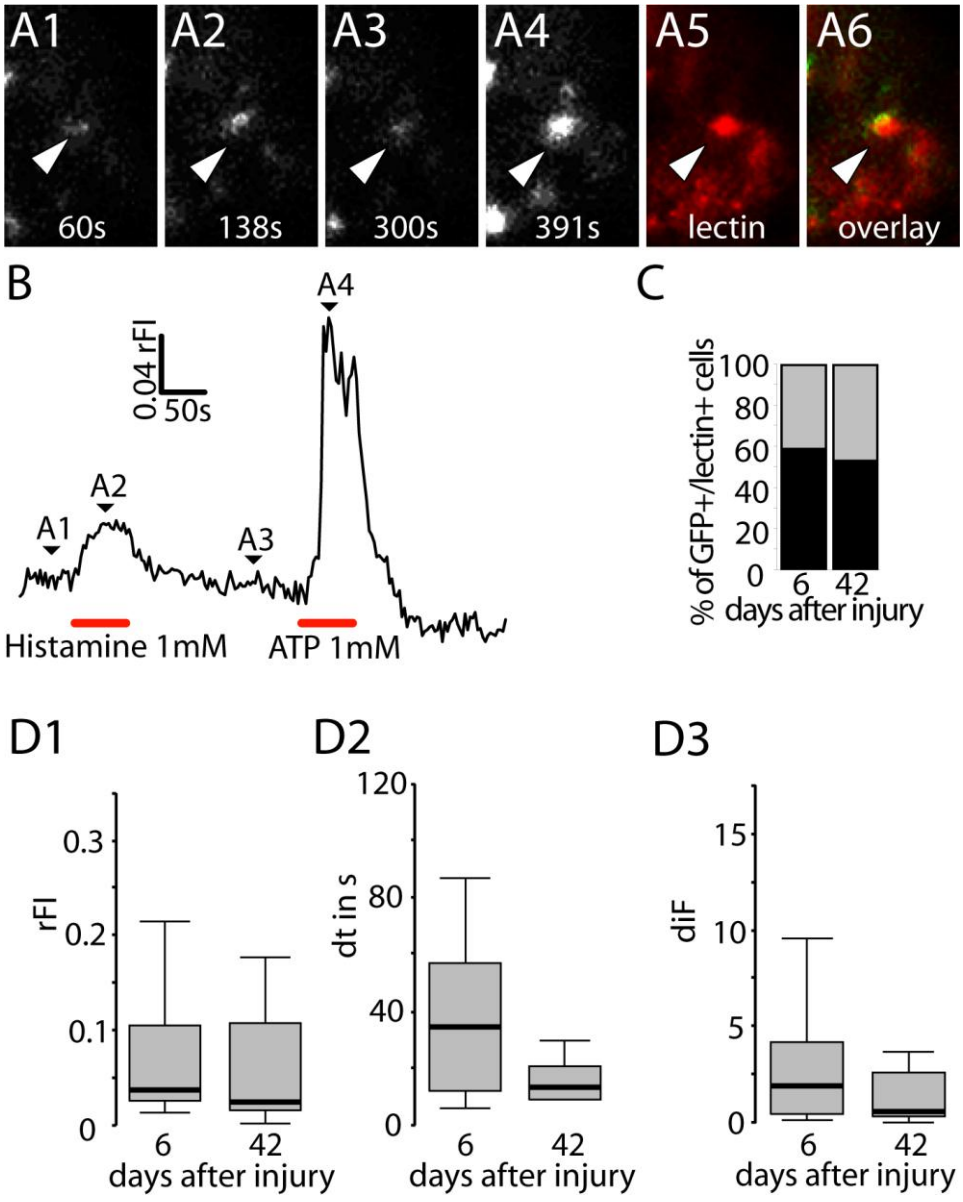


Figure 5. 12 Microglia *in situ* respond to histamine with an increase in intracellular calcium. A, Fluorescence images before (A1) and during (A2) application of 1 mM histamine and before (A3) and during (A4) application of 1 mM ATP. A5, Tomato lectin staining. A6, Overlay of the fluorescence of the calcium sensor during application of histamin (A2) and tomato lectin staining (A5). B, Trace shows changes in the relative fluorescence intensity (rIF) obtained from a microglial cell at day 6 after stab wound injury, indicated by a white arrowhead in A. Bars indicate the application of histamin and ATP for 60 s. Triangles indicate

when images A1-4 were recorded. C, Graph shows the percentage of cells that responded to histamin (black bar) normalized to the ATP responsive population (=100 % gray bar) at day 6 and day 42. Pearson Chi-square test. D, Quantification of the microglial calcium in response to histamin. Analysis of rFI (D1), dt (D2) and diF (D3). Mann-Whitney U-test.

Days after stab wound injury	Nr of cells/ experiments/ animals	Value	Max rFI[a]	dt in s[a]	diF[a]	% of ATP responders[b]
6	37/9/3	median	0.035	34.578	1.901	64
		75th percentile	0.114	57.387	4.861	
		25th percentile	0.023	12.000	0.431	
42	12/7/4	median	0.023	13.500	0.553	53
		75th percentile	0.112	22.558	2.592	
		25th percentile	0.013	9.012	0.274	

Table 5. 10 Quantification of microglial calcium signaling in response to Histamine. [a]Mann-Whitney U-test. [b]Pearson-Chi-Square test.

5.4.5 Calcium transients in response to serotonin

I tested the reactivity of tomato lectin positive cells to serotonin at 6 and 42 days after stab wound injury. Serotonin was applied at a concentration of 1 mM for 60 s followed after 4.5 min by an application of 1 mM ATP for 60 s. Figure 5.13 A shows a tomato lectin positive cell responding with a fluorescence signal to application of 1 mM serotonin. The corresponding trace is shown in Figure 5.13 B. Calcium signals in response to serotonin application (1 mM) elicited a calcium transient in 61 % of cells at day 6 and 38 % at day 42 (Figure 5.13 C).The number of responding cells to serotonin was not significantly different between day 6 and day 42. When I compared max rFI, dt and diF of the fluorescence transients, I did not find a significant difference of the responses to serotonin recorded at day 6 and day 42 (Table 5.12).

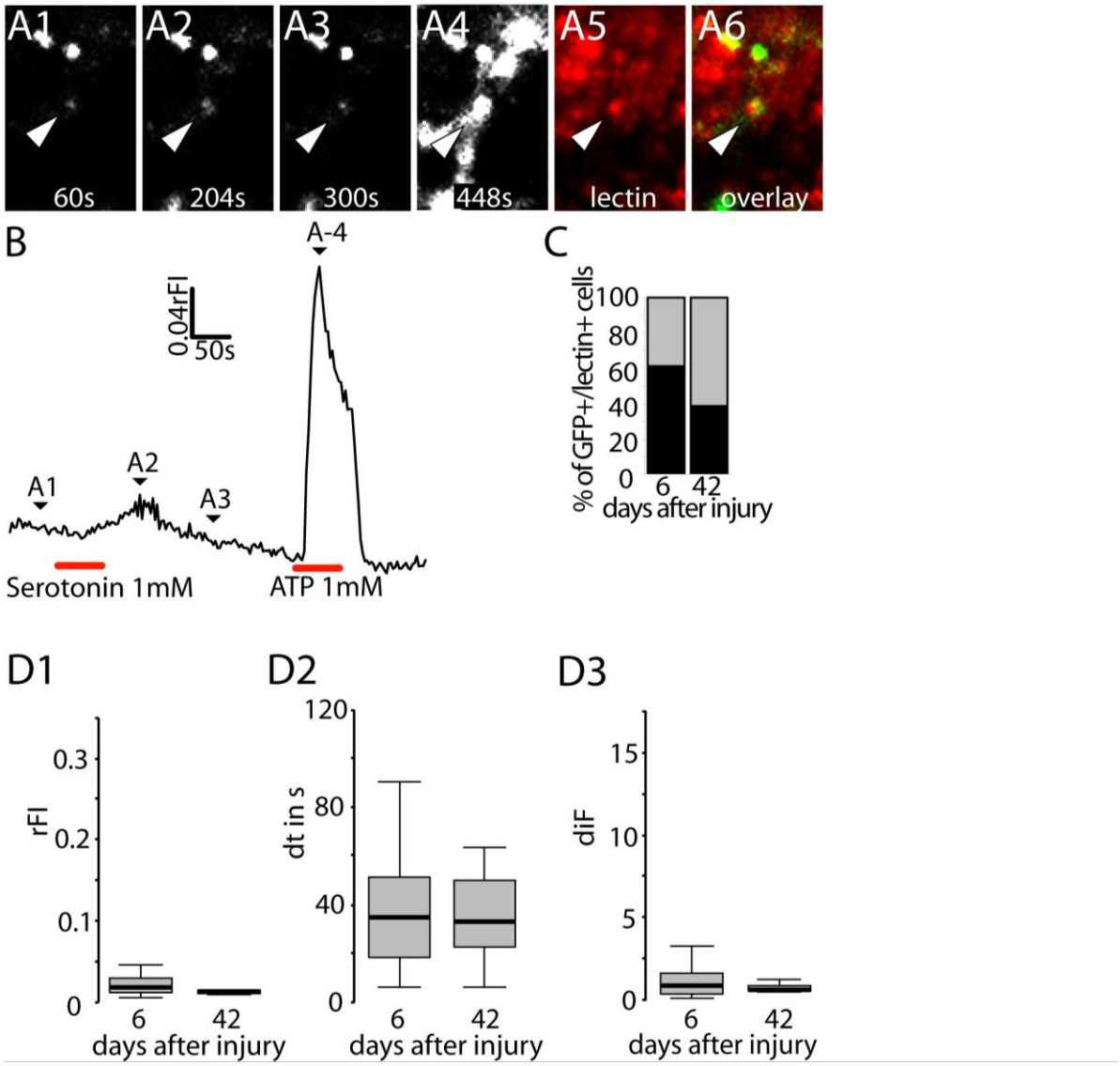


Figure 5. 13 Microglia *in situ* respond to serotonin with increase in intracellular calcium. A, Fluorescence images before (A1) and during (A2) application of 1 mM serotonin and before (A3) and during (A4) application of 1 mM ATP. A5, Tomato lectin staining. A6, Overlay of the fluorescence of the calcium sensor during application of serotonin (A2) and tomato lectin staining (A5). B, Trace on the left shows changes in the relative fluorescence intensity (rIF) obtained from a microglial cell at day 6 after stab wound injury, indicated by a white arrowhead in A. Bars indicate the application of serotonin and ATP for 60 s. Triangles indicate when images A1-4 were recorded. Graph on the right shows the percentage of cells that responded to serotonin (black bar) normalized to the ATP responsive population (=100 % gray bar) at day 6 and day 42. Pearson Chi-square test. D, Quantification of the microglial calcium in response to serotonin. Analysis of rFI (D1), dt (D2) and diF (D3). Mann-Whitney U-test.

Days after stab wound injury	Nr of cells/ experiments/ animals	Value	Max rFI[a]	dt in s[a]	diF[a]	% of ATP responders[b]
6	59/9/3	median	0.019	34.711	0.811	61
		75th percentile	0.031	51.414	1.598	
		25th percentile	0.013	18.344	0.299	
42	26/16/4	median	0.014	33.063	0.565	38
		75th percentile	0.016	51.657	0.950	
		25th percentile	0.012	21.110	0.450	

Table 5. 11 Quantification of microglial calcium signaling in response to Serotonin. [a]Mann-Whitney U-test. [b]Pearson-Chi-Square test

5.5 Microglia *in vitro* respond to serotonin but not to substance P

I tested the responsiveness of cultured microglial cells to substance P and serotonin while responses to ATP, endothelin-1 and histamine have been previously reported and quantified (Bader *et al*., 1994; Möller *et al*., 1997; Boucsein *et al*., 2003). None of the cultured microglial cells responded to substance P with an increase in calcium, while 19% responded to 1mM serotonin. The population of serotonin responsive cells varied considerably among different cultures ranging between 0% and 55% (n=688 cells; Figure 5.14).

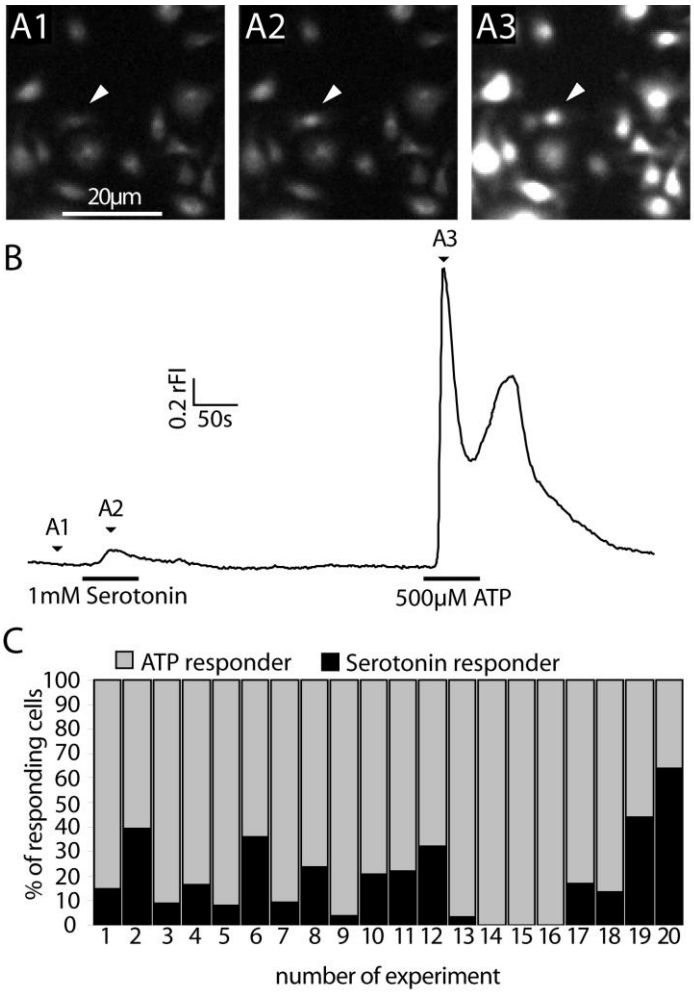


Figure 5. 14 Microglia *in vitro* respond to serotonin application with an increase in intracellular calcium. A, Fluo-4 loaded primary microglia before (A1), during 1 mM serotonin application (A2) for 60 s and 1 mM ATP (A3) for 60 s. B, Trace of relative fluorescence intensity (rIF) of the microglial cell indicated by an arrow in A. Triangles refer to the time points of the pictures in A. C, Quantification of the percentage of cells responding in each of 20 experiments. Gray bars indicate ATP (=100 %), black bars percentage of serotonin responsive cells.

6. Discussion

6.1 Comparison of adult microglia from different preparations

So far the most electrophysiological studies on microglia were done on cultured microglia prepared from neonatal pups (P 0-3). A few studies of non-isolated adult microglia from brain slice preparations revealed that adult resting microglia were very different in terms of their current expression (Boucsein *et al*., 2000; Lyons *et al*., 2000). Studies on cultured adult microglia cells are needed to clarify if the difference in current expression is due the age of the mice or due to culture conditions. Here microglia from the brain of adult mice were isolated by two different methods and characterized by patch clamp technique. First microglia cells were isolated by Percoll gradient and investigated after culturing periods of 3-8 h and 22-36 h. The second preparation method allowed us to study adult microglia after a culturing period of 2 weeks grown on whole brain cell cultures. Adult microglia cells of both methods were compared to non-isolated adult microglia from brain slices and cultured microglia from preparations of neonatal pups. All adult microglial cells showed smaller inward and outward currents then postnatal microglia cells, indicating that inward rectifying currents are a feature of neonatal microglia. Microglia currents are described to regulate proliferation and migration, microglial features needed in development (Schlichter et al., 1996b; Schilling et al., 2004; Pannasch et al., 2006; Nutile-McMenemy et al., 2007). Also invading amoeboid microglia of P 6-8 mice in acute brain slices show inward currents (Brockhaus *et al*., 1993). Microglial cells invade in fountains in the brain at that age (Del Rio-Hortega P, 1932; Brockhaus *et al*., 1993).

3-8 h after isolation microglia cells from the adult brain showed even smaller inward and outward currents then microglia from adult brain slices. Apparently this microglia loose or down regulate currents during the isolation process. After 22-36 h in culture 51 % of the adult isolated microglia developed a significant inward current, comparable to microglia from brain slices. On the other hand microglia of the corpus callosum were not able to express significant inward currents after 22-36 h in cell culture, indicating that there might be a difference in sensitivity to signaling for example by receptor expression or a delay in potassium channel expression between

both regions. Microglia show region specific phenotypes (Schwartz *et al*., 2006). Microglia from white matter for example are found to be less dense, which influenced the intensity of neuronal cell death evoked by LPS induced inflammation (Kim *et al*., 2000). Differences of the expression of several surface receptors were found to be region dependent (de Haas *et al*., 2008).

When isolated adult microglia cells were cultured for longer time on whole brain cultures for 2 weeks microglia developed large inward and outward currents. It was proposed that receptor mediated signaling can alter microglial activation status and orchestrate microglial functions (Hanisch & Kettenmann, 2007). Missing signalling events and changes in the homeostasis evoked by the culturing process therefore lead to microglia activation which is accompanied by the expression of inward currents. The current could be induced by astrocytes from the whole brain cultures, since the cells are participating in the current induction of microglia when co cultured (Schmidtmayer *et al*., 1994). In this study comparison of Percoll isolated adult microglia and adult microglia 2 weeks in culture show, that the inward currents were not directly induced by the isolation process, but developed under cell culture conditions. Nevertheless the inward currents of cultured microglial cells from neonatal pups showed larger inward currents then adult cultured microglial cells.

Electrophysiological studies in vitro have revealed dramatic changes in the expression levels of potassium channels in microglia during the process of activation. Upon LPS stimulation microglial cells from neonatal pups develop large outward currents and reduced inward currents (Prinz *et al*., 1999). In this study current profiles of microglia from juvenile (P 22) and microglia from adult (P 49-56) animals were recorded and compared with microglia from neonatal pups, before and upon LPS stimulation. The inward conductances of non-stimulated microglia was declining in an age dependent manner. Outward conductances of non-stimulated microglia were the small and independent of age. Adult microglial cells showed the smallest inward currents before stimulation but were able to reduce these currents further and induced outward currents upon LPS stimulation. This change in current profile is also typical for microglia from neonatal animals. Microglia from juvenile animals developed surprisingly no significant outward currents and did not reduce their inward currents upon LPS stimulation. The reduced sensitivity upon LPS stimulation

in juvenile microglia needs to be further investigated. Just to mention, juvenile mice from our experiments were weaned (ablactated) at day 21 ± 1 day. Neither the amplitude nor the conductance did change significantly in microglia from juvenile animals upon LPS stimulation.

Microglia inward currents are coded by Kir 2.1 mRNA which leads to the expression of inward rectifying potassium channels (Schilling *et al.*, 2000). The functional relevance of these channels is largely unknown, but plays an important role in the stabilization of the membrane potential (Franchini *et al*., 2004). In microglia expressing only inward currents the membrane potential is low, which leads to an increase of the driving force of calcium. A blockage with Ba^{2+} ions leads to a depolarization of the potential (Visentin *et al*., 1995; Chung *et al*., 1999). The blockage of the inward potassium currents also leads to a reduction of proliferation in microglia cells (Schlichter et al., 1996b). Upon LPS stimulation the inward current as well as proliferation were reduced (Schilling *et al*., 2000). On the other hand microglia express outward currents when stimulated with LPS. These currents were shown to be of Kv 1.5 and 1.3 potassium channel type (Kotecha & Schlichter, 1999; Schilling et al., 2000; Pannasch et al., 2006). It was shown that microglia outward currents are regulating respiratory burst, NO release and release of pro inflammatory signaling molecules (Pyo et al., 1997; Khanna et al., 2001b; Pannasch et al., 2006; Kaushal et al., 2007; Wu et al., 2009)

6.2 Retroviral genetransfer of GCaMP2 as a tool to study microglia

Receptor mediated signaling plays a central role in microglia activation and deactivation. Microglia are able to sense a bunch of molecules that are influencing microglia reactivity. For further investigation of adult microglia cells I develop a tool that allowed me to measure calcium signals of adult microglia cells and identify microglial ligands *in situ*. I therefore cloned a genetically encoded calcium sensor into a retroviral vector system. Retroviral particles were used to transduce microglia cells *in vivo* to express the calcium sensor GCaMP2. Microglia calcium signals were investigated upon application of different neurotransmitter and neuropeptides.

Before starting the *in vivo* experiments I tested the expression of the retroviral

construct in cultured microglial cells from neonatal pups. Proliferation of microglia was low in culture and needed to be stimulated. I used conditioned medium of the fibroblast cell line L929. Nearly all cells showed 2 nuclei giving evidence that the cells were still in the process of proliferation after 48 h treatment with the virus. L929 conditioned medium stimulated microglial proliferation in about 60 %. When microglial cells were treated with L929 and virus conditioned medium together approximately one third of the cultured microglial cells expressed the calcium sensor protein GCaMP2. That means that half of the proliferating microglial cells were able to express the calcium sensor. Nevertheless already at 24 h application of 500 µM ATP induced a transient increase in intracellular calcium 84 % of the GCaMP2 expressing cells. This responsiveness is comparable with studies from Fluo-4 (conventional calcium dye) stained cells, which showed responsiveness in 87 % (Möller *et al*., 2000). GCaMP2 is a eGFP based calcium sensor and was expressed in the cytosol, with a low basic fluorescence. Fluorescence intensity was found to increase about 1.6 fold when ATP was applied.

Microglia cells are immune competent cells of the brain and are able to sense a huge number of pathogens via Toll-like receptors (TLRs). Also specialized TLRs for viral RNAs and DNAs are existent in microglia (Lehnardt, 2010). TLR signaling in microglia ultimately induces of NF-kappaB activation, leading to transcription of chemokines, proinflammatory cytokines, NO release and proliferation (Olson & Miller, 2004; Ribes *et al*., 2010). In our experiments I inspected, if microglial activation properties are changed by the retroviral transduction relevant for this work. pMP71 vectors are already used in gene therapy for ex vivo modification of T cells (Engels *et al*., 2003). Proliferation, release of the proinflammatory cytokines Il-1beta, TNF-alpha and IL-6 nor the release of NO was altered when primary microglia were treated with the retrovirus. Viral genetransfer is therefore a useful method to express a calcium sensor in microglial cells.

6.3 Expression of eGFP *in vivo*

Since microglial expression of the retroviral constructs was dependent on microglial proliferation I first induced a stab wound into the right frontal cortex of mice. When

eGFP virus was directly applied to the brain (without a stab wound before) I could not find any eGFP expression in the cortex. By application of the eGFP virus 2 days after stab wound, I found eGFP labeled cells in the vicinity of the injury. This proves that proliferation of the cells was induced by the stab wound and was necessary for the expression of the transgene eGFP *in vivo*. Furthermore I conclude that there were no proliferating cells at the time of the induction of the stab wound injury in the cortex.

eGFP positive cells were found at 3, 6, 21 and 42 days after stab wound (which means 1, 4, 19 and 39 days after virus injection). For identification and characterization of eGFP labeled cells I used whole cell patch clamp technique. Current profiles of the eGFP positive cells identified them mainly as microglia or macrophages (see discussion below). I investigated eGFP positive microglia 3, 6, 21 and 42 days after stab wound and compared these cells to microglia from control animals that received no stab wound, but expressed eGFP under the microglia/macrophage specific promoter of CX3CR1 gene. Control microglia showed only small inward and outward currents, as described already before. 3 days after stab wound the eGFP positive cells exhibited prominent inward and outward currents. These results are consistent with the data obtained from activated microglia in the model of facial nerve axotomy and after MCAO (Boucsein *et al*., 2000; Lyons *et al*., 2000). Microglial cells accumulate at the site of injury, either by migration or proliferation within 3 days *in vivo* (Ivacko *et al*., 1996; Kreutzberg, 1996). Microglial cells after facial nerve axotomy showed current profiles comparable to resting after 7 days (Boucsein *et al*., 2000). I found that microglial inward currents were still elevated at day 21 after stab wound. That means that microglial cells in my model of cortical stab wound showed prolonged activation of inward currents. The advantage of my technique is that I can investigate the proliferating subpopulation of microglia after injury. These microglia cells might be longer activated compared to non proliferating microglia. A correlation of proliferation and current expression is already evident since microglia proliferation is reduced, when potassium channels are blocked (Schlichter et al., 1996b; Pannasch et al., 2006). The prolonged activation of inward currents could be also evoked by regional differences, since here I investigated cortical microglia, whereas in the model of facial nerve axotomy microglia of the facial nucleus were investigated. In experiments were I analyzed the

inward current activation of adult isolated microglia in cell culture obtained from corpus callosum and cerebral cortex I found that microglia from corpus callosum were not able to induce inward currents like cerebral microglia after 22-36 h in culture. On the other hand the two injury models differ for example in terms of blood brain barrier disruption which might lead to prolonged microglia activation.

Besides the patch clamp experiments I performed some immuno histology experiments for quantification of the eGFP expressing cells. I found that with my method I mainly transduced microglia (66-86 % of all eGFP positive cells). For identification of microglial cells in fixed slices I stained for the intracellular marker protein Iba-1 (Köhler, 2007). The number of eGFP/Iba-1 expressing microglia was high at 3 and 6 days after stab wound, whereas the number of eGFP positive microglia was reduced to very low numbers at day 21 and 42. It is still not known if the disappearance of microglial cells at later time points is due to apoptosis since only one study showing caspase activity in microglia after endorhinal cortex lesion (Wirenfeldt *et al*., 2007).

However specific markers for the distinction of microglia cells from blood derived macrophages are not yet available. At the present literature it is still under debate, if microglia can be seen as an own population of cells or as a subpopulation of macrophages (brain specific macrophage). Nevertheless it is now clear that a significant portion of up to 10% of microglia/macrophages originate from bone marrow derived stem cells and infiltrate the brain throughout adulthood also without lesion of the brain tissue (Lassmann *et al*., 1993; Simard & Rivest, 2004). Significant numbers of microglia can be replaced by macrophages in lesions depending on the severity and type of brain injury (Popovich & Hickey, 2001; Priller et al., 2001b; McMahon et al., 2002; Ladeby et al., 2005; Kokovay & Cunningham, 2005; Schilling et al., 2005). Invading macrophages share many properties of microglia. The intrinsic microglia are electrophysiologically indistinguishable from the blood derived macrophages (Lyons *et al*., 2000). Therefore the GFP positive cells in this study could be microglia as well as macrophages.

6.4 Expression of a calcium sensor *in vivo*

6.4.1 Retroviral genetransfer as a tool to study microglia *in situ*

After identification and characterization of eGFP-transduced microglial cells in the stab wound model, I expressed the calcium sensor protein GCaMP2 instead of eGFP. I present a new approach to measure calcium signaling of microglial cells from acute brain slices. In this study I focused on a highly defined population of glial cells that have a number of properties which identify them as microglia and which distinguish them from astrocytes, oligodendrocytes and neurons. They proliferate in a certain time window after injury, are positive for the markers Iba-1 and tomato lectin and develop characteristic in- and outward currents after injury. Microglia cells are immune cells and surveying their environment for adverse conditions in the CNS, such as neurodegeneration, trauma, inflammation and infection. There is growing appreciation of the ability of resident glial cells to initiate inflammation following trauma or infection in the central nervous system. Controversially discussed is if microglia activation can be controlled through “ON” and “OFF” signaling factors (Hanisch & Kettenmann, 2007). Neurotransmitters are described to drive different functions in microglia cells.

Activation of neurotransmitter receptors involves Ca^{2+} dependent pathways. Neuropeptides and Neurotransmitter may play a role in the initiation and/or progression of inflammation within the CNS following injury or infection. Traumatic injury and infectious agents can elicit marked inflammatory responses within the CNS. While such responses may represent protective immune responses to certain pathogens, inflammation elicited by infectious agents often results in progressive damage to the CNS. A hallmark of developing immune responses is the synergistic interactions between cells and their products, which can amplify the response. Such amplification and positive feedback loops serve to recruit cells to the site of infection, while promoting activation signals which continue to expand the response. Following initiation of inflammation within the CNS, infiltrating immune cells including macrophages and dendritic cells can contribute to the production of proinflammatory signals such as the cytokines, IL-6, TNFalpha and IL1-beta (Hanisch, 2002).

Viral transfer of genetically coded calcium sensors is a tool to study Ca^{2+} signaling in microglia *in situ*. Potentially this approach could also be used for *in vivo*

imaging. Nevertheless I found some restrictions in this approach. I only labeled a population of microglial cells/brain macrophages which has undergone proliferation in response to brain injury. Thus I select a subpopulation of these cells. 42 days after the injury these cells may still be different from the resting microglial cells, despite the fact that their membrane current profile and the morphological features are more reminiscent of resting, ramified microglial cells than of the amoeboid phenotype in an injured tissue. As discussed in Hanisch & Kettenmann (2007) microglial cells may still keep the memory of a previous activation by a distinct phenotype. The transformation of the microglia into a resting phenotype after injury was shown following facial nerve axotomy (Boucsein *et al*., 2000).

Microglial cells showed almost no spontaneous calcium signals but calcium transients in response to application of ATP, Endothelin-1, Substance P, Histamine and Serotonin could be recorded. Thanks to this approach I was able to record calcium transients of all substances investigated at different time points after stab wound, in which microglia displayed a more activated or resting phenotype, allowing the identification of specific answer of microglia at different activation states. The approach I developed allows to distinguish between two microglial populations, one resembling activated microglia which I studied 6 days after the injury and a second which might be considered as deactivated microglia which I studied 42 days after the injury.

6.4.2 ATP and substance P sensitivity differ between activated and deactivated microglia/brain macrophages

For serotonin, endothelin-1 and histamine I found no significant difference in the fraction of the cell population responding to the given transmitter nor in a change in the max rFI (relative fluorescence intensity), dt (duration) or diF (integrated fluorescence) when comparing the time points 6 days and 42 days. For purinergic signaling, there was a striking increase in the response at day 6 just after the peak of the microglial activation after the injury. This could be due to an upregulation of purinergic receptors in activated microglial cells. Indeed, P2X receptors are upregulated after spinal cord injury (Tsuda *et al*., 2008; Ulmann *et al*., 2008),

ischemia (Franke *et al*., 2004) and in Alzheimer´s disease (McLarnon *et al*., 2006). In cultured microglia, however, calcium signals by P2 receptor activation were reduced after microglial activation by LPS (Möller *et al*., 2000).

Inwardly rectifying K^+ channels influence Ca^{2+} entry due to nucleotide receptor activation in microglia. With each additionally expressed K^+ channel, the membrane potential is stabilized at more negative values and thus activation correlates with membrane hyperpolarization (Boucsein *et al*., 2003). Since the reversal potential for Ca^{2+} is at a positive membrane potential, microglial activation also correlates with an increased driving force for Ca^{2+} across the cell membrane (Franchini *et al*., 2004).

Activation of P2X purinergic receptors and subsequent depolarization was shown to inhibit store-operated entry of Ca^{2+} mediated by P2Y receptors (Wang *et al*., 2000). In contrast K^+ could be elevated after brain injury. This was demonstrated in animal models of ischemia (Petzold *et al*., 2005). Depolarization by high external K^+ or Cl^- decreased calcium transients through store operated channels in cultured microglia (McLarnon *et al*., 2000). Therefore microglia possibly lower their membrane potential by expressing inward currents to regulate the Ca^{2+} driving force.

Substance P sensitivity revealed another difference between activated and deactivated microglia. I found that all cells responded to substance P 6 days after the injury, while after 42 days only half of the population was responsive. There is, of course, a major limitation in our study: The calcium signals depend on the expression levels of the genetically encoded sensor and it cannot be excluded that the expression levels change over time. Moreover, the signal also depends on the concentration of the sensor and the cell volume. Resting microglia cells have very little cytoplasm and the soma is almost entirely filled with the nucleus. The processes are thin and are unlikely to lead to large calcium signals. On the other hand, the decrease in responsiveness was not a general phenomenon since I did not observe a significant change in the signal for serotonin, histamine and endothelin-1 or the population of cells responding to these agonists (see below).

6.4.3 A larger population of microglial cells *in situ* is sensitive to endothelin-1, histamine, substance P and serotonin as compared to culture

The present study shows that microglial cells *in situ* express functional neuropeptide and transmitter receptors. Interestingly, the population of sensitive cells is larger than those described in cell culture. Only 13% of the microglial cells in culture responded to Endothelin-1 (Möller *et al*., 1997) while I found 47% to 56% responders. 30% of cultured microglial cells responded to histamine (Bader *et al*., 1994), while in slices 53-59% responded. I found a calcium increase in 19% of the primary microglia to application of serotonin, whereas *in situ* 38-61% of the cells were responsive. Serotonin receptors have been previously described in microglia (Mahé *et al*., 2005). Substance P (NK-1) receptor expression has been reported for cultured microglia cells (Rasley *et al*., 2002), but calcium signaling in response to substance P was not described. I therefore tested the sensitivity of cultured microglial cells to substance P and found that the cells did not respond *in vitro*. In contrast, all microglia *in situ* responded to Substance P 6 days after injury, whereas the number of responding cells was reduced to 55% at 42 days. Moreover in my analysis, I included only those microglial cells which also responded to ATP. In expression studies of GCaMP2 in culture I found that the majority (87%) of the transduced microglial cells respond to ATP similar to that previously reported (Möller *et al*., 2000).

These results indicate that cultured primary microglia differ from adult microglia of the brain parenchyma in their responsiveness to neurotransmitters and neuropeptides. This might be either due to the age of the animal or the different environment of the culture versus tissue.

6.4.4 The role of microglial neurotransmitter and –hormone sensitivity in physiology

There is evidence that all the receptor systems described here for microglia could be activated under physiological and pathological conditions. The concept of volume transmission postulates that neurotransmitter and –hormones can spread through the extracellular space and can activate populations of cells (Agnati *et al*., 2010). There could even be an autocrine mechanism involved since microglia produce endothelin-1 (Wu *et al*., 2009; Li *et al*., 2010) and histamine (Katoh *et al*., 2001). Astrocytes could be the source of ATP (Werry *et al*., 2006) and Endothelin-1 (Li *et al*., 2010)

under physiological conditions. Whereas neuronal serotonin (Marek & Aghajanian, 1998) and substance P (Vruwink *et al*., 2001) projections in the cortex are widespread. Histaminergic neurons are known to be located in the tuberomammillary nucleus of the posterior hypothalamus nucleus and also project to almost all regions of the brain (Watanabe *et al*., 1984). Histamine participates in many physiological functions in the brain such as thermoregulation, circadian rhythms, neuroendocrine regulation, catalepsy, locomotion, and aggressive behavior (Prell & Green, 1986; Sawada *et al*., 1990; Wada *et al*., 1991).

6.4.5 The role of microglial neurotransmitter and –hormone sensitivity in pathology

All the signaling system described here play also an important role in pathology. It may be advantageous for microglial cells to express such receptors also in the resting state so that they can rapidly sense pathologic changes related to an increase or decrease in these substances. Indeed microglial cells do not only respond to a new or an increase in a signal ('on signal'), but can also respond to the absence or decrease of a signal ('off signal') as recently reviewed (Biber *et al*., 2007). ATP is massively released from injured tissue and is considered to be an important danger signal (Abbracchio *et al*., 2009). Endothelin-1 concentration in cerebrospinal fluid is elevated in stroke patients, as well as in patients with subarachnoid hemorrhage (Kessler et al., 2005). Similarly, ischemia or trauma in experimental animals results in an elevation of the endothelin-1 level in the central nervous system (Petrov et al., 2002; Li et al., 2010). Histamine is released after trauma, ischemia, seizures, and inflammation. Substance P expression is increased after traumatic brain injury (Zacest *et al*., 2010) and middle cerebral artery occlusion, an animal model for stroke (Stumm *et al*., 2001). These receptor systems can control some functional parameters in microglial cells. From culture studies it is known that substance P induces chemotaxis (Maeda *et al*., 1997) and induces the production of ROS (Block *et al*., 2006) and increases the release of IL-1 after LPS activation (Martin *et al*., 1993). Substance P also augments Borrelia burgdorferi-induced prostaglandin E2 and TNF-alpha production in murine cultured microglia (Rasley *et al*., 2004; Chauhan *et al*., 2008). Whether all these features are also relevant in microglia *in situ* remains to be determined.

Glial Ca^{2+} signaling may have great significance in neuropathology. The release of neurotransmitters by neural cells provides a potential communication pathway for the communication of glia cells, which may be the basis of the extensive reactive astrogliosis, myelin disintegration and microglial cell activation that characterize the glial response to CNS injury.

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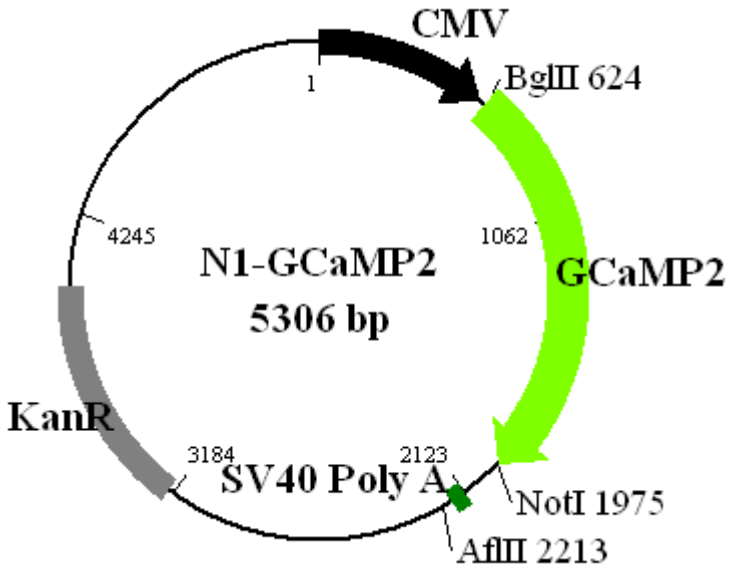
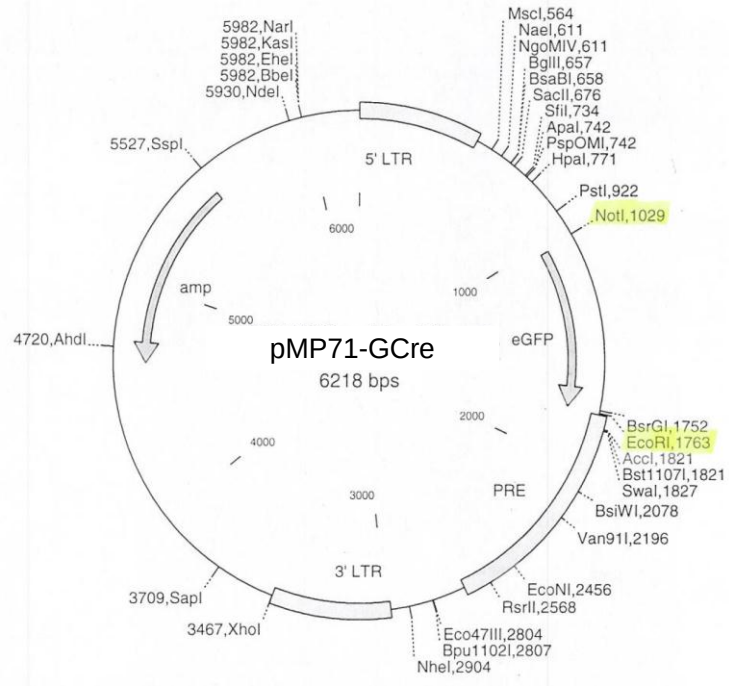
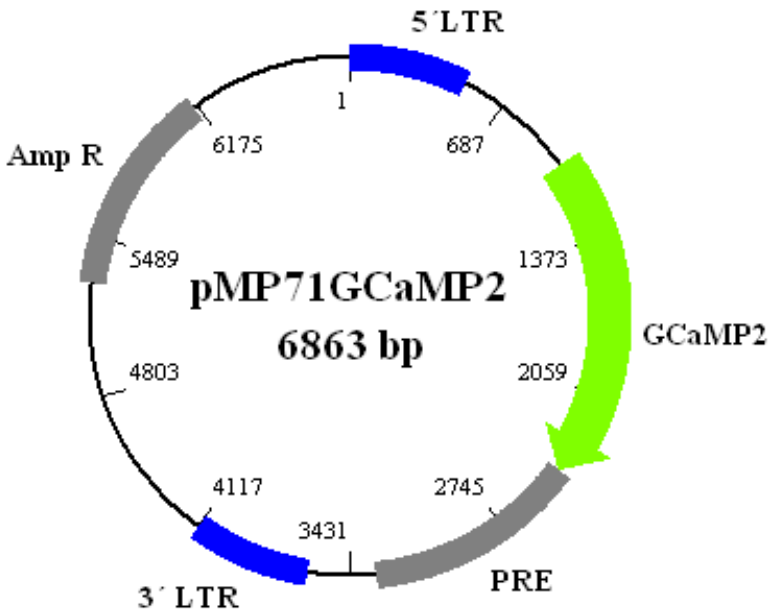
8. Appendix



Vector map of N1-GCaMP2



Vector map of pMP71-GCre



Vector map of pMP71-GCaMP2

Curriculum Vitae

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Education

2006-today PhD candidate of the Freie Universität, Berlin, Germany

2006-2009 Stipend of the Deutsche Forschungsgesellschaft (German science society), member of the Graduate School 1258 “Neuroinflammation”

2000-2006 Diploma (MS equivalent) in Biology, Freie Universität, Berlin, Germany

Research experience

2006-today PhD candidate, Department of Cellular Neuroscience, Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany; Project: “Characterization of microglia properties by the genetically encoded calcium sensor GCaMP2 in the injured brain”; Advisor Prof. Dr. H. Kettenmann, Mentor Prof. Dr. med U. Dirnagl

2005-2006 Research period, Department of Neurobiology, Freie Universität Berlin, Berlin, Germany; Project: “Blocking of Kennyon cell spike activity by

local anesthetics"; Advisor Prof. Dr. B. Grünewald, Lab of Prof. Dr. R. Menzel

2003-2005 Diploma student, Department of Neurobiology, Freie Universität Berlin, Berlin, Germany; Project: "Ca2+-stimulation of CREB in primary Kennyon cells in the honey bee, Apis mellifera". Thesis advisored by Dr. D. Eisenhard and Prof. Dr. R. Menzel

Technical skills

Physiology	Patch clamp (whole-cell recordings) and Calcium imaging techniques with genetically encoded calcium sensors (Pericam, GCaMP2, inversePericam, TN-XXL) and calcium indicator dyes (Fura-2 and Fluo-4) in cell culture and acute brain slices
Biochemistry	Immunohistochemistry approaches, SDS-PAGE, Western Blot, ELISA, Nitric oxide assay
Molecular Biology	Molecular cloning, PCR amplification, Transfection methods: Transformation, Retroviral transduction, Calcium/Phosphate transfection, Electroporation (AMAXA) and Lipofectamine 2000, Transfection of cell lines, primary cell cultures and in vivo, DNA/RNA extraction
Cellular Biology	Preparation of primary microglia cell culture, brain slice culture, primary astrocyte cell culture, handling of different cell lines (fibroblasts, virus producer HEK cell line, microglia cell line), Chemotaxis assay, Phagocytosis assay
Microscopy	Light, Fluorescence, Confocal and Two-photon microscopy
Animal experiment	local injections (ip, im, iv), blood sampling, stereotactic injections in anesthetized mice, organ sampling, preparation of acute brain slices, isolation methods

Affiliation to scientific society

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Forum of the European Neuroscience Society

Member of the graduate school 1258 "Neuroinflammation"

Publications

Seifert S, Pannell M, Uckert W, Färber K and Kettenmann H: Transmitter- and hormone-activated Ca2+ responses in adult microglia in situ recorded after viral transduction of a recombinant Ca2+ sensor (submitted)

Ifuku M, Okuno Y, Yamakawa Y, Izumi K, Seifert S, Kettenmann H, Noda M: Functional importance of inositol-1,4,5-triphosphate-induced intracellular Ca2+ mobilization in galanin-induced microglial migration (accepted in Journal of Neurochemistry)

Scheffel J, Regen T, van Rossum D, Ribes S, Seifert S, Parsa R, Harris R, Kohsaka S, Brück W, Nau R, Kettenman H and Hanisch UK: Functional ex vivo analyses of microglia reveal diversity of responses to Toll-like receptor challenges in an age- and region-dependent manner (in preparation)

Communications

Abstracts/Posters

Seifert S, Färber K, Uckert W and Kettenmann H (2008): Expression of a calcium sensor protein in microglia in vivo. SfN 2009, Chicago, USA

Seifert S, Färber K, Uckert W and Kettenmann H (2008): Expression of a calcium sensor protein in microglia in vivo. Berlin Brain Days 2009, Berlin, Germany

Seifert S, Färber K, Uckert W and Kettenmann H (2008): Characterization of newly born cells in the corpus callosum after stab wound injury. Berlin Brain Days 2008, Berlin, Germany

Seifert S, Färber K, Uckert W and Kettenmann H (2008): Expression of a calcium sensor protein in microglia. Berlin Neuroscience Forum 2008, Berlin, Germany

Seifert S, Färber K, Uckert W and Kettenmann H (2007): Expression of a calcium sensor protein in microglia. Berlin Brain Days 2007, Berlin, Germany

Seifert S, Fröse A, Grünewald B and Eisenhardt D (2006): AmCREB response to calcium stimulation of cultered honeybee Kenyon cells and to induction of long term memory in vivo . FENS 2006, Vienna, Austria

Grünewald B, Seifert S, Blunk A (2006): Mapping complex olfactory learning tasks within the honeybee brain by procaine injections. Berlin Neuroscience Forum 2006, Berlin, Germany

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