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„Intracellular modification of microglial membrane currents and their heterogeneity in different brain regions in acute mouse brain slices"

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II LIST OF ABBREVIATIONS

ACSF	Atrificial cerebrospinal fluid
ATP	Adenosine triphosphate
et al.	Et alii (lat.: and others)
e.g.	Exempli grantia (lat.: for example)
cAMP	3',5'-cyclic adenosine monophosphate
DAG	Diacyl glycerol
eGFP	Enhanced green fluorescent protein
GDP	Guanosine diphosphate
GIRK	Inwardly rectifying G-protein-activated potassium channel
GTPγS	Guanosine 5'-O-3-thiotriphosphate
HSIS	High sodium intracellular solution
i.e.	Id est (lat.: that is to say)
ISI	Intracellular solution
I	Current
IP_3	Inositol 1,4,5-triphosphate
NHE	Na^{2+}/H^{+} exchanger
pF	Picofarad (measure of electrical capacitance)
PIP_2	Phospholipid phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
U	Voltage
V_m	Membrane potential

III FIGURE LEGEND

1 Introduction

1.1 Microglia in the context of the CNS

The central nervous system (CNS) consists mainly of neurons as well as astrocytes and oligodendrocytes, also known as macroglia (Kandel, 2013). Recent advances suggest that all three cell types might be derived from a common precursor, polydendrocytes (also known as NG2 cells; Nishiyama et al., 2009). NG2 cells were first characterized in the 1980s because they displayed properties that resemble both glia and neurons (see **Fig. 1**). The fifth cell type of the CNS however, which makes up about 15% of the population, are microglia – the immunocompetent cells of the CNS. Although microglia have long been regarded as pro-inflammatory and cytotoxic, it is now widely accepted that they are involved in homeostasis and repair mechanisms. Microglia can be found in the entire CNS, including the eye and optic nerve (Kettenmann et al., 2011). That microglia are not generated within the nervous system was already established 1932 by Pío del Río Hortega, who noticed "fountains of microglia" during early brain invasion (Verkhratski and Butt, 2013). Incidentally, Río Hortega was also the first to use the name microglia (Kandel, 2013). Based on their divergent functions in healthy and distressed brain, two states of microglia have been defined. In the healthy brain, microglia are regarded as "resting". In this state, ramified microglial use their processes to survey their surroundings in fixed territories that do not overlap. Upon encountering noxious stimuli microglia rapidly undergo a shift in activity states. Their activity is temporally and spatially restricted in order to maintain the delicate structure and environment of the brain. These divergent cellular states are widely believed to be the result of adaptations to the exceptional conditions in the brain (Hanisch and Kettenmann, 2007). Although meningeal lymphatic vessels have recently been discovered (Louveau et al., 2015), the brain is relatively isolated from its surroundings via structures like the blood-cerebrospinal fluid and blood-brain barrier. At these strategic sites, mononuclear cells as macrophages and dendritic cells fulfil sentinel functions. In addition to this protective screen, ramified microglia survey their surroundings by constant movement of their processes. Although microglia are regarded as resident macrophages in the brain and share haematopoietic stem cells as common precursors, they are distinct from macrophages in other tissues. Particularly fate mapping studies have demonstrated that microglia are not just brain macrophages. In contrast to macrophages that are derived from the bone marrow, microglia are generated in the yolk sac (Ginhoux et al., 2010). Furthermore, unlike macrophages, microglia are long-lived and possess the ability of self-renewal, which means that no further cell invasion into the CNS takes place for replacement or cell expansion. Despite being of the same cellular origin as macrophages, ramified microglia show no phagocytic capacity or expression of receptors necessary for induction of macrophage functions in the healthy brain (Aloisi, 2001). It has been demonstrated that substances released from healthy neurons and their activity is sufficient to maintain micro-

glia in their ramified, resting state (Neumann et al., 1996). However, microglia are opulently equipped to detect acute physiological disturbances originating inside or outside of the CNS, as damage, neurodegeneration or pathogens. One such proinflammatory signal for microglial activation is ATP, which is released by damaged CNS cells (Inoue, 2002). Microglia then extend their processes toward the source of the proinflammatory signal and are induced to adapt an "activated", amoeboid phenotype. As a result, microglia migrate toward the source of insult and act as scavengers. By release of pro-inflammatory cytokines they recruit the innate immune cells, induce apoptosis of damaged cells and phagocytize cell debris. Once the physiological disturbance has been resolved, microglia are also involved in tissue repair by releasing anti-inflammatory cytokines and growth factors (Farber and Kettenmann, 2005). Starting in the 1980s, glial cells have become of interest in electrophysiological research. While microglia have long been disregarded in favour of astrocytes and oligodendrocytes, they were electrophysiologically characterized over the last decade. Making use of these past studies, the current experiment was designed to investigate the intracellular modification of microglial membrane currents and their heterogeneity in different brain areas.

1.2 Heterogeneity of microglia

As mentioned before, substances released from healthy neurons and their activity are sufficient to maintain microglia in their ramified, resting state. In order to do so, microglia are equipped with numerous neurotransmitter and –peptide receptors (Kettenmann et al., 2011). Based on the diverse utilization of signaling molecules in the brain, e.g. in distinct brain regions or circuits, the hypothesis has been posed that microglia in distinct brain regions might be characterized by corresponding receptor expression patterns (Hanisch, 2013). In an experiment with freshly isolated murine microglia all cells were found to exhibit an ATP response. In contrast, about 26% of cells showed a response to substance P and only 1% responded to endothelin. Percental responses to other neurotransmitters and –peptides lay between these values (Pannell et al., 2014). Furthermore microglia are not uniformly distributed (Lawson et al., 1990) and functional differences have been uncovered between microglia populations. For example, subpopulations of microglia are engaged in the removal of myelin-laden exosomes from oligodendrocytes (Fitzner et al., 2011). In these cells macropinocytosis of exosomes is not associated with an immune response and they remained immunologically inactive in a t cell activation assay. Throughout adulthood, microglia of the subventricular zone not only pertain their proliferative potential but also support neurogenesis (Walton et al., 2006). It is to date unknown if these functional or expressional differences in microglia are determined by local environmental cues or lineage commitment. Nevertheless, the concept of microglial heterogeneity is worth more consideration. In the present study microglia were therefore compared between the cortex, thalamus and cerebellum.

1.3 Electrophysiology

At the beginning of the 20th century Julius Bernstein proposed with his "membrane hypothesis" that the membrane potential is based on a diffusion potential. This is set up by the partially permeable cell membrane and the resulting tendency of positively charged ions to diffuse across their concentration gradient into the extracellular solution, while other ions are retained. In the central nervous system, electrical signaling and excitation depend on the movement of ions through specialized pores in the cell membrane, known as ion channels. Selective, but high, permeability to one or more of the four principal ions (sodium, potassium, calcium and chloride) as well as responsiveness to specific stimuli (voltage changes, chemical excitation e.g. with neurotransmitters, pH and many more) that induce a conformational change (closed to open or vice versa) are main characteristics of ion channels. Ion fluxes thereby represent current changes that directly affect membrane potential (Hille, 2001). The voltage clamp technique was therefore devised by Meech and Standen in the 1970s to study changes in current responses of membrane channels by manipulation of membrane voltage irrespective of the ionic currents. Based on the voltage clamp technique, Neher and Sakman shortly after developed it further into the patch-clamp technique. For this, Neher and Sakman were subsequently awarded with the Nobel Prize in 1991 (Neher, 1992; Sigworth, 1986). In contrast to the voltage clamp technique, patch clamp utilizes the formation of a strong seal between the patch pipette and the cell membrane by applying weak negative pressure at the end of the pipette. The electrical resistance between the opening of the patch pipette and the cell membrane increases to more than one gigaohm and is therefore also called gigaohm seal. Furthermore, in order to gain electrical control of the whole cell, the membrane patch under the pipette is removed by gentle suction, while the gigaohm seal remains intact. This allows for clamping the membrane to a command voltage and subsequently to study changes in current responses (Molleman, 2003).

1.3.1 Electrophysiology of (micro)glial cells

Starting in the 1980s, the whole cell patch clamp technique made small glial cells accessible to recording (Kettenmann et al., 1982; Sonnhof et al., 1982). Three general principles that emerged during this time are that 1) glial cell membranes are foremost potassium permeable, 2) they possess linear current-voltage relationships, i.e. they are electrically passive, 3) they are not excitable and appear to lack sodium channels. Not all of these principles still hold true today for glial cells in general (Barres et al., 1990).

First of all, the electrical passiveness of glial cells has only partly pertained. Although about 50% of the oligodendrocyte population has been reported to possess linear current-voltage relationships based on their preferential permeability to potassium ions, this cannot be generalized (Sontheimer and Waxman, 1993). Glial current-voltage plots are mainly determined by inward currents. As will be discussed in the next paragraph, these currents become primarily visible at

negative voltages between -120 and -170 mV. However, early voltage-clamp experiments were based on protocols derived from neurons and thus barely clamped to voltages below -100 mV (Barres et al., 1988; Sontheimer et al., 1988). The non-linear nature of current-voltage plots was therefore disregarded for a time. In microglia, the non-linear current-voltage relationship is determined by voltage-gated and inward rectifying (K_{ir}) potassium as well as chloride channels (Newell and Schlichter, 2005).

The prominent role of potassium currents in glia can be observed in **Fig. 1e**. Inward current (represented by downward deflections) is mainly passed at negative currents (lower traces) and is not transmitted at voltages positive to the resting potential (upper traces that do not show downward deflection). For activation of all K_{ir} channels phosphatidylinositol 4,5-bisphosphate (PIP_2) is necessary. Delayed rectifying potassium channels are mainly upregulated as a result of cytokine or chemokine exposure during microglial activation, which is also accompanied by a K_{ir} downregulation (Farber and Kettenmann, 2005). In contrast to K_{ir} currents, delayed rectifier currents are triggered at voltages positive to -40 mV and their amplitude is correlated with the depolarization (Eder, 1998). Delayed rectifier current is outward current, represented by upward deflections in the current profile in **Fig. 1e**. Due to the resting phenotype of this microglia, delayed rectifier current is limited. In acute slices stored for five to six hours however, induction of delayed rectifier current can be observed due to the trauma inflicted by cutting. Additionally pronounced delayed rectifier currents can be observed in neuron (**Fig. 1a**, left panel) and NG2 cell (**Fig. 1b**). This relationship of inward and outward current distinguishes microglia from any other glial cell type. Due to the near absence of outward current, the microglial membrane is easily depolarized by small inward currents (Kettenmann et al., 1993). Even though potassium channels are most prominent in microglia, expression of numerous other channels has been demonstrated. The afore-mentioned microglial chloride channels have been implicated in the induction and execution of proinflammatory functions (Newell and Schlichter, 2005). Even expression of voltage-gated sodium channels, a bastion of cell excitability, has been demonstrated in rat microglial cells (Black et al., 2009). However, it has to be noted that excitability is still prevented in glia by the low density of sodium channels as well as the high potassium permeability. For example, when astrocytes were isolated from human epileptogenic foci, expression of voltage-gated sodium channels was dramatically increased. As a result, these cells exhibited excitability and injections of current produced action potential-like responses (O'Connor et al., 1998). Furthermore, all major ion channels known from neurons have also been discovered in glia. Apart from the already mentioned channels, these include calcium and proton channels (Schilling and Eder, 2007; Walz and Bekar, 2001).

Although many of these ion channels have first been described in cultured glial cells, they can also be found in acutely isolated cells and in acute brain slices and are therefore considered to

bear functional significance. Furthermore, glia-specific ion channel isoforms have been discovered. Apart from the pronounced potassium conductances in glial cells, which are relevant for glial proliferation, spatial K^+ buffering and contribute to the negative membrane potential, other ion channels are important for nervous system homeostasis. Comparable to neurons, calcium acts as second messenger in cellular processes mediated by calcium channels. Apart from the ubiquitous Na^{2+}/K^+-ATPase, sodium channels also provide the substrate for the Na^{2+}/H^+ exchanger (NHE), which is implicated in pH regulation in glia and induction of a pro-inflammatory state in microglia. In addition to NHE, stretch-activated cation channels and chloride channels also mediate regulation of cell volume (De Vito, 2006). Furthermore, electrophysiological studies in vertebrates revealed the existence of voltage-dependent and even some neurotrasmitter-gated ion channels. Glial cells in the central nervous system are thus able to sense neuronal activity and signaling and respond to it. This largely dismisses the notion that voltage-gated ion channels are solely associated with cell-cell communication of excitable cells. Just like neuronal ion channels, glial channels are prone to functional regulation by G-proteins and second-messenger pathways. This intracellular modification of microglial membrane currents is the subject of the present project.

1.3.1.1 Characterization of the microglial ATP response

Resting microglia continuously monitor their surroundings with highly dynamic processes. Pathological conditions can induce a switch from immune surveillance to release of pro- or anti-inflammatory cytokines and chemokines, chemotaxis toward injured areas and phagocytosis of cell debris. One of the substances mediating microglial activation and chemotaxis is ATP, a signaling molecule released from damaged brain tissue (Davalos et al., 2005; Neary, 1996). Apart from P2 adenosine receptors, ionotrophic P2X and metabotrophic P2Y receptors are the major ATP receptors in the brain. Other than P2Y11 and P2Y14 which are not expressed, there is at least either functional or mRNA evidence for microglial expression of the other P2Y receptor subtypes in microglia (Fields and Burnstock, 2006). Similarly, all P2X receptors except P2X5 and P2X7 are expressed in the central nervous system. Activation of the P2Y purinergic receptors can induce a number of cell signaling molecules and second messenger systems, including cAMP, calcium, IP_3 and PLC. This signaling complexity is based on the broad spectrum of cellular functions mediated by ATP signal transduction. In response to ATP, microglia in acute slices as well as cell culture have been found to exhibit both inward and outward currents (Boucsein et al., 2003; Haas et al., 1996). In detail, a previous study in acute brain slices of C57/Bl6 mice found that 1mM ATP applied extracellularly induced an increase in membrane currents at depolarizing potentials positive to -70 mV. Since the reversal potential is close to the potassium equilibrium potential it is believed that potassium currents represent the main ion flux (Boucsein et al., 2003). In contrast, when the non-selective potassium channel blocker quinine was applied, 3mM

ATP could not induce outward currents in fractalkine knockout mice (Wu et al., 2007). In a second population of cells, modestly inwardly rectifying current was observed. In a small number of cells both inwardly and outwardly rectifying potassium conductances were observed. The observed inward current has been attributed to ionotrophic non-selective cation channels (P2X), while the outward current has been ascribed to potassium channels linked to metabotropic purinergic receptors (P2Y, Visentin et al., 1999). A pharmacological screening using purinergic ligands furthermore revealed that the P2Y1, P2Y2 and P2Y4 receptor subtypes are likely involved in the observed outward K^+ currents (Boucsein et al., 2003).

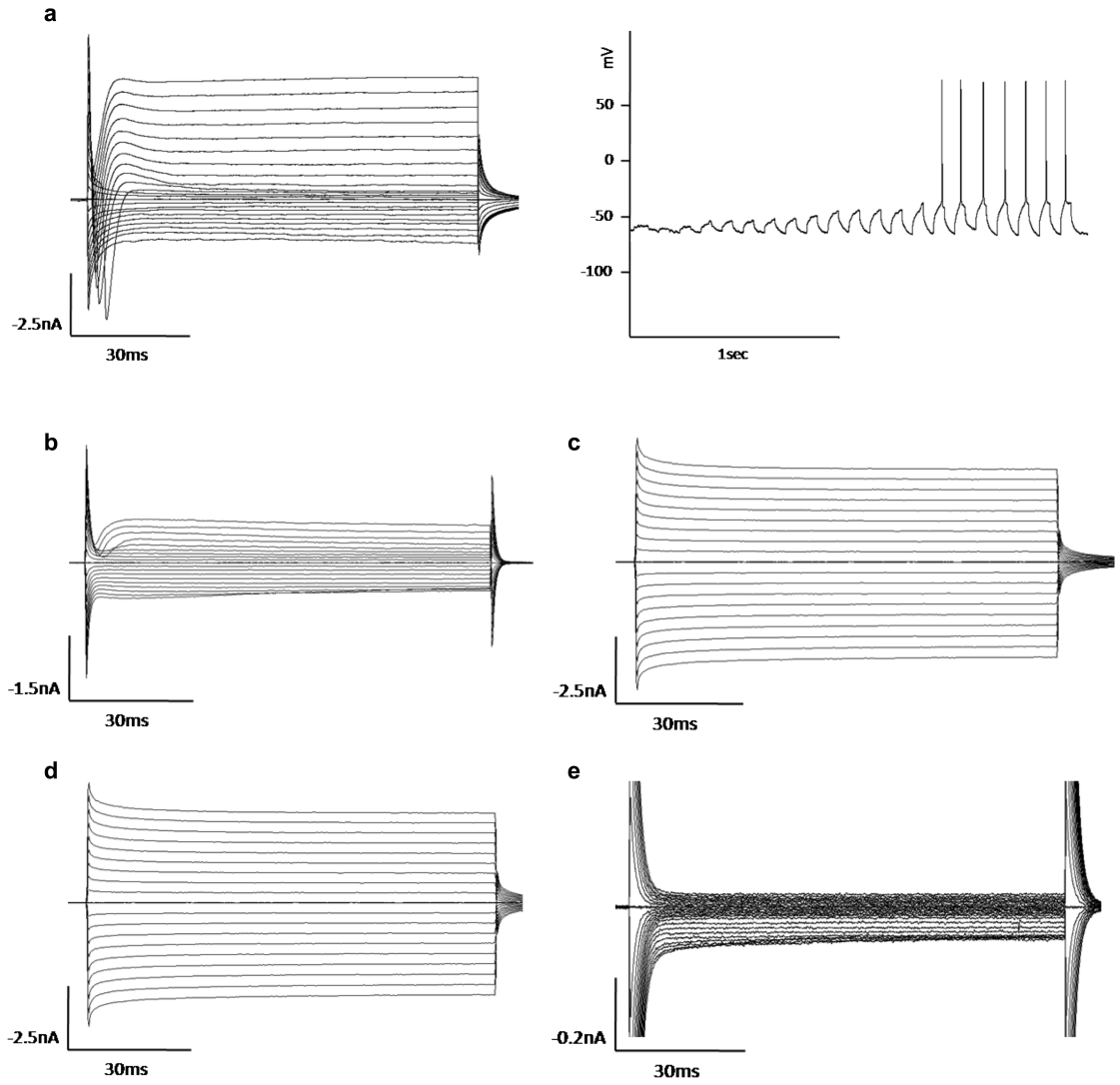

Figure 1: Comparison of current profiles of electrically active and passive cells in the cerebral cortex of the mouse current profilse of **a)** cortical neuron (left) and current clamp trace of action potential initiation (right) **b)** cortical NG2 cell **c)** oligodendrocyte and **d)** astrocyte were obtained by clamping from a holding potential of -70mV to 10 de- and hyperpolarizing voltage steps in 10mV increments **e)** microglia, clamped from a holding potential of -20mV to 6 depolarizing and 17 hyperpolarizing voltage steps in 10mV increments

1.4 G-proteins and intracellular second messengers

G-protein coupled receptors (GPCR), a superfamily of cell-surface receptors that comprises more than 900 members, represent one of the major targets of biomedicine (Pierce et al., 2002). As the name suggests, these receptors are coupled to intracellular G-proteins, which consist of three subunits (Gα, Gβ, and Gγ). Upon receptor activation, Gα binds to guanosine triphosphate (GTP), which triggers dissociation of Gα from the Gβγ dimer. The activity of other intracellular proteins is then modulated by both Gα and Gβγ. Upon hydrolysis of GTP to guanosine diphosphate (GDP), Gα associates with Gβγ and receptor activation is terminated (Lattin et al., 2007). G-proteins mediate a myriad of functions via activation or inhibition of adenylyl cyclase, stimulation of phosphoinositide hydrolysis (cleavage of PIP_2 to IP_3) and direct regulation of ion channels. In general, GPCR activation requires both, the appropriate ligand of the receptor and GTP. However, a response mimicking receptor activation can be elicited by non-hydrolizable GTP analogues, as guanosine 5'-O-3-thiotriphosphate (GTPγS) or 5'-Guanylyl imidodiphosphate (Gpp[NH]p; Gilman, 1987).There are four specific α subunit mechanisms: $G_{\alpha s}$ (G_s), $G_{\alpha i}$ (G_i), $G_{\alpha q/11}$ (G_q) and $G_{\alpha 12/12}$. In addition the Gβγ dimer can exert functions, e.g. activation of G protein-coupled inwardly-rectifying potassium channels (Hibino et al., 2010). The specific effects of the relevant α subunit mechanisms will be discussed in the upcoming sections 1.41 and 1.4.2.

1.4.1 GTPγS

GTPγS is a non-hydrolysable GTP analogue, which is sufficient to evoke intracellular responses in lieu of extracellular G-protein receptor activation (Gilman, 1987). Negative cooperativity has been established for the binding of Gα to Gβγ and GTPγS. In other words, GTPγS facilitates dissociation of the G-protein subunits (to Gα and Gβγ dimer; Sternweis et al., 1981). GTPγS can stimulate three distinct G-proteins: G_s, G_i and G_q. GTPγS acts on the G_s G-protein, the positive regulator of adenylyl cyclase. Adenylyl cyclase can therefore be directly activated by the Gsα-GTPγS complex (Northup et al., 1983). In contrast, association of GTPγS with Gi leads to inhibition of adenylyl cyclase. Activation of G_q G-proteins leads to activation of phospholipase C. PLC leads to the cleavage of PIP_2 (phospholipid phosphatidylinositol 4,5-bisphosphate) into DAG (diacyl glycerol) and IP_3 (inositol 1,4,5-trisphosphate). IP_3 results in a subsequent increase in intracellular calcium. Calcium and DAG in turn, activate protein kinase C, which exerts its function by phosphorylation of proteins (Nishizuka, 1988). Since protein expression patterns vary between cell types, PKC actions are cell-type specific (Newton, 1995). In microglia, PKC activation has been mainly implicated in activation and proliferation (Ryu et al., 2000; Sawada et al., 1990).

1.4.2 cAMP

3',5'-cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger molecule. In a physiological setting, intracellular cAMP levels are therefore balanced by finely coordinating

production by adenylyl cyclase with degradation by phosphodiesterases (Kopperud et al., 2003). Furthermore, intracellular cAMP levels can be modulated by extracellular mechanisms through G-protein coupled receptors. Intracellular cAMP levels can be modulated by activation of G_i as well as G_s G-proteins, which exhibit opposing actions. The general function of G_i is to inhibit cAMP-dependent/adenylyl cyclase pathway, which decreases the generation of cAMP from ATP. In contrast, activation of the G_s pathway can be mimicked by an increase in intracellular cAMP, as done in the present study. Activation of the G_s protein bound to the α receptor subunit results in activation of adenylyl cyclase via binding to it. Adenylyl cyclase then mediates the conversion of ATP to cAMP. The resulting increases in intracellular cAMP concentration may result in the activation of cAMP-dependent protein kinase A (PKA), cyclic nucleotide-gated ion channels and exchange proteins induced by cAMP (Bos, 2003; Kaupp and Seifert, 2002). Additionally, other mechanisms have been described, these do, however, bear no relevance in the context of microglia (Simrick et al., 2013). While the present study was designed to investigate the effects of intracellularly applied cAMP throughout the cytoplasm as a mimic of receptor activation, it has to be noted that current research indicates that under physiological conditions, alterations in cAMP concentrations occur in microdomains of individual cell compartments only (Cooper, 2003). It is therefore possible that application of cAMP throughout the whole cytoplasm induced activation of several signaling systems.

1 Aim

Although microglia have been electrophysiologically characterized over the past decade, many questions remain unresolved. This is at least in part due to the fact that microglia in culture seem to exhibit different properties than in acute slices (Farber and Kettenmann, 2005). It is therefore not clear if all channels and receptors described in culture can be found in microglia in acute slices. Apart from the pronounced potassium conductance in glial cells, other ion channels are important for nervous system homeostasis (Kettenmann et al., 2011). As all ion channels, microglial channels are prone to functional regulation by G-proteins and second-messenger pathways. Since the compositions of both the extra- and intracellular solution are predefined in whole-cell patch clamp experiments, specific modifications are possible. In order to test this effect of functional ion channel regulation on microglial membrane currents, a high sodium concentration, a near-universal G-protein activator and ubiquitous second messenger molecule were individually added to the intracellular solution. Since high intracellular sodium concentration is believed to act on a multitude of intracellular targets, it was used to test if microglial currents could be modified intracellularly. GTPγS and cAMP were chosen based on their capacity to activate intracellular targets. This intracellular modification of microglial membrane currents in whole-cell patch clamp recordings is the subject of the first part of the present project. Further-

more, recent observations suggest that microglia exhibit functional and therefore expressional heterogeneity (Gertig and Hanisch, 2014). It has been speculated that this heterogeneity is based on adaptations to specific signaling molecule milieus. In the second part of this project, microglia were therefore electrophysiologically compared between the well-characterized cerebral cortex and two novel areas, namely the cerebellum and thalamus.

2 Materials and methods

2.1 Animals

For the present study *Csf1r* ^{EGFP} transgenic mice on C57BL/6 background were used as well as Cx3cr1$^{GFP/wt}$ mice on 129SvEv and C57BL/6 mixed genetic background. *Csf1r* ^{EGFP} mice are characterized by enhanced green fluorescent protein (EGFP) expression in all cells of the mononuclear phagocyte system, including microglia, due to an EGFP insert in the *c-fms* gene, which encodes the macrophage colony-stimulating factor (CSF-1) receptor (Sasmono and Williams, 2012). Due to this EGFP expression they are commonly referred to as MacGreen mice. CX3CR1 is also known as the fractalkine receptor gene, which encodes a chemokine receptor primarily expressed by monocytes, microglia and subsets of natural killer and dendritic cells (Cook et al., 2001; Jung et al., 2000). In the Cx3cr1$^{GFP/wt}$ mice, one allele of the CX3CR1 gene is replaced by a green fluorescent protein (GFP) reporter gene. For all experiments regarding the modification of the intracellular solution a total of 11 MacGreen (seven male, four female) mice were used. For the experiments comparing brain regions, a total of 7 MacGreen and 3 Cx3cr1$^{GFP/wt}$ animals (four male, seven female) were used. For the current profiles of oligodendrocyte and astrocyte, one 3 weeks old PLP^{EGFP} xGRFU^{MRFP} mouse was used (Fuss et al., 2000; Hirrlinger et al., 2005). In this mouse model, the PLP promotor (of the CNS myelin proteins proteolipid protein) is linked to EGFP and the GRFU promoter to MRFP (monomeric red fluorescent protein). Animals were maintained under constant environmental conditions (12 h light-dark cycle with lights on at 06.00 h, ambient temperature 20-22 °C, relative humidity 50-60%) and food and water were available *ad libitum*. All experiments were conducted according to the German law for animal protection and with approval of the Landesamt für Gesundheit und Soziales (LaGeSo) of the Berlin Senate (G0438-12).

2.1 Solutions

2.1.1 Cold buffer

For preparation of acute brain slices, 134 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 1.26 mM K_2HPO_4, 10 mM glucose as well as 43 mM NaHCO3 were used with a pH of 7,4 at 4°C under continuous gassing with carbogen (95% O_2, 5% CO_2).

2.1.2 Artificial cerebro-spinal fluid (ACSF)

The standard ACSF contained 134 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 1.26 mM K_2HPO_4, 10 mM glucose and 26 mM NaHCO3 (adjusted from Schipke et al., 2008). The pH of the solution was adjusted to 7.4 by continuous gassing with carbogen. During experiments, the perfusate was pumped through the recording chamber at a rate of 3-6 mL/min.
For recording of ATP responses, ATP (1 µM) was applied extracellularly via the perfusate.

2.1.3 Intracellular solutions

For microglia and neurons, intracellular solutions containing 130 mM KCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM HEPES, 5 mM EGTA, 2mM Na-ATP and 1µM sulforhodamine 101 were used. pH of the intracellular solutions was either adjusted with potassium hydroxide (15.1mM KOH, pH 7.4, osmolarity 272 mosmol/l) to generate standard intracellular solution (SIS) or sodium hydroxide (11.5 mM NaOH, pH 7.31, osmolarity 286 mosmol/l) to generate high sodium intracellular solution (HSIS). For intracellular G-protein and second messenger effector molecule experiments an additional 100 µM of GTPγS or 500 µM of cAMP were added. For astroctes and oligodendrocytes, the intracellular solution contained 30mM KCl, 1mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 mM Glucose, 100 mM Gluconate, 10 mM HEPES, 5 mM EGTA and 3mM Na-ATP (pH 7,2, osmolarity 313 mosmol/l). 1µM Lucifer Yellow was used to check pipette outflow.

2.2 Preparation of acute brain slices

Acute brain slices were prepared as previously described by Boucsein et al. (2000). In summary, (Boucsein et al., 2000)mice were killed by cervical dislocation and the brain was subsequently removed and cooled down in cold buffer. The forebrain and/or cerebellum was then glued to a metal block and suspended in cold buffer while coronal sections of 200µm were cut by a microtome (Thermo Scientific, Braunschweig, Germany) under continuous gassing with carbogen. Depending on the brain area to be patch-clamped, the thickness of the slices ranged from 200 to 250 µm. Until used, the slices were stored at room temperature in a recovery chamber submerged in ACSF and continuously perfused with carbogen. All experiments were performed within five hours after sacrifice of the animals.

2.3 Patch-clamp recordings

The whole cell patch-clamp technique for recording membrane currents (Hamill et al., 1981) was conducted using an EPC 10 patch-clamp amplifier and TIDA 5.25 software (HEKA Elektronik, Lambrecht, Germany). For recordings, the slices were kept at room temperature in an ACSF perfused holding chamber. In the holding chamber, a grid of nylon threads attached to an U-shaped platinum wire held the slices in place. Cells in the acute slices were selected by their fluorescence under 488nm (microglia and oligodendrocytes) or 585nm (astrocytes) illumination, by the aid of a polychromator set to the appropriate wavelength (Till photonics, Gräfelfing,

Deutschland). Patch pipettes with resistances ranging from 4 to 6 megaohm (MOhm) were pulled from borosilicate capillaries (Hilgenberg, Malsfeld, Germany) with a pipette puller (Sutter Instrument, Novato, California) and filled with intracellular solution. Subsequently, cells were patched under bright-field illumination (overlaid with fluorescence in the thalamus and cerebellum due to poor visibility) using the 63× objective (Zeiss; Oberkochen, Germany) of an upright microscope (Zeiss Axioskop 2 FS plus, Zeiss, Oberkochen, Germany; **Fig. 2**) coupled to a digital camera (PCO Computer Optics GmbH, Kelheim, Germany). In order to obtain whole-cell configuration, cell-attached patches were formed by reaching a gigaohm seal and capacitative transients were compensated by TIDA 5.25 software. The cell membrane under the pipette was ruptured by gentle suction.

2.3.1 Stimulation protocols

Stimulation protocols were used to record current responses after whole-cell voltage-clamp configuration was established. The holding potential was set to -20mV before rupture of the microglial cell membrane underneath the patch pipette. Immediately after "opening" the cell and determining the membrane potential in current-clamp configuration, a series of voltage steps ranging between -160 mV and 60 mV, in 10 mV increments, were imposed on the cell using holding potentials of -20, -40 and -70 mV. For the experiments concerning the modification of intracellular solutions, current profiles were measured every minute for up to seven minutes to monitor dialysis of the cytoplasm with the intracellular solution. The actual stimulation protocol can be found in Appendix section 6.3. However, since cell viability after this sequence was low and the -70 mV current profiles were found to be most conclusive, the current profiles at -20 and -40 mV holding potential were omitted from the stimulation protocol and data analysis. Low cell viability was probably due to the rigorous clamping to various holding potentials while recording current profiles for several minutes. As a result, most cells died during the seven minutes stimulation protocol during the recording of ATP responses. When the stimulation protocol recorded current profiles at -70 mV holding potential only, cells survived long enough to record ATP responses afterwards. For extracellular application of ATP through the perfusate (standard ACSF), the cells were repeatedly and alternately clamped to the holding potential of -20 mV and predefined voltage steps. The series of voltage steps ranged between -140 and 60 mV. This time, 20 mV increments were used and the alternating series of 100 millisecond pulses was repeated every 5 seconds for six minutes. The protocol for recording of ATP responses can also be found in Appendix section 6.3

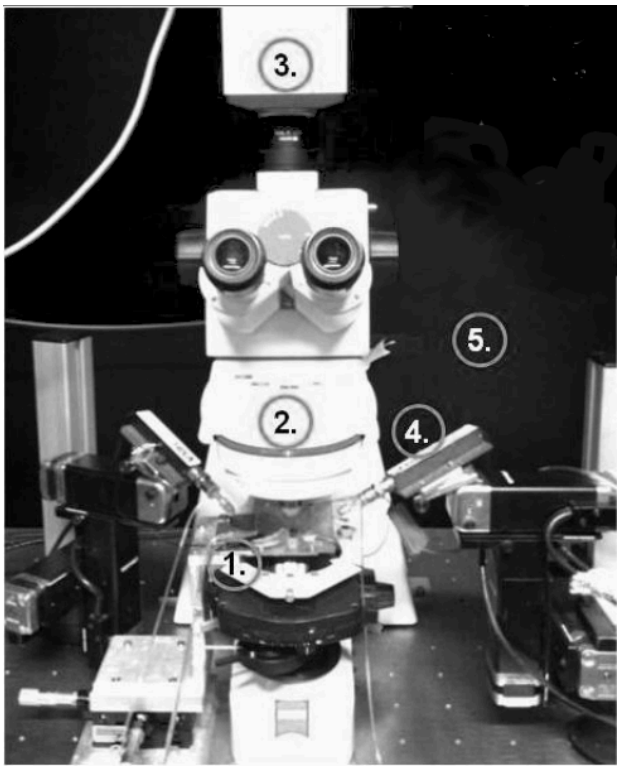

Figure 2: Setup comprising perfused holding chamber **(1)**, fluorescence filters **(2)** of the upright light microscope, digital camera **(3)**, pipette holder directly connected to amplifier **(4)**, faraday cage for electrical isolation **(5)**, adapted from Richter, 2015

2.4 Data analysis

For each cell, membrane capacitance was used as an estimate of membrane area, assuming a membrane capacitance of 1µF/cm^2. In every current profile, the current values recorded between 0,343 and 0,354 seconds were used for the generation of a current-voltage (I/U) plot. Current responses were normalized to membrane capacitance, as an estimate of membrane area and expressed as current density.

Between groups, current densities for each voltage step were compared by 2-way ANOVA with repeated measures. To compare capacitance, membrane resistance, series resistance and reversal potential, unpaired t-tests with equal standard deviations or ordinary one-way ANOVA with multiple comparisons were performed, depending on the number of experimental groups. Conductance was calculated in a defined area of the I/U plot with linear relationship (e.g. between -120 and -90 mV) and statistical analysis was conducted by using ordinary one-way ANOVA with multiple comparisons. Where used, box plots represent median as well as first and third quartile while whiskers indicate minimum and maximum. For ATP application experiments, the normalized current response for every pulse was plotted against time. Stable base line readings were then subtracted from the peak response. Further data analysis was conducted by 2-way ANOVA. For all analyses, Bonferroni's multiple comparison post-hoc tests were used where applicable.

3 Results

3.1 Comparison of intracellular solutions

In patch clamp whole-cell recordings, the intracellular/pipette solution is matched to the cytoplasmic milieu in both ionic composition and pH. This way, washout of ions via dialysis of the cytoplasmic and the pipette solution, taking place during recording, is prevented. Since the intracellular ionic composition of microglial cells is to date unknown, data of microglia patched with established standard intracellular solution (SIS; Richter et al., 2014; Schilling and Eder, 2013; Schipke et al., 2008) provided by Stefan Wendt was compared to intracellular solution high in sodium (HSIS). To achieve this difference in ionic composition while maintaining similar pH (7.4 for standard intracellular solution and 7.31 for high sodium intracellular solution) and osmolarity (271 and 286 mosmol/l, respectively), the high sodium intracellular solution was pH adjusted with sodium hydroxide (NaOH) instead of the potassium hydroxide (KOH) commonly used. This way, the intracellular solutions diverged in both potassium as well as sodium concentrations. However, the additional 15.1mM KOH (for adjusting the pH) used in the standard intracellular solution represent a decrease of potassium concentration in the high sodium intracellular solution of 11.62%. In contrast, 11.5mM NaOH were used for HSIS pH adjustment, which represents an increase of 287% in sodium. The reduction in potassium in the high sodium intracellular solution can therefore be considered as negligible. While membrane potentials (determined in current-clamp mode) between the groups did not differ significantly immediately after achieving whole-cell patch clamp configuration ($p=0.0624$, data not shown), dialysis of SIS into the cytoplasm resulted in a decrease in membrane potential compared to membrane potentials of HSIS dialysed cells ($p=0.075$, **Figure 3a**). Furthermore, membrane capacitance, a measure of cell area, of microglial cells was significantly different between the groups ($p= 0.0456$, **Figure 3b**). **Figure 3c** shows normalized mean current voltage curves of microglial cells dialysed with either high sodium (grey) or standard (black) intracellular solution in response to voltage steps ranging from -160 to +60 mV (see Methods section 2.3.1). Conspicuously, microglia dialysed with HSIS show a significantly increased response to negative voltage steps ranging between -160 and -100 mV ($p<0.0001$ to $p=0.0121$, respectively) at a holding potential of -20mV, when compared to SIS. Similarly, significantly more current is passed through the membrane in response to the 60mV pulse in cells dialysed with HSIS ($p=0.0027$).

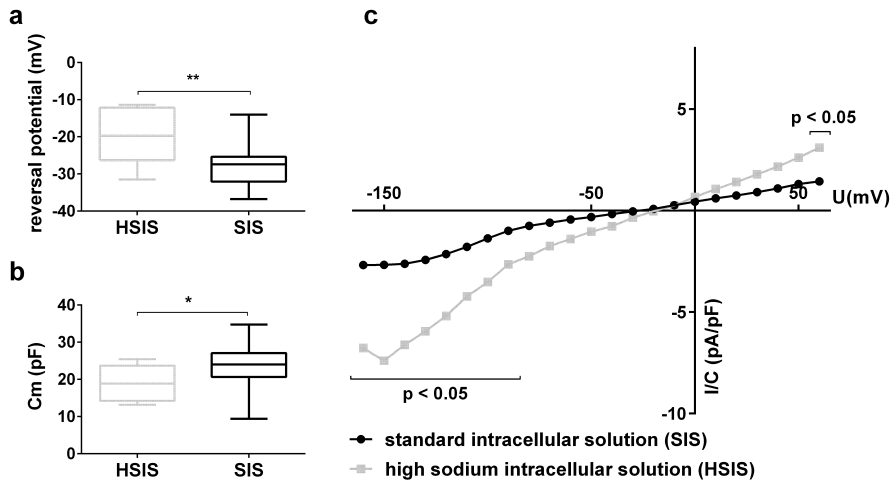

Figure 3: Comparison of intracellular solutions Box plots comparing the **a)** reversal potential (mV) and **b)** capacitance (pF) of high sodium (HSIS, grey) versus standard intracellular solution (SIS, black); unpaired t –tests with equal SD, p= 0.0456 and p= 0.0075, respectively; boxes indicate median, first and third quartile, whiskers indicate minimum and maximum. **c)** current density–voltage plot comparing the two intracellular solutions in response to a series of voltage steps ranging between -160 mV and 60 mV, in 10 mV increments, last nine pulses from -160 mV to -80 mV significantly different, $p<0.0001$ for -160 to -100 mV pulses, $p<0.01$ for -90 mV pulse, $p<0.05$ for -80 mV pulse, $p<0.01$ for 60 mV pulse; 2-way ANOVA with Bonferroni's multiple comparison test; n= 9 for HSIS, n= 18 for SIS; SIS data provided by Stefan Wendt

3.1 G-protein and intracellular second messenger – activated membrane currents

To test whether G-protein second messenger molecules affect the electrophysiology of microglial cells 100 µM GTPγS or 500 µM cAMP were added into the intracellular solution (this time, only HSIS was used). Since it was expected that the full intracellular effect of added effector molecules would be observed once the cytoplasm was completely dialysed with the intracellular solution, a TIDA 5.25 script was conceived to allow continuous recording of current profiles every minute for seven minutes. According to Kostyuk et al. (1975), seven minutes were chosen as recording duration as it was assumed that by then full dialysis must have occurred. Furthermore, a comparable time frame was chosen for an electrophysiological intracellular application experiment in oligodendrocytes (Karschin, 1993). Initially, current profiles were recorded every minute at -20, -40 and -70 mV holding potential (for script, see Appendix). Since preliminary data analyses indicated that the responses were largest at -70mV (data not shown), current profiles were then recorded at -20mV holding potential initially after obtaining whole-cell configuration as well as at -70mV holding potential during the seven minutes sequence. No significant differences were found in capacitance, reversal potential, membrane resistance and series resistance between groups at either time point as well as between time points (data not shown). **Figures 4a** and **4b** show the change in normalized current densities in response to 10mV volt-

age steps ranging from -160 mV to +60 mV at one minute and after seven minutes, respectively. Initially, inward current responses in GTPγS as well as cAMP treated cells were found to be significantly decreased at voltage steps between -160 mV and -120 mV ($p<0.0001$ to $p=0.0079$ and $p=0.0024$ to 0.0332, respectively) when compared to the control. After seven minutes, the current response of GTPγS was significantly decreased between the -160 mV and -110 mV pulses ($p<0.0001$ to $p=0.0126$), as well as at the 60 mV pulse ($p=0.0334$), when compared to the control. Also after seven minutes of dialysis, the inward current response of microglia dialysed with intracellular cAMP was also decreased between the -160 mV and the -120 mV pulses ($p=0.002$ to $p=0.0206$). These effects are also illustrated in exemplary current profiles for each of the conditions and time points (**Fig. 4c**). Incidentally, when normalized current-voltage relationships were compared between the one and seven minutes time points, no significant differences were found for control and cAMP-treated cells. In contrast, GTPγS-dialysed microglia exhibited a significant decrease in outward current when one and seven minutes current profiles were compared ($p=0.0002$). This effect is also reflected in **Figure 4d**, which compares inward (upper panel; between -120 mV to -90 mV) and outward (lower panel; between 60 mV to 0 mV) conductance for each of the three groups between the time points.

Finally, after the 7 minutes sequences were recorded, still viable cells were challenged with 1mM of ATP added to the perfusate. ATP is not only used as a substrate for energy in the brain, it is also released from injured neurons and thus serves as a signaling molecule for microglial activation (Davalos et al., 2005; Neary, 1996). Well-characterized ATP responses can be elicited in virtually all microglia and are therefore commonly used as a control of cell vitality (Boucsein et al., 2003). Microglia exposed to ATP in acute slices as well as cell culture have been found to exhibit both inward and outward currents (Boucsein et al., 2000; Boucsein et al., 2003; Haas et al., 1996). In the present study it was of particular interest to what extent the addition of G-protein receptor second messenger molecules would interfere with the microglial ATP response. The protocol used for recording ATP (Methods section 2.3.1) was six minutes long. For one minute, a base line reading was obtained followed by one minute of extracellular ATP application and four minutes of recovery. As described before, HSIS and ATP alone induced pronounced outward currents at voltages above and slight inward currents at voltages below -60 mV (left panel of **Fig. 4e**). When cAMP was added to the intracellular solution, it did not significantly affect this result (**Fig. 4e**, right panel). However, when GTPγS was added to the intracellular solution, only the slight inward current observed in the other two experimental groups remained, while the outward current induced at voltages above -60 mV was abolished. Instead, an inward current could be observed (see middle panel, **Fig. 4e**), that was significantly different from both control (0 to 60 mV pulse; $p=0.003$ to $p=0.0011$, respectively) and cAMP-treated (-20 to 60 mV; $p=0.0137$ to 0.0007, respectively) cells.

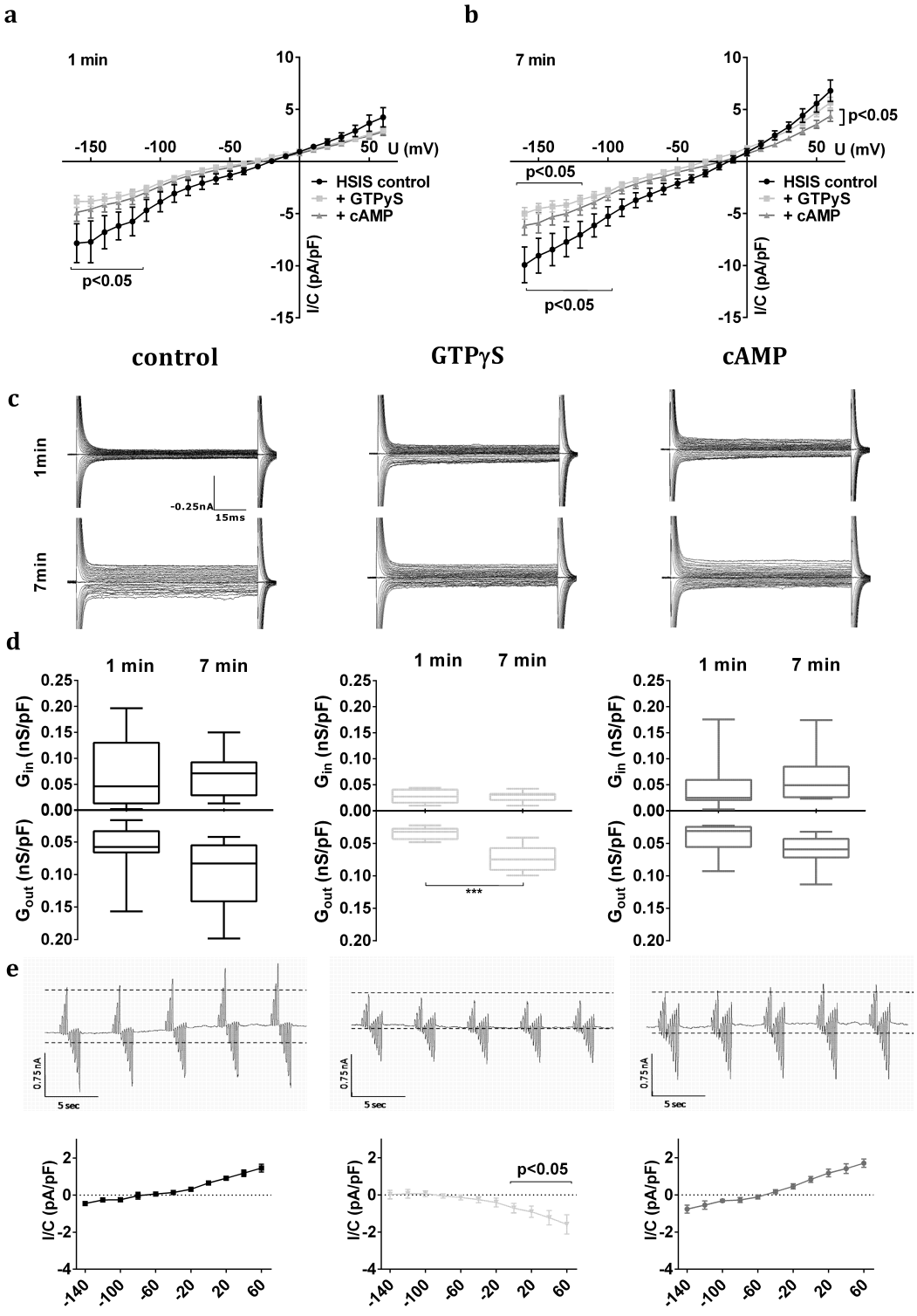

Figure 4: Intracellular manipulation of potassium currents a) Current densities of cells treated with high sodium intracellular solution (see Figure 2, n=9), 100 µM GTPγS (n=8) and 500 µM cAMP (n=9) elicited by voltage steps ranging from -160 mV to 60 mV from a holding potential of -70 mV at 1 minute; GTPγS significantly different from control, -160 and -150 mV pulses p<0.0001, -140 mV to -120 pulses p<0.01; cAMP significantly different from control, -160 to -140 mV pulses p<0.01, -130 and -120 mV pulses p<0.05, 2-way ANOVA with Bonferroni's multiple comparison test **b)** Current densities of cells treated with HSIS (n=9, left panel) GTPγS (n=7, middle panel) and cAMP (n=8, right panel) elicited by voltage

steps ranging from -160 mV to 60 mV from a holding potential of -70 mV after seven minutes; GTPγS significantly different from control, -160 to 140 mV pulses $p<0.0001$, -130 mV $p<0.001$, -120 mV $p<0.01$, -110 mV $p<0.05$; cAMP significantly different from control, -160 mV $p<0.001$, -150 and -140 mV pulses $p<0.01$, -130 and -120 mV pulses $p<0.05$; ; 2-way ANOVA with Bonferroni's multiple comparisons test **c)** exemplary current profiles of HSIS control (left panel), GTPγS (middle panel) and cAMP (right panel) at one minute (upper traces) and after seven minutes (lower traces) **d)** inward conductance (upper panel; from -120 mV to -90 mV) and outward conductance (lower panel; from 60 mV to 0 mV) at one (n= 9 for control and n= 11 for cAMP) and after 7 minutes (n=9 for control and n= 7 for cAMP); no significant differences were found between groups, outward conductance of GTPγS was significantly ($p=0.0002$) reduced after seven minutes (n=7) as compared to one minute (n=8); unpaired t tests **e)** responses to 1mM extracellularly applied ATP of cells treated intracellularly with GTPγS (middle, n=5) and cAMP (right, n=6) compared to NaOH adjusted control (left, n=5): 2-way ANOVAs with Bonferroni's multiple comparison corrections

3.2 Comparison of microglial current responses in different brain areas

In the last set of experiments, it was investigated if microglial current responses could not only be manipulated intracellularly, but if they also differed between brain regions (cortex, cerebellum and thalamus). Microglial membrane currents have been mainly studied in the cortex and are therefore well described in this region (Boucsein et al., 2000; Farber and Kettenmann, 2005; Richter et al., 2014). For this reason, cortical microglia were chosen as a control. Additionally, cerebellar and thalamic microglial currents were investigated because they have to date not been characterized in acute slices. However, past research in cultured microglia has suggested that heterogeneous populations might exist (Hagino et al., 2004; Kuhn et al., 2004). For this purpose, microglia were patch clamped in whole-cell configuration with pipettes filled with standard intracellular solution. The same voltage clamp protocol as described in section 2.3.1 was used. For all three regions, current profiles exhibited classical microglial parameters. They lacked macroscopic sodium, calcium and chloride currents but exhibited pronounced potassium inwardly rectifying as well as small delayed outward currents. They were also all characterized by a high input resistance. Interestingly, the membrane capacitance of cerebellar microglia was found to be significantly lower than cortical microglia membrane capacitance ($p=0.0002$, **Fig. 5a**), which was also visible from the cerebellar current profiles (not shown). This indicates that microglial cells in the cerebellum possess a smaller membrane area than cortical microglia. Furthermore, microglial reversal potential between the three brain areas was not found to be significantly different (**Fig. 5b,** $p=0.3391$ for cerebellum vs. cortex, $p=0.9996$ for thalamus vs. cortex and $p=0.4288$ for cerebellum vs. thalamus). Similarly, no differences were found in series resistance ($p=0.4198$ for cerebellum vs. cortex, $p=0.6734$ for thalamus vs. cortex and $p=0.428$ for cerebellum vs. thalamus) and membrane resistance ($p=0.3936$ for cerebellum vs. cortex,

p=0.8091 for thalamus vs. cortex and p=0.5126 for cerebellum vs. thalamus). **Figure 5c** finally depicts the comparison of conductances normalized to membrane capacitance between the three groups. No significant differences in either inward (top panel, -120 to -90 mV) as well as outward (bottom panel, 60 to 0 mV) conductances were found between cortex and thalamus (p=0.5003 and 0.8830, respectively). In contrast, inward as well as outward conductances were significantly increased in the cerebellum compared to the thalamus (p=0.007 and p=0.0297, respectively) and the cerebral cortex (p<0.0001 and p=0.0007, respectively)

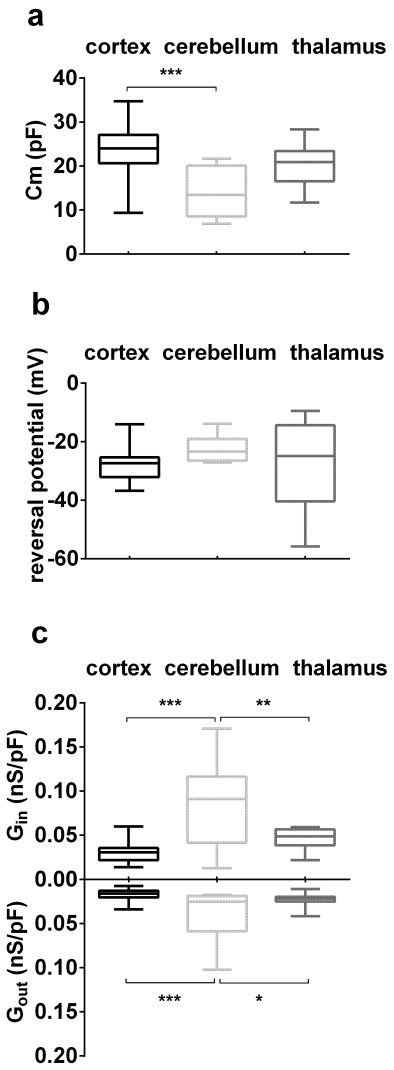

Figure 5: Comparison of microglial currents in cerebral cortex, cerebellum and thalamus a) comparison of membrane capacitance between cortex (n=18), cerebellum (n=11) and thalamus (n=11), capacitance in the cerebellum was significantly (p<0.001) lower than in the cortex; Ordinary one-way ANOVA with Bonferroni's multiple comparisons test **b)** no significant differences in reversal potential were found between the three brain areas compared; Ordinary one-way ANOVA with Bonferroni's multiple comparisons test **c)** comparison of inward (upper panel, from -120 to -90 mV) and outward (bottom panel, from 60 to 0 mV) conductance; inward conductance was significantly increased in the cerebellum (n=11) compared to cortex (p<0.001, n=18) and thalamus (p<0.01), outward conductance was significantly increased in the cerebellum (n=11) compared to cortex (p<0.001, n=18) and thalamus (p<0.05, n=11); Ordinary one-way ANOVA with Bonferroni's multiple comparisons test

4 Discussion

4.1 Comparison of two intracellular solutions

In the present experiment, an increase of 287% of sodium in the high sodium intracellular solution (HSIS) resulted in a marked increase in outward current density when compared to cells treated with standard intracellular solution (SIS). Furthermore, membrane potential as well as capacitance were significantly decreased in cells treated with HSIS.

The exact intracellular ionic composition of microglia is to date unknown. Therefore established intracellular solutions for microglia are largely based on intracellular solutions used in neuronal electrophysiological experiments (Legendre et al., 1993). Intracellular sodium concentration was thus increased to investigate how important and tightly controlled intracellular sodium concentration is (i.e. if it would yield another effect as SIS) and in what way it would affect microglial membrane currents in comparison to SIS. In the present experiment, an increase of 287% of sodium in the HSIS resulted in a marked increase in outward current density when compared to cells treated with SIS. Based on these differential results between the groups, additional intracellular sodium was most likely not exported out of the cell (e.g. via sodium-proton exchange) or imported into cell organelles in concentrations high enough to abolish effects on membrane currents in the HSIS group. The observed decrease in reversal potential could be expected based on the Nernst equation (Hille, 2001). Other effects induces in increased intracellular sodium cannot be explained as easily. Intracellular sodium could exert its effect on a number of channels, receptors and transporters. A reduction in intracellular sodium concentration resulted in a concomitant reduction of sodium-dependent potassium conductance in patch-clamp experiments of feline ventricular myocytes (Harvey and Ten Eick, 1988). In guinea pig ventricular cells, intracellular sodium was found to reduce the amplitude of the outward current in a voltage-dependent manner (Matsuda, 1993). In mushroom body Kenyon cells of the cricket, dependence of outward currents on sodium influx was demonstrated. Sodium dependent potassium channels were found to be stimulated by sodium influx (Takahashi and Yoshino, 2015). However, it has to be noted that the ionic composition of these intracellular solutions differed considerably from the present study. For example, Matsuda et al. used an intracellular solution that was both high in sodium and low in potassium. Furthermore, the diversity of the cell types investigated could result in diverging results. It has also been described that the effects of both sodium and proton concentration on inward-rectifying potassium currents are orchestrated by sodium-proton exchange (Harvey and Ten Eick, 1989; Moody and Hagiwara, 1982). The sodium-proton exchanger (NHE) regulates cell volume and intracellular pH by exchanging sodium for protons. In microglia the NHE isoform 1 was implicated in the induction of a pro-inflammatory state (De Vito, 2006). The possibility that NHE-1 might have been affected by the increased intracellular concentration of sodium is corroborated by the fact that the capacitance differed sig-

nificantly between the two experimental groups in the present study, which means that cell volume was affected.

4.2 G-protein and intracellular second messenger – activated membrane currents

4.2.1 GTPys

In response to intracellular GTPγS, inward current responses were significantly decreased at voltages negative to -120mV (at one minute) and -110mV (after seven minutes), when compared to control. Concomitantly, outward current was significantly decreased when the two time points were compared.

GTPγS can stimulate three distinct G-proteins: G_s, G_i and G_q. GTPγS acts on the G_s G-protein, the positive regulator of adenylyl cyclase. Adenylyl cyclase can therefore be directly activated by the Gsα-GTPγS complex (Northup et al., 1983). In contrast, association of GTPγS with Gi leads to inhibition of adenylyl cyclase. The specific mechanism underlying this effect has been a matter of debate (Gilman, 1987). Since both GTPγS and cAMP exerted a comparable decrease in both inward and outward current in current density-voltage plots (**Fig. 4a** and **b**) however, the effects of the compounds are not expected to be opposing. As a consequence, it is highly unlikely that inhibition of adenylyl cyclase and resulting inhibition of cAMP production would yield similar results as an increase in intracellular cAMP levels. G_i-mediated effects of GTPγS can therefore be excluded as mechanisms of action. In a G_s-mediated pathway, intracellular calcium concentration is elevated via cleavage of PIP_2 to IP_3 by phospholipase C. Phospholipase C in turn, is mobilized by guanine nucleotides. In 1988, Brock et al. published that GTPγS stimulates phospholipase C and subsequent increases in IP_3 formation and turnover as well as calcium mobilization in epithelial cells. The ability of GTPγS (but not GDP or ATP) to hydrolyse PIP_2 has also been described in neurtrophils (Lowe et al., 1996). In contrast, GDPβS could not stimulate PIP_2 hydrolysis or increase IP_3 formation, while it did stimulate intracellular calcium concentration (Brock et al., 1988). Increases in intracellular calcium could therefore, at least theoretically, be responsible for the decrease in inward current observed in response to intracellularly applied GTPγS.

Activation of G_q G-proteins ultimately leads to phosphorylation of proteins via activity of protein kinase C. Since protein expression patterns vary between cell types, PKC actions are cell-type specific (Newton, 1995). In microglia, PKC activation has been mainly implicated in activation and proliferation (Ryu et al., 2000; Sawada et al., 1990). From the results obtained in the current experiment, it seems that the effects induced by GTPγS were already visible after one minute (current density significantly reduced when compared to control from -120 mV pulse downwards, **Fig. 4a**). After seven minutes, the results were not much different (current density signif-

icantly reduced from -110 mV pulse downwards, **Fig. 4b**). It is therefore fairly unlikely, that this fast effect was induced by phosphorylation events.

Another possible mode of action of GTPγS represents G-protein-mediated inhibition of K^+ inward rectifiers, which is considered to be an ubiquitous signal conduction mechanism in excitable, but also non-excitable cells (Kurtz and Penner, 1989). In this context, Schilling and Eder postulated in 2013 that the effect of intracellularly applied GDPβS was mediated by the inhibition of inwardly rectifying G-protein-activated potassium channels (GIRKs). While most GPCRs are modulated via G-protein second messenger cascades, GIRKs are the only ion channels known to be directly activated by the Gβγ dimer. As GTPγS would exert an opposite effect to GDPγS, an increase in inwardly rectifying potassium currents would be expected; contrary to what was observed in the present study. However, at least one member of the GIRK family ($K_{ir}2.3$) is inhibited by Gβγ. Similarly, an inward potassium rectifier, speculated to be of the $K_{ir}2$ family, has been found to be inhibited by GTPγS in cultured rat oligodendrocytes (Karschin, 1993). Similar to the current experiment, 100µM GTPγY were allowed to dialyse with the cytoplasm for 400 seconds after obtaining whole-cell configuration. With a second patch pipette, responses were then recorded in cell-attached mode. Within this time frame, current responses were observed to decline (Karschin, 1993). Altough GIRKs are widely distributed throughout the central nervous system; they have to date not been described in microglia (Dascal, 1997; Yamada et al., 1998).

4.2.2 cAMP

In response to intracellular cAMP, inward current responses were significantly decreased at voltages negative to -120mV (at one minute and after seven minutes) when compared to control. However, no differences could be found when the two time points were compared.

In neurons of *Aplysia*, increased intracellular cAMP induced a decrease in membrane conductance following iontophoresis (Scholz and Byrne, 1988). In rabbit vascular smooth muscle cells on the other hand, increases in intracellular cAMP concentration rapidly produced an elevation in Kv1.2 current amplitude, while not affecting channel kinetics (Aiello et al., 1995). In microglia (Aronoff et al., 2005), macrophages (Ydrenius et al., 2000) and neutrophils (Makranz et al., 2006) modulation of intracellular cAMP levels has been implicated in the regulation of phagocytosis. Intracellular cAMP levels can be modulated activation of G_i as well as G_s G-proteins, which exhibit opposing actions. The general function of G_i is to inhibit cAMP-dependent/adenylyl cyclase pathway, which decreases the generation of cAMP from ATP. In contrast, activation of the G_s pathway can be mimicked by an increase in intracellular cAMP, as done in the present study. Activation of the G_s protein bound to the α receptor subunit results in activation of adenylyl cyclase via binding to it. Adenylyl cyclase then mediates the conversion of ATP to cAMP. The resulting increases in intracellular cAMP concentration may result in the activation of

cAMP-dependent protein kinase A (PKA), cyclic nucleotide-gated ion channels and exchange proteins induced by cAMP (Bos, 2003; Kaupp and Seifert, 2002). Additionally, other mechanisms have been described, these do, however, bear no relevance in the context of microglia (Simrick et al., 2013). While the present study was designed to investigate the effects of intracellularly applied cAMP throughout the cytoplasm as a mimick of receptor activation, it has to be noted that current research indicates that under physiological conditions, alterations in cAMP concentrations occur in microdomains of individual cell compartments only (Cooper, 2003). It is therefore possible that application of cAMP throughout the whole cytoplasm induced activation of several signaling systems.

Most effects conveyed by cAMP are mediated by PKA induced phosphorylation. PKA is a cAMP-dependent serine/threonine kinase. As was the case with PKC, the proteins available for phosphorylation by PKA are dependent upon the protein expression in the respective cell type. It has been found however, that a number of potassium channels possess phosphorylation sites. For example, in the mammalian *Shaker* superfamily of potassium channels phosphorylation sites for PKA have been identified. In Kv1.2, PKA phosphorylation is believed to induce an increase in passed current (Jonas and Kaczmarek, 1996). Similarly, when *Xenopus oocytes* were used as an expression system for rat potassium *Shab* superfamily Kv2.1 channel, a cAMP analog stimulated an increase in current amplitude (Wilson et al., 1994). Furthermore, ion channels can be directly altered in their properties via phosphorylation by serine/threonine kinases as well as tyrosine kinases (Wilson and Kaczmarek, 1993). Tyrosine kinase can rapidly modulate ion channel function with mechanisms similar to those of serine/threonine kinases, namely changes in current amplitudes or changes in the rate of inactivation. For example K^+ current ampitudes can be acutely suppressed by receptor tyrosine kinase in potassium channels expressed in *Xenopus oocytes* (Kononenko et al., 1983). In contrast, Huang et al. (1993) found that a tyrosine phosphorylation site was responsible for suppression of Kv1.2 current amplitude, when co-expressed with m1 muscarinic acetylcholine receptors. This effect was mediated by an increase in intracellular calcium concentration and could be imitated by PKC activation. Furthermore, Kv1.3 has been found to possess tyrosine phosphorylation sites, activation of which resulted in a decrease in current amplitude (Jonas and Kaczmarek, 1996). In rat adipocytes, indirect increases of intracellular cAMP levels via forskolin or glucagon resulted in attenuation of mitogen-activated protein kinase activation, which leads to the activation of serine/threonine kinase (Sevetson et al., 1993).

As mentioned above, there are also actions of cAMP that occur independently of PKA. The direct regulation of ion channels by cAMP is not only used in olfactory receptor cells (Liman and Buck, 1994), where it gates a nonselective cation channel, but there are also cyclic nucleotide ion channels in other brain regions (Torgersen et al., 2002). Cyclic nucleotide gated channels are

ubiquitously expressed in the CNS; for example cerebellum, cerebral cortex, basal ganglia of the rat (el-Husseini et al., 1995). Hereby, cAMP binds to the cyclic nucleotide-binding (CNB) domain, a highly conserved singnaling module. Other than in bacteria, where the CNB domain is linked to a DNA-binding domain and mediates gene transcription, it is linked to phosphodiesterastes, cAMP-regulated guanine nucleotide exchange factors (Epac = exchange protein directly activated by cAMP = Rap1 guanine exchange factor), protein kinases and cyclic nucleotide gated ion channels in mammals (Johnson et al., 2001). In both human and mice, EPAC protein 1 is ubiquitously expressed and high levels are expressed in some regions of the brain in mice (Kawasaki et al., 1998).

The effects induced by intracellular application of cAMP could therefore be attributed to a plethora of mechanisms. Initially after gaining electrical control over the microglia, intracellular application of cAMP resulted in a decrease in current density when compared to control. Since this effect was observed so quickly, it is probably not due to phosphorylation events. Activation of cyclic nucleotide gated ion channels and Epac by cAMP occurs indepently of PKA (de Rooij et al., 1998). Cyclic nucleotide gated channels mediate Ca^{2+} entry, which is, independently of membrane voltage, regulated by intracellular cyclic nucleotide concentration (Zufall et al., 1997). An increase in cAMP concentration in microglia might therefore result in Ca^{2+} entry, which also occurs under physiological conditions, as it is also necessary for microglia to develop features of activation (Hoffmann et al., 2003). If this was true, the calcium chelator BAPTA should prevent the effects induced by cAMP and restore the signaling efficacy to control levels. However, when full dialysis was achieved after seven minutes of incubation with intracellularly applied cAMP, only the outward current density at the +60 mV pulse was significantly decreased compared to the control. It is of note that this difference was not statistically significant when the current-voltage plots or the conductances of the two time points were compared within the cAMP group. It is therefore unclear if intracellular application of 500µM cAMP truly elicited an effect in microglia following 7 minutes of dialysis. Since a previous study investigating intracellular modification of glial currents successfully used a similar time frame, it is unlikely that the effects induced by cAMP could have been to slow to develop within 7 minutes. However, based on the fact that the intracellular cAMP levels are also implicated in phagocytosis in microglia (Makranz et al., 2006), it is possible that the concentration of cAMP is tightly controlled. The added cAMP might therefore have been degraded by phosphodiesterases and could therefore not potentiate its effect over the course of the experiment. Another possibility is that the effect exerted by cAMP was induced faster than expected. Because cAMP actions are usually exerted in microdomains only (Cooper, 2003), it is possible that fully dialysis of cAMP with the cytoplasm was not necessary to induce an effect. To test these hypotheses, BAPTA should be used as an additional negative control.

4.2.3 ATP responses

As has been reviewed in the Introduction (see Introduction 1.3.1.1), the purinergic response to ATP is mediated by ionotrophic P2X and metabotrophic P2Y channels. Activation of the P2Y purinergic receptors on the other hand can induce a number of cell signaling molecules and second messenger systems, including cAMP, calcium, IP_3 and PLC. This signaling complexity is based on the broad spectrum of cellular functions mediated by ATP signal transduction. In response to ATP, microglia in acute slices as well as cell culture have been found to exhibit both inward and outward currents (see Introduction; Boucsein et al., 2003; Haas et al., 1996). The results obtained in the present study closely resemble what was previously described. In the control group the current-voltage relationship of the ATP response reflected an outward potassium conductance, which reversed at about -70mV and was hyperpolarizing. Additionally, a modest inward current was observed at hyperpolarizing voltages below -70mV. The observed inward current has been attributed to ionotrophic non-selective cation channels (P2X), while the outward current has been ascribed to potassium channels linked to metabotropic purinergic receptors (P2Y, Visentin et al., 1999). A pharmacological screening using purinergic ligands furthermore revealed that the P2Y1, P2Y2 and P2Y4 receptor subtypes are likely involved in the observed outward K^+ currents (Boucsein et al., 2003). Activation of all three of these receptors is linked to G_q - mediated signaling and PLC activation (Inoue, 2006). Furthermore, G_i G-protein signaling mechanisms have been proposed for P2Y2 and P2Y4 (Fields and Burnstock, 2006). Activation of P2Y receptors therefore to induces phospholipase C activation and mobilization of intracellular calcium (G_q - mediated signaling; Neary, 1996; Visentin et al., 1999) or inhibition of adenylate cyclase-mediated activation of cAMP in microglia (Gi - mediated signaling; Inoue, 2002). The hyperpolarizing nature of the P2Y receptor activation has been hypothesized to be linked to IP_3 – mediated exhaustion of intracellular calcium stores and the subsequent activation of Ca^{2+} - dependent potassium channels (Norenberg et al., 1997). In the present study and similar to previous reports, cortical microglia where characterized by an increase in outward membrane current at voltage steps positive to about -70 mV following extracellular application of 1mM ATP through the bathing solution.

4.2.3.1 GTPγS

In contrast, addition of 100µl GTPγS to the intracellular solution abolished the outward current in favour of an inward current. Furthermore, the modest inward current at hyperpolarizing voltage steps was also abolished. Since Gq and possibly Gi mechanisms can theoretically mediate both the observed outward current induced by ATP in controls as well as the general effect of GTPγS, additional activation of these pathways in response to GTPγS should have resulted in an increase in outward current. Instead, the observed outward current was abolished and replaced by an inward current. In a previous experiment for example, endothelial cells treated with

100μM of extracellular ATP exhibited a marked increase in intracellular calcium concentration, which was inhibited by GTPγS. Furthermore, intracellularly applied GTPγS was found to inhibit IP_3 formation as well as calcium mobilization in response to ATP. It was therefore hypothesized that intracellular GTPγS stimulated the continuous activation of phospholipase C via generation of free α receptor subunits. This way, maximal phospholipase C activation was hypothesized limit the ability of ATP receptor activation to lead to even more phospholipase C activation (Brock et al., 1988). However, this would only explain the abolition of the outward current, but not why an inward current was observed instead. Similarly, GDPβS, another non-hydrolysable guanine nucleotide analogue and the enzymatically stable analogue of GDP, has been found to block the effects of agonists at G-protein coupled receptors (Norenberg et al., 1997). For example, when GDPβS was applied intracellularly to activated rat microglial cells, the outward current induced by the P2Y1 and P2Y12 receptor agonist 2-MeSATP was abolished (Langosch et al., 1994). However, since GDPβS cannot stimulate PIP_2 hydrolysis or increase IP_3 formation, the ATP response seen in GTPγS treated cells is likely not solely based on a G_q - mediated mechanism. (Norenberg et al., 1997). It is therefore possible that the signaling pathways utilized by ATP and GTPγS were not identical, but still interfered. In this context, the G_s G-protein activated by GTPγS can be excluded as a mode of action, because the actions of cAMP and GTPγS differed. However, based on the fact that GTPγS is an ultimately universal signaling molecule, it is hard to pin down its effects based on the present experiments.

4.2.3.2 cAMP

In contrast to the results observed following intracellular application of GTPγS, cAMP did not significantly affect the microglial ATP response. This finding is reflected by the fact that the affected P2Y receptors are G_q and/or G_i coupled, while cAMP activates G_s and G_i mechanisms. It therefore seems likely, that the signaling pathway induced by intracellular cAMP did not interfere with the signaling pathway activated by extracellularly applied ATP. In accordance with this notion, Gyoneva et al. (2009) found that both G_i and G_s mechanisms control the response of microglia to ATP. More specifically they postulated that two purinergic signaling cascades exhibit opposing actions on microglia: the G_s-coupled adrenergic A_{2A} receptor as well as the G_i-coupled $P2Y_{12}$ receptor. Immediate activation of the G_i-coupled $P2Y_{12}$ receptor induces ramified, resting microglia to migrate towards the source of ATP. As a result, microglia slowly adopt an activated phenotype and downregulation of $P2Y_{12}$ receptors takes place, while the expression of A_{2A} receptors is upregulated. As ATP is broked down to adenosine, it results in microglial process retraction and adaption of an amoeboid phenotype (Orr et al., 2009). According to this model, microglia in the current study were not equipped with the right receptors (G_s-coupled A_{2A} receptors) to adapt their ATP response when faced with increased intracellular cAMP concentration.

4.1 Comparison of microglial current responses in different brain areas

In the present study, cell area as measured by capacitance was found to be significantly lower in the cerebellum than in the cerebral cortex. Furthermore, inward and outward conductances were increased in the cerebellum, when compared to the cortex and thalamus.

Microglia are situated throughout the whole brain, but their distribution is not uniform (Lawson et al., 1990). The density of microglia in immunohistochemical stainings can vary more than five-fold between brain regions, whereby the cerebellum is considered one of the regions less densely populated (Vela et al., 1995). In contrast, the population density of microglia in the cerebral cortex and the thalamus is intermediary (Lawson et al., 1990). In the first and second layer of the cerebral cortex, microglial cell body perimeter was found to be 34.43 ± 0.98 and 33.81 ± 1.13 µm (Kongsui et al., 2014). In contrast, Vela et al. (1995) found that the diameter of microglia in the molecular and granular layer of the cerebellum ranged from 5.5 to 13 µm, which calculates to a perimeter of 28.5 ± 11.48 µm. This variance in soma perimeter corresponds to the variance in capacitance seen in the cerebellum (**Fig. 5a**). Additionally, the microglial capacitance was significantly lower than in the cortex. As a consequence, both previous comparisons of cell morphology as well as data from the current study indicate that cerebellar microglia might be smaller than microglia in the cerebral cortex. Both inward and outward conductances were significantly greater in the cerebellum, when compared to the cortex and thalamus (**Fig. 5c**). Since cerebellar microglia have not yet been characterized electrophysiologically, this has not been described in the literature. It could be speculated, that smaller membrane area in cerebellar microglia is compensated by higher membrane conductance. The current experiments in different brain areas demonstrate that heterogeneous populations of microglia exist. This heterogeneity was found to be based purely on differing electrophysiological characteristics. To investigate the basis of these differences, pharmacological experiments could provide clarification. Based on the functional differences (e.g. expression of different sets of receptors) hinted at in the past in experiments with cultured microglia (Hagino et al., 2004; Kuhn et al., 2004), intracellular application of G-protein activators or second messenger molecules in different brain areas provides an exciting perspective.

5 Appendix

5.1 Abstact

Although microglia have been electrophysiologically characterized over the past decade, many questions remain unresolved. This is at least in part due to the fact that microglia in culture seem to exhibit different properties than in acute slices (Farber and Kettenmann, 2005). It is

therefore not clear if all channels and receptors described in culture can be found in microglia in acute slices. Apart from the pronounced potassium conductance in glial cells, other ion channels are important for nervous system homeostasis (Kettenmann et al., 2011). As all ion channels, microglial channels are prone to functional regulation by G-proteins and second-messenger pathways. Since the compositions of both the extra- and intracellular solution are predefined in whole-cell patch clamp experiments, specific modifications are possible. In order to test the effect of functional ion channel regulation on microglial membrane currents, a high sodium concentration, a near-universal G-protein activator and ubiquitous second messenger molecule were individually added to the intracellular solution during a seven minutes recording to ensure full dialysis. Since high intracellular sodium concentration is believed to act on a multitude of intracellular targets, it was used to test if potassium currents could be modified intracellularly. Interestingly, microglia responded with a significantly increased current density ($p<0.0001$ to $p=0.0121$) in response to negative voltage steps in response to high intracellular sodium when compared to standard intracellular solution. Additionally, cells treated with high intracellular sodium also exhibited a lower capacitance ($p=0.0456$) and membrane potential ($p=0.075$) than control cells. In a next step, intracellular modulation of G-protein coupled receptors was attempted with the nearly universal Gα protein activator GTPγS. Additionally, the ubiquitous intracellular second messenger molecule cAMP was applied intracellularly. While both GTPγS and cAMP significantly reduced the current density ($p<0.05$) when compared to the control, only GTPγS exhibited a reduction in outward conductance ($p=0.0002$) between the one and seven minute recordings. In contrast to cAMP, GTPγS also affected the microglial ATP response. In contrast to the usually observed outward current (Boucsein et al., 2000) at voltages positve to -60 mV, intracellularly applied GTPγS induced an inward current, which was significantly different from control and cAMP ATP responses ($p=0.003$ to 0.0007). Furthermore, recent observations suggest that microglia exhibit functional and therefore expressional heterogeneity (Gertig and Hanisch, 2014). It has been speculated that this heterogeneity is based on adaptations to specific signaling molecule milieus. In the second part of this project, microglia were therefore compared between the well-characterized cerebral cortex and two novel areas, namely the cerebellum and thalamus. Surprisingly, inward ($p=0.007$ and $p<0.0001$ compared to thalamus and cortex, respectively) and outward ($p=0.0297$ and $p=0.0007$) membrane conductance was significantly increased in the cerebellum. Additionally, membrane capacitance of cerebellar microglia was found to be significantly decreased ($p=0.0002$). While the smaller membrane area of cerebellar microglia has already been noted in immunohistochemical studies (Lawson et al., 1990; Vela et al., 1995), the increased conductance is a novel oberservation. This current project therefore yields new insight into the electrophysiology of murine microglia. Especially the intracellular modification of membrane currents is a relatively new approach in microglia. Furthermore, the characterization of microglial membrane currents in different brain areas demonstrates that

heterogeneous microglia populations do exist. The underlying mechanisms of this heterogeneity should be further investigated.

5.2 Zusammenfassung

Obwohl Mikroglia in den letzten 10 Jahren elektophysiologisch charakterisiert wurden, bleiben viele Fragen offen. Das liegt auch daran, dass kultivierte Mikroglia und solche in akuten Hirnschnitten verschiedene Eigenschaften aufweisen (Farber and Kettenmann, 2005). Es ist daher nicht sicher, ob alle in Zellkultur beschriebenen Rezeptoren und Ionenkanäle auch in Mikroglia in akuten Schnitten zu finden sind. Neben Kaliumkanälen, die Mikroglia elektrophysiologisch prägen, tragen andere Ionenkanäle wichtige Rollen (Kettenmann et al., 2011). Wie alle Ionenkanäle, können jene in Mikroglia Membranen durch G-proteine und sekundäre Botenstoffe funktionell reguliert werden. Da bei der whole-cell patch clamp Methode sowohl die intra- als auch die extrazelluläre Flüssigkeit genau definiert sind, sind gezielte Manipulationen möglich. Um den Effekt der funktionellen Ionenkanalregulation auf Mikroglia Membranströme zu bestimmen, wurde die intrazelluläre Lösung modifiziert. Dazu wurden eine höhere Natriumkonzentration, der beinahe universelle G-Protein Aktivator GTPγS sowie der allgegenwärtige sekundäre Botenstoff cAMP der intrazellulären Lösung beigemengt. Da die Dialyse des Zellinneren mit der Intrazellulären Lösung langsam voranschreitet, wurde ein sieben Minuten langes patch clamp Protokoll erarbeitet. Nachdem Natrium eine Vielzahl von intrazellulären Zielen aufweist, wurde es benutzt um zu testen, ob Kaliumströme intrazellulär modifiziert werden können. Mikroglia reagierten mit einer signifikant höheren Stromdichte als Antwort auf negative Spannungsschritte ($p<0.0001$ bis $p=0.0121$). Weiters wiesen Mikroglia mit höherer intrazellulärer Natriumkonzentration eine niedrigere Zellkapazität ($p=0.0456$) und niedrigeres Membranpotenzial ($p=0.075$) auf als Kontrollzellen. Im nächsten Schritt wurde die intrazelluläre Modulation von G-protein gekoppelten Rezeptoren mit dem beinahe universellen Gα Protein Aktivator GTPγS, sowie dem sekundären Botenstoff cAMP getestet. Sowohl GTPγS als auch cAMP führten zu einer signifikanten Reduktion der Stromdichte ($p<0.05$). Bei einem Vergleich der auswärts Leitfähigkeit zwischen den beiden Messpunkten bei einer und nach sieben Minuten wurde diese jedoch nur von GTPγS signifikant reduziert ($p=0.0002$). Weiters führte GTPγS zu einer Änderung der ATP Antwort. Anstatt des üblichen Auswärtsstromes (Boucsein et al., 2000) bei Spannungen positiver als -60 mV, resultierte Zugabe von GTPγS in die Intrazelluläre Lösung in einem Einwärtsstrom. Aktuellen Beobachtungen zufolge sind Mikroglia durch funktionelle Heterogenität charakterisiert, die sich in verschiedenen Proteinexpressionsmustern wiederspiegeln könnte (Gertig and Hanisch, 2014). Es wurde spekuliert, dass dieser Heterogenität spezifische Anpassung auf lokal begrenzte Signalbotenstoffe zugrunde liegen. Im zweiten Teil dieser Arbeit wurden daher bereits charakterisierte Mikroglia im cerebralen Cortex mit Mikroglia im Cerebellum und Thalamus verglichen. Erstaunlicher Weise wiesen Mikroglia im Cerebellum eine signifikant

höhere ein einwärts ($p=0.007$ und $p<0.0001$ verglichen mit Thalamus und Cortex) und auswärts ($p=0.0297$ und $p=0.0007$) Leitfähigkeit sowie eine niedrigere Zellkapazität ($p=0.0002$) als Zellen im Cortex auf. Während die kleinere Membranoberfläche von Mikroglia im Cerebellum bereits in histologischen Studien (Lawson et al., 1990; Vela et al., 1995) auffiel, ist die höhere Leitfähigkeit eine neue Observation. Diese Arbeit liefert daher neue Erkenntnisse über die Elektrophysiologie von Mikroglia. Insbesondere die intrazelluläre Modifikation von Membranströmen ist ein Ansatz, dem in Mikroglia bisher nur wenig Beachtung geschenkt wurde. Die Charakterisierung von Mikroglia in verschiedenen Arealen zeigt, dass heterogene Mikrogliapopulationen bestehen. Besonders die unterliegenden Mechanismen dieser Heterogenität sollten in der Zukunft weiter analysiert werden.

5.3 Stimulation files

This loop was repeated six times in order to record current profiles at -20, -40 and -70mV holding potential every minute for seven minutes for the intracellular modification experiments:

```
Channels 1 7
PPS 100000

REM -20
For 9
ACQ
DA 4 0
WAIT 5
DA 4 0 *
WAIT 50
DA 4 0
WAIT 100
SACQ
NEXT 0.1

For 16
ACQ
DA 4 0
WAIT 5
DA 4 0 *
WAIT 50
DA 4 0
WAIT 100
SACQ
NEXT -0.1
SAVE
```

```
OWR
DA 4 -0.2
WAITSEC 10

REM -40
For 11
ACQ
DA 4 -0.2
WAIT 5
DA 4 -0.2 *
WAIT 50
DA 4 -0.2
WAIT 100
SACQ
NEXT 0.1

For 14
ACQ
DA 4 -0.2
WAIT 5
DA 4 -0.2 *
WAIT 50
DA 4 -0.2
WAIT 100
SACQ
NEXT -0.1
SAVE

OWR
DA 4 -0.5
WAITSEC 10

REM -70
For 14
ACQ
DA 4 -0.5
WAIT 5
DA 4 -0.5 *
WAIT 50
DA 4 -0.5
WAIT 100
SACQ
NEXT 0.1
```

For 11

ACQ

DA 4 -0.5

WAIT 5

DA 4 -0.5 *

WAIT 50

DA 4 -0.5

WAIT 100

SACQ

NEXT -0.1

SAVE

DA 4 0

WAITMIN 2

...

All other current profiles were recorded with stimulation files following the same principle according to the respective protocols described.

ATP responses were recorded between +60 and -140mV with the following protocol from a holding potential of -40mV for six minutes.

CHANNELS 1 7
PPS 50000
ACQ

DA 4 0

FOR 6

DA 4 0.0
Wait 100
DA 4 0.4 *
Wait 100
DA 4 0.0
Wait 100
NEXT 0.2

FOR 14

DA 4 0.0
Wait 100
DA 4 -1.0 *
Wait 100
DA 4 0.0
Wait 100
NEXT -0.2
Wait 5000

NEXT
SACQ

END

5.4 Curriculum vitae

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AUSBILDUNG

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Sept. 2014 – Feb. 2015	**Praktikum** in der Forschungsgruppe Neuroimmunologie am Center for Brain Research, Ass. Prof. Monika Bradl, Medizinische Universität Wien
Sept. 2013 – Okt. 2015	**Masterstudium** Verhaltens-, Neuro- und Kognitionsbiologie, Universität Wien
März 2012 - Juni 2012	Gaststudentin Medizinische Universität Wien
Nov. 2011 - März 2012	Praktikum in **Marketing/Produktentwicklung bei GA Generic Assays in Dahlewitz, Berlin**, im Bereich Ganglioside und autoimmune Neuropathien
Sept. 2009 - Juni 2013	**BSc (Hons.) in Neuroscience, University of Nottingham** Bachelorarbeit „Caffeine counteracts hypothermic and anxiogenic actions of mephedrone following co-administration, extends its effect on locomotion and induces long-term increases in dopamine and serotonin levels in the hippocampus in rats„
2004 - Juni 2008	Begabungsförderungsmodell Sir Karl Popper-Schule Wiedner Gürtel 68, 1040 Wien

ARBEIT

Oktober 2010 - Juni 2011	Catering Assistant, Sherwood Hall, University of Nottingham (CIEH Level 2 Food Safety Certificate)
Oktober 2008 - Juni 2009	Lernhilfe in der Volksschule Eslarngasse 23, 1030 Wien, Arbeit mit Kindern mit AD(H)S und Autismus Spektrum
Juli - September 2008	Praktikantin in der Steuerberatungskanzlei Mag. Dr. Walter Stefan Weinhandl, Kettenbrückengasse 5a, 1040 Wien

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Sept. 2012 - Juni 2013	Session Coordinator, University of Nottingham student union, University of Nottingham
Juni 2011 - Juli 2011	Musikfestival „Luft & Liebe“ in Berlin (Getränkebestellung, Aufbau sowie Bestockung von 6 Bars für das 3-tägige Festival mit insgesamt 10.000 Besuchern, Einschulung und Supervision des Barpersonals)

Sept. 2010 - Juni 2011	Publicity Officer des International Student Bureau (gewählte Studentenvertretung), University of Nottingham student union, University of Nottingham
2006 - 2009	Mitglied & Vizepräsidentin im karitativ tätigen LEO/LIONS Club Wien St.Stephan

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