Aus dem Max-Delbrück-Centrum für Molekulare Medizin Berlin

DISSERTATION

Neurotransmitter and neurohormone receptors on microglia

zur Erlangung des akademischen Grades
Doctor of Philosophy (PhD)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

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Datum der Promotion: 22.06.2014

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Abbreviations

Alzheimer's disease (AD)

Antigen presenting cells (APCs)

Beta-amyloid (Aβ)

Blood-brain barrier (BBB)

Bovine serum albumin (BSA)

Calcium (Ca^{2+})

Carbondioxide (CO2)

Central nervous system (CNS)

Copy DNA (cDNA)

Degree Celsius (^{o}C)

Desoxyribonucleic acid (DNA)

Dimethyl sulfoxide (DMSO)

Dulbecco's Modified Eagle Medium (DMEM)

Enhanced green fluorescent protein (EGFP)

Enzyme-Linked Immuno Sorbent Assay (ELISA)

Experimental autoimmune encephalomyelitis (EAE)

Fetal calf serum (FCS)

G-protein coupled receptors (GPCRs)

High grade (HG)

Horseradish peroxidase (HRP)

Immunoglobulin (Ig)

Immunoglobulin superfamily (IgSF)

Insulin-degrading enzyme (IDE)

Insulin-like growth factor 1 (IGF-1)

Interferon-gamma (IFN-γ)

Interleukin (IL-)

Ionized calcium-binding adaptor molecule (Iba)

Lipopolysaccharide (LPS)

Magnetic activated cell sorting (MACS)

Membrane type-1 matrix metalloprotease (MT1-MMP)

Messenger RNA (mRNA)

Microlitre (µl)

Micrometer (µm)

Micromolar (μM)
Middle cerebral artery occlusion (MCAo)
Millilitre (ml)
Millimolar (mM)
Multiple sclerosis (MS)
Myelin basic protein (MBP)
Myelin oligodendrocyte glycoprotein (MOG)
n-acetyl tryptophan (NAT)
Nanomolar (nM)
Neurofibrillary tangles (NFTs)
Nitric oxide (NO)
Normal goat serum (NGS)
Oxygen-glucose deprivation (OGD)
Parkinson's disease (PD)
Phosphate buffer saline (PBS)
Phosphate buffer saline-Triton X-100 (PBS-T)
Polymerase chain reaction (PCR)
Proteolipid protein (PLP)
Reactive oxygen species (ROS)
Receptor protein kinases (RPKs)
Rennin-angiotensin system (RAS)
Reverse transcriptase PCR (RT-PCR)
Revolutions per minute (rpm)
Ribonucleic acid (RNA)
Room temperature (RT)
Stress inducible protein 1 (STI1)
Transforming growth factor beta (TGF-β)
Translocator protein 18 kDa (TSPO)
Tumor necrosis factor alpha (TNF-α)
Uridine-5'-triphosphate (UTP)
Wild type (wt)
World health organization (WHO)

1. Introduction

1.1 Microglia, the caretakers of the central nervous system

The Spanish neuroscientist Pio del Rio-Hortega first identified microglia in the 1932 milestone publication “Cytology and Cellular Pathology of the Nervous System” (Del Rio-Hortega, 1932). Among his many observations, all of which continue to hold true today, he stated that microglia enter the brain during early development, have amoeboid morphology and originate from the mesoderm. Since this article was published, a great deal of research has been carried out highlighting the critical role that microglia play in the healthy and diseased brain.

Microglia invade the brain during the embryonic and early postnatal stage of life. They are derived from circulating monocytes and monocyte-macrophage precursor cells from the bone marrow. The immigrating cells have an amoeboid morphology and are phagocytic, helping to clear cellular debris associated with brain development. (Barron, 1995).

As del Rio-Hortega also observed, microglial cells are the resident immune cells of the brain and respond to any pathologic event with a change in morphology and function (Hanisch and Kettenmann, 2007). In the normal, healthy brain microglial cells have a ramified morphology and were termed “resting microglia”. It has, however, become evident that these cells constantly move their processes and seem to scan their environment (Nimmerjahn et al., 2005). Recent evidence has indicated that they interact with synapses and are possibly involved in plasticity processes (Kettenmann et al., 2013; Tremblay and Majewska, 2011). Indeed, microglial cells have the capacity to respond to synaptic activity since they express a variety of neurotransmitter receptors such as those for glutamate, GABA, serotonin and adrenaline (Kettenmann et al., 2011; Krabbe et al., 2012; Pocock and Kettenmann, 2007). Most studies on microglial transmitter receptors were performed *in vitro* and for only some of these receptors, the functional expression could be confirmed *in situ* such as for purinergic or $GABA_B$ receptors (Boucsein et al., 2003; Kuhn et al., 2004). The expression of neurotransmitter and

neurohormone receptors on microglia enables neurons to interact with and control distinct microglial functions. A 2007 review discussed this ability in depth, describing the activation of receptors through “on” and “off” signals (Biber et al., 2007). Purines such as ATP and UTP are such “on” signals and are released from damaged neurons. They initiate microglial phagocytosis upon activation of P2Y6 (Koizumi et al., 2007) or cause rapid process movement towards an injury site through activation of P2Y12 (Davalos et al., 2005; Haynes et al., 2006). Glutamate is another “on” signal whose release is associated with neurodegenerative disease. Its release not only results in neuronal death, but activates glutamate receptors on microglia. For example, activation of mGlu2 has been shown to result in release of TNF-alpha which contributes to microglial neurotoxicity through activation of neuronal TNF receptor 1 and caspase-3 (Taylor et al., 2005).

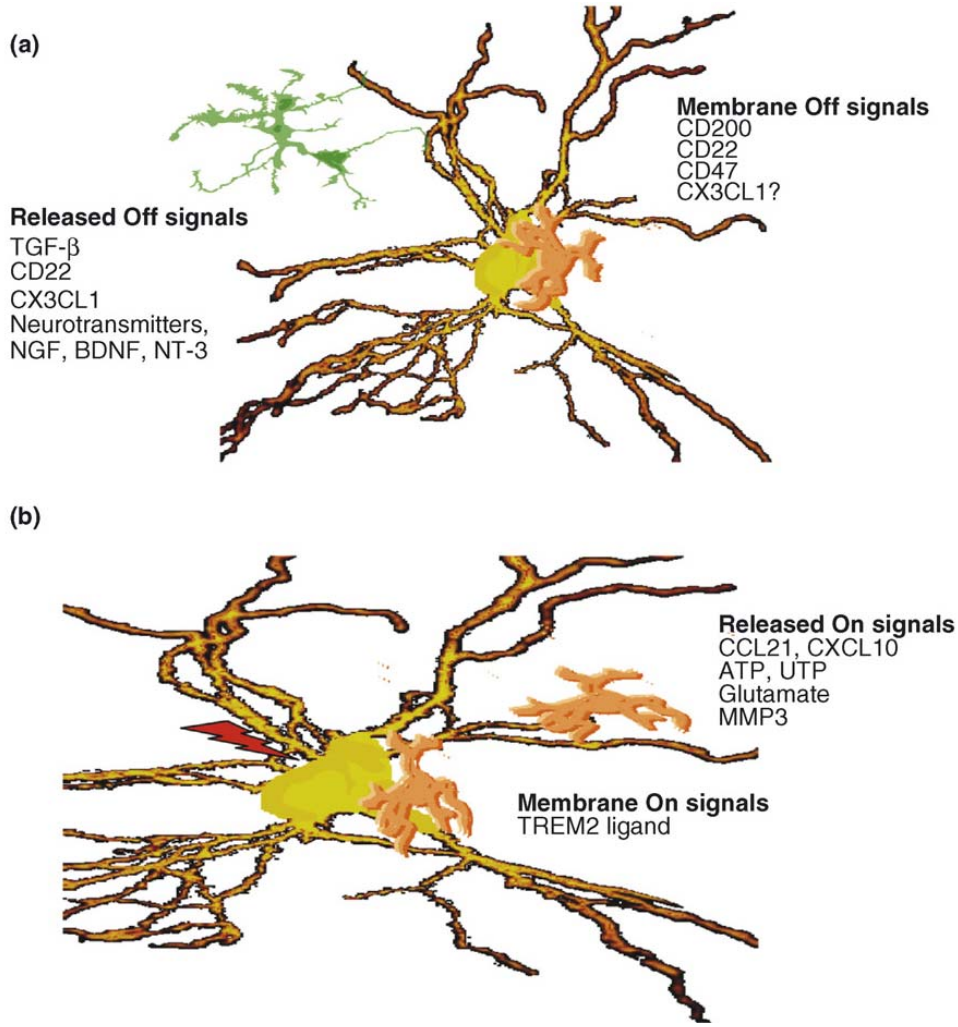


Figure 1. Microglia respond to “on’’ and “off’’ signals released by neurons which control their function (Biber et al., 2007)

In order to ensure that various functions of microglia which have been initiated in pathology do not become detrimental, “off” signals can be released by neurons. CD200 is a membrane off signal which belongs to the immunoglobulin superfamily (IgSF) and is expressed on neuronal membrane surfaces, while the receptor is located on microglia (Hoek et al., 2000). Its inhibitory function was demonstrated by Wright et al, who blocked the receptor using antibodies, resulting in exacerbation of disease in EAE in rats, as well as an increase in macrophage infiltration of the spinal cord (Wright et al., 2000). A number of studies have been carried out on other neurotransmitters and neurohormones, examining the expression of receptors on microglial cells as well as the impact that activation has on various functions. Current research concerning the receptors and ligands of interest in this study has been summarised from section 1.3.

1.2 Calcium imaging as a tool to determine functional receptor expression

Ca^{2+} is a major intracellular messenger that is involved with cellular responses such as exocytosis, metabolic processes, gene expression, ion transport systems, cell growth, proliferation, cell death, apoptosis and cell migration. The activation of microglial cells results in an increase in intracellular calcium going from a cytoplasmic calcium concentration of ~50-150nM in resting microglia to μM concentrations in a matter of milliseconds (Farber and Kettenmann, 2006). The predominant mechanism of calcium release in microglia is through ligand interaction with G-protein coupled receptors (GPCRs) of the Gq family or receptor protein kinases (RPKs) on the plasma membrane. This results in the conversion of phosphatidylinositol (4,5)-biphosphate into inositol 1,4,5 triphosphate ($InsP_3$) by phospholopase C. $InsP_3$ then binds to the $InsP_3$ receptor on the endoplasmic reticulum which results in the release of calcium from the internal stores (Moeller, 2002). Figure 2 illustrates the G-protein coupled signalling pathways for the five subtypes of muscarinic acetylcholine receptors (Eglen et al., 2001), one of the receptor systems investigated in this study.

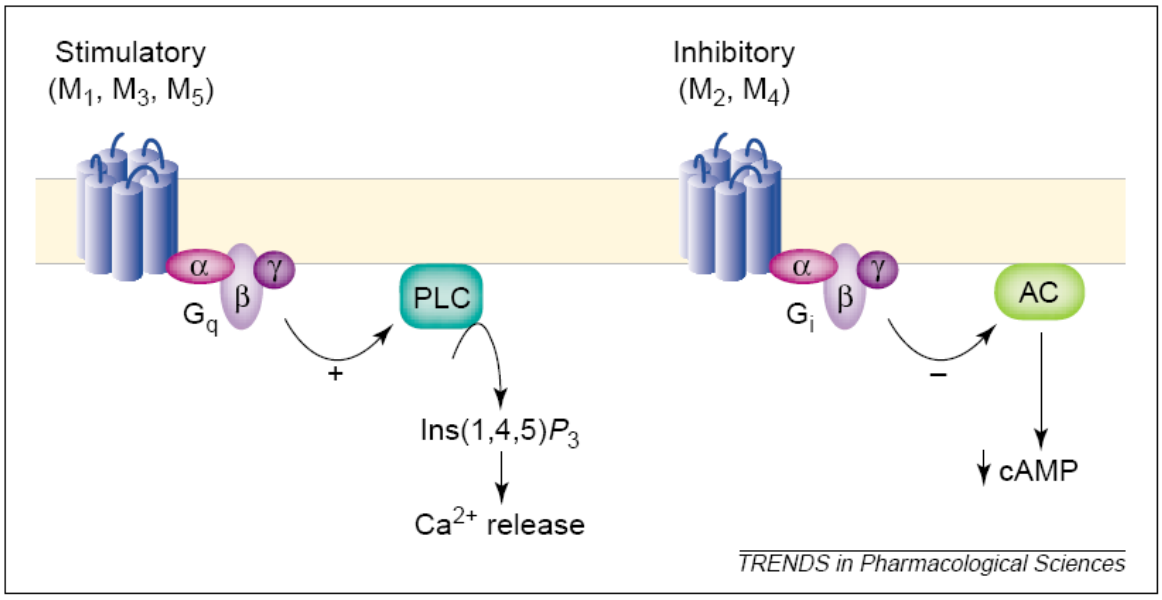


Figure 2. Gq and Gi protein coupled receptor signal transduction for muscarinic acetylcholine receptors. Subtypes M1, M3 and M5 are G_q coupled resulting in an increase in intracellular calcium, while M2 and M4 subtypes are G_i coupled and are inhibitory, negatively modulating adenylyl cyclase (AC) resulting in a reduction of cytoplasmic concentrations of cAMP (Eglen et al., 2001)

Fluorescence microscopy is one method which enables the increase in intracellular calcium to be visualised. Light of the correct excitation wavelength is focused on the specimen. This excites the flurophore resulting in fluorescent light to be emitted, which is then picked up by the detector. Figure 3 illustrates the basic principles of fluorescence microscopy.

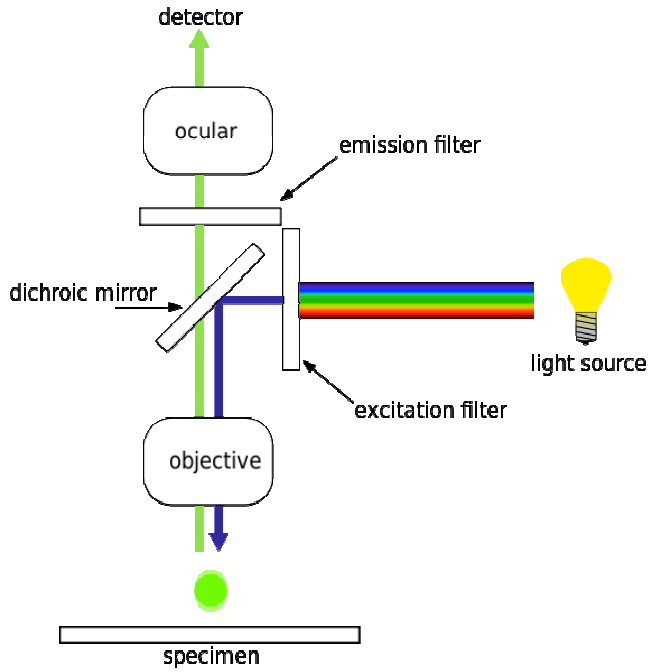


Figure 3. Basic principle of fluorescence microscopy. Adapted from a talk by Henry Mühlpfordt

For this research, the fluorescent dye Fluo-4 (Invitrogen) was used in isolated microglia. Fluo-4 is a green-fluorescent calcium indicator, which is used in the non-fluorescent acetoxymethyl ester form, Fluo-4 AM. This is then cleaved within the cell to give the free, fluorescent form, which is then excited at 488nm and increases in fluorescence upon binding with Ca^{2+}. Figure 4 shows an example of a microglia cell, stained with Fluo-4 during excitation.

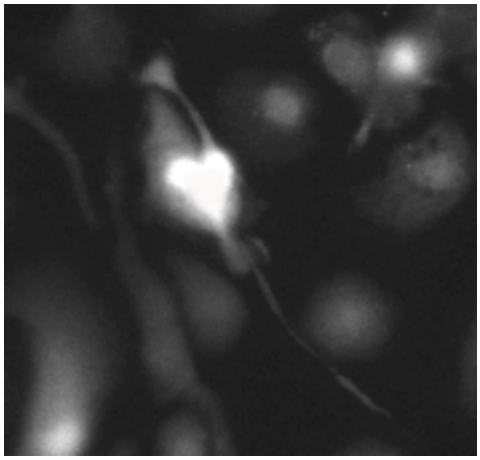


Figure 4. Neonatal cultured microglia showing baseline fluorescence after staining with Fluo-4 am and excitation at 488nm

1.3 Endothelin

Endothelin, a potent vasoconstrictive peptide, was first discovered in 1988 (Yanagisawa et al., 1988) from the medium of cultured porcine endothelial cells. There are 3 endogenous endothelin isoforms found in humans: ET1, ET2 and ET3 and 4 receptor subtypes, ETA and ETB1, ETB2 and ETC. Activation of the ETA and ETB2 receptor is associated with prolonged vasoconstriction while activation of ETB1 results in vasodilation. The physiological role of ETC, however, remains uncertain (Pollock et al., 1995). Endothelin and its receptors play a significant role in the central nervous system. ET1 and ET3 are produced by neurons and glial cells, as well as by the central nervous system vasculature. ET receptors are also widely expressed in the brain (Khimji and Rockey, 2010), with ETA and ETB both G_q coupled receptors, where activation leads to an increase in Phospholipase C stimulation and a rise in intracellular calcium.

Previous studies have shown that CSF and plasma levels of ET-1 and ET-3 are low when the CNS is uninjured (Suzuki et al., 1989; Yoshizawa et al., 1990), but significantly increase following traumatic brain injury (Hama et al., 1997), spinal cord trauma (McKenzie et al., 1995), and stroke (Lampl et al., 1997). Cultured mouse microglia have been shown to express mRNA for the ETB receptor (Moeller et al., 1997). Neuronal injury after damage to the optic nerve in rats and rabbits was found to cause an upregulation in the expression of the ETB on microglia (Rogers et al., 1997). Microglia were also found to be a source of ET-1 after cerebral ischemia, resulting in an immediate constriction of blood vessels after interaction with ETA receptors, as well as interacting with microglial and astrocytic ETB receptors resulting in cytokine and chemokine release (Li et al.).

1.4 Histamine

As well as its role in local immune function and regulation of physiological function in the gut, histamine, an endogenous biogenic amine, also acts as a neurotransmitter in the CNS. The main sources of histamine in the brain are neurons and mast cells (Garbarg et al., 1976). There are 4 subtypes of the histamine receptor, all of which can be found within the central nervous system. H1 is located post synapytically, and is a G_q coupled receptor and stimulates the

activity of phospholipase C (Leurs et al., 1994) and is associated with arousal and appetite suppression (Passani et al., 2011). H2 receptors are G_s coupled, activation of which leads to stimulation of adenylyl cyclase and production of cAMP. H2 and H1 are both widely expressed in the CNS and activation of these receptors leads to excitatory effects as a result of the blockade of calcium dependant potassium channels and modulation of hyperpolarisation activated cation channel (Brown et al., 2001). H3 are G_i coupled, resulting in inhibition of adenylyl cyclase. Activation of this receptor subtype results in inhibition of histamine as well as other neurotransmitters, including acetylcholine, serotonin and glutamate, via the inhibition of presynaptic calcium channels. H4 receptors also belong to the G_i family and have a role in chemotaxis. Microglia have recently been shown to express all receptor subtypes in a microglial cell line and primary murine microglia, and treatment with histamine has been shown to trigger microglial motility and to obstruct LPS induced microglial migration and IL-1B release (Ferreira et al., 2012). Calcium imaging experiments showed that histamine induces a calcium increase in 30% of cultured MG through IP3 induced calcium release from ER (Bader et al., 1994).

1.5 Substance P

Substance P belongs to a family of peptides known as the neurokinins (NKs) which are widely distributed in the CNS. Substance P is released from the terminals of specific sensory nerves and functions as a neuromodulator and neurotransmitter, where its roles include the regulation of affective behaviour and emesis in the brain, and pain processes in the spinal cord (Mantyh, 2002). Substance P mediates these functions via its specific receptor, the NK1 receptor, although only a small proportion of neurons in particular areas of the CNS express this receptor. The NK1 receptor is a G_q coupled receptor, activation of which stimulates Phospholipase C.

NK1 receptors have been found to be expressed in both mouse (Rasley et al., 2002) and human microglia. It was also found that human microglia from fetal tissue produce significant amounts of substance P (Lai et al., 2000). Activation of NK1 receptors on microglia may regulate the production of cytokines in microglial

cultures and microglia in vivo. Substance P in combination with LPS was found to increase IL1 production in rat microglia, however this was not increased by substance P alone, demonstrating the collaborative role of substance P in inflammatory diseases (Martin et al., 1993). Substance P was also found to increase microglial proliferation in a rat model of traumatic brain injury, while treatment with an NK1 antagonist n-acetyl tryptophan (NAT), was found to be neuroprotective through the inhibition of microglia (Carthew et al., 2012). High levels of substance P were also found to be detrimental in a rat model of Parkinson's disease, leading to blood brain barrier breakdown and the activation of microglia and astrocytres, while treatment with NAT resulted in an improvement of motor function, conserved the integrity of the blood brain barrier, reduced neuroinflammation and protected dopaminergic neurons. (Thornton and Vink, 2012).

1.6 Serotonin

Serotonin, also known as 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter, primarily produced in the brain by the neurons of the Raphe nuclei which project to all brain regions including the cortex and brain stem. Its role as a neurotransmitter has been linked to various functions such as sleep, behaviour, mood and appetite, as well as functioning as a neuromodulator in the CNS and the periphery (Mohammad-Zadeh et al., 2008). There are a distinct number of serotonin receptors 5-HT1, 5-HT2, 5-HT3, 5-HT4, 5-HT5, 5-HT6 and 5-HT7 which can be further categorised into subtypes. The 5-HT2 receptor is a G_q linked G-protein coupled receptor, increasing cellular levels of IP3 and DAG, while 5-HT1 and 5-HT5 are G_i -protein coupled, 5HT3 is a ligand-gated Na+ and K+ cation channel and 5HT4,-6 and -7 are G_s-protein coupled. Microglia from primary cultured neonatal mice, as well as freshly isolated microglia from the brains of adult mice, were shown to express mRNA for serotonin receptors. Freshly isolated adult microglia were found to express class 2, 5-HT5a and 7 serotonin receptors but not 5-HT1b, 5-HT3a, 5-HT5b, 5-HT6. Neonatal primary cultured microglia were only found to express the 5-HT2b receptor. Serotonin was also found to impact microglial function, causing an increase in process motility towards a laser lesion

in acute brain slices, increasing the chemotactic response to ATP in vitro and decreasing phagocytic activity in vitro and in situ (Krabbe et al., 2012).

1.7 Galanin

Galanin is a neuropeptide widely expressed in the brain, spinal cord and gut, although knowledge of its function remains limited. Its predominant role in the CNS is the modulation and inhibition of action potentials in neurons, but it is also known to have a physiological role in functions such as nociception, arousal/sleep regulation and cognition as well as various neuroendocrine functions (Mechenthaler, 2008). There are 3 classes of galanin receptors GALR1, GALR2 and GALR3. GALR1 is Gi/Go-protein coupled, GALR2 is G_q coupled while the signal transduction pathway of GALR3 remains unknown. Galanin is up-regulated following neuronal axotomy and inflammation and was shown to increase the migration of cultured rat microglia (Ifuku et al., 2011). Galanin has also been shown to be upregulated in microglia in multiple sclerosis and Alzheimer's disease which is believed to reduce the severity of disease progression, while loss of function in the gene expression of galanin or the GALR2 genes exacerbates the disease outcome (Wraith et al., 2009).

1.8 Somatostatin

The regulatory peptide hormone somatostatin is produced by inflammatory, immune and neuroendocrine cells and acts via its G-protein coupled receptors as an inhibitory regulator of secretion and proliferation of target cells in the brain and periphery (Patel, 1999). There are 5 subtypes of the somatostatin receptor, SSTR1-5, all of which are expressed in the brain, with SSTR2, SSTR3 and SSTR5 G_q coupled and SSTR1 and SSTR4 G_i coupled. SSTR2, 3 and 4 were found to be expressed in both rat (Feindt et al., 1998) and mouse microglia (Fleisher-Berkovich et al., 2010), where the latter publication also reported the inhibition of the basal, GM-CSF and IL-3 induced proliferation of microglial cells by somatostatin, as well as the induction of tyrosine phosphorylation of a 95 kDa protein in microglia. Somatostatin was also found to cause a concentration dependant increase in the phagocytosis of amyloid β plaques in cultured primary

microglia as well as in the BV2 microglial cell line (Fleisher-Berkovich et al., 2010). The role of somatostatin in affecting microglial function was also shown through its ability to increase the expression and secretion of the Insulin-degrading enzyme (IDE), an important enzyme for Alzheimer's research due to its ability to degrade Aβ (Tundo et al., 2012). Somatostatin also inhibited the LPS induced prostaglandin E2 synthesis in rat microglia (Grinshpun et al., 2008), highlighting the numerous beneficial effects somatostatin may have on controlling detrimental microglial functions in neurodegenerative disease.

1.9 Angiotensin II

Angiotensin II is a peptide hormone which forms part of the rennin-angiotensin system (RAS) which controls blood pressure through the regulation of body water (Wright and Harding, 2013). It is a vasoactive octapeptide which is converted from angiotensin I by the angiotensin-converting enzyme. The brain also has its own RAS where angiotensin II has 3 main functions: the regulation of body fluid as a result of a decrease in volume of blood plasma, a role in the activity of gonadotropic hormone releasing hormones and pituitary hormone in reproduction, and the interaction of angiotensin II with neurotransmitters and synapses (Phillips, 1987). Angiotensin II influences its target cells via 2 G-protein coupled receptors, AT1 and AT2. The AT1 receptor can be both G_i and G_q coupled, and activation of this receptor can result in effects such as smooth muscle cell contraction, aldosterone, ADH and endothelin release, and central and peripheral sympathetic stimulation. The AT2 receptor is G_i coupled and is less widespread than AT1. Unstimulated rat microglia have been shown to express mRNA for AT2 but not AT1 receptors. However, after stimulation with LPS for 6 hours, AT1 mRNA was found to be expressed (Miyoshi et al., 2008). This study also found that the AT1 antagonist losartan suppressed morphological activation of MG and reduced production of NO and IL 1B. Losartan also inhibited LPS induced activation of NF kB.

1.10 Vasopressin

Arginine vasopressin (AVP), also known as vasopressin and argipressin orantidiuretic hormone (ADH) is a neurohormone found in mammals. Its primary function is the retention of water by increasing absorption in the collecting ducts of the kidney nephron. It is also involved in peripheral vascular resistance resulting in an increase in arterial blood pressure. Vasopressin is also widely expressed in the central nervous system and is synthesised by neurons in the hypothalamus. Its functions include memory formation and the amplification of post traumatic production of pro inflammatory mediators (Chodobski et al., 2011). There are 3 subtypes of vasopressin receptors, V1a, V1b and V2. V1a and V1b belong to G_q family, where binding results in an increase in intracellular calcium, while the V2 receptor belongs to the G_s family. Very little research has been carried out relating to vasopressin receptors on microglia. Activated microglia/brain macrophages were shown to synthesize vasopressin following traumatic brain injury in rats, identified by double immunostaining for CD11B and vasopressin (Szmydynger-Chodobska et al., 2011), however, evidence of functional vasopressin receptors on microglia has yet to be demonstrated.

1.11 Neurotensin

Neurotensin is a neuropeptide widely distributed in the central and peripheral nervous system which is involved in the regulation of luteinizing hormone and prolactin release, as well as interacting with the dopaminergic system. Neurotensin producing neurons are widely expressed in the CNS along with the three subtypes of the neurotensin receptor. NTS1 and NTS2 receptor subtypes are both members of the G_q family, while NTS3/sortillin receptor is located intracellularly (St-Gelais et al., 2006) and is the only receptor subtype that has been shown to be expressed on microglia (Martin et al., 2005). The NTS3 receptor has already been implicated in the function of microglia. A study using the murine microglial cell line found that activation of this receptor on microglia led to the expression of several cytokine and chemokine genes including macrophage inflammatory protein (MIP)-2, monocyte chemotactic protein (MCP)-1, interleukin (IL)-1beta and tumor necrosis factor (TNF)-alpha (Dicou et al., 2004). Expression of this receptor was also found

in the human microglial cell line C13NJ and that its activation induced migration (Martin et al., 2003).

1.12 Dopamine

Dopamine is a catecholaminergic transmitter in the CNS which was discovered more than 50 years ago (Carlsson et al., 1957). It is involved in a number of important functions in the brain including sleep, attention, working memory, voluntary movement, reward and learning. Dysfunction of the dopaminergic pathway has been shown in a number of diseases including Parkinson's and Huntington's chorea. There are five subtypes of the dopamine receptor, D1-D5, all of which are G-protein coupled. D1 and D5 stimulate cAMP production through the G_S family of G proteins while D2, D3, and D4 are G_i family coupled and resulting in the inhibition of Adenylate cyclase. D1 also has a secondary transduction mechanism which results in the opening of a calcium channel (Arias-Montano et al., 2007). A number of publications have found evidence of functional dopamine receptors on microglia from healthy mice and rats. Specific ligands for the D1 and D2 receptors triggered the inhibition of constitutive potassium inward rectifier and activated potassium outward currents in a subpopulation of microglia. Stimulation of the dopamine receptor chronically also resulted in an increase in migratory activity and reduced the LPS stimulated nitric oxide release (Farber et al., 2005). Dopamine induced chemotaxis was also found in aged microglia from human brains. The same study also demonstrated the presence of the dopamine receptor subtypes D1-D4 through immunostaining and RT-PCR of microglia from the substania nigra of Parkinson's patients (Mastroeni et al., 2009).

1.13 The cholinergic system

Acetylcholine was the first neurotransmitter to be identified (Loewi, 1924) and has a number of different functions in both the peripheral and central nervous system. In the CNS, acetylcholine and its associated neurons form the cholinergic system, which have an involvement in functions such as sleep, learning and memory, arousal (Jones, 2005) and attentiveness (Himmelheber et al., 2000). The impairment of the cholinergic system is well documented in diseases such as

Alzheimer's (Francis et al., 1999) and Parkinson's disease (Bohnen and Albin, 2011) and is therefore an important area of research. Early experiments by Sir Henry Dale suggested the presence of two acetylcholine receptor classes (Dale, 1934) which were named muscarinic and nicotinic according to their sensitivity to the agonists muscarine and nicotine.

1.13.1 Muscarinic Acetylcholine receptors

Evidence for functional muscarinic receptors was found for cultured human and rat microglia. The muscarinic acetylcholine receptor agonist carbachol triggered a transient increase in intracellular calcium in microglia cultured from early postnatal rat cortex. This response was due to the release of Ca^{2+} from intracellular stores (Whittemore et al., 1993). A similar response was described for microglia cultured from human foetal tissue (Zhang et al., 1998). So far, there is neither evidence for expression of these receptors in the adult brain, nor for an impact on microglial function. Muscarinic acetylcholine receptors (mAChRs) are G-protein coupled receptors with 5 subtypes described for mammalian cells. The M1, M3 and M5 subtypes couple to the G-proteins of the G_q family, while the subtypes M2 and M4 couple to G_i -type G-proteins (Wess et al., 2007).

1.13.2 Nicotinic acetylcholine receptors

In contrast to the muscarinic acetylcholine receptors, nicotinic acetylcholine receptors are not members of the G-protein coupled family of receptors but are ligand gated ion channels. They have a molecular mass of ~290 kDa and are integral allosteric membrane proteins which are made up of 5 subunits symmetrically arranged around a central ionic channel (Taly et al., 2009). Binding of the endogenous agonist Ach results in a conformational change of the subunits, followed by opening of the ion channel and influx of cations, which may include Ca^{2+}. As with mAch receptors, nAch receptors are found in both the PNS and the CNS and are involved in the rapid effect of Ach after release. Activation of nAch receptors results in the release of neurotransmitters such as serotonin, dopamine, glutamate and GABA. The expression of nicotinic acetylcholine receptor subunits has already been demonstrated on microglia from mouse (Shytle et al., 2004), rat

(De Simone et al., 2005), rhesus monkey (Liu et al., 2009) and human (Rock et al., 2008).

1.14 Functions of microglia

1.14.1 Phagocytosis

Phagocytosis is a common feature of activated microglia and a form of endocytosis where a solid particle is engulfed and forms an internal phagosome. These phagosomes then fuse with enzyme-containing lysosomes which enable breakdown of the internalised particle (Kinchen and Ravichandran, 2008). It is the main mechanism used by many cells to remove pathogens, such as bacteria, as well as other cellular debris. Phagocytosis is controlled by signals located on the target cell which may be “eat me’’ signals or “don’t eat me’’ signals which will determine whether phagocytosis takes place (Ravichandran, 2011). Phagocytic microglia can be neuroprotective, through the removal of pathogens (Ribes et al., 2013) or engulfment of Aβ plaques (Bard et al., 2000), but have also been shown to be detrimental, resulting in neuronal death as a result of viable neurons being phagocytosed (Neher et al., 2012). The mechanism of phagocytiosis is made up of 3 steps: find me, eat me and digest me (Savill et al., 2002). “Find me’’ signals released by the target activate receptors on microglia which initiates phagocytosis. These signals can include extracellular nucleotides such as ATP and UTP released by apoptotic cells which bind to purinergic receptors on microglia (Koizumi et al., 2007), as well as fractalkine/CX3CL1, which is also released by apoptotic cells and binds to the CX3CR1 receptor on microglia (Noda et al., 2011). Once the microglia has reached its phagocytic target, “eat me” signals enable the cell to discriminate the phagocytic particle from living cells, which express “don’t eat me” signals (Ravichandran, 2010). These “eat me’’ signals include receptors which may tether the phagocyte to the target, or trigger internalisation (Underhill and Goodridge, 2012). Scavenger receptors in conjunction with toll-like receptors allow the detection of pathogen-associated molecular patterns (PAMPS), for example the CD14/TLR4 complex (Sierra et al., 2013). Activation of TLRs such as TLR-4 by ligands including *Streptococcus pneumoniae* initiates phagocytosis, which can be mimicked in culture through LPS stimulation (Ribes et al., 2009).

Triggering receptor expressed on myeloid cells-2 (TREM2) is an internalisation signal, and it was shown that loss of function of this receptor resulted in a prevention of phagocytic activity of microglia (Hsieh et al., 2009). In this research, phagocytosis is triggered through soluble opsonins which bind to microglial receptors, triggering internalisation. Incubation of latex beads with fetal calf serum causes proteins of the complement system such as C3b and antibodies such as IgG to bind to compliment receptor 3 and Fc receptors allowing phagocytosis to be mediated (Underhill and Goodridge, 2012). Once internalisation has taken place and the phagocytic target has been engulfed, “digest me’’ signals cause maturation of the phagosome, resulting in the formation of phagolysosomes which contain numerous proteins for digestion of the phagocytosed substance (Desjardins et al., 1994).

1.14.2 Chemotaxis

Chemotaxis is the process by which cells direct their movements according to certain stimuli in order to carry out specific functions, such as the movement of sperm towards the egg during fertilization, or the migration of neurons during early development. For microglia, chemotaxis is an important function for recruitment of cells towards, for example, a pathogenic event which may require microglia to release inflammatory substances or to become phagocytic in order to clear cellular debris (Koizumi et al., 2007; Kreutzberg, 1996). In order for eukaryotic cells to respond to a chemotactic signal and initiate motility, a chemotactic gradient is sensed, normally by G-protein coupled receptors on the surface of the cell, which results in a signalling cascade and the polymerisation of actin filaments (Bagorda and Parent, 2008). The role of ATP as a trigger of microglial chemotaxis and motility has already been established through the activation of the P2Y12 receptor (Irino et al., 2008), and chemokines such as CCL21 have also been shown to act as a chemoattractant for microglia (Biber et al., 2001). In this research we have focused on the receptor expression of neurotransmitters and neurohormones on microglia, and the effect of this receptor activation on function. We have specifically focused on the activation of the muscarinic acetylcholine receptor and its effects on microglial chemotaxis, however, a small amount of research has

been carried out on our other ligands of interest, which has been briefly described in the previous section.

1.15 The impact of microglia in pathology

Chronic neuroinflammation is a common feature in neurological diseases such as multiple sclerosis, Huntington's, Parkinson's, Alzheimer's and stroke, and since microglia are the resident immune cells of the CNS, their activation has been implicated in the progression of a number of diseases. Microglia can be activated by a vast number of substances including many proteins associated with neurodegenerative disease such as amyloid-beta (Jana et al., 2008), alpha-synuclien (Zhang et al., 2005) and prions (Szpak et al., 2006), as well as through injury (Maeda et al., 2010), ischemia (Hur et al., 2010) and infection (Xu et al., 2009). Activation of microglia does not always lead to detrimental effects. During traumatic injury, microglia have been shown to clear glutamate, whose excessive release results in excititoxicity in stroke, ALS and autism among others, without evoking inflammatory mediators (Shaked et al., 2005). During axotomy of the optic nerve, microglia have also been shown to efficiently clear myelin debris (Battisti et al., 1995). In this study we have focused on the expression of various receptors on microglia from mouse models of glioma, multiple sclerosis, Alzheimer's disease and stroke. The function and behaviour of microglia in these diseases is summarised below.

1.15.1 Glioma

High-grade gliomas are the most common type of primary brain tumor in adults and have a disproportionally poor survival rate. The median survival rate for patients with glioblastoma is 12-15 months (Wen and Kesari, 2008). Microglia/brain macrophages commonly accumulate around the tumor which leads to increased malignancy and poor prognosis (Vinnakota et al., 2013). Microglia have been shown to play a supportive role in glioma initiation and progression by creating an optimal microenvironment for the tumor (Markovic et al., 2005). IL-10 release also results in immunosupression further promoting glioma expansion (Huettner et al., 1997). Microglia have been shown to demonstrate antitumor abilities, but only in vitro, where IFN-γ and endotoxin stimulation led to tumor cell

cytotoxicity and production of tumor necrosis factor alpha (Frei et al., 1987). This demonstrates the potential for the development of therapies which can alter microglial function to steer them to a more neuroprotective phenotype. However, in the case of glioma, the majority of research indicates that glioma release substances which suppress protective functions of microglia, for example suppressing the release of proinflammatory cytokines such as TNF-α, IL-1 or IL-6 (Hussain et al., 2006), while upregulating the production of enzymes beneficial to tumor expansion and growth (Konnecke and Bechmann, 2013). Membrane bound and secreted proteases such as membrane type-1 matrix metalloprotease (MT1-MMP) and MMP9 have been extensively studied in recent years. Their role in degrading the extracellular matrix to support tumor growth has been linked to microglia. MT1-MMP was shown to be upregulated in tumor associated microglia, which was triggered by factors released by the glioma via microglial toll-like receptors and the p38 MAPK pathway. MT1-MMP was not upregulated in glioma cells. The resulting upregulation in microglia leads to activation of glioma-derived pro-MMP-2 which in turn promotes glioma expansion (Markovic et al., 2009). Microglia have also been shown to modulate MMP-9 activity through the release of the co-chaperone stress inducible protein 1 (STI1) (Fonseca et al., 2012). Higher densities of microglia/brian macrophages in glioma tissue also positively correlates towards increased grade and invasiveness of the tumor (Markovic et al., 2005). In order to study glioma and glioma-associated microglia, there are a number of animal models of this disease which can effectively mimic the human condition when human samples are unavailable or limited. Mouse models of glioma can include genetic models (Chen et al., 2012), where genes such as those for cell-cycle control or tumor suppression are knocked out. For this study, we have used a stab wound model of glioma. This model involves the induction of a stab-wound injury, followed by the injection of a glioma cell line and is discussed in more detail in the methods section.

1.15.2 Stroke

Stroke is one of the major causes of death and disability in industrialised countries, ischaemic stroke being the most common type, accounting for about 80% of all cases (Rosamond et al., 2007). Activated microglia/brain macrophages have been

implicated in stroke, having been shown to have both neuroprotective and neurotoxic functions. Expression of the translocator protein 18 kDa (TSPO) is upregulated in activated microglia and has been used as a marker in imaging studies to determine the presence and quantity of activated microglia in human and animal models. One such study, using positron emission tomographic imaging showed that activated microglia/macrophages appear in the ischemic core 1-2 days after stroke, gradually expanding into the peri-infarct zones. This may be an indicator of secondary damage or could enable the microglia/macrophages to be targeted for therapeutic intervention (Thiel and Heiss, 2010). Oestrogen has been shown to be neuroprotective in stroke (Simpkins et al., 1997), as well as in multiple sclerosis (Offner and Polanczyk, 2006) and Parkinson's disease (Dluzen, 1997) and this oestrogen-mediated neuroprotection was found to be dependant on IGF-1 signaling, where the primary source was microglia (Sohrabji and Williams, 2013). Microglia were also found to be neuroprotective in an in vitro model of ischemic injury. Application of the microglial cell line BV2 onto organotypic hippocampal slice cultures 24 hours before oxygen-glucose deprivation led to a decrease in OGD-induced neuronal damage (Neumann et al., 2006). A study by Hu et al. also found that microglia assume a neuroprotective M2 phenotype in the early stages of stroke but then gradually transformed into a neurotoxic, M1 phenotype. They showed that M1 markers including the gene expression of iNOS, CD16 and CD32 gradually increased from day 3 after stroke, while M2 markers including the expression of CD206, CCL22 and IL-10 was induced at days 1-3, peaked at days 3-5, started to decrease by day 7 and were at pre-stroke level at day 14 (Hu et al., 2012). Another study found that microglia were activated by glutamate released by OGD neurons. Microglia became neurotoxic and killed naive neurons through an apoptotic mechanism mediated by TNF-alpha which also involved the activation of both caspase-8 and caspase-3 (Kaushal and Schlichter, 2008). For this study we used a focal ischemia mouse model of stroke. MCAo was induced for 30 mins using a silicone-coated, 8-0 monofilament, described in more detail in the methods section (Endres et al., 2004).

1.15.3 Alzheimer's disease

Alzheimer's disease (AD) was first described in 1901 by the German psychiatrist Alois Alzheimer and is the most frequently occurring neurodegenerative disease which is characterised by the loss of neuronal activity in all brain regions. Early symptoms begin with short-term memory loss, often mistakenly attributed to old age, eventually leading to confusion, aggression, irritability, long-term memory loss, loss of self-awareness and speech impairment, followed by withdrawal from society, loss of bodily function and death. In 2006, there were 26.6 million sufferers worldwide and it is predicted to affect 1 in 85 by 2050 (Brookmeyer et al., 2007). AD is characterised by 2 features, the presence of plaques made up of extracellular β-amyloid (Aβ) aggregates and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein (Figure 5). These features can be seen years before clinical symptoms appear. There is currently no cure for Alzheimer's disease, and existing treatments only help with the symptoms. Although the exact cause and progression of Alzheimer's is still not fully understood, it is believed that there are pathogenic interactions between features which lead to clinical symptoms and disease progression. Aβ aggregation and accumulation in the brain impairs synaptic function and signalling pathways, alters neuronal activity and triggers the release of neurotoxic substances from glial cells. ApoE4 is a lipid transport protein which has also been implicated in AD by impairment of Aβ clearance. Tau proteins, which normally function to stabilize microtubules and are abundant in the CNS, particularly in axons, become localised in the soma of neurons, and form NFTs. α-synuclein has also been implicated in AD, and is also able to form pathogenic oligomers forming larger aggregates called Lewy bodies. Release of both Tau and α-synuclein into the extracellular space enables the spread of these neurotoxic substances to other cells (Huang and Mucke, 2012).

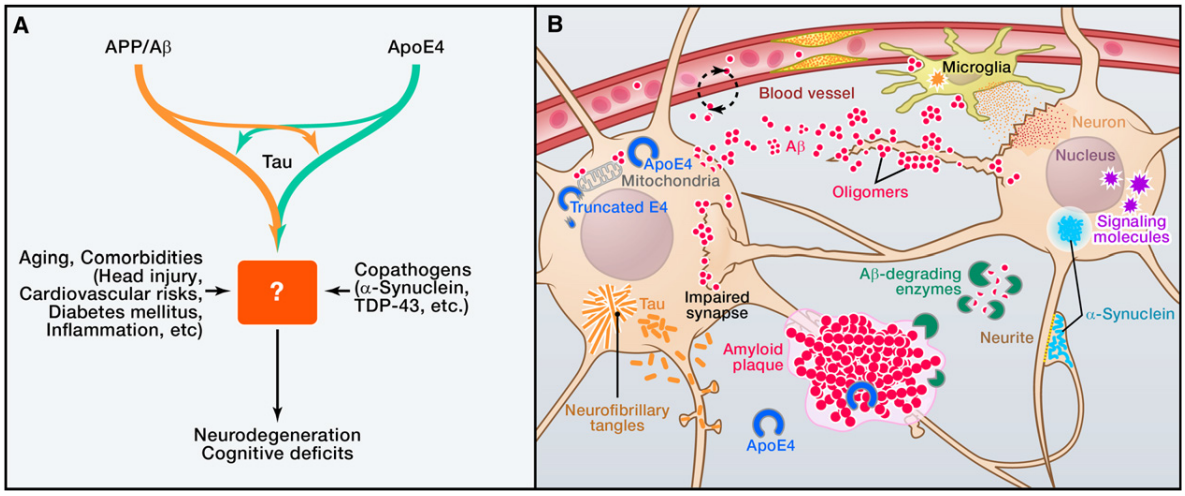


Figure 5. Contributing factors leading to the pathogenesis of Alzheimer's disease. (Huang and Mucke, 2012)

A number of recent studies have examined the role of microglia in AD. Activated microglia were first identified in post-mortem brains of Alzheimer's patients through the increased expression of the class II cell surface glycoprotein of the human histocompatibility complex, HLA-DR. This glycoprotein is normally associated with an immune response, and its main function is to signal to T-helper cells that a foreign antigen is being presented. The presence of HLA-DR- positive microglia was particularly concentrated in areas of Aβ plaque formation, and in the hippocampus, HLA-DR- positive cells were also positively correlated with the number of senile plaques (McGeer et al., 1987). The inflammatory cytokine IL-1 was also found to be upregulated in microglia from Alzheimer's brains (Griffin et al., 1989), providing further evidence that microglia are activated during this disease. Microglia have also been shown to be attracted to Aβ plaques, extending their processes and migrating towards plaques (Bolmont et al., 2008), as well as causing the activation of cell surface immune and adhesion molecules such as CD45, CD40, CD36 and integrins, resulting in signalling cascades that result in the production of cytokines and chemokines (Ho et al., 2005). Aβ plaques were also found to trigger microglial phagocytosis, allowing for the removal of senile plaques (Koenigsknecht and Landreth, 2004), however, phagocytic activity, as well as process motility was found to be impaired in 2 mouse models of Alzheimer's disease. This impairment could be decreased through the use of an Aβ specific antibody, indicating that the lowering of the plaque burden can reverse the

impairment of microglial function (Krabbe et al., 2013). In order to study microglia in Alzheimer's disease, we used a transgenic mouse model of cerebral amyloidosis, APPPS1 mice that were provided by Mathias Jucker, University of Tübingen, Germany. These mice express human APP with the Swedish mutation (KM670/671NL) and human mutated PS1 (PS1-L166P) (Radde et al., 2006). These mice have cerebral amyloidosis starting from 6-8 weeks of age, with amyloid associated pathologies including gliosis, dystrophic synaptic boutons and hyperphosphorylated tau-positive neuritic structures.

1.15.4 Multiple sclerosis

Multiple sclerosis is an inflammatory disease which affects the brain and spinal cord through the immune mediated damage of the myelin which surrounds and insulates nerve cells. The damaged myelin then forms scar tissue, know as sclerosis, which most commonly affect the white matter in the optic nerve, basal ganglia, brain stem and spinal cord. Myelin loss results in the impaired ability of neurons to effectively transmit electrical signals (Compston and Coles, 2002), leading to a variety of symptoms including muscle weakness, problems with balance and coordination, visual impairment and memory problems (Compston and Coles, 2008). During the early stages of the disease, remyelination is able to occur, as the oligodendrocytes attempt to repair the damage. However, as the disease progresses, this repair becomes less effective, leading to worsening symptoms and an increase in lesions (Chari, 2007). Multiple sclerosis lesions are characterised by a T lymphocyte inflammatory response. Since these cells are members of the adaptive immune system, they require antigen presentation from members of the innate immune system. Microglia, brain macrophages and B cells are also present in the immune response to MS lesions, and these cells act as antigen presenting cells (APCs), which allow neural antigen-reactive T cells to persist in the CNS during the course of the disease (Jack et al., 2005). Activated microglia/brain macrophages are know to be detrimental to the progression of MS due to the destructive phagocytic activity of these cells (Huizinga et al., 2012). Axonal damage was also positively correlated to local microglial activation in MS (Howell et al., 2010), and microglia/macrophages were also found to contribute to

oligodendrocyte loss within MS lesions from human autopsy material (Lucchinetti et al., 1999). Based on the assumption that microglia are detrimental to the progression of MS, a recent study used dipyridamole as an inhibitor of microglial activity in a mouse model of MS (EAE) (Sloka et al., 2013). The authors found that daily dipyridamole treatment from day 7 postimmunisation reduced the clinical severity of the disease during the chronic phase, although it had no effect on the clinical signs during the initial onset and peak of the disease. Dipyridamole has also been used clinically for secondary prevention in stroke (Shulga and Bornstein, 2011), while another inhibitor of microglia activity, the broad spectrum antibiotic minocycline, has also been shown to improve the outcome of diseases such as epilepsy (Abraham et al., 2012), stroke (Hayakawa et al., 2008) and Alzheimer's (Fan et al., 2007). This demonstrates the vast potential for improving the outcome of numerous neurodegenerative diseases through the control of microglial activity. Multiple sclerosis does not occur naturally in species other than humans. In order to carry out animal studies to research the disease, experimental autoimmune encephalomyelitis (EAE) can be induced. In our research we used a mouse model of EAE. In order to mimic the demylination of MS, myelin protein such as myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) is injected into the animal, along with an adjuvant which results in an inflammatory response to the protein. In the mice used for this study PLP was injected along with complete Freund's adjuvant and heat-inactivated Mycobacterium tuberculosis. Pertussis toxin was also administered to break down the BBB and allow immune cells to enter the CNS. The protocol is described in more detail in the methods section.

1.16 Aim of this research

The aims of this research were as follows;

- To determine whether murine microglia express functional receptors for endothelin, histamine, substance P, serotonin, galanin, somatostatin, angiotensin II, vasopressin, neurotensin, dopamine and acetylcholine (nicotinic and muscarinic Ach receptors) using calcium imaging

- To assess whether the specific neurotransmitter and neurohormone sensitive populations differ in primary cultured neonatal and adult microglia, as well as the novel freshly isolated adult microglia preparation
- To determine whether freshly isolated microglia from murine models of disease vary in their receptor repertoire compared to those from healthy mice, and whether this can be mimicked in cultured cells through treatment with pro and anti-inflammatory substances
- To focus specifically on one receptor system, namely the muscarinic acetylcholine receptor system, and investigate in more detail the role this receptor system plays in the phagocytic and chemotactic function of microglia

2. Materials

2.1 Drugs and reagents	Company
Angiotensin II	Sigma-Aldrich, Munich, Germany
Aqua-Poly/Mount	Polysciences Europe GmbH, Eppelheim, Germany
ATP	Sigma-Aldrich, Munich, Germany
Bordetella pertussis toxin	List Biological Laboratories, USA
Bovine serum albumin (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Carbachol	Sigma-Aldrich, Munich, Germany
Chlodronate	Calbiochem, Darmstadt Germany
Complete Freund's Adjuvant	H37Ra, Difco,, USA
dNTP	Invitrogen, Karlsruhe, Germany
Dopamine	Sigma-Aldrich, Munich, Germany
Dulbecco's Modified Eagle Medium (DMEM)	GIBCO®Media, Invitrogen, Darmstadt, Germany
Dulbecco's Phosphate-Buffered Saline (DPBS)	GIBCO®Media, Invitrogen, Darmstadt, Germany
Endothelin-1	PolyPeptide group, Torrance, CA, USA
Fetal calf serum (FCS)	GIBCO®Serum, Invitrogen, Darmstadt, Germany
Fluoresbrite microspheres	Polysciences Europe GmbH, Eppelheim, Germany
Galanin	PolyPeptide group, Torrance, CA, USA
Glucose	Merck, Damstadt, Germany
Go Taq qPCR Master Mix	Promega, Mannheim, Germany
Hanks Balanced Solution (HBSS)	GIBCO®Products, Invitrogen, Darmstadt, Germany
Heat-inactivated Mycobacterium tuberculosis	H37Ra, Difco,, USA
HEPES for cell culture	GIBCO®Products, Invitrogen, Darmstadt, Germany
Histamine	Sigma-Aldrich, Munich, Germany
IFN-gamma	R&D systems, Minneapolis, MN USA
IL-4	Peprotech, Hamburg, Germany
LPS (Lipopolysaccharide from *E.coli*)	Enzo, Lörrach, Gemany
MACS CD11b beads	Miltenyi Biotech, Bergisch Gladbach, Germany
MACS Myelin beads	Miltenyi Biotech, Bergisch Gladbach, Germany
Neurotensin	Sigma-Aldrich, Munich, Germany
Nicotine	Sigma-Aldrich, Munich, Germany
Normal goat serum (NGS)	GIBCO®Products, Invitrogen, Darmstadt, Germany

oligo-dT primer	Invitrogen, Karlsruhe, Germany
PBS (Phosphate buffered saline)	GIBCO®Products, Invitrogen, Darmstadt, Germany
Percoll	GE Healthcare, Munich, Germany
PLP139–151	Pepceuticals Ltd., UK
Puromycin	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
RNase-Inhibitor	Invitrogen, Karlsruhe, Germany
Serotonin	Sigma-Aldrich, Munich, Germany
Somatostatin	Sigma-Aldrich, Munich, Germany
Substance P	polypeptide group
Superscript II Transcriptase	Invitrogen, Karlsruhe, Germany
Trition X-100	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Vasopressin	Sigma-Aldrich, Munich, Germany

2.2 Media, buffers and solutions	**Composition**
0.9 % saline	0.9% NaCl, Aqua dest. sterile
Blocking buffer (immunohistochemistry)	2% Triton X-100 2% BSA 10% donkey serum in 0.1 M phosphate buffer PB
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)
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
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
Paraformaldehyde	4% paraformaldehyde in A. dest. heated to facilitate dissolution
Trypsin/DNase	10 mg Trypsin, 0.5 mg DNase per ml PBS

2.3 Fluorescent probes, enzymes and antibodies	**Company**
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
Alexa® 594 goat anti rabit	Invitrogen, Karlruhe, Germany
Cholinergic receptor, Muscarinic 3 (CHRM3) antibody	Antikoerper-online.de
Fluo-4 AM, cell permeant	Invitrogen, Karlruhe, Germany
Iba-1 antibody	Wako Pure Chemical Industries, Ltd., Japan
Secondary antibodies conjugated to fluorescein isothiocyanate (FITC), rhodamine red or Cy5	Jackson ImmunoResearch Europe Ltd., Suffolk, UK

2.4 Commericial kits	**Company**
First strand cDNA kit	Invitrogen, Karlsruhe, Germany
MACS tissue dissociation kit	Miltenyi Biotech, Bergisch Gladbach, Germany
RNeasy micro extraction kit	Qiagen, Hilden, Germany

2.5 Devices	**Company**
40x Achroplan water immersion objective	NA 0.75, Zeiss, Jena
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
Sonicator	Dr. Hielscher GmbH, Teltow, Germany
Stereotactic alignment system	David Kopf Instruments, Tujunga, USA
Vibratome VT 1000 S	Leica, Heidelberg, Germany

2.6 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
R software	R Foundation for Statistical Computing, Vienna, Austria
TIDA	HEKA electronics, Lambrecht/Pfalz, Germany

2.7 Cells and mice	**Company**
Adult microglia cells	Isolation from C57/Bl6 mice
GL261 glioma cells	NCI, NIH, USA
Neonatal microglia cells	Isolation from C57/Bl6 mice
APPPS1 mice	Frank Heppner, Stefan Prokop (Charité, Berlin) and Mathias Jucker (University of Tübingen)
EAE mice	SJL/J mice (Janvier, France), provided by Sonia Waiczies, MDC, Berlin
Freshly isolated microglia (control)	Isolation from C57/Bl6 mice
Glioma mice	In house mouse model using C57/Bl6 mice
Stroke mice	MCAo on C57/Bl6 mice, provided by Mathias Endres, Charité, Berlin

2.8 Tools	**Company**
10ml syringe	B Braun Melsungen AG, Germany
23 gauge needle	B Braun Melsungen AG, Germany
24-well Cell Culture Plate	BD Biosciences, Heidelberg, Germany
27 gauge needle	B Braun Melsungen AG, Germany
40x10mm dishes	TPP Techno Plastic Products AG, Trasadingen, Switzerland
4-well Cell Culture Plate	BD Biosciences, Heidelberg, Germany
6-well Cell Culture Plate	BD Biosciences, Heidelberg, Germany
8.0 nylon monofilament coated with a silicone resin/hardener mixture	Xantopren M Mucosa and Activator NF Optosil Xantopren, Haereus Kulzer
Cells strainer, 40 µm	BD Biosciences, Heidelberg, Germany
Cells strainer, 70 µm	BD Biosciences, Heidelberg, Germany
Glass bottom culture dish, 35mm, 14mm Microwell	MatTek, Ashland, MA, USA
Menzel glass cover slip 24x50 mm	Thermo Fisher Scientific, Walldorf, Germany
Minisart single use filter unit, 0.2 and 0.45µm	Sartorius AG, Goettingen, Germany
T75 Cellstar culture flasks	Greiner Bio-One GmbH, Frickenhausen, Germany

3. Methods

3.1 Preparation of freshly isolated microglia

Microglia from adult C57BL/6 mice (P49-56) were acutely isolated for calcium imaging using magnetic activated cell sorting (MACS). Pure microglia were magnetically isolated from cortical and subcortical cell suspensions using CD11b microbeads from Miltenyi Biotech (Bergisch Gladbach, Germany), according to the manufacturer's instructions and as previously described (Nikodemova and Watters, 2012). In brief, adult mice were sacrificied by cervical dislocation and the brain was removed. After removal of the cerebellum and olfactory bulbs, the remaining cortex and midbrain was cut into small pieces ($1mm^3$) and dissociated using the Miltenyi Biotec Neural Tissue Dissociation Kit (Trypsin). After dissociation using a glass Pasteur pipette, microglia were passed through a 40µm cell strainer followed by centrifugation for 10mins at 300g and 4^oC. Microglia were then incubated for 15mins with Miltenyi Biotec myelin removal beads, followed by magnetic separation using LS columns, with 2 columns used per brain. Following myelin removal, the myelin free flow-through was incubated with CD11B beads for 15mins, and the cell suspension was applied to an MS column (Miltenyi Biotec) and washed three times with HBSS/0.5% BSA/2mM EDTA. Labelled CD11b positive cells (microglia) within the column were then flushed out and plated onto glass coverslips, followed by 15 minutes incubation to allow adherence. Calcium imaging was then carried out as described below. This protocol is summarised in figure 6.

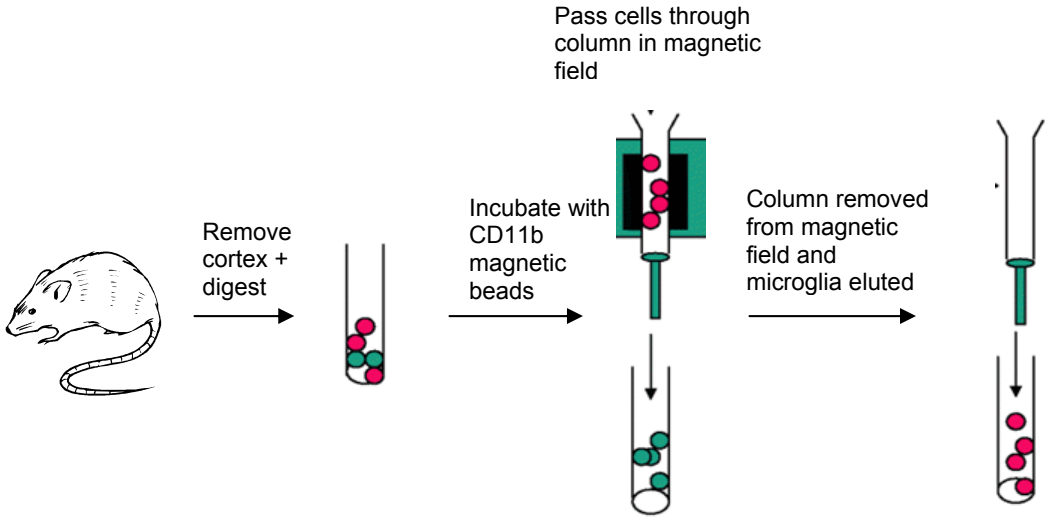


Figure 6. Preparation of freshly isolated adult microglia using MACS

3.2 Induction of cerebral ischemia

Left middle cerebral artery occlusion (MCAO)/reperfusion was performed as described previously (Endres et al., 2004). In brief, C57BL/6 mice were anaesthetized for induction with 1.5% isoflurane and maintained in 1.0% isoflurane in 69% N_2O and 30% O_2 using a vaporizer. Transient brain ischaemia was induced using an 8.0 nylon monofilament coated with a silicone resin/hardener mixture (Xantopren M Mucosa and Activator NF Optosil Xantopren, Haereus Kulzer). The filament was introduced into the internal carotid artery up to the anterior cerebral artery resulting in occlusion of the middle cerebral artery and anterior choroidal arteries. The filament was removed after 30 minutes to allow reperfusion. Core temperature was monitored and maintained at 36.5 ± 0.5°C with a feed-back temperature control unit until 1 hour after reperfusion using a heating pad. Microglia were isolated one week after MCAO from a 4 mm coronal section through the cortex and striatum using MACS as described before. Microglia from the same brain region were isolated from healthy C57BL/6 mice to serve as a control.

3.3 Mouse model of glioma

Twenty thousand GL261 glioma cells in a volume of 1 µl were injected into the right frontal cortex of adult C57BL/6 mice (6-10 weeks). Mice were sacrificed after

approximately 20 days and a 3 mm^3 section was cut around the tumor. Microglia were isolated from the tumor using MACS as described above.

3.4 Mouse model of Alzheimer's disease

APPPS1 mice were used as an Alzheimer's mouse model, and were sacrificed around P90. Microglia were isolated from the cortex and midbrain using MACS as described above.

3.5 Mouse model of autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis was induced as previously described (Waiczies et al., 2012). In brief, 6-8 week old female SJL/J mice (Janvier, France) were subcutaneous immunised with 250 mg PLP139–151 (Pepceuticals Ltd., UK; purity 95%) along with Complete Freund's Adjuvant and heat-inactivated Mycobacterium tuberculosis (H37Ra, Difco,, USA). Bordetella pertussis toxin (250 ng; List Biological Laboratories, USA) was administered intraperitoneally on days 0 and 2. Mice were assigned a daily clinical score: 0, no disease; 1, tail weakness; 2, paraparesis; 3, paraplegia; 4, paraplegia with forelimb weakness; 5, moribund or dead animals. Microglia were isolated from the cortex and midbrain as before.

3.6 Preparation of cultured microglia

Primary microglia cultures were prepared from the cerebral cortex and midbrain of newborn C57BL/6 mice (P0-P3) as described previously (Giulian and Baker, 1986). The microglia were seeded at 1-1.5 × 10^5 cells per glass coverslip (14 mm diameter). Cultures usually contained >95% microglia as detected by isolectin B4 (*Griffonia simplicifolia*) and were used for experiments within 1 day after plating.
Microglia were also cultured from adult mice (P49-56) (Scheffel et al., 2012). Cortical and midbrain tissue was freed of blood vessels and meninges in Hank's balanced salt solution (HBBS), mechanically dissociated into 1mm^3 pieces and trypsinized in 1% trypsin and 0.05% deoxyribonuclease for 5 min at room temperature, as described for neonatal microglia. Digested tissue was dissociated using a fire-polished pipette and washed twice in HBBS. The feeder layer of

astrocytes were depleted of neonatal microglial cells using clodronate (200 µg/ml) before the adult microglia were added. The adult mixed glial cultures received fresh complete DMEM medium every other day and were treated with 33% L929-conditioned medium after 7 days or once cells became confluent. Microglia were shaken off 1 week later and were used for experiments within 1 day of plating. Figure 7 summarises the main steps for this protocol.

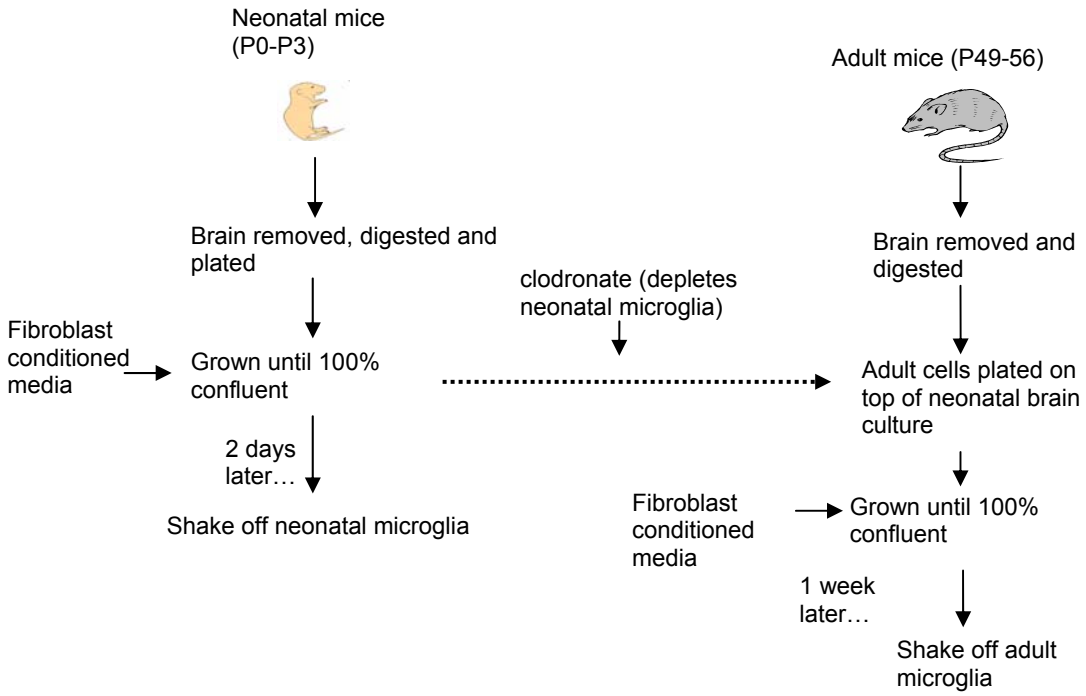


Figure 7. Culturing protocol for neonatal and adult primary microglia

3.7 Isolation of Peritoneal Macrophages

Macrophages from C57BL/6 adult mice (P49-56) were freshly isolated for calcium imaging (Zhang et al., 2008). Mice were killed by cervical dislocation and skin around the peritoneum was disinfected with 70% ethanol and carefully removed. 4-5ml of sterile PBS was injected into the peritoneum using a 27 gauge needle and massaged for 2-3 minutes. The injected PBS was then removed from the peritoneum using a 23 gauge needle and syringe and centrifuged for 10 minutes at 200 g. 150 000 cells were plated on a coverslip (14 mm diameter). Cells were allowed to adhere for 15 minutes and subsequently loaded with 5 µM fluo-4/AM (Invitrogen, USA) for calcium imaging.

3.8 Isolation of Bone Marrow Derived Macrophages

Bone marrow macrophages were isolated from C57BL/6 adult mice (P49-56) as previously described (Marim et al., 2010). In summary, mice were killed by cervical dislocation and the femurs were removed by cutting through the tibia near the pelvic bone and below the knee. Any muscle connected to the bone was carefully removed. Sterile PBS was slowly flushed through the bone and the contents collected in a sterile 15 ml polypropylene tube. After centrifugation for 10 minutes at 200 g, red blood cells were lysed by adding 3-10 ml ammonium chloride solution. After a 2-3 minute recovery period, the suspension was centrifuged for 10 minutes at 200 g and the supernatant was removed. Cells were plated in a 10 cm dish with 33% L929-conditioned media to allow differentiation. After 7 days cells were trypsinized and were plated onto coverslips (150 000 cells/coverslip). Calcium imaging was carried out the following day as described below.

3.9 Activation of cultured microglia

Microglia cultured from neonatal and adult cells were incubated with 100ng/ml lipopolysaccaride (LPS) (Enzo, Lörrach, Germany) or 20U/ml IFN-γ (R&D systems, Minneapolis, USA). An anti-inflammatory M2 phenotype was induced by incubating with 30 ng/ml IL-4 (Peprotech, Hamburg, Germany). Stimulating agents were added to cultures approximately 5 hours after plating microglia on glass coverslips. Incubation was carried out overnight for approximately 18-24 hours.

3.10 Calcium imaging

Freshly isolated cells were loaded with 5 μM fluo-4/AM (Invitrogen, USA) in the presence of the detergent 0.02% Pluronic F-127 in HEPES buffer which contained (in mM) NaCl 150.0, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 2.0, HEPES 10.0, glucose 10.0, adjusted with NaOH to pH 7.4 supplemented with 1% tomato lectin conjugated Alexa Fluor®594 for 40 min at room temperature. After staining, coverslips were washed for 10 minutes in HEPES buffer. Cultured microglia were stained with 5 μM fluo-4/AM for 40 minutes at room temperature followed by a 10 minute wash in HEPES buffer without the addition of tomato lectin. Coverslips were then

transferred to a recording chamber and buffer flow was adjusted to 3-4 ml/min. Fluorescence pictures were taken every 3 s at 488 nm excitation wavelength produced by a Polychrome II monochromator (Till Photonics, Martinsried, Germany) using 40x Achroplan water immersion objective (NA 0.75, Zeiss, Jena). A perfusion system was installed to change the solution within seconds. Fluorescence of tomato lectin was detected after excitation with 560 nm (emission filter at 630 nm).

3.11 Immunohistochemistry

Freshly isolated microglia from adult C57BL/6 were stained for Iba-1 as a marker for microglial cells and Hoechst 33258 for identification using confocal microscopy (Leica SPE). Fixed cells on glass coverslips were incubated in blocking buffer for four hours at room temperature, followed by a 48 hours incubation with 0.75µg/ml Iba-1 antibody in 10% blocking buffer (diluted with 0,1 M PB) at 4°C. For detection 6.25µg/ml donkey anti rabbit Cy3 secondary antibody in 10% blocking buffer was used. After washing, cells were incubated for 30 min with Hoechst 33258 1:10000 in 0.1M PB and mounted in Aqua polymount for further analysis using confocal microscopy.

3.12 Migration assay

The chemotaxis activity was monitored using the agarose spot assay (Wiggins and Rappoport, 2010) . 0.1 g of agarose with a low melting point was dissolved in 20 ml PBS to obtain a 0.5% agarose solution. Four spots (10 µl) were placed onto 35 mm glass plates (MatTek Corporation, Massachusetts, USA) with three containing carbachol and one containing PBS only. 10^6 microglial cells in a 2 ml suspension were added to the plates and incubated at 37°C in 5% CO_2. After 4 hrs and 6 hrs, cells under the spot were counted.

3.13 Phagocytosis assay

Yellow green fluorescent Fluoresbrite carboxylated microspheres (3 µm diameter, Polysciences Europe GmbH, Eppelheim, Germany) were coated with FCS by

shaking at 1000 rpm for 30 min at room temperature. After centrifugation for 2 min at 900 g, the supernatant was discarded and microspheres were washed and resuspended in DMEM. 8.4 x 10^6 microspheres in a solution containing either 10 or 100 µM carbachol or control (HBSS) were applied on each coverslip with primary microglia for 30 min and incubated at 37°C. After vigorous washing with 0.1 M PB, coverslips were fixed with 4% paraformaldehyde. Coverslips were finally stained with blue membrane stain for 1 hour, followed by a final 0.1M PB wash and mounted for cell counting. For cell counting, each coverslip was divided into four 7 μm^2 quadrants. The number of beads phagocytozed by each cell within the quadrants was counted. Cells were grouped to 1-4, 5-7, 8-10 and >10 microspheres per cell and the percentage of cells in each group was multiplied by the corresponding grade of phagocytosis (1-4:1, 5-7:2, 8-10:3, >10:4). The sum of the products in each group was then given as the phagocytosis index.

3.14 RNA Isolation and PCR

Total RNA was isolated from C57BL/6 neonatal microglia (± IFN-γ treatment), C57BL/6 adult microglia (± IFN-γ treatment), C57BL/6 freshly isolated adult microglia and C57BL/6 whole brain tissue using the RNeasy Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before RNA extraction, freshly isolated whole brain tissue was sonicated (Dr. Hielscher GmbH, Teltow, Germany) to access intracellular compartments.

First-strand cDNA was synthesized from up to 1µg RNA by reverse transcription reaction (Invitrogen, Karlsruhe, Germany). For quantitative PCR we used Go Taq qPCR Master Mix (Promega, Mannheim, Germany) based on SYBR Green and primers shown in table 7 to amplify 4 ng of total cDNA. These primers were tested for their efficiency to apply the delta/delta c_t-method for analysis. To ensure the specificity of the PCR product we analysed the melting curves of each product.

3.15 Statistical analysis

All data for calcium imaging represent the percentage of cells responding from at least 3 mice for freshly isolated microglia or 3 preparations for cultured microglia. The total number of cells responding to ATP was calculated for all experiments for

each condition. The total number of cells responding to a given substance in a given condition was then calculated as a percentage of the ATP responding population. Data for calcium imaging was analyzed using the 2-sample test for equality of proportions with continuity correction in R software (*R Foundation for Statistical Computing, Vienna, Austria*) and the differences were considered statistically significant at $p<0.05$. Data for phagocytosis and chemotaxis assays represent the average of at least triplicate samples. Error bars represent the standard error of the mean. Values are presented as means +/- standard error of the mean. Data were analyzed using the Mann-Whitney test in SPSS (IBM SPSS Statistics, USA) and the differences were considered statistically significant at $p<0.05$.

4. Results

4.1 Subpopulations of freshly isolated microglia respond to defined neurotransmitters/-hormones

We isolated microglia from adult mouse cortex and midbrain by MACS using CD11b microbeads to specifically purify microglia. Isolated cells had a round morphology with a diameter of 2.8 µm and no apparent processes (Figure 8A). The cells were loaded with the Ca^{2+}-sensor Fluo-4 for 40 min and labelled in parallel with 1% tomato lectin conjugated Alexa Fluor®594 to verify that the cells were microglia. Figure 8D shows that the isolated cell population is positive for the microglial marker tomato lectin. Figures 8 E-F show staining of freshly isolated microglia with Hoechst, Iba-1 and an overlay of the two images.

Each neurotransmitter/-hormone was applied for one minute and after a 5 min wash, ATP (500 µM) was applied for 30 seconds. We selected only those cells which showed a clear response to ATP which were, in most experiments, all cells in the selected field. Of those cells which responded to ATP, 1% of cells responded to endothelin application, 10% to histamine, 26% to substance P, 9% to serotonin, 14% to galanin, 11% to somatostatin, 7% to angiotensin II, 5% to vasopressin, 8% to neurotensin, 6% to dopamine, 6% to nicotine and 16% to carbachol with a transient increase in Fluo-4 fluorescence corresponding to an increase in Ca^{2+}. Figure 9 shows examples of a positive response for each substance. Table 1 describes the mean integral and amplitude of the calcium responses for each substance compared to the ATP response, as well as the duration of the calcium transients. The mean amplitudes and integral (which combines the data of amplitude and duration) were typically smaller when compared to the ATP response, while the duration was calculated as the mean number of seconds from the beginning of the increase in fluorescence, which was four standard deviations higher than the baseline, to the end of the signal.

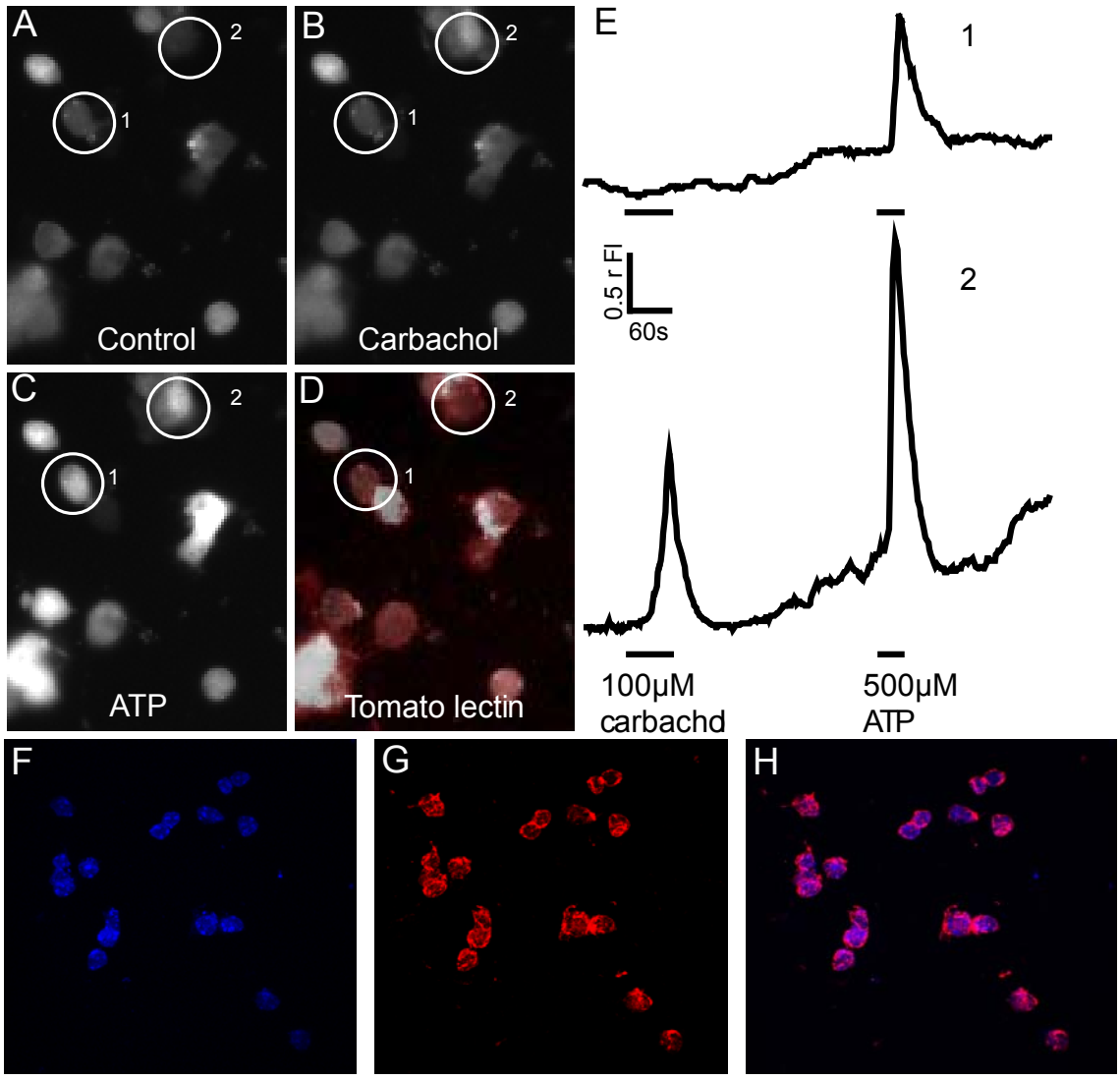
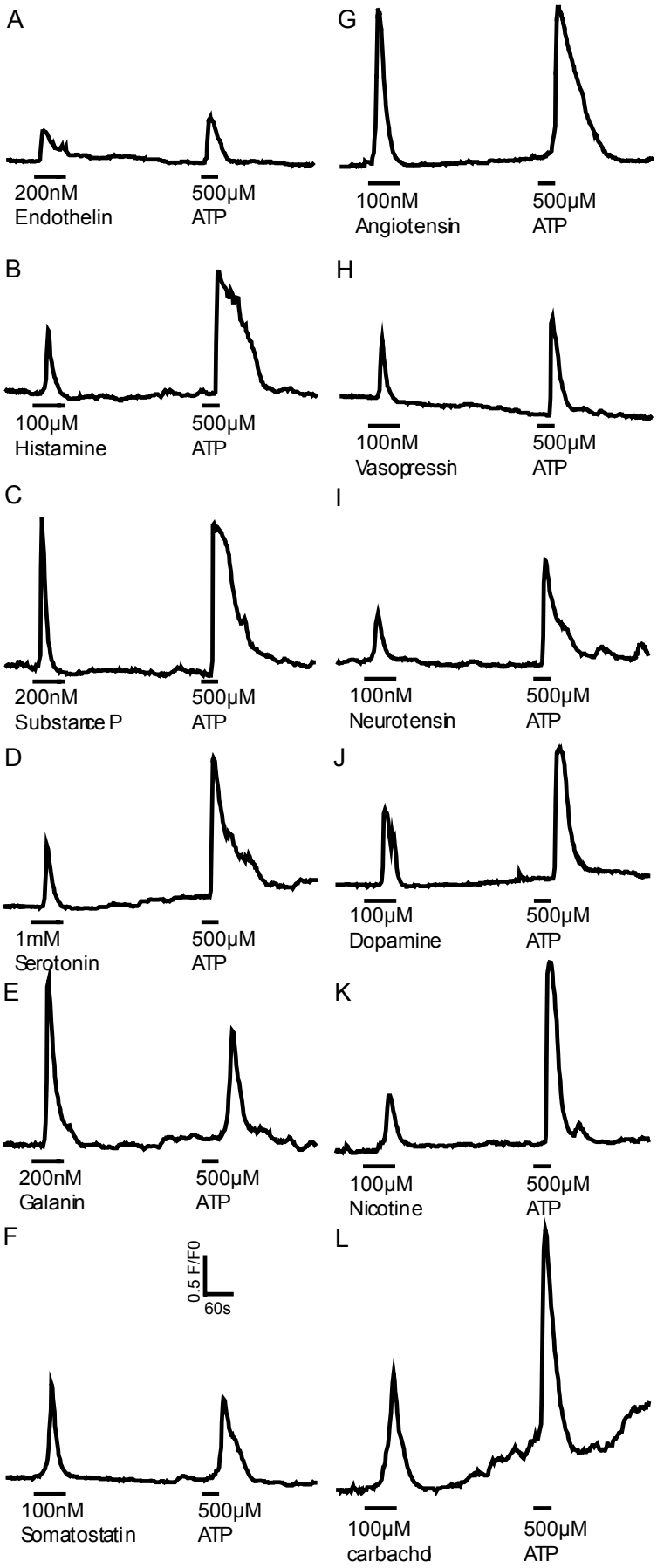


Figure 8. A subpopulation of adult microglia responds to carbachol

Freshly isolated adult microglia respond to carbachol and ATP with an increase in intracellular calcium (A) Baseline fluorescence of freshly isolated microglia after incubation with 5 µM fluo-4/AM. (B) Application of 100 µM carbachol results in an increase in fluorescence intensity in a small number of cells (C) Application of 500 µM ATP as a positive control shows all cells responding to application with an increase in fluorescence intensity (D) Tomato lectin labeling confirms that the cells are microglia. (E) Traces of relative fluorescence increase (rFI) from regions 1 and 2 (as indicated in A, B. C and D) shows a carbachol-responsive and -unresponsive microglial cell. (F) Hoechst staining of nuclei from freshly isolated microglia (G) Iba-1 staining identifies isolated cells as microglia, (H) Overlay of pictures F and G.


A
200nM
Endothelin
500μM
ATP
G
100nM
Angiotensin
500μM
ATP
B
100μM
Histamine
500μM
ATP
H
100nM
Vasopressin
500μM
ATP
C
200nM
Substance P
500μM
ATP
I
100nM
Neurotensin
500μM
ATP
D
1mM
Serotonin
500μM
ATP
J
100μM
Dopamine
500μM
ATP
E
200nM
Galanin
500μM
ATP
K
100μM
Nicotine
500μM
ATP
F
0.5 F/F0
60s
100nM
Somatostatin
500μM
ATP
L
100μM
carbachol
500μM
ATP

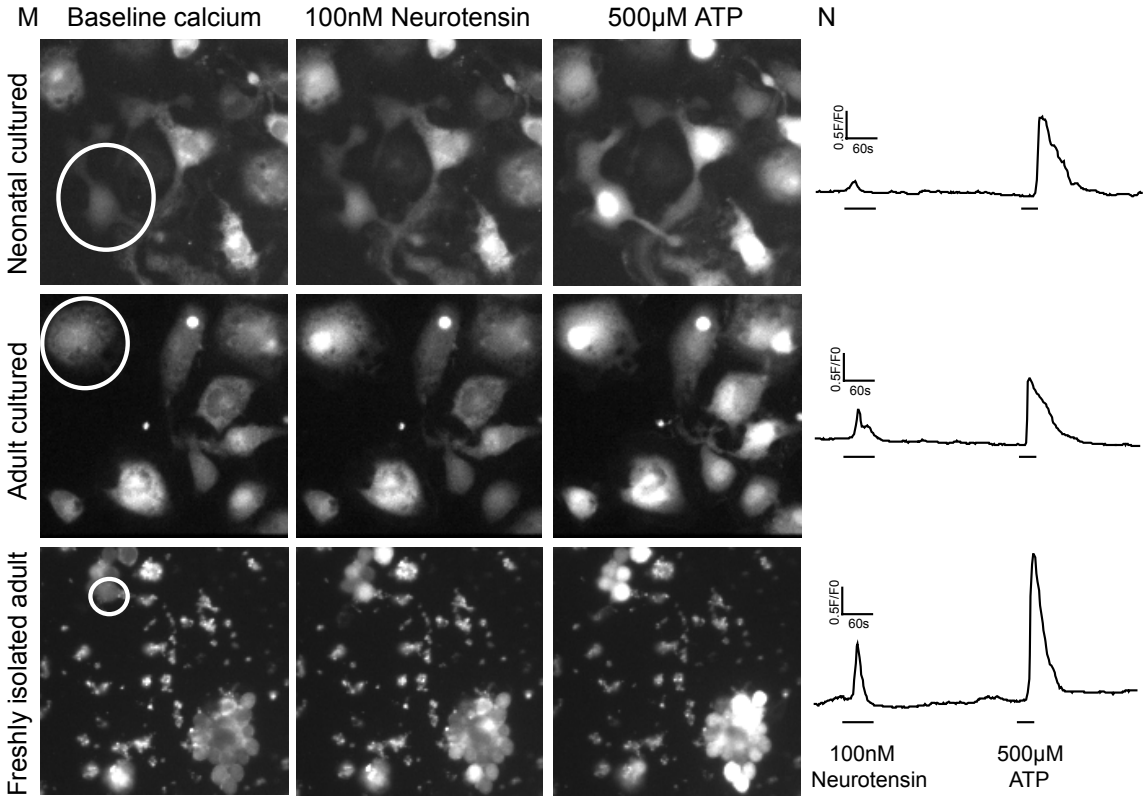


Figure 9. A subpopulation of freshly isolated adult microglia respond to neurotransmitters and neurohormones

Representative examples of intracellular calcium transients induced in freshly isolated adult microglia cells by 60 s application of endothelin (A), histamine (B), substance P (C), serotonin (D), galanin (E), somatostatin (F), angiotensin II (G), vasopressin (H), neurotensin (I), dopamine (J), nicotine (K) and carbachol (L). After a 5min washout, 500μM ATP was applied for 30 s as a positive control. M shows an example of neonatal and adult cultures, and freshly isolated microglia responding to 100nM application of neurotensin and 500μM ATP with corresponding calcium traces (N).

	Mean integral of fluorescence change as a % of ATP response (±SEM)	Mean amplitude as a % of ATP response (±SEM)	Mean duration (s) (±SEM)
Endothelin	57 (18)	85 (4)	53 (16)
Histamine	58 (9)	61 (5)	84 (9)
Substance P	51 (7)	93 (7)	58 (7)
Serotonin	41 (8)	74 (6)	72 (9)
Galanin	80 (11)	100 (6)	90 (6)
Somatostatin	56 (6)	70 (4)	85 (8)
Angiotensin II	48 (5)	69 (3)	76 (5)
Vasopressin	67 (7)	71 (4)	69 (7)
Neurotensin	52 (6)	66 (5)	73 (6)
Dopamine	60 (7)	68 (6)	82 (6)
Nicotine	56 (9)	62 (6)	83 (7)
Carbachol	50 (5)	72 (4)	61 (4)

Table 1. Mean integral, amplitude and duration of neurotransmitter/-hormone induced Ca2+ transients for freshly isolated adult microglia

4.2 The size of the endothelin, somatostatin, angiotensin II, vasopressin and carbachol sensitive subpopulation is affected by pathology

To study changes in microglial responses under pathological conditions we used mouse models for glioma, stroke, and multiple sclerosis (Figure 11A-L). Since it is known that monocytes from the periphery infiltrate the glioma and during late phases of stroke the infarct area, we use the term microglia/brain macrophages when referring to these pathologies. In 6-10 week old mice inoculated for 20 days with 20,000 glioma cells, the glioma tissue was dissected out and microglia/brain macrophages were isolated by MACS. Glioma associated microglia/macrophages contained an increased population of cells sensitive to endothelin, which was 5% of the population (Figure 11A). The histamine (Figure 11B) and angiotensin II (Figure 11G) sensitive populations were also significantly increased to 19 and 13% respectively, while the substance P (C), galanin (E), somatostatin (F) and carbachol (L) sensitive populations were significantly decreased to 16%, 9%, 5% and 10% when compared to microglia from control mice. Responses of glioma isolated microglia/brain macrophages to the other substances were not significantly different when compared to control. Microglial cells/brain macrophages isolated from a 4 mm coronal section through the cortex and

striatum of mice which had a transient middle cerebral artery occlusion 8-10 days prior to isolation showed a significant increase in the endothelin (Figure A), histamine (B), somatostatin (F), angiotensin II (G), vasopressin (H) and carbachol (L) sensitive populations and a decrease in the substance P (C) and neurotensin (I) sensitive populations. We also carried out detailed analysis on the carbachol transients from microglia isolated from stroke and compared the amplitude, intergral and duration of the signal to those from control mice. The amplitude of the Ca^{2+} response to carbachol relative to ATP was larger at 106 ±3 % as compared to control animals at 72 ±4 % ($p<0.001$) (Figure 10). The average duration of this signal (65 ± 5 s), was comparable to the average duration of controls (61 ± 4 s).

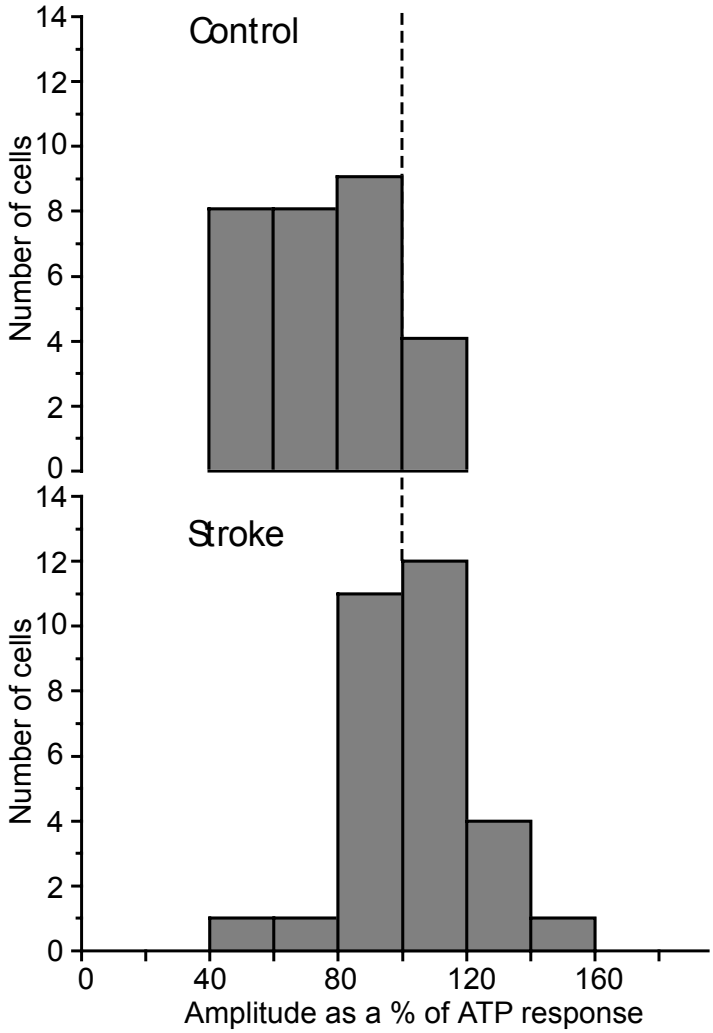


Figure 10. Amplitude histograms of the Ca2+ transients as a percentage of ATP response in microglia isolated from control and stroke tissue.

Experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, was induced through subcutaneous immunisation with 250 mg PLP139–151 along with Complete Freund's Adjuvant and Mycobacterium tuberculosis. This was followed by intraperitoneal injection of Bordetella pertussis toxin at days 0 and 2 after immunisation. Microglia were isolated at the peak of disease, between 12 and 14 days after immunisation. The populations of endothelin, serotonin, somatostatin, angiotensin II and dopamine sensitive microglia were significantly increased while the histamine, substance P and carbachol sensitive populations were significantly decreased. Responses of microglia to all other substances from EAE tissue did not significantly differ when compared to microglia from control animals (Figure 11). Table 2 summarizes the proportion of the microglia populations sensitive to each of the neurotransmitter/hormones from the different pathological models.

As with the freshly isolated microglia from healthy BL6 mice, only 2 types of responses were seen in microglia isolated from disease models: single peak and complex responses. Oscillating cells were not seen in any of the freshly isolated preparations from any of the disease models.

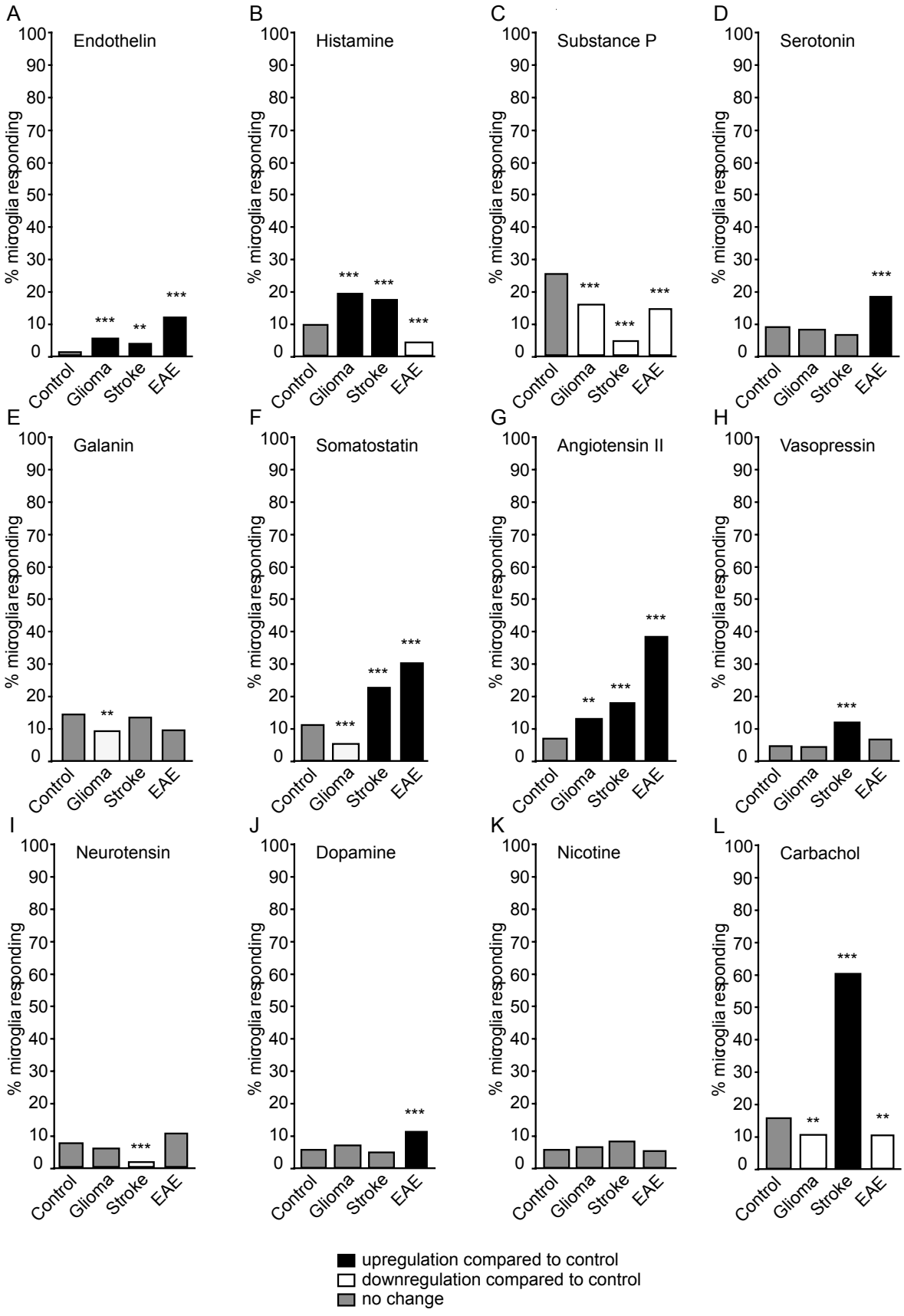


Figure 11. The pattern of responsiveness to specific ligands changes in pathology

Percentages of freshly isolated microglia responding to neurotransmitter/-hormone application with an increase in intracellular Ca^{2+} (A-L). Cells were obtained from control tissue and from murine disease models of glioma, stroke and multiple sclerosis. Data are presented as the percentage of cells to respond to a given substance (the ATP responsive population is taken as 100%) from at least 10 investigated microscopic fields from at least 3 independent cell preparations and statistical significance is shown as *** for $p \leq 0.001$, ** for $p \leq 0.01$ and * for $p \leq 0.05$.

	Control	Glioma	Stroke	EAE
Endothelin	1	5	4	12
Histamine	10	19	18	5
Substance P	26	16	4	15
Serotonin	9	8	7	19
Galanin	14	9	14	11
Somatostatin	11	5	23	31
Angiotensin II	7	13	18	39
Vasopressin	5	4	12	7
Neurotensin	8	6	2	11
Dopamine	6	7	5	12
Nicotine	6	7	9	5
Carbachol	16	10	60	11

Table 2. Percentage of microglia/brain macrophages isolated from healthy, glioma, stroke and EAE mice that respond to the application of each test substance

4.3 The size of the carbachol-sensitive subpopulation is increased in a mouse model of Alzheimer's

To further study changes in microglial responses to carbachol under pathological conditions we also used a mouse model of Alzheimer's disease. Microglial cells isolated from cortices of the APPPS1 mice, a mouse model of Alzheimer's disease, showed a significantly larger carbachol-sensitive subpopulation (25% $p<0.001$) than healthy control animals (Figure 12).

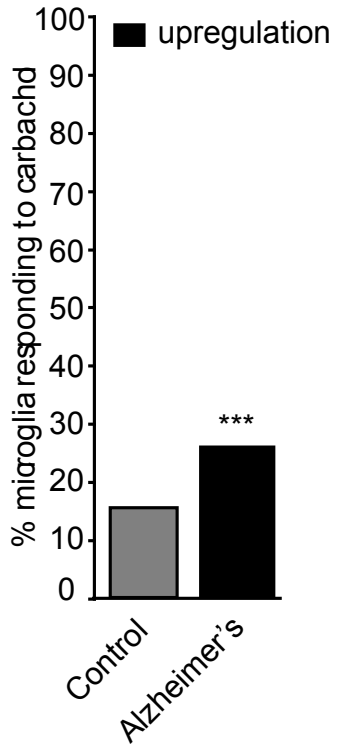


Figure 12. Percentage of microglia freshly isolated from APPPS1 mice to respond to the application of carbachol with an increase in intracellular calcium compared to microglia freshly isolated from healthy mice.

4.4 A small population of macrophages isolated from healthy mice respond to carbachol application with an increase in intracellular calcium

Macrophages isolated from the peritoneum and bone marrow from healthy BL6 mice were also tested for their response to carbachol. The population of peritoneal and bone marrow macrophages to respond to carbachol was significantly smaller than freshly isolated microglia with 9% of peritoneal macrophages and 5% of bone marrow macrophages responding with a calcium signal (Figure 13). As with freshly isolated microglia, only single peak and complex signals were seen in response to carbachol application. Similarly, as for the analysis of microglia, only those macrophages which responded to ATP were analysed. In all experiments, the majority of macrophages in the selected field responded to ATP.

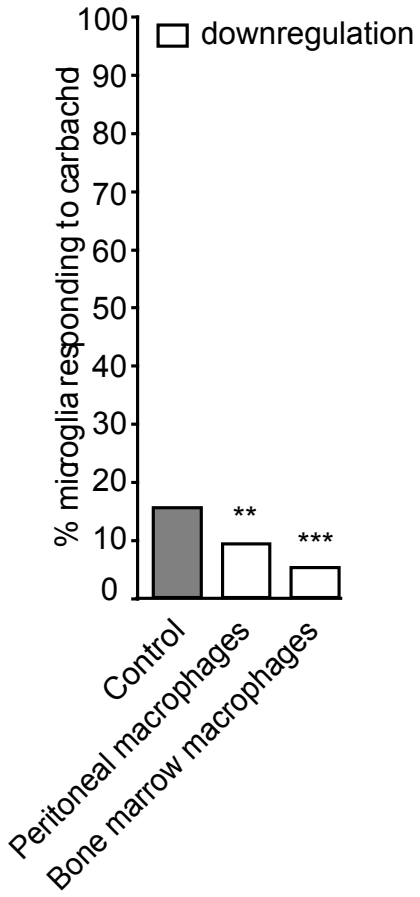


Figure 13. Percentage of peritoneal and bone marrow macrophages to respond to carbachol application with an increase in intracellular calcium. These populations were significantly downregulated compared to freshly isolated microglia from healthy Bl/6 mice.

4.5 Small subpopulations of cultured neonatal and adult microglia respond to neuropeptides/-hormones

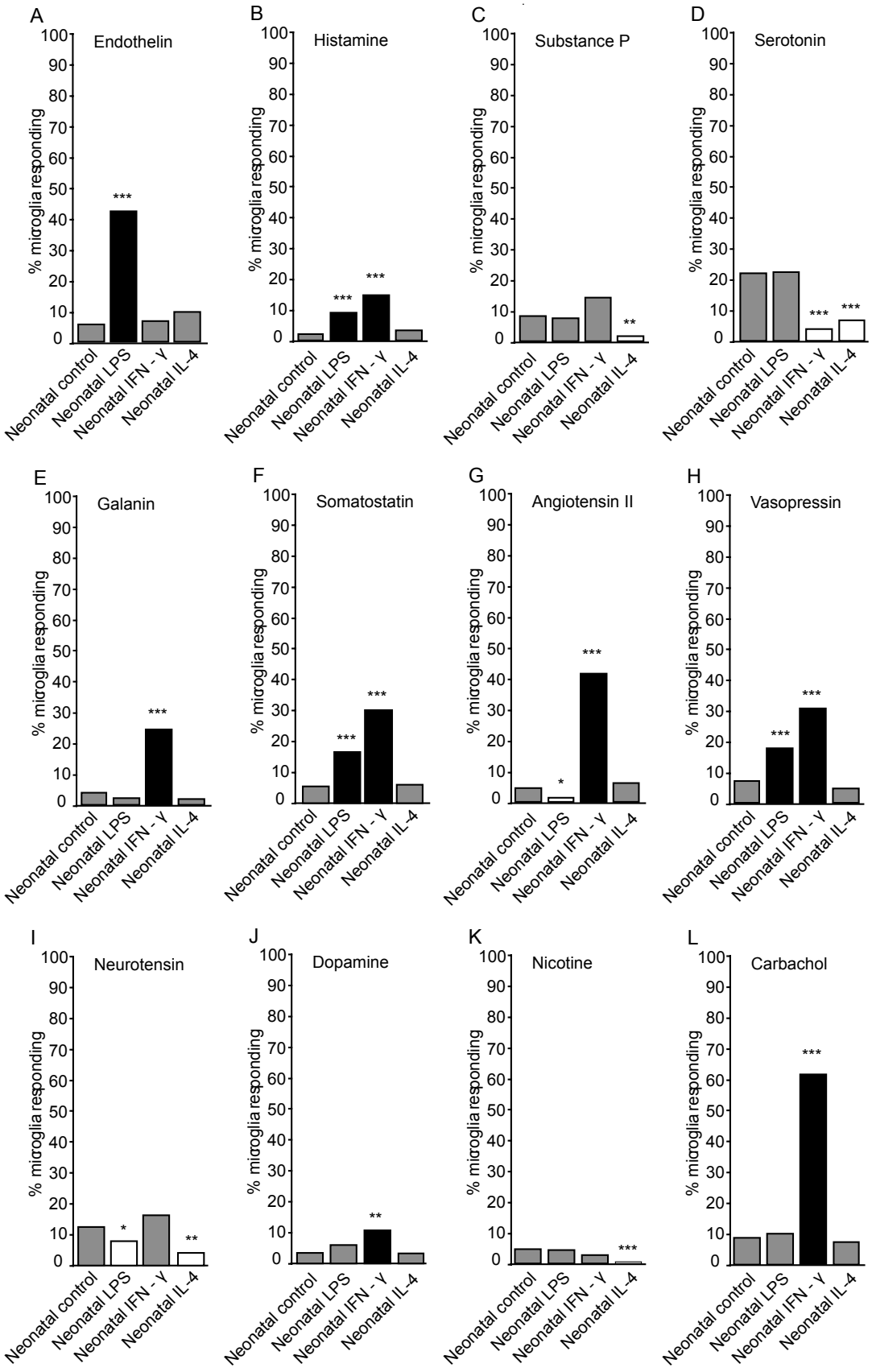
Endothelin-1, histamine, substance P, serotonin, galanin, somatostatin, angiotensin II, vasopressin, neurotensin, dopamine, nicotine and carbachol were all applied to cultured neonatal (postnatal day 1) microglia using the same calcium imaging protocol described above for freshly isolated cells. ATP was used as a positive control. Of those cells which responded to ATP, 6% of cells responded to endothelin application (Figure 14A), 2% to histamine (B), 9% to substance P (C), 22% to serotonin (D), 4% to galanin (E), 6% to somatostatin (F), 5% to angiotensin II (G), 8% to vasopressin (H), 13% to neurotensin (I), 4% to dopamine (J), 5% to

nicotine (K) and 9% to carbachol (L) with a transient increase in Fluo-4 fluorescence corresponding to an increase in Ca^{2+}.

Microglia from adult brain were cultured for approximately 4 weeks until confluent, then shaken off and plated onto glass coverslips after 1 week and tested for functional receptor expression using calcium imaging as before. The cells responding to endothelin (13%; $p<0.001$) and nicotine (17%; $p<0.001$) application was significantly higher than the percentage of responders in freshly isolated adult cells (Figure 15A). The population responding to histamine (3%; $p=0.0002$), substance P (1%; $p<0.001$) and carbachol (8%; $p<0.001$) was significantly lower than the percentage of freshly isolated cells. The population responding to serotonin (6%), to galanin, to somatostatin (12%), to angiotensin II (11%), to vasopressin (2%), to neurotensin (6%), and to dopamine (7%) did not significantly differ when compared to freshly isolated adult cells (Figure 11 A-L).

4.6 Distinct neurotransmitter/-hormone sensitive populations can be increased by treatment with IFN-y, LPS and IL-4

A pro-inflammatory phenotype was induced in cultured neonatal microglia by 18h treatment with 100 ng/ml LPS or 20 U/ml IFN-γ. An anti-inflammatory phenotype was induced through overnight treatment with 30 ng/ml IL-4. LPS treatment resulted in a significantly higher population of endothelin (42%), histamine (9%), somatostatin (17%) and vasopressin (18%) sensitive cells and a decrease in angiotensin (1%) and neurotensin (8%) sensitive cells, while IFN-γ treatment resulted in a significantly larger histamine (15%), galanin (24%), somatostatin (31%), angiotensin II (42%), vasopressin (31%), dopamine (11%) and carbachol (62%) sensitive population and a significant decrease in the serotonin sensitive population (4%). IL-4 treatment did not result in an increase in any of the neurotransmitter/-hormone sensitive populations but led to a significant decrease in the substance P (2%), serotonin (7%), neurotensin (4%) and nicotine (1%) sensitive populations (Figure 14 A-L).


A
Endothelin
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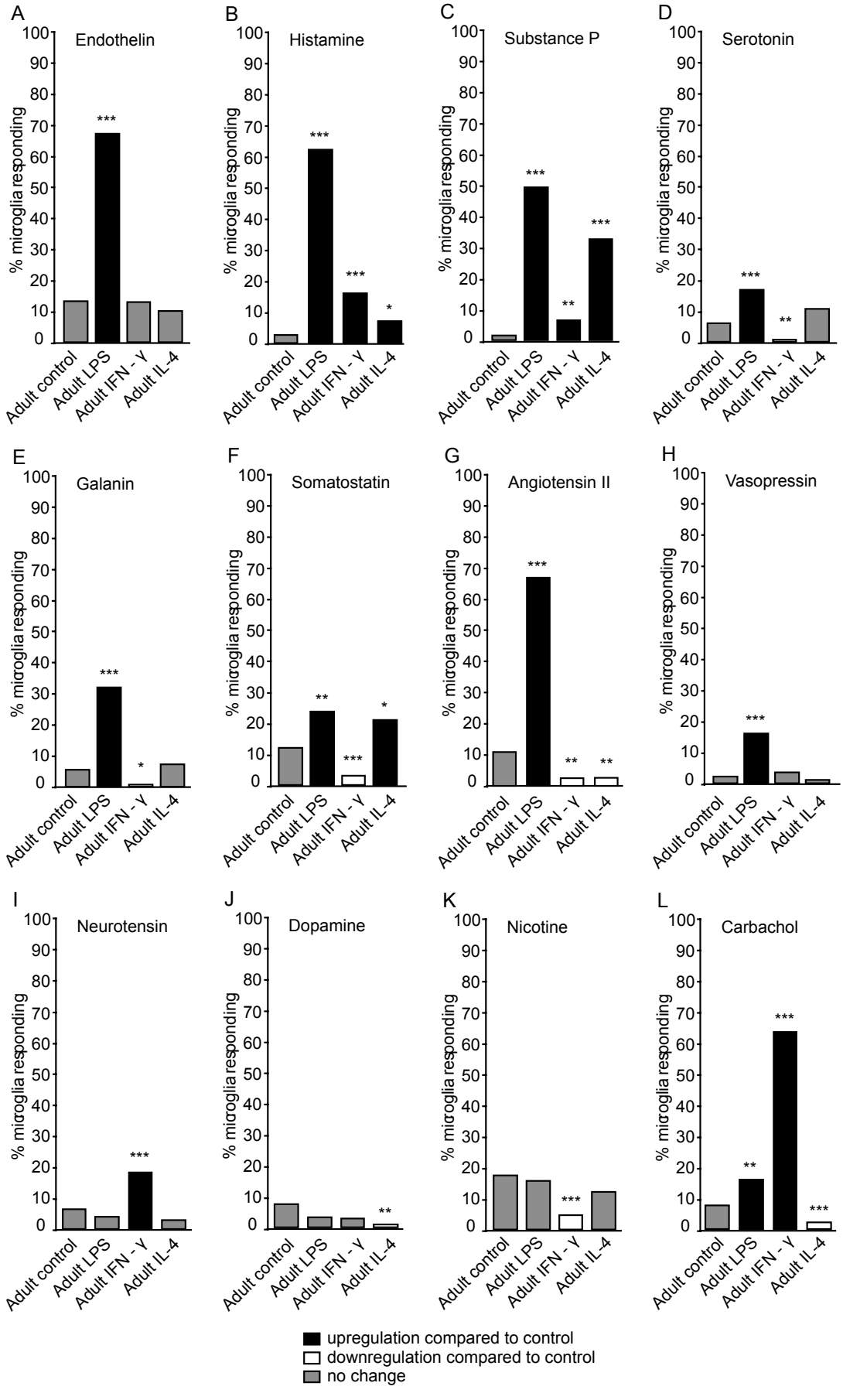
Histamine
C
Substance P
D
Serotonin
E
Galanin
F
Somatostatin
G
Angiotensin II
H
Vasopressin
I
Neurotensin
J
Dopamine
K
Nicotine
L
Carbachol
% microglia responding
Neonatal control
Neonatal LPS
Neonatal IFN - γ
Neonatal IL-4
upregulation compared to control
downregulation compared to control
no change

Figure 14. Treatment of neonatal cultured microglia with LPS, IFN- γ and IL-4 has distinct effects on the receptor repertoire

Percentage of neurotransmitter/hormone responsive cultured neonatal cells (A-L). Untreated neonatal microglia (control) are compared to cultures treated with LPS (100ng/ml), IFN-γ (20U/ml) and IL-4 (30ng/ml). Data are presented as the percentage of cells which responded to a given substance (the ATP responsive population is taken as 100%) from at least 10 investigated microscopic fields from at least 3 independent cell preparations and statistical significance is shown as *** for $p \leq 0.001$, ** for $p \leq 0.01$ and * for $p \leq 0.05$.

Incubation of cultured adult microglia with 100 ng/ml LPS resulted in a significantly higher population of endothelin (68%), histamine (63%), substance P (50%), serotonin (17%), galanin (33%), somatostatin (25%), angiotensin II (68%), vasopressin (17%) and carbachol (16%) sensitive populations. Treatment of adult microglia with 20 U/ml IFN-γ resulted in a significant increase in the histamine (17%), substance P (7%), neurotensin (19%) and carbachol (63%) sensitive populations, and a significant decrease in the serotonin (1%), galanin (1%), somatostatin (3%), angiotensin II (3%) and nicotine (5%) sensitive populations. Responses to all other substances were not significantly different to untreated adult microglia. Treatment of adult microglia with 30 ng/ml IL-4 resulted in a significant increase in the histamine (8%), substance P (33%) and somatostatin (21%) sensitive populations and a significant decrease in the angiotensin II (2%), dopamine (1%) and carbachol (2%) sensitive populations (Figure 15A-L).


A
Endothelin
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Histamine
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Galanin
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Somatostatin
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Angiotensin II
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Vasopressin
I
Neurotensin
J
Dopamine
K
Nicotine
L
Carbachol
% microglia responding
Adult control
Adult LPS
Adult IFN - γ
Adult IL-4
upregulation compared to control
downregulation compared to control
no change

Figure 15. Treatment of adult cultured microglia with LPS, IFN- γ and IL-4 has distinct effects on the receptor repertoire

Percentage of neurotransmitter/-hormone responsive cultured adult cells (A-L). Control, LPS (100ng/ml), IFN-γ (20U/ml) and IL-4 (30ng/ml). Data are presented as the percentage of cells which responded to a given substance (the ATP responsive population is taken as 100%) from at least 10 investigated microscopic fields from at least 3 independent cell preparations and statistical significance is shown as *** for $p \leq 0.001$, ** for $p \leq 0.01$ and * for $p \leq 0.05$.

Table 3 summarises all the results for calcium imaging experiments from freshly isolated and cultured microglia with fold change and significance.

	Freshly isolated			Neonatal cultured			Adult cultured		
	Glioma	Stroke	EAE	LPS	IFN-γ	IL-4	LPS	IFN-γ	IL-4
Endothelin	4.1	3.1	9.5	7.3	1.1	1.6	5.3	1.0	0.8
Histamine	1.9	1.8	0.5	3.7	6.0	1.4	24.9	6.5	3.0
Substance P	0.6	0.2	0.6	0.9	1.6	0.2	34.4	4.7	22.4
Serotonin	0.9	0.8	2.1	1.0	0.2	0.3	2.8	0.2	1.7
Galanin	0.6	1.0	0.7	0.6	5.6	0.4	5.9	0.3	1.3
Somatostatin	0.5	2.0	2.7	2.7	4.9	1.0	2.0	0.3	1.7
Angiotensin II	1.8	2.5	5.5	0.3	8.8	1.3	6.2	0.3	0.2
Vasopressin	0.9	2.5	1.4	2.3	4.0	0.6	7.5	1.9	0.6
Neurotensin	0.8	0.2	1.4	0.6	1.3	0.3	0.8	3.0	0.5
Dopamine	1.2	0.9	2.0	1.6	2.8	0.8	0.5	0.5	0.2
Nicotine	1.1	1.4	0.9	0.9	0.6	0.2	1.0	0.3	0.7
Carbachol	0.6	3.8	0.7	1.1	6.7	0.8	2.0	7.7	0.2

***	
**	Downregulation
*	
	Not significant
*	
**	Upregulation
***	

	Freshly isolated		Adult cultured
	APPPS1 microglia	Peripheral macrophages	Bone marrow macrophages
Carbachol	1.6	0.6	0.3

Table 3. A summary of fold change and statistical significance (greyscale) of microglial populations responsive to the specific ligands in glioma, MCAo and EAE freshly isolated microglia, and LPS, IFN-γ and IL-4 treated neonatal and adult cultured cells compared to controls.

4.7 Consecutive application of three neurotransmitters/hormones shows heterogeneity in the expression pattern

We addressed the question of whether different transmitter receptors are expressed in a coordinated fashion or at random. We therefore applied galanin,

somatostatin and angiotensin II to IFN-γ stimulated neonatal microglia since a high amount of cells responded under these conditions. When tested alone, 24, 30 and 45% of the cells responded to application of these substances, respectively. We consecutively applied galanin, somatostatin and angiotensin II to one given cell, with a 5 minute washout in between each substance and a 30 second application of ATP as a positive control at the end of the experiment (Figure 16). We found that 31% of cells responded to only one ligand, 8% to two and 2% to all three. 59% of cells did not respond at all (except to ATP). Fourteen percent responded to galanin, 3% to somatostatin and 14% to angiotensin II alone. The order of application was also changed. We subsequently tested somatostatin, followed by angiotensin II and then galanin; we found that 62% of cells did not respond to any of the ligands, 18% to only one, 16% to two and 3% to all three. Similarly, when the order of application was switched to angiotensin II – galanin – somatostatin, 67% did not respond, 28% to 1 ligand, 4% to 2 and 1% to all three. The data are summarized in Table 4. Based on the percentage of cells responding to two given transmitters we calculated the probability that they co-respond and compared it to the measured value. These values were fairly similar indicating that the expression of the different receptors is independently regulated. When calculating the probability that a cell responds to all three different transmitters, we found that the measured value was higher than the calculated value indicating that there is a low cooperativity of expression for triple sensitive cells.

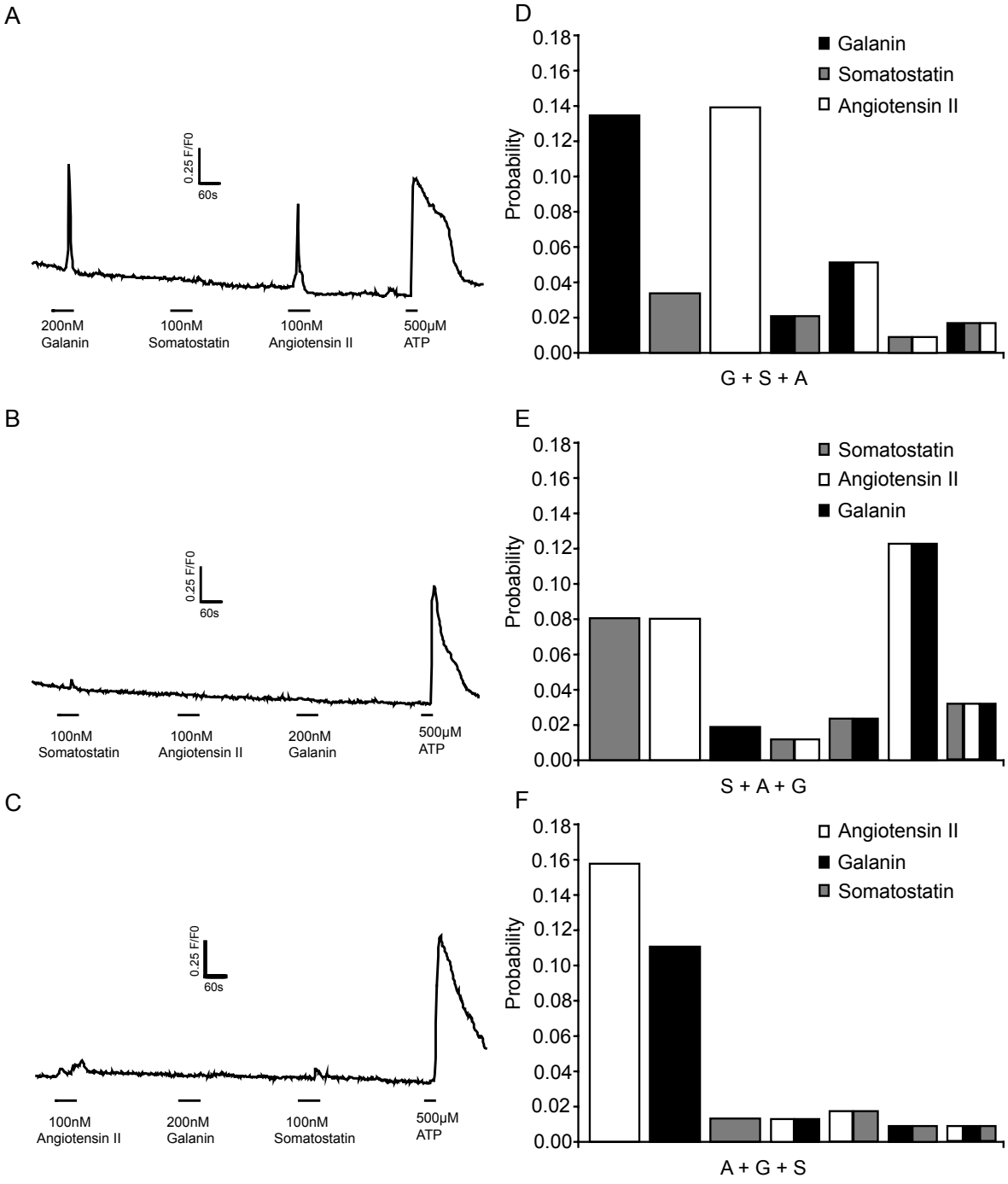

Figure 16. Consecutive application of galanin, somatostatin and angiotensin II shows heterogeneity in the expression pattern

Calcium imaging for these substances was carried out on IFN-γ treated neonatal microglia. An example trace for each experimental sequence shows (A) the cell responds to both galanin and angiotensin II after the sequence galanin – somatostatin – angiotensin II, (B) a response to somatostatin only after the application sequence somatostatin – angiotensin II – galanin, (C) a response to angiotensin II and somatostatin after the sequence angiotensin II – galanin – somatostatin. (D, E, F) Bar charts show the probability that a cell will respond to each given

substance, alone and in combination with a response from one or more other substances. The probability changes depending on the order of application.

								All cells responding to -		
	Somatostatin only	Galanin only	Angiotensin II only	Angiotensin II + Somatostatin	Angiotensin II + Galanin	Somatostatin + Galanin	Angiotensin II + Somatostatin + Galanin	Somatostatin	Galanin	Angiotensin II
G + S + A	0.034	0.135	0.139	0.008 *(0.017)*	0.051 *(0.048)*	0.021 *(0.018)*	0.017 *(0.0039)*	0.080	0.224	0.215
S + A + G	0.081	0.021	0.081	0.013 *(0.038)*	0.123 *(0.051)*	0.025 *(0.031)*	0.034 *(0.0078)*	0.153	0.203	0.250
A + G + S	0.014	0.112	0.158	0.019 *(0.010)*	0.014 *(0.029)*	0.009 *(0.007)*	0.009 *(0.0015)*	0.051	0.144	0.200

Table 4. Probability of a single or a combination of responses to galanin, somatostatin and angiotensin II depending on the order of application. Measured probabilities were calculated by dividing the percentage response by 100. Calculated probabilities are shown in italics.

A similar series of experiments was carried out on adult cultured microglia treated with LPS (Figure 17). We consecutively applied endothelin, histamine and substance P since a large amount of LPS stimulated cells responded under these conditions. We also tested the sequence histamine – substance P – endothelin, and substance P – endothelin –histamine. We made the same observations as for the neonatal IFN-γ stimulated cells.

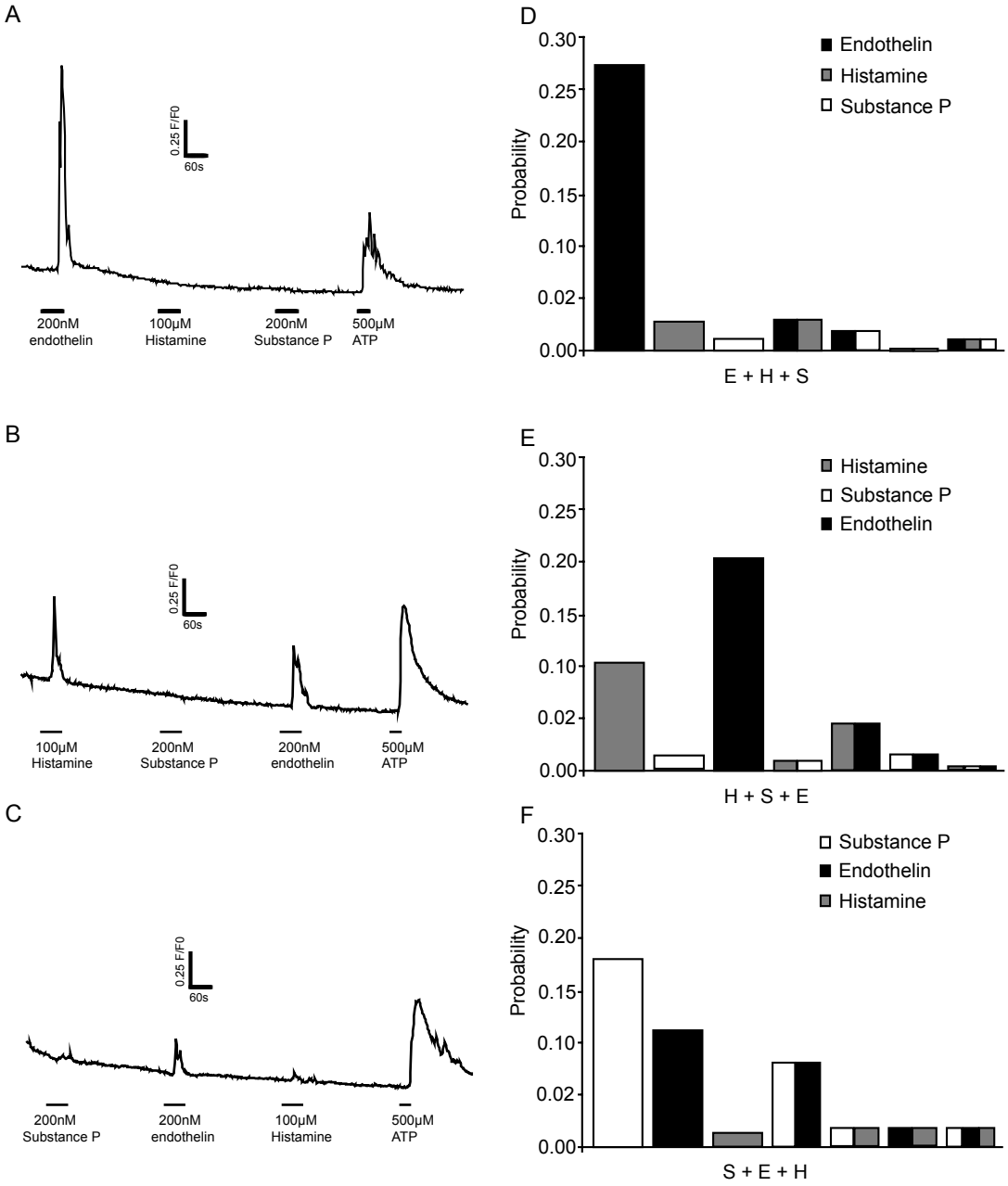

Figure 17. Consecutive application of endothelin, histamine and substance P shows heterogeneity in the expression pattern

Calcium imaging for these substances was carried out on LPS treated adult microglia. An example trace for each experimental sequence shows (A) the cell responds only to endothelin after the sequence endothelin – histamine – substance P, (B) a response to histamine and endothelin after the application sequence histamine – substance P – endothelin, (C) a response to all 3 ligands after the sequence substance P – endothelin – histamine. (D, E, F) Bar charts show the probability that a cell will respond to each given substance, alone and in combination with a response from 1 or more other substances. The probability changes depending on the order of application.

Table 5 contains a summary of the data.

									All cells responding to -		
	Endothelin only	Histamine only	Substance P only	Endothelin + Histamine	Endothelin + Substance P	Histamine + Substance P	Endothelin + Histamine + Substance P		Endothelin	Histamine	Substance P
E + H + S	0.275	0.026	0.011	0.030 *(0.024)*	0.019 *(0.015)*	0.004 *(0.003)*	0.011 *(0.0011)*		0.335	0.071	0.045
H + S + E	0.202	0.103	0.013	0.045 *(0.043)*	0.013 *(0.011)*	0.009 *(0.007)*	0.004 *(0.0017)*		0.265	0.161	0.040
S + E + H	0.112	0.013	0.179	0.018 *(0.015)*	0.080 *(0.067)*	0.018 *(0.02)*	0.018 *(0.0045)*		0.228	0.067	0.295

Table 5. Probability of a single or a combination of responses to endothelin, histamine and substance P depending on the order of application. Measured probabilities were calculated by dividing the percentage response by 100. Calculated probabilities are shown in italics.

4.8 Characterisation of carbachol-induced calcium transients

We analysed the carbachol-induced calcium transients for freshly isolated adult microglia and cultured neonatal and adults microglia. For the freshly isolated adult from healthy mice and those from mouse models of pathology, we either recorded single Ca^{2+} peaks or more complex responses which were comprised of several peaks and lasted up to 105 s. We did not observe spontaneous Ca^{2+} oscillations in the freshly isolated cells.

For cultured neonatal and adult microglia, with respect to peak and time course of the carbachol response, we found cells with simple and complex responses similar to freshly isolated microglia (Figure 18). In addition some cells showed Ca^{2+} oscillations which were defined as a response with multiple peaks (>2). The Ca^{2+} transients following the first peaks usually were smaller in amplitude (Figure 18). Integral, amplitude and duration of the calcium signal from all cells responding to carbachol and ATP were calculated for neonatal untreated, neonatal + IFN-γ, adult untreated, adult + IFN-γ and freshly isolated adult. The average carbachol-induced Ca^{2+} amplitude of untreated neonatal and adult cells was similar (94 ± 4% and 100 ± 6 %, respectively; where the amplitude in each experiment is taken as a percentage of the ATP signal). Average duration for neonatal and adult was 76 ±4 s and 55 ± 5 s, respectively. For neonatal and adult microglia treated with IFN-γ, the average amplitude was 88 ± 4 % (not significant compared to neonatal control) and 133 ± 6 %, (significant compared to adult control: p=0.002) and indicates that

IFN-γ potentiates the calcium signal in adult but not in neonatal cells. The average duration was significantly longer than control at 94 ±5 s (p=0.02) and 112 ± 8 s (p<0.001), respectively (Table 6).

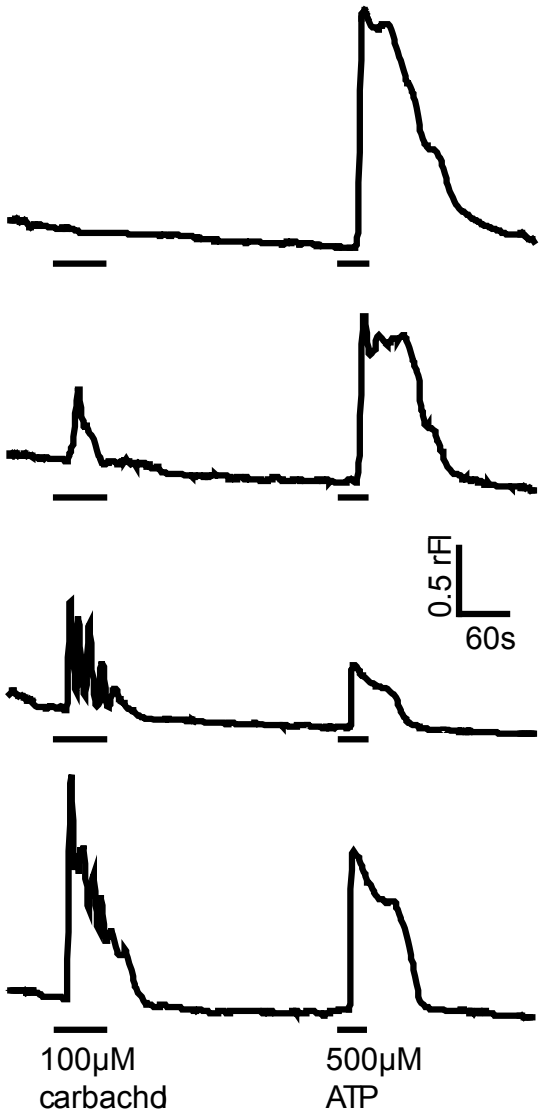

Figure 18. Properties of the carbachol induced Ca2+ response

Four types of calcium responses can be seen after carbachol application in adult cultured microglia: no transient increase, a single calcium peak, an oscillating response and a more complex response.

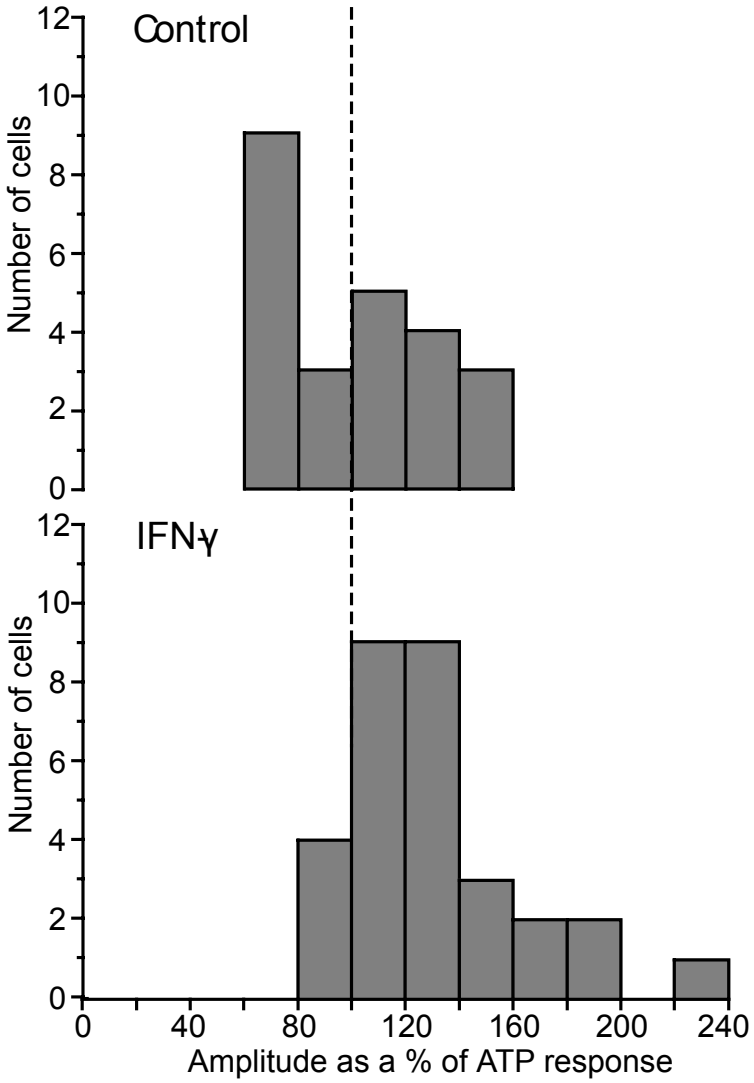

Figure 19. IFN-γ treatment increased the carbachol-sensitive microglial population
Amplitude histograms of the fluorescence increase (relative to the ATP response) in control cultured adult microglia and cultured adult microglia treated for 18h with interferon-γ.

	Mean integral as a % of ATP response (±SEM)	Mean amplitude as a % of ATP response (±SEM)	Mean duration (s) (±SEM)
Neonatal control	76 (6)	94 (4)	76 (4)
Neonatal IFN-γ	85 (7)	88 (4)	94 (5)
Adult control	56 (6)	100 (6)	55 (5)
Adult IFN-γ	129 (12)	133 (6)	112 (8)
Freshly isolated adult	50 (5)	72 (4)	61 (4)
Freshly isolated stroke	87 (6)	106 (3)	65 (5)

Table 6. Mean area, amplitude and duration of carbachol induced Ca2+ transients for primary cultured neonatal and adult microglia and freshly isolated adult microglia.

4.9 The IFN-γ-induced upregulation of the carbachol-sensitive population depends on protein synthesis

To study the time course by which the Ca^{2+} sensitive population is changed following treatment with IFN-γ, we measured the Ca^{2+} response 6, 8, 10, 11, 12 and 18 hours after treatment with IFN-γ. As shown in Figure 20A, the population of responsive cells increased significantly after 11 h, reached a plateau after 12 h (70%) and remained stable at 18 h (62%).

Inhibition of protein synthesis by incubation with 10 µM cycloheximide (CHX) of IFN-γ treated cells reduced the carbachol-sensitive population to 5%. Treatment of cells with 10 µM puromycin in combination with IFN-γ as an alternative protein synthesis inhibitor also reduced the responsive population to 3% (Figure 20B). Importantly, cells were shown to be viable since they all responded to the application of ATP with a Ca^{2+} transient.

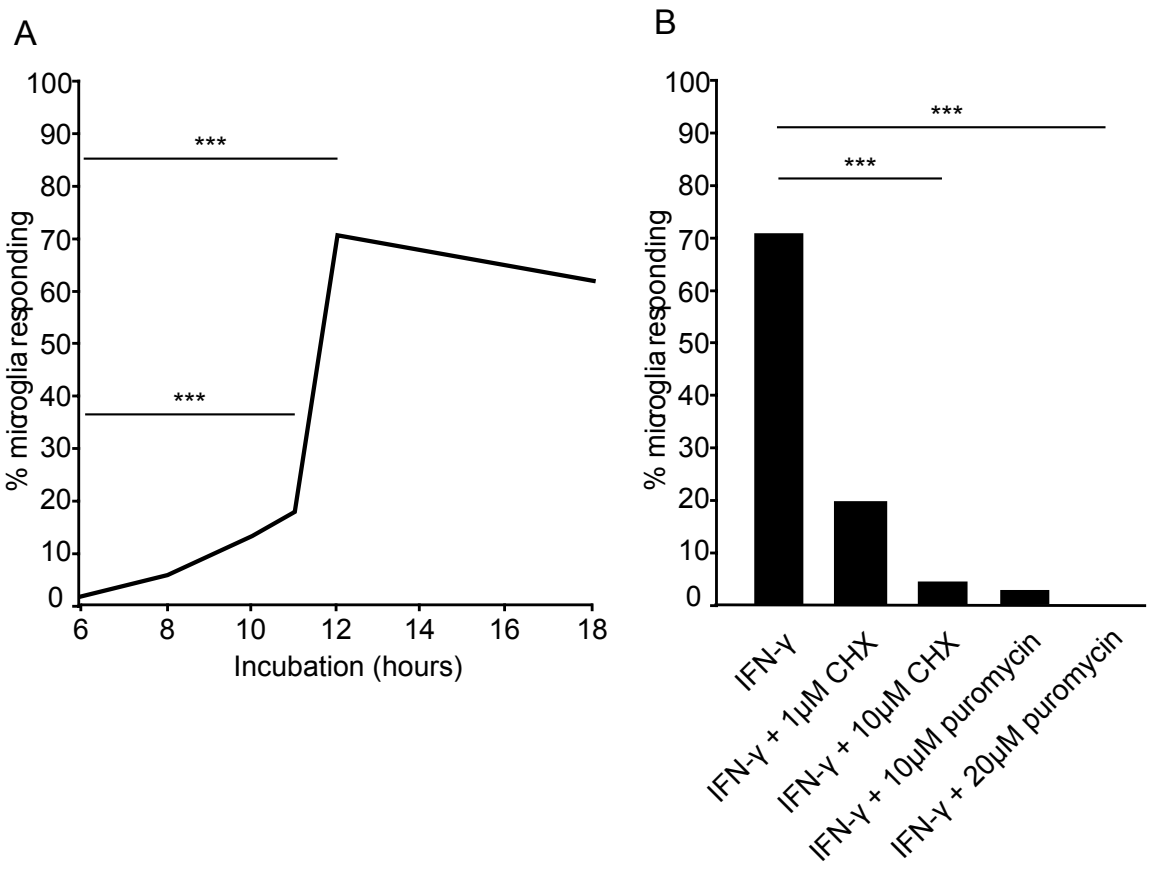

Figure 20. Time course and properties of the IFN- γ induced change in the carbachol-sensitive microglial population

(A) Neonatal cultured microglia were incubated with IFN-γ for different time points and the population of carbachol-sensitive cells was determined. The increase is significant after 11 h and reaches a peak after 12 h. (B) Inhibition of protein synthesis with cycloheximide (CHX, 1 µM and 10 µM) and puromycin (10 µM and 20 µM) significantly reduces the population of carbachol sensitive microglia.

4.10 The Ca^{2+} signal depends on release from intracellular Ca^{2+} stores

To study the desensitization of the muscarinic acetylcholine receptor, 100 µM carbachol was applied to IFN-γ treated neonatal microglia for 1 minute. After 5 minutes, carbachol was applied a second time for one minute. The percentage of cells responding to the first stimulation was 73%. The percentage responding to the first and second was 57%. The amplitude of the second signal was on average 43% as compared to the first signal (n=90).

To distinguish whether the increase in intracellular Ca^{2+} is due to an influx via the plasma membrane or to release from intracellular stores, we compared carbachol responses in Ca^{2+}-containing and Ca^{2+}-free solution. After application of carbachol in normal Ca^{2+}, the perfusion was changed to nominally 0 µM calcium. After 5 min and after 10 min in zero calcium, carbachol was applied and resulted in a second response, which was reduced to 64% of the first response. The third application did not trigger a Ca^{2+} response. Calcium containing buffer was then reintroduced for 5 min which resulted in a Ca^{2+} increase that slowly returned to baseline. Five minutes after reintroduction of Ca^{2+}, carbachol triggered a Ca^{2+} response, which was 87% of the first control (Figure 21). At the end of the experiment ATP was applied for 30 s to test for the viability of the cell.

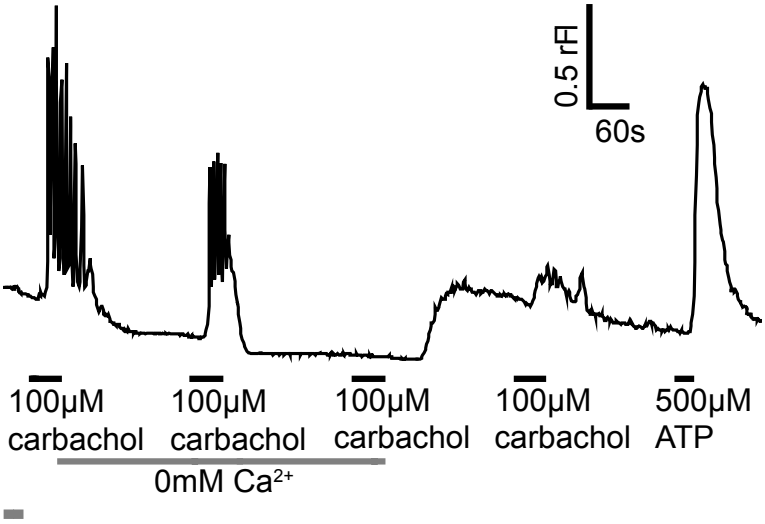

Figure 21. The Ca2+ signal depends on release from intracellular Ca2+ stores
Carbachol was applied 3 times for 1 min with a 5 min interval in nominally 0mM calcium. Note that the third application of carbachol did not trigger a Ca^{2+} response. Re-addition of Ca^{2+} into the bath led to a transient increase in cytosolic Ca^{2+}. In the Ca^{2+}-containing solution, carbachol triggered a small Ca^{2+} increase. Application of ATP served as a control. rFI, relative fluorescence increase.

4.11 Carbachol regulates microglial functions – chemotaxis

To test the effect of muscarinic acetylcholine receptor activation on microglial functions, we determined the effect of carbachol on microglial chemotactic behaviour. We used purified microglial cultures in an agarose spot assay. Agarose spots containing PBS (as control) or 10, 50 and 100 µM carbachol were placed on glass-bottomed Petri dishes and microglial cells in suspension were subsequently added (Figure 22A). After 4 and 6 hour incubation times, the images of the agarose spots were recorded under the microscope and the accumulated microglial cells were quantified. Microglial invasion into the carbachol-containing agarose spots was significantly higher as compared to the control spots (at 4 hours 100 µM carbachol spot: 896 ± 34 cells/spot; PBS spot: 21 ± 8 cells/spot; p=0.00001) (Figure 22B).

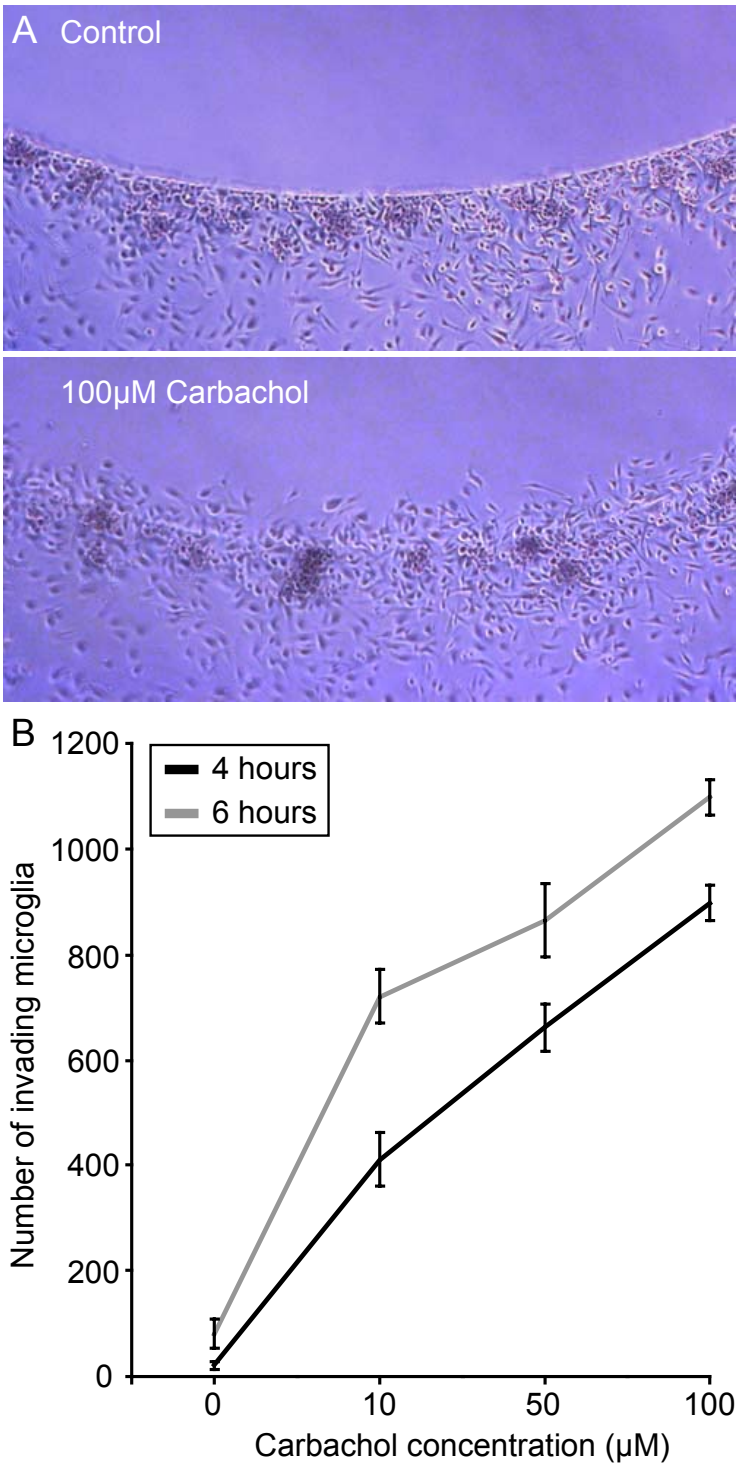

Figure 22. Carbachol is a chemoattractant for microglia

(A) Agarose spot assay without carbachol shows low microglial invasion after 6 hours incubation. An agarose spot containing 100 μM carbachol showed significant invasion after 6 hours. (B) Average invasion activity from data as shown in A with increasing concentrations of carbachol measured after 4 and 6 hours incubation.

4.12 Carbachol regulates microglial functions – phagocytosis

To study the influence of carbachol on phagocytic activity, we quantified the uptake of FCS coated yellow-green fluorescent microspheres under control conditions or in the presence of 10 µM and 100 µM carbachol in neonatal and adult primary cultured microglia. The phagocytic activity of cultured microglial cells was tested by application of microspheres for 30 min with or without addition of 10 µM or 100 µM carbachol. The phagocytic activity, calculated as the phagocytic index of unstimulated neonatal and adult microglia was 103 ±10 and 204 ±15, respectively. Stimulation with 100 µM carbachol resulted in a significant decrease of the phagocytosis index, to 70 ± 9 (p=0.019) and 140 ± 7 (p=0.002) respectively, while 10 µM carbachol resulted in a decrease, which was not significant (Figure 23).

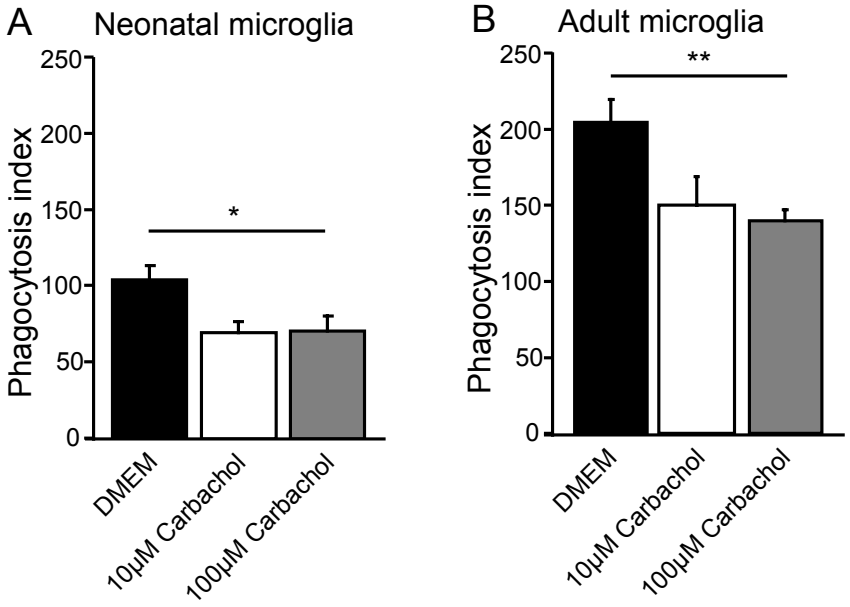

Figure 23. Carbachol decreased phagocytosis activity

Phagocytosis of latex beads by primary cultured microglia is significantly decreased with 100 µM carbachol in both neonatal (A) and adult (B) cultured microglia. The phagocytosis index is defined in Materials and Methods.

4.13 Microglia express mRNA for muscarinic acetylcholine receptors

We performed qPCR on primary neonatal and adult microglia unstimulated and stimulated with IFN-γ, freshly isolated adult microglia and whole brain tissue from C57BL/6 mice (Figure 24). We tested all primers for efficiency and specificity on cDNA samples from whole brain tissue of C57BL/6 mice.

In order to determine relative expression of cholinergic receptor, muscarinic (chrm)1, 2, 3, 4 and 5 subtypes in whole brain and in freshly isolated microglia, we used chrm2 from whole brain as the reference value. Similarly, for neonatal expression we used the value for chrm2 in control cells as a reference for chrm 1, 2, 3, 4 and 5 expression in control and IFN-γ treated cells. The same method was used for expression in adult cultured microglia.

In whole brain tissue as well as primary adult and neonatal microglia, we observed that mRNA for all muscarinic acetylcholine receptors were expressed (chrm1-chrm5) (Figure 24). Freshly isolated adult microglia showed low mRNA expression for all receptor subtypes (Figure 24A). Upon overnight treatment with IFN-γ, both neonatal and adult primary microglia showed an increase in the expression of chrm3 mRNA (Figure 24B, C).

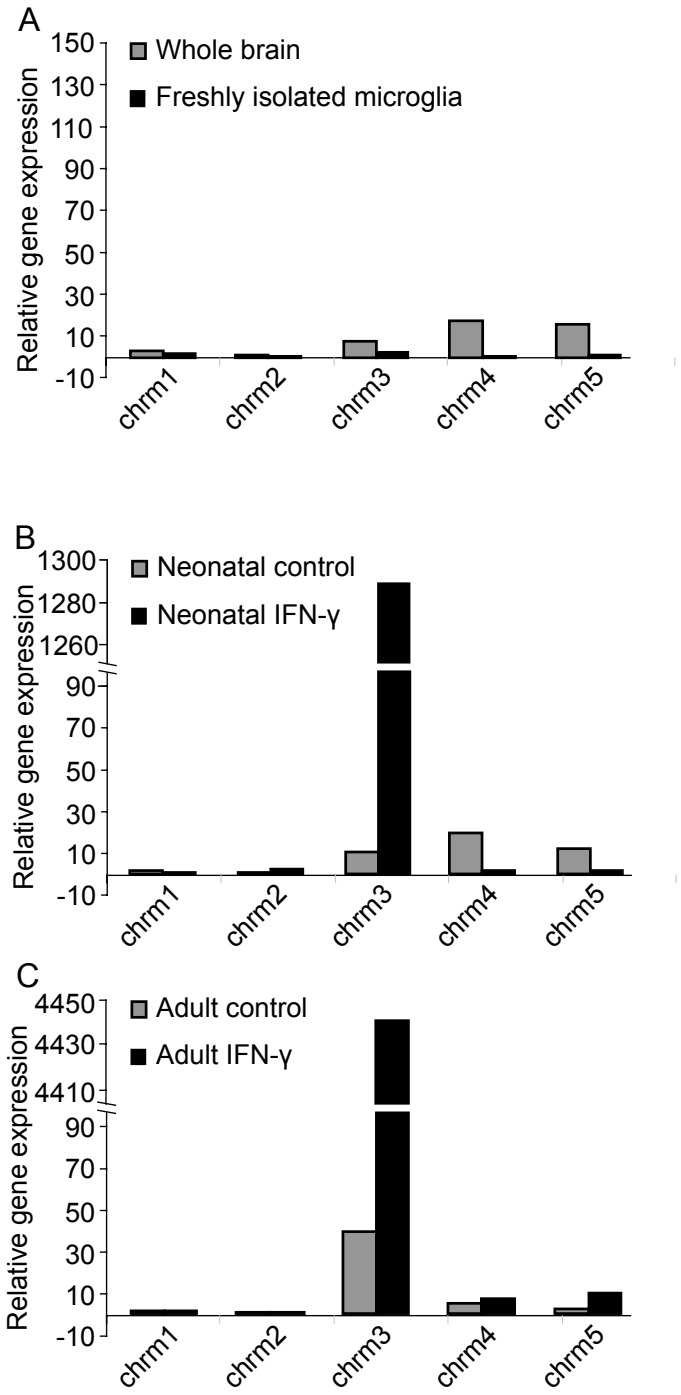

Figure 24. Expression of muscarinic receptor subtypes in microglia

Microglial cells express mRNA for muscarinic acetylcholine receptors (mAChR). Expression of the 5 muscarinic acetylcholine receptors (mAChR) subtypes was quantified in neonatal and adult cultured, and freshly isolated adult microglia, normalized to the expression of the housekeeping gene beta actin. (A) For whole brain chrm2 expression was used as reference for chrm1,3,4,5. For freshly isolated cells, whole brain chrm2 was used as a reference for expression of chrm1,2,3,4,5 . (B, C) chrm2 from control was used as reference for the expression of subtypes in neonatal and adult control and IFN- γ-treated. All samples were isolated from C57BL/6 mice.

Name	Size	Sense Primer	Antisense primer
ß-Actin	238	CCCTGAAGTACCCCATTGAA	GTGGACAGTGAGGCCAAGAT'
Chrm1	105	AGTCCCAACATCACCGTCTTG	CAGGTTGCCTGTCACTGTAGC
Chrm2	143	TGCCATTGCGGCTTTCTATCT	CTTGCACTAGACTCGGAGACA
Chrm3	158	ACCTGTTCACGACCTACATCA	AGTGAGTGGCCTGGTAATAGAAA
Chrm4	163	AGATGGTGTTCATTGCGACAG	GAGAACGCCCCTATGATGAGA
Chrm5	160	CCTCTACACGACCTACATCCTC	GTATGTCAGTGGTCTTGTGATGG

Table 7. Primer sequences used for quantitative PCR

5. Discussion

5.1 Microglia comprise a heterogeneous population with respect to their responsiveness to neurotransmitter/-hormones

In the past, microglia were not thought to have a role in communication within the neural network. However, in recent years, there has been increasing evidence that a large number of receptors for neurotransmitters are expressed on microglia, allowing neurons to control their function (Biber et al., 2007).

In this research, we used calcium imaging as readout to determine whether microglia expressed functional receptors for endothelin, histamine, substance P, serotonin, galanin, somatostatin, angiotensin II, vasopressin, neurotensin, dopamine and acetylcholine (nicotinic and muscarinic receptors). In a previous study (Seifert et al. 2011), we transduced a genetically encoded Ca^{2+} sensor, GCaMP2, by a retroviral approach, and carried out calcium imaging on microglia in acute brain slices, however this required a pathological insult to stimulate microglial proliferation in situ, therefore activating the cells and changing their phenotype. In this research we have therefore utilized a freshly isolated preparation of microglia cells from adult mice, isolated within 4 hours of death, in order to better examine Ca^{2+} signaling in microglia from healthy brains. We have also compared the neurotransmitter/-hormone sensitive population to those from microglia cultured from neonatal and adult mouse brains. Since cultured microglia have been isolated from the mouse brain for up to 4 weeks, freshly isolated microglia, which have been removed from the brain environment for a shorter period of time, enable us to validate the results we obtain from cultured cells since they should more closely resemble the physiology of microglia within the healthy brain.

We have studied the microglial Ca^{2+} response to endothelin, histamine, substance P, serotonin, galanin, somatostatin, angiotensin II, vasopressin, neurotensin, dopamine, nicotine and carbachol in our three different preparations: freshly isolated adult cells, cultured adult and cultured neonatal cells. We found that only

a subpopulation of less than 20% of cells responded to any of these ligands, suggesting that small heterogeneous subpopulations of microglia exist that are characterized by distinct functional receptor expression. We found that all three preparations showed a similar functional expression pattern. Previous studies have also used calcium imaging as a tool to test for functional receptor expression. Endothelin was previously applied to both mouse and human cultured microglia and the change in intracellular calcium was measured. A 1997 study by Moeller et al. found that 13% of cultured neonatal murine microglia responded to endothelin application with a calcium transient (Moeller et al., 1997) whilst McLarnon et al. found that 86% of human microglia isolated from fetal brain tissue responded (McLarnon et al., 1999). Although the endothelin sensitive population from Moeller et al. is slightly higher than from our experiments, it is still within a low range and therefore supportive of our conclusion that unstimulated microglia show a lower expression of this receptor. The results from McLarnon, however, demonstrate the differences in neurotransmitter/hormone receptor expression across species. This is also supported by research by Bader et al. which found that 30% of neonatal microglia cultured from rat brain responded to the application of histamine with a calcium increase (Bader et al., 1994). This was significantly higher than the 10%, 3% and 2% of microglia which responded from the respective freshly isolated, adult cultured and neonatal cultured populations that we observed in our research. Cultured neonatal rat microglia also had a slightly higher galanin sensitive population, with 13.4% of cells responding to galanin application during calcium imaging experiments (Ifuku et al., 2011), which was also higher than the 4% of neonatal cultured murine microglia which responded in our experiments, again indicating that receptor expression differs across species, but is generally expressed in a smaller population of unstimulated cells. While the use of calcium imaging has been carried out in various studies in order to test for the expression of functional receptors, the majority of the research has been carried out in microglia cultured from neonates. In our research, we not only tested our substance on neonatal murine microglia, for the first time we also compared these results with the population of adult cultured microglia which responded. Calcium imaging on adult cultured microglia is not routinely carried out and our freshly

isolated microglia preparation is at present, a new and novel way of carrying out calcium imaging studies on microglia isolated from the brain for such a short amount of time. This method has been used in previous studies to isolate microglia, and a study from 2013 utilised the MACS procedure to isolate human microglia from post-mortem retina samples (Carter et al., 2013). This research also included calcium imaging experiments, however, these experiments were carried out on microglia after at least 24hours post isolation, thus removing the benefit of having them "freshly isolated". Since our protocol has not been used to date to carry out calcium imaging experiments on microglia from adult mice, we can only compare our results to those obtained from research on cultured cells or *in vivo* experiments. In our isolation procedure to obtain freshly isolated microglia, it appears that we may strip the cells of their processes, since the cells are significantly smaller than those from neonatal and adult cultured preparations and have a small, round morphology (Figure 8). If the receptors are predominantly located on the processes, the cells might lose their sensitivity. While we can not exclude that possibility, a study on virally transduced microglia did not provide any evidence that Ca^{2+} signals are stronger in processes as compared to the soma (Seifert et al., 2011). In addition, we found that cultured microglia both from adult and neonatal tissue also show a similar response pattern as the freshly isolated cells. We thus assume that our data reflect the response pattern of microglia in the tissue and that untreated cultured microglia reflect the properties of "resting" microglia, where receptors for neurotransmitters and neurohormones are not so widely expressed. While there is evidence for region-specific heterogeneity in microglia with respect to the expression of immunoregulatory proteins (De Haas, Boddeke, and Biber 2008), our data suggest that "resting" microglial cells also comprise a functionally heterogeneous cell population.

Isolation of microglia from tissue will always result in activation of the cells through the sometimes vigorous isolation procedure. Carrying out Ca^{2+} imaging on completely naive, resting microglial cells with our test substances is not yet possible. The calcium sensor stab wound model previously discussed (Seifert et al., 2011) recorded microglial Ca^{2+} responses to ATP, endothelin-1, substance P, histamine and serotonin. About half of the ATP-sensitive population responded to

these four ligands and this population was not altered 42 days after the injection. In this preparation one has to take into account that these microglial cells have undergone an activation process and that they must be distinct from naïve microglia. A different approach was used by Eichhoff et al., by delivering the Ca^{2+} indicator dye Oregon green BAPTA 1 to microglial cells *in vivo* with an electroporation technique (Eichhoff et al., 2011). They found that 20% of the cells responded to glutamate, but none to carbachol. The electroporation technique, however, might also have an impact on the properties of the cells.

Other transmitter receptors were studied with the patch-clamp technique in acute slices. About half of the microglial cells in rat or mouse responded to the $GABA_B$ receptor ligand SKF 97541 with the induction of an outwardly rectifying K^+ conductance (Kuhn et al., 2004). The use of $GABA_B$ receptor-specific antibodies indicated that the population of $GABA_B$ receptor expressing microglia was lower than 50% and this discrepancy can be explained by the fact that patch-clampers make an individual selection of cells. In the facial nerve lesion model, activated microglia show an increase in $GABA_B$ receptor expression. Microglial cells of early postnatal brain also only partially responded to dopamine and adrenergic receptor agonists when studied with the patch-clamp technique in acute slices from postnatal day 8 animals; about a third of the cells responded to dopamine with a change in K^+ conductance, and a fourth to adrenergic agonists (Farber et al., 2005). Despite differences in the preparation and activation state of the microglia, all of these studies show that only a subpopulation of microglia expresses functional receptors for a given neurotransmitter/ neurohormone. Regardless of age, isolation procedure and stimulation, we have been able to support this data and thus conclude that microglia constitute a rather heterogeneous cell population with respect to their response to neurotransmitters and neurohormones.

5.2 Pathology alters the population of microglia sensitive to different neurotransmitter/-hormones

Our study provided evidence that the population of microglial cells sensitive to particular neurotransmitters and neurohormones expands considerably in defined pathologies. Microglia freshly isolated from MCAo, EAE and glioma mice

demonstrated that the upregulation of defined receptors differs depending on the disease type. In all three disease models, macrophages from the periphery enter the brain indicating that we actually investigated a mixture of intrinsic microglia and peripheral macrophages. The change in the chemosensitive microglial / brain macrophage populations was quite diverse in the different pathologies and there is no uniform pattern for most of the receptors. Endothelin and angiotensin II were the only substances that showed an increase in the responding microglial populations in all three pathologies.

Upregulation of the angiotensin II receptors on microglia is supported by Stegbauer (Stegbauer et al., 2009) which found a general upregulation of angiotensin II receptors in the inflamed spinal cord and in a mouse model of EAE. TGF-beta upregulation in microglia during EAE was also blocked with the inhibitor of the angiotensin II type 1 receptor, candesartan, which also improved the outcome of the disease, further demonstrating the therapeutic possibilities of targeting the renin-angiotensin system in neurodegenerative diseases (Lanz et al., 2010). The renin-angiotensin system has also been shown to play a role in stroke since cerebral edema and mortality in gerbils following stroke was increased after intravenous infusion of angiotensin II (Hosomi et al., 1999). Moreover, the ischemic area in mice where the angiotensin II receptor was knocked out was significantly larger compared to wildtype (Iwai et al., 2004). The angiotensin II type 1 receptor blocker eprosartan has already been employed in clinical trials; high-risk hypertensives with cerebral events during the last 24 months showed a reduced mortality and morbidity when treated with eprosartan (Schrader et al., 2005).

The endothelin-1 receptor has also been implicated in the pathogenesis of EAE, stroke and glioma. Intrathecal administration of the ET1 antagonist BQ-123 during the peak stage of EAE in rats from day 8 resulted in a reduction in EAE paralysis (Shin et al., 2001) and the combined use of antagonists for both the ETA and ETB receptor resulted in a reduction in edema and infarct volume in rats after MCAo (Moldes et al., 2012). The use of ETB receptor antagonists also reduced the proliferation and viability of a glioma cell line (Paolillo et al., 2010).

The somatostatin sensitive population was increased in both MCAo and EAE, but was decreased in glioma, which hints towards a more dominant role of somatostatin under pro-inflammatory conditions than under conditions associated with glioma growth. The role of the somatostatin receptor in MCAo and EAE has already been established in a number of studies. Somatostatin has a role as an inhibitory regulator of secretion and proliferation of target cells in the brain and periphery (Patel, 1999), including the downregulation of cytokines including IFN-γ, the reduction of lymphocyte proliferation and Ig production. The expression of the somatostatin 2 receptor was found to be reduced in the early recovery phase of EAE in mice (Baranzini et al., 2005), while the somatostatin analogue SMS 201-995 increased the susceptibility of rats to EAE (Muhvic et al., 2005). The somatostatin receptor 2 has also been implicated in stroke since mice deficient in the receptor showed a reduction in infarct size and postischemic neurodegeneration after MCAo (Stumm et al., 2004).

The vasopressin sensitive population was significantly increased in MCAo only. As for the angiotensin, somatostatin and endothelin receptors, the use of selective antagonists for the vasopressin receptor was also found to act as a potent neuroprotective agent when used in an embolic focal ischemia model in rats (Shuaib et al., 2002). Our data can partially explain the treatment effect in these studies through the regulation of the given receptors in microglia.

Isolation of microglia from a mouse model of stroke and Alzheimer's showed a significant increase in the carbachol-sensitive population. Microglia isolated from a mouse model of glioma and autoimmune encephalomyelitis showed a significant decrease in the carbachol sensitive population. This suggests that activation of microglia in certain pathologies results in an increase in the expression of functional muscarinic acetylcholine receptors, however, since the population of carbachol sensitive cells was decreased in glioma and EAE, this suggests that receptors may be up or down regulated depending on the pathology and mechanism of stimulation of microglia and points towards possible functional changes of the cell depending on the disease state. Muscarinic acetylcholine receptors play different roles in the CNS, including the control of many cognitive, autonomic, motor and sensory functions (Wess et al., 2007). Impairment of the

cholinergic system is well known in a number of CNS disorders including schizophrenia (Scarr and Dean, 2009), manic depression (Rowntree et al., 1950), Parkinson's disease (Rinne et al., 2008), Alzheimer's disease (Armstrong, 2013) and stroke (Kataoka et al., 1991; Togashi et al., 1996), however the involvement and expression of microglial muscarinic acetylcholine receptors in these diseases, as well as in multiple sclerosis and glioma, has not been widely studied. The general use of a selective antagonist for the M1 and M3 receptor, however, did offer neuroprotection in cerebral ischaemia, although the precise mechanism is still unclear (Ma et al., 2013). Since our data shows that microglia isolated from stroke tissue have an increased population sensitive to carbachol, therefore suggesting an upregulation of the muscarinic acetylcholine receptor, more widespread activation of this receptor could then result in an "on" signal with potentially harmful functional changes.

5.3 Manipulation of cultured microglia with LPS, IFN-γ nor IL-4 alters the functional expression pattern of neurotransmitter/-hormones

LPS, IFN-γ and IL-4 triggered defined changes in the functional expression pattern of the receptors which we studied. There were some changes which reflected those in the disease models, such as the increase in the histamine sensitive population in both adult and neonatal microglia after treatment with both LPS and IFN-γ, and the IL-4 treated adult cells. We observed differences between adult and neonatal cultures such as the increase in the angiotensin II sensitive population in adult, but not neonatal LPS treated microglia or the increase in the angiotensin II sensitive population only in neonatal cells after IFN-γ treatment. Neither LPS, IFN-γ nor IL-4 triggered an altered expression pattern which mimics that of any of the pathologies which we studied. Thus we could not recognize a uniform pattern in the change in chemosensitive microglial populations in different pathologies or activation paradigms. We could mimic the expansion of the carbachol-sensitive population seen microglia isolated from stroke and Alzheimer's models in neonatal

and adult cultures by overnight treatment with IFN-γ. This increased the carbachol-sensitive population to 62 and 60 % respectively. It is, however, unlikely that IFN-γ is the only signal upregulating the carbachol-sensitive population in the context of Alzheimer's disease since IFN-γ is released by T cells, NK cells and B cells, and the invasion of these cells in the Alzheimer's context is rather low (Stalder et al. 2005). In contrast, there is a considerable invasion of T cells and release of IFN-γ in EAE (Chu, Wittmer, and Dalton 2000; Flügel et al. 2001), yet we did not find an upregulation of the carbachol-sensitive population in this disease context.

5.4 Microglia do not show a correlated expression of receptors

One could speculate that there is one small population of microglia which expresses all the receptors and a large population which does not respond at all. Alternatively, each cell could express a distinct subset resulting in a large number of distinct subpopulations. We addressed this question experimentally and found evidence for the latter. We selected galanin, somatostatin and angiotensin II in IFN-γ treated neonatal cells, and endothelin, histamine and substance P application in LPS treated adult cells, since under these conditions the percentage of responding cells was much higher as compared to control cells. The probability of a given cell to respond to two transmitters could be calculated by multiplying the probability of the population to respond to each of the two, assuming that the expression is not correlated. The actual determined probability value matched that of the predicted value confirming that the expression of galanin, somatostatin and angiotensin II or endothelin, histamine and substance P is not correlated. Only the cell population responding to all three of the tested ligands was slightly higher than that of an uncorrelated expression. This indicates that there is an immense diversity of microglia with respect to their neurotransmitter/-hormone sensitivity.

5.5 Further investigation of the role of muscarinic acetylcholine receptors on microglia

Calcium imaging experiments on untreated neonatal and adult cultured microglia, as well as freshly isolated microglia from adult mice, showed that a subpopulation of these microglia express functional muscarinic acetylcholine expression, as previously discussed. Activation of these receptors also resulted in the release of calcium from intracellular stores. Treatment of both neonatal and adult cultured microglia with interferon-γ gave us a robust and significant increase in the carbachol sensitive population during calcium imaging experiments. Since carbachol was the only substance to give an almost identical response in both neonatal and adult IFN-γ treated microglia, we decided to explore the expression and function of these receptors in further detail. Analysis of the amplitude of the carbachol induced calcium transient in both untreated and IFN- γ treated microglia showed that the amplitude of the signal (as a percentage of the ATP signal) was increased after pro-inflammatory activation with IFN- γ. This shift was also seen in the calcium transients of microglia isolated from stroke as compared to control. A time course analysis showed that the expansion of the carbachol-sensitive neonatal population occurred after 12 hours of IFN-γ treatment. Inhibition of protein synthesis using both cycloheximide and puromycin abolished the increase in the carbachol-sensitive population and even led to a decrease in this population. This indicates that protein synthesis is required for the increase in the carbachol-sensitive cell population and points to novel synthesis of cholinergic receptors triggered by IFN-γ stimulation.

5.5.1 Treatment of cultured neonatal and adult microglia with IFN- γ results in an increase in the expression of the calcium linked chrm3 receptor

Quantitative PCR of IFN-γ stimulated cells showed that IFN-γ treatment led to a marked upregulation in cholinergic receptor chrm3 mRNA expression in both neonatal and adult treated cells, as compared to untreated cells. It has been shown that the M3 muscarinic acetylcholine receptor belongs to the G_q family (Wess, Eglen, and Gautam 2007). Activation of this receptor results in an

upregulation of phospholipase C and inositol trisphosphate, resulting in an increase in intracellular calcium, which corresponds to the calcium imaging data where the number of cells responding to carbachol with a calcium signal increases. mRNA expression of chrm1-chrm5 in freshly isolated microglia was found to be low when compared to the expression of all subtypes in whole brain. This also corresponds to our calcium imaging data where the subpopulation of microglia to respond to carbachol was also relatively low (16%). We would hypothesize that based on the calcium imaging results for microglia isolated from stroke, mRNA for the chrm3 receptor, or the calcium linked chrm1 or chrm5 receptor, should be increased compared to freshly isolated microglia from controls. Unfortunately, mRNA yields from the MACS freshly isolated microglia protocol was in general low, and a large amount of cells from several brains was needed in order to obtain good quality RNA. For this reason we were unable to obtain enough stroke brains and therefore cells to carry out RNA isolation. Future studies could therefore look into the changes in gene expression in microglia from stroke, including the changes in mAChR expression.

5.5.2 Activation of muscarinic acetylcholine receptors results in functional changes of microglial cells

In this study we could establish that activation of the muscarinic acetylcholine receptor modulates microglial function, namely migration and phagocytic activity. An increase in carbachol concentration resulted in an increase in chemotaxis in neonatal microglia but resulted in a decrease in phagocytic activity in both neonatal and adult cultured microglia. It is however, unclear whether this effect on function would result in a protective or destructive phenotype. Microglia have been shown to be involved in synaptic pruning (Paolicelli et al., 2011), therefore the ability of microglia to sense neurotransmitter release from synapses allows them to determine whether the synapses are functioning normally. The decrease in phagocytic activity with increased concentrations of carbachol supports the hypothesis that an increase in neurotransmitter release indicates that the synapse is active and functional, therefore synaptic pruning or phagocytosis of dysfunctional neurons is not necessary (Schafer and Stevens, 2010). The ability of microglia to increase their chemotactic ability as a response to increasing

concentrations of carbachol indicates that this is a signal for them to move to the source of the release, however, in order to determine whether their presence results in detrimental effects, further experiments such as pro-inflammtory cytokine release or iNOS are needed.

The calcium imaging data carried out on microglia from pathology indicates that mACh receptors in stroke and Alzheimer's disease are upregulated which might also modulate their functional repertoire. The specific induction of a neuroprotective phenotype of microglia in neuroinflammatory diseases via modulation of these functions could promote therapeutic effects. We propose that the muscarinic acetylcholine receptor on microglia could be a candidate target for such intervention.

5.6 Conclusions

We have demonstrated here that only a small subpopulation, at around 20%, of microglia isolated from the brain of healthy mice respond to the application of endothelin, histamine, substance P, serotonin, galanin, somatostatin, angiotensin II, vasopressin, neurotensin, dopamine, nicotine and carbachol. Similarly, calcium imaging experiments carried out on untreated neonatal and adult cultured microglia show a similar, small population of microglia which are sensitive to these substances. The receptor repertoire of microglia has also been shown to vary greatly depending on pathology or inflammatory stimulus, and stimulation of neonatal and adult microglia with anti- and pro-inflammatory agents does not result in the same pattern of up and downregulation, further showing how the behaviour of microglia alters with age. Experiments using the muscarinic acetylcholine receptor agonist carbachol has demonstrated that as well as expressing functional receptors, activation of these receptors can influence the functional behaviour of microglia. This research demonstrates the vast potential for further investigation into the impact of the expression of these receptors on microglial function. The up and down regulation of these receptors in different pathologies could lead to therapeutic interventions which could have an impact on reducing the harmful effects of microglial activity during neurological diseases.

6. Summary

Neurotransmitters/-hormones have recently been identified as factors controlling the function of microglia, the immune cells of the central nervous system. In this study we compared the responsiveness of microglia to neurotransmitters/-hormones; we freshly isolated microglia from healthy adult mice and those from murine models of stroke (MCAo), multiple sclerosis (EAE), glioma and Alzheimer's disease. Using calcium imaging, we found that only a small fraction (1 - 20 %) of microglia isolated from control mice responded to the application of endothelin, histamine, substance P, serotonin, galanin, somatostatin, angiotensin II, vasopressin, neurotensin, dopamine, nicotine and carbachol. The population of angiotensin II- and endothelin-sensitive cells was increased in microglia freshly isolated from stroke, EAE and glioma, while some populations increased in only defined disease models. In cultured microglia from neonatal and adult mice, a similarly small population of cells responded to these neurotransmitters/-hormones. To induce activation into a pro-inflammatory phenotype, we applied LPS or IFN-γ to the cultures for 24 h. Several of the responding populations increased, but there was no uniform pattern when comparing adult with neonatal microglia or LPS with IFN-γ treatment. IL-4 as an anti-inflammatory substance increased the histamine, substance P and somatostatin sensitive populations only in microglia from adult, but not in neonatal cells. We also found that the expression of different receptors was not strongly correlated indicating that there are many different populations of microglia with a distinct set of receptors.

We also carried out a more detailed investigation on the expression and function of muscarinic acetylcholine receptors on microglia. Microglia isolated from a mouse model of stroke had a significantly higher carbachol-sensitive population compared to those from healthy mice, where the sensitive population increased from 16% to 60%. Microglia isolated from APPPS1 mice, a mouse model of Alzheimer's disease, also had a significantly higher population of carbachol-sensitive cells (25%). Treatment of neonatal and adult cultured microglia with IFN-γ also significantly increased the carbachol-sensitive population to 62 and 63%

respectively. Treatment of neonatal microglia with IFN- γ was sensitive to blockers of protein synthesis. Functional assays demonstrated that carbachol was a chemoattractant for microglia and decreased their phagocytic activity. Quantitative PCR showed that IFN- γ treatment resulted in an increase in the expression of the calcium linked M3 receptor in both adult and neonatal cultures. Our results indicate that microglial cells are a heterogeneous population with respect to their sensitivity to neurotransmitters/-hormones and that they are more sensitive in pathology and in defined activation states.

7. Zusammenfassung

Neue Forschungsergebnisse haben gezeigt, dass Neurotransmitter/-hormone das Verhalten von Mikroglia beeinflussen. In dieser Studie untersuchten wir das Verhalten von *frisch isolierten* Mikroglia von gesunden adulten Mäusen, sowie pathologischen Mäusen wie Schlaganfall (MCAo), Multiple Sklerose (EAE), Gliom und Alzheimer, unter dem Einfluss verschiedener Neurotransmitter/-hormone.

Wir konnten in gesunden adulten Mäuseneine Subpopulation von 1 – 20 % aller Mikroglia identifizieren, welche auf die Stimulation mit Endothelin, Histamin, Substanz P, Serotonin, Galanin, Somatostatin, Angiotensin II, Vasopressin, Neurotensin, Dopamine, Nicotin und Carbachol mit einem Anstieg von intrazellulärem Kalzium reagierte. Die Subpopulation von Angiotensin II- und Endothelin-sensitiven Zellen vergrößerte sich in mehreren Pathologiemodellen (Schlaganfall, Multiple Sklerose und Gliom), wohingegen sich andere Subpopulation nur in einzelnen Pathologien wesentlich vergrößerten. Die Resultate von den *frisch isolierten* gesunden adulten Mäusen ließen sich in kultivierten neonatelen und adulten Mikroglia wiederholen. Des Weiteren untersuchten wir die Reaktion von Mikrogliaproinflammatorischpolarisiertem Phänotyps auf jene Neurotransmitter/-hormone. Sowohl die Stimulation von LPS als auch IFN-γ für 24 Stunden vergrößerte mehrere Subpopulationen. Im Vergleich von LPS und IFN-γ stimulierten, wie auch im Vergleich von adulten und neonatalen Mikroglia konnten wir jedoch kein einheitliches Muster erkennen. Die Polarisation in einen anti-inflammatorischen Phänotyp durch Stimulation mit IL-4 vergrößerte die Population jener Zellen welche sensitiv auf Histamin, Substanz-P und Somatostatin reagierten, in adulten jedoch nicht in neonatalen kultivierten Mikroglia. Die Analyse der Expression der einzelnen Rezeptoren ergab ein relativ heterogenes Muster, was auf differenzierte Mikrogliasubpopulation schließen lässt, jede mit einem unterschiedlichen Repertoire an Rezeptoren.

In einem zweiten Schritt unserer Studie untersuchten wir die Expression und Funktion der muskarinischenAcetylcholinrezeptoren auf Mikroglia. Im Vergleich zur gesunden Maus erhöhte sich die Carbachol sensitive Mikrogliasubpopulation signifikant um 16 – 60% und im Alzheimer modell APPPS1 signifikant um 25%.

Den gleichen Effekt erreichten wir nach der Stimulation von kultivierten neonatalen und adulten Mirkogliazellen mit IFN-γ, wobei die Carbachol sensitive Mikrogliasubpopulation um 63%, bzw. 62% zunahm. In kultivierten neonatalen Mikroglia konnte die Auswirkung der Stimulation mit IFN-γ durch Unterbinden der Proteinsynthese verhindert werden. In weiteren Untersuchungen konnten wir nachweisen, dass Carbachol sowohl als Chemoattractant wirkt, als auch die Phagozytoseaktivität hemmt. Mit der quantitativen PCR konnten wir zeigen, dass die Stimulation mit IFN-γ sowohl in kultivierten Mikroglianeonatalen als auch adulten Ursprungs die Expression vom „calciumlinked M3 receptor“ erhöht.
Die Ergebnisse unserer Studie deuten auf eine sehr heterogene Mikrogliapopulation hin, in der sich die Subpopulationen in ihren Reaktionen auf Neurotransmitter/-hormoneunterscheiden. Des Weiteren konnten wir zeigen, dass Mikroglia unter pathologischen Bedingung eine erhöhte Sensibilität ausweisen.

8. References

Abraham, J., Fox, P.D., Condello, C., Bartolini, A., and Koh, S. (2012). Minocycline attenuates microglia activation and blocks the long-term epileptogenic effects of early-life seizures. Neurobiol Dis *46*, 425-430.

Arias-Montano, J.A., Floran, B., Floran, L., Aceves, J., and Young, J.M. (2007). Dopamine D(1) receptor facilitation of depolarization-induced release of gamma-amino-butyric acid in rat striatum is mediated by the cAMP/PKA pathway and involves P/Q-type calcium channels. Synapse *61*, 310-319.

Armstrong, R.A. (2013). What causes alzheimer's disease? Folia Neuropathol *51*, 169-188.

Bader, M.F., Taupenot, L., Ulrich, G., Aunis, D., and Ciesielski-Treska, J. (1994). Bacterial endotoxin induces [Ca2+]i transients and changes the organization of actin in microglia. Glia *11*, 336-344.

Bagorda, A., and Parent, C.A. (2008). Eukaryotic chemotaxis at a glance. J Cell Sci *121*, 2621-2624.

Baranzini, S.E., Bernard, C.C., and Oksenberg, J.R. (2005). Modular transcriptional activity characterizes the initiation and progression of autoimmune encephalomyelitis. J Immunol *174*, 7412-7422.

Bard, F., Cannon, C., Barbour, R., Burke, R.L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K.*, et al.* (2000). Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat Med *6*, 916-919.

Barron, K.D. (1995). The microglial cell. A historical review. J Neurol Sci *134 Suppl*, 57-68.

Battisti, W.P., Wang, J., Bozek, K., and Murray, M. (1995). Macrophages, microglia, and astrocytes are rapidly activated after crush injury of the goldfish optic nerve: a light and electron microscopic analysis. J Comp Neurol *354*, 306-320.

Biber, K., Neumann, H., Inoue, K., and Boddeke, H.W.G.M. (2007). Neuronal 'On' and 'Off' signals control microglia. Trends in Neurosciences *30*, 596-602.

Biber, K., Sauter, A., Brouwer, N., Copray, S.C., and Boddeke, H.W. (2001). Ischemia-induced neuronal expression of the microglia attracting chemokine Secondary Lymphoid-tissue Chemokine (SLC). Glia *34*, 121-133.

Bohnen, N.I., and Albin, R.L. (2011). The cholinergic system and Parkinson disease. Behav Brain Res *221*, 564-573.

Bolmont, T., Haiss, F., Eicke, D., Radde, R., Mathis, C.A., Klunk, W.E., Kohsaka, S., Jucker, M., and Calhoun, M.E. (2008). Dynamics of the microglial/amyloid interaction indicate a role in plaque maintenance. J Neurosci *28*, 4283-4292.

Boucsein, C., Zacharias, R., FÃ¤rber, K., Pavlovic, S., Hanisch, U.-K., and Kettenmann, H. (2003). Purinergic receptors on microglial cells: functional expression in acute brain slices and modulation of microglial activation in vitro. The European Journal of Neuroscience *17*, 2267-2276.

Brookmeyer, R., Johnson, E., Ziegler-Graham, K., and Arrighi, H.M. (2007). Forecasting the global burden of Alzheimer's disease. Alzheimers Dement *3*, 186-191.

Brown, R.E., Stevens, D.R., and Haas, H.L. (2001). The physiology of brain histamine. Prog Neurobiol *63*, 637-672.

Carlsson, A., Lindqvist, M., and Magnusson, T. (1957). 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. Nature *180*, 1200.

Carter, D.A., Balasubramaniam, B., and Dick, A.D. (2013). Functional analysis of retinal microglia and their effects on progenitors. Methods Mol Biol *935*, 271-283.

Carthew, H.L., Ziebell, J.M., and Vink, R. (2012). Substance P-induced changes in cell genesis following diffuse traumatic brain injury. Neuroscience *214*, 78-83.

Chari, D.M. (2007). Remyelination in multiple sclerosis. Int Rev Neurobiol *79*, 589-620.

Chen, J., McKay, R.M., and Parada, L.F. (2012). Malignant glioma: lessons from genomics, mouse models, and stem cells. Cell *149*, 36-47.

Chodobski, A., Zink, B.J., and Szmydynger-Chodobska, J. (2011). Blood-brain barrier pathophysiology in traumatic brain injury. Transl Stroke Res *2*, 492-516.

Compston, A., and Coles, A. (2002). Multiple sclerosis. Lancet *359*, 1221-1231.

Compston, A., and Coles, A. (2008). Multiple sclerosis. Lancet *372*, 1502-1517.

Dale, H. (1934). Chemical Transmission of the Effects of Nerve Impulses. Br Med J *1*, 835-841.

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

De Simone, R., Ajmone-Cat, M.A., Carnevale, D., and Minghetti, L. (2005). Activation of alpha7 nicotinic acetylcholine receptor by nicotine selectively up-regulates cyclooxygenase-2 and prostaglandin E2 in rat microglial cultures. J Neuroinflammation *2*, 4.

Del Rio-Hortega, P. (1932). Cytology and Cellular Pathology of the Nervous System, Vol 2 (New York, NY, USA: P. B. Hoeber).

Desjardins, M., Huber, L.A., Parton, R.G., and Griffiths, G. (1994). Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J Cell Biol *124*, 677-688.

Dicou, E., Vincent, J.P., and Mazella, J. (2004). Neurotensin receptor-3/sortilin mediates neurotensin-induced cytokine/chemokine expression in a murine microglial cell line. J Neurosci Res *78*, 92-99.

Dluzen, D. (1997). Estrogen decreases corpus striatal neurotoxicity in response to 6-hydroxydopamine. Brain Res *767*, 340-344.

Eglen, R.M., Choppin, A., and Watson, N. (2001). Therapeutic opportunities from muscarinic receptor research. Trends Pharmacol Sci *22*, 409-414.

Eichhoff, G., Brawek, B., and Garaschuk, O. (2011). Microglial calcium signal acts as a rapid sensor of single neuron damage in vivo. Biochim Biophys Acta *1813*, 1014-1024.

Endres, M., Biniszkiewicz, D., Sobol, R.W., Harms, C., Ahmadi, M., Lipski, A., Katchanov, J., Mergenthaler, P., Dirnagl, U., Wilson, S.H.*, et al.* (2004).

Increased postischemic brain injury in mice deficient in uracil-DNA glycosylase. The Journal of clinical investigation *113*, 1711-1721.

Fan, R., Xu, F., Previti, M.L., Davis, J., Grande, A.M., Robinson, J.K., and Van Nostrand, W.E. (2007). Minocycline reduces microglial activation and improves behavioral deficits in a transgenic model of cerebral microvascular amyloid. J Neurosci *27*, 3057-3063.

Farber, K., and Kettenmann, H. (2006). Functional role of calcium signals for microglial function. Glia *54*, 656-665.

Farber, K., Pannasch, U., and Kettenmann, H. (2005). Dopamine and noradrenaline control distinct functions in rodent microglial cells. Mol Cell Neurosci *29*, 128-138.

Feindt, J., Schmidt, A., and Mentlein, R. (1998). Receptors and effects of the inhibitory neuropeptide somatostatin in microglial cells. Brain Research. Molecular Brain Research *60*, 228-233.

Ferreira, R., Santos, T., Goncalves, J., Baltazar, G., Ferreira, L., Agasse, F., and Bernardino, L. (2012). Histamine modulates microglia function. J Neuroinflammation *9*, 90.

Fleisher-Berkovich, S., Filipovich-Rimon, T., Ben-Shmuel, S., Hulsmann, C., Kummer, M.P., and Heneka, M.T. (2010). Distinct modulation of microglial amyloid beta phagocytosis and migration by neuropeptides (i). J Neuroinflammation *7*, 61.

Fonseca, A.C., Romao, L., Amaral, R.F., Assad Kahn, S., Lobo, D., Martins, S., Marcondes de Souza, J., Moura-Neto, V., and Lima, F.R. (2012). Microglial stress inducible protein 1 promotes proliferation and migration in human glioblastoma cells. Neuroscience *200*, 130-141.

Francis, P.T., Palmer, A.M., Snape, M., and Wilcock, G.K. (1999). The cholinergic hypothesis of Alzheimer's disease: a review of progress. J Neurol Neurosurg Psychiatry *66*, 137-147.

Frei, K., Siepl, C., Groscurth, P., Bodmer, S., Schwerdel, C., and Fontana, A. (1987). Antigen presentation and tumor cytotoxicity by interferon-gamma-treated microglial cells. Eur J Immunol *17*, 1271-1278.

Garbarg, M., Barbin, G., Bischoff, S., Pollard, H., and Schwartz, J.C. (1976). Dual localization of histamine in an ascending neuronal pathway and in non-neuronal cells evidenced by lesions in the lateral hypothalamic area. Brain research *106*, 333-348.

Giulian, D., and Baker, T.J. (1986). Characterization of ameboid microglia isolated from developing mammalian brain. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience *6*, 2163-2178.

Griffin, W.S., Stanley, L.C., Ling, C., White, L., MacLeod, V., Perrot, L.J., White, C.L., 3rd, and Araoz, C. (1989). Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. Proc Natl Acad Sci U S A *86*, 7611-7615.

Grinshpun, J., Tveria, L., and Fleisher-Berkovich, S. (2008). Differential regulation of prostaglandin synthesis in neonatal rat microglia and astrocytes by somatostatin. Eur J Pharmacol *584*, 312-317.

Hama, H., Kasuya, Y., Sakurai, T., Yamada, G., Suzuki, N., Masaki, T., and Goto, K. (1997). Role of endothelin-1 in astrocyte responses after acute brain damage. J Neurosci Res *47*, 590-602.

Hanisch, U.-K., and Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nature Neuroscience *10*, 1387-1394.

Hayakawa, K., Mishima, K., Nozako, M., Hazekawa, M., Mishima, S., Fujioka, M., Orito, K., Egashira, N., Iwasaki, K., and Fujiwara, M. (2008). Delayed treatment with minocycline ameliorates neurologic impairment through activated microglia expressing a high-mobility group box1-inhibiting mechanism. Stroke *39*, 951-958.

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

Himmelheber, A.M., Sarter, M., and Bruno, J.P. (2000). Increases in cortical acetylcholine release during sustained attention performance in rats. Brain Res Cogn Brain Res *9*, 313-325.

Ho, G.J., Drego, R., Hakimian, E., and Masliah, E. (2005). Mechanisms of cell signaling and inflammation in Alzheimer's disease. Curr Drug Targets Inflamm Allergy *4*, 247-256.

Hoek, R.M., Ruuls, S.R., Murphy, C.A., Wright, G.J., Goddard, R., Zurawski, S.M., Blom, B., Homola, M.E., Streit, W.J., Brown, M.H.*, et al.* (2000). Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science *290*, 1768-1771.

Hosomi, N., Mizushige, K., Kitadai, M., Ohyama, H., Ichihara, S.I., Takahashi, T., and Matsuo, H. (1999). Induced hypertension treatment to improve cerebral ischemic injury after transient forebrain ischemia. Brain Res *835*, 188-196.

Howell, O.W., Rundle, J.L., Garg, A., Komada, M., Brophy, P.J., and Reynolds, R. (2010). Activated microglia mediate axoglial disruption that contributes to axonal injury in multiple sclerosis. J Neuropathol Exp Neurol *69*, 1017-1033.

Hsieh, C.L., Koike, M., Spusta, S.C., Niemi, E.C., Yenari, M., Nakamura, M.C., and Seaman, W.E. (2009). A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. J Neurochem *109*, 1144-1156.

Hu, X., Li, P., Guo, Y., Wang, H., Leak, R.K., Chen, S., Gao, Y., and Chen, J. (2012). Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke *43*, 3063-3070.

Huang, Y., and Mucke, L. (2012). Alzheimer mechanisms and therapeutic strategies. Cell *148*, 1204-1222.

Huettner, C., Czub, S., Kerkau, S., Roggendorf, W., and Tonn, J.C. (1997). Interleukin 10 is expressed in human gliomas in vivo and increases glioma cell proliferation and motility in vitro. Anticancer Res *17*, 3217-3224.

Huizinga, R., van der Star, B.J., Kipp, M., Jong, R., Gerritsen, W., Clarner, T., Puentes, F., Dijkstra, C.D., van der Valk, P., and Amor, S. (2012). Phagocytosis of neuronal debris by microglia is associated with neuronal damage in multiple sclerosis. Glia *60*, 422-431.

Hur, J., Lee, P., Kim, M.J., Kim, Y., and Cho, Y.W. (2010). Ischemia-activated microglia induces neuronal injury via activation of gp91phox NADPH oxidase. Biochem Biophys Res Commun *391*, 1526-1530.

Hussain, S.F., Yang, D., Suki, D., Aldape, K., Grimm, E., and Heimberger, A.B. (2006). The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses. Neuro Oncol *8*, 261-279.

Ifuku, M., Okuno, Y., Yamakawa, Y., Izumi, K., Seifert, S., Kettenmann, H., and Noda, M. (2011). Functional importance of inositol-1,4,5-triphosphate-induced intracellular Ca(2+) mobilization in galanin-induced microglial migration. Journal of Neurochemistry.

Irino, Y., Nakamura, Y., Inoue, K., Kohsaka, S., and Ohsawa, K. (2008). Akt activation is involved in P2Y12 receptor-mediated chemotaxis of microglia. J Neurosci Res *86*, 1511-1519.

Iwai, M., Liu, H.W., Chen, R., Ide, A., Okamoto, S., Hata, R., Sakanaka, M., Shiuchi, T., and Horiuchi, M. (2004). Possible inhibition of focal cerebral ischemia by angiotensin II type 2 receptor stimulation. Circulation *110*, 843-848.

Jack, C., Ruffini, F., Bar-Or, A., and Antel, J.P. (2005). Microglia and multiple sclerosis. J Neurosci Res *81*, 363-373.

Jana, M., Palencia, C.A., and Pahan, K. (2008). Fibrillar amyloid-beta peptides activate microglia via TLR2: implications for Alzheimer's disease. J Immunol *181*, 7254-7262.

Jones, B.E. (2005). From waking to sleeping: neuronal and chemical substrates. Trends Pharmacol Sci *26*, 578-586.

Kataoka, K., Hayakawa, T., Kuroda, R., Yuguchi, T., and Yamada, K. (1991). Cholinergic deafferentation after focal cerebral infarct in rats. Stroke *22*, 1291-1296.

Kaushal, V., and Schlichter, L.C. (2008). Mechanisms of microglia-mediated neurotoxicity in a new model of the stroke penumbra. J Neurosci *28*, 2221-2230.

Kettenmann, H., Hanisch, U.-K., Noda, M., and Verkhratsky, A. (2011). Physiology of microglia. Physiological reviews *91*, 461-553.

Kettenmann, H., Kirchhoff, F., and Verkhratsky, A. (2013). Microglia: New Roles for the Synaptic Stripper. Neuron *77*, 10-18.

Khimji, A.K., and Rockey, D.C. (2010). Endothelin--biology and disease. Cell Signal *22*, 1615-1625.

Kinchen, J.M., and Ravichandran, K.S. (2008). Phagosome maturation: going through the acid test. Nat Rev Mol Cell Biol *9*, 781-795.

Koenigsknecht, J., and Landreth, G. (2004). Microglial phagocytosis of fibrillar beta-amyloid through a beta1 integrin-dependent mechanism. J Neurosci *24*, 9838-9846.

Koizumi, S., Shigemoto-Mogami, Y., Nasu-Tada, K., Shinozaki, Y., Ohsawa, K., Tsuda, M., Joshi, B.V., Jacobson, K.A., Kohsaka, S., and Inoue, K. (2007). UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis. Nature *446*, 1091-1095.

Konnecke, H., and Bechmann, I. (2013). The Role of Microglia and Matrix Metalloproteinases Involvement in Neuroinflammation and Gliomas. Clin Dev Immunol *2013*, 914104.

Krabbe, G., Halle, A., Matyash, V., Rinnenthal, J.L., Eom, G.D., Bernhardt, U., Miller, K.R., Prokop, S., Kettenmann, H., and Heppner, F.L. (2013). Functional impairment of microglia coincides with Beta-amyloid deposition in mice with Alzheimer-like pathology. PLoS One *8*, e60921.

Krabbe, G., Matyash, V., Pannasch, U., Mamer, L., Boddeke, H.W.G.M., and Kettenmann, H. (2012). Activation of serotonin receptors promotes microglial injury-induced motility but attenuates phagocytic activity. Brain, Behavior, and Immunity *26*, 419-428.

Kreutzberg, G.W. (1996). Microglia: a sensor for pathological events in the CNS. Trends Neurosci *19*, 312-318.

Kuhn, S.A., van Landeghem, F.K.H., Zacharias, R., FÃ¤rber, K., Rappert, A., Pavlovic, S., Hoffmann, A., Nolte, C., and Kettenmann, H. (2004). Microglia express GABA(B) receptors to modulate interleukin release. Molecular and cellular neurosciences *25*, 312-322.

Lai, J.P., Zhan, G.X., Campbell, D.E., Douglas, S.D., and Ho, W.Z. (2000). Detection of substance P and its receptor in human fetal microglia. Neuroscience *101*, 1137-1144.

Lampl, Y., Fleminger, G., Gilad, R., Galron, R., Sarova-Pinhas, I., and Sokolovsky, M. (1997). Endothelin in cerebrospinal fluid and plasma of patients in the early stage of ischemic stroke. Stroke *28*, 1951-1955.

Lanz, T.V., Ding, Z., Ho, P.P., Luo, J., Agrawal, A.N., Srinagesh, H., Axtell, R., Zhang, H., Platten, M., Wyss-Coray, T., and Steinman, L. (2010). Angiotensin II sustains brain inflammation in mice via TGF-beta. J Clin Invest *120*, 2782-2794.

Leurs, R., Traiffort, E., Arrang, J.M., Tardivel-Lacombe, J., Ruat, M., and Schwartz, J.C. (1994). Guinea pig histamine H1 receptor. II. Stable expression in Chinese hamster ovary cells reveals the interaction with three major signal transduction pathways. J Neurochem *62*, 519-527.

Li, J.J., Wu, L.H., Cao, Q., Yuan, Y., Yang, L., Guo, Z.Y., Kaur, C., Sivakumar, V., Ling, E.A., and Wu, C.Y. Endothelins-1/3 and endothelin-A/B receptors expressing glial cells with special reference to activated microglia in experimentally induced cerebral ischemia in the adult rats. Neuroscience *167*, 665-677.

Liu, J., McGlinn, A.M., Fernandes, A., Milam, A.H., Strang, C.E., Andison, M.E., Lindstrom, J.M., Keyser, K.T., and Stone, R.A. (2009). Nicotinic acetylcholine receptor subunits in rhesus monkey retina. Invest Ophthalmol Vis Sci *50*, 1408-1415.

Loewi, O. (1924). Uber humorale Ubertragbarkeit der Herznervenwirkung. Pfluger's Archiv fur die gesamte Physiologie des Menschen und der Tiere *204*, 629-640.

Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M., and Lassmann, H. (1999). A quantitative analysis of oligodendrocytes in multiple sclerosis lesions. A study of 113 cases. Brain *122 (Pt 12)*, 2279-2295.

Maeda, M., Tsuda, M., Tozaki-Saitoh, H., Inoue, K., and Kiyama, H. (2010). Nerve injury-activated microglia engulf myelinated axons in a P2Y12 signaling-dependent manner in the dorsal horn. Glia *58*, 1838-1846.

Mantyh, P.W. (2002). Neurobiology of substance P and the NK1 receptor. J Clin Psychiatry *63 Suppl 11*, 6-10.

Marim, F.M., Silveira, T.N., Lima, D.S., and Zamboni, D.S. (2010). A Method for Generation of Bone Marrow-Derived Macrophages from Cryopreserved Mouse Bone Marrow Cells. PLoS ONE *5*.

Markovic, D.S., Glass, R., Synowitz, M., Rooijen, N., and Kettenmann, H. (2005). Microglia stimulate the invasiveness of glioma cells by increasing the activity of metalloprotease-2. J Neuropathol Exp Neurol *64*, 754-762.

Markovic, D.S., Vinnakota, K., Chirasani, S., Synowitz, M., Raguet, H., Stock, K., Sliwa, M., Lehmann, S., Kalin, R., van Rooijen, N.*, et al.* (2009). Gliomas induce and exploit microglial MT1-MMP expression for tumor expansion. Proc Natl Acad Sci U S A *106*, 12530-12535.

Martin, F.C., Anton, P.A., Gornbein, J.A., Shanahan, F., and Merrill, J.E. (1993). Production of interleukin-1 by microglia in response to substance P: role for a non-classical NK-1 receptor. J Neuroimmunol *42*, 53-60.

Martin, S., Dicou, E., Vincent, J.P., and Mazella, J. (2005). Neurotensin and the neurotensin receptor-3 in microglial cells. J Neurosci Res *81*, 322-326.

Martin, S., Vincent, J.P., and Mazella, J. (2003). Involvement of the neurotensin receptor-3 in the neurotensin-induced migration of human microglia. J Neurosci *23*, 1198-1205.

Mastroeni, D., Grover, A., Leonard, B., Joyce, J.N., Coleman, P.D., Kozik, B., Bellinger, D.L., and Rogers, J. (2009). Microglial responses to dopamine in a cell culture model of Parkinson's disease. Neurobiol Aging *30*, 1805-1817.

McGeer, P.L., Itagaki, S., Tago, H., and McGeer, E.G. (1987). Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. Neurosci Lett *79*, 195-200.

McKenzie, A.L., Hall, J.J., Aihara, N., Fukuda, K., and Noble, L.J. (1995). Immunolocalization of endothelin in the traumatized spinal cord: relationship to blood-spinal cord barrier breakdown. J Neurotrauma *12*, 257-268.

McLarnon, J.G., Wang, X., Bae, J.H., and Kim, S.U. (1999). Endothelin-induced changes in intracellular calcium in human microglia. Neurosci Lett *263*, 9-12.

Mechenthaler, I. (2008). Galanin and the neuroendocrine axes. Cell Mol Life Sci *65*, 1826-1835.

Miyoshi, M., Miyano, K., Moriyama, N., Taniguchi, M., and Watanabe, T. (2008). Angiotensin type 1 receptor antagonist inhibits lipopolysaccharide-induced stimulation of rat microglial cells by suppressing nuclear factor κB and activator protein-1 activation. European Journal of Neuroscience *27*, 343-351.

Moeller, T. (2002). Calcium signaling in microglial cells. Glia *40*, 184-194.

Moeller, T., Kann, O., Prinz, M., Kirchhoff, F., Verkhratsky, A., and Kettenmann, H. (1997). Endothelin-induced calcium signaling in cultured mouse microglial cells is mediated through ETB receptors. Neuroreport *8*, 2127-2131.

Mohammad-Zadeh, L.F., Moses, L., and Gwaltney-Brant, S.M. (2008). Serotonin: a review. J Vet Pharmacol Ther *31*, 187-199.

Moldes, O., Sobrino, T., Blanco, M., Agulla, J., Barral, D., Ramos-Cabrer, P., and Castillo, J. (2012). Neuroprotection afforded by antagonists of endothelin-1 receptors in experimental stroke. Neuropharmacology *63*, 1279-1285.

Muhvic, D., Barac-Latas, V., Rukavina, D., and Radosevic-Stasic, B. (2005). Induction of experimental allergic encephalomyelitis in a low-susceptible Albino Oxford rat strain by somatostatin analogue SMS 201-995. Neuroimmunomodulation *12*, 20-28.

Neher, J.J., Neniskyte, U., and Brown, G.C. (2012). Primary phagocytosis of neurons by inflamed microglia: potential roles in neurodegeneration. Front Pharmacol *3*, 27.

Neumann, J., Gunzer, M., Gutzeit, H.O., Ullrich, O., Reymann, K.G., and Dinkel, K. (2006). Microglia provide neuroprotection after ischemia. FASEB J *20*, 714-716.

Nikodemova, M., and Watters, J.J. (2012). Efficient isolation of live microglia with preserved phenotypes from adult mouse brain. J Neuroinflammation *9*, 147.

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma in Vivo. Science *308*, 1314-1318.

Noda, M., Doi, Y., Liang, J., Kawanokuchi, J., Sonobe, Y., Takeuchi, H., Mizuno, T., and Suzumura, A. (2011). Fractalkine attenuates excito-neurotoxicity via

microglial clearance of damaged neurons and antioxidant enzyme heme oxygenase-1 expression. J Biol Chem *286*, 2308-2319.

Offner, H., and Polanczyk, M. (2006). A potential role for estrogen in experimental autoimmune encephalomyelitis and multiple sclerosis. Ann N Y Acad Sci *1089*, 343-372.

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

Paolillo, M., Russo, M.A., Curti, D., Lanni, C., and Schinelli, S. (2010). Endothelin B receptor antagonists block proliferation and induce apoptosis in glioma cells. Pharmacol Res *61*, 306-315.

Passani, M.B., Blandina, P., and Torrealba, F. (2011). The histamine H3 receptor and eating behavior. J Pharmacol Exp Ther *336*, 24-29.

Patel, Y.C. (1999). Somatostatin and Its Receptor Family. Frontiers in Neuroendocrinology *20*, 157-198.

Phillips, M.I. (1987). Functions of angiotensin in the central nervous system. Annu Rev Physiol *49*, 413-435.

Pocock, J.M., and Kettenmann, H. (2007). Neurotransmitter receptors on microglia. Trends in Neurosciences *30*, 527-535.

Pollock, D.M., Keith, T.L., and Highsmith, R.F. (1995). Endothelin receptors and calcium signaling. The FASEB Journal *9*, 1196-1204.

Radde, R., Bolmont, T., Kaeser, S.A., Coomaraswamy, J., Lindau, D., Stoltze, L., Calhoun, M.E., Jaggi, F., Wolburg, H., Gengler, S.*, et al.* (2006). Abeta42-driven cerebral amyloidosis in transgenic mice reveals early and robust pathology. EMBO Rep *7*, 940-946.

Rasley, A., Bost, K.L., Olson, J.K., Miller, S.D., and Marriott, I. (2002). Expression of functional NK-1 receptors in murine microglia. Glia *37*, 258-267.

Ravichandran, K.S. (2010). Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. J Exp Med *207*, 1807-1817.

Ravichandran, K.S. (2011). Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. Immunity *35*, 445-455.

Ribes, S., Ebert, S., Regen, T., Agarwal, A., Tauber, S.C., Czesnik, D., Spreer, A., Bunkowski, S., Eiffert, H., Hanisch, U.K.*, et al.* (2009). Toll-like receptor stimulation enhances phagocytosis and intracellular killing of nonencapsulated and encapsulated Streptococcus pneumoniae by murine microglia. Infect Immun *78*, 865-871.

Ribes, S., Riegelmann, J., Redlich, S., Maestro, B., de Waal, B., Meijer, E.W., Sanz, J.M., and Nau, R. (2013). Multivalent Choline Dendrimers Increase Phagocytosis of Streptococcus pneumoniae R6 by Microglial Cells. Chemotherapy *59*, 138-142.

Rinne, J.O., Ma, S.Y., Lee, M.S., Collan, Y., and Roytta, M. (2008). Loss of cholinergic neurons in the pedunculopontine nucleus in Parkinson's disease is related to disability of the patients. Parkinsonism Relat Disord *14*, 553-557.

Rock, R.B., Gekker, G., Aravalli, R.N., Hu, S., Sheng, W.S., and Peterson, P.K. (2008). Potentiation of HIV-1 expression in microglial cells by nicotine: involvement of transforming growth factor-beta 1. J Neuroimmune Pharmacol *3*, 143-149.

Rogers, S.D., Demaster, E., Catton, M., Ghilardi, J.R., Levin, L.A., Maggio, J.E., and Mantyh, P.W. (1997). Expression of Endothelin-B Receptors by Gliain Vivols Increased after CNS Injury in Rats, Rabbits, and Humans. Experimental Neurology *145*, 180-195.

Rosamond, W., Flegal, K., Friday, G., Furie, K., Go, A., Greenlund, K., Haase, N., Ho, M., Howard, V., Kissela, B.*, et al.* (2007). Heart disease and stroke statistics--2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation *115*, e69-171.

Rowntree, D.W., Nevin, S., and Wilson, A. (1950). The effects of diisopropylfluorophosphonate in schizophrenia and manic depressive psychosis. J Neurol Neurosurg Psychiatry *13*, 47-62.

Savill, J., Dransfield, I., Gregory, C., and Haslett, C. (2002). A blast from the past: clearance of apoptotic cells regulates immune responses. Nat Rev Immunol *2*, 965-975.

Scarr, E., and Dean, B. (2009). Role of the cholinergic system in the pathology and treatment of schizophrenia. Expert Rev Neurother *9*, 73-86.

Schafer, D.P., and Stevens, B. (2010). Synapse elimination during development and disease: immune molecules take centre stage. Biochem Soc Trans *38*, 476-481.

Scheffel, J.r., Regen, T., Van Rossum, D., Seifert, S., Ribes, S., Nau, R., Parsa, R., Harris, R.A., Boddeke, H.W.G.M., Chuang, H.-N.*, et al.* (2012). Toll-like receptor activation reveals developmental reorganization and unmasks responder subsets of microglia. Glia *60*, 1930-1943.

Schrader, J., Luders, S., Kulschewski, A., Hammersen, F., Plate, K., Berger, J., Zidek, W., Dominiak, P., and Diener, H.C. (2005). Morbidity and Mortality After Stroke, Eprosartan Compared with Nitrendipine for Secondary Prevention: principal results of a prospective randomized controlled study (MOSES). Stroke *36*, 1218-1226.

Seifert, S., Pannell, M., Uckert, W., FÃ¤rber, K., and Kettenmann, H. (2011). Transmitter- and hormone-activated Ca(2+) responses in adult microglia/brain macrophages in situ recorded after viral transduction of a recombinant Ca(2+) sensor. Cell Calcium *49*, 365-375.

Shaked, I., Tchoresh, D., Gersner, R., Meiri, G., Mordechai, S., Xiao, X., Hart, R.P., and Schwartz, M. (2005). Protective autoimmunity: interferon-gamma enables microglia to remove glutamate without evoking inflammatory mediators. J Neurochem *92*, 997-1009.

Shin, T., Kang, B., Tanuma, N., Matsumoto, Y., Wie, M., Ahn, M., and Kang, J. (2001). Intrathecal administration of endothelin-1 receptor antagonist ameliorates autoimmune encephalomyelitis in Lewis rats. Neuroreport *12*, 1465-1468.

Shuaib, A., Xu Wang, C., Yang, T., and Noor, R. (2002). Effects of nonpeptide V(1) vasopressin receptor antagonist SR-49059 on infarction volume and recovery of function in a focal embolic stroke model. Stroke *33*, 3033-3037.

Shulga, O., and Bornstein, N. (2011). Antiplatelets in secondary stroke prevention. Front Neurol *2*, 36.

Shytle, R.D., Mori, T., Townsend, K., Vendrame, M., Sun, N., Zeng, J., Ehrhart, J., Silver, A.A., Sanberg, P.R., and Tan, J. (2004). Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. J Neurochem *89*, 337-343.

Sierra, A., Abiega, O., Shahraz, A., and Neumann, H. (2013). Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis. Front Cell Neurosci *7*, 6.

Simpkins, J.W., Rajakumar, G., Zhang, Y.Q., Simpkins, C.E., Greenwald, D., Yu, C.J., Bodor, N., and Day, A.L. (1997). Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. J Neurosurg *87*, 724-730.

Sloka, S., Metz, L.M., Hader, W., Starreveld, Y., and Yong, V.W. (2013). Reduction of microglial activity in a model of multiple sclerosis by dipyridamole. J Neuroinflammation *10*, 89.

Sohrabji, F., and Williams, M. (2013). Stroke neuroprotection: Estrogen and IGF-1 interactions and the role of microglia. J Neuroendocrinol.

St-Gelais, F., Jomphe, C., and Trudeau, L.E. (2006). The role of neurotensin in central nervous system pathophysiology: what is the evidence? J Psychiatry Neurosci *31*, 229-245.

Stegbauer, J., Lee, D.H., Seubert, S., Ellrichmann, G., Manzel, A., Kvakan, H., Muller, D.N., Gaupp, S., Rump, L.C., Gold, R., and Linker, R.A. (2009). Role of the renin-angiotensin system in autoimmune inflammation of the central nervous system. Proc Natl Acad Sci U S A *106*, 14942-14947.

Stumm, R.K., Zhou, C., Schulz, S., Endres, M., Kronenberg, G., Allen, J.P., Tulipano, G., and Hollt, V. (2004). Somatostatin receptor 2 is activated in cortical neurons and contributes to neurodegeneration after focal ischemia. J Neurosci *24*, 11404-11415.

Suzuki, N., Matsumoto, H., Kitada, C., Yanagisawa, M., Miyauchi, T., Masaki, T., and Fujino, M. (1989). Immunoreactive endothelin-1 in plasma detected by a sandwich-type enzyme immunoassay. J Cardiovasc Pharmacol *13 Suppl 5*, S151-152.

Szmydynger-Chodobska, J., Zink, B.J., and Chodobski, A. (2011). Multiple sites of vasopressin synthesis in the injured brain. J Cereb Blood Flow Metab *31*, 47-51.

Szpak, G.M., Lewandowska, E., Lechowicz, W., Wierzba-Bobrowicz, T., Kulczycki, J., Bertrand, E., Pasennik, E., and Dymecki, J. (2006). The brain immune response in human prion diseases. Microglial activation and microglial disease. I. Sporadic Creutzfeldt-Jakob disease. Folia Neuropathol *44*, 202-213.

Taly, A., Corringer, P.J., Guedin, D., Lestage, P., and Changeux, J.P. (2009). Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system. Nat Rev Drug Discov *8*, 733-750.

Taylor, D.L., Jones, F., Kubota, E.S., and Pocock, J.M. (2005). Stimulation of microglial metabotropic glutamate receptor mGlu2 triggers tumor necrosis factor alpha-induced neurotoxicity in concert with microglial-derived Fas ligand. J Neurosci *25*, 2952-2964.

Thiel, A., and Heiss, W.D. (2010). Imaging of microglia activation in stroke. Stroke *42*, 507-512.

Thornton, E., and Vink, R. (2012). Treatment with a substance P receptor antagonist is neuroprotective in the intrastriatal 6-hydroxydopamine model of early Parkinson's disease. PLoS One *7*, e34138.

Togashi, H., Kimura, S., Matsumoto, M., Yoshioka, M., Minami, M., and Saito, H. (1996). Cholinergic changes in the hippocampus of stroke-prone spontaneously hypertensive rats. Stroke *27*, 520-525; discussion 525-526.

Tremblay, M.-Ã.v., and Majewska, A.K. (2011). A role for microglia in synaptic plasticity? Communicative & Integrative Biology *4*, 220-222.

Tundo, G., Ciaccio, C., Sbardella, D., Boraso, M., Viviani, B., Coletta, M., and Marini, S. (2012). Somatostatin modulates insulin-degrading-enzyme metabolism: implications for the regulation of microglia activity in AD. PLoS One *7*, e34376.

Underhill, D.M., and Goodridge, H.S. (2012). Information processing during phagocytosis. Nat Rev Immunol *12*, 492-502.

Vinnakota, K., Hu, F., Ku, M.C., Georgieva, P.B., Szulzewsky, F., Pohlmann, A., Waiczies, S., Waiczies, H., Niendorf, T., Lehnardt, S., *et al.* (2013). Toll-like receptor 2 mediates microglia/brain macrophage MT1-MMP expression and glioma expansion. Neuro Oncol.

Waiczies, H., Millward, J.M., Lepore, S., Infante-Duarte, C., Pohlmann, A., Niendorf, T., and Waiczies, S. (2012). Identification of Cellular Infiltrates during Early Stages of Brain Inflammation with Magnetic Resonance Microscopy. PLoS ONE *7*.

Wen, P.Y., and Kesari, S. (2008). Malignant gliomas in adults. N Engl J Med *359*, 492-507.

Wess, J.r., Eglen, R.M., and Gautam, D. (2007). Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nature reviews. Drug discovery *6*, 721-733.

Whittemore, E.R., Korotzer, A.R., Etebari, A., and Cotman, C.W. (1993). Carbachol increases intracellular free calcium in cultured rat microglia. Brain research *621*, 59-64.

Wiggins, H., and Rappoport, J. (2010). An agarose spot assay for chemotactic invasion. Biotechniques *48*, 121-124.

Wraith, D.C., Pope, R., Butzkueven, H., Holder, H., Vanderplank, P., Lowrey, P., Day, M.J., Gundlach, A.L., Kilpatrick, T.J., Scolding, N., and Wynick, D. (2009). A role for galanin in human and experimental inflammatory demyelination. Proc Natl Acad Sci U S A *106*, 15466-15471.

Wright, G.J., Puklavec, M.J., Willis, A.C., Hoek, R.M., Sedgwick, J.D., Brown, M.H., and Barclay, A.N. (2000). Lymphoid/neuronal cell surface OX2 glycoprotein recognizes a novel receptor on macrophages implicated in the control of their function. Immunity *13*, 233-242.

Wright, J.W., and Harding, J.W. (2013). The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases. Pflugers Arch *465*, 133-151.

Xu, Y., Xue, Y., Wang, Y., Feng, D., Lin, S., and Xu, L. (2009). Multiple-modulation effects of Oridonin on the production of proinflammatory cytokines and

neurotrophic factors in LPS-activated microglia. Int Immunopharmacol *9*, 360-365.

Yanagisawa, M., Kurihara, H., Kimura, S., Goto, K., and Masaki, T. (1988). A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle Ca2+ channels. J Hypertens Suppl *6*, S188-191.

Yoshizawa, T., Shinmi, O., Giaid, A., Yanagisawa, M., Gibson, S.J., Kimura, S., Uchiyama, Y., Polak, J.M., Masaki, T., and Kanazawa, I. (1990). Endothelin: a novel peptide in the posterior pituitary system. Science *247*, 462-464.

Zhang, L., McLarnon, J.G., Goghari, V., Lee, Y.B., Kim, S.U., and Krieger, C. (1998). Cholinergic agonists increase intracellular Ca2+ in cultured human microglia. Neuroscience letters *255*, 33-36.

Zhang, W., Wang, T., Pei, Z., Miller, D.S., Wu, X., Block, M.L., Wilson, B., Zhou, Y., Hong, J.S., and Zhang, J. (2005). Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. FASEB J *19*, 533-542.

Zhang, X., Goncalves, R., and Mosser, D.M. (2008). The Isolation and Characterization of Murine Macrophages. Current protocols in immunology / edited by John E. Coligan ... [et al.] *CHAPTER*, Unit-14.11.

9. Acknowledgments

I would first like to thank Professor Helmut Kettenmann for the opportunity to work on a fantastic project in such a great lab. I am very grateful for all the invaluable support and supervision which I received for this project from Prof. Kettenmann, Dr Susanne Wolf, Dr Vitali Matyash and Dr Marina Matyash. Special thanks to the wonderful technicians, Regina Piske and Irene Haupt who provided excellent technical assistance during my research and thanks to Frank Heppner, Stefan Prokop, Mathias Jucker, Matthias Endres, Golo Kronenberg, Vincent Prinz and Sonia Waiczies for providing all the mouse models which were a key element of my project. I would also particularly like to thank all my fellow PhD students, without whom this experience would not have been the same. It has been an amazing few years, and all the help and support, both intellectually and socially, has made it incredibly enjoyable. Thanks especially to Frank Szulzewsky, Felipe Sassi, Daniele Mattei, Petya Georgieva, Nadine Richter and Philipp Jordan, who were always available when I needed to drown my sorrows. I love you guys. You are amazing. I would also like to thank all my former colleagues not listed here and my former and current students Maria Almut Meier and Anne Hagen for all their hard work. This thesis is dedicated to my sister Catherine, who has provided overwhelming support during my PhD and has made these last few years in Berlin some of the greatest of my life. Chance made us sisters, hearts made us friends.

10. Appendix

10.1 Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

10.2 Publications

Pannell M, Szulzewsky F, Meier MA, Matyash V, Endres M, Kronenberg G, Prinz V, Waiczies S, Wolf SA and Kettenmann H. The subpopulation of microglia expressing functional muscarinic acetylcholine receptors expands in stroke and Alzheimer's disease
(Manuscript submitted)

Pannell M, Szulzewsky F, Matyash V, Wolf SA and Kettenmann H. 2013. The subpopulation of microglia sensitive to neurotransmitters/-hormones is modulated by stimulation with LPS, Interferon-γ and IL-4**.** Glia. 62(5):667-79

Seifert S, **Pannell M**, Uckert W, Färber K, Kettenmann H. 2011. Transmitter- and hormone-activated Ca(2+) responses in adult microglia/brain macrophages in situ recorded after viral transduction of a recombinant Ca(2+) sensor. Cell Calcium 49(6):365-375.

10.3 Abstracts

Pannell M, Szulzewsky F, Matyash V, Endres M, Heppner FL, Kronenberg G, Prinz V, Prokop S, Waiczies S, Wolf SA, Kettenmann H. The subpopulation of microglia expressing functional muscarinic acetylcholine receptors expands in stroke and Alzheimer's disease. GLIA 61:S49–S216 (2013). Presented as a poster at Euroglia 2013, July 3rd-6th, Berlin, Germany

10.4 Posters

Pannell M, Szulzewsky F, Matyash V, Endres M, Heppner FL, Kronenberg G, Prinz V, Prokop S, Waiczies S, Wolf SA, Kettenmann H. (2013) The subpopulation

of microglia expressing functional muscarinic acetylcholine receptors expands in stroke and Alzheimer's disease. Gordon Research Conference 2013, March 3rd-8th, Ventura, California, USA

Pannell M, Wolf SA, Matyash V, Kettenmann H (2012) Responses of adult and neonatal microglia to neurotransmitters/-hormones. Berlin Neuroscience Forum 2012, July 5th-6th, Liebenwalde, Brandenburg, Germany

Pannell M, Seifert S, Wolf SA, Kettenmann H (2011) Responses of adult and neonatal microglia in vitro to neurotransmitters/-hormones. Berlin Brain Days 2011, December 7th-9th, Berlin, Germany

10.5 Talks

Pannell M, Szulzewsky F, Matyash V, Endres M, Kronenberg G, Prinz V, Waiczies
S, Wolf SA, Kettenmann H (2013) Neurotransmitter/-hormone receptors on microglia. Berlin Brain Days 2013, November 20th-22nd, Berlin, Germany

Pannell M, Wolf SA, Matyash V, Kettenmann H (2012) Neurotransmitter/-hormone receptors on microglia. Berlin Neuroscience Forum 2012, July 5th-6th, Liebenwalde, Brandenburg, Germany

Pannell M, Seifert S, Kettenmann H (2011) Neuropeptides, neurotransmitters and microglial activation. The 13th MDC and FMP joint PhD Retreat 2011, September 1st-3rd, Liebenwalde, Brandenburg, Germany

11. Affidavit

"I, Maria Pannell certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "*Neurotransmitter and neurohormone receptors on microglia*" I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature