
# Microglial properties in health and disease

## Modulation of microglial phagocytic activity and migration by monoamine neurotransmitters or presence of amyloid beta peptides

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# 1 Introduction

## 1.1 Microglia - Immune defense of the brain

The immune system can mount innate and adaptive immune responses. In the healthy central nervous system (CNS), microglial cells are the immune cells belonging to the innate part of the immune system. Therefore, microglia represent the first line of defense in the CNS (Kreutzberg 1996). Microglial immune response can not only be triggered by stimuli entering the brain via the blood brain barrier but also by signals coming from the choroid plexus and circumventricular organs expressing immune molecules like Toll-like receptor 4 (TLR4) and CD14 (Laflamme and Rivest 2001). Upon activation microglia upregulate the expression of surface molecules like CD14, major histocompatibility complex (MHC) and chemokine receptors (Block, Zecca et al. 2007) followed by a complex immune response involving neurotoxic and therefore detrimental or beneficial, neuroprotective signals (Hanisch and Kettenmann 2007). Beneficial functions of microglia include release of trophic and anti-inflammatory factors (Morgan, Taylor et al. 2004; Liao, Bu et al. 2005) and clearance of cellular debris (Tanaka, Ueno et al. 2009). In contrast, microglial cells can exhibit detrimental capacities involving overproduction of neurotoxic factors like nitric oxide, superoxide radicals and tumor necrosis factor alpha (TNF-α) (Colton and Gilbert 1987; Liu, Gao et al. 2002; Bi, Zhu et al. 2011). Hence, microglial activation is not an “all or nothing” process but rather a temporally and spatially well concerted expression of pro- and anti-inflammatory phenotypes (Hanisch and Kettenmann 2007).

### *1.1.1 Origin of microglial cells*

Recently, it was shown that microglial cells derive from primitive myeloid progenitor cells that arise before embryonic day E8 within the yolk sac. These cells migrate from the yolk sac to the brain via blood vessels between E8.5 and E9.5. Primitive myeloid progenitors labeled between E7.25 and E7.5 can still be found as microglia in the adult mouse brain but do not contribute to macrophage pools in other tissues (Ginhoux, Greter et al. 2010). In newborn mice (postnatal day P6-9) one can find amoeboid microglia, that is characterized by enlarged cell somata and short

processes, also called an amoeboid morphology (Figure 1). It is thought that amoeboid microglia populate the brain from the meninges, the choroid plexus and the ventricular zone, they exhibit proliferative capacities, high phagocytic activity and express immune relevant molecules like MHCII (Rezaie and Male 2002; Kaur, Dheen et al. 2007). They accumulate around fiber tracts within the white matter and can be found in clusters in the corpus callosum region. This tight association to fiber tracts suggests an active role in developmental processes like axonal guidance and synaptogenesis (Rezaie and Male 2002; Verney, Monier et al. 2010). During development amoeboid microglia colonize the whole brain and settle as resting, ramified microglia (Figure 1) (Perry, Hume et al. 1985). These cells are characterized by small cell somata and long branched processes. Ramified microglia are distributed equally throughout the whole CNS, occupying non-overlapping territories, and constantly survey their surrounding for concentration changes of endogenous substances released by neighboring cells or the presence of exogenous substrates (Nimmerjahn, Kirchhoff et al. 2005). They express a variety of receptors for cytokines, chemokines and neurotransmitters that enables them to integrate various signals from the microenvironment and process a concerted reaction which depends on the signal reaching the microglia cell (Schwartz 2003; Pocock and Kettenmann 2007).

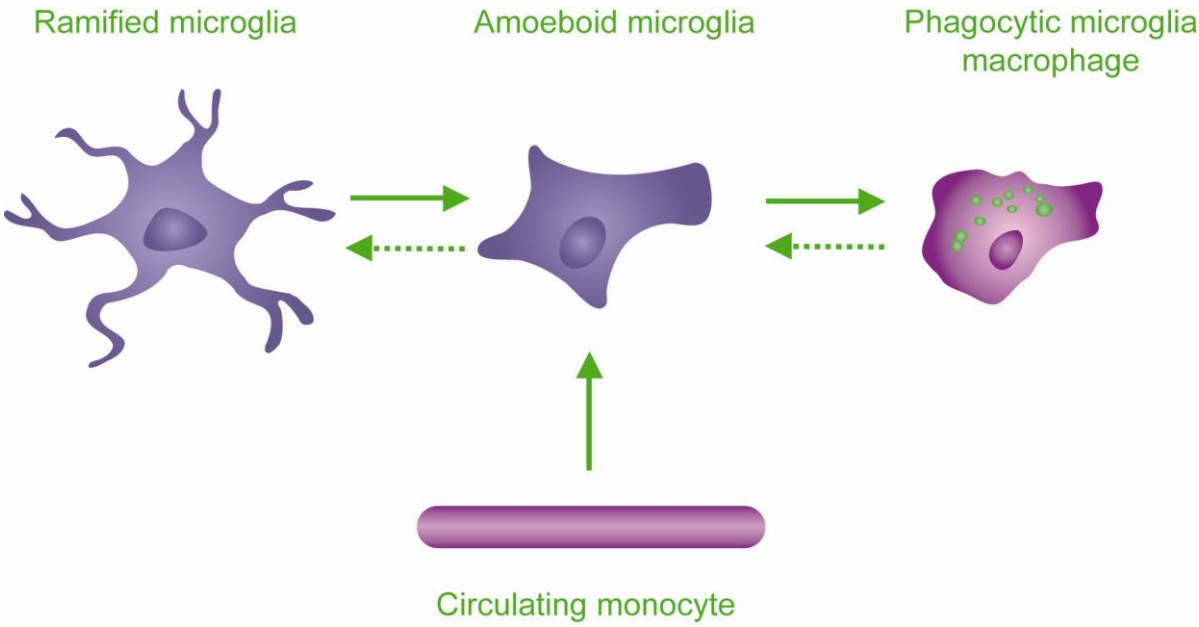


Figure 1 Microglial phenotypes in development and activation.

Myeloid progenitors populate the brain early in development (around E8) from the blood stream showing an amoeboid morphology characterized by enlarged cell soma and short thin processes (middle). During development these cells invade all brain regions and settle as ramified microglia showing long ramified processes and small cell soma. Activation of this resting phenotype leads again to transformation to the amoeboid shape. Both, the amoeboid form of microglia in development and upon activation show high phagocytic activity and vacuolization. Illustration was modified from http://missinglink.ucsf.edu (http://missinglink.ucsf.edu/lm/introductionneuropathology/Response%20_to_IInjur/Injury_Images/microglial%20activation3w.jpg).

### *1.1.2 Microglial phagocytosis*

Microglia, as part of the innate immune system, fulfills also a function as phagocytes within the brain. The internalization of extracellular material is called endocytosis and includes three different mechanisms. All three processes lead to internalization of extracellular material into vesicles that are transferred into the cytosol (Napoli and Neumann 2009). Microglial cells are able to bind extracellular particles by specific receptors that lead to receptor-mediated uptake of these particles. This process, called receptor-mediated endocytosis, involves coated pits on the cell membrane responsible for vesicle formation (Figure 2 left) (Goldstein, Anderson et al. 1979). A second process needed for the uptake of extracellular fluid containing single proteins and small molecules is called pinocytosis (Figure 2 middle) (Glenn, Booth et al. 1991). Macropinocytosis was shown to be involved in the internalization of soluble

amyloid beta protein (Aβ) in a model of Alzheimer's disease (Mandrekar, Jiang et al. 2009). Phagocytosis is the third mechanism for particle uptake in microglial cells (Figure 2 right). It is characterized by the internalization of solid particles and intracellular digestion in phagosomes that fuse with lysosomes which provide enzymes necessary for particle break down (Kinchen and Ravichandran 2008).

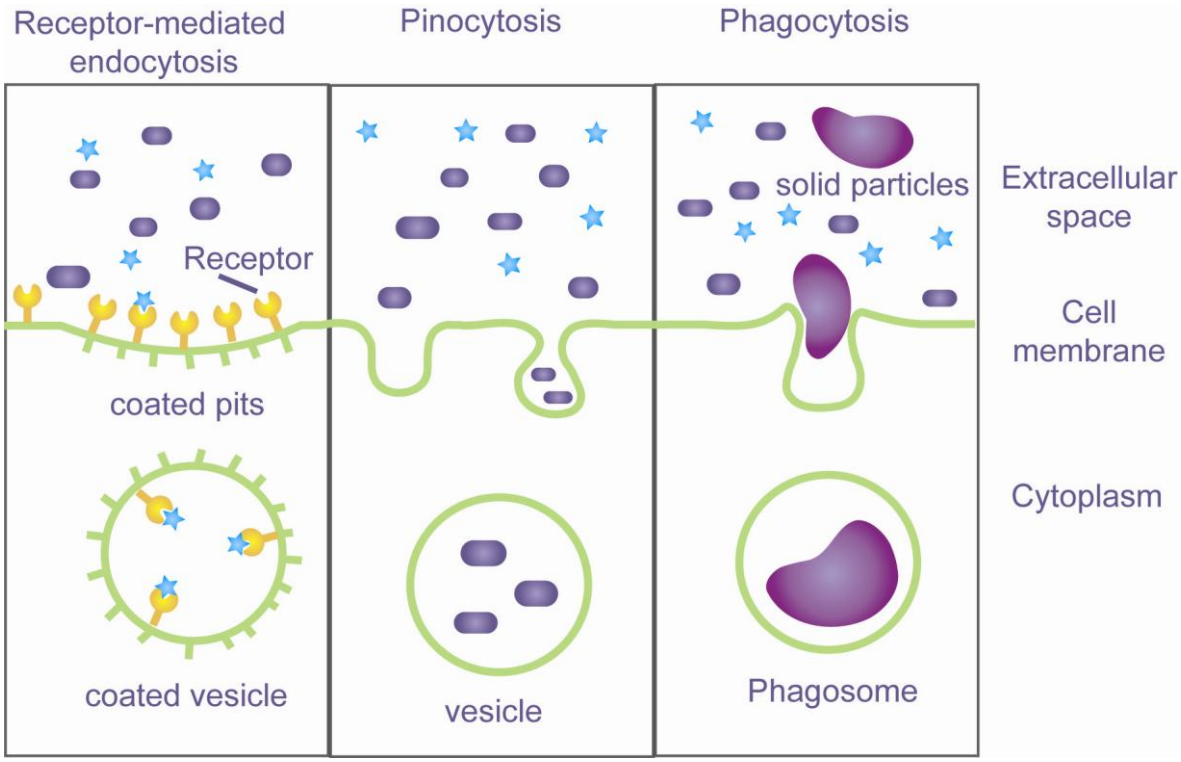


Figure 2 Uptake mechanisms in microglial cells

Microglial cells are able to take up extracellular material or pathogens by engulfing them with their cell membrane. There are three mechanisms studied for endocytosis. Particles can be engulfed by receptor mediated endocytosis involving coated pits (left) leading to the formation of coated vesicles that are transported to the cytosol. A second process involved in uptake of extracellular fluid containing proteins and other small molecules is called pinocytosis (middle). Solid particles like microbes are internalized by phagocytosis (right) leading to the formation of vesicles that are fused to lysosomes containing degrading enzymes to form phagosomes. Illustration was modified from (Napoli and Neumann 2009).

Different receptors are involved in the uptake of extracellular particles thereby mediating pro- or anti-inflammatory processes (Figure 3). Phagocytic receptors recognize "eat-me" signals that present non-self or toxic materials and consist of pathogen-associated molecular patterns (PAMPs) for non-self agents and apoptotic cell-associated molecular pattern (ACAMPs) for toxic self agents (Elward and Gasque 2003). Probably the best studied PAMP is phosphatidylserine (PtdSer),

which is located in the inner leaflet of the plasma membrane under healthy conditions. Upon cell death, different lipids, proteins or sugars like PtdSer are modified or newly exposed on the extracellular side of the cell membrane, recognized by scavenger receptors (SR) and T cell immunoglobin mucin 4 (TIM-4) on macrophages and internalized (Fadok, Voelker et al. 1992; Miyanishi, Tada et al. 2007). Other receptors involved in clearance of apoptotic cell debris are the purinergic receptor P2Y6 (Koizumi, Shigemoto-Mogami et al. 2007), the Fc receptor (FcR) and triggering receptor expressed on myeloid cells (TREM2) (Takahashi, Rochford et al. 2005). These receptors share the property to trigger anti-inflammatory response in microglial cells.

In contrast, receptors mediating the uptake of non-self agents like microbial pathogens cause pro-inflammatory signaling leading to release of cytokines like TNF-α and nitric oxide which in turn recruits peripheral immune cells and reactive microglia (Elward and Gasque 2003). The most studied receptors involved in this scenario include Toll-like receptors (TLR), scavenger receptors and complement receptors (CR) (Napoli and Neumann 2009). The latter one is also involved in the microglial engulfment of redundant synaptic structures for synaptic refinement during development (Stevens, Allen et al. 2007).

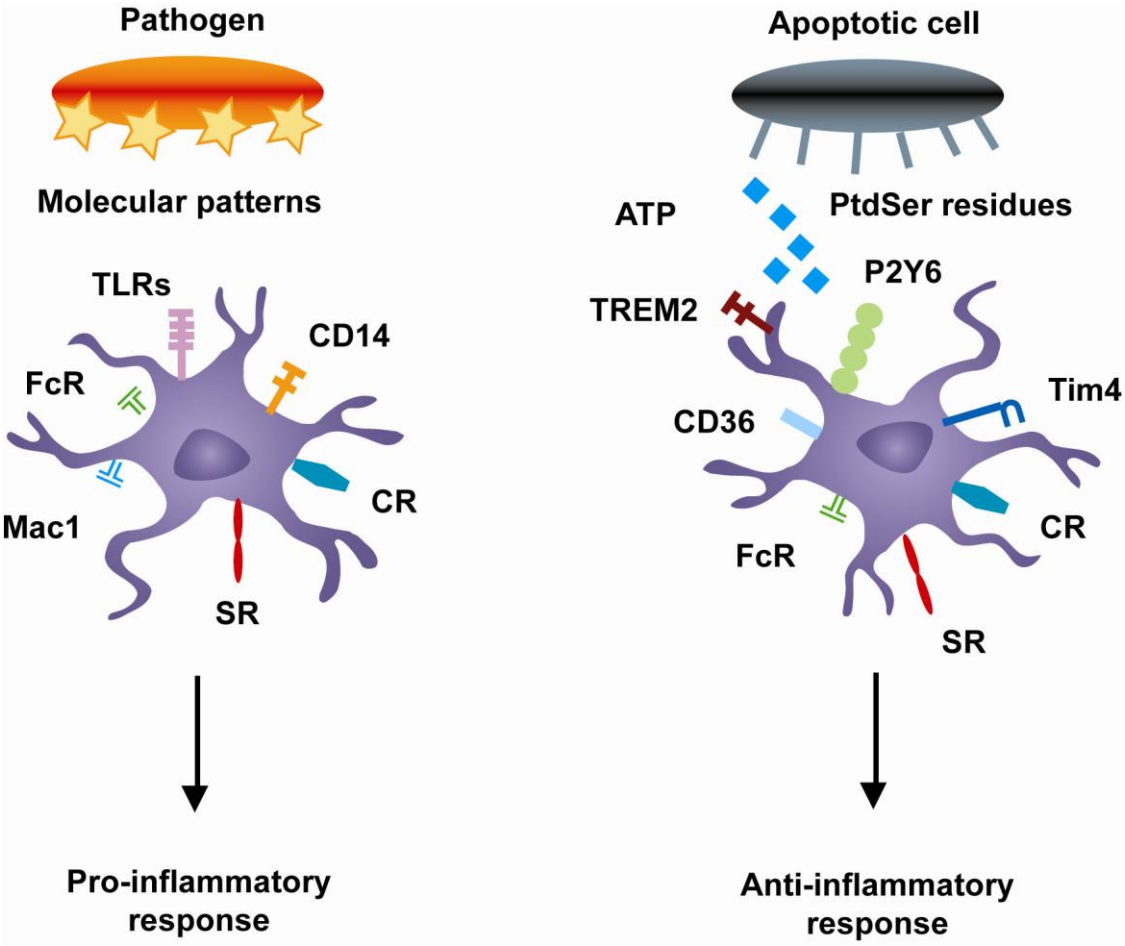


Figure 3 Phagocytic receptors expressed by microglial cells.

Microglia recognize specific structural patterns by different classes of phagocytic receptors leading to either pro- or anti-inflammatory phenotype. Different structural patterns from microbial pathogens summarized as PAMPs are recognized by microglial receptors like Toll-like receptors (TLRs), Fc receptor (FcR), complement receptor (CR) or scavenger receptors (SR) leading to pro-inflammatory signaling (left). ACAMPs, including phosphatidylserine (PtdSer) expressed by apoptotic cells act via microglial receptors like triggering receptor expressed on myeloid cells (TREM2), purinergic receptors (P2Y6), FcR, Tim4 or SR and lead to engulfment of those apoptotic cells. Here, phagocytosis is associated with an anti-inflammatory microglial response. Illustration was modified from (Napoli and Neumann 2009).

### *1.1.3 Microglial motility*

Within the brain microglial cells are equally distributed and constantly survey their surroundings for any changes in homeostasis. It has been estimated that it takes few hours for microglia to scan the whole brain parenchyma by constant extension and

retraction of microglial processes (Nimmerjahn, Kirchhoff et al. 2005). To be mobile, microglia need to screen their surroundings, change their shape and penetrate the tissue.

Upon injury of the adult brain there are two phases of response. 1. Immediate movement of the fine processes towards the lesion site to encapsulate the site of injury and prevent further destruction which takes place within the first hours after injury. This is not accompanied by any cell displacement. 2. Complete translocation to the site of injury is accompanied by a change of morphology towards an amoeboid shape. Here, microglial cells display an activated state acting as phagocytes and releasing inflammatory molecules.

Experimentally, acute injury can be induced by focusing a two-photon laser beam to a small area that results in tissue damage. It was shown that in this experimental condition microglial cells sense the release of ATP by dead cells in the injured tissue (Davalos, Grutzendler et al. 2005). It is suggested that microglial expression of P2Y12 purinergic receptor propagates microglial attraction to this free ATP, upon which they send their processes towards the lesion and shield the site of injury (Haynes, Hollopeter et al. 2006). This response can be diminished by volume sensitive chloride channel blockers like NPPB, tamoxifen and DIDS (Hines, Hines et al. 2009). Following this, a whole activation cascade can be triggered which finally can lead to the transformation into an amoeboid morphology.

To penetrate through the tissue, microglia cleave the surrounding extracellular matrix by e.g. matrix metalloproteases (MMP). MMPs are enzymes that can be expressed by microglia or astrocytes and are either synthesized as membrane-bound or releasable proteins. Expression of MMPs can be stimulated by various signals such as focal ischemia that results in modified MMP activity on microglial cells and their translocation to the injured site (del Zoppo, Milner et al. 2007).

## 1.2 Microglia in neurodegenerative diseases

### *1.2.1 General aspects and examples*

The role of microglial cells in progression of neurodegenerative diseases is under investigation for a long period but is still controversial (Graeber and Streit 2010). Microglia were suggested to play neuroprotective as well as neurotoxic roles in disease progression (Giulian, Haverkamp et al. 1996; Streit 2005). Aging is one of

strongest risk factors for development of neurodegenerative diseases as it is accompanied by a significant increase in glial activation (Miller and Streit 2007; Lucin and Wyss-Coray 2009). Interestingly, Iba-1 staining, a marker for microglia/macrophages, recently revealed rather fragmented microglial phenotype which depends on the presence of pathological signs. In contrast, microglia express ramified morphology in regions that do not show pathology (Streit, Braak et al. 2009). Together this suggests a dystrophic phenotype for microglia in brain regions affected by neurodegeneration.

Nevertheless, neuroinflammation is a prominent pathological feature in different neurodegenerative diseases including amyotrophic lateral sclerosis (ALS). ALS is characterized by a rapid destruction of motor neurons leading to denervated neuromuscular junctions followed by weakness and atrophy of limb musculature (Boillee, Vande Velde et al. 2006). Isolation of mutations in the gene encoding superoxide dismutase 1 (SOD1), as one cause of familiar ALS, allowed the development of transgenic mouse models for ALS (Bowling, Schulz et al. 1993; Dal Canto and Gurney 1994). These mice develop a chronic progressive motor neuron disease accompanied by inflammation of the spinal cord involving microglia and infiltrating T-cells and dendritic cells (Alexianu, Kozovska et al. 2001). Again, the role of microglia was shown to be controversial. It was suggested that microglial overproduction of neurotoxic substances like reactive oxygen species (ROS) leads to neurodegeneration (Boillee and Cleveland 2008) whereas others could show that ablation of proliferating microglia did not modulate disease progression (Gowing, Philips et al. 2008). Hence, if microglia play a neurotrophic or neurotoxic role in ALS still has to be elucidated.

Further aspects of microglial involvement in neurodegenerative diseases could be studied in Parkinson' disease (PD) which is characterized by loss of dopaminergic neurons in the Substantia nigra leading to motor impairment like rigidity, tremor, slowed motion and inability to induce movements (Heisters 2011). Microglial activation and intracytoplasmic accumulation of α-synuclein in dopaminergic neurons, called Lewy bodies, can be detected at early stages of disease progression (Spillantini, Schmidt et al. 1997). Using a mouse model of PD combined with *in vitro* studies, it was suggested that neuron derived α-synuclein activates microglial cells leading to increased release of cytotoxic molecules like TNF-α and ROS (Su, Maguire-Zeiss et al. 2008). Most studies on disease progression were carried out

using mouse models where PD is induced by injection of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which is oxidized to $MPP^+$ leading to PD symptoms, neurotoxicity and microglial activation (Czlonkowska, Kohutnicka et al. 1996). Although there is obvious contribution of microglial activation to PD progression, direct proof of microglial involvement in the induction of the disease is still missing. Furthermore the availability of representative animal models is controversially debated as MPTP mouse models reflect only the symptoms and not the full cellular characteristics of the disease (Graeber and Streit 2010).

Experimentally, the right choice of a representative mouse model to study a disease is highly important. For investigations of the sensitivity of the CNS to traumatic brain injury, Georg Kreutzberg introduced the facial nerve axotomy model (Tetzlaff and Kreutzberg 1984) displaying facial nerve degeneration and regeneration depending on the severity of nerve injury (crush injury, transection). In this model there occurs no direct CNS trauma or blood brain barrier opening but T-cell infiltrate, accumulate around activated microglia and remove neuronal debris (Raivich, Jones et al. 1998). Upon injury of peripheral nerve axons glial cells become activated (Barron, Marciano et al. 1990). Microglia start to proliferate at the site of injury (Graeber, Tetzlaff et al. 1988) and upregulate the expression of immune relevant molecules like MHC II, components of the complement cascade like complement receptor 3 and intercellular adhesion molecule-1 (ICAM-1) (Graeber, Streit et al. 1988; Kiefer and Kreutzberg 1991; Werner, Kloss et al. 1998). Expression of ICAM-1 was detectable in a biphasic manner 1-2 days and 14 days after facial nerve axotomy and accompanied neuronal cell death and microglia phagocytosis of neuronal debris (Werner, Kloss et al. 1998). Hence, the model of facial nerve axotomy also gives insight into microglial involvement in neuronal repair as microglia contributes actively to clearance from neuronal debris and therefore provides space for regeneration.

### *1.2.2 Microglia in Alzheimers disease*

As one of the most common neurodegenerative diseases AD is a well-studied but not yet understood disorder affecting 20 to 30 millions of people worldwide (Brookmeyer, Johnson et al. 2007). There are two major characteristics for AD: the presence of extracellular β-amyloid (Aβ) aggregations, also called senile plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau protein. As the presence of both toxic protein aggregates was also found in other degenerative diseases without any

cognitive impairment other factors that lead to full establishment of the disease need to be identified (Lee, Goedert et al. 2001). *In vivo*, Aß exists in a soluble and a fibrillary form within the brain and is produced and cleared in the human and mouse brain at an equivalent rate (Bateman, Munsell et al. 2006). Any disturbance of this homeostasis can lead to an increase in Aß and results in the formation of Aß plaques. According the “amyloid hypothesis” AD pathology is primarily caused by Aβ deposition followed by formation of neurofibrillary tangles, neuronal loss and dementia (Hardy and Selkoe 2002). The predominant forms found in the brain are Aβ 40 and Aβ 42 that subsequently fibrillize and aggregate as plaques.

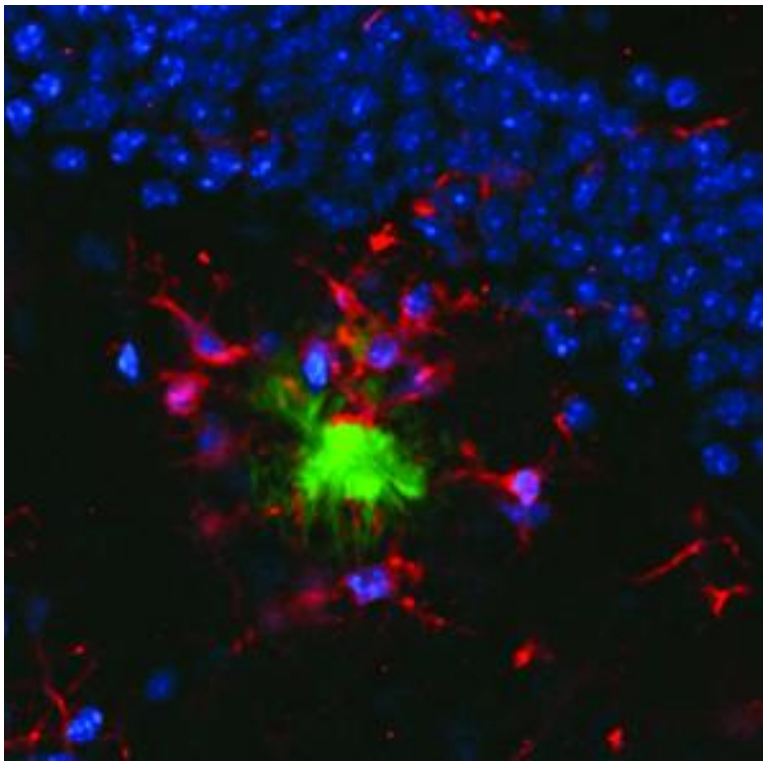

Figure 4 Microglia accumulate around senile plaques

Microglia are attracted by Aβ aggregations within days and accumulate around them rapidly. Picture was taken from the hippocampus area of acute brain slices of 9 months old *APPPS1* mice by confocal microscopy stained for microglia (Iba-1, red), Aβ (Thiazine Red, green) and cell nuclei (Hoechst 33258, blue).

A combination of aspects of AD can be studied by introduction of human disease related genes into mice that display a transgenic mouse model for cerebral amyloidosis. In these mouse models of AD, microglial cells accumulate at Aβ plaques and show typical hypertrophic cell bodies and shortened cellular processes resembling an activated phenotype (Figure 4) (Frautschy, Yang et al. 1998; Bolmont,

Haiss et al. 2008). Since expression of pro-inflammatory cytokines and reactive oxygen species is found to be increased in microglia from AD animal models (Simard, Soulet et al. 2006), it has been assumed that the change in microglial morphology in AD represents the transition to an “activated” functional state. Furthermore the number of released pro-inflammatory molecules potentially contributes to further neuronal dysfunction and finally death. But still the role of microglial cells in AD has been the subject of a number of studies (Perry, Nicoll et al. 2010). *In vivo* imaging studies suggested that microglial cells become attracted by newly formed plaque deposits, reach them within 1-2 days and accumulate around the plaque (Meyer-Luehmann et al. 2008). Some reports suggested that microglia even aid to restrict Aβ plaque growth (Bolmont, Haiss et al. 2008) as plaque associated microglia were shown to internalize systemically injected amyloid-binding dye. In contrast, it was recently shown that temporary ablation of microglia has no obvious effect on the formation and maintenance of Aβ plaques (Grathwohl, Kalin et al. 2009). The authors ablated microglia in transgenic animals expressing CD11b-driven thymidine kinase (TK) crossbred to a mouse model for AD, by two to four weeks ganciclovir treatment. Investigation of plaque size, Aβ40-42 content and amount of dystrophic neuritis after treatment did not show differences to AD mice containing microglial cells suggesting a dysfunctional phenotype of microglia under these conditions.

As microglial activation is not an “all or none” process depending on a combination of signaling substrates in physiology but also in a variety of pathologies like stroke, brain tumor or neurodegenerative diseases, the beneficial or detrimental role of microglia needs to be elucidated in each condition (Figure 5) (Hanisch and Kettenmann 2007).

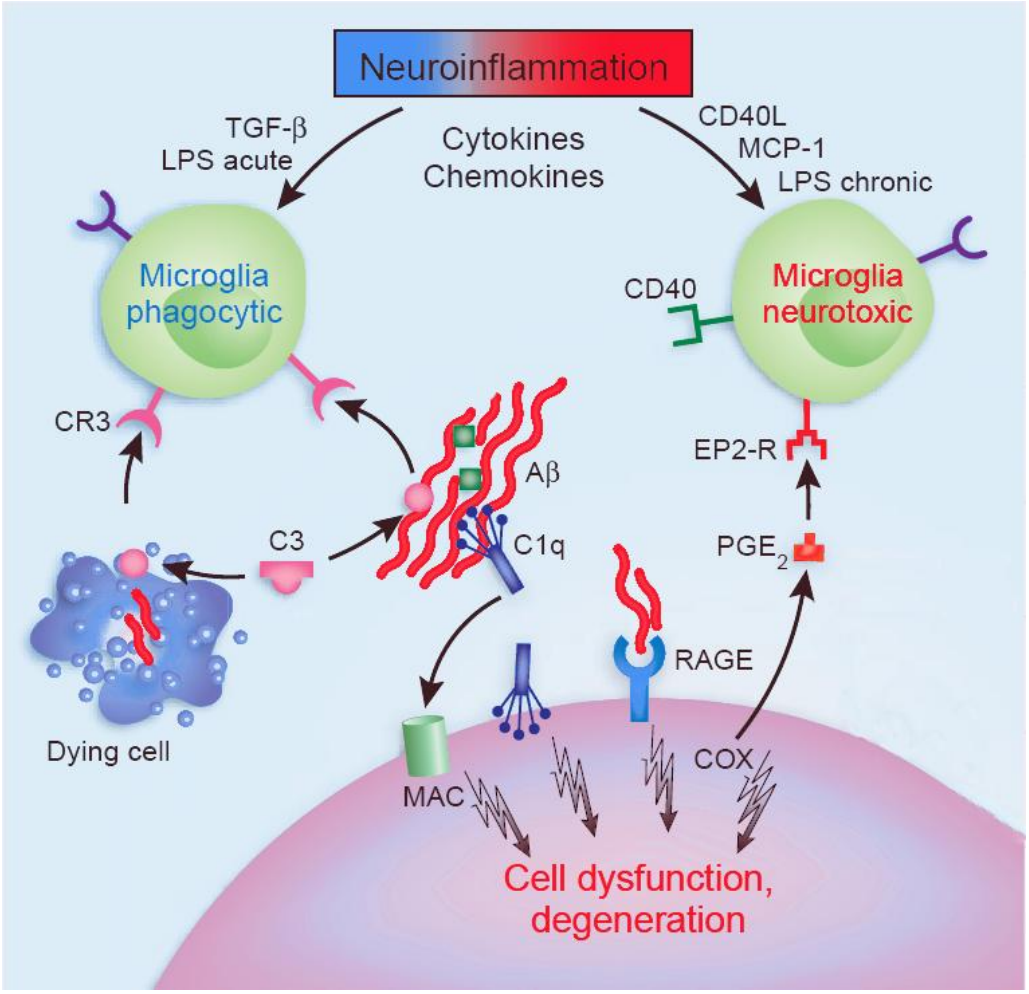


Figure 5 Beneficial and detrimental effects of microglia in AD

Inflammation during progression of AD triggered by TGFβ or acute LPS treatment may induce beneficial responses including activation of microglia to phagocytose dying cells or Aβ aggregates via complement receptor 3 (CR3). Inflammation may also induce detrimental effects of microglia in disease course. Cd40L, MCP-1 and chronic LPS treatment favors secretion of neurotoxic factors from microglia like the complement factor C1q that promote Aβ accumulation, directly affect neurons or damage neurons via membrane attack complex (MAC). Neurodegeneration can be triggered by Aβ binding to RAGE expressed by neuronal cells or COX induction and PGE signaling via microglial EP2 receptor. Illustration modified from (Wyss-Coray 2006).

Microglial cells are considered to be the professional phagocytes of the brain. A number of studies investigated phagocytic capacities of microglial cells in AD context. *In vitro,* microglia take up fibrillary Aβ by a receptor-mediated non-classical mechanisms involving B-class scavenger receptor CD36, alpha6beta1 integrin and CD47 (Koenigsknecht and Landreth 2004) which can be stimulated by addition of the neurotransmitter norepinephrine (Heneka, Nadrigny et al. 2010). Interestingly, active immunization to Aβ and therefore stimulating Fc receptor-mediated uptake of Aβ prevented plaque formation and reduced plaque burden in a mouse model of AD.

Furthermore, externally applied soluble Aβ was shown to be engulfed *in vivo* and *in vitro* by macropinocytosis independent from receptor-mediated processes (Mandrekar, Jiang et al. 2009). Additionally, microglial phagocytic machinery was shown to be impaired as the expression of Aβ binding enzymes as well as degrading enzymes are reduced in microglia isolated from aged AD mice (Hickman, Allison et al. 2008). This and the presence of a pro-inflammatory environment including cytokines like IL-1β and TNFα and nitric oxide which was shown to impair microglial uptake of fibrillary Aβ *in vitro* (Koenigsknecht-Talboo and Landreth 2005) possibly causes impaired elimination of Aβ aggregates leading to extensive plaque deposition. But so far, it was not shown that microglia are actively involved in plaque clearance *in vivo*. Therefore, I studied this subject together with the group of Prof. Frank Heppner (Institute for Neuropathology, Charité, Berlin) that provided different mouse models for AD. In this project I used acute coronal brain slices from *APPPS1* and *APP23* transgenic animals and wildtype littermates. I investigated *in situ* motility and phagocytic activity in these different AD mouse models utilizing the laser-lesion model. I studied different ages and brain areas to link microglia activity to total plaque load and/or distance to the plaque.

## 1.3 Microglia-Neuron interaction

It is known that micoglial cells express neurotransmitter receptors for example for dopamine, norepinephrine and GABA (Pocock and Kettenmann 2007). Stimulation of dopamine receptors modulates microglial migration and cytokine release (Farber, Pannasch et al. 2005).

In the first postnatal days synapses are strengthened and maintained or eliminated depending on synaptic activity and input strength. Microglia play a critical role in this process as they eliminate complement tagged synapses during the first days after birth (Stevens, Allen et al. 2007). Mice deficient for C1q or C3 show synapse refinement disturbances leading to increased synapse number until adulthood. A tight interaction between microglial cells and neuronal structures could be also shown for adult mice (Wake, Moorhouse et al. 2009). Here, adult microglia constantly survey synapses by contacting them about five minutes, detach and possibly contact the

same structure again. Upon traumatic events, like cerebral ischemia, these contacts are prolonged to one hour and mediate the elimination of these neuronal structures.

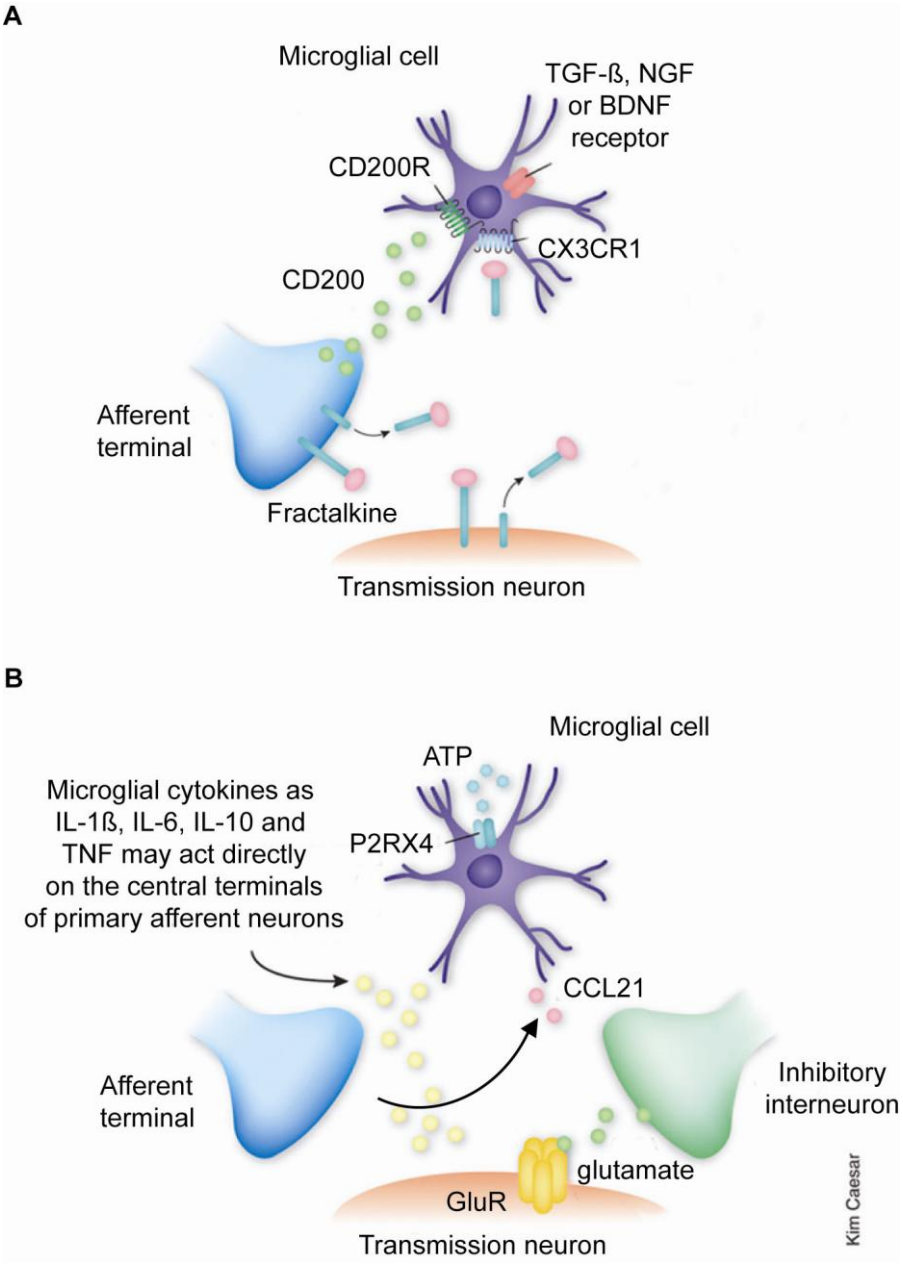


Figure 6 “On” and “Off” signaling in microglia-neuron interaction

(A) Neurons secrete signals like the chemokine Fractalkine (CX3CL1) that acts on microglial CX3CR1 to keep microglia in a quiescent state. Further signals released by neurons acting as “Off” signal for microglia are CD200 and growth factors like TGF-β, NGF or BDNF. These signals act on disappearance. (B) Microglia can be activated by various stimuli like purines acting on e.g. P2X4 or chemokines like CCL21 which upregulates purinergic receptors in microglial cells. In turn, microglia release cytokines like IL-1β, IL-6 or TNF that acts on neuronal structures. The neurotransmitter glutamate was shown to have also “On” signaling capacities in contrast to other neurotransmitters like norepinephrine or dopamine that rather attenuate LPS induced cytokine release *in vitro*. The illustration was modified from (Scholz and Woolf 2007).

These contacts to synapses are activity-dependent so that the interaction between microglia and neurons seems to be important throughout all stages of development. Relevant signals in the healthy but also diseased brain that are involved in such interaction are those that initiate protective or activated phenotype of microglia, so called “On”-signals, and “Off”-signals which keep microglia in a quiescent state (Biber, Neumann et al. 2007). The interplay between these two kinds of signals needs to be integrated by microglial cells by the expression of respective receptors and leads to a variable and adaptive response appropriate to the input situation. “On” and “Off”-signals can be released or expressed by neuronal cells. “On”-signals include membrane bound TREM2 or released chemokines like CCL21 and transmitter like ATP or glutamate. In contrast, neurons release CD200, CX3CL1 and growth factors like TGF-β, NGF and BDNF which act as “Off”-signals on microglia (Figure 6). Different neurotransmitters, especially monoamines like dopamine, norpeinephrine and serotonin also contribute to “Off” signaling in the non-inflamed brain (Farber and Kettenmann 2005).

### *1.3.1 Neurotransmitters and glia-transmission*

In the brain one can distinguish between synaptic and non-synaptic transmission. The classical neurotransmitters like GABA, glycine and glutamate are released into the synaptic cleft where they act on low affinity ligand-gated ion channels located on the postsynaptic membrane (Vizi 2000). These ion channels only open if high concentrations of their ligand are present which is achieved upon stimulated release into the synaptic cleft of synapses (Figure 7). To terminate signaling, neurotransmitters are either cleaved to non-active metabolites and/ or taken up by transporters located on the presynaptic membrane. Neurotransmitter molecules can be recycled, packed into vesicles and released upon stimulation. Monoamines as well as acetylcholine are known to be released predominantly into the extrasynaptic space and act on receptors located at the extrasynaptic parts of dendrites, cell bodies but also axons (Figure 7) (Hensler 2006). This non-synaptic transmission, also known as paracrine transmission, is suggested to involve a large amount of transmitter molecules as it has to diffuse through the distance between release and receptor site. Needed amounts of neurotransmitters are stored in vesicles ready to be released not only at presynaptic terminals but also from the somata, dendrites and

axonal varicosities even in the absence of postsynaptic structures (Descarries and Mechawar 2000; De-Miguel and Trueta 2005).

This suggests that besides the synaptic transmission via pre- and postsynaptic terminals there is a large amount of transmitter molecules diffusing in the extracellular space released from extrasynaptic neuronal parts which can therefore act on neurotransmitter receptors expressed by glial cells.

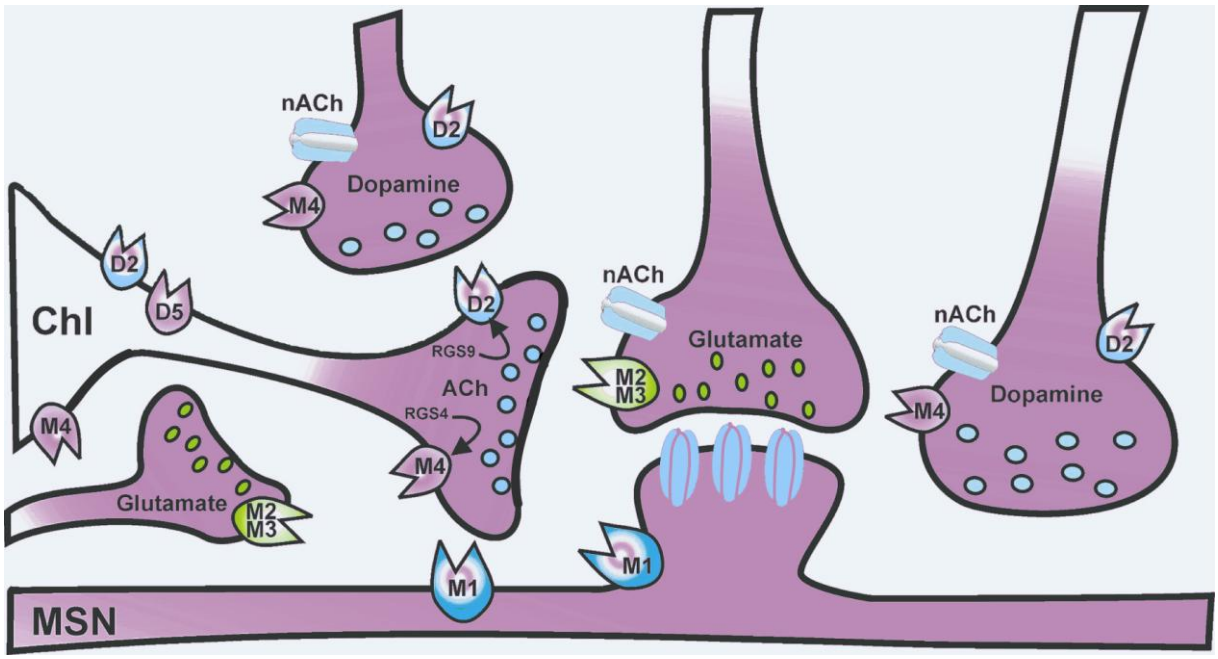


Figure 7 Mechanism of synaptic and volume transmission.

Neurotransmitters like glutamate can be released from the presynaptic membrane of neuronal cells into the synaptic cleft where they act on low affinity receptors located on the post-synaptic membrane (synaptic transmission). In contrast, neurotransmitters like dopamine and acetylcholine (Chl) can be released from different neuronal structures including cells soma and axons and act on receptors not only expressed on postsynaptic membrane but also cells soma, axons and extrasynaptic parts of dendrites (M1-4, nACh, D2/5) which already open upon presence of low amounts of neurotransmitters. This extrasynaptic transmission is also known as volume transmission. Illustration was modified from (Bonsi, Cuomo et al. 2011).

The most prominent neurotransmitter acting on microglial cells is ATP. It is released as a co-transmitter from synaptic terminals, as so called “gliotransmitter” from astrocytes or by damaged cells (Su 1983; Butt 2011; Tozaki-Saitoh, Tsuda et al. 2011). Microglial cells express a variety of purinergic receptors that can be divided into ionotropic P2X receptors and metabotropic P2Y receptors whose activation leads to the release of immune relevant molecules like cytokines and chemokines,

chemotaxis, modulation of phagocytosis and other microglial executive functions (Farber and Kettenmann 2006; Orr, Orr et al. 2009). Furthermore, microglial cells specifically express enzymes for the degradation of ATP, the ectonucleotidases CD39 and CD73 (Braun, Sevigny et al. 2000). Degradation products of ATP like ADP and AMP can act as more specific agonists on purinergic receptors or are fully degraded to adenosine which may act on microglial adenosine receptors (Farber and Kettenmann 2006).

Monoamines, including serotonin, dopamine and norepinephrine, are a widespread family of hormones that are also present in the brain where they act as neurotransmitters. They were found to have impact on executive functions of peripheral immune cells like release of chemo- and cytokines, proliferation and migration (Nijhuis, Olivier et al. 2010; Sarkar, Basu et al. 2010).

Serotonin (5-HT) is a monoamine neurotransmitter primarily found in the gut and the brain. The principal source of serotonin in the brain is the neurons of the Raphe nuclei projecting to nearly all brain regions including the cortex and the brain stem (for review see (Filip and Bader 2009). Serotonin is involved in a variety of physiological and behavioral functions and the dysregulation of the serotonergic system can lead to many psychiatric and neurological disorders. In mice there are 14 different serotonin receptors divided into 7 classes. All classes, except class 3, are G-protein coupled receptor leading to the release of internal calcium stores (Gi and Gq) or the activation of cAMP mediated signaling cascades (Gs). So far, 5-HT7 is the only receptor published to be expressed by microglial cells (Mahe, Loetscher et al. 2005). In several studies it could be shown that serotonin acts as an anti-inflammatory component on peripheral immune cells since serotonin has an influence on cytokine release (Muller, Durk et al. 2009), maturation to dendritic cells (Katoh, Soga et al. 2006) and apoptosis induction (Katoh, Soga et al. 2006).

Another monoamine, dopamine, was shown to act on different immune cells of the peripheral and central nervous system modulating the release of cytokines like IL-4 and IFN gamma (Farber, Pannasch et al. 2005; Huang, Qiu et al. 2010; Matalka, Attallah et al. 2011). Dopamine receptors are divided into D1-like receptors including D1 and D5 that belong to Gs-protein coupled receptors whereas D2, D3 and D4 are summarized to D2-like receptors that trigger signaling via Gi/Go-proteins (Ogawa 1995). Human microglial cells were shown to express D1, D2, D3 and D4 receptors

(Mastroeni, Grover et al. 2009). In contrast, in rat neonatal microglia the dopamine receptors D1, D2, D4 and D5 could be detected (Farber, Pannasch et al. 2005).

Norepinephrine is the most studied monoamine on microglial functions (Carnevale, De Simone et al. 2007). It induces a neuroprotective phenotype in microglia *in vitro* and *in situ* by regulating the expression of cytokine receptors like IL-1ra or the release of chemo- and cytokines (McNamee, Ryan et al. 2010). *In vitro* LPS induced cytokine release is attenuated upon norepinephrine stimulation (Farber, Pannasch et al. 2005). Peripheral immune cells but also microglia are known to express α- as well as β-adrenergic receptors that are Gi/Gq coupled and Gs coupled receptors, respectively (Kohm and Sanders 2001; Mori, Ozaki et al. 2002). Both, dopamine as well as norepinephrine modulates membrane currents, cytokine- and nitric oxide release and migration in primary neonatal microglial cells (Farber, Pannasch et al. 2005).

Microglial functions in health and disease have been investigated for a long time. Nevertheless, the regulation of microglial effector functions like phagocytosis and motility upon neuronal activity and during acute injury are still unclear. Therefore I investigated these microglial capacities upon neurotransmitter application utilizing C57BL/6 mice for *in vitro* and *in situ* approaches. Acute microglial activation was triggered by laser induced injury *in situ* for investigating microglial motility.

# 2 Material and Methods

## 2.1 Materials

### *2.1.1 Reagents and dyes*

| Reagent | Company |
|---|---|
| Acetylcholine | Sigma-Aldrich, Munich, Germany |
| Alexa Fluor®594 –conj. tomato lectin | 0.72 mg/ml *Lycopersicon esculentum* (tomato) Lectin (Vector Laboratories, Burlingame, USA) 18.2 µg/ml Alexa® 594, Succinimidester (Invitrogen, Karlsruhe, Germany) 5 mM Phosphate 15 mM NaCl 0.1 mM $CaCl_2$ |
| Aqua polymount | Polysciences Europe GmbH, Eppelheim, Germany |
| Bovine serum albumin (BSA) | Roth, Karlsruhe, Germany |
| 4′,6-Diamidin-2-phenylindol (DAPI) | Sigma-Aldrich, Munich, Germany |
| Diff-Quik staining set | Medion Diagnostics, Langen, Germany |
| Dihydrexidine | Tocris Bioscience, Missouri, USA |
| Dithiothreitol | Invitrogen, Karlsruhe, Germany |
| dNTP | Invitrogen, Karlsruhe, Germany |
| DOI | Tocris Bioscience, Missouri, USA |
| Donkey serum | Sigma-Aldrich, Munich, Germany |
| Dopamine | Tocris Bioscience, Missouri, USA |
| Dulbecco's Modified Eagle Medium | GIBCO®, Invitrogen, Darmstadt, Germany |
| Ethidium bromide | Roth, Karlsruhe, Germany |
| Fluoresbrite microspheres | Polysciences Europe GmbH, Eppelheim, Germany |
| Glucose | Roth, Karlsruhe, Germany |
| Glutamate | Sigma-Aldrich, Munich, Germany |
| Go Taq qPCR Master Mix | Promega, Mannheim, Germany |
| Go Taq PCR Master Mix | Promega, Mannheim, Germany |

| | |
|---|---|
| Hank's balanced salt solution | GIBCO®, Invitrogen, Darmstadt, Germany |
| HEPES | Roth, Karlsruhe, Germany |
| Histamine | Sigma-Aldrich, Munich, Germany |
| Hoechst 33258 | Sigma-Aldrich, Munich, Germany |
| Isoproterenol | Sigma-Aldrich, Munich, Germany |
| Ketanest | Bela-Pharm, Vechta, Germany |
| Lipopolysaccharide (LPS) | Alexis biochemicals, Lausen, Switzerland |
| Metaraminol | Sigma-Aldrich, Munich, Germany |
| Norepinephrine | Sigma-Aldrich, Munich, Germany |
| oligo-dT primer | Invitrogen, Karlsruhe, Germany |
| Percoll | GE Healthcare, Munich, Germany |
| Phosphate Buffered Saline (PBS) | GIBCO®, Invitrogen, Darmstadt, Germany |
| Quinpirol | Sigma-Aldrich, Munich, Germany |
| Rhomputin | Bayer Vital, Mannheim, Germany |
| RNase-Inhibitor | Invitrogen, Karlsruhe, Germany |
| SeaKem® LE Agarose | Lonza, Cologne, Germany |
| Serotonin | Sigma-Aldrich, Munich, Germany |
| Superscript II Transcriptase | Invitrogen, Karlsruhe, Germany |
| Thiazine Red | Sigma-Aldrich, Munich, Germany |
| Triton X-100 | Roth, Karlsruhe, Germany |

Table 1 List of reagents and dyes

### *2.1.2 Media and solutions*

| Name | composition |
|---|---|
| 0.9% saline | 0.9% NaCl<br>Aqua Destilled (A. dest.) |
| Artificial cerebrospinal fluid<br>ACSF | NaCl 134 mM; KCl 2.5 mM; $MgCl_2$ 1.3 mM; $CaCl_2$ 2 mM; $K_2HPO_4$ 1.25 mM; $NaHCO_3$ 26 mM; D-glucose 10 mM; pH 7.4. The buffer solution was saturated with carbogen (95% $O_2$, 5% $CO_2$), purchased from Roth, Karlsruhe, Germany |
| Blocking buffer<br>(immunohistochemistry) | 2% Triton X-100<br>2% BSA<br>10% donkey serum<br>in 0.1 M phosphate buffer PB |
| Dulbecco's Modified Eagle Medium<br>DMEM | supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine,100 u/ml penicillin, 100 µg/ml streptomycin |
| Griess reagent | Solution A: 1 mg/ml Naphthylethylene in A. dest.<br>Solution B: 1 g/ml Sulfanilamid, 60 µl/ml $H_3PO_4$ (85%) in A. dest.<br>To obtain Griess reagent solution A and B were freshly mixed 1:2. |
| L929 conditioned medium | L929 mouse fibroblast cells at 80% confluency were overlayed with 30 ml supplemented DMEM. After 2 days conditioned medium was collected, filtered and frozen until usage. |

| | |
|---|---|
| Medium A | Hank's balanced salt solution (HBSS) supplemented with:<br>15 mM HEPES<br>0.5% Glucose |
| Paraformaldehyde | 4% paraformaldehyde in A. dest. heated to facilitate dissolution |
| Percoll solution | nine parts Percoll added to one part 10x HBSS further diluted with PBS to indicated concentrations |
| Trypsin/DNase | 10 mg Trypsin, 0.5 mg DNase per ml PBS |

Table Table 23 List of media and solutions

### *2.1.3 Antibodies*

| Antibody | Company |
|---|---|
| Donkey Anti-Mouse IgG DyLight 649 | Jackson ImmunoResearch Europe Ltd., Suffolk, UK |
| Donkey Anti-Rabbit IgG (H+L) Cy3 | Jackson ImmunoResearch Europe Ltd., Suffolk, UK |
| Mouse Anti-β-amyloid, 17-24 (4G8). | Signet, Dedham, USA |
| Rabbit Anti-Iba-1 | Wako Pure Chemicals, Japan |

Table 4 List of antibodies

### *2.1.4 Tools*

| Tools | Company |
|---|---|
| 24-well Cell Culture Plate | BD Biosciences, Heidelberg, Germany |
| 6-well Cell Culture Plate | BD Biosciences, Heidelberg, Germany |
| 96-well Cell Culture Plate | BD Biosciences, Heidelberg, Germany |
| Cells strainer, 70 µm | BD Biosciences, Heidelberg, Germany |
| Double-edged razor blade | Thermo Fisher Scientific, Walldorf, Germany |
| Minisart Filter for syringe | Sartorius Stedim biotech, Göttingen, Germany |
| Menzel glass cover slip 24x50 mm | Thermo Fisher Scientific, Walldorf, Germany |
| 1 µl Syringe 7101KH | Th. Geyer, Renningen, Germany |
| Microchemotaxis chamber (Boyden) | Neuro Probe, Inc., Gaithersburg, USA |
| polycarbonate membrane (5µm pore size) | Neuro Probe, Inc., Gaithersburg, USA |
| Potter-Elvehjem PTFE pestle and glass tube (P7859) | Sigma-Aldrich, Munich, Germany |
| Single-edged razor blade | Apollo Herkenrath, Solingen, Germany |
| Stereotactic alignment system | David Kopf Instruments, Tujunga, USA |

Table 5 List of tools

### *2.1.5 Commercial Kits*

| Commercial Kits | Company |
|---|---|
| Enzyme linked immunosorbent Assay (ELISA) for MIP-1α, IL-6, TNFalpha | R&D Systems, Wiesbaden, Germany |
| InviTrap Spin Universal RNA Mini Kit | Invitek, Berlin, Germany |
| peqLAB DirectLyse Tail Kit | peqlab, Erlangen, Germany |
| RNeasy Micro Kit | Qiagen, Hilden, Germany |
| RNeasy Mini Extraction Kit | Qiagen, Hilden, Germany |

Table 6 List of commercial kits

### *2.1.6 Devices*

| Device | Company |
|---|---|
| Centrifuge Eppendorf 5403 | Eppendorf, Hamburg, Germany |
| Centrifuge Eppendorf 5417R | Eppendorf, Hamburg, Germany |
| Centrifuge Eppendorf 5810R | Eppendorf, Hamburg, Germany |
| Eppendorf BioPhotometer plus | Eppendorf, Hamburg, Germany |
| Eppendorf Thermomixer 5355 | Eppendorf, Hamburg, Germany |
| Axioplan Fluorescent microscope | Carl Zeiss, Jena, Germany |
| Leica SPE confocal microscope | Leica, Wetzlar, Germany |
| Microplate reader Infinite M200 | Tecan, Männedorf, Switzerland |
| Microscope Axiovert 25 | Carl Zeiss, Jena, Germany |
| realplex$^2$ Mastercycler | Eppendorf, Hamburg, Germany |
| G24 Shaker incubator | New Brunswick Scientific, Edison, USA |
| Sonicator UP 50 H | Dr. Hielscher GmbH, Teltow, Germany |
| Stuart see-saw rocker SSM4 | Bibby Scientific Limited, Staffordshire, UK |
| T3000 thermocycler | Biometra, Göttingen, Germany |
| Two photon microscope | Till Photonics, Martinsried, Germany |
| Vibratome Microm HM 650V | Thermo Fisher Scientific, Walldorf, Germany |

Table 7 List of devices

### *2.1.7 Software*

| Software | Company |
|---|---|
| Eppendorf Mastercycler ep realplex | Eppendorf, Hamburg, Germany |
| GraphPad Prism | GraphPad Software Inc., La Jolla, USA |
| Image J Macbiophotonics | http://www.macbiophotonics.ca/index.htm |
| Leica LAS AF | Leica, Wetzlar, Germany |
| SPSS 11.5 | SPSS Inc., IBM, Chicago, USA |
| Thomson Reuters EndNote® | Thomson Reuters, Carlsbad, USA |
| Volocity LE | PerkinElmer, Waltham, USA |

Table 8 List of software

## 2.2 Methods

### *2.2.1 Animals*

For investigations of the impact of neurotransmitter application on microglial properties C57BL/6 mice were obtained from the animal facility of the MDC. Mice were kept in a 12 h/12 h dark-light cycle and food and water *ad libitum*.
Mouse models for Alzheimer's disease, namely *APPPS1 and APP23,* were provided and maintained by Prof. Frank Heppner, Department of Neuropathology, Charité - Universitätsmedizin Berlin. *APPPS1* is a transgenic mouse model of cerebral amyloidosis in which expression of human APP with the Swedish mutation (KM670/671NL) and human mutated PS1 (PS1-L166P) is restricted to Thy1 positive neurons (Radde, Bolmont et al. 2006). Data was compared to another mouse model for cerebral amyloidosis, *APP23*, which only expresses mutated human APP and therefore develops Aβ plaques later than *APPPS1*, namely at around 9 months of age (Sturchler-Pierrat, Abramowski et al. 1997). I used heterozygous mice and wildtype littermates for phagocytosis experiments. *APPPS1* mice were also bred with *Cx3Cr1*-GFP to visualize microglia in imaging experiments (Jung, Aliberti et al. 2000). Expression of GFP driven by the Cx3Cr1 promoter is restricted to microglia in CNS and monocytes in the periphery as they specifically express the Fractalkine receptor Cx3Cr1. I performed laser lesions either in mice heterozygous for *APPPS1* and *Cx3Cr1*-GFP or littermates only heterozygous for *Cx3Cr1*-GFP (control).
Due to restrictions in breeding I used male and female mice for all of my experiments.

### *2.2.2 Genotyping Cx3cr1-GFP mouse strain*

Animals were kept for breeding in the MDC animal facility under approved housing conditions (TVV 0014/08, according to LAGESO). DNA for genotyping was isolated from tail cuts by using peqLABDirectLyse Tail Kit according to manufacturer's instructions. Briefly, 100 µl Direct Lyse together with 10 µl Proteinase K was added to the tail tip and incubated for 3 h at 55°C followed by an incubation for 45 min at 85°C to inactivate the enzymes.

The following primers were used for genotyping:

| Name | Sequence |
|---|---|
| **IMR3945** | 5'-TTC ACG TTC GGT CTG GTG GG-3' |
| **IMR3946** | 5'-GGT TCC TAG TGG AGC TAG GG-3' |
| **IMR3947** | 5'-GAT CAC TCT CGG CAT GGA CG-3' |

Table 9 Primer sequences for genotyping *Cx3cr1*-GFP mouse strain.

| | knockout | | wildtype |
|---|---|---|---|
| **H2O** | 17,25 µl | **H2O** | 17,25 µl |
| **10x PCR buffer** | 2,5 µl | **10x PCR buffer** | 2,5 µl |
| **MgCl2** | 1,5 µl | **MgCl2** | 1,5 µl |
| **dNTP** | 0,5 µl | **dNTP** | 0,5 µl |
| **IMR 3946** | 0,5 µl | **IMR 3946** | 0,5 µl |
| **IMR 3947** | 0,5 µl | **IMR 3947** | 0,5 µl |
| **Taq Polymerase** | 0,25 µl | **Taq Polymerase** | 0,25 µl |
| | | | |
| **Subtotal** | 23 µl | | 23 µl |
| **DNA** | 2 µl | | 2 µl |
| | | | |
| **PCR product size** | **1200 bp** | | **970 bp** |

Table 10 PCR composition for genotyping *Cx3cr1*-GFP mouse strain.

PCR amplification parameters were set as follows:

Lid 95°C
1. 94°C 3 min
2. 94°C 30 s
3. 60°C 30 s
4. 72°C 2 min

} x35

5. 72°C 2 min
6. 10°C ∞

### *2.2.3 Induction of a stab wound injury to activate microglia in vivo*

In order to acutely activate microglia, a stab wound was induced by inserting a needle into the cortex (TVV G0289/07, according to LAGESO). The stab wound was performed by a trained and approved technician in the lab. Adult C57BL/6 mice (8-12 weeks) were anesthetized by intraperitoneal injection ofRhomputin (20 mg/ml, 9.2 mg/kg KG) and Ketanest (100 mg/ml, 131mg/kg KG). During surgery, animals were restrained using a stereotactic alignment system. A 1 cm transsection was cut into the scalp. Scull and meninges were drilled through by a cannula (18 ga). A 1µl Hamilton syringe (22 ga, Ø 0.7 mm, blunt tip) was inserted into the right frontal cortex (1.5 mm deep, Bregma coordinates: +2 mm lateral, +2 mm frontal) for 3 min. After removing the needle the skin was closed by saturation. After six days animals were used for acute brain slice preparation.

### *2.2.4 Microglial cell culture*

Microglial cultures were prepared from cerebral cortex of newborn C57BL/6 mice. The whole isolation procedure was performed on ice. Brains from newborn mice were collected in Hank's Balanced Salt solution (HBSS). Forebrains were carefully freed of blood vessels and meninges. After washing three times with HBSS cortical tissue was incubated for 2 min with a Trypsin/DNase mix. The reaction was stopped by addition of Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Finally, cell mixture was incubated with DNase, dissociated with a fire-polished pipette and washed twice. Mixed glial cells were cultured in complete DMEM in T75 flasks until confluency. Cultures need to be washed carefully every third day to remove cell debris by several replacements of the medium with PBS and strong shaking. After establishment of an astrocytic monolayer, checked by morphology under a microscope, the medium was changed to DMEM (10% FCS, supplemented with L-glutamine, penicillin and streptomycin) containing 30% L929 conditioned medium and incubated for 3 days. Microglial cells were then separated from the underlying astrocytic layer by gentle shaking of the flasks for one hour at 37 °C in a shaker-incubator (100 rpm). The cells were seeded on glass coverslips in 24-well plates, in 6-well plates or 96-well plates at a density of 5 x $10^4$/cover slip, $10^6$

cells/well, 2 x $10^4$ or $10^5$ cells/well, respectively. Cultures usually contained $>$ 95 % microglial cells, which can be checked by staining with tomato lectin, a marker for microglia. Cultures were used for experiments 1 to 3 days (d) after plating.

### *2.2.5 Microglia isolation from adult mouse brain*

Isolation of microglia from adult mice was adapted from de Haas et al. 2007 (de Haas, Boddeke et al. 2007). 8 weeks old C57BL/6 mice were transcardially perfused with 0.9% saline solution to clear the intravascular compartment of blood cells. Brains were isolated by careful opening of the skull not to destroy any part of the brain to facilitate dissection in different brain regions. Brains were stored in ice-cold medium A throughout the whole isolation procedure. Brains were either used as whole brain to isolate microglia or dissected into cortex, hippocampus, striatum, thalamus, medulla oblongata and cerebellum (Figure 8). Brain regions were pooled from three mice to increase the microglial number.

Tissue was cut with a blade and mechanically grinded in a tissue homogenizer (glass potter) and subsequently filtered through a 70 µm cell strainer. Single cell suspension was pelleted at 300 g for 10 min at 4°C with low brake and resuspended in ice-cold 75% Percoll. This was gently overlayed with ice-cold 25% Percoll solution followed by one layer PBS. This density gradient was centrifuged at 800 g for 25 min at 4°C with low acceleration and no brake. After removing the myelin layer at the 0/25% Percoll interface with a Pasteur pipette, the 25%/75% Percoll interface containing mononuclear cells was collected using a fresh Pasteur pipette. For centrifugation cell-Percoll suspension was filled up maximally with ice-cold PBS. Centrifugation at 300 g for 20 min at 4°C with low brake resulted in a cell pellet which was subsequently used for RNA isolation using the QiagenRNeasy micro kit according to the manufacturer's instructions.

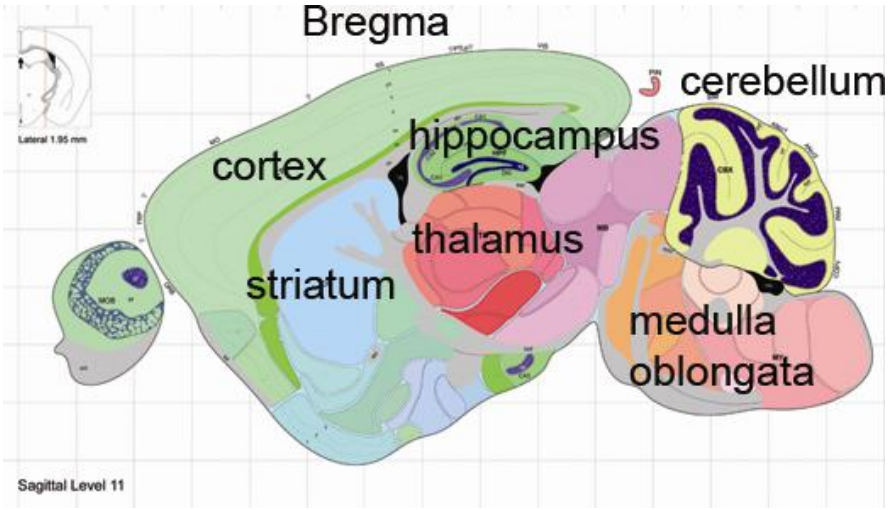


Figure 8 Brain regions taken for acute isolation of adult microglial cells.

Sagittal sections through mouse brain taken from Allen Brain Atlas (http://www.brain-map.org/). Adult microglial cells were isolated for RNA preparation from whole brain, cortex, striatum, thalamus, hippocampus, Medulla oblongata including Nucleus Raphe and cerebellum.

### *2.2.6 RNA Isolation and PCR*

For *in vitro* studies, total RNA was isolated from C57BL/6 primary microglial andC57BL/6 primary astrocytes using the RNeasy Mini Extraction Kit or InvitekInviTrap Spin Universal RNA Mini Kit according to the manufacturer protocol. For primary cells, $10^6$ cells were seeded into a six-well plate and allowed to adhere for six hours. Primary microglial cells were then stimulated with DMEM with or without 10 µM neurotransmitter or specific agonist for 24 h. C57BL/6 whole brain tissue was used for comparison. Therefore fresh whole brain tissue was cut with single-edged razor blade and sonicated at full power for three times ten seconds. Tissue homogenate was immediately used for RNA isolation using the RNeasy Mini Extraction Kit according the manufacturer's instructions.

First-strand cDNA was synthesized from up to 1 µg RNA by reverse transcription reaction incubating the RNA with 0.5 µg oligo-dT primer and 0.5 mM dNTPs in a volume of 20 µl for 5 min at 65°C. After adding First-Strand Buffer, 10 mM dithiothreitol (DTT), 100u RNase Inhibitor and 200 u Superscript II to a final volume of 28 µl, reaction was incubated for further 50 min at 42°C.

For quantitative PCR I used Go Taq qPCR Master Mix based on SYBR Green and primers shown in Table 11. These primers were tested for their efficiency to apply the

delta delta $c_t$-method for analysis. Therefore concentration curves (dilution 1:10) of whole brain cDNA was used to determine the slope of a qPCR standard curve. Slopes between -3.1 and -3.6 were accepted so that an efficiency of 2 could be estimated for the PCR condition which is necessary to apply the delta $c_t$-method. For the analysis $c_t$ values (Figure 9 A) of the house keeping gene (HKG) were subtracted from the $c_t$ values of the gene of interest (GOI) to calculate the delta $c_t$ value. Assuming an amplification efficiency of 2 per PCR cycle one can calculate for the expression:

$$\text{Expression (GOI)} = 2^{(c_t\ \text{GOI}-c_t\ \text{HKG})}$$

To compare the expression of a gene of interest upon stimulation with a neurotransmitter, I applied the delta delta $c_t$ method. Here, one subtracts the $c_t$ value of control treatment from the delta $c_t$ value to obtain the delta delta $c_t$ value. The fold change in expression of the GOI can be again calculated by:

$$\text{Change in expression (GOI)} = 2^{((c_t\ \text{GOI}-c_t\ \text{HKG})-c_t\ \text{GOI control})}$$

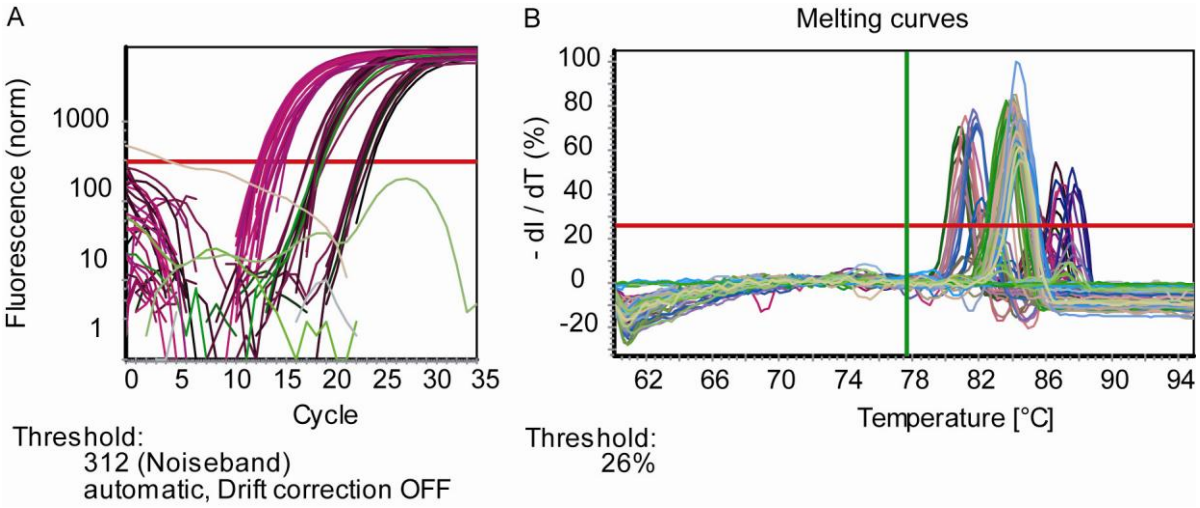


Figure 9 Analyzed parameters for qPCR.

(A) SYBR Green fluorescence of each sample is measured after each PCR cycle. The cycle at which the the fluorescences passes a threshold (red line) is given as $c_t$ value of this sample. (B) At the end of the whole run all samples are heated to 95°C and cooled stepwise. The melting temperature of each amplified PCR product can be assessed by measuring the SYBR Green fluorescence that only intercalates into double stranded PCR products.

The following primers were used in PCR and qPCR approaches:

| Name | size | Sense Primer | Anti-sense Primer |
|---|---|---|---|
| **HMBS** | 106 | CCGAGCCAAGCACCAGGATA | CTCCTTCCAGGTGCCTCAGA |
| **GAPDH** | 203 | CATCAAGAAGGTGGTGAAGC | ACCACCCTGTTGCTGTAG |
| **CD11b** | 65 | TACCGTCTACTACCCATCTGGC | TTGGTGAGCGGGTTCTGG |
| **GFAP** | 65 | GGAGGTGGAGAGGGACAAC | GTTTCATCTTGGAGCTTCTGC |
| **htr1a** | 210 | TCAGCTACCAAGTGATCACCTCT | GTCCACTTGTTGAGCACCTG |
| **htr1b** | 122 | ATGGAGGAGCAGGGTATTC | GCGATGGAGTCCTGGTAA |
| **htr1f** | 133 | CGCTTGTATTGATATTGATCCTCTA | CTCTTCTCACCGCTCTCC |
| **htr2a** | 112 | TCTCCACACTTCATCTGCTA | CGAGTCGTCACCTAATTGC |
| **htr2b** | 120 | CTGTGAGTGTCTGGTAGGT | GATGAAGCCATTCGCCTT |
| **htr2c** | 123 | GCCTGTCTATCCCTTACCTT | ACTATTCTAAGCGGACGAAGA |
| **htr3a** | 129 | TTCCTCATCATCGTGTCAGA | CAGCCGCACAATGAAGAT |
| **htr3b** | 106 | ACATAGACCTGGGCTTCC | TGGTAAGTAGAGGACACAGAA |
| **htr4** | 111 | AATTCAGCCACAACTCTAACTC | CCAGCACCATGAGGAGAA |
| **htr5a** | 130 | CATCGGTCGCAAACATCT | AAGAGGAGGGAGTAGAGAGA |
| **htr5b** | 110 | TCTCTTGCTTCTCTTGCTTC | GCCTGAGAGGTTAGAAACTT |
| **htr6** | 200 | TTATGTCCTCGTGGCGTC | TGTCAGCAGACTCCATCC |
| **htr7** | 137 | GCTCAGAATGTGAACGATGA | CTGGCGGCCTTGTAAATC |
| **SERT** | 140 | CCATAACACTGAGAGGAGATTC | CTTACACTCTGACAGCACTT |
| **TPH1** | 161 | TCTCTTGGCTGAACCCAGTT | CAGCTGTCCATCTTGTTTGC |
| **TPH2** | 206 | GCCATGAACTCTTGGGACAC | TCCGATGGACGAAAGTAACC |

Table 11 Primer sequences for expression analysis of different neurotransmitter receptors.

The qPCR as well as PCR reaction was prepared to a total volume as follows and placed in an appropriate thermocycler to run the following program:

| | |
|---|---|
| GoTaq qPCR/PCR 2x master mix | 10 µl |
| Primer mix (concentration 10 pMol) | 1 µl |
| cDNA | 1 µl |
| DEPC water | 8 µl |

PCR program:

| | | | |
|---|---|---|---|
| Lid | 95°C | | |
| 1. | 94°C | 3 min | |
| 2. | 94°C | 30 s | 40x |
| 3. | 60°C | 45 s | |
| 4. | 72°C | 30 s | |
| 5. | 72°C | 3 min | |
| 6. | 4°C | ∞ | |

PCR was carried out in a Biometra T3000 thermocycler whereas qPCR was performed in an Eppendorf realplex mastercycler that allowed analysis of the amount of amplified PCR product after each cycle by measuring the SYBR Green fluorescence (Figure 9 A). After completing the whole run each well was heated to 95°C and stepwise cooled to 62°C to measure the melting temperature of the amplified PCR product and control for specificity (Figure 9 B).

### *2.2.7 Microchemotaxis assay*

*In vitro*, motility as well as migration can be studied by various assays. One of those is the Boyden chamber assay (Figure 10). Here, cells on top of fine-pored membrane are exposed to either gradient or uniform application of chemoattractants. Therefore chemotaxis is defined as the migration along a gradient of the chemoattractant whereas chemokinesis is characterized by undirected motility stimulated by the substance applied. The Boyden chamber assay allows discrimination of these two movements by placing the chemoattractant either in the bottom well or in both, the bottom and top well.

Chemotaxis of primary microglial cells was studied using a 48-well microchemotaxischamber. Therefore serum free DMEM containing chemoattractiveagents, like serotonin and ATP were added to the lower wells in quadruplicates and covered by a polycarbonate membrane with pores of 5µm. After complete mounting of the chamber, it was put into an incubator (37°C) for 30 min to equilibrate. A number of 2.5 x $10^4$ cells in serum free DMEM were added to the upper wells and incubated for 3 h at 37°C. Finally membranes were fixed and stained using Diff-Quick. Non-migrated cells on the upper surface of the filter were wiped off using

a cotton swab. The rate of migration was determined by counting four fields of view per well (in total 16 fields per stimulation) and numbers were normalized to a control group (serum free DMEM).

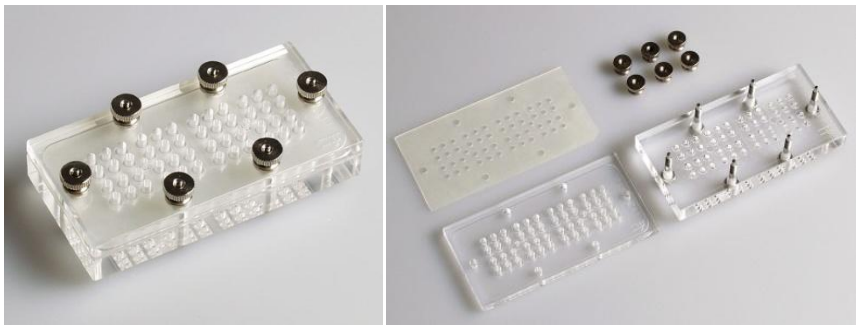

Figure 10 Microchemotaxis chamber (Boyden chamber) used for chemotaxis assays *in vitro.*

Boyden chamber consists of 3 parts, a lower compartment for the chemoattractant, a soft membrane and an upper compartment to fill the cells. During the experiment cells migrate through a membrane which was placed right above the lower compartment. Only cells that migrated through the membrane are counted for analysis. Cells sitting on top are washed away with a cotton swab.

### *2.2.8 ELISA for cytokine release*

Cells (plated in 96 well plates, $10^5$ cells/well) were stimulated for 24 h with LPS (100 ng/ml) alone or in combination with 0.01, 1 or 100 µM serotonin. The negative control was treated with DMEM containing 10% FCS. Quantification of tumor necrosis factor (TNF-alpha), macrophage inflammatory protein 1 alpha (MIP-1 alpha) and Interleukin 6 (IL-6) content in supernatants was performed using mouse-specific antibody pairs and mouse protein standards designed for ELISA application according to the manufacturer's instructions. The colorimetric reaction was analyzed in a microplate reader as absorbance at 450 nm with 540 nm as reference.

### *2.2.9 Nitric oxide release assay*

$10^5$ microglial cells plated in a 96-well format were stimulated for 24 h with DMEM (containing 10% FCS) or LPS (100 ng/ml) with or without 0.01, 1 and 100 µM serotonin. Nitric oxide (NO) production was assessed in culture supernatants by the Griess reaction for the accumulation of nitrite ($NO_2^-$), a breakdown product of NO. 100 µl of each supernatant were mixed with 100 µl of Griess reagent, incubated at room temperature for 10 min and the optical density was determined in a microplate reader at 540 nm.

### *2.2.10 Preparation of acute brain slice*

Acute brain slices were prepared from either young C57BL/6 mice (P6-9) or adult C57BL/6 (8-12 weeks) mice as previously described (Haas, Brockhaus et al. 1996). In brief, mice were decapitated and brains were carefully removed and washed in ice cold artificial cerebrospinal fluid (ACSF, Figure 11 A). Coronal slices of 200 µm for P6-9 animals and 130 µm for adult animals were prepared at 4°C using a vibratome (Figure 11 B). Brain slices were kept in ACSF at room temperature (21 - 25°C) for 2h until phagocytosis experiment or used immediately for two-photon microscopy.

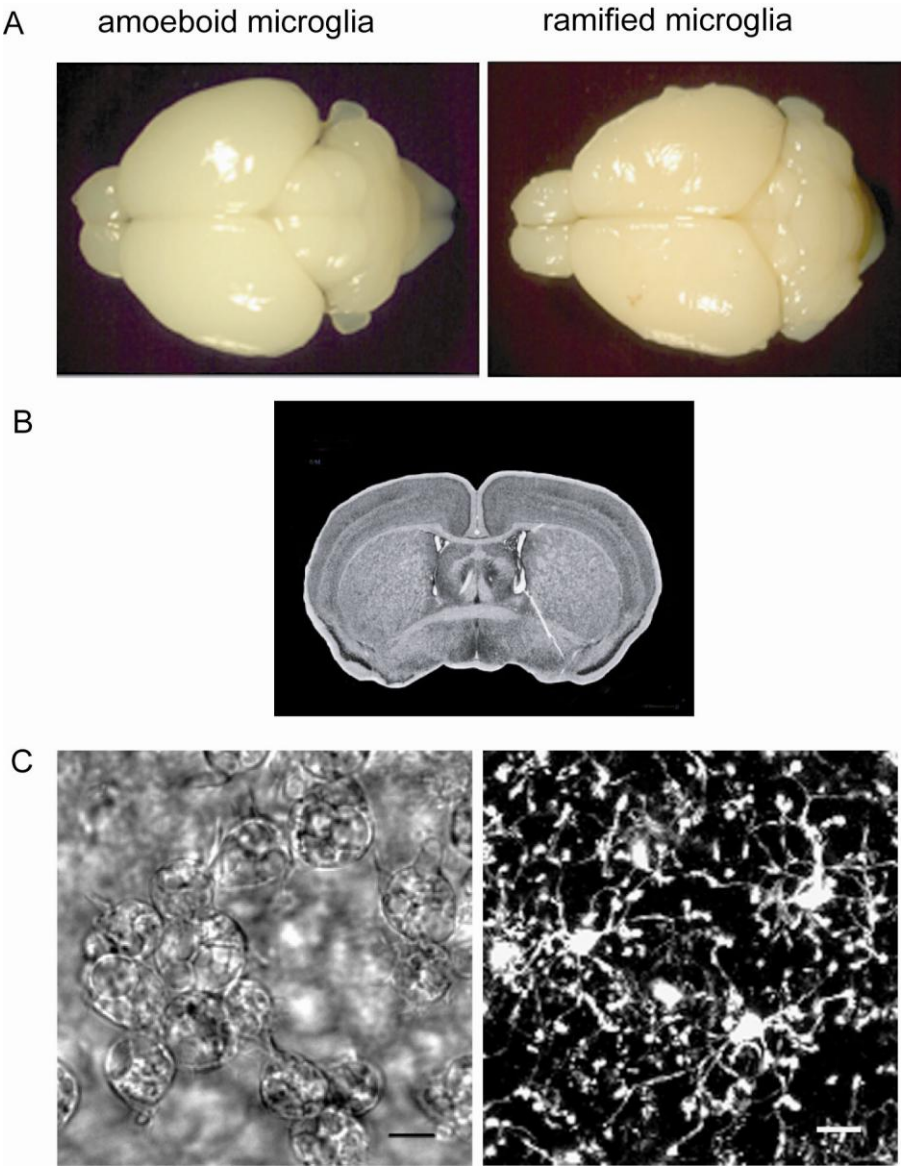


Figure 11 Acute brain slice preparation from P6-9 and adult C57Bl/6 mice.

(A) Brains of P6-9 old mice (left) or adult (right) mice are carefully isolated. (B) Coronal brain slices are prepared from the frontal cortex of freshly isolated whole brain including the corpus callosum using a vibratome. (C) Here, amoeboid microglia accumulate after two hours incubation in ACSF. Ramified microglia display a territorial organization in acute brain slices. Scale bar represents 10 µm.

### *2.2.11 Phagocytosis experiment in vitro*

In this project microglial phagocytic activity was examined using fluorescent monodispersed polystyrene microspheres with a diameter of 3 µm. Carboxylate groups on the surfaces were covalently coupled to proteins present in heat inactivated fetal calf serum. Similar microspheres were used by different groups to study phagocytosis of microglia or other immune cells *in vitro* (Mitrasinovic, Vincent

et al. 2003; Mandrekar, Jiang et al. 2009). Recently, microspheres were injected into the brain of an animal model for prion disease to assess the effect of disease progression on microglial properties (Hughes, Field et al. 2010). Usage of different sizes and opsonization allows a quantitative analysis of phagocytic activity of different cell types in various conditions.

Primary microglia was used one day after plating on glass cover slips in a 24 well format. Yellowgreen fluorescent Fluoresbrite carboxylated microspheres (3µm diameter) were coated with heat inactivated fetal calf serum (FCS) by shaking at 1000 rpm for 30 min at room temperature. After centrifugation for 2 min at 3000 rpm supernatant was discarded and microspheres washed and resuspended in DMEM. $8.4x10^6$ microspheres with or without neurotransmitters were applied on each cover slip for 30 min and incubated at 37°C in an incubator. As a negative control, the experiment was also done at 4°C. After strong washing with 0.1 M PB cells on the cover slips were fixed with 4% Paraformaldehyde and stained with a thiazide containing solution to enhance the contrast of the cell membrane in bright field microscopy in order to count the number of microspheres per cell. In additional experiments cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to measure the fluorescence of microspheres (excitation: 488 nm; emission: 530 nm) normalized to DAPI fluorescence (cell density; excitation: 360 nm; emission: 450 nm) in a plate reader.

### *2.2.12 Phagocytosis experiment in situ*

Acute brain slices from P6-9 or 8-12 weeks old mice were used 2 h after preparation. After that time amoeboid microglia in acute brain slices from younger animals accumulate on the slice surface in the area of the corpus callosum and are easily detectable by their amoeboid morphology (Figure 12 A). In acute brain slices from older animals I investigated phagocytic properties of adult, ramified microglia (Figure 12 B). Again, FCS coated microspheres with or without neurotransmitters in HBSS were applied on acute brain slices for 30 min (amoeboid microglia) or 60 min (ramified microglia) and incubated at 37°Cfollowed by washing with 0.1 M PB and fixing in 4% Paraformaldehyde to stop any engulfment process. After washing, slices were stained to visualize microglial cells and cell nuclei. Acute slices from young animals were stained for 30 min with Hoechst 33258 (1:10000 in 0.1 M PB) and

tomato lectin (1:100 in 0.1 M PB), which labels microglia and endothelial cells of the blood vessels, that can be easily distinguished by their morphology.
Acute slices from adult mice were stained as indicated in the immunhistochemistry section. Slices were mounted with Aqua polymount on glass slides and used for analysis using fluorescent microscopy.

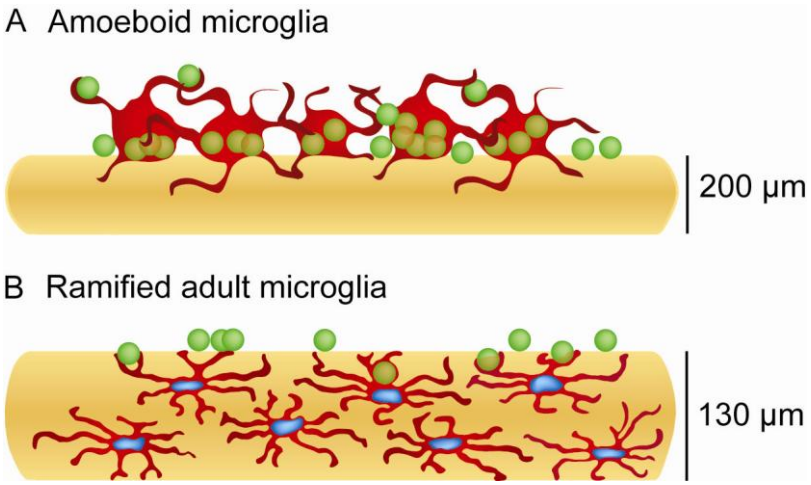


Figure 12 Schematic presentation of phagocytosis experiments *in situ.*

(A) Amoeboid microglia accumulate on the slice surface and are highly phagocytic. They take up material by their fine and short processes. (B) Adult microglia are evenly distributed in the slice. They send their processes towards the slice surface and take up microspheres. Slices were prepared in 200 µm or 130 µm thickness for amoeboid and adult microglia investigations, respectively.

### *2.2.13 Phagocytic Index calculation*

After counting the number of microspheres per cell per field of view, 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 phagocytic index.

### *2.2.14 Immunohistochemistry*

Fixed slices from adult C57BL/6 were stained for Iba-1 as a marker for microglial cells and Hoechst 33258 after phagocytosis experiment for quantification using confocal microscopy (Leica SPE). Therefore slices 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% permeabilization buffer was used. After washing slices 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.

### *2.2.15 Fluorescent microscopy*

Acute brain slices from P6-9 mice were analyzed using fluorescent microscopy together with xxx filters. Both, microglia in red and microspheres in green are visible. I counted the number of microspheres per cell in at least 8 fields of view per condition at 40x magnification and calculated the phagocytic index as indicated above.

### *2.2.16 Confocal microscopy*

Confocal laser scanning microscopy was performed using Leica SPE confocal microscope and LAS AF software to quantify the phagocytosis activity in acute brain slices from adult mice. I performed z-stacks of 20µm thickness with a step size of 1µm beginning from the top of the slice were the microspheres are located. Beads per cell were counted per touch count function using Image J Macbiophotonics cell counter plugin ensuring that only beads inside a cell were counted positive. The phagocytosis index was calculated as described above.

### *2.2.17 Two-photon imaging and laser lesion*

For two-photon experiments 300 µm brain slices were obtained from *Cx3Cr1*-GFP mice. For all experiments I used heterozygous mice for which was shown that microglial properties are not affected (Jung, Aliberti et al. 2000). Slices were used directly after slicing and were superfused with ACSF throughout the whole experiment. GFP positive microglia were imaged at a wavelength of 950 nm with a two-photon laser scanning microscope directly coupled to a Chameleon ultrafast laser. During imaging no photo bleaching could be observed over a period of one hour. In order to create a discrete laser lesion the laser was focused to the highest

extent at a wavelength of 730 nm to -35 µm depth and scanned the tissue until autofluorescence was visible. By that technique I could obtain comparable lesions with a size of 19 ± 6µm (between 10 and 34 µm, measured as longest dimension of the lesion) in the middle of the observed region. For monitoring the response to the laser lesion we imaged a 60µm thick z-stack with a step size of 3 µm covering a field of 307.2 x 307.2µm.

In order to investigate the influence of serotonin on the microglial response to the laser lesion I applied concentrations of 10µM, 100µM and 1mMserotonin via the perfusion system and incubated the slice at least 10 min before starting the experiment.

Image J MacBiophotonics was used for data analysis adapted from Davalos et al. (Davalos, Grutzendler et al. 2005). Before analysis the sequences of 3D image stacks were converted into sequences of 2D images by maximum intensity projection algorithm. For quantification, microglial response to focal lesion was defined as a shift of GFP fluorescence (white pixels), associated with microglial processes, from distal into proximal circular region, immediately surrounding the focal lesion. To eliminate signal intensity differences between different records, grayscale images were first converted into binary form using built-in function of Image J software. In resulting images all fluorescent structures were set to the maximum grayscale value (255) and the background to 0. Proximal to lesion region was defined as circle with the center at the center of a lesion and the diameter equal to 2x of the lesion diameter, distal region was defined as concentric circle with diameter 4x of the lesion (Figure 13). We calculated the pixels entering the inner circle in each frame over time subtracting the pixels of the first picture to only focus of new processes migrating to the site of injury. This was then related to the pixels of the outer diameter taken from the first picture directly after laser lesion to correct for the cell density around the lesion site. The microglial response is therefore given as $R(t)=(Rx(t)-Rx(0))/Ry(0)$.

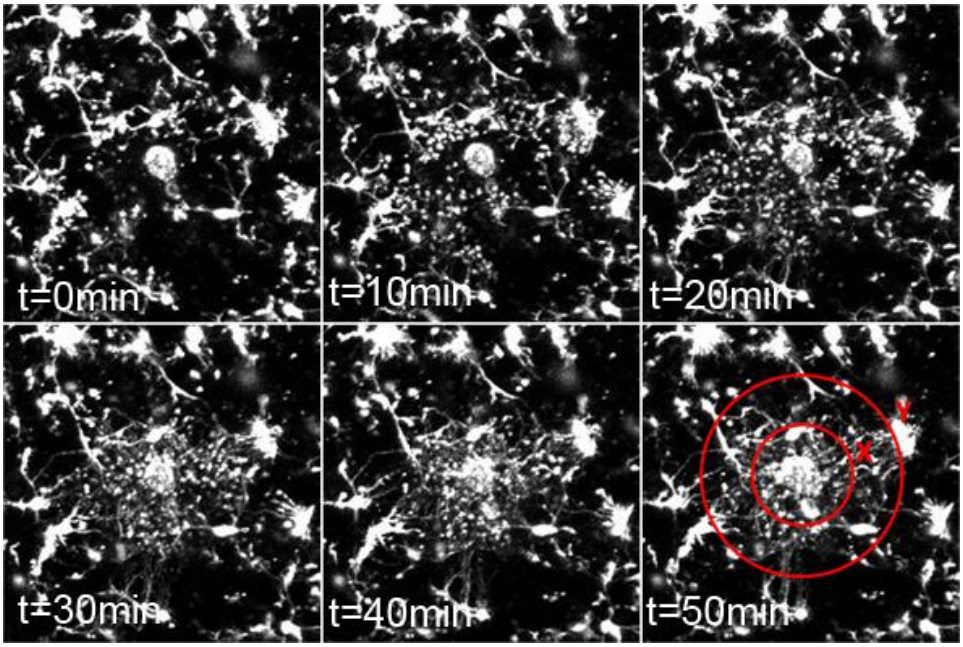


Figure 13 Analysis of live imaging experiments.

Live imaging of acute brain slices after laser induced lesion was performed over one hour. Binarized pictures were evaluated by two concentric circles having either two times diameter (X) or 4 times diameter (Y) of the laser induced lesion. Pixel entering the inner circle from the outer circle were counted and normalized to the first picture to control for different cell densities. Displayed are representative pictures of maximum projected z-stack from 0, 10, 20, 30, 40 and 50 min of the recorded time. Settings of the circles for analysis are displayed in red.

### *2.2.18 Statistical analysis*

Data sets were tested for normality by Shapiro-Wilks Test using SPSS 11.5 or GraphPad Prism. Two-sided levels of significance were determined by using the non-parametric Mann-Whitney U-Test or the parametric T-Test according to the distribution and are depicted as *: $p < 0,05$, **: $p < 0,01$, ***: $p < 0,001$. Data are presented as mean ± s.e.m.

# 3 Results

## 3.1 Modulation of microglial properties by neurotransmitter stimulation

It is of great interest to understand microglia – neuron interaction and the impact of neurotransmitters, the neuronal means of communication, on microglial properties. In this project I determined the phagocytic activity and migratory response towards an ATP gradient *in vitro* and *in situ* upon stimulation with various neurotransmitters like serotonin, norepinephrine, dopamine and glutamate.

### *3.1.1 Microglial baseline activity is age dependent*

I quantified the uptake of green fluorescent microspheres in three different microglial preparations: 1. Primary neonatal microglia from P0 mice, 2. Amoeboid microglia in acute brain slices from P6-9 mice and 3. Adult microglia in acute brain slices from 2 months old mice. Analysis of microsphere uptake resulted in a significant change of baseline phagocytic activity in adult microglia as measured by the percentage of phagocytic cells per field of view (Figure 14 A) and engulfed microspheres per phagocytic cell (Figure 14 B).

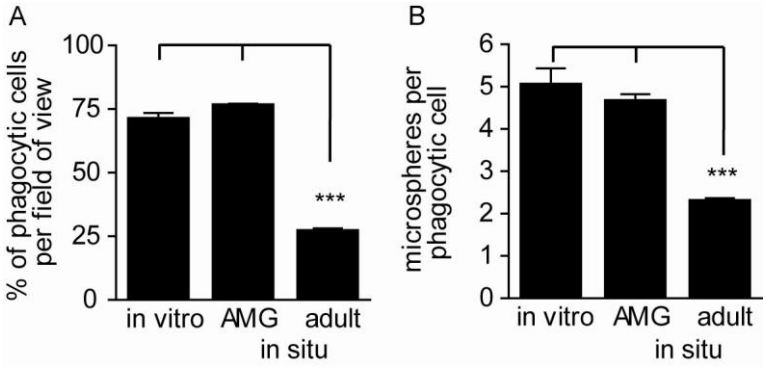


Figure 14 Phagocytic activity is higher in young than in adult microglia.

Percentage of phagocytic cells per field of view (A) and the number of microspheres per phagocytic cell (B) for neonatal microglia (*in vitro*), amoeboid microglia (AMG) and ramified microglia (adult) *in situ* show a significant reduction in adult microglia. Data is presented as mean ± s.e.m., *** $p<0.001$.

In average 71 ± 3% and 76 ± 1% (mean ± s.e.m.) of neonatal and amoeboid microglia (young microglia) per field of view phagocytosed one or more microspheres, respectively, which was significantly lower in adult microglia as only 27 ± 2% of analyzed cells were phagocytic. Additionally I determined the number of microspheres in adult microglia in cortex of acute brain slices as being in average 2.27 ± 0.1 per phagocytic cell whereas young phagocytic microglia take up significantly more microspheres engulfing in average 5.02 ± 0.42 (neonatal) and 4.63 ± 0.19 (amoeboid) microspheres per phagocytic cell (mean ± s.e.m.).
Comparison of microglial phagocytic activity *in situ* in 2 months, 4 months, 9 months and 20 months old *APPPS1* or *APP23* wildtype mice (C57BL/6) revealed a significant increase of phagocytic activity, measured as phagocytic index, from 33 ± 3 in 2 months old mice to 62 ± 4 in 9 months old mice which drops again in the oldest investigated animals to 48 ± 4 (mean ± s.e.m.). There is already a tendency of increase in 4 months old mice (46 ± 3) which is not yet significant (Figure 15 A).

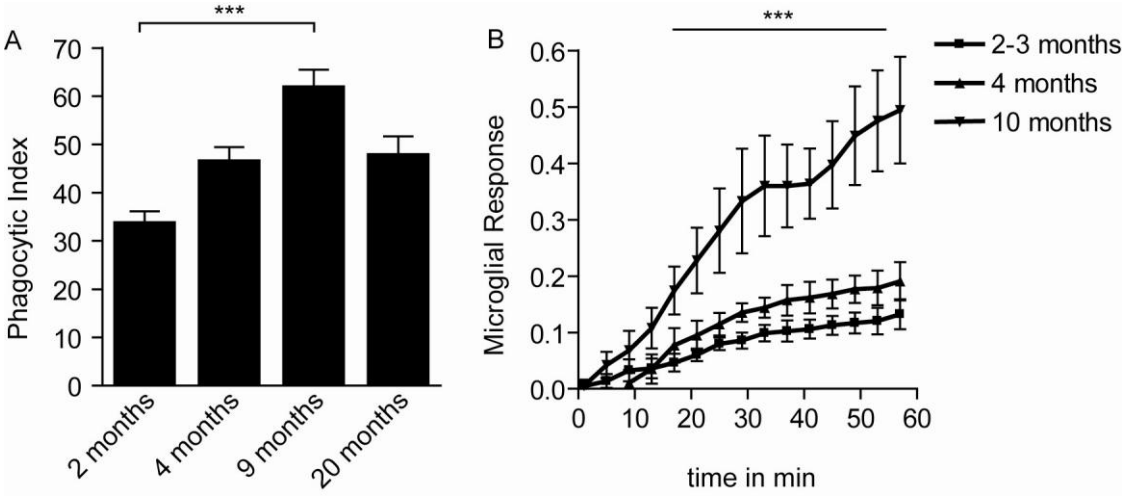


Figure 15 Microglial properties in different ages

(A) Absolute values of microglial phagocytic activity in 2 months, 4 months, 9 months and 20 months old C57BL/6 mice. (B) Microglial response towards a laser induced lesion in 2-3 months, 4 months and 10 months old *Cx3cr1-GFP* mice is displayed. Data are mean ± s.e.m., *** p<0.001.

Additionally, I determined the response towards a laser induced lesion of microglial cells in 2-3 months, 4 months and 10 months old *Cx3Cr1*$^{+/GFP}$ mice. Comparison revealed that there is slight increase of the response in 4 months old mice in respect

to 2-3 months old mice and a very strong increase in 10 months old mice (Figure 15 B) which is significant from minute 35 of observation.

### *3.1.2 Microglial phagocytic activity is reduced upon stimulation with neurotransmitters of the monoamine family in vitro*

Primary microglia derived from mixed glial cultures of P0 C57BL/6 mice were used to assess microglial phagocytic activity upon neurotransmitter application (Figure 16 A) measured by the uptake of microspheres. In experiments without stimulation 70 ± 3% of the analyzed cells were phagocytic cells thereby engulfing in average 5 ± 0.4 microspheres. Application of 100 µM serotonin resulted in a decreased phagocytic activity of 76 ± 5% of control conditions. Also application of 100 µM dopamine or norepinephrine together with the microsphere solution lowered microglial phagocytic activity to 81 ± 5% and 83 ± 4% of control condition, respectively (mean ± s.e.m., Figure 16 B).

I tested the specific agonist for the serotonin receptor 2 family (5-HT2) DOI for phagocytosis experiments because it was shown in previous studies in our group that DOI evokes current changes in microglia in electrophysiological recordings (data not published). Application of DOI resulted in a concentration dependent decrease of microglial phagocytic activity as application of 10 µM DOI lowered the phagocytic index to 71 ± 3% compared to control conditions whereas application of 100 µM reduced phagocytic activity to 60 ± 2% of control (Figure 16 C).

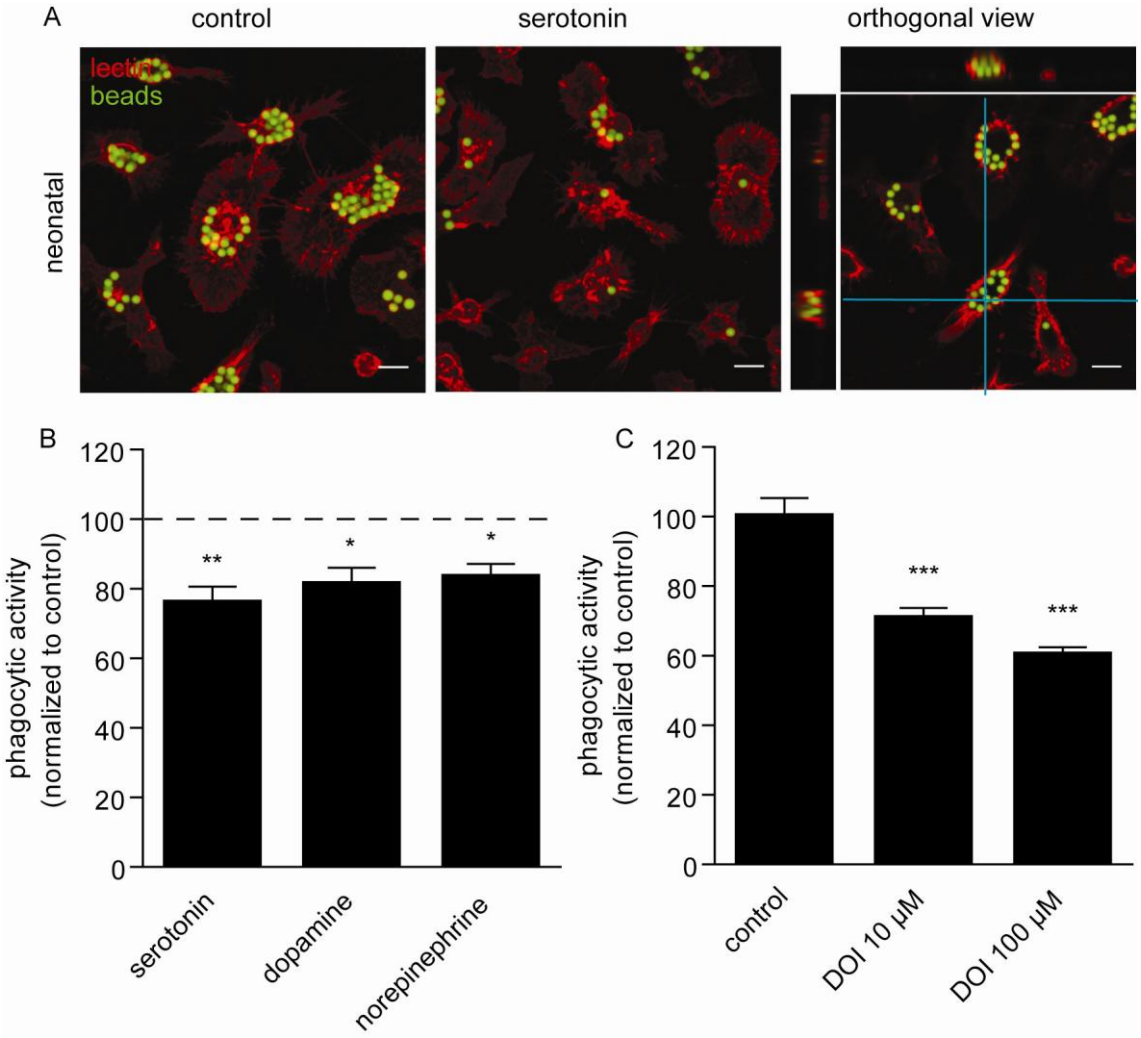


Figure 16 Phagocytic activity of neonatal mouse microglia upon neurotransmitter receptor stimulation.

(A) Representative pictures of confocal z-stacks of neonatal primary microglia after incubation with microspheres with (middle) or without (left) neurotransmitter (serotonin) for 30 min. Right panel shows orthogonal view of 10 µm z-stack. Cells were stained with tomatolectin to visualize microglia. (B) Serotonin, dopamine and norepinephrine were applied at a concentration of 100 µM to primary microglia together with microsphere solution to quantify phagocytic activity and normalized to unstimulated control. (B) Quantification of phagocytic activity of primary microglia upon stimulation with 10 and 100 µM of the 5-HT2 agonist DOI. Values are mean ± s.e.m. and were normalized to untreated control, *p≤0.05, **p≤0.01, *** p≤0.001. Scale bar represents 10 µm.

To verify the duration of microsphere-neurotransmitter application, I performed a time series experiment using 10 µM and 100 µM serotonin for stimulation. I applied the serotonin-microsphere solution for 5, 10, 30 and 60 min to primary microglia plated in a 24-well format, washed carefully and immediately fixed the cells after the indicated incubation time to stop any uptake mechanism (Figure 17). Results show that 30 min incubation of 100 µM had the strongest effect in decreasing phagocytic activity

significantly to 32 ± 4% compared to 67 ± 8% in the corresponding control (mean ± s.e.m.). Longer incubation of the serotonin-microsphere solution did not enhance this effect.

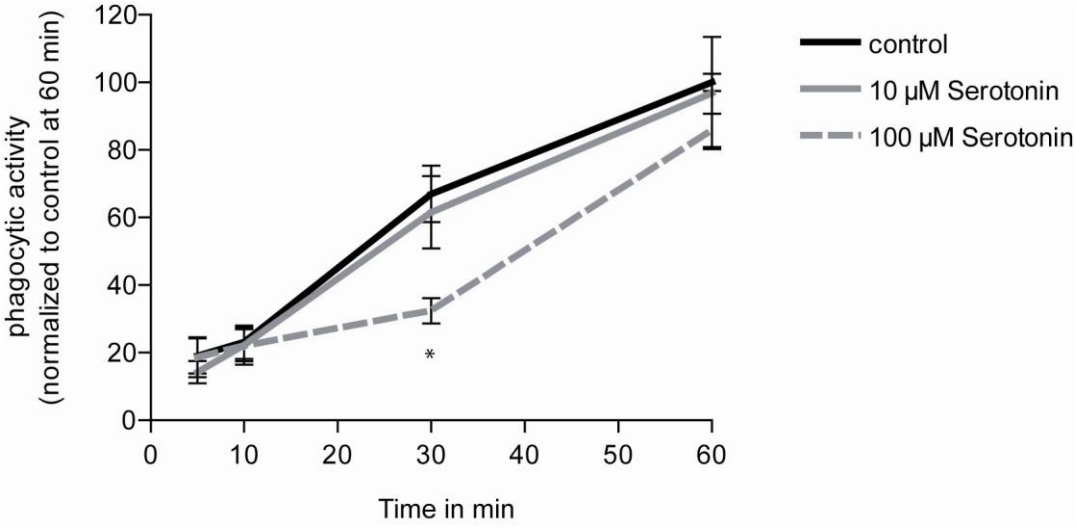


Figure 17 Time series of phagocytosis experiment in neonatal primary microglia.

Microsphere solution was applied with or without serotonin (10 µM, 100 µM) to neonatal primary mouse microglia in a 24-well plate and incubated at 37°C. Phagocytic activity is depicted as ratio of microsphere fluorescence per DAPI fluorescence after 5, 10, 30 and 60 min incubation. Data are represented as mean ± s.e.m., *p≤0.05

### *3.1.3 Neurotransmitter application leads to a decrease in phagocytic activity of amoeboid microglia in situ*

Amoeboid microglial cells *in situ* are highly phagocytic (Figure 18 A) as 68 ± 4% of the analyzed cells were phagocytic cells engulfing 3 ± 0.19 microspheres in average. Application of 100 µM serotonin, dopamine and norepinephrine decreased phagocytic activity significantly to 80 ± 5%, 76 ± 4% and 70 ± 6%, respectively. Application of 100 µM glutamate, a classical neurotransmitter, did not show any significant effect (mean ± s.e.m, n=20 from 10 mice, Figure 18 B).

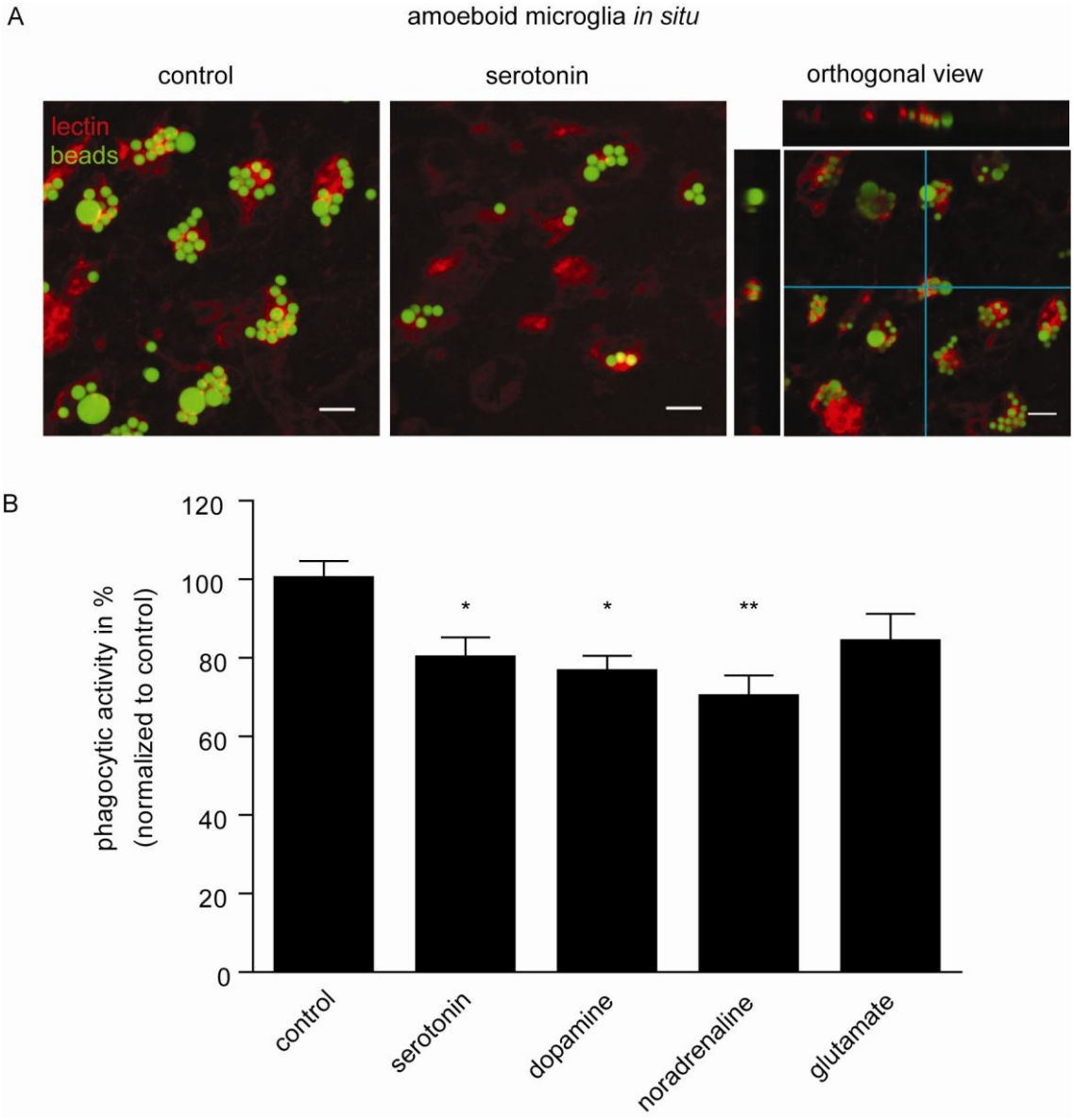


Figure 18 Phagocytic activity of amoeboid microglial cells upon neurotransmitter stimulation.

(A) Representative confocal pictures of amoeboid microglia after application of microsphere solution with or without serotonin stimulation. Slices were stained for tomatolectin (red) to visualize microglial cells. (B) Quantification of the phagocytic activity of amoeboid microglia *in situ* upon stimulation with 100 µM of serotonin, dopamine, norepinephrine and glutamate. Values are mean ± s.e.m. and were normalized to untreated control, * p≤0.05, ** p≤0.01.

### *3.1.4 Microglial phagocytic activity in acute brain slice from adult mice is only modulated by high concentrations of dopamine*

In a first set of experiments different concentrations of serotonin, dopamine, norepinephrine and glutamate were tested for their effect on phagocytic activity of ramified microglial cells in acute brain slices from adult C57BL76 mice (Figure 19 A). Here, slices were also incubated two hours after slicing procedure to compare results to amoeboid microglia in slices from younger animals. I incubated slices with the neurotransmitter-microsphere solution for one hour as it was not expected that ramified microglia are as active as amoeboid microglia in taking up these particles. In control conditions 27 ± 2% of the investigated cells were phagocytic cells engulfing in average 2.3 ± 0.1 microspheres per cell.

Application of 100 µM or 1 mM dopamine resulted in a decrease of phagocytic activity to 77 ± 9% and 43 ± 8% compared to control, respectively. Also norepinephrine showed a tendency to inhibit the uptake of microspheres to 80 ± 10% and 64 ± 10% of control conditions by application of 100 µM and 1 mM, respectively. Application of either 100 µM or 1 mM serotonin or glutamate did not show any significant effect (mean ± s.e.m., n>15 from >3 mice, Figure 19 B).

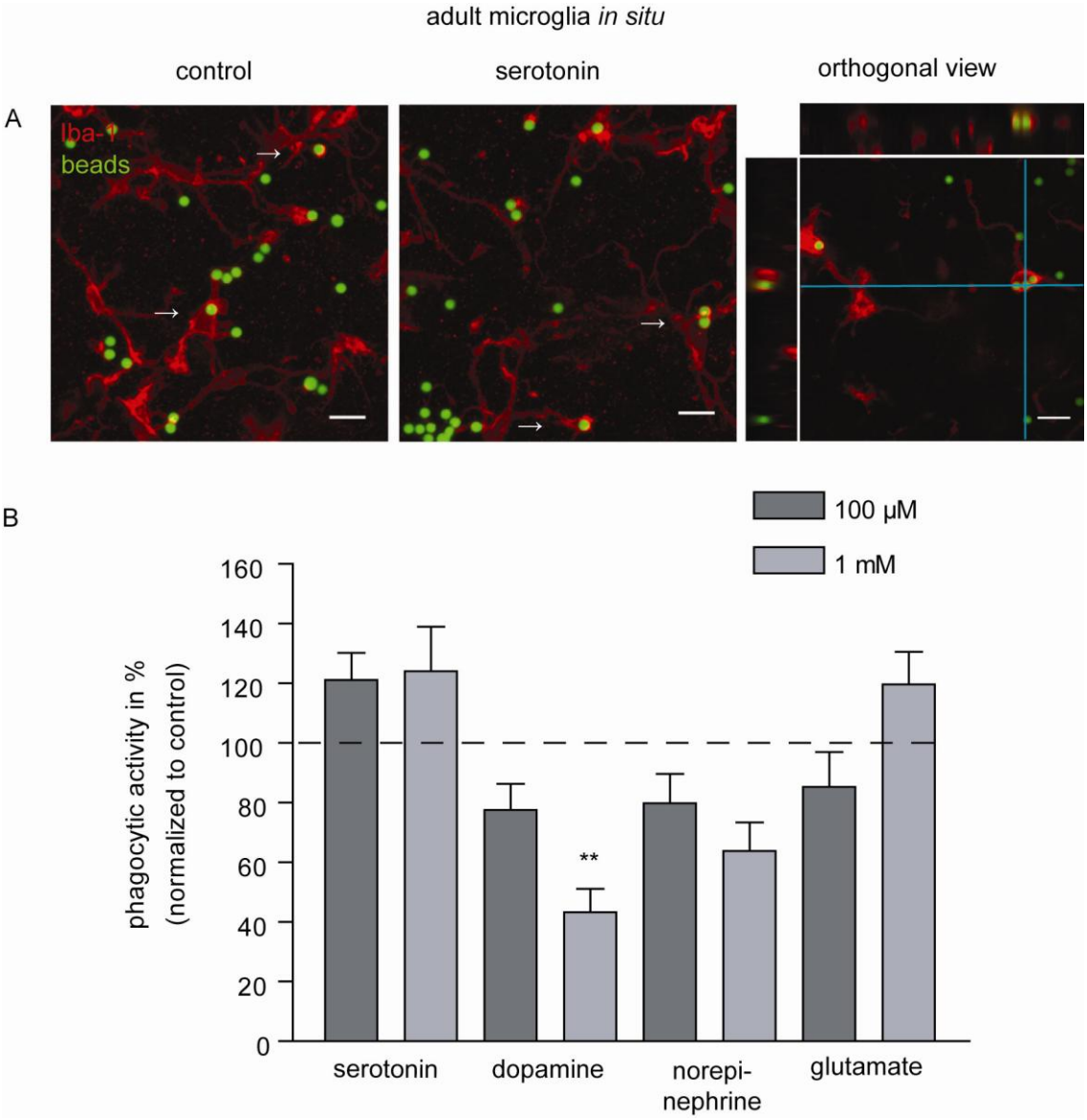


Figure 19 Phagocytic activity of adult microglia cells *in situ* upon neurotransmitter application.

Phagocytic activity was measured after exposure of acute adult brain slices to neurotransmitter-microsphere solution for one hour and subsequent fixation and staining for Iba-1 (microglia, red). Representative confocal stacks show that Iba-1 positive cells take up microspheres (green). Orthogonal view of confocal stacks illustrates complete engulfment of fluorescent micropsheres. Quantification of phagocytic activity of adult microglia after application of 1 mM or 100 µM of serotonin, dopamine, norepinephrine and glutamate together with microspheres reveal a significant decrease with 1 mM dopamine. Values are mean ± s.e.m. and were normalized to untreated control, **p≤0.01.

### 3.1.5 *Adult microglial phagocytic activity in slices is not changed by dopaminergic or adrenergic receptor agonists*

I aimed to verify the effect of dopamine and norepinephrine on microglial phagocytic activity in adult slices by using specific agonists for dopaminergic and adrenergic

receptors alone or in combination. This part of the project was carried out by a Bachelor student Lars Krieger from the University of Konstanz. Since only norepinephrine and dopamine influence adult microglial phagocytic activity, but were shown to be oxidized rapidly exhibiting modified effects (Takakura, Xiaohong et al. 2003; Zoccarato, Toscano et al. 2005), we tested dihydrexidine (D1, D5 receptor, 100 µM) and quinpirole (D2, D3, D4 receptors, 10 µM) as well as metaraminol (α-adrenergic receptor, 100 µM) and isoproterenol (β-adrenergic receptor, 50 µM) for their ability to modulate microglial phagocytic activity in concentrations that were shown to mediate membrane currents changes in microglia (Farber, Pannasch et al. 2005). Neither application of dopaminergic receptor agonists alone or in combination nor adrenergic agonists in combination modulated significantly phagocytic activity in acute brain slices when exposed together with green fluorescent microspheres (n=24 from 4 mice, Figure 20).

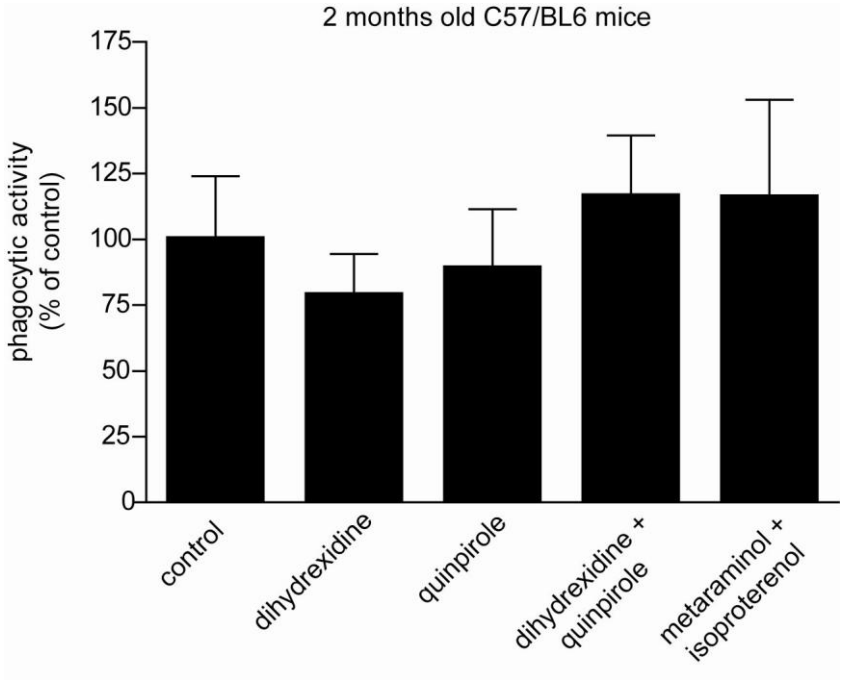


Figure 20 Specific agonists for adrenergic and dopaminergic receptors do not modify microglial phagocytic activity.

The dopaminergic agonists dihydrexidine (10 µM) and quinpirole (10 µM) were applied alone or in combination on acute brain slices from 2 months old C57BL/6 mice together with fluorescent microspheres to quantify microglial phagocytosis. In parallel experiments the adrenergic agonists metaraminol (100 µM) and isoproterenol (50 µM) were also tested in combination for their effect on microglial phagocytic activity. Neither dopaminergic nor adrenergic agonists showed significant modulation of microglial phagocytic activity. Data is presented as mean ± s.e.m.

### *3.1.6 Serotonin facilitates ATP induced microglial migration in vitro and in situ*

Live imaging experiments of the microglial response towards laser induced injury were performed on acute brain slices taken from heterozygous *Cx3Cr1*-GFP mice with or without neurotransmitter application. Data was obtained after creating laser lesions in acute brain slices with an average diameter of 19 ± 6 µm (mean ± s.d.). For control recordings I determined an average peak response of 0.12 ± 0.02 (n = 6). Application of 100 µM and 1 mM serotonin resulted in similar modulation of microglial response towards acute injury by increasing microglial process movements towards injury to an average peak response to 0.20 ± 0.03 (n = 7) and 0.21 ± 0.02 (n = 13), respectively whereas 10 µM serotonin did not change the response significantly (mean ± s.e.m., Figure 21 A, B).

To verify whether this was a direct effect on microglia or if the application of serotonin triggers the release of other molecules that act on microglia, I performed migration experiments *in vitro* using primary microglia. Application of 100 µM serotonin alone resulted in an increased migration rate of 155 ± 5% (n = 3) compared to control. The chemoattractant ATP at a concentration of 100 µM triggered a migratory response of 350 ± 8% (n = 4) compared to control conditions. To investigate if serotonin could further stimulate the migratory response of microglial cells towards an ATP gradient I applied 0.01 µM, 1 µM and 100 µM together with 100 µM ATP which further increased ATP induced migration rate to 387 ± 8%, 391 ± 9% and 430 ± 10%, respectively (mean ± s.e.m., n=4, Figure 21 C).

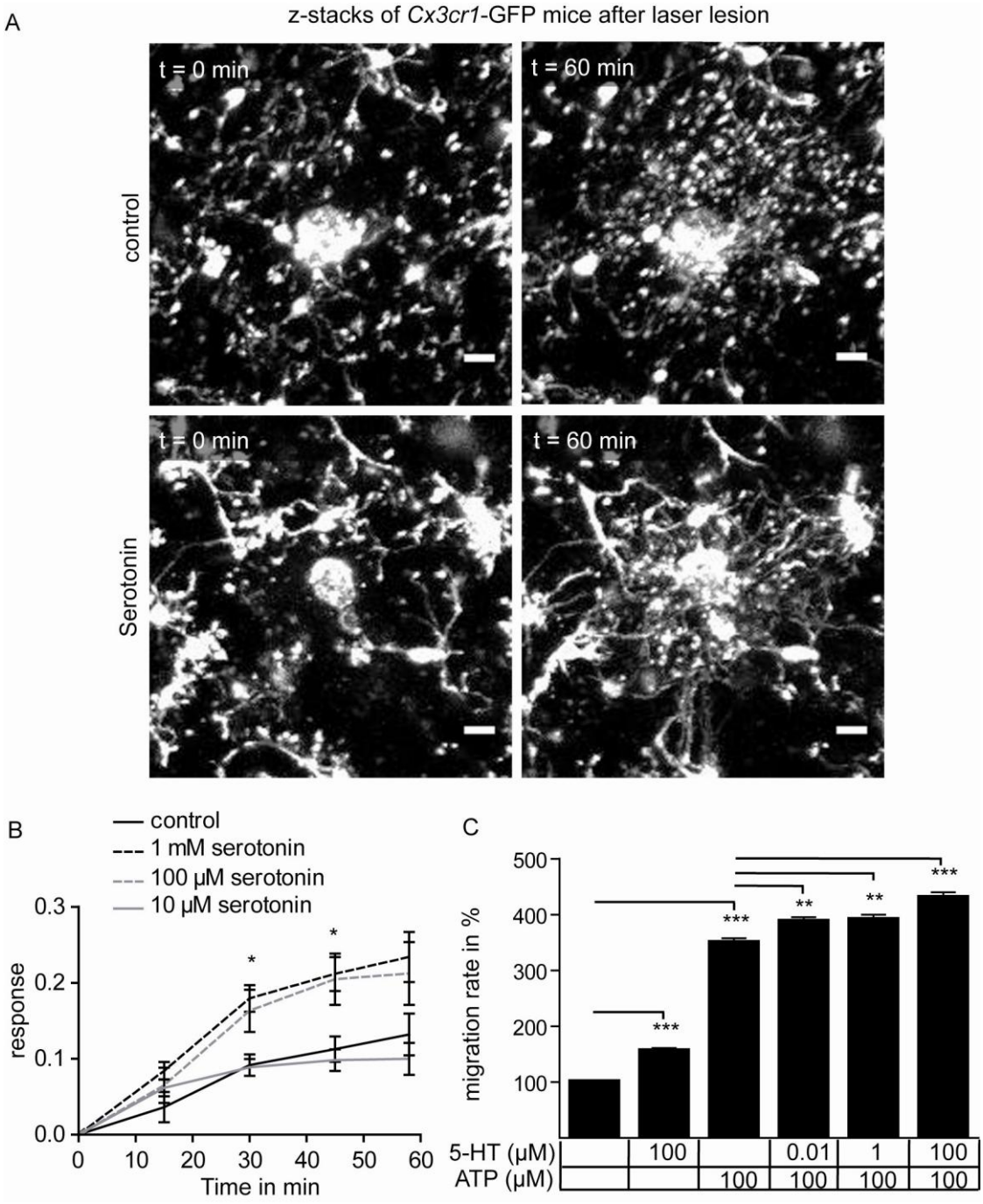


Figure 21 Serotonin stimulates ATP induced microglial migration.

(A) Representative pictures of 60 µm z-stacks of two-photon imaging in control (upper panel) and upon serotonin (lower panel) application. Pictures of t=0 min (left panel) and t=60 min (right panel) are displayed. (B) Time course of the microglial response in control conditions and after stimulation with serotonin. Application of 100 µM and 1 mM serotonin significantly enhanced microglial process movements. (C) *In vitro* chemotaxis experiments using neonatal primary microglia. Data is normalized to unstimulated control. 100 µM serotonin triggered microglial migration significantly. Application of increasing concentration of serotonin to an ATP gradient further stimulated ATP induced microglial chemotaxis. Values are mean ± s.e.m, *p≤0.05, **p≤0.01, *** p≤0.001. Scale bar represents 10 µm.

### *3.1.7 Norepinephrine but not dopamine or adrenergic and dopaminergic agonists modify microglial response towards laser induced acute injury*

Neurotransmitters like dopamine were shown to stimulate chemotactic movements of neonatal microglial cells (Farber, Pannasch et al. 2005). Therefore I tested the effect of dopamine as well as norpeinpehrine on microglial movement induced by acute injury in acute brain slices derived from adult *Cx3cr1*-GFP mice. Neurotransmitters were applied at concentration of 100 µM to ensure that also microglia in deeper layers of the brain slice can be stimulated. Application of norepinephrine resulted in a significant reduction of microglial process movements towards laser induced injury by lowering the average peak response from 0.12 ± 0.02 (n = 6) for control recordings to 0.05 ± 0.02 (n = 11) upon norepinephrine stimulation. In contrast, application of 100 µM dopamine did not change microglial response significantly although there is a tendency in increasing microglial response as the average peak response could be determined as 0.21 ± 0.07 (n = 6, mean ± s.e.m., Figure 22 A).

Further studies to evaluate modulation of microglial response towards acute injury by adrenergic and dopaminergic receptors were included in the bachelor project of Lars Krieger which he performed under my supervision. Acute brain slice were stimulated throughout the observation of microglial response to acute injury using a combination of either adrenergic receptor agonists metaraminol (100 µM, α-adrenergic agonist) and isoproterenol (50 µM, β-adrenergic agonist) or dopaminergic agonists dihydrexidine and quinpirol (both 10 µM, D1-like and D2-like receptor agonists, respectively, Figure 22 B). An average peak response was determined for unstimulated control of 0.28 ± 0.06 (n=8). Application of dopaminergic agonists did not change microglial movements to the injury site significantly as an average peak response of 0.34 ± 0.11 (n=5) was calculated. In contrast to stimulation with norpepinephrine which reduced significantly microglial process movements, combination of adrenergic agonists tends to result in an increased microglial response to acute injury as the average peak response is increased to 0.51 ± 0.11 (n=8, mean ± s.e.m.).

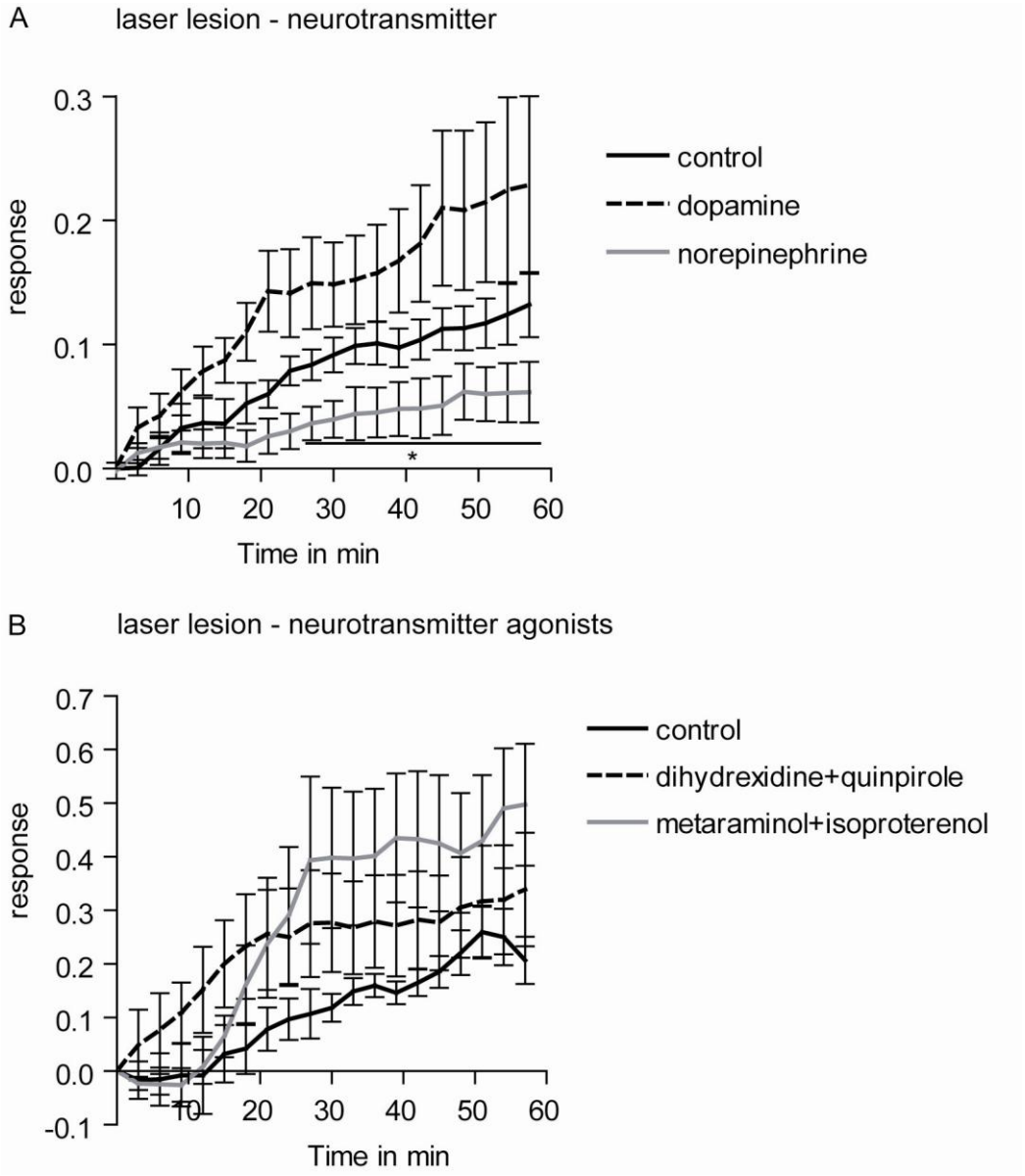


Figure 22 Norepinephrine but not dopamine or specific agonists modify microglial response towards laser lesion.

Laser lesion experiments were performed on acute brain slices from *Cx3cr1*-GFP mice in control conditions and upon constant perfusion with neurotransmitter receptor agonists. (A) Time course of microglial process movements towards laser induced lesion. 100 µM of neurotransmitters norepinephrine and dopamine were applied throughout the observation. Only norepinephrine significantly reduced microglial response compared to unstimulated control. (B) Dihydrexidine and quinpirole (both 10 µM) or metaraminol (50 µM) and isoproterenol (100 µM) were applied in combination on acute brain slices to stimulate either dopaminergic or adrenergic receptors, respectively, during response to acute injury. Observation of microglial process movements was not significantly changed upon neurotransmitter agonist treatment compared to unstimulated control. Data is presented as mean ± s.e.m., *p≤0.05.

### *3.1.8 Serotonin does not alter LPS induced release of cytokines and nitric oxide*

Dopamine as well as norepinephrine was shown to attenuate lipopolysaccharide (LPS) induced release of cytokines like Interleukin-6 (IL-6) and TNF-α and nitric oxide in neonatal microglia (Farber, Pannasch et al. 2005). I tested different concentrations of serotonin together with 100 ng/ml LPS on the release of nitric oxide and cytokines like macrophage inflammatory protein-1 α (MIP-1α), TNF-α and IL-6 measured by Griess reaction and ELISA, respectively and compared the results to unstimulated control. LPS application triggered a significant release of nitric oxide (Figure 23 A) as well as MIP-1α (Figure 23 B), TNF-α (Figure 23 C) and IL-6 (Figure 23 D). Neither the release of nitric oxide nor the release of the analyzed cytokines was changed upon treatment with 0.1, 1 or 100 µM serotonin compared to LPS stimulation alone.

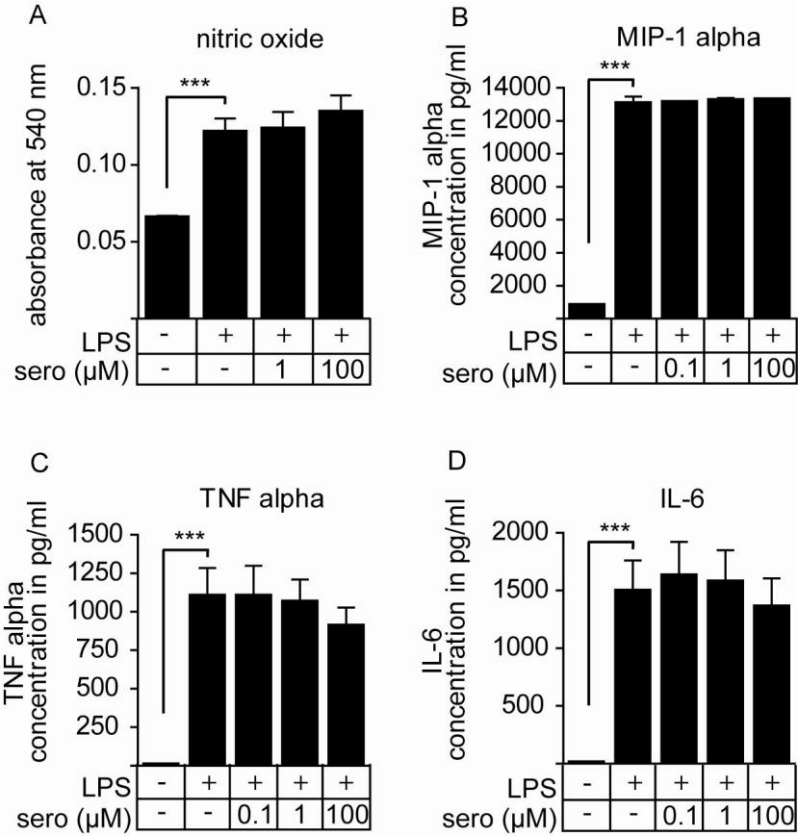


Figure 23 Serotonin application did not modify LPS induce cytokine and nitric oxide release in neonatal cultured microglia.

Microglial cells in 96 well plates were stimulated for 24 h with different concentrations of serotonin in the presence of 100 ng/ml LPS. Nitric oxide release upon LPS stimulation together with 1 µM or 100 µM serotonin was quantified in the supernatant by Griess reaction. Release of cytokines like MIP-1 alpha, TNF alpha and IL-6 after stimulation with LPS alone and together with 0.1, 1 or 100 µM serotonin were measured in the supernatant and compared to unstimulated control using ELISA assay. Data is presented as mean ± s.e.m., *** $p<0.001$.

## 3.2 Neurotransmitter receptor expression in microglial cells

Functional characterization of the impact of monoamine neurotransmitters on microglial properties like migration, phagocytosis and release of immune relevant molecules suggests an expression of functional dopaminergic, adrenergic and serotonergic receptors in microglial cells. There is already proof that microglial cells from different species express mRNA for dopaminergic and adrenergic receptors but studies on the expression of serotonin related genes are still missing for microglial cells (Mori, Ozaki et al. 2002; Farber, Pannasch et al. 2005; Mastroeni, Grover et al. 2009).

### *3.2.1 The serotonin receptor repertoire differs in neonatal versus adult microglial preparations*

Quantitative PCR was performed on primary microglia and adult microglia acutely isolated from C57BL/6 mice. In mice, there are 14 serotonin receptor and the serotonin transporter SERT expressed. Before analyzing microglial samples, primers were tested for efficiency and specificity on cDNA samples from whole brain tissue of C57BL/6 mice. Primers for 5-HT1d had to be excluded because also different tested primer sets did not meet the criteria to apply the delta $c_t$ method for analysis. In whole brain tissue, I observed that most serotonin receptors (5-HT1a, 5-HT1f, 5-HT2a, 5-HT2b, 5-HT2c, 5-HT3a, 5-HT3b, 5-HT4, 5-HT5a, 5-HT5b, 5-HT7) are expressed but detected only very low levels of 5-HT1b and 5-HT6 and the serotonin transporter SERT (Figure 24). For analysis of microglia samples I first tested their purity by the expression of a microglial marker CD11b and GFAP for contamination in relation to the expression of the housekeeping gene HMBS. In all microglia samples there was a high expression of the microglia/macrophage marker CD11b and virtually no GFAP expression as a sign for astrocyte contamination. For comparison, there were moderate levels for CD11b and high levels for GFAP in whole brain samples. In adult microglia samples from whole brain but also from different brain regions like cortex, cerebellum and striatum I could detect a reasonable expression of class 2 serotonin receptors but also expression of 5-HT5a and 7. I did not detect expression of 5-HT1b, 5-HT3a, 5-HT5b, 5-HT6 and SERT in any of the microglia samples. But I could detect

a low expression of 5-HT1a in microglia samples from adult whole brain and 5-HT1f in microglia samples isolated from adult mouse whole brain, cortex and striatum.

To compare these results to primary glial cells I used primary microglia and astrocytes from mixed glial cultures of P0 C57BL/6 mice. Primary microglia expressed the serotonin receptor of 5-HT2b but no other tested genes. In primary astrocytes I could detect the expression of 5-HT2a but also low levels of the microglial marker CD11b probably due to microglia contamination.

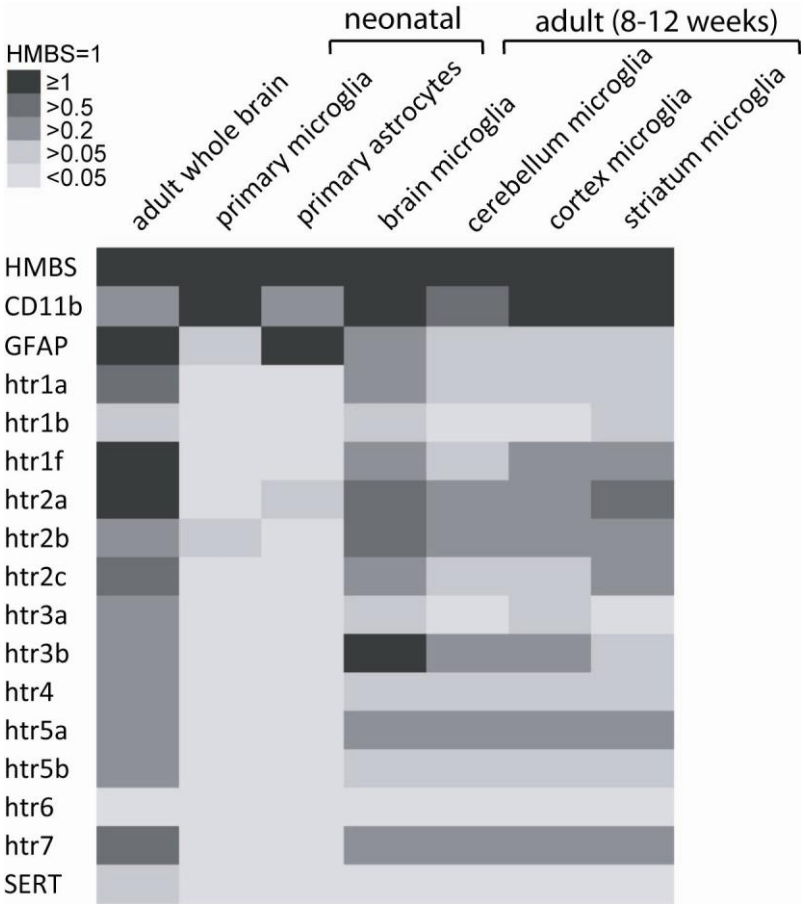


Figure 24 Serotonin receptor and transporter expression *versus* HMBS.

Quantification of mouse serotonin receptors was performed by qPCR and is displayed as expression versus the housekeeping gene HMBS. Marker for monocytes (CD11b) and astrocytes (GFAP) were used for analysis of purification. Receptor expression in primary neonatal microglia (P0) and acutely isolated adult microglia (8-12 weeks) from whole brain, cerebellum, cortex and striatum is displayed in comparison to adult whole brain and neonatal astrocytes. Data was obtained from ≥3 independent preparations.

### *3.2.2 Microglial cells do not express the essential enzyme for serotonin synthesis*

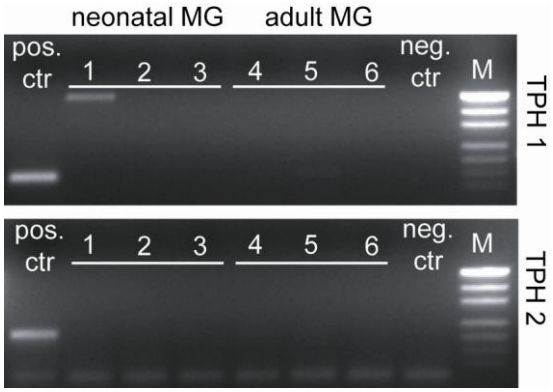


Figure 25 Microglia do not express TPH1 or TPH2.

Expression of the serotonin synthesis enzyme tryptophan hydroxylase (TPH1 and TPH2) was tested in 3 samples of neonatal (1, 2, 3) and freshly isolated adult microglia from cortex (4, 5, 6) of C57BL/6 mice. Positive controls (TPH1: P815, TPH2: cortex tissue) give clear bands and the right size (M: marker, neg. ctr: A.dest).

To investigate whether microglial cells have the capacity to synthesize serotonin, I studied the expression of tryptophan hydroxylase (TPH) in different microglial preparations (Figure 25). There is TPH1 and TPH2, selectively expresses in the periphery or brain, respectively, that catalyzes the rate limiting step of serotonin synthesis, namely the formation of 5-hydroxytryptamine from tryptophan (Walther, Peter et al. 2003). I could not detect any message for TPH1 and TPH2 neither in three independent samples from C57BL/6 neonatal microglia nor three independent isolations of adult C57BL/6 microglia. As positive controls I used P815 (for TPH1) mouse mastocytoma cell line and brain cortex (for TPH2) tissue in which I clearly obtained a signal.

## 3.3 Microglial properties in a mouse model of Alzheimer's Disease

Alzheimer's disease is accompanied by accumulation of microglial cells around amyloid-β (Aβ) plaques. To investigate functional parameters of plaque associated microglia I performed phagocytosis and imaging experiments using mouse models of Alzheimer's disease, *APPPS1* and *APP23*.

### *3.3.1 Plaque load in APPPS1 mice differs with age and brain region*

In *APPPS1* mice formation of cortical Aβ plaques occurs in the cortex at around 8 weeks of age so that 7 weeks old mice show rarely and 9 weeks old mice show some Aβ accumulation stained by Thiazine Red. These animals show a robust cortical plaque load at 4 months of age and excessive cortical Aβ accumulation at 9 months of age (Figure 26 A). Furthermore *APPPS1* mice are absolutely plaque free in hippocampus at the earlier time points but show robust Aβ accumulation starting from 4 months of age (Figure 26 B). At this age the cerebellum is still plaque free and can be used for comparative analysis (Figure 26 C).I studied the phagocytic activity of microglial cells *in situ* in different brain areas and various time points by the application of fluorescent microspheres.

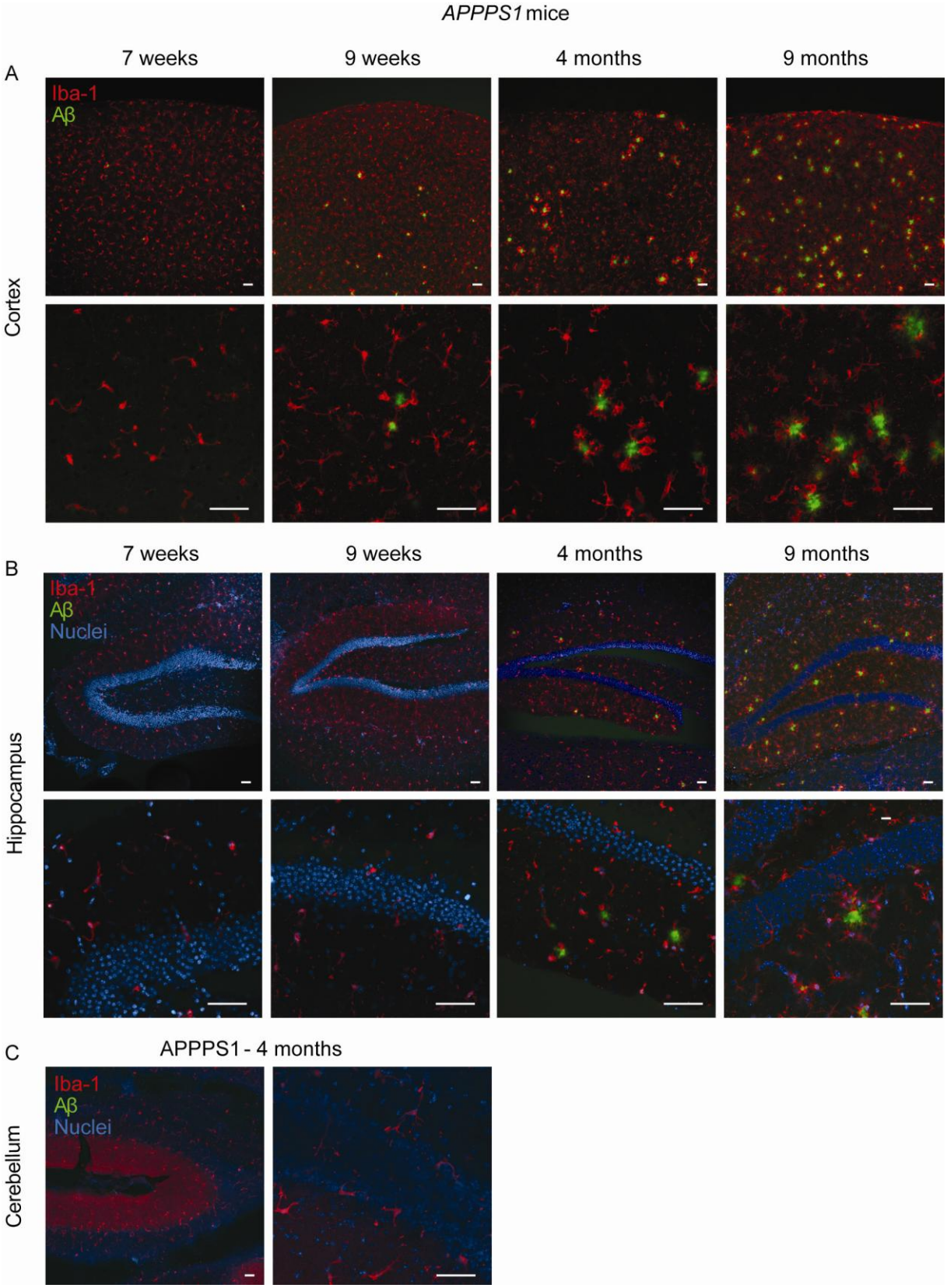
APPPS1 mice
A
7 weeks
9 weeks
4 months
9 months
Iba-1
Aβ
Cortex
B
7 weeks
9 weeks
4 months
9 months
Iba-1
Aβ
Nuclei
Hippocampus
C
APPPS1 - 4 months
Iba-1
Aβ
Nuclei
Cerebellum

Figure 26 Confocal images of 7 weeks, 9 weeks, 4 months and 9 months old *APPPS1* mice display different degrees of plaque deposition in investigated brain areas at different ages.

(A) Shown are representative images of acute coronal brain slices from *APPPS1* mice stained for microglia (Iba-$1^+$; red) and Aβ (Thiazine Red; green). At 7 weeks, *APPPS1* mice show almost complete absence of amyloid plaques in the cortex whereas at 9 weeks of age, first Aβ plaques can be detected. At 4 month, *APPPS1* mice exhibit significant cortical plaque burden, which is even more pronounced in 9 months old *APPPS1* mice. (B) Representative confocal images of the hippocampal region of *APPPS1* mice depicting microglia (Iba-$1^+$; red), Aβ (Thiazine Red; green) and nuclei (Hoechst 33258; blue). In 7 and 9 weeks old *APPPS1* mice hippocampus is plaque-free. Aβ plaque formation is clearly detectable in hippocampal slices from 4 months and 9 months old *APPPS1* mice. (C)Representative images taken from 4 months old *APPPS1* mice show the absence of Aβ plaque deposition in cerebellar slices. Scale bars: 50 µm.

### *3.3.2 Phagocytic activity is increased in a model of acute injury*

To evaluate if microglial phagocytic activity is enhanced upon activation I analyzed the uptake of fluorescent microspheres in acute brain slices taken from mice 6 days after stab wound injury. Here, microglial cells/macrophages accumulate around the lesion site showing an amoeboid morphology. I determined the phagocytic activity of these microglial cells compared to the contralateral hemisphere which is not affected by the stab wound so that microglial cells display a ramified morphology (Figure 27 A). Activated microglia in the lesion site are highly phagocytic and show a significantly increased phagocytic activity of 42 ± 5 compared to 25 ± 3 at the contralateral site (absolute values for phagocytic indices, n = 42 out of 4 mice, Figure 27 B). Furthermore significantly more cells of the area investigated are phagocytic cells (35 ± 3% compared to 24 ± 2% in the contralateral site) thereby engulfing 2.6 ± 0.14 microspheres in comparison to 2.1 ± 0.14 micropsheres in the contralateral hemisphere (Figure 27 C and D, data is presented as mean ± s.e.m.).
Thus I felt confident to apply the same paradigm to chronically activated microglia of *APPPS1* mice.

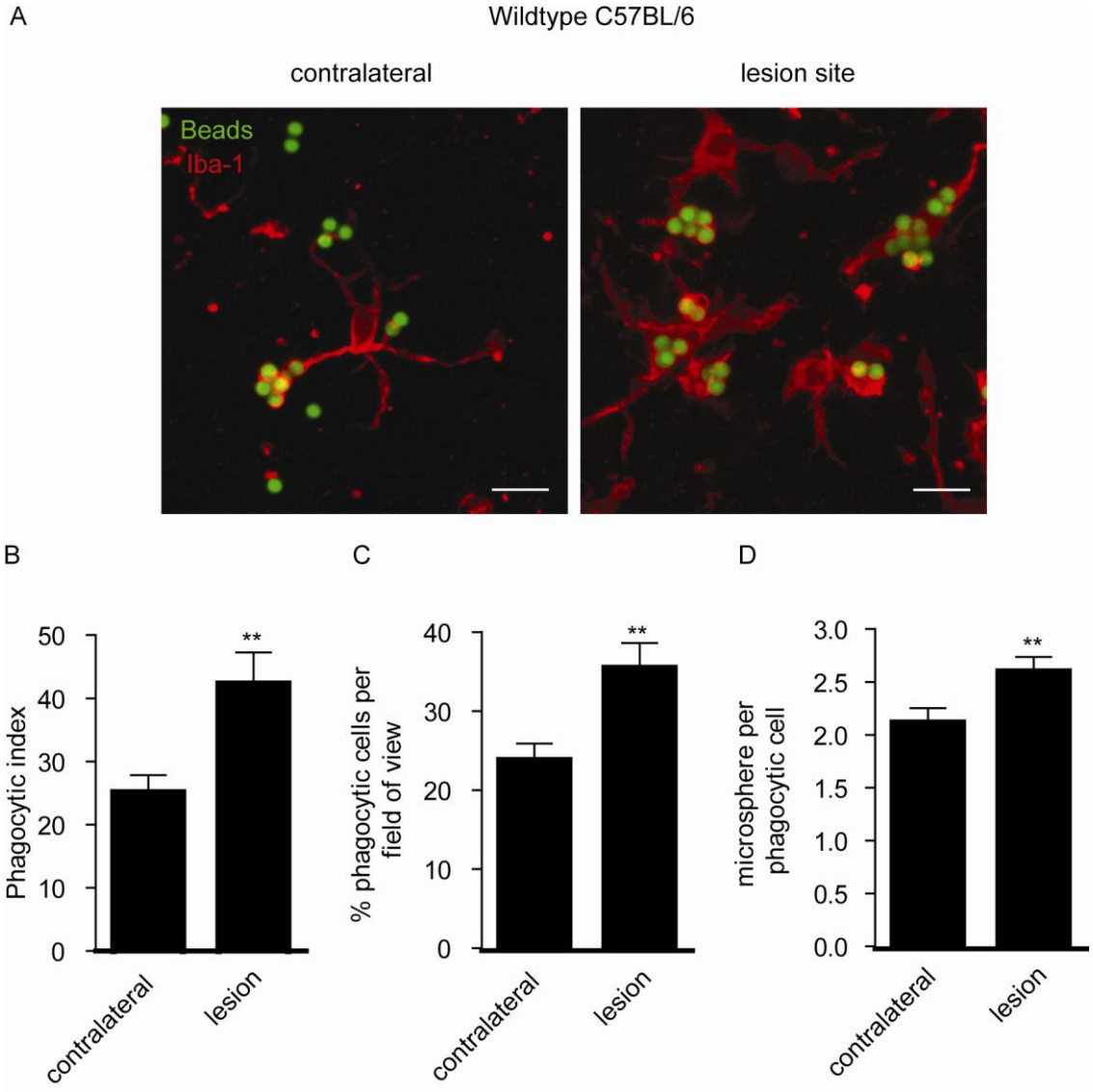


Figure 27 Phagocytic capacity is increased in microglia next to the lesion site upon acute stab wound lesion.

(A) Representative confocal images depicting microglia (Iba-1$^+$, red) with phagocytosed fluorescent microspheres (green) at the lesion site compared to the contralateral hemisphere. (B) Absolute values of microglial phagocytic indices upon lesioning the brain by a stab wound 6 days after injury. (C) and (D) display the mean percentage of phagocytic cells and the number of engulfed beads per phagocytic cell, respectively. Data are mean ± s.e.m, **p<0.01. Scale bars: 10 µm.

### *3.3.3 Microglial phagocytic activity is changed in cortex in different mouse models of Alzheimer'disease*

I analyzed the phagocytic activity of microglial cells surrounding Aβ plaques in cerebral cortex in acute brain slices of 9 months old *APPPS1* transgenic animals and compared them to microglial cells in corresponding regions in slices derived from wildtype littermates. The phagocytic activity was drastically decreased to 23 ± 2 of microglia in slices derived from *APPPS1* mice compared to 62 ± 4 in control littermates (Figure 28 A, absolute values of phagocytic indices, n>50 from 3 mice per genotype). I compared my results obtained from *APPPS1* mice to another mouse model of cerebral amyloidosis, namely *APP23*, as it was shown that mutations in *Presenilin 1* by itself leads to impaired phagocytic activity (Lee, Yu et al. 2010; Farfara, Trudler et al. 2011). *APP23* mice start to develop Aβ plaques at 9 months of age and were used for phagocytosis experiments at 20 months age. At this time point mice show a robust cortical plaque load which is comparable to that seen in 9 months old *APPPS1* mice. Quantification of phagocytic activity revealed a strong reduction to 13 ± 2 compared to 48 ± 4 in wildtype mice (Figure 28 B, absolute values of phagocytic indices, n>25 from 3 mice per genotype).

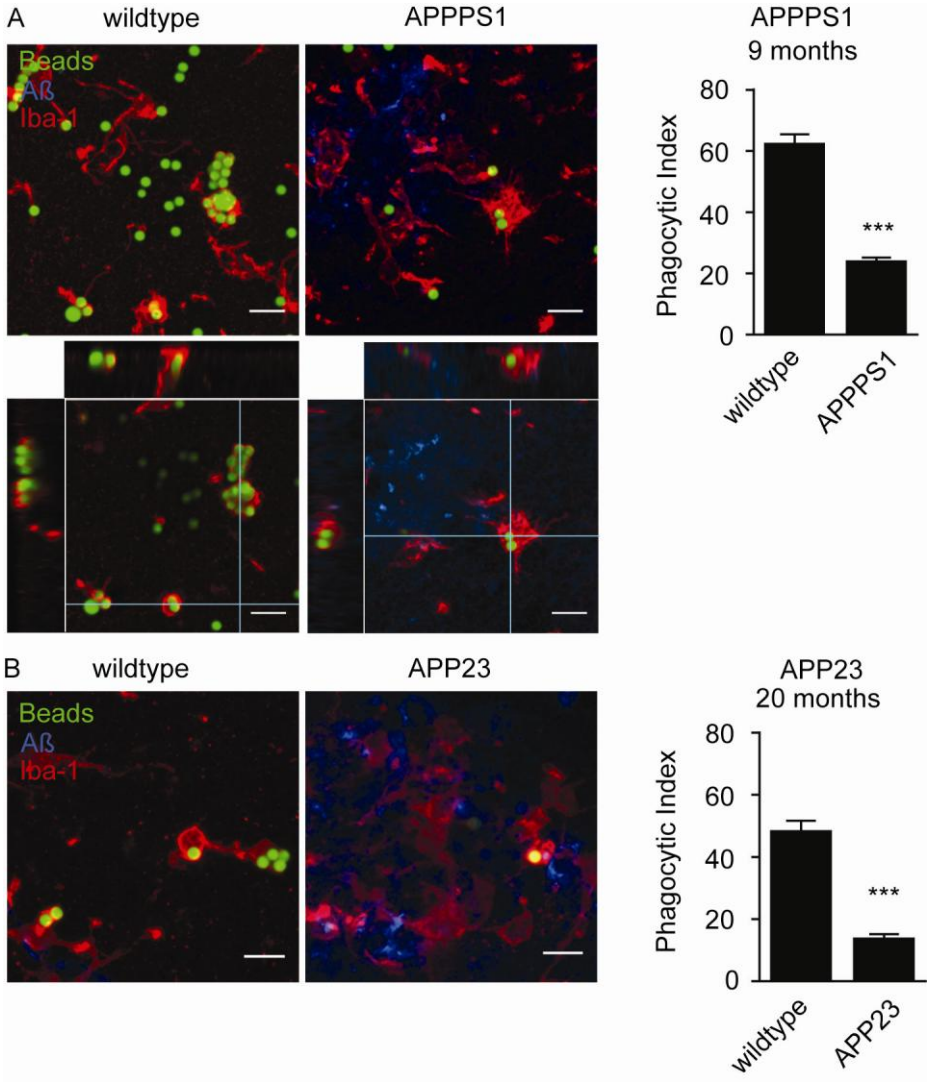


Figure 28 Impairment of microglial phagocytosis in AD mice depends on the presence of Aβ plaques.

(A) Representative confocal pictures (projections of z-stacks) of acute coronal brain slices and phagocytic indices from 9 months old *APPPS1* and wildtype mice (n > 50 from 3 mice per genotype). Iba-1$^+$-microglia (red), Aβ (4G8, blue) and internalized fluorescent microspheres (green) are shown (top panel). Orthogonal views of z-stacks display fluorescent microspheres internalized by Iba-1$^+$-cells (lower panel). Only microspheres engulfed by microglia were included for phagocytic index calculation. (B) Absolute values of cortical microglial phagocytic indices from 20 months old *APP23* and control mice (n > 25 from 3 mice per genotype, right). Representative confocal images (left) display the uptake of microspheres (green) by Iba-1$^+$ microglia (red) in the absence or presence of Aβ (blue). All data are mean ± s.e.m; ***p<0.001. Scale bars: 10 µm.

To determine the impact of Aβ plaque load on phagocytic activity of cortical microglia I analyzed acute brain slices of younger *APPPS1* mice. Therefore I used mice at 7-8 weeks which show no plaque deposition, 8-9 weeks old mice with low plaque deposition and 4 months old mice with robust cortical plaque formation and normalized data to the corresponding wildtype. Phagocytic activity in 7-8 weeks old

*APPPS1* mice was not changed whereas Aβ plaque occurrence in 8-9 weeks old mice significantly decreased phagocytic activity to 51 ± 6% compared to wildtype littermates. In 4 months old mice analysis of plaque associated microglia revealed a significantly decreased phagocytic activity of 36 ± 4%, a level comparable to 9 months old mice (38 ± 3%, Figure 29).

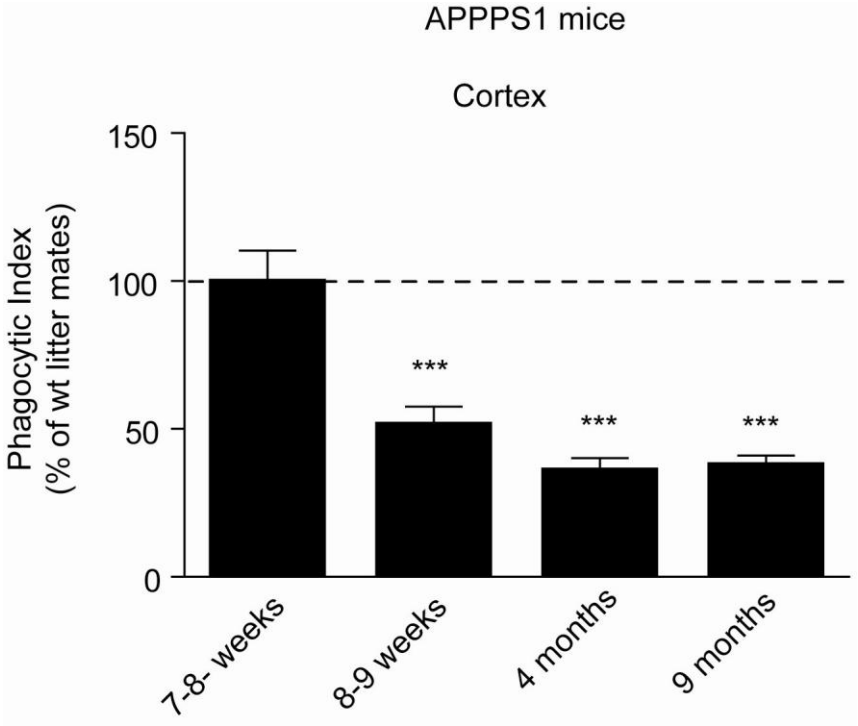


Figure 29 Phagocytic activity is impaired in cortical microglial cells associated to Aβ plaques.

Phagocytic indices of cortical microglia from *APPPS1* mice normalized to respective wildtype at different ages (n > 27 from 4 mice per group) where plaques are detectable in 8-9 weeks, 4 months and 9 months old mice. Microglial uptake of fluorescent microspheres was significantly reduced in plaque bearing cortices (*** $p < 0.001$) whereas microglial phagocyitc activity in 7-8 weeks old *APPPS1* mice was unchanged. Data are mean ± s.e.m.

### *3.3.4 Phagocytic activity is impaired by plaque presence*

To determine if extracellular Aβ plaque formation triggers impaired phagocytic activity or if microglia from transgenic animals are generally dysfunctional over time I used two approaches. I quantified phagocytic activity of primary microglia isolated from P0 *APPPS1* transgenic animals or wildtype littermates (Figure 30 A). I did not find any change in phagocytic activity in these microglia preparations (Figure 30 B).

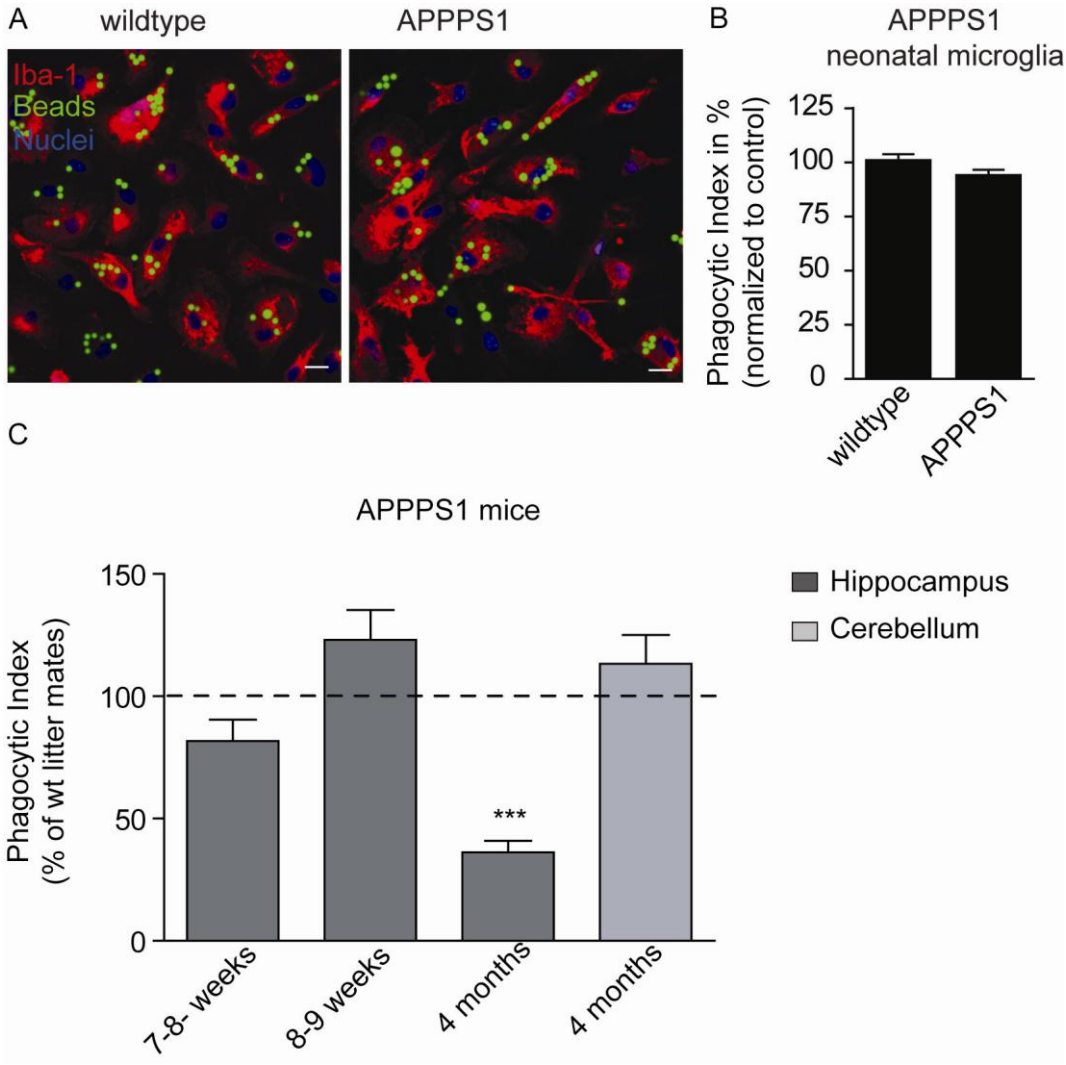


Figure 30 Microglial phagocytic activity is only affected in *APPPS1* microglia upon plaque presence.

(A) Representative confocal images of primary neonatal microglial cultures from wildtype and *APPPS1* mice displaying microglia (Iba-1$^+$, red), microglial nuclei (DRAQ5, blue) and internalized microspheres (green; left panel). (B) Average phagocytic indices (n > 24 from 3 independent experiments) of neonatal microglia derived from *APPPS1* mice and nontransgenic littermates do not differ (p=0.559). (C) Microglial phagocytic index in plaque-free brain areas (hippocampus in 7-8 and 8-9 weeks old *APPPS1* mice, cerebellum in 4 months old *APPPS1* mice) is unchanged relative to respective wildtype whereas microglia in plaque-bearing brain areas (hippocampus in 4 months old *APPPS1* mice) show reduced phagocytic activity compared to respective wildtype (n > 21 from 4 mice per group, ***p<0.001). Data are displayed as mean ± s.e.m. Scale bars: 10 µm.

As a second approach to test for Aβ plaque deposition as an external factor to inhibit microglial phagocytic activity I analyzed different brain regions of acute brain slices taken from *APPPS1* and wildtype littermates. Quantification of the uptake of fluorescent microparticles resulted in unchanged phagocytic indices for 7-8 and 8-9 week old *APPPS1* mice in the hippocampus which is plaque free at this age whereas

microglial phagocytic activity was lowered to 36 ± 5% in 4 months old mice in this region as it shows robust plaque formation compared to control littermates. In these mice the cerebellum was defined as plaque free region and showed no impairment of phagocytic activity in microglial cells (Figure 30 C).

### *3.3.5 Microglial response to a laser induced lesion in cortical brain slices is reduced in 10 months but not 4 months old APPPS1 mice*

To determine microglial capability to respond to an acute injury in a degenerative context, I observed microglia in acute brain slices from *APPPS1;Cx3Cr1*$^{+/GFP}$ mice and *Cx3Cr1*$^{+/GFP}$ littermates after induction of a laser lesion. I found a significant decrease of the microglial response in 10 months old *APPPS1;Cx3Cr1*$^{+/GFP}$ mice compared to *Cx3Cr1*$^{+/GFP}$ littermates independent of distance to a plaque (Figure 31 B and D). In non-plaque areas I determined an average peak response of 0.16 ± 0.02 (n = 11) to the lesion which was similar to that found for lesions close to plaques (0.18 ± 0.05, n = 8) compared to 0.49 ± 0.09 in *Cx3Cr1*$^{+/GFP}$ littermates (n = 7). In contrast, average peak response was unchanged in 4 months old *APPPS1;Cx3Cr1*$^{+/GFP}$ although they show similar plaque burden and microglial morphology as 10 months old mice (Figure 31 A and C). I calculated an average peak response of 0.19 ± 0.03 for *Cx3Cr1*$^{+/GFP}$ mice (n = 9) compared to 0.16 ± 0.04 for lesion close to a plaque (n = 8) and 0.19 ± 0.04 for lesion distant to plaques (n = 8).

In direct comparison of average traces of 4 month old and 10 months old *Cx3Cr1*$^{+/GFP}$ animals it is noteworthy that the response in 10 months old *Cx3Cr1*$^{+/GFP}$ is increased compared to lesions placed in plaque bearing cortex and therefore leading to a significant difference compared to 10 months old *APPPS1;Cx3Cr1*$^{+/GFP}$ mice.

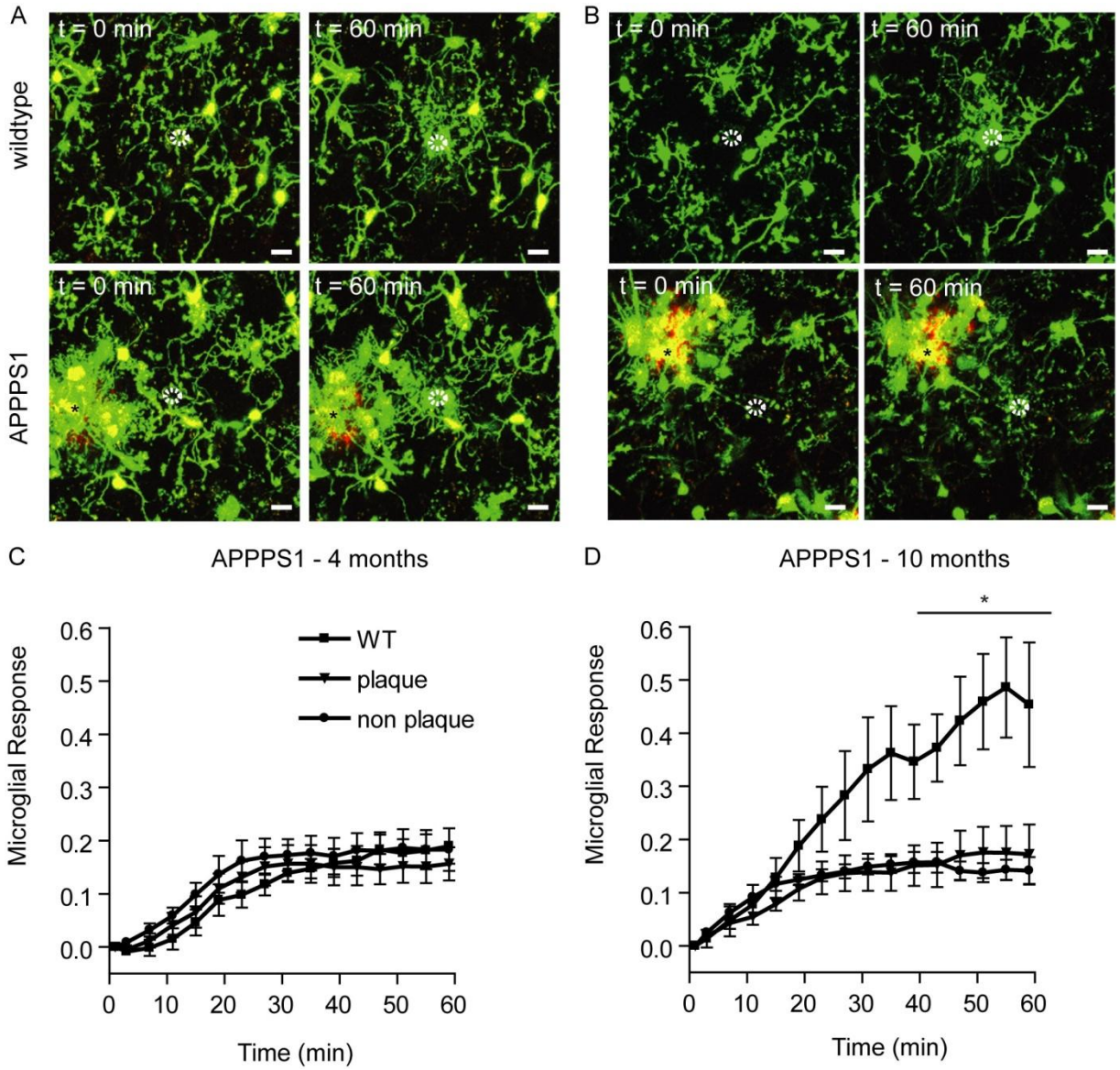


Figure 31 Acute microglial response towards an acute injury is impaired in *APPPS1*;*Cx3cr1*$^{+/GFP}$ mice.

Representative two-photon microscopy images of 60 µm z-stacks projections after laser lesion (dashed circle) in 4 months (A) old and 10 months old (B) *APPPS1* and non-transgenic wildtype littermates crossbred to *Cx3cr1-GFP* mice at t = 0 min and t = 60 min. Microglia are visualized by transgenic expression of GFP under the *Cx3cr1* promoter (green), Aβ plaques are stained with Thiazin Red (red, *). Time course of microglial processes movement towards the laser lesion site in 4 months old (C) and 10 months old (D) *APPPS1* mice and *Cx3cr1* littermates is displayed as relative response. Data are mean ± s.e.m, * $p < 0.05$. Scale bars: 10 µm.

# 4 Discussion

## 4.1 Impact of neurotransmitters on microglial properties

Findings of the presented work indicate that neonatal, amoeboid as well as adult microglia express functional receptors for monoamine neurotransmitters. Activation of serotonin receptors enhanced process motility in adult microglia in acute brain slices whereas activation of adrenergic receptors led to the opposite effect. Interestingly, application of dopamine, norepinephrine as well as serotonin resulted in a decreased phagocytic activity in young microglia. Reduction of phagocytic acitivity in adult ramified microglia by dopamine application seems to be a rather unspecific effect since it could not be reproduced by dopamine receptor agonists. It is known that microglia constantly move their fine processes to scan their surroundings and were shown to directly contact neuronal synapses, sites for neurotransmitter release, for minutes before process retraction (Wake, Moorhouse et al. 2009). In ischemic brains, these synaptic contacts were prolonged over one hour (Wake, Moorhouse et al. 2009). Microglial cells could therefore sense neurotransmitter release upon neuronal activity. In addition, monoamine neurotransmitters like dopamine, serotonin and norepinephrine can be released in a non-synaptic fashion into the extrasynaptic space and can act on extrasynaptic receptors (Hensler 2006). They are stored in vesicles ready to be released not only at presynaptic terminals but also from the somata, dendrites and axonal varicosities even in the absence of postsynaptic structures (Descarries and Mechawar 2000; De-Miguel and Trueta 2005). This non-synaptic transmission was termed volume transmission as opposed to wiring transmission (for review see (Agnati, Guidolin et al. 2010)) and acts over a much larger range than synaptic transmission. It not only stimulates neuronal receptors, but also receptors which are potentially expressed on glial cells, which could be confirmed in this study.

### *4.1.1 Microglial phagocytosis and migration in different microglial preparations depend on age*

In the past, studies on microglial phagocytic activity were carried out mainly in cell culture systems using different approaches like application of fluorescent proteins and bacteria or yeast particles (Koenigsknecht and Landreth 2004; Heneka, Nadrigny et al. 2010; Mairuae, Connor et al. 2011) thereby focusing either on neonatal or immortalized microglia not reflecting microglial properties in tissue environment. Recently, fluorescent microspheres were not only applied to primary microglia but also injected into the brain of prion diseased mice to analyze their phagocytic activity in disease progression (Hughes, Field et al. 2010). In this project I aimed to characterize microglial phagocytic capacities upon treatment with monoamine neurotransmitters like serotonin, norepinephrine and dopamine. Therefore I investigated primary neonatal microglia, amoeboid microglia in acute brain slices from P6-9 mice and ramified microglia in acute brain slices of adult mice. Control experiments with unstimulated primary or tissue microglia revealed significant differences in basic phagocytic activity. A higher percentage of observed young microglia like primary neonatal and amoeboid microglia in P6-9 brain slices phagocytosed a higher number of microspheres although cells were incubated for a shorter period compared to ramified microglia in adult brain slices. Interestingly, neonatal and amoeboid microglia display similar phagocytic activity comparing percentage of phagocytic cells per field of view and number of microspheres per phagocytic cell as well as phagocytic index suggesting that cultivation of neonatal microglia does not alter high phagocytic capacities of young microglia which is necessary in this developmental stage to participate in synaptic rearrangements (Stevens, Allen et al. 2007). Further studies of basic phagocytic capacities of ramified microglia show increasing uptake of microspheres in 2, 4 and 9 months old mice which declines in aged, 20 months old animals. This indicates a higher reactivity during aging balancing in aged animals. This observation was supported by studies on directed movement of microglial processes in acute brain slices of 2, 4 and 10 months old mice induced by acute injury as experiments revealed significant higher responses in the oldest investigated (10 months old) mice compared to 2 months old mice. However, 20 months old mice were not investigated in this

context so that it would be interesting to know whether microglial response towards laser induced lesion would further increase or stabilize in aged animals.

### *4.1.2 Monoamine modulation of phagocytic activity in different microglia preparations*

Monoamine neurotransmitters were shown to have impact on a number of microglial functions (Farber, Pannasch et al. 2005; Pocock and Kettenmann 2007) like chemotaxis and cytokine release in the non-diseased brain, but studies on other than neonatal or immortalized microglia can be rarely found. Recently it was shown that induced degeneration of the Locus ceruleus, the major source for norepinephrine in the brain, in a mouse model of Alzheimer's disease affected microglial properties e.g. increasing the expression of pro-inflammatory markers like cyclooxygenase-2 (COX-2), decreasing Aβ uptake and reducing the recruitment to Aβ plaques. In this project I aimed to close the gap in understanding the impact of monoamines on microglial properties in the non-diseased brain. I studied microglial phagocytic activity in different microglial preparations by determining the uptake of fluorescent microspheres upon treatment with different neurotransmitters or specific neurotransmitter receptor agonists. Application of dopamine, norepinephrine and serotonin together with microspheres resulted in a significant reduction of microsphere uptake in young microglia including neonatal and amoeboid microglia. The same effect was detected when I applied the 5-HT2 agonist DOI on neonatal microglia. Only high concentrations of dopamine changed the microsphere uptake by ramified microglia. In my studies using single or combined application of dopaminergic or adrenergic receptor agonists I could not confirm this observation. Microglial processes in adult mice were shown to establish contacts to neuronal structures like synapses and dendritic spines indicating the abilitiy to sense neurotransmitter release (Wake, Moorhouse et al. 2009). Differences in the regulation of phagocytic activity of young *versus* adult microglia by monoamine neurotransmitters can be explained by a different neurotransmitter receptor profile at different stages of microglial development as was shown in this study. For example, microglia are involved in synaptic pruning (Stevens, Allen et al. 2007) by eliminating weak or inappropriate synapses during development (Schafer and Stevens 2010). It is crucial that microglia are able to distinguish between functional and less functional

or active synapses and to eliminate weak ones at this developmental stage. A possible mechanism how microglia distinguish between weak and strong synapses could be via the neurotransmitter release of these synapses. My data indicates that the neurotransmitter dependent phagocytic activity is differentially regulated in adult ramified microglial cells compared to their younger counterparts.

Besides, it was shown that monoamines oxidize easily. Although there is evidence that only 11% of initial dopamine is oxidized in a cell free system within one hour (Garner and Nachtman 1989) one has to admit that oxidation of dopamine or other monoamines is facilitated by the expression of converting enzymes by cellular systems therefore leading to modified properties of the applied neurotransmitter (Takakura, Xiaohong et al. 2003; Zoccarato, Toscano et al. 2005). This could explain the observed reduction of phagocytic activity of adult microglia as these cells were investigated in acute brain slices and stimulated for one hour with neurotransmitters together with microspheres. Experiments in tissue environment make it difficult to distinguish between directly mediated effects of applied substances and indirect effects mediated by surrounding cells as already observed recently where application of the GABA(A) receptor agonist muscimol only induced transient current changes in amoeboid microglial cells attached to the slice surface but not if cells were lifted up during patch clamp experiments (Cheung, Kann et al. 2009).

### *4.1.3 Serotonin does not alter LPS induced cytokine release*

Neurotransmitters like norepinephrine were shown to attenuate LPS induced release of immune relevant molecules like TNF-α, IL-6 and nitric oxide in neonatal cultured mouse microglia (Farber, Pannasch et al. 2005) while dopamine attenuated only LPS induced nictric oxide release. Studies using different serotonin-reuptake inhibitors like fluoxetine in ischemia mouse models as well as on microglial cell lines suggested anti-inflammatory capacities for these substances as they attenuate Interferon-γ induced release of IL-6 and nitric oxide (Hashioka, Klegeris et al. 2007; Lim, Kim et al. 2009). Interestingly, data obtained in this project shows that in contrast to dopamine and norepinephrine, different concentrations of serotonin do not alter LPS induced release of TNF-α, IL-6, MIP-1α and NO in mouse neonatal microglia. Hence, these anti-inflammatory capacities of serotonin-reuptake inhibitors are obviously not mediated by the impaired uptake of released serotonin and therefore accelerated

serotonin concentrations but anti-inflammatory properties of serotonin-reuptake inhibitors itself (Lim, Kim et al. 2009). This leads to the conclusion that dopamine, norepinephrine and serotonin differentially modulate release of immune relevant molecules in neonatal microglia.

### *4.1.4 Modulation of migration by monoamines and receptor agonists*

Microglial cells scan their environment by constant extension and retraction of their fine processes and respond to an insult, like laser induced injury, by directed movement of these processes towards the injury site (Nimmerjahn, Kirchhoff et al. 2005) probably induced by released ATP from damaged cells (Davalos, Grutzendler et al. 2005). Further it was shown that dopamine application stimulated migratory behavior of neonatal microglia which could be confirmed by using the dopamine receptor agonists dihydrexidine and quinpirole (Farber, Pannasch et al. 2005). In this project I demonstrated that serotonin not only attracts microglial cells in a chemotaxis experiment but also enhances migration of neonatal microglial cells towards an ATP gradient. Moreover, ramified microglial process extension induced by acute injury is promoted by serotonin application in ramified microglia in acute brain slices from adult *Cx3cr1*-GFP mice. In contrast, microglial response to acute injury in acute brain slices was reduced by application of norepinephrine and unchanged upon dopamine treatment. Interestingly, application of either dopamine receptor agonists or adrenergic agonists did not show this effect indicating a rather unspecific effect of norepinephrine on microglial migratory behavior. A recent report investigating the capacity of local application of different chemokines, neuromodulators and neurotransmitters like serotonin and norepinephrine to induce motility of spinal cord microglia showed that application of ATP is the only stimulus investigated that induces process extension in spinal cord microglia (Chen, Koga et al. 2010). Accordingly, this indicates that monoamine neurotransmitters do not induce migration behavior in microglia *in situ* themselves but rather regulate ATP induced microglial process extension.

### *4.1.5 Serotonin receptor are differentially expressed in the investigated microglial preparations*

The data obtained on the influence of neurotransmitter application on microglial effector functions like phagocytic activity and migration in response to an ATP gradient suggest a distinct regulation of microglial properties by monoamine neurotransmitters depending on the activation state or developmental stage of microglial cells. There are already reports about the expression of dopaminergic and adrenergic receptors in elderly human as well as rat microglial cells showing that microglia express not only α1A, α2A, β1 and also β2 adrenergic but also D1-D4 or D1, D2, D4 and D5 dopaminergic receptors, respectively (Mori, Ozaki et al. 2002; Farber, Pannasch et al. 2005; Mastroeni, Grover et al. 2009). Therefore I tested the expression of serotonergic receptors by quantitative PCR in mouse neonatal cultured and freshly isolated adult microglia. I used primers detecting the expression of 13 serotonin receptor subtypes, the serotonin transporter SERT and the limiting enzymes of serotonin synthesis TPH1 and TPH2. Only for 5HT1d I could not find primers that meet the criteria I set for data analysis. I detected a much broader spectrum of serotonin receptors expressed by adult microglia including 5HT2, 5HT5a and 5HT7 compared to neonatal microglia only expressing 5HT2b supporting the distinct regulation of microglial effector functions by serotonin at different developmental stages. A recent report about $Ca^{2+}$ responses in a subpopulation of activated microglial cells in acute brain slices of adult C57BL/6 mice triggered by serotonin (Seifert, Pannell et al. 2011) further substantiates the expression of functional serotonin receptors in adult microglial cells. In further expression studies I did not detect any mRNA for the serotonin transporter SERT or tryptophan hydroxylase TPH1 or TPH2 expressed by neonatal or adult microglia. In contrast, it was reported that other tissue macrophages in the peritoneum and intestine were found to express SERT as well as TPH1 (Rudd, Nicolas et al. 2005; Nakamura, Sato et al. 2008) indicating again a spatially and temporally regulated expression of genes involved in serotonin dependent mechanisms.

In conclusion, this work substantiates the role of neurotransmitters like serotonin, dopamine and norepinephrine as important regulators of microglial properties. Modulation of microglial functions like phagocytosis and migration, especially by serotonergic receptors, are fundamental for the central nervous system since microglia can influence the balance of synaptogenesis and neuronal death during development and in pathology (Bessis, Bechade et al. 2007). It will be now interesting to see whether monoamine neurotransmitters and especially serotonin have beneficial impact on microglial properties in context of pathology as not only neuronal release is a potential source of serotonin but leakage of blood brain barrier, as seen in many pathologies (Pluta 2006; McQuaid, Cunnea et al. 2009), can provide high amounts of peripheral serotonin acting on glial cells.

## 4.2 Microglial properties in AD

Already in the first observations of glial accumulations around senile plaques in AD tissue these cells were considered as being activated (Alzheimer, 1911). Later, accumulating cells were identified as activated microglia in the human brain with AD (McGeer, Itagaki et al. 1987). A large number of *in vitro* studies have manifested the view that activated microglial cells release a battery of pro-inflammatory mediators including nitric oxide and TNFa. When cultured microglia are stimulated with Aß peptides they produce molecules which are toxic for neurons (Meda, Bonaiuto et al. 1995; Giulian, Haverkamp et al. 1996; Combs, Johnson et al. 1999). Thus AD was considered as a disease with a neuroinflammatory component and microglia as a harmful element (Akiyama, Arai et al. 2000).

This work showed in two different mouse models of AD that microglia display reduced phagocytic activity depending on the presence of Aβ plaques by investigating the engulfment of fluorescent microspheres in different brain areas and different stages of disease development. Moreover, microglia accumulating around senile plaques in aged AD mice respond less to an acute injury induced by a laser lesion in the cortex of acute brain slices compared to the healthy control group. Microglial impaired phagocytic as well as migratory response to acute stimuli in the vicinity of senile plaques indicates an overall hampered phenotype as proposed recently in a review by Streit (Graeber and Streit 2010) arguing that data from *in vitro* models cannot be simply extended to the human brain and that the microglial response in AD is unlike that of an acute inflammation as seen in multiple sclerosis. He considers microglia in the Alzheimer diseased brain rather dystrophic or senescent with fragmented processes when colocalized with neuronal structures positive for neurofibrillary tangles and neuritic plaques in brains of AD patients (Lopes, Sparks et al. 2008; Streit, Braak et al. 2009). Even independent of AD, microglia might undergo a senescence in the aging brain (Streit 2006).

### *4.2.1 Microglial cells around senile plaques do not represent phagocytic phenotype found in acute activation*

Acute injury can be induced using the stab wound model to investigate the impact of classical activation on microglial engulfment of fluorescent microspheres. Within

three days after inducing a stab wound microglia get activated, migrate to the site of injury and accumulate (Fujita, Yoshimine et al. 1998). Microglia as well as infiltrating monocytes display classical signs of activation like amoeboid morphology, proliferation and enhanced expression of CD68 (Amat, Ishiguro et al. 1996). In fact, microglial cells in the stab wound region display enhanced phagocytic activity verified by the number of engulfed microspheres and the percentage of phagocytic cells per area compared to the contralateral hemisphere making this a valid method for characterization of activation-dependent phagocytosis. Surprisingly, the same approach used in acute brain slices from different mouse models for AD, *APPPS1* and *APP23*, resulted in a reduced uptake of microspheres by microglial cells in plaque bearing cortex of these mice pointing to a dysfunctional phagocytic activity although cells clearly display amoeboid morphology. Indeed, recent studies more and more addressed the question whether microglia activation needs to be an “all or nothing” process or if it is rather a distinct phenotype depending on the pathology which can also change during the course of pathology (Hanisch and Kettenmann 2007).

### *4.2.2 Microglial phagocytic activity in AD*

Recent reports identified an essential role for the enzyme presenilin-1 (PS1) in phagocytosis mechanism. Not formation but maturation of phagolysosomes was impaired in PS1 knockout cells by a failure in v-ATPase trafficking. As a result, loss of phagolysosome acidification prevented protein degradation (Lee, Yu et al. 2010). Additionally, deletion of PS1 or PS2, as being parts of the γ-secretase catalytic site, in microglia resulted in a dysfunctional phenotype leading to reduced uptake of Aβ in *in vitro* as well as *in situ* approaches (Farfara, Trudler et al. 2011). In this project I used for the majority of experiments a double-transgenic mouse model for AD, *APPPS1* mice, that in addition to expressing the human mutated APP carry a mutation in the *presenilin-1* gene under the Thy1 minipromoter, leading to an early-onset, aggressive form of AD (Radde, Bolmont et al. 2006). To answer the question whether microglial phagocytic impairment results from exogenous presence of Aβ accumulations or from endogenous dysfunction of presenilin-1, I used different approaches. First of all, presenilin-1 should not be affected in microglial cells as

expression of mutated PS1 as well as human mutated APP is restricted to Thy1 positive neurons. Nevertheless, analysis of phagocytic activity of cortical microglia in acute brain slices of diseased *APP23* mice, not carrying a mutation in *presenilin-1* gene, showed similar impairment compared to microglia in plaque bearing cortex of acute brain slices from *APPPS1* mice. Moreover, neonatal microglia derived from *APPPS1* mice show comparable phagocytic activity as neonatal microglia from non-transgenic littermates. These data support the proper function of the phagocytosis machinery in *APPPS1* mice and indicates that impaired microsphere engulfment is dependent on Aβ plaque presence. This was further substantiated by the fact that only microglia in the plaque bearing brain areas of *APPPS1* mice showed reduced uptake of microspheres whereas microglia in plaque free brain areas in the same mouse showed comparable phagocytic activity to non-transgenic littermates. This stays in contrast to *in vivo* observations that microglia take up injected, soluble Aβ by macropinocytosis (Mandrekar, Jiang et al. 2009) and internalize systemically injected Aβ binding dye (Bolmont, Haiss et al. 2008) in AD diseased mouse models. Endogenous Aβ uptake was never observed in the investigated mouse models. But my data underlines recent reports that specific ablation of microglial cells in a mouse model of AD neither alters plaque size, Aβ40-42 content nor the amount of dystrophic neurits (Grathwohl, Kalin et al. 2009) and that the expression of crucial members of the microglial phagocytic machinery is reduced in a mouse model of AD (Hickman, Allison et al. 2008). There has been also the attempt to show that blood-born myeloid cells enter the brain during the course of AD and participate in neuroinflammatory processes (Simard, Soulet et al. 2006). These cells, in contrast to endogenous microglial cells, have been suggested to be involved in Aβ plaque restriction and release of pro-inflammatory molecules. Even so, this work indicates that microglia/brain macrophages display a dysfunctional phagocytic phenotype induced by the presence of Aβ plaques during disease progression.

### *4.2.3 Microglial response to laser lesion is only reduced in AD*

Acute injury induces a directed movement of microglial processes towards the injury site. In case of long-lasting exposition to inflammatory stimuli microglial cells are attracted and subsequently translocate to the origin of inflammation like a stab wound area (Fujita, Yoshimine et al. 1998). Recently, it was reported that microglial cells

become attracted also by newly formed Aβ plaques and reach them within 1-2 days where they accumulate (Bolmont, Haiss et al. 2008; Meyer-Luehmann, Spires-Jones et al. 2008). These cells still show rapid process and membrane movements in the vicinity of the plaque (Bolmont, Haiss et al. 2008). In my study, imaging of microglial response to acute injury in *APPPS1* mice crossbred to *Cx3cr1*-GFP mice was performed after full establishment of Aβ plaques (4 months of age) and in aged mice (9 months of age) displaying persistent exposure to Aβ plaques and compared to the corresponding *Cx3cr1*-GFP littermate. I can show that in plaque bearing cortex of aged *APPPS1;Cx3cr1*$^{+/GFP}$ mice microglial cells respond less to acute injury induced by a laser lesion whereas there is no difference in the younger animals also showing extensive plaque formation. Detailed analysis reveals that microglia in plaque bearing cortex show similar response at both time points of disease progression but the response between the control groups differs significantly. Similarly, recent reports indicate that during aging the innate immune system shows enhanced responsiveness to acute stimuli and that microglia seemed to get primed most probably by increased oxidative stress during aging and deficits in microglial regulation by chemokines, neurotransmitters and neuropeptides following reduced expression of e.g. CD200R and CX3CR1 that transmit microglial “Off” signals (Wynne, Henry et al. 2009). *Ex vivo* analysis of LPS stimulated microglia revealed increased expression of neuroinflammatory signals like IL-6 and MHCII in elderly microglia compared to younger counterparts (Frank, Barrientos et al. 2010). On the background of exaggerated inflammatory reactivity of microglial cells during aging my data suggests a hyperreactive phenotype of aged microglia towards acute injury in non-diseased animals which is attenuated in AD mice leading to the difference in responsiveness. It would be now interesting to know whether reactivity is delayed or altered in AD and how this process continues during further ageing. Again, these observations favor a dysfunctional phenotype of microglial cells in AD context as cells in the plaque bearing cortex fail to respond as quick as their control counterparts to an acute injury regardless if the lesion was placed in proximity to microglia surrounding an Aβ plaque or involving microglia that do not surround a plaque.

In conclusion, my data supports the view that microglial functions in the presence of Aβ plaque deposits are impaired suggesting that resident microglia in AD brain morphologically and phenotypically resemble rather dysfunctional than classically activated immune cells. It would be now interesting to learn whether dysfunctional microglia from Aβ containing brain regions of AD mice will return back to normal functionality if they were liberated from this environment. Additionally, one needs to understand whether dysfunction of microglia in respect to their phagocytic activity and migratory response negatively impacts on disease progression and if fully functional microglia would aid to restrict Aβ plaque burden and therefore display a tool to manipulate plaque deposition and inflammatory consequences of AD.

# 5 Summary

Microglia are the immune cells of the brain and are involved in developmental as well as regenerative processes of the brain. As being part of the immune system they survey their surroundings by constant process extension and retraction for any insult and can act as phagocytes engulfing cellular debris and pathological invaders of the brain. In this project I studied the phagocytic activity as well as process motility

1. upon stimulation with different monoamine neurotransmitters like serotonin, norepinephrine and dopamine to investigate microglia - neuron communication and
2. in the context of Aβ plaque deposition in mouse models of Alzheimer's disease.

Therefore I used different microglial preparations, namely primary neonatal microglia, amoeboid microglia on top of acute brain slices of P6-9 mice and ramified microglia in acute brain slices of adult mice from healthy C57BL/6 mice or mutated *APPPS1* and *APP23* mice, mouse models for Alzheimer's disease, respectively.

In the first part of my work I found phagocytic uptake of fluorescent microsphere to be reduced by monoamine neurotransmitters mainly in young microglia including neonatal as well as amoeboid microglia whereas adult microglia only respond to high concentrations of dopamine with reduced particle uptake. In contrast to adult microglia where application of specific dopaminergic or adrenergic receptor agonists did not show the same reduction, application of the specific 5HT2 receptor agonist DOI leads in neonatal microglia to a concentration dependent decrease of particle engulfment. Moreover, serotonin was found to be a potent stimulator of ATP induced microglial migration as it not only triggers microglial migration towards an applied ATP gradient *in vitro* but also enhances process motility towards a laser induced injury that was suggested to be a source for ATP released from damaged cells.

Secondly, my data shows that microglial phagocytic activity is lowered in the cortex of diseased *APPPS1* as well as *APP23* mice. Experiments on microglial microsphere uptake in unaffected brain regions revealed fully functional microglial phenotype providing evidence that microglial impairment is dependent on the presence of Aβ

plaques. In addition, microglial response towards acute injury induced by a cortical laser lesion increases during aging comparing 2, 4 and 10 months old mice which is attenuated in aged *APPPS1* mice but not in younger, plaque bearing mice supporting functional impairment of microglia by the presence of Aβ plaques.

In conclusion, this work provides data that microglia express functional neurotransmitter receptors for serotonin, norepinephrine and dopamine whose activation leads to modification of substantial effector functions involved in synapse rearrangement during development as well as regeneration in pathology. Moreover, I can show that these functions are impaired upon Aβ plaque presence supporting the view of a dysfunctional microglial phenotype during neurodegenerative disease progression.

# 6 Zusammenfassung

Mikrogliazellen sind eingebunden in verschiedenste Vorgänge im Gehirn, sowohl während der Entwicklung als auch bei Regeneration nach Verletzung. Als Teil des Immunsystems sind sie in der Lage ihre Umgebung mittels ihrer feinen Fortsätze ständig abzutasten, aber auch als Phagozyten Zelltrümmer und Pathogene aufzunehmen. In diesem Projekt sollte sowohl die Phagozytoseaktivität als auch die Bewegung der Zellfortsätze

1. Nach Stimulation mit verschiedenen Monoaminneurotransmittern wie Serotonin, Noradrenalin und Dopamin zur Charakterisierung der Mikroglia - Neuron Interaktion untersucht werden.
2. Im Zusammenhang mit Aβ-Ablagerungen in verschiedenen Mausmodellen der Alzheimer Krankheit untersucht werden.

Für meine Experimente nutzte ich verschiedene Mikrogliapräparationen, wie neonatale Mikroglia, amöboide Mikroglia auf der Oberfläche akuter Hirnschnitte von 6-9 Tage alten Mäusen und ramifizierte Mikroglia in akuten Hirnschnitten adulter Mäuse jeweils von gesunden C57BL/6 Mäusen oder mutierten *APPPS1* bzw. *APP23* Mäusen, als Mausmodell der Alzheimer Krankheit.
Im ersten Teil dieser Arbeit konnte gezeigt werden, dass vor allem junge Mikroglia, also neonatale und amöboide Mikroglia, mit einer reduzierten Aufnahmefähigkeit von Mikropartikeln nach Neurotransmitterapplikation reagieren, während adulte Mikroglia nur bei hohen Konzentrationen von Dopamin diesen Effekt zeigen. Applikation von spezifischen dopaminergen oder adrenergen Rezeptoragonisten führte allerdings nicht zu einer reduzierten Phagozytoseaktivität im Gegensatz zu neonatalen Mikroglia, die aufgrund der Applikation des 5HT2-spezifischen Rezeptoragonisten DOI mit einer konzentrationsabhängigen Reduktion der Phagozytoseaktivität reagierten. Darüber hinaus konnte Serotonin als potenter Stimulator ATP-induzierter Mikrogliabewegung identifiziert werden, da es nicht nur die ATP-induzierte Migration von Mikroglia *in vitro*, sondern auch die Bewegung mikroglialer Fortsätze zu einer akuten Verletzung steigert. Es wurde bereits gezeigt, dass akute Verletzung mittels Laserläsion zur Freisetzung von ATP aus verletzten Zellen führt.

Der zweite Teil meiner Arbeit zeigt, dass die Phagzytoseaktivität kortikaler Mikroglia in erkrankten *APPPS1* und *APP23* Mäusen reduziert ist. Experimente zur Partikelaufnahme durch Mikroglia in plaquefreien Regionen erkrankter Mäuse ergaben eine vollständige Funktionsfähigkeit der Mikroglia. Demzufolge scheint die Phagozytoseaktivität von Mikroglia abhängig von Aβ-Ablagerungen reduziert zu sein. Zusätzlich konnte gezeigt werden, dass beim Vergleich von 2, 4 und 10 Monate alten Mäusen die mikrogliale Antwort auf kortikale akute Verletzungen im Alter ansteigt. In gealterten *APPPS1* Mäusen zeigen plaque-assoziierte Mikroglia allerdings eine reduzierte Bewegung der Fortsätze. Dies unterstützt die Ansicht, dass Mikroglia durch Aβ-Ablagerungen funktionell beeinträchtigt werden.

Diese Arbeit verdeutlicht, dass Mikrogliazellen funktionelle Neurotransmitter-rezeptoren für Serotonin, Noradrenalin und Dopamine besitzen, dessen Stimulation zu Veränderungen substantieller Effektorfunktionen führt, die sowohl während der Entwicklung in den Umbau neuronaler Synapsen, als auch in Regenerations-prozesse nach Verletzung involviert sind. Darüber hinaus können diese Mikrogliafunktionen durch Aβ-Ablagerungen beeinträchtigt sein. Dies unterstützt den Ansatz, dass Mikroglia einen dysfunktionellen Phänotyp im Verlauf neurodegenerativer Erkrankungen ausbilden.

# 7 Eidestattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit dem Thema „Microglial properties in health and disease - Modulation of microglial phagocytic activity and migration by monoamine neurotransmitters or presence of amyloid beta peptides" eigenständig und ohne unerlaubte Hilfe angefertigt habe. Es wurden von mir ausschließlich die angegebene Quellen und Hilfen in Anspruch genommen. Die Arbeit wurde weder in dieser noch in ähnlicher Form bei keiner anderen Institution eingereicht.

Berlin, den 18.11.2011

Grietje Krabbe

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# 10 Appendix

## 10.1 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten

## 10.2 Communications

### *10.2.1 Publications*

1. **G. Krabbe**, V. Matyash, U. Pannasch, L. Mamer., H.W.G.M. Boddeke, H. Kettenmann. Serotonin promotes microglial injury-induced migration but attenuates phagocytic activity of microglia. In Review in Brain, Behavior, and Immunity.

2. **G. Krabbe[1]**, A. Halle[1], U. Bernhardt, V. Matyash, H. Kettenmann[2], F.L. Heppner[2], Severe impairment of microglia by Alzheimer's disease beta-amyloid plaques. In preparation. [1, 2] contributed equally. In preparation.

3. D. Rosskopf, A. Bornhorst, C. Rimmbach, C. Schwahn, A. Kayser, A. Kruger, **G. Tessmann**, I. Geissler, H.K. Kroemer, H. Volzke, 2007. Comment on "A common genetic variant is associated with adult and childhood obesity". Science 315, 187; author reply 187.

4. M. Schurks, T. Kurth, I. Geissler, **G. Tessmann**, H.C. Diener, D. Rosskopf, 2006. Cluster headache is associated with the G1246A polymorphism in the hypocretin receptor 2 gene. Neurology 66, 1917-1919.

5. M. Schurks, T. Kurth, I. Geissler, **G. Tessmann**, H.C. Diener, D. Rosskopf, 2007. The G1246A polymorphism in the hypocretin receptor 2 gene is not associated with treatment response in cluster headache. Cephalalgia 27, 363-367.

6. M. Schurks, V. Limmroth, I. Geissler, **G. Tessmann**, I. Savidou, J. Engelbergs, T. Kurth, H.C. Diener, D. Rosskopf, 2007. Association between migraine and the G1246A polymorphism in the hypocretin receptor 2 gene. Headache 47, 1195-1199.

### *10.2.2 Abstracts/Talks*

**G. Tessmann**, V. Matyash, K. Färber, H. Kettenmann (2010). Neurotransmitter receptors on microglial cells. Network meeting of GRK 1167, 1326, 1258, September 27th, Rangsdorf, Germany

### *10.2.3 Selected Abstracts/Posters*

1. **G. Tessmann**, V. Matyash, K. Färber, H. Kettenmann (2011). Serotonin modulates microglial phagocytosis and motility. Gordon Research Conference, Glial Biology: Functional Interactions among Glia & Neurons, March 6th-11th, Ventura, California.

2. **G. Tessmann**, V. Matyash, K. Färber, H. Kettenmann (2010). Serotonin modulates microglial phagocytosis and motility. ISNI 2010, October 26th-30th, Sitges, Spain

3. **G. Tessmann**, V. Matyash, K. Färber, H. Kettenmann (2010). Modulation of microglial phagocytosis by neurotransmitter receptors. FENS 2010, July 3rd-7th Amsterdam, Netherlands.

4. **G. Tessmann**, V. Matyash, K. Färber, H. Kettenmann (2010). Modulation of microglial phagocytosis by neurotransmitter receptors. Joint PhD Retreat Molecular Neurobiology Berlin & UCL London in Split, Croatia

5. **G. Tessmann**, K. Färber, H. Kettenmann (2009). Neurotransmitter modulate microglial function. Berlin Brain Days 2009, December 10th-12th Berlin, Germany.