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NGF steers microglia toward a neuroprotective phenotype

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Contents

Abstract

Microglia are the resident immune cells of the Central Nervous System (CNS). Beside classic inflammatory activities shared with macrophages, microglia actively participate in activity-dependent plasticity and learning processes [1] [2], as sculpting the neuronal circuitry during development [3] [4]. Microglia have been shown to be key players in the pathogenesis and progression of many neurodegenerative disorders and they are responsible for brain homeostasis and monitor the brain environment with their ever-moving processes [5] [6]. However, their role, either promoting or preventing pathology, is debated. On one hand, excessive activation of microglia leads to oxidative stress, neuroinflammation, and eventually neuronal death [7]. On the other hand, microglial activation might be harnessed to carry out protective activities in the brain, such as phagocytosis of aggregates, synaptic pruning and formation, and the maintenance of healthy neuronal circuits [8]. Therefore, it is important to identify and modulate selectively the neuroprotective activities of microglia. The idea of microglia cells as *the natural scavengers of the brain* becomes especially interesting when dealing with diseases with the loss of proteostasis such as Alzheimer's disease.

In the search of neuroprotective agents against neurodegeneration, neurotrophins have been historically considered as potential therapeutic candidates but usually with actions targeted to natural neuronal

population. In this thesis I tested the hypothesis that microglia represent a new target cell for Nerve Growth Factor (NGF) in the brain. So far sparse experiments in the literature suggest this insight.

In the literature microglia cells are known to be a source of neurotrophins [9] [10][11], most notably the Brain Derived Neurotrophic Factor (BDNF) which has been shown to promote synapse formation [1] and NGF [12] [13]. However, the extent of the modulation NGF might exert on physiological microglial functions and how this effect might come into play in neurodegenerative disorders has not been investigated yet.

Indeed, the main cellular targets of the neurotrophin Nerve Growth Factor (NGF) [14] in the central nervous system are considered to be the cholinergic neurons of the basal forebrain (BFCNs) [15], while its sources are mainly cortical and hippocampal neurons [16].

Consistently, interference with NGF signaling (trkA-NGF signalling) in the adult brain leads to deficits of the cholinergic system that has been related to the mechanisms driving neurodegeneration, as in the AD11 transgenic mouse model [17] [18]. The expression of anti-NGF antibodies selectively neutralizing mature NGF in the adult brain determines a progressive comprehensive neurodegeneration with neuroinflammation as the earliest observed change, at a presymptomatic phase [19] [20]. A similar progressive neurodegeneration is observed in transgenic mice expressing a neutralizing anti TrkA antibody in the adult brain [21]. Changes in NGF homeostasis in the brain, with particular regard to the ratio of NGF to proNGF levels, have also been linked to Alzheimer's disease [22].

However the overall neurodegenerative picture induced by anti-NGF or anti-TrkA antibodies in those transgenic models is much broader than what one would expect on the basis of an action of the antibodies on the BFCNs exclusively. Moreover, the loss of NGF-TrkA signaling in the CNS, obtained by conditionally deleting NGF or TrkA genes in CNS cells derived from nestin-positive cells, has proven not to

be sufficient in inducing severe cognitive impairments or neurodegeneration in mice [23]. Altogether, this body of results has motivated our search for non neuronal targets of NGF in the adult brain. Microglia was a strong candidate, because (1) previous work had suggested that NGF could modulate some aspects of microglial cells in culture [12] and (2) transcriptomic studies in the AD11 mouse model expressing anti-NGF had shown that neuroinflammation is the earliest phenotypic alteration, already at a presymptomatic phase (1 month of age; [19] [20]).

In this thesis I show that microglia cells are true target of NGF both in vivo and in vitro and that the activity carried out by this neurotrophin on these myeloid cells might result neuroprotective in the context of Alzheimer's Disease.

Chapter 1

Introduction

Microglia constitute around 10% of the total cells in the adult CNS [24]. Microglia are distributed throughout the CNS and vary in density in both rodents and humans, with subtle variations in morphology in different cytoarchitectural regions [25]. The word *glia* was coined in the mid 19th century and defined as *the nerve glue*. For decades, it was assumed to be a uniform matrix, until cell theorists raised the *neuron doctrine* which stipulated that nervous tissue was composed of individual cells. The term *astrocytes* was introduced in the late 19th century as a synonym for glial cells and later Santiago Ramon y Cajal defined a *third element* distinct from astrocytes and neurons.

Pio del Rio-Hortega, an alumnus of the Cajal School, introduced the modern terms and the concept of microglia that we use today. Microglia was defined cellular element of the central nervous system in a book chapter called *Microglia*[26] of the book *Cytology and Cellular Pathology of the Nervous System* edited by Wilder Penfield in 1932. He thoroughly described both *oligodendrocytes* and *microglia* to clearly distinguish them from astrocytes. Rio-Hortega described microglia postulating the following visionary concepts: 1) microglia enter into the brain during early development 2) these invading cells

have amoeboid morphology and are of mesodermal origin. 3) They use vessels and white matter tracts as guiding structures for migration and enter all brain regions. 4) They transform into a branched, ramified morphological phenotype in the more mature brain (known today as the resting microglia). 5) In the mature brain, they are found almost evenly dispersed throughout the central nervous system and display little variation. 6) Each cell seems to occupy a defined territory. 7) After a pathological event, these cells undergo a transformation. 8) Transformed cells acquire amoeboid morphology similar to the one observed early in development. 9) These cells have the capacity to migrate, proliferate and phagocytose [27] [28].

Microglia cells are often described as the macrophages of the central nervous system but a series of recent findings in the mouse has established that microglia are a unique cell population distinct from macrophages. Fate mapping has revealed that adult microglia are derived from precursors that leave the yolk sac on E8.5 E9.0, entering the neural tube via the primitive bloodstream [29] [30] as in figure 1.1.

As the progenitor cells journey to the CNS, they gain lineage-specific gene expression and ultimately differentiate into mature microglia. The yolk-sac-derived microglia remain throughout life, with the population being maintained by self-renewal in the healthy CNS with little contribution from bone-marrow macrophages [31] [24]. Microglia are not macrophages that migrate into the brain but instead they are known to represent a distinct population of resident tissue mesenchymal cells that populate the CNS during early development [32] [33]. Remarkably, since the origins and responses of microglia and macrophages are different, the roles they play in mitigating or propagating pathology could be different as well. Microglial are derived from primitive erythromyeloid progenitors in the yolk sac, distinct from the definitive hematopoiesis from which the majority of macrophages is derived.

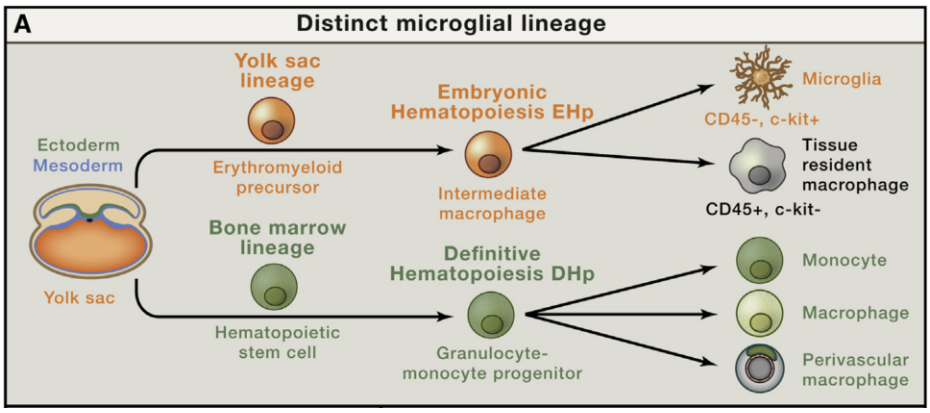

Figure 1.1: Microglia have a distinct lineage and molecular signature (A) The genesis and progression of yolk-sac-derived cell lineages are illustrated. Microglial are derived from primitive erythromyeloid progenitors in the yolk sac (embryonic hematopoiesis, indicated in yellow), distinct from the definitive hematopoiesis (shown in green) from which the majority of macrophages are derived. Figure from Salter and Beggs 2014 [24].

1 Microglia Cell Physiology

Thus, microglia cells are distinct from macrophages. In the brain, microglia, as macrophages in the other body regions, are the primary immune effector and they are key players in brain injury and disease. However, their role in the intact postnatal brain has remained elusive for a long time. *Resting* microglia are extremely dynamic in vivo, perpetually changing their morphology by extending and retracting highly motile processes on a time scale of minutes [34] [5]. This unexpected finding led to a series of discoveries suggesting potential roles of microglia in postnatal development, adult neuronal plasticity, and circuit function. In the next chapters some of these physiological mi-

croglial activities and their implications for normal brain function will be described. In particular the focus will be on microglia morphology and motility, the activation state (inflammation and the engulfing ability by microglia) and the interaction between microglia and neurons. Then how microglia physiology changes in pathological conditions will be discussed.

In Figure 1.2 from the paper *The Role of Microglia in the Healthy Brain* by Marie-Eve Tremblay et al [35], three different microglial functions are represented:(1) the continuous scanning of their local environment by highly motile microglial processes, (2) the structural and functional interactions with synaptic elements through direct contacts and exchanges of molecular signals, and (3) the contribution to restructuring the neuronal circuits by phagocytosing synaptic elements and newborn cells.

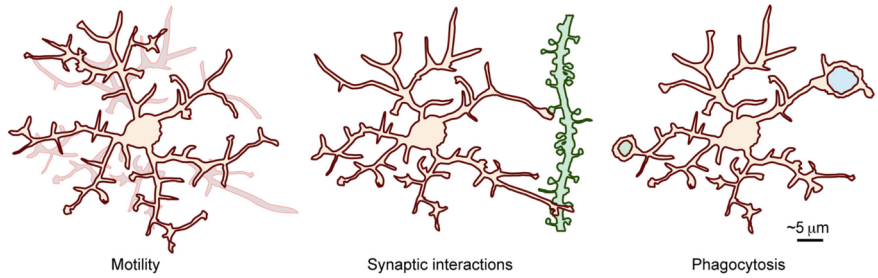

Figure 1.2: Overview of microglial behavior in the healthy brain. (1) Motility, (2) Synaptic interaction (3) Phagocytosis. In green are shown dendritic branch and spines, while in blue are shown cellular inclusions.

1.1 Morphology and Motility

In 1996 Georg W. Kreutzberg published the Review *Microglia: a sensor for pathological events in the CNS* [36]. Here the microglial rapid

activation in response to minor pathological changes in CNS was described for the first time. This ability to respond quickly to a variety of signalling represents a state of vigilance to changes in their extracellular space. In response to any kind of brain damage or injury, microglial cells become activated and undergo morphological as well as functional transformations. In the healthy brain, microglia are characterized by cellular processes branching off from the small soma with further distal arborisation. This morphological state is typical of *ramified* microglia, called also *resting* microglia, the latter definition implying an intimate link between morphology and function [28]. The idea that microglia assume various morphological appearances correlated with distinct functional states arose through studies conducted in the facial nerve system [37]. It was demonstrated that depending on whether facial motoneurons regenerate after a reversible injury (facial nerve axotomy) or degenerate after irreversible injury (ricin intoxication), microglia cells will respond in characteristic fashion. In figure 1.3 the original illustration from article [38] explains the new concept of *functional plasticity of microglia*.

Injured or diseased neurons cause resting microglia to become activated by emitting injury signals, as ATP [38] [34]. Microglia state of activation changes with the severity of neuronal damage. The mildest injuries cause hyper-ramification of microglia [39], that represents an intermediate stage between the resting and the reactive form. In the hyper-ramification state microglia start to become hypertrophic. This morphology correspond to the state of activation in vivo. Many types of neuronal damage shift resting microglia to a reactive phenotype. Nolte et al, in their paper published in 1996, investigated the regulation of microglia motility induced by different inflammatory mediators using time-lapse video microscopy. In this paper it was demonstrated how microglia cells exhibit a high resting motility characterized by intense ruffling of cell membranes followed by lamellipodia extension within few seconds. It was shown that this process is accompanied by

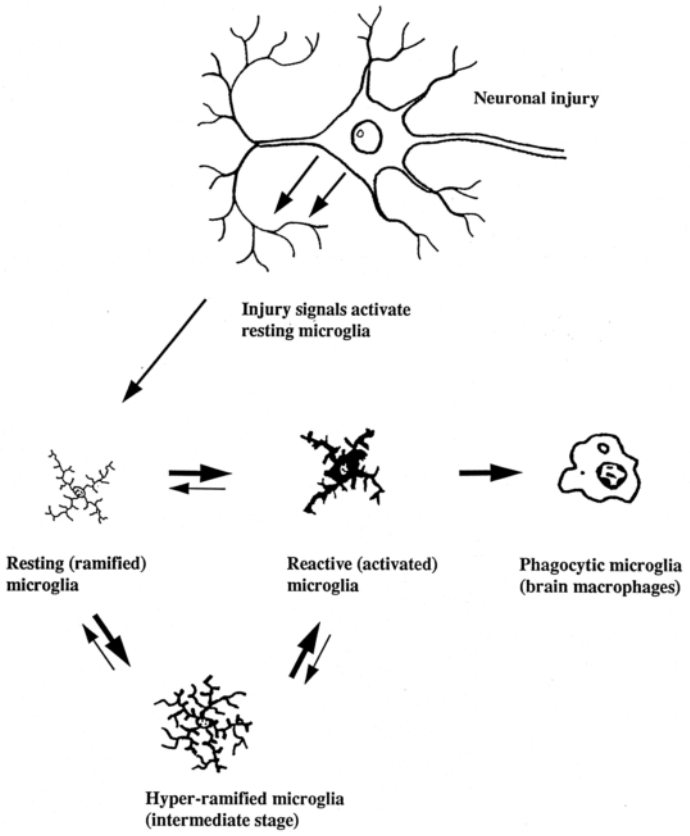

Figure 1.3: Different states of activation of microglia from Streit et al 1999.

a rapid rearrangement or the actin cytoskeleton. Later, Stence and colleagues studied microglia dynamics in rat brain tissue slices using stacks of confocal optical sections taken at intervals of 2-5 min for several hours [40]. During this time microglia became activated and shifted from a ramified phenotype to amoeboid macrophages. Ramified microglia progress to amoeboid macrophages through a stereo-

typical sequence of three steps, as described in figure 1.4.

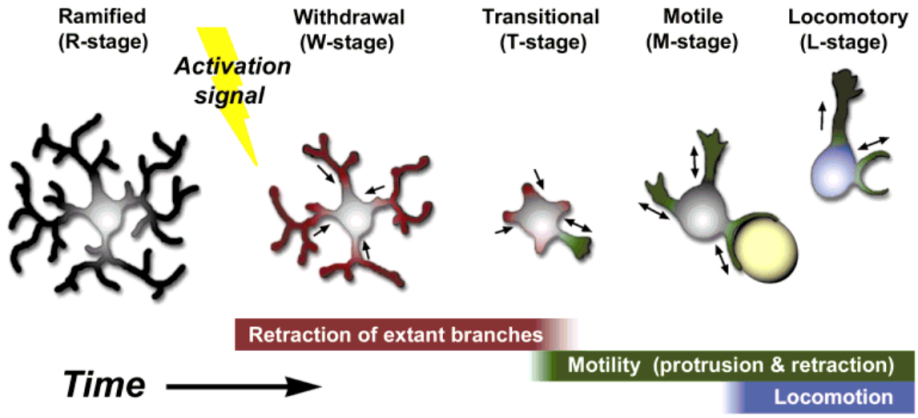

Figure 1.4: Multistep model of microglial activation dynamics from Stence Glia 2001 [40].

First, in the withdrawal stage, the existing ramified branches of activating microglia do not actively extend or engulf other cells, but instead retract back (mean rate, 0.5-1.5 μm/min) and are completely readsorbed into the cell body. Second, in the motility stage, a new set of dynamic protrusions, which can exhibit cycles of rapid extension and retraction (both up to 4 μm/min), abruptly emerges. Sometimes new processes begin to emerge even before the old branches are completely withdrawn. Third, in the locomotory stage, microglia begin translocating within the tissue (up to 118 μm/h). The translocation starts only after the new protrusions emerge. From these experiments, it was concluded that the rapid conversion of resting ramified microglia to active amoeboid macrophages is accomplished not by converting quiescent branches to dynamic ones, but rather by replacing existing branches with an entirely new set of highly motile protrusions.

This suggests that the ramified branches of resting microglia are

normally incapable of rapid morphological dynamics necessary for the function of activated microglial. More generally, these observations identified changes in the dynamic behavior of activating microglia and thereby helped definining distinct temporal and functional stages of activation [40].

As described above resident microglial cells in the healthy brain are thought to rest in a dormant state, whereas activation is associated with structural changes, such as motile branches or migration of somata [40] [41] [38]. Most tissue preparations represent traumatic injuries but, using time-lapse two-photon imaging of microglia, it has been demonstrated that the fine terminal of microglial processes are highly dynamic not only during the activation but also in their presumed resting state, continually surveying their microenvironment with extremely motile processes and protrusions in the intact mouse cortex [34] [5] as shown in figure 2.12.

Upon traumatic brain injury, for example when blood-brain barrier disruption occurs [5] or after ATP injection [34], microglial processes rapidly and autonomously change as described above on the site of injury without cell body movement, creating a barrier between the healthy and injured tissue. Thus microglia can be defined as busy and vigilant housekeepers in the adult brain [34]. In vivo two-photon microscopy revealed that microglial processes and arborizations are highly mobile [34] [5]. This microglia ability to monitor the environment probably depends on purinergic stimulation and involve a support from astrocytes [34] [42].

In vitro microglia cells usually do not show the ramified structure characteristic of the tissue microglia. Microglia show ameboid cell morphology, no polarity, many short processes that they extend into lamellipodia to even round cells. Some cells show morphological reorganization after LPS administration [43]. Moreover, ameboid microglial cells progressively ramify when co-cultured with astrocytes. Thus, microglia undergo morphological transformations from *ramified*

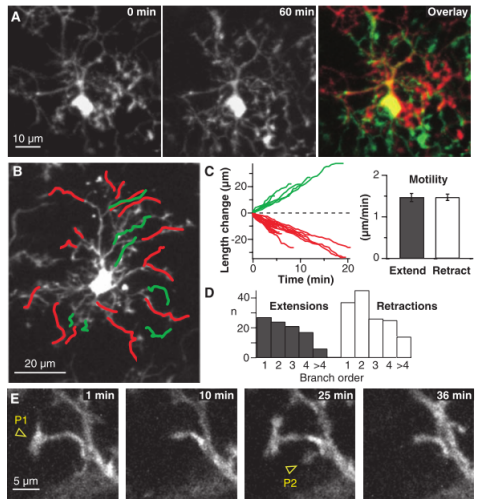

Figure 1.5: Microglial cells are highly dynamic in the resting state in vivo. (A) Maximum-intensity projections of an individual microglial cell (45 to 75 mm below the pia) at the beginning (left) and 1 hour after (center) the start of a transcranial time-lapse recording. (Right) Overlay showing extensive formation (green) and deletion (red) of microglial processes. (B) Extensions (green) and retractions (red) of processes over the time course of 20 min. (C) Length changes of the processes shown in (B) as a function of time. (Right) Mean motility values in mm/min for extensions and retractions. (D) Branch motility occurred at every branch order. (E) Example images of microglial protrusions (arrowheads) from a time-lapse recording. (F) Length changes over time of the two protrusions P1 and P2 indicated in (E). Vertical dashed lines mark the acquisition times of the images shown in (E). Arrows indicate protrusion lifetime. (G) Lifetime histogram of protrusions [5].

to *bipolar* to *tripolar* and *ameboid* states in accordance with local en-

vironmental cues associated with astrocytes in subconfluent cultures [44].

It was through these studies on morphological changes that a microglial role in the diseased CNS was recognized. The theory of microglia as sensors of pathologic changes was formulated in 1996 by Kreutzberg [36].

Given the importance of microglia morphology for its physiological role, it is important to study the changes in microglia morphology, but it is not clear from the literature how activated microglia might be accurately described. Recently, two articles, that describe an analysis of microglia morphology in vivo were published. In the CNS, microglia are the only cells that express CX3CR1, also known as the fractalkine receptor [45], so Dan R. Littman and colleagues developed in 2000 a knock-in mouse model CX3CR1-GFP/+ to study the role of fractalkine [46], but later the same model was used to study microglia morphology. Franck Verdonk and colleagues developed a 3D automated confocal tissue imaging system coupled with morphological modelling of many thousands of microglial cells. This study revealed a precise and quantitative assessment of major microglia cell features: cell density, cell body area, cytoplasm area and number of primary, secondary and tertiary processes. The use of two morphological criteria, the complexity index (CI) and the covered environment area (CEA), allowed to establish an innovative approach lying in (i) an accurate and objective study of morphological changes in healthy or pathological conditions, (ii) an in situ mapping of microglial distribution in different neuroanatomical regions and (iii) a study of the clustering of numerous cells, allowing us to discriminate different subpopulations. Using clustering analysis, the authors highlighted that, at resting state, microglial cells are distributed in four microglial subpopulations defined by their CI and CEA with a regional pattern and a specific behaviour. These results suggest that microglial cells are distributed in different defined sub-populations that present specific

behaviour after pathological challenge, allowing to postulate for a cellular and functional specialization [47]

More recently, María del Mar Fernández-Arjona and colleagues described a new method to classify microglia by their morphology, using images obtained by section scanning individual microglial cells from various regions (septofimbrial nucleus, hippocampus and hypothalamus) at different times (2, 4 and 12 h) post-injection of a single dose of the enzyme neuraminidase (NA) within the lateral ventricle (LV). Each cell yielded a set of 15 morphological parameters. This method allowed classifying microglia population in four clusters. A linear discriminant analysis (LDA) suggested three specific parameters to objectively classify any microglia by a decision tree. In addition, a principal component

analysis (PCA) revealed two extra valuable variables that allowed to further classifying microglia in a total of eight sub-clusters or types. The spatio-temporal distribution of these different morphotypes in a rat inflammation model allowed to correlate them to microglial activation status and brain location [48].

1.2 Resting (monitoring) state

It is probably reductive to apply the term *resting* to the non-activated microglia since they are continually involved in surveillance with their processes, which are largely responsible for this function, and therefore continually motile. The cell body of resting microglia is not motile, there is minimal expression of cell surface markers and release of cytokines and chemokines, and the cells are not involved in phagocytosis. However, as they clearly are not at rest, microglia under these circumstances might be better described as *monitoring microglia* [49]. It is probably accurate to state that the function of resting microglia is largely unknown although key roles in homeostasis, host defense and repair have been attributed to these cells [50]. It has been hypothe-

sized that microglia are responsible for dealing with the microdamage that occurs commonly in the brain and that this ranges from forms of plasticity which are associated with resculpting (perhaps eliminating) synapses to responders to capillary damages [51]. As the major resident immunocompetent cells in the brain [52] [53], microglia are responsible for sampling the microenvironment and play a role in removing cell debris [50]. This surveying role is likely to be an important factor in maintenance of homeostasis; microglia possess an array of ion channels, and expression and activation of potassium channels, in particular have been suggested to be important in production of inflammatory mediators in response to stressors [54] and neuronal survival/death [55]. Similarly, at least in certain circumstances, microglia, like astrocytes, express the glutamate uptake protein, GLT-1, suggesting that they may play a role in protection against glutamate toxicity [56]. The role for microglia in repair has been associated with their ability to produce neurotrophic factors, although it is also unclear whether release of neurotrophins can be achieved by *resting* cells [57]. What is clear is that microglia are exquisitely sensitive to stressors and rapidly change their morphology and function in response to all forms of insult. Microglia can be stimulated and may then express particular cell surface markers which promote chemotaxis or infiltration of circulating cells into the CNS which permit interaction of microglia with other cells. They may be alternately non-phagocytic cells producing soluble proinflammatory molecules or they may be phagocytic and motile [36] [58]. Intermediate states may also exist: for example, it has been proposed that expression of major histocompatibility complexes (MHC) enables phagocytic function and that engulfment of pathogens or cell debris promotes release of soluble proinflammatory molecules [59] shown in figure 1.6.

Description	Morphology/function
Resting (monitoring)	Extensive processes
	Sampling/surveying environment
Phagocytic	Ameboid structure with retracted processes, large cell body, and nucleus
	Motile
	CD68-expressing (indicative of lysosomal activity)
	Probably express TLR, Iba-1
Expressing cell surface markers	Antigen presentation
	Chemotaxis
	Facilitates influx of peripheral cells
Cytokine producing	Neuroprotective when release is limited
	Neurotoxic when release is persistent
Neurotrophin producing	Neuroprotective
	Neurosupportive
	Response to insult

Figure 1.6: At least five functional states of microglia exist but intermediate states, and states which are multifunctional, probably also exist.

1.3 Activation state

As cited previously, microglia in healthy brain have a ramified morphology, with a small resting soma with fine cellular processes that scan and monitor the environment. Microglial activation is the transition from *resting* cells to an activated state that occurs upon dis-

turbance of tissue homeostasis. This transition should be considered a change in functional phenotype. Cells shift from the surveillance mode (one type of activity) to a reactive profile, withstanding with altered homeostasis. The adjustments occur rapidly, in minutes or also seconds: cells change their chemotactic reorientations through non-transcriptional modifications. Significant transcriptional modifications take place within a few hours [51].

Infection, trauma, ischemia, neurodegenerative diseases, or altered neuronal activity, ie. any disturbance or loss of brain homeostasis indicating real or potential danger to the CNS, can evoke rapid and profound changes in the microglial cell shape, gene expression and functional behavior which summarily is defined as *microglial activation* [28] Phenotypically, the complexity of the cellular processes is reduced, and microglia revert to an amoeboid appearance. Microglia can become motile and actively move to a lesion or herd of infectious invaders following chemotactic gradients. Local densities of microglia can also increase by proliferation, to provide more cells for the defense against invading germs and to organize for the protection and restoration of tissue homeostasis. Induction and rearrangement of surface molecules to support cell-cell and cell-matrix interactions, changes in intracellular enzymes as well as release of multiple factors and compounds with proinflammatory and immuno-regulatory effects are additional elements of the activation process. Microglia can unfold their phagocytotic activities to clear tissue debris, damaged cells, or microbes. The release of chemoattractive factors by microglia recruits and guides immune cell populations to the CNS. Presentation of antigens by microglia to T cells can subsequently aid the adaptive immunity in fight against viral or bacterial invasion. The range of microglial activities also covers the production of neurotrophic factors and the physical association with endangered neurons.

Various kinds of stimulators have been predicted for microglia activation: some kinds of molecular and cellular mechanisms are shown

in figure 1.7.

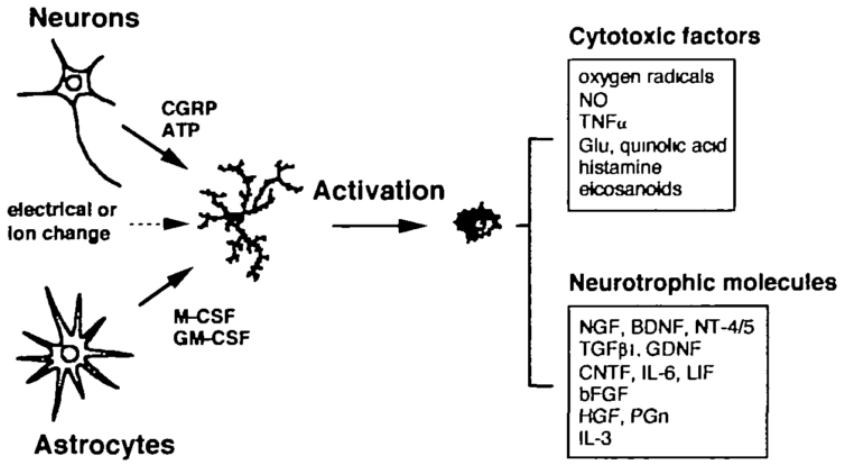

Figure 1.7: Putative molecules for microglial activation and activated microglia-derived cytotoxic and neurotrophic molecules. Figure from "Microglia: Activation and Their Significance in the Central Nervous System" Nakajima et al 2001 [60].

One category is represented by non-material stimulators, the electrical potential change resulting from neuronal injury and changes in the ion medium around injured neurons are candidates. The other category is that of biologically active substances, which may include low molecular weight molecules such as peptides and hormones. Growth factors or cytokines may also be able to activate microglia. Among the cytokines are macrophage colony-stimulating factors (M)-CSF and granulocyte-macrophage colony-stimulating factors (GM)-CSF [61]. As for other activators, calcitonin gene-related peptide(CGRP) and ATP [62] induce immediate early gene mRNA expression and translation in microglia. Likewise, ATP can cause biological responses such

as chemotaxis [63], release of plasminogen (PGn) and interleukin IL-1β, and activation of nuclear factor (NF) KB in cultured microglia [64]. Therefore, it is likely that some molecules derived from neurons and/or astrocytes trigger the activation of microglia in vivo as shown in figure 1.7.

Microglial activation can be accompanied by proliferation. The factor(s) necessary for mitogenesis in microglia were clarified by an in vitro study. Microglia can proliferate in response to GM-CSF, M-CSF, and multi-CSF (IL-3) [65]. Moreover, CSFs, IL-2, IL-4, IL-5, and tumor necrosis factor (TNF)α are reported to induce proliferative activity in cultured microglia. In vivo, CSF or CSF-like factors may be responsible for the proliferation and activation of microglia.

As described above, many molecules and conditions can trigger a transformation of resting (or surveying) microglia to activated (alerted or reactive) states. These have in common that they indicate a threat to the structural and functional integrity of the CNS. Microglial cells are prepared to recognize a wide range of signs for homeostatic surveillance, independent of their biochemical nature (peptides, lipoproteins, glycolipids, nucleotides) or diverse (patho)physiological implications. The list is shown in figure 1.8

The activation of microglia may have serious consequences on neuronal and astroglial activity and survival. The effects of activated microglia on neuronal survival is debated. On one hand, activated microglia in culture have been shown to produce several potentially cytotoxic molecules, including superoxide anion, nitric oxide, and proinflammatory cytokines [66] [67], shown in figure 1.7. Lipopolysaccharide (LPS), interferon γ (INFγ), and β-amyloid [68] are among the stimulators for the production of harmful factors from microglia. Reactive oxygen species (ROS) including superoxide anions, hydroxy radicals, and hydrogen peroxide are generally hazardous, particularly to myelin and its forming cells (oligodendrocytes) owing to their capability of inducing lipid peroxidation. LPS and phorbol-12-mynstate-

Table 1 Examples of signals and modulators of microglial activation

Class of compound	Examples
Surface structures and DNA/RNA of viral, bacterial or fungal origin	Agonists of members of the pattern recognition receptor families, notably TLR1/2, TLR3, TLR4,TLR6/2 and TLR9, such as bacterial LPS or cell wall proteoglycans and lipoteichoic acid (LTA), gp41, gp120 (the TLR4-agonistic LPS serving as a common model agent)
Abnormal endogenous proteins	β-amyloid (aggregates), Aβ25–35, Aβ40, Aβ42, prion protein (PrP)
Complement	Complement factors C1q, C5a
Antibodies	Immunoglobulin of various classes and isotypes (IgA, IgG, IgM), presented in immune complexes
Cytokines	Colony stimulating factors (M-CSF, GM-CSF), IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-15, IL-18, IFN-γ, TGF-β, TNF-α
Chemokines	Ligands for chemokine receptors: CCR3, CCR5, CXCR2, CXCR, CXCR4, CX3CR1, IL-8R
Neurotrophic factors	Brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), NT-4
Plasma components	Albumin, fibronectin, fibrinogen, thrombin
Other proteins and peptides	Apolipoprotein E (ApoE), heat shock proteins hsp60 and hsp70, CD40L, melanocyte-stimulating hormone (MSH), endothelin, S100 proteins, vasoactive intestinal peptide (VIP)
Neurotransmission-related compounds	ATP (and related purines), β-adrenergic agonists, glutamate, kainate, NMDA
Ions	K^+, Mn^{2+}
Other compounds	Cannabinoids, ceramide, gangliosides, lysophosphatidic acid (LPA), melatonin, opioids (endomorphines), platelet-activating factor (PAF), prostaglandin E_2 (PGE_2), steroid hormones, vitamin D_3

[1]Chemokine receptors can accept several chemokines owing to their promiscuous nature.

Figure 1.8: Exemples of signals and modulators of microglia activation adapted from Uwe-Karsten Hanisch, Helmut Kettenmann 2007 [51].

13-acetate (PMA) are stimulators of ROS production from cultured microglia. Nitrogen oxides such as NO are highly reactive free radicals, of which nitrite peroxide is the strongest species. These radicals are believed to inhibit respiratory enzymes, oxidize the SH group of proteins, and enhance DNA injury, finally resulting in neuronal cell

death. LPS and β-amyloid are known stimulators of NO production from microglia. In the presence of INFγ, β-amyloid synergistically stimulates the production of NO and TNFα [69] in microglia.

Besides, recently it was demonstrated by Liddlelow and colleagues a direct role of microglia on the astrocytes activation. They demonstrated that a subtype of reactive astrocytes, named in their paper A1, is induced by classically activated neuroinflammatory microglia. They show that activated microglia induce A1 astrocytes by secreting Il-1a, TNFα and C1q, and that these cytokines together are necessary and sufficient to induce A1 astrocytes. A1 astrocytes lose the ability to promote neuronal survival, outgrowth, synaptogenesis and phagocytosis, and induce the death of neurons and oligodendrocytes. Death of axotomized CNS neurons in vivo is prevented when the formation of A1 astrocytes is blocked. Finally, they have shown that A1 astrocytes are abundant in various human neurodegenerative diseases including Alzheimer's, Huntington's and Parkinson's disease, amyotrophic lateral sclerosis and multiple sclerosis [70]. *Effector* microglia might better describe cells executing a number of adaptive responses to a given challenge.

However, on the other hand, co-cultures of microglia and neurons revealed that microglia did not kill healthy neurons in vitro, indicating that their phagocytic properties are not necessarily dangerous for neurons. Secondly, the effects of conditioned microglial medium (CMM) on neurons were examined. The results showed the CMM expressed no neurotoxicity. Rather, CMM significantly enhanced neuronal survival and the neurite outgrowth of neocortical and mesencephalic neurons [60].

In conclusion, activated microglia act primarily in the defence of the brain, as brain scavengers, and in tissue remodeling, an immune and/or immunoeffector role has also been proposed. Activated microglia are implicated in pathogenesis and inflammation. They have also been shown to produce a variety of biological factors. Recent bio-

chemical and neurobiological studies have revealed that activated microglia produce and secrete not only cytotoxic/harmful molecules but also neurotrophic molecules including neurotrophins and neurotrophic cytokines. As a whole, activated microglia appear to play a significant role in pathological and regenerative states of the brain, expressing both or either of two potentially opposing functions: cytotoxic and neurotrophic actions.

1.3.1 Inflammation markers

Neuroinflammation, and to the same degree all inflammation, is a fundamental immune response designed to protect the body from harm, arising from both endogenous and exogenous sources. Being the sentinel immune cell of the brain, microglia play a role as first responders to infections or tissue injuries and initiating an inflammatory response. Using a full array of immune receptors, such as toll-like receptors (TLRs), nucleotide binding oligomerization domains (NODs), NOD-like receptors, and many scavenger receptors [71] [72], microglia (as well as other CNS cells, such as astrocytes) are able to recognize harmful stimuli and respond by producing inflammatory cytokines such as TNFα, IL-6, IL-1β, interferon-γ (IFNγ), and several chemokines [73]. This cytokine production is essential for the polarization of microglia into what has been termed a classically activated, M1, state [74]. Due to this ability to respond to inflammatory cytokines, as macrophages do, the microglia activation state started to be named in the same way of macrophages. In particular, the term M1 parallels the Th1 terminology used for T cells, and underscores the close relationship between T cells and macrophages in the periphery. Interferon-γ produced from Th1 cells was found to be instrumental in polarizing macrophages to M1 state [74]. However, the ability to produce these cytokines does not rest solely with T cells. Microglia and astrocytes have also been observed to fill this role [75], demonstrating, at least in part, that

microglia can control their own polarization through autocrine and paracrine means. For the evolution of these theories on macrophages polarization see figure 1.9.

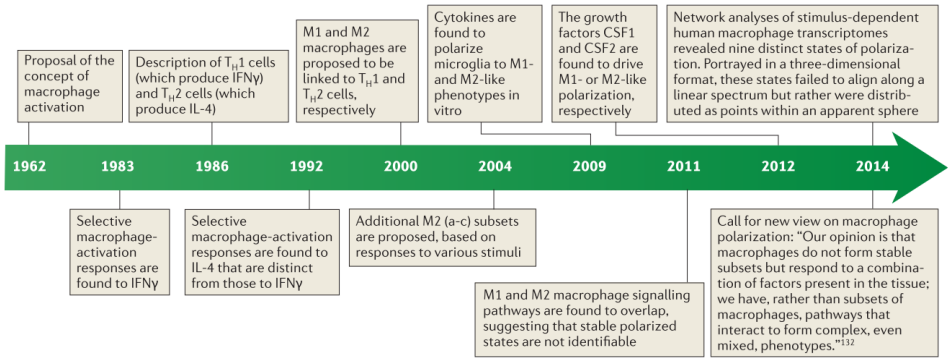

Figure 1.9: M1 and M2: the concept of macrophage polarization [76].

In many cases, this response is protective and is downregulated once the damage or pathogen has been dealt with. However, dysregulated, long-term, or chronic inflammation can lead to tissue destruction [77] [7] as in the rappresentative figure 1.10.

Differently to proinflammatory M1 cells, alternatively activated macrophages (M2) express cytokines and receptors that are implicated in inhibiting inflammation and restoring homeostasis. This includes: IL-10 to downregulate inflammatory cells, extracellular matrix protecting proteins like YM1, ornithine, and polyamines for wound repair, and higher levels of receptors associated with phagocytosis, such as scavenger receptors [78]. Just as the Th1 cytokine IFNγ has been associated with induction of proinflammatory M1 macrophages, the Th2 cytokine IL-4 has been associated with M2, or alternative, activation. Interestingly, it appears that when there is a lack of M2 cell differentiation in the CNS, problems can arise. To properly understand

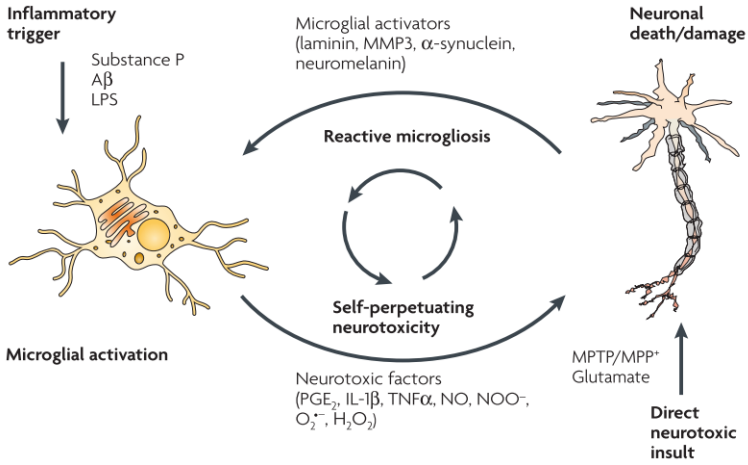

Figure 1.10: Reactive microgliosis drives progressive neurotoxicity: microglia can become overactivated and cause neurotoxicity through two mechanisms. First, microglia can initiate neuron damage by recognizing pro-inflammatory stimuli, such as lipopolysaccharide (LPS), becoming activated and producing neurotoxic pro- inflammatory factors. Second, microglia can become overactivated in response to neuronal damage (reactive microgliosis), which is then toxic to neighbouring neurons, resulting in a perpetuating cycle of neuron death. Reactive microgliosis could be an underlying mechanism of progressive neuron damage across numerous neurodegenerative diseases, regardless of the instigating stimuli [7].

the role microglia play in neurodegeneration, understanding their phenotypes is important. The functional effects of classical activation are geared towards antigen presentation and the killing of intracellular pathogens. Therefore, upregulation of many associated receptors and enzymes reflects that purpose. For example, MHC II, CD86, and Fcγ receptors are upregulated to allow for antigen-presenting activity of

microglia and increased crosstalk with other immune cells [79]. In addition, the ratio of particular cytokines has been used to identify inflammatory macrophages and this observation could extend to microglia. For example, since M1 macrophages were found to be a key source of IL-12 [80], it was suggested that IL12 High, IL-10 Low production is a simple way to distinguish inflammatory cells [81]. Another potential distinction and an important component of M1 microglia is their ability to produce reactive oxygen species and reactive nitrogen species [82]. A key microglial enzyme associated with this process is inducible nitric oxide synthase (iNOS), which utilizes arginine to produce nitric oxide [83]. However, even though it seems straightforward to identify M1 cells based on these characteristics, classifying these cells in vivo has proven to be more challenging, reflecting the plastic nature of microglia. Even for M2 cells the classification is not clear. In fact, there are many efforts to identify unique subgroups with different functions. Division of M2 cells is based on observations that stimulation with various cytokines yields different sets of receptor profiles, cytokine production, chemokine secretion, and function [81]. Even though the profiles of these cells are diverse, the one feature that places them all in the M2 classification is that they express mediators or receptors able to downregulate, repair, or protect the body from inflammation [84]. The original alternatively activated macrophage was classified based on expression of the mannose receptor [85]. Since then a panel of different markers has been identified as M2 specific. One of the best characterized markers is the enzyme arginase 1 (Arg1) [86], which converts arginine to polyamines, proline, and ornithines that can contribute to wound healing and matrix deposition [87]. Thus, iNOS and Arg1 represent a relatively straightforward set of markers to distinguish M1 from M2 phenotypes, as shown figure 1.11.

Other markers used for identifying M2 cells include Ym1, a heparin-binding lectin [89], FIZZ1, which promotes deposition of extracellular matrix [90], and CD206, a mannose receptor [85]. Despite the benefit

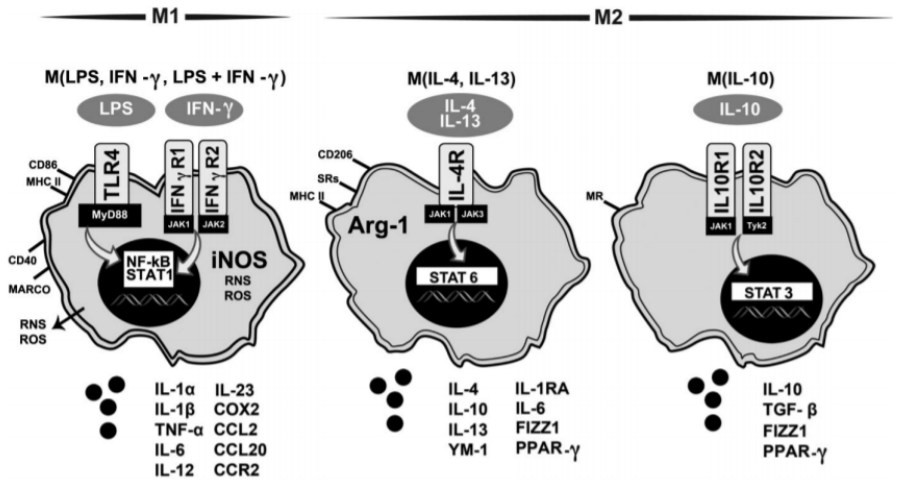

Figure 1.11: Diagram of activation states of microglia based on inflammatory profile and effector function. Based upon peripheral macrophage nomenclature, M1 and M2 polarization states of microglia have been proposed as a framework to evaluate the heterogeneity of responses with recent evaluations suggesting a framework focused on the inducing stimuli [88].

of having specific markers, using just one or two is limiting and ignores the overall diversity of M2 phenotypes. Another way to classify the function and phenotype of M2 cells is based on the cytokines that induce them. The prototypical cytokine used to first induce alternative activation was IL-4 [85]. Both IL-4 and the closely related cytokine IL-13 signal through IL-4Rα to induce a host of downstream processes that lead to potent anti-inflammatory functions, such as Arg1 upregulation, inhibition of NF-kB isoforms, and production of scavenger receptors for phagocytosis [79] [91] [92]. This type of activation has been classified as M2a. The main function of this response appears to be suppression of inflammation. A second state of alternative acti-

vation is based on macrophages exposed to IL-10, glucocorticoids, or TGF-β. This phenotype is classified as M2c [81] [93]. Originally, this state was described as being deactivated but that is not a particularly useful description. Instead of having no function, deactivated M2c macrophages appear to be involved in tissue remodeling and matrix deposition after inflammation has been downregulated [81]. A third sub-class of M2 activation has been observed following exposure to immune complexes and stimulation of TLR. This class is termed M2b or Type II [81] [94]. Of these three classes, M2b macrophages are the least understood. Interestingly, they more closely resemble M1 macrophages, owing to the lack of any M2 specific markers, such as Arg1, YM1, or FIZZ1. However, they do express the typical IL-10 High, IL-12 Low M2 cytokine profile [95]. Moreover, they have higher levels of MHCII and CD86, suggesting that they retain their ability to stimulate T cells [94].

An important consideration regarding M2 phenotypes is that these states were typically elucidated in vitro following exposure to one or two stimuli. This does not represent the complex environmental milieu seen in tissue. Therefore, some investigators have cautioned against this classification into distinct subtypes and instead propose that M2 cells should be viewed as a spectrum of phenotypes [95]. This detailed classification of M2 cells has been primarily carried out in the periphery. Whether or not this will extend to brain resident microglia is yet to be seen. Furthermore, microglia are not macrophages that migrate into the brain, but instead are known to represent a distinct population of resident tissue mesenchymal cells that populate the CNS during early development [32] [33]. Importantly, because the origins and responses of microglia and macrophages are different, the roles they play in mitigating or propagating pathology could be different as well.

Moreover, some other markers have been variously used as indicators of microglial activation but the limitations of their use are seldom

recognized. Important among these limitations is that several (if not all) of the markers are not unique to microglia and that, except in particular circumstances for example where pure microglial cultures are used, their upregulation may not reflect changes in microglia alone (figure 1.12).

Cell surface marker	Proposed function	Expressed on other cells?
MHCII	Hallmark of activated cells; enables antigen presentation in presence of costimulatory molecules; probably modulates phagocytosis	Macrophages; astrocytes
CD86	Costimulatory molecule; probably required for antigen presentation	Activated astrocytes
CD80	Costimulatory molecule; probably required for antigen presentation	Macrophages
CD11b	Constituitively expressed on microglia; upregulation is indicative of cell activation; plays a role in chemotaxis and modulates leukocyte adhesion and migration	Monocytes
CD40	Marker of activation; contributes to restimulation of T cells; probably affects phagocytosis	Macrophages; endothelial cells; astrocytes
ICAM	Constituitive expression is low but increased upon stimulation; plays a role in cell adhesion	Endothelial cells; macrophages; leukocytes
Iba1	Calcium-binding protein specific to microglia and macrophages; may play a role in phagocytosis	Macrophages
CD200R	Activated by CD200; decreases microglial activation	Cells of the myeloid lineage
Fractalkine R	Activated by fractalkine; decreases microglial activation	Macrophages; leukocytes NK cells; astrocytes; neurons

Figure 1.12: Proposed cell surface markers of microglial activation [49].

It is also important to recognize that, to a large extent, studies report changes in expression of cell surface markers under various conditions with minimal assessment of functional change and therefore the coupling of phenotype and function remains a challenge.

1.3.2 Phagocytosis and pinocytosis

Microglial cells are the professional phagocytes of the CNS tissue. This function is important for the normal brain, during brain development, and in pathology and regeneration [28]. As for other microglia functions, such as the inflammatory cytokines secretion, also for phagocytosis there is no obvious concordance with whether it is beneficial

or detrimental for tissue health. As I described previously, microglia cells are highly sensible to different stimuli of the environment. As these cells change their motility, their surface receptors, the release of soluble chemokines and cytokines, they also change the ability of up-taking debrees. Based on the type of debris, microglia can use phagocytosis or pinocytosis to take them up [28].

Endocytosis can be classified in two types: the clathrin-dependent and clathrin independent endocytic pathways. The clathrin-dependent endocytic pathway requires the recruitment of endocytosed molecules into clathrin-coated pits that are around 100 nm in diameter [96]. Phagocytosis and pinocytosis are two clathrin-independent endocytic processes that occur in phagocytes, and both create large endocytic vacuolar compartments ($\geq$0.2 μm) through organized membrane movements and actin polymerization [97]. Nevertheless, distinct models and molecular mechanisms have been suggested for the formation of phagosomes and pinosomes [97]. The well-studied Fc receptor-mediated phagocytosis is guided by a zipper-like progression of receptor-initiated membrane invagination that is shaped by the geometry of the internalized particle, whereas pinosomes, which may vary from 0.2 to 10 μm in diameter, are suggested to form spontaneously or in response to growth factor receptor activation from membrane ruffles that close at their distal margins to engulf extracellular fluid [97] [98]. Phagocytosis is applied in many physiological and pathological processes, including development, innate immunity, and the entry of pathogens into host cells [97] [98].

The activity of microglial phagocytosis relies on "eat me" signals expressed on the cell surface of dying cells [99] and on specific receptors expressed on microglia cell surface. Downstream signaling pathways are activated and contribute to the reorganization of actin protein and engulfment of harmful microparticles (Fig.1.13).

Microglial phagocytosis may need different types of receptors to initiate function [100], there are two distinctive types of receptors:

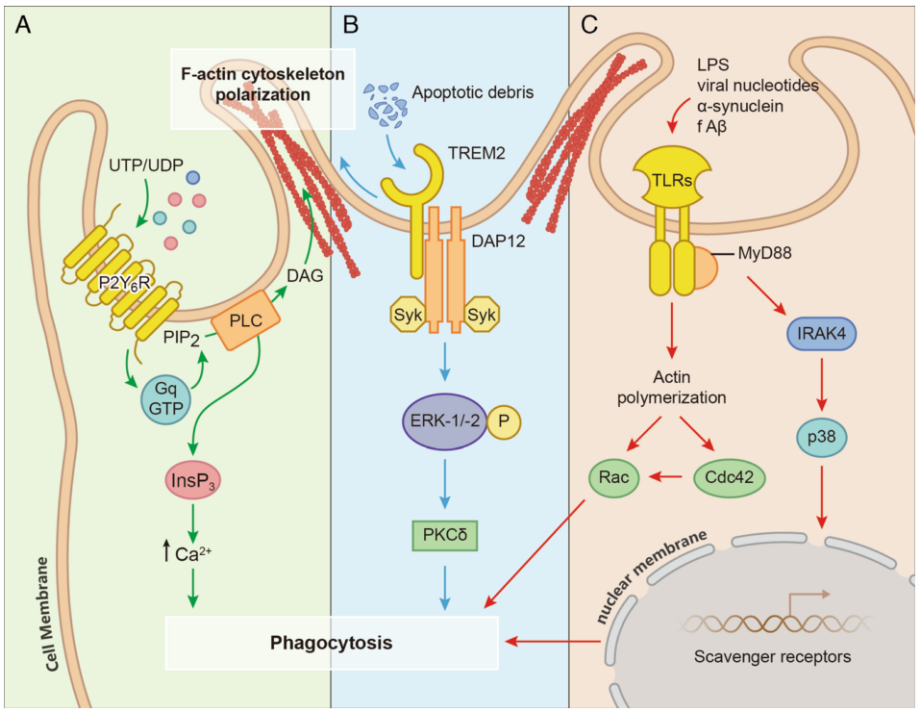

Figure 1.13: Signaling pathways involved in microglial phagocytosis. A Extracellular nucleotides, such as UDP and UTP, trigger microglial phagocytosis through P2Y6R/ PLC/InsP3 pathway. B Apoptotic debris induces phagocytosis via TREM2/DAP12/ERK/PKC pathway. C Endogenous or ectogenic detriments, such as LPS, viral nucleotides, α- synuclein, and f-Aβ, provoke phagocytosis by microglia via TLRs through activation of MyD88-dependent IRAK4/p38/ scavenger receptors pathway or MyD88-independent actin- Cdc42/Rac signaling pathway.

- the first with a high affinity for foreign microbial pathogens and which simultaneously stimulates a pro-inflammatory response in the

phagocytes [101], such as Toll-like receptors (TLRs). - The second type of receptors can, recognize apoptotic cellular substances and stimulate an anti-inflammatory response in phagocytes. Triggering receptor expressed on myeloid cells 2 (TREM2) is an example of this second type of receptors. The phagocytosis triggered by TREM2 takes place without inducing inflammation and it is one of the major beneficial functions of phagocytes (figure 1.13).

TREM2 is a kind of pattern receptor specific for polyanions and locates mainly on the cell surface of osteoclasts in bones and in microglia of the CNS [102].

TREM2 [103] is a 40-kDa type I membrane glycoprotein with a single extracellular immunoglobulin-like domain, one trans-membrane domain, and a short cytoplasmatic tail. Its transmembrane domain interacts with the adaptor protein TYROBP/DNAX-activating protein of 12 kDa (DAP12) via electrostatic interaction [103]. DAP12 is a type I transmembrane protein, which acts as a signaling adaptor protein for TREM2 and for other cell surface receptors.

TREM2 seems to be involved in two signaling pathways in microglia: phagocytosis and proinflammatory cytokines secretion. Indeed, increased expression of TREM2 on microglia has been associated with enhanced phagocytosis and increased alternatively activated M2 protective microglia, while absence of TREM2 expression on microglia does exactly the opposite, impairing phagocytosis and increasing the proinflammatory phenotype [104]. TREM2 phagocytic function is not associated with inflammation. In addition to up-regulating of chemokine synthesis and mediating protective phagocytosis of apoptotic cell debris, activation of TREM2 receptors suppresses secretion of pro-inflammatory factors such as cytokines and ROS [102]. On microglia TREM2 via binding to DNAX- activation protein 12 (DAP12), an ITAM-containing adaptor protein, triggers the reorganization of F-actin and phosphorylation of ERK/MAPK, mediating the clearance of apoptotic neurons [105] [106].

The paramount importance of these receptors has recently become clear as patients with a loss of function mutation of either TREM2 or DAP12 develop an inflammatory neurodegenerative disease leading to death at the fourth or fifth decade of life. Nasu-Hakola disease, a systemic bone cystic disorder with progressive presenile dementia followed by extensive sclerosis in the front-temporal lobe and the basal ganglia, occurs due to genetic mutation of TREM2 and DAP12 resulting in aberrant TREM2/DAP12 signaling pathway. Thus, even though the ligand of TREM2 is unknown, microglial TREM2/DAP12-mediated phagocytosis appears to be an essential function for CNS tissue homoeostasis [104].

Besides these two types of receptors, some others including Fc receptors, complement receptors [107], scavenger receptors (SR), pyrimidinergic receptor P2Y, G-protein coupled, 6 (P2RY6), macrophage antigen complex 2 (MAC-2), mannose receptor [108], and low-density lipoprotein receptor-related protein (LRP) receptor also participate in microglial clearance of misfolded, apoptotic cells and dead neurons in both acute and chronic brain injury [109].

Furthermore, TLRs are a class of proteins that play a key role in the innate immune system and the digestive system. TLRs are single, membrane-spanning, non-catalytic receptors usually expressed in peripheral sentinel cells, such as macrophages and dendritic cells, that recognize structurally conserved molecules derived from microbes. An example is proposed by TLRs 1-9, which belong to interleukin (IL)-1R super-family and that are expressed exclusively on antigen presenting cells including microglia [110], macrophages, antigen presenting dendritic cells, and on cerebral parenchyma cells including neurons, oligodendrocytes, and astrocytes. TLRs not only trigger the recognition of pathogen-associated molecular patterns (PAMPs), such as LPS or viral nucleotides, but also recognize so called danger-associated molecular patterns (DAMPs), such as deposited amyloid β (Aβ) fibril and α-synuclein [111] [112], they are host biomolecules that can initiate

and perpetuate a noninfectious inflammatory response. In contrast, PAMPs initiate and perpetuate the infectious pathogen-induced inflammatory response. TLR4-, TLR2-, and TLR9-dependent signaling pathways are involved in mediating microglial phagocytosis of neurotoxic Aβ deposits in AD brain and exert a protective role in nerve regeneration [113] [114]. It has been reported that TLRs regulate phagocytosis through myeloid differentiation factor 88(MyD88)-dependent and MyD88-independent signaling pathways. The MyD88-dependent pathway is triggered by TLRs through activation of IL-1 receptor-associated kinase (IRAK)-4 and MAP p38, resulting in up-regulation of scavenger receptors [115]. On the other hand, TLRs also regulate phagocytosis by a MyD88-independent actin-Cdc42/Rac pathway [116].

Pinocytosis can be triggered by "drink me" signals, since in the pinosomes particles are small and a part of the extracellular fluid is also included. It can be subdivided in micro-and macro-pinocytosis.

Micropinocytotic vesicles are no larger than 0.1 μm in diameter and may be caveolin-coated. Micropinosome formation is independent of actin and occurs within cholesterol-rich lipid domains of the plasma membrane [117], while macropinocytosis results in the formation of vesicles that are between 0.2 μm and 5.0 μm in diameter and are created by the enclosure of membrane ruffles. Macropinocytosis was first described morphologically by Warren Lewis in 1931 [96] and it is a clathrin-independent endocytic route, a regulated form of endocytosis that mediates the non-selective uptake of solute molecules, nutrients and antigens. Macropinocytosis is a signal-dependent process that normally occurs in response to growth factor stimulation, such as macrophage colony-stimulating factor-1 (CSF-1), epidermal growth factor (EGF) and platelet-derived growth factor or tumour-promoting factor, such as phorbol myristate acetate (PMA) [118] [119]. However, some specialised cell types such as antigen-presenting cells are capable of constitutive macropinocytosis [120] [121]. Macropinocyto-

sis involves actin- mediated membrane ruffling of the plasma membrane leading of the formation of lamellipodia. Most of these retract back into the cell. However, a subset of lamellipodia may fold back onto themselves and fuse with the basal membrane creating large, irregular shaped vesicles named macropinosomes (Figure 1.14). However, there is, no consensus on the proportion of membrane ruffles that end up forming macropinosomes. Macropinosomes are distinct from other forms of endocytic vesicles. Macropinocytic vesicles have no apparent coat structures and although heterogenous in size, they are generally considered to be $\geq$0.2 μm in diameter [122] [123], a size considerably larger than clathrin coated vesicles. Owing to the large size of macropinosomes, which can be up to 5 μm in diameter, macropinocytosis provides cells with a way to non-selectively internalise large quantities of solute and membrane. Thus, in addition to its size, macropinosomes can also easily be identified through the use of fluid phase markers, such as Lucifer Yellow, horseradish peroxidase and dextran. Once formed, macropinosomes undergo a maturation process. However, this maturation is unlikely to occur through a pathway common to all cells (see in figure 1.14).

Macropinosomes form in response to treatment with CSF-1 [124]. They acquire and loose different endocytic protein markers as they migrate centripetally toward the lysosome. Within 1 min of colony-stimulating factor-1 treatment, macropinosomes become positive for transferrin receptors. These are considered to be macropinosomes at an early maturation stage, within 2-4 min and the macropinosomes are devoid of transferrin receptors and begin to acquire Rab7, a marker of the late endosomes. Rab7-staining coincides with the presence of lysosomal glycoprotein A (lgp-A). Finally, the macropinosomes merge with the existing tubular lysosomal compartments [125] and fuse with the plasma membrane, recycling its content to the exterior of the cell [122] [126].

As macropinocytosis is associated with actin-dependent ruffling of

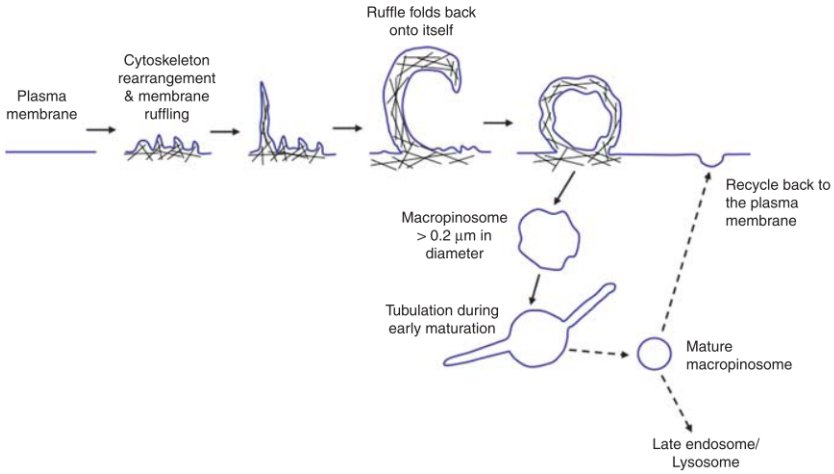

Figure 1.14: Pathway of macropinocytosis. Macropinocytosis involves actin cytoskeleton rearrangement at the plasma membrane leading to the formation of membrane ruffles. Ruffles may fold back onto themselves and fuse at the base of plasma membrane, trapping solute and soluble substances in macropinosomes. Early maturation of macropinosomes involves extensive tubulation resulting in mature macropinosomes that are more spherical. Content of the macropinosomes are then either degraded at the late endosome/lysosome or recycled back to the plasma membrane. The cytoskeleton is depicted as black lines [96].

the plasma membrane, this pathway has been implicated in cell motility. Macropinocytosis is also considered important in the chemotactic response of highly mobile cells.

A key difference between clathrin-dependent endocytosis and macropinocytosis is that the latter requires actin cytoskeleton reorganisation. Treatment with phosphoinositide 3-kinase (PI3-kinase) inhibitors or cy-

tochalasin, an actin-disrupting agent [127] has been shown to inhibit macropinocytosis but not the clathrin-dependent endocytic pathway [128] [129]. The dynamics of actin cytoskeleton during macropinocytosis has been visualised in cells expressing fluorescently labelled actin probes such as coronin. Coronin is an actin-associated protein, which has a role in controlling actin dynamics during processes such as cell motility, phagocytosis and cytokinesis [130]. In a separate experiment, the dynamics of actin cytoskeleton was observed using GFP-actin-binding domain probe binding specifically to F-actin, a structure also recognised by phalloidin [131] [132]. The F-actin probe remained associated with the macropinosome but was completely lost 30-50 seconds after vesicle closure [131].

Araki et al. [133] investigated the distribution of F-actin in mouse macrophages using actinin-4, a non-muscle form of α-actinin [133]. α-Actinin is an actin-binding protein, which cross-links F-actin into bundles or connects F-actin to the plasma membrane. Immunofluorescence analyses revealed that lamellipodia stained positive for actinin-4. Actinin-4 was also associated with dextran-labelled macropinosomes and this association was found to decrease as the macropinosome matures [133]. These examples clearly depict the importance of actin cytoskeleton reorganisation during macropinocytosis. Therefore, components of signal transduction pathways linking receptor stimulation to actin cytoskeleton remodelling are also implicated as regulators of macropinocytosis as in table 1.15.

The remodelling of the cytoskeleton during macropinocytosis requires also the activity of PI3-kinase [133] [134] [135]. In the case of bone marrow-derived macrophages, PI3-kinase activity was not required for cell surface ruffling but rather for the closure of macropinosomes [133]. PI3-kinase is a core signalling component in multiple signal transduction pathways such as endocytosis, membrane traffic and cell proliferation and inhibition of its activity will result in a range of other effects. Amiloride or its more potent counterpart, dimethyl amiloride,

Regulator	*Localisation during macropinocytosis*	*References*
Ras	Plasma membrane	57,58
Src	Plasma membrane and macropinosome	60,98–101
PI3-kinase	Plasma membrane[34]	48,59,60
Phosphoinositide 5-kinase (PIKfyve)	Macropinosomes	102
Phospholipase C (PLC)	Unknown	60
SH3-containing guanine nucleotide exchange factor (SGEF)	Plasma membrane	103
Rab34	Plasma membrane and macropinosome	104,105
Rab5	Plasma membrane and macropinosome	106–108
Rabankyrin-5	Macropinosome	108
ADP-ribosylation factor 6 (ARF6)	Plasma membrane	109–112
Rac	Plasma membrane[109] and macropinosome[113]	34,113–115
Cdc42	Unknown	34,116
p21-activated kinase 1 (PAK1)	Plasma membrane and macropinosome	11,117
C-terminal-binding protein-1/brefeldinA-ADP ribosylated substrate (CtBP1/BARS)	Plasma membrane	118,119
Switch-associated protein 70 (SWAP-70)	Macropinosome	113
Sorting nexin 1 (SNX1)	Macropinosomes	70
Sorting nexin 5 (SNX5)	Plasma membrane and macropinosomes	18,62,66
Sorting nexin 9 (SNX9)	Macropinosomes	70
Sorting nexin 18 (SNX18)	Macropinosomes	70
Sorting nexin 33 (SNX33)	Cytosolic	70

Regulators of macropinocytosis and their respective subcellular localisation during macropinocytosis.

Figure 1.15: Regulators of macropinocytosis and their respective subcellular localisation during macropinocytosis [96].

is an inhibitor of the Na+/H+ exchanger pump in the plasma membrane. It has been reported to selectively block macropinocytosis, and not the clathrin-dependent endocytic pathway, when used at the appropriate concentration [126] [136]. Thus, amiloride is gaining pop-

ularity as a tool to study macropinocytosis. It has recently been proposed that macropinocytosis is not directly sensitive to amiloride; rather, macropinocytosis is affected by acidification from excessive localised H+ production at the site of macropinocytosis when the regulatory action of Na+/H+ exchanger pump is impaired by amiloride. Acidification does not inhibit receptor activation or PI3-kinase localisation and activation but it does affect downstream localisation and activation of Rac1 and Cdc42, GTPases required for actin remodelling [136]. Members of the sorting nexin (SNX) family have been localised to macropinosomes and also implicated in macropinosome formation. There is a direct relationship between the level of cell surface SNX5 protein and macropinocytic activity, highlighting the role for this sorting nexin in driving this signal-activated endocytic pathway. In a recent study, 12 members of the SNX-PX-BAR family were screened in an assay to determine the effect of their overexpression on macropinosome formation [137]. SNX1, SNX5, SNX9, SNX18 and SNX33 were all found to enhance macropinosome formation, an effect that appeared dependent on the increase in PI(3,4,5)P3 levels, which is regulated by PI3-kinase activity [137]. PI3-kinase, Ras, Rac1 and Cdc42, as well as sorting nexins, are some examples of macropinocytic regulators. Additional molecules, which are components of the signal transduction pathway, and which have been implicated in macropinocytosis are listed in table 1.15. Nonetheless it is unclear how these different components are collectively organised to regulate the process of macropinocytosis.

1.4 Microglia and neurons interaction: Spine pruning and Synaptic Stripper

Recent findings challenge the concept that microglia solely function in disease states in the CNS. Rather than simply reacting to CNS injury,

infection, or pathology, emerging lines of evidence indicate that microglia sculpt the structure of the CNS, refine neuronal circuitry and network connectivity, and contribute to plasticity. These physiological functions of microglia in the normal CNS begin during development and persist into maturity. Furthermore, both *resting* and *activated* microglia (as defined by a morphological phenotype) have physiological functions even in the absence of pathologies. Consequently, the concept of resting and activated microglia is misleading because multiple phenotypic stages of these cells can influence the neuronal structure and function to maintain neural circuits.

In the absence of pathological insults, the highly dynamic motility of microglia is specifically targeted to synaptic structures as shown in figure 1.16.

In the somatosensory and visual cortex two-photon microscopy revealed that microglial processes make brief, repetitive contacts with synapses at a frequency of about one per hour. These interactions were visualized by in vivo two-photon imaging in the Iba-1-EGFP/Thy-1-GFP double-transgenic mice in which microglial cells and neuronal structures can be simultaneously visualized. Wake et al. in 2009 reported that the microglial processes appear in a close proximity to presynaptic boutons, where they remain for about 5 min and then retract. In the visual cortex, these structural interactions are activity-dependent, as their frequency was reduced by decreased neuronal activity following either binocular eye enucleation, injection of tetrodotoxin into both retinae or a reduction of body temperature [138]. By combining two-photon in vivo imaging with immunohistochemistry and three-dimensional reconstructions obtained from serial section electron micrographs as shown in figure 1.16B, a rather specific apposition of microglial processes to pre as well as postsynaptic compartments was found in the visual cortex of juvenile mice [139]. Process extrusions were typically associated with small and transiently growing dendritic spines. Again, neuronal activity modulated microglial behavior: light

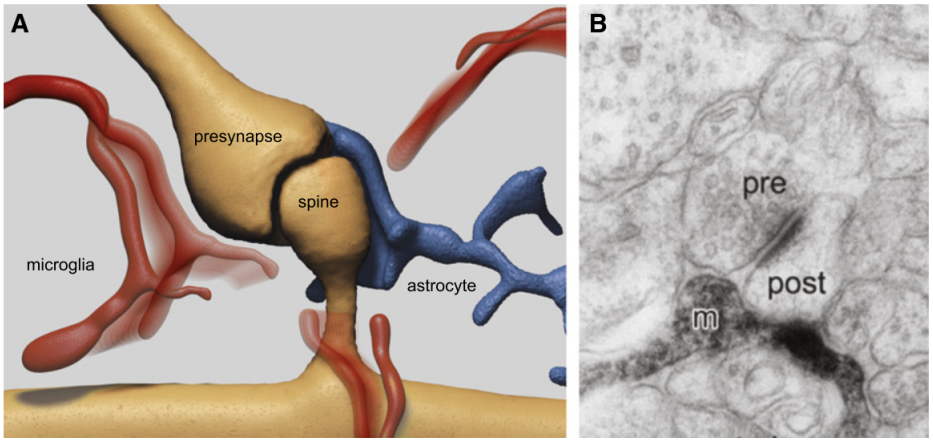

Figure 1.16: Dynamic Interaction of Microglial Processes with the Quadripartite Synapse: (A) Microglial processes (red) dynamically contact the cellular compartments of the tripartite synapse: pre- and postsynaptic neuronal terminals (in brown) as well as the enwrapping perisynaptic astroglial process (in blue). (B) The electron micrograph (EM) specifically shows a microglial process (m) contacting both the pre and postsynaptic compartment. The EM image is modified from Wake et al. (2009) [138].

deprivation reduced the motility of microglial processes and increased their association with larger dendritic spines. Re-exposure to light reversed these behaviors and microglial processes enwrapped synapses more extensively [139]. In response to neuronal activity, microglia steer their processes toward active synapses (as shown in Figure1.17A), which facilitates contact with highly active neurons. The mechanism of this steering of microglia to active synapses is currently not known.

Thus microglial cells respond to altered sensory experience and it remains an open question whether the interactions play any role

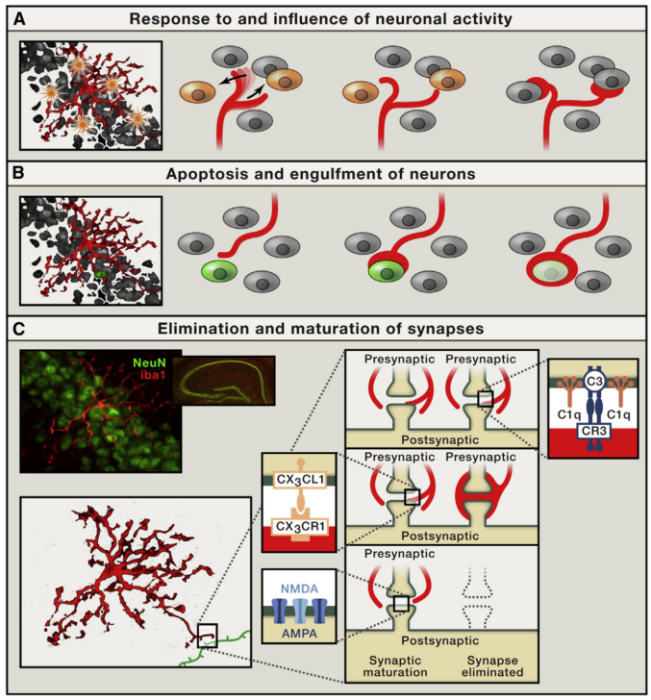

Figure 1.17: Microglia Control Neuronal Activity, Programmed Cell Death, and Synapse Connectivity. Taken from [24].

in experience-dependent modification or elimination of synapses, in the developing as well as in the adult CNS. The molecular cues that attract microglial processes to the synapses remain largely unknown. Considering these synapse modification or elimination that could be affected by microglia during experience-dependent plasticity, the term tripartite synapse was changed in quadripartite synapse, where pre-post synaptic neurons, astrocytes and microglia modulate the synaptic activity together.

Conceptually, microglial cells can affect neural networks either

through removal of cellular and subcellular elements (by phagocytosis) or through secreting various factors with transmitter, trophic or neuroprotective properties. The variety of neuroactive agents that can be secreted by microglia cells has been mainly studied in cell culture, an environment that triggers transformation of microglial cells into an activated state. Thus, these factors are considered to be representative of an activated state, with possible relevance to a more pathological activation. These include several types of cytokines (e.g., TNFα or ligands for receptors such as CCR1, CCR3, CCR5, and CCR7 and CXCR1 or CXCR3), trophic factors like brain derived neurotrophic factor (BDNF), the gaseous transmitter NO or neurotransmitters (ATP and glutamate) (for review, see Kettenmann et al. 2011 [28]). Some of these substances were reported to rapidly modulate neuronal function by changing excitability and synaptic strength. Moreover, several factors known from pathology can mediate the interactions of microglia with synapses in the healthy brain. Recent findings indicate that some of these pathological factors mediate neuron-microglia crosstalk in the developing or uninjured adult brain.

As described in the first chapter, the primitive myeloid progenitors originating from the extra-embryonic yolk sac enter the nervous tissue very early in embryonic development, being the first existing glial cells (as both astrogliogenesis and oligodendrogliogenesis occur later in a perinatal period) [29]. This initial migration coincides with the first wave of embryonic synaptogenesis (which occurs, in rodents, around embryonic day 14-15) that proceeds in the absence of astrocytes (which assist and are indispensable for postnatal formation of synapses). In this phase microglia can assist and even promote early synaptogenesis through secretion of growth factors [140].

Miyamoto et al. in 2016 [141] demonstrated how the microglia-neurons contact induces synapse formation in the developing somatosensory cortex. Using in vivo multiphoton imaging of layer 2/3 pyramidal neurons in the developing somatosensory cortex, they demonstrated

that microglial contact with dendrites directly induces filopodia formation. This filopodia formation occurs only around postnatal day 8-10, a period of intense synaptogenesis and when microglia have an activated phenotype. Filopodia formation is preceded by contact-induced Ca^{2+} transients and actin accumulation, as shown in figure 1.18 [141].

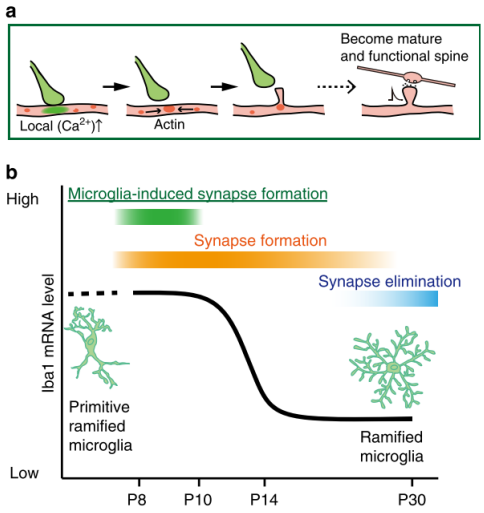

Figure 1.18: Scheme of microglia functions during synaptogenesis. (a) Sequence of proposed cellular events during synapse formation: Microglial contact initiates a rise in local $[Ca^{2+}]$i resulting in actin accumulation and filopodia formation. Some filopodia find presynaptic partners and mature into functional synapses. (b) Schematic graph depicting a change in microglia phenotype and function, from immature or activated microglia inducing filopodia formation and enhancing specific circuit synapse formation during early synaptogenesis, followed by a putative role in synapse elimination by more mature, quiescent microglia in the latter period of circuit formation [141].

Inhibition of microglia by genetic ablation decreases subsequent spine density, functional excitatory synapses and reduces the relative connectivity from layer 4 neurons [141] demonstrating how microglia cells are crucial elements to the spine formation.

During later, pre and postnatal development, microglial processes actively engulf synaptic structures and exert a major role in controlling the number of synapses through synaptic pruning. The chemokine fractalkine plays a role in chronic pain, inflammation, and Alzheimer's disease and it is released by neurons and endothelial cells [142] [143]. In the brain, fractalkine receptors are expressed specifically by microglia and fractalkine receptor-driven EGFP expression has become a reliable marker for identifying microglial cells [46]. In mice deficient for this receptor, there is a transient increase in the spine density during development, suggesting an underlying deficit in synaptic pruning. Depletion of fractalkine receptor also increased the frequency of miniature excitatory postsynaptic currents recorded in the hippocampus. This demonstrates that deficits in microglia function result in synaptic changes [3].

Cunningham and colleagues investigated the function of microglial cells in the developing cerebral cortex of prenatal and postnatal macaques and rats showing that microglia limit the production of cortical neurons by phagocytosing neural precursor cells. They show that microglia selectively colonize the primate sub ventricular zone (SVZ) and phagocytose neural precursor cells as neurogenesis nears completion. These data demonstrate that microglia play a fundamental role in regulating the size of the precursor cell pool in the developing cerebral cortex [144]. Paradoxically, eliminating microglia is found to increase apoptosis of lamina V cortical neurons [145].

As microglia are phagocytic, it has long been assumed that their primary role in CNS development is to engulf and clear the bodies of neurons that die as a result of programmed cell death, a mechanism that eliminates the excess of neurons generated during normal develop-

ment [146]. However, microglia are not simply reactive waste collectors in development, but rather, they are active neuronal killers, assassinating neurons inducing apoptosis (figure 1.17B). Several molecular mechanisms by which microglia can directly instruct neuronal apoptosis have been identified in different CNS regions: (1) the release of superoxide ions in the cerebellum [146], (2) the secretion of nerve growth factor in the retina [147], or (3) production of TNFα in the spinal cord [148]. In the hippocampus of neonatal mice, microglia-induced neuronal apoptosis is dependent upon complement receptor 3 CR3 [149] and upon other cell surface receptors that transduce *kill-me* and *eat-me* signals [150] expressed by neurons that are committed to die. Activation of these microglial cell surface receptors initiates intracellular downstream signaling through KARAP/DAP12, ultimately triggering the release of neurotoxic agents. The commitment of a particular neuron to die may be made cell autonomously. Alternatively, microglial cells may also participate actively in the induction of kill-me and eat-me signals in the neurons [146]. The quantity of neuronal precursors, as well as of differentiated neurons, are regulated by microglia [144].

Thus, it could be speculated that a microglial dysfunction or any factor that alters the number or activation state of microglia can profoundly affect neural development and could be responsible for developmental disorders.

Importantly the microglia physiological and pathological phagocytosis show morphological specificity: the phagocytosis of synaptic material or of apoptotic cells is performed by microglial processes or *en passant* branches forming *ball-and-chain* structures [151] without affecting the ramified microglial phenotype.

During development, CNS initially produces excessive numbers of neurons and neuronal axons typically make exuberant synaptic connections to more target neurons than those that are maintained in the fully developed nervous system. The projections are refined and

sculpted to the mature architecture as large numbers of synapses are eliminated. Synaptic *pruning* has long been known to be dependent on neuronal activity [152], but over the past decade, microglia have emerged as critical for this process (figure 1.17C). Indeed, in the cortex the peak of microglial density coincides with the peak of synaptogenesis [153]. Developmental synaptic pruning has been extensively investigated in the retinogeniculate system where the initial widespread and overlapping projections of retinal ganglion cells (RGCs) are progressively segregated into stereotyped eye-specific territories [150]. Pruning of inappropriate RGC synapses in the lateral geniculate nucleus (LGN) is achieved by microglial engulfment of the synapses, both pre- and postsynaptic elements, in a retinotopically appropriate manner. The phagocytosis of inappropriate synapses is lost in mice lacking complement receptor 3 (CR3), which is activated through the well-known classical complement cascade that begins with C1q and proceeds to opsonization of C3. In the CNS, CR3 is expressed only by microglia. Mice lacking C1q, CR3, or C3 have sustained defects in eye-specific segregation [154] [155], implicating complement-dependent signaling as necessary for the synaptic elimination.

In addition to synaptic pruning, microglia are required for proper maturation of excitatory synaptic transmission. In CA1 hippocampal pyramidal neurons, the frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) are increased in CX3CR1 null mice as compared with wild-type littermates around postnatal day 13 (P13) [3]. Mice lacking CX3CR1 show persistent alterations in excitatory transmission, but no differences have been found with inhibitory synaptic transmission [156].

The impairments caused by lack of CX3CR1 are suggestive of a role for microglia in maturation of synaptic functioning (Figure1.17 C).

Moreover, several studies indicate that microglial cells can influence synaptic plasticity. Examples include modulation of the NMDA

receptor glycine binding site by microglia [157], signaling by fractalkine and its receptor [3] [158], modulation of Cl- gradient in neurons through microglial BDNF release [159] and purinergic signaling [160]. However, the best documented role of microglia in controlling neural network functions comes from the analysis of the effects of the proinflammatory cytokine TNFα on synaptic connectivity.

Incubating hippocampal slices with TNFα increased the ratio of AMPAR- to NMDAR-mediated synaptic currents without affecting long-term potentiation (LTP) or long-term depression (LTD). By using cocultures of neurons and glia from wild-type or TNFα knockout mice, the source of TNFα was attributed to glia. Thus, by modulating TNFα levels, neuroglia actively participates in the homeostatic activity-dependent regulation of synaptic connectivity [161].

More recent data [162] demonstrate that the TNFα is exclusively generated by microglia, and not by the astrocytes. Microglial TNF-α and microglial ATP act in concert to trigger adjacent astrocytes to release ATP. Astroglial ATP subsequently amplifies the microglial signal and promotes astroglial release of glutamate that directly affects synaptic transmission through presynaptic metabotropic glutamate receptors [162]. These experiments performed in the hippocampus provide important insights into microglia role in modulating synaptic plasticity.

A similarly complex function of microglial ATP release, purinergic receptor activation, Ca2+ signaling, and subsequent glutamate release has been also described in zebrafish, though not in physiological conditions but during the onset of an acute injury [163].

Sipe and colleages demonstrate that microglia respond to monocular deprivation during the critical period, altering their morphology, motility and phagocytic behaviour as well as interactions with synapses. To explore the underlying mechanism, they focused on the P2Y12 purinergic receptor which is selectively expressed in non-activated microglia and mediates process motility during early injury

responses. They found that the disruption of this receptor alters the microglial response to monoculardeprivation and abrogates ocular dominance plasticity. These results suggest that microglia actively contribute to experience-dependent plasticity in the adolescent brain [2].

In different pathologies microglia acquire distinct functional states and, during the disease progression, microglial cells modify and change their activated phenotype and their interaction with neurons. Activated microglia specifically interact with neurons and influence their survival either in a positive or in a negative direction. Microglia can physically contact injured neurons and remove synapses, a process termed synaptic stripping: in some instances, entire dendritic trees are eliminated. During the activation state microglial cells release diverse substances such as reactive oxygen species, cytokines or growth factors, which can influence the pathological process during acute and chronic phases as well as during subsequent regeneration. This process was first recognized in the facial nerve injury model [164]. Lesion to the facial nerve (that carries axons of motoneurons located in the facial nucleus) triggers microglial activation, manifested by upregulation of cytokines, cell adhesion molecules, extracellular matrix proteins, transcription factors, and proteins of the major histocompatibility complex. Activated microglial cells release neurotrophic factors such as nerve growth factor (NGF), neurotrophin (NT)-4/5, transforming growth factor (TGF)-β1, glial-derived neurotrophic factor (GDNF), fibroblast growth factor (FGF), and interleukin (IL)-3, which affect neuronal survival [165]. Microglia-derived factors also include proinflammatory mediators such as tumor necrosis factor TNFα, IL-6, or nitric oxide (NO), which confer neurotoxicity. Microglial secretory activity can vary depending on the pathologic context [51]. The specific role of NGF release from activated microglia has not been investigated and totally understood, but it is discussed in more detail the chapter Microglia and Neurotrophins.

Conceptually, two types of signals control microglial behavior in response to injury: *find-me* signals, attracting microglial cells to the damaged site, and *eat-me* signals, allowing microglia to identify the target and trigger phagocytosis. Another classification distinguishes between *on signaling*, which includes factors newly appearing in the pathologic context or are upregulated, and *off signaling* which includes factors that disappear or are downregulated in pathology [166].

In the next chapters how microglia cells change their physiological functions into pathological situation will be described in more detail.

2 Microglia in the Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder of the elderly. It is characterized by progressive memory decline and cognitive dysfunction, manifested histologically by the parenchymal deposition of amyloid-beta (Aβ) plaques, the formation of neurofibrillary tangles in the brain (particularly in the hippocampus and cerebral cortex) and neuroinflammation [167] [168] [167] [168].

The senile plaques described by Alois Alzheimer in his original case report result from the abnormal extracellular accumulation and deposition of the Aβ; it is a fragment of a larger membrane-spanning glycoprotein called amyloid precursor protein (APP) [169]. The physiological function of APP protein in neurons is still unknown, probably it has some role in plasticity [170]. Aβ is generated through proteolytic processing of APP by β and γ secretase. The predominant forms of Aβ in AD are 40 and 42 aminoacid peptides that undergo conformational changes leading to the formation of intracellular and extracellular fibrils [169] (see figure 1.19). Because of its higher rate of oligomerization, Aβ 42 is more toxic than Aβ40. The cellular components of the senile plaque include dystrophic neurites, astrocytes and activated microglia [171].

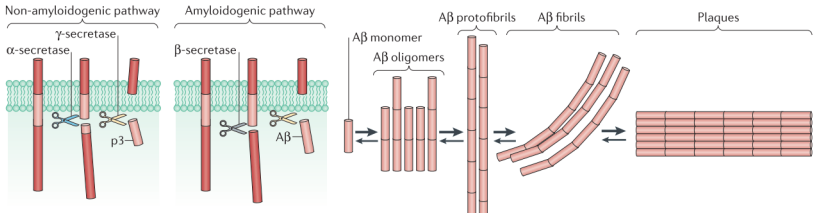

Figure 1.19: The plaque formation: The increase in production and/or reduced clearance of Aβ, which is derived from the β-amyloid precursor protein (APP), is thought to be a central event in Alzheimer disease (AD). Cleavage of APP occurs either in a non-amyloidogenic (physiological) or in an amyloidogenic (pathological) fashion; only the latter results in the production of Aβ. In the non-amyloidogenic pathway, APP is cleaved first by α-secretase and then by γ-secretase, whereas in the amyloidogenic pathway, γ-secretase cleavage of APP is preceded by β-secretase cleavage, releasing Aβ into the extracellular compartment. The cleavage site used by γ-secretase in the amyloidogenic pathway determines whether the predominant Aβ40 or the more aggregation-prone and neurotoxic Aβ42 species of the peptide is generated. Aβ monomers may then go on to form oligomers or other arrays, depending on mutations in the Aβ coding region of APP and post-translational modificationsThe arrow thickness indicates the likelihood of conversion of Aβ species or arrays [76].

Plaque-associated neuritic dystrophies represent the most notorious evidence of Aβ-induced neurotoxicity and feature many of the pathophysiological processes downstream Aβ.

The neurofibrillary tangles (NFTs) were first described by Alois Alzheimer in his original autopsy case report as intraneuronal filamentous inclusions within the perikaryal region of pyramidal neurons.

NFTs (present in neuronal cell bodies) and neuropil threads (present

in neuronal processes) are intracellular structures composed predominantly of a hyperphosphorylated, aggregated form of the microtubule binding protein, tau [172] [173] [174]. Tau is synthesized and produced in all neurons and is also present in glia. The normal function of tau is to bind to tubulin and stabilize microtubules. However, in AD, tau becomes hyperphosphorylated and this form of tau dissociates from microtubules and has a tendency to self-aggregate, thus forming NFTs in cell bodies and dystrophic neurites. In summary, the major constituent of NFTs is the microtubule-associated protein tau, which is aberrantly misfolded and abnormally hyperphosphorylated. Invariably accompanying NFTs are the neuropil threads, which are thought to result from the breakdown of dendrites and axons of the tangle-bearing neurons.

In addition to plaques and tangles, other neuropathological and neurochemical hallmarks of AD include loss of synapses and selective neuronal cell death as well as decreases in markers for certain neurotransmitters. Neurons that are particularly vulnerable in AD include those in layer II of the entorhinal cortex, the pyramidal layers (e.g. CA1) of the hippocampus, and certain areas of the temporal, parietal, and frontal neocortex [168]. Although the majority of the vulnerable neurons use glutamate, the major neurotransmitter in the brain, there is also loss/dysfunction of certain subcortical projection neurons such as basal forebrain cholinergic neurons and noradrenergic neurons in the locus ceruleous. The dysfunction of cholinergic neurons [175] [176], which are involved in attention and memory, has been the basis for cholinergic therapy in AD.

It is important to note that much of the cognitive dysfunction in AD is not due to loss of one neurotransmitter but rather to disruption of network connections between several key brain regions within the limbic system and specific areas of the neocortex [177] [178].

Early neurochemical analyses of AD brain tissue revealed that the enzymes that generate and metabolize acetylcholine are substantially

depleted [176]. This finding fits well with the fact that the defining lesions of AD -the Aβ-containing neuritic plaques and the tau-containing neurofibrillary tangles- are present in septal hippocampal and basal forebrain-neocortical pathways that are cholinergic, and with the fact that frank cell loss is observed in the projection neurons of these pathways [179]. Although deficits in numerous neurotransmitters (including corticotropinreleasing factor, somatostatin, GABA, and serotonin) accrue as the disease progresses, the early symptoms appear to correlate with dysfunction of cholinergic and glutamatergic synapses. Morphometric study of temporal and frontal cortical biopsies, performed within an average of 2 to 4 years of the onset of clinical AD [180], revealed a 25 to 35% decrease in the numerical density of synapses in biopsied AD cortex, and a 15 to 35 % decrease in the number of synapses per cortical neuron. Even at the end of the disease, quantitative correlations of postmortem cytopathology with premortem cognitive deficits indicate that the latter is more correlated with synapse loss than with the numbers of plaques or tangles, degree of neuronal perikaryal loss, or extent of cortical gliosis [181].

Also in mice model the comparisons of transgenic lines having varying APP expression suggest that decreases in presynaptic terminals are critically dependent on cortical Aβ levels, not on Aβ plaque burden or APP levels [182]. Coherently with this finding, presynaptic terminals are already significantly depleted in 2- to 4-month-old APP transgenic mice as their soluble Aβ- levels rise, but before Aβ deposition (i.e., plaque formation) begins. This animal work fits nicely with growing evidence that memory and cognitive deficits in MCI and AD patients correlate far better with cortical Aβ levels than with plaque numbers [183] and correlate best with the soluble pool of cortical Aβ, which includes soluble oligomers [184] [185]. Even in very mildly impaired patients, soluble Aβ levels in the cortex show a significant correlation with degree of synaptic loss [185].

Moreover, several electrophysiological studies of young mice trans-

genic for human APP with AD-causing mutations have revealed significant deficits in basal synaptic transmission and/or long-term potentiation (LTP, an electrophysiological correlate of synaptic plasticity) in the hippocampus, well before the development of microscopically detectable Aβ deposits.

Another important element in AD pathology is represented by ϵ4 APOE allele on chromosome 19. It is the strongest genetic risk factor for developing Alzheimer's disease. APOE is involved in the normal catabolism of triglyceride-rich lipoproteins. One of the first reports linking APOE to Alzheimer's disease pathology was APOE immunoreactivity in Aβ deposits and neurofibrillary tangles which, as described before, are hallmarks of Alzheimer's disease pathology [186]. In addition, polymorphisms in the transcriptional regulatory region of APOE have been associated with Alzheimer's disease [187]. APOE is a 299 aminoacid protein that has three common isoforms in humans that only differ by 1 or 2 aminoacids: APOE2 (Cys112 and Cys158), APOE3 (Cys112 and Arg158) and APOE4 (Arg112 and Arg158).

The aminoacid substitutions affect the total charge and structure of APOE, thereby altering binding to both cellular receptors and lipoprotein particles, and possibly changing the stability and rate of production and clearance. APOE has high expression in the brain, where it is produced primarily by astrocytes and microglia. Under certain conditions, some APOE production can occur in neurons [188].

Population studies have demonstrated that APOE4 increases the risk of developing Alzheimer's disease (one allele imparts a threefold increase in risk and two alleles impart a 12-fold increase in risk) [189] and is also associated with an earlier age of Alzheimer's disease onset [190].

Many studies using cell culture and transgenic animals have investigated the potential mechanisms by which APOE4 may contribute to Alzheimer's disease. These include studies investigating the role that APOE4 has as a pathological chaperone for Aβ, thus affecting the

clearance and deposition of Aβ and ultimately contributing to plaque formation [191] [192]. Other studies investigate alterations in tau phosphorylation, in neurofibrillary tangle formation [193] [194] and in lipid metabolism causing inhibition of neurite extension [195]. The APOE4 isoform does not alter Aβ synthesis [192], but it can dramatically increase Aβ deposition in animal models of Alzheimer's disease [191]. In accordance, in ApoE knock-in mice, clearance of Aβ40 from the CNS to plasma is inhibited in an allele-specific manner (ApoE4>ApoE3 or ApoE2), demonstrating the role of APOE in the clearance of Aβ; the capacity of which depends on the isoform [196].

Collectively, these studies indicate that APOE is a contributing factor for Alzheimer's disease by acting as a chaperone for Aβ, which affects the clearance and deposition of Aβ, ultimately contributing to plaque formation. Any profound change in APOE production and clearance, aspects that are affected differently in the various isoforms, are likely to have a large effect on Aβ deposition and Alzheimer's disease pathogenesis. Moreover, as described APOE is produced primarily by astrocytes and microglia, pointing to a central role of these cells in the AD etiopathology.

The contribution of microglia in Alzheimer's disease etiopathology will be discussed in the next chapter, with special attention to the multiple role of these cells and how the inflammation mediated by microglia is crucial for the disease.

In this very complex system, who is the first effector of this disease? Or better, is there one principal effector for this disease?

There are several hypotheses that could explain the onset of Alzheimer's disease but they will not be discussed into more detail in this thesis.

2.1 Microglia Activation: β-Amiloide and Inflammation

In this section, the important roles of β amiloide and inflammation on microglia activity are discussed due to their important effect for AD pathology.

A lot of literature describe the inflammation as a part of the causal role in Alzheimer's disease pathogenesis, and understanding and control of interactions between the immune system and the nervous system might be a key for the prevention or delay of most late-onset CNS diseases.

In fact, AD pathology is also characterized by an escalating inflammatory response, which is primarily driven by microglia. For more than a decade, there have been data indicating that the immune system may have a role in AD. However, the importance of inflammation to AD pathogenesis has only very recently been appreciated, and inflammation is now thought to contribute to and exacerbate AD pathology [197] [198] [199] [200] [201] [202].

Neuroinflammation was assumed to occur only at late to end stages of AD and possibly to represent merely an epiphenomenon. In particular, glial cell activation was thought to accompany but not significantly contribute to amyloid pathology [202]. However, the spectrum of glial cell actions and other immunerelated changes in AD had not been fully dissected, and it is still far from being well understood. In the last years, preclinical, genetic and bioinformatic data have shown that activation of the immune system accompanies AD pathology and contributes to the pathogenesis of this disease [203]. The identification of associations between AD and mutations in genes encoding triggering receptor expressed on myeloid cells 2 (TREM2) [204] [205] and myeloid cell surface antigen CD33 [206] proved to be conceptually powerful, as it was the first time that the link between immune alterations and AD pathogenesis was supported beyond the purely descriptive level.

Correlative analyses of the clinical symptoms that precede AD (namely mild cognitive impairment (MCI)) and the presence of inflammatory changes (for example, in the cerebrospinal fluid (CSF)) have indicated a much earlier involvement of the immune system [207] [208]. Moreover, one study [209] showed that systemic immune challenge triggered and drove the development of AD-like neuropathology comprising Aβ plaques, tau aggregation, microglia activation and reactive gliosis in wild-type mice, suggesting that immune actions can precede AD-like pathology and are sufficient to cause it. The modulation of the neurodegenerative disease courses by specific immune molecules in pre-clinical experimental approaches and the upregulation of inflammatory genes in arrays on tissues derived from patients with degenerative CNS diseases also point to a relationship between inflammation and neurodegenerative disorders (including AD), and implicate immune actions early in the pathogenic process [198] [200] [210] [211] [212] [213]. These observations imply that immune processes may at least at a given time point drive AD pathology independently of Aβ deposition and sustain increased Aβ levels, thus exacerbating pathology and culminating in a vicious, pathophysiological cycle, see figure 1.20; suggesting a very crucial role in the pathology for the mediators of inflammation, as microglia cells.

Indeed, it was demonstrated that the microglia numbers increase in the AD brain [214]. Nowadays it is well known that microglia in the AD brain are located around plaques. The evidence for the presence of microglia in senile plaques derives from immunohistochemical studies that examined also the brains of AD patients [215]. In contrast to normal brains where microglia are distributed uniformly throughout the gray and white matter [215], in AD brains microglia are clustered in and around Aβ deposits [214]. Also the electron microscopy studies have revealed that microglia are closely apposed to Aβ [216]. In some instances, microglia contain intracellular deposits of Aβ, indicating a possible role for these cells in the clearance and/or processing of

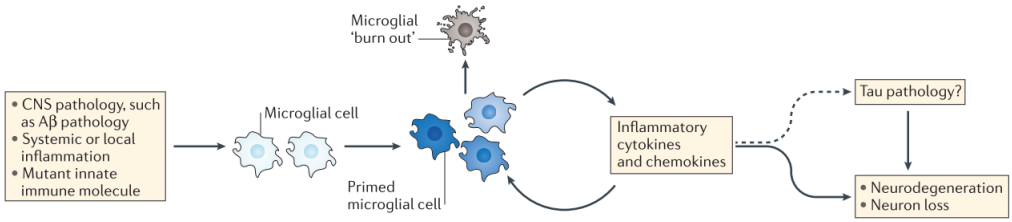

Figure 1.20: The presence of Aβ can *prime* microglial cells; namely, Aβ makes these cells susceptible to a secondary stimulus and/or promotes their activation. Priming results in various functional microglia phenotypes (indicated by different colours), presumably accompanied with no or only minor morphological alterations and/or no (major) cell-surface marker differences. In AD, Aβ sustains chronic activation of primed microglia, which results in a constant production of inflammatory cytokines and chemokines; the cytokines and chemokines maintain activation of the primed cells. This process results in a vicious circle, which ultimately impairs microglia; moreover, it affects surrounding CNS resident cells (astrocytes, oligodendrocytes and neurons), possibly aggravating tau pathology, and finally causing neurodegeneration and neuron loss. If these processes perpetuate over a prolonged period, it forces microglia into a senescent, *burn-out*-like (dystrophic) phenotype which is thought to be irreversible [76].

Aβ [217], as discussed in the next section. Similar to their association with AD lesions in humans, microglia cluster around Aβ deposits in the brains of transgenic mice with AD-like pathology. Quantitative analysis of microglia as a function of distance from the center of senile plaques in transgenic mice revealed a 2- to 5-fold increase in microglia numbers in areas with plaques compared with neighboring regions [218]. These studies in transgenic mice confirm that microglia

accumulation in senile plaques is an integral part of the disease process in AD and that microglia might have a role in the pathogenesis of AD.

Moreover, microglia in senile plaques are activated as a result of their interactions with Aβ. In AD brains, microglia associated with senile plaques express several markers of microglia activation. When compared with microglia in normal brains or those in AD brains not interacting with Aβ, senile plaque associated with microglia selectively expressed the MHC class II molecule HLA-DR, a marker for mononuclear phagocyte activation [215].

The appearance of amyloid plaques in the brain coincides with a dramatic phenotypic activation of the surrounding microglia which also display increased immunoreactivity as CD11b, CD68, complement receptor 3 and CD45 [219]. Postmortem brains from AD patients, as well as brains from APP transgenic animals, display increased levels of inflammatory cytokines as well as chemokines including IFNγ and TNFα, IL-1β, IL-6, TGF-β, a peptide growth factor and chemokines, macrophage inflammatory proteins -1α, -1β, -2, and most prominently CCL2/monocyte chemotactic protein 1 [220] [221] [221] [214] [222].

Interaction of microglia with Aβ also leads to secretion of chemokines, such as monocyte chemotactic protein-1 (MCP-1, also known as CCL2) [223] and induction of mRNA for several additional chemokines, including CCL4 and CXCL2 [223]. Aβ-stimulated microglia also produce reactive oxygen species (ROS) and reactive nitrogen species. These cells also elaborate a number of other immune mediators including macrophage colony stimulating factor and the complement protein, C1q [224].

As in other pathological conditions, resident microglia in AD have been shown to produce either inducible nitric oxide synthase (NOS2) or arginase, as described in the chapter 1.3.1. The NOS2 expression and arginase production were suggested to be marker of M1 and of M2 phenotypes respectively [225].

Moreover, microglia produce a large diversity of mediators, some

of which may be attractive targets for modulating neuroinflammation in AD and thus, potentially, for ameliorating the disease course.

A study by Shaftel et al. [226] took an alternative approach and examined the effects of sustained inflammatory signaling by over-expressing the pro-inflammatory cytokine IL-1β in astrocytes. The sustained IL-1β activation, which was expected to have detrimental effects, resulted in a significant decrease in amyloid deposition in the hippocampus of these mice. They found that IL-1β over-expression led to an increase in the number of activated plaque-associated microglia that were heterogeneous in their phenotype and were positive for either Iba1 or both Iba1 and MHCII and this phenotypic diversity arose from the IL-1β-stimulated recruitment of blood-borne leukocytes into the brain [226]. Similarly, in another study conducted by Chakrabarty et al. [227], in which the pro-inflammatory cytokine IL-6 was over-expressed in the brain of murine models of AD using an adenoviral vector, it was found that amyloid deposition was attenuated in these animals. IL-6 expression resulted in a dramatic increase in gliogenesis that was associated with enhanced microglial phagocytosis of Aβ, without affecting APP expression or processing [227]. From these studies we can conclude that while inflammation has been shown to be detrimental to the brain, some components of this system may be involved in amelioration of AD-related pathology.

In studies in transgenic mouse models of AD, TNFα6 released by microglia in response to Aβ was triggered by an interaction of CD40 with CD40L or by TLR4 engagement [228] [229]. Besides the production of inflammatory mediators due to binding of Aβ to various microglia receptors, Aβ has been shown to be cleared by microglia in vitro through receptor-mediated phagocytosis and degradation [197] as described in the next section. In some other studies in animal model of AD the transient depletion of microglia that have acquired a dysfunctional phenotype has no impact on Aβ burden [230]. Microglial impairment might paradoxically be sustained by inflammatory cytokines

such as TNFα, IL-1, IL-12 and IL-23 [231] [232] [233]. This idea suggests that AD pathology could be accelerated through this negative feedback loop. Therefore, the idea of neuroinflammation in AD goes beyond the degree of mere alterations in microglial morphology and instead it implies changes in the phenotype and function of these cells, with relevant consequences. Rare structural variants of genes encoding the immune receptors TREM2 [203] [204] [234] [235], CD33 [206] [236] and CR1 [237], all of which are expressed on microglia and other myeloid cells, have been found to be associated with a higher risk of AD. These findings support the concept of altered microglial function in AD.

In particular, TYROBP/DAP12 the gene encoding the TREM2 protein described in the previous chapter 1.3.2, was found to be strongly associated with the pathophysiology of late onset AD (LOAD) as a key network regulator [203].

Several rare variants of TREM2 in the heterozygous state have recently been identified by exome sequencing to increase the risk of late onset Alzheimer disease (LOAD) [238] [205]. This risk factor is nothing but negligible. In fact, one particular TREM2 variant, R47H, though not so frequent in the population, has an effect on individual risk of developing AD comparable with the apolipoprotein $\epsilon 4$ allele [205] [204].TREM2 was also found to be up-regulated in microglia of amyloid precursor protein transgenic mouse models [234]. Moreover, in post-mortem temporal cortices of AD patients, intense TREM2 immunoreactivity was observed in the microglia associated with amyloid plaques and in neuritic pathology-enriched areas [239].

In addition, in the recent publication of Philip W.Brownjohn and colleagues [240] about microglia derived from human stem cells, provides tool for understanding microglial biology. These cells are phenotypically and functionally comparable with primary microglia. The authors studied the consequences of missense mutations of TREM2 protein and they found that mutant TREM2 accumulated in its imma-

ture form, does not undergo typical proteolysis, and is not trafficked to the plasma membrane. However, in the absence of plasma membrane TREM2, microglia differentiate normally, respond to stimulation with lipopolysaccharide and they are phagocytically competent.

Aβ itself has been shown to have proinflammatory properties: microglia surrounding Aβ plaques show elevated production of inflammatory factors [241]. The inflammatory nature of amyloid has been recognized as a potential mechanism of disease progression. Interestingly, inflammation can promote accumulation of Aβ by elevating APP levels and the activity of cleavage enzymes [242].

The literature to date highlights the link between Alzheimer's disease and inflammation with a rather defined involvement also by microglia cells, modulators of the inflammation itself, but what was really interesting were the results coming out our laboratory.

Indeed in our laboratory it was developed the mouse model AD11 that produces specific antibodies against the mature murine form of NGF, αD11 [243] [244]. αD11 is a monoclonal antibody, produced in rat, that has the ability of effectively neutralize NGF both in vivo [245] [18] and in vitro [246] [245].

The AD11 mouse shows loss of BFCNs and presents a form of neurodegeneration very similar to AD with amyloid plaques derived from the endogenous APP gene, hyperphosphorilated tau, NFTs, loss in synaptic plasticity and memory [17] [247] [248]. The AD11 mice display a comprehensive neurodegeneration that is reminiscent of the sporadic forms of AD. In our laboratory it was also demonstrated changes in global gene expression, these were assessed by microarray mRNA analysis plus RT-PCR validation, and surprisingly, interesting differences could be detected already at P30 mice, when no overt pathology is present [20].

Moreover, the most significant differentially regulated mRNAs cluster in three families: Wnt signaling, synaptic neurotransmission and inflammation/immune response.

Due to these evidences, it was crucial for me trying to understand if in addition to inflammation response, microglia in this mouse model was damaged in the early stages as well and whether these alterations were caused by neurotrophin deprivation or not, as it was important to understand the role played by the NGF neurotrophin on microglia neuroinflammation to understand if NGF could somehow aggravate or improve the inflammation activated by Aβ.

But before start to describe the results of this thesis, it is important to focus on the other microglia activities as microglia's Aβ clearance and the relationship between microglia and neurotrophins.

2.1.1 Clearance of toxic peptides: Beneficial or Detrimental role of Microglia

In this chapter I will describe the different types of mechanisms used by microglia to clear Aβ peptides and how different microglia inflammatory phenotype can modify its activities, thus making the role of microglia not always clearly protective. The beneficial or detrimental microglia role in the Alzheimer's Disease is still discussed in litterature.

There are two principal mechanisms for removal of Aβ from the brain: efflux of intact soluble Aβ (sAβ) to the peripheral circulation and proteolytic degradation of both soluble Aβ and fibrillar forms of Aβ (fAβ). The efflux of sAβ can occur through a number of different routes, including efflux across the blood-brain-barrier (BBB) into the circulation mediated by LRP1 (low-density lipoprotein receptor-related protein 1), the bulk flow of interstitial fluid (ISF)/cerebrospinal fluid (CSF) into the lymphatic system and transport via the P- glycoprotein (PgP) efflux pump across the BBB. The efflux of Aβ through these mechanisms has been postulated to be facilitated or inhibited by its binding to chaperone proteins, such as ApoE, ApoJ, α2-macroglobulin, transthyretin and albumin [249] [250] [251] [252]. A growing body of

studies about Aβ clearance has demonstrated that Aβ interacts with immune cells through both innate and antibody-mediated adaptive immune responses.

The endocytosis encompasses three primary mechanisms: phagocytosis, receptor-mediated endocytosis and pinocytosis. Phagocytosis and receptor-mediated endocytosis of Aβ are described in the chapters above 1.3.2, the former involves the uptake of large particles whose internalization is stimulated through its interaction with cell surface receptors, engaging the cell's phagocytic machinery [253] and the second, the receptor-mediated endocytosis, is a mechanistically distinct process, elicited by ligand binding to a receptor on the cell surface, resulting in the internalization of the receptor and its ligand within clathrin-coated or uncoated vesicles [254] [255] [256].

The pinocytosis can occur through two separate pathways, micropinocytosis or macropinocytosis, and it is typically associated with the uptake of solutes from the extracellular medium as described in the previously chapters 1.3.2.

The mechanism by which soluble or small oligomeric forms of Aβ are removed from the environment, was well demonstrated by Mandrekar et al in 2009 [257].

It was demonstrated that macropinocytic uptake of sAβ and its subsequent proteolytic degradation represents a significant mechanism mediating Aβ clearance from the extracellular milieu. Although internalization of the soluble peptide is not limited to microglia, but these cells are the most efficient in the endocytosis of the peptide in vitro. Microglia internalize sAβ from the extracellular milieu through a nonsaturable, fluid phase macropinocytic mechanism that is distinct from phagocytosis and receptor-mediated endocytosis both in vitro and in vivo. The uptake of sAβ is dependent on both actin and tubulin dynamics and it does not involve clathrin assembly, coated vesicles or membrane cholesterol. Upon internalization, fluorescently labeled sAβ colocalizes to pinocytic vesicles. Microglia rapidly traffic these sol-

uble peptides into late endolysosomal compartments where they are subject to degradation. Additionally, it was demonstrated that the uptake of sAβ and fAβ occurs largely through distinct mechanisms and upon internalization the peptides are segregated into separate subcellular vesicular compartments. Remarkably, it was found that upon proteolytic degradation of fluorescently labeled sAβ, the fluorescent chromophore is retained by the microglial cell. These studies identify an important mechanism through which microglial cells participate in the maintenance of Aβ homeostasis, through their capacity to constitutively clear sAβ peptides from the brain [257].

Engulfment of fAβ by microglia through receptor-mediated phagocytosis and its targeting to the endosome lysosomal pathway have been investigated in detail [217] [218] [261] [262]. Microglia are able to engulf and phagocyte fAβ readily; the question of whether fAβ can be degraded intracellularly remains controversial. Early studies showed that primary mouse microglia release fAβ after they have internalized it [263]. Paresce et al., found that microglia retain fAβ for a period of weeks without degrading the peptides [264]. A subsequent study conducted by Majumdar et al., suggested that microglia have to be activated to enhance their ability to degrade fAβ, as microglia in a nonactivated state were unable to degrade fAβ. However, stimulating microglia with macrophage colony-stimulating factor (M-CSF) enabled them to degrade fAβ efficiently through acidification of lysosomes [265]. Microglia directly interact with and ingest fAβ via an ensemble of cell surface receptors, including pattern recognition receptors (PRRs). PRRs, and most prominently the Toll like receptors (TLRs), are commonly used by the innate immune system to identify pathogen-associated molecular patterns (PAMPs) of bacteria and viruses. The cell surface receptor complex of fAβ (SR-A), the class B scavenger receptors CD36 consists of class A scavenger receptor $\alpha 6\beta 1$ integrin, CD14, CD47 and TLR2, TLR4, TLR6 and TLR9 [266] [262] [267] [268]. Upon binding of fAβ, the receptor ensemble initiates the

activation of intracellular signaling cascades leading to the induction of phagocytic activity by microglia. SRs were the first receptors reported to be involved in fAβ uptake. Paresce et al., demonstrated that microglial uptake of fAβ microaggregates was reduced by competitive ligands for SRs [262]. A study conducted by Hickman et al. reported that the microglial mRNA levels of SR-A, CD36 and RAGE were progressively and significantly reduced as mice aged [269]. This suggests that the advance of AD pathogenesis may result from the decreased ability of microglia to clear Aβ. TLRs and their coactivator CD14 were shown to stimulate the phagocytosis of fAβ. Activation of TLRs (TLR2, TLR4 or TLR9) with their specific ligands significantly enhanced the uptake of fAβ by clonal BV-2 microglial and primary microglia. However, microglia carrying defective TLR4 were less efficient than wild-type microglia in their ability to take up fAβ after being stimulated by lipopolysaccharide (LPS). The results suggest that TLR signaling stimulates microglial phagocytosis of fAβ. Interestingly, fAβ itself activates microglia and induces their phagocytic activity through TLR signaling [270] [266] [267]. Blocking TLRs signaling by interfering with receptor-ligand interaction or their downstream effectors also reduced fAβ - induced phagocytosis and signaling [270] [266]. Microglia deficient in TLR2, TLR4 or their coreceptor CD14 failed to induce phagocytosis in response to fAβ stimulation [267]. These results highlight the key function of these PRRs in fAβ uptake and their stimulation of phagocytosis. Moreover, this process is thought to be part of the inflammatory reaction in Alzheimer's disease, the binding of Aβ with CD36, TLR4, and TLR6 results in activation of microglia, which start to produce proinflammatory cytokines and chemokines as shown in figure 1.21.

As said one way to clear Aβ is via phagocytosis and degradation by resident CNS immune cells, such as microglia [114].

This particular clearance pathway shows microglia not only as the primary source of inflammatory factors, but also as a crucial element

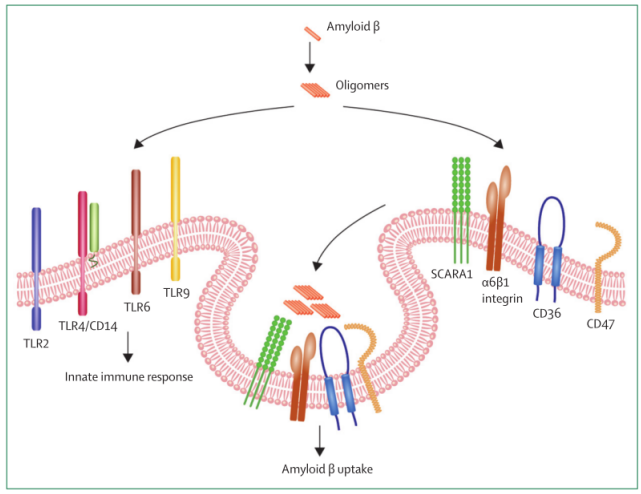

Figure 1.21: Activation of microglia by Amyloid-β: Aβ aggregates (oligomers) act on several Toll-like receptors on the microglial surface, triggering reactions of the innate immune system, including production of pro-inflammatory cytokines and chemokines. Aβ oligomers are internalised by microglia, aided by SCARA1, $\alpha 6\beta 1$ integrins, CD36, and CD47 [171].

for removal of harmful material in the CNS [114]. Thus, the failure of microglia to carry out homeostatic functions possibly underscores one mechanism of increased Aβ accumulation during disease.

It was demonstrated how the different microglia inflammatory phenotype change microglia ability to phagocyte Aβ peptides: for example, in vitro treatment of microglia with the pro M1 activator lipopolysaccharide inhibited microglial phagocytosis of Aβ [270]. Other proinflammatory cytokines, such as IFNγ and TNFα not only inhibited uptake of Aβ, but also prevented internalized Aβ degradation [271]. This demonstrates that M1 microglia might be less able to

properly take up and degrade Aβ. While M1 microglia appear to be impaired in their ability to remove Aβ, M2 microglia have been demonstrated to be efficient phagocytes. Treatment with the pro M2 activating cytokine IL-4 can effectively block lipopolysaccharide induced inhibition of Aβ phagocytosis [270] and similar data have been obtained using IL-10 [270]. This effect also extends to degradation of the internalized Aβ. These studies showing that inflammation limits the phagocytic potential of microglia were performed in vitro [270], but they have been extended in vivo. In the 6-month old APP/PS1 Alzheimer's disease mouse model Jimenez et al. [272] observed that when Aβ begins to accumulate there were YM1+ cells present in the CNS. However, by 18 months of age YM1 mRNA levels decreased and there was a massive upregulation in inflammatory factors, suggesting a switch from M2 to M1 as pathology worsened [272]. This is consistent with the idea that microglia become less responsive to M2 induction signals as they age, perhaps due to an age-associated decrease in IL-4R levels. Correspondingly, in older, non-diseased mice there is downregulation of receptors associated with Aβ engulfment, such as scavenger receptor A, the Aβ degradation enzymes neprilysin (Nep), insulin degrading enzyme (IDE), and matrix metallopeptidase 9 (MMP-9) [269]. These observations suggest that the Aβ induced inflammatory environment, combined with age-associated effects on microglia, leads to a situation where M1 cells predominate and microglia lose the ability to switch phenotypes and mitigate damage. Several groups have utilized animal models of Alzheimer's disease to demonstrate that altering microglial activation state can be beneficial.

It was also demonstrated that IFNγ and TNFα not only have toxic effects on neurons but they also reduce levels of insulin degrading enzyme, a key Aβ degrading protease. This may be a secondary mechanism through which inflammation could increase amyloid deposition. Furthermore, both TNFα and INFγ have been shown to increase the production of Aβ from APP expressing cortical neurons as well as

impair the ability of microglial cells to degrade Aβ [214].

The generation of mouse models lacking inflammatory signaling elements has provided insight into the role of inflammation in AD pathogenesis and Aβ clearance in vivo. AD transgenic mouse models lacking expression of either the IFNγ receptor type 1 or TNF type 1 receptor display significant decreases in amyloid deposition as well as microglial activation [273] [274]. Deletion of TNF type 1 receptor also alleviated Aβ-associated cognitive deficits [275]. However, it cannot be concluded that inhibition of cytokine signaling promoted microglial clearance of Aβ in the animal models since both these studies documented decreases in beta-site APP-cleaving enzyme 1 activity as well. Since the deletion of these receptors is global it is difficult to assess their specific contribution in the inflammatory response and clearance of Aβ in microglia [275] [276].

The emerging role of microglia activation in Alzheimer's disease pathogenesis makes these cells a legitimate therapeutic target. However, depending on the circumstances, microglia activation can have both beneficial and detrimental effects.

As discussed above, the phagocytic activity of microglia is attenuated by proinflammatory cytokines [270], indicating that microglia committed to an inflammatory response may have a lower phagocytotic capacity. In studies with anti-inflammatory drugs, suppression of the inflammatory response by microglia attenuates symptoms in a mouse model of Alzheimer's disease [51].

On the other hand, Schenk et al., reported that active immunization to Aβ in a mouse model of AD prevented plaque formation in younger animals and reduced the plaque burden and associated neuropathy in animals with established plaque pathology [277]. Introduction of anti-Aβ antibodies directly into the brain or peripherally resulted in a robust phagocytic response of microglia and consequently the dissolution of Aβ deposition in the brain [278] [279]. These data argue that the uptake and degradation of Aβ by microglia were a re-

sult of the Aβ-antibody complex interaction with Fc receptors (FcR) which stimulated the phagocytic uptake of Aβ.

However, the role of endogenous anti-Aβ antibodies in AD pathogenesis remains unclear. Indeed, the relative levels of anti-Aβ antibodies in AD patients and healthy individuals are highly variable. The complement system has also been reported to be involved Aβ clearance [280] [281]. Fibrillar Aβ is a strong stimulator of the complement system and can activate the classical (antibody-dependent) pathway by binding Clq and the alternative (antibody- independent) pathway by binding C3b [282] [283] [284] [280] [285]. Upon opsonization of Aβ by complement, microglia elicited more aggressive phagocytosis via complement receptors (1.5-fold increase over fAβ alone) [286] [281] [287]. Interestingly, the association of the complement component C1q with Aβ inhibited microglial phagocytosis [288]. Thus, C1q may have opposing effects on ingestion of Aβ via the SR- and FcR-mediated pathways, inhibiting naked Aβ uptake and enhancing the Aβ immune complex uptake. The study conducted by Maier et al. demonstrated that complement C3 deficiency in APP mice resulted in elevated cerebral Aβ levels and amyloid plaque burden. The authors also noticed that the activation status of microglia was switched from the classical activation M1 state to the alternative activation M2 state, suggesting the important role of complement system in Aβ clearance and microglia activation [289].

Nevertheless, the role of microglia in AD is not clearly established. There are studies indicating that microglia promote disease, whereas other studies indicate that they are protective. In vitro data demonstrate that activated microglia can cause neuronal damage. When microglia or monocytes are incubated with neurons in culture [290], or with organotypic cultures of rat brains [291], a significant increase in the ratio of dead to live neurons was detected in cultures containing Aβ-stimulated monocytes or microglia compared with cultures containing monocytes or microglia stimulated with control peptides. In

some studies, the presence of microglia was required for Aβ-associated neuronal damage to occur [290]. When microglia were not included in the culture, Aβ alone had no significant effects on neuronal survival [290]: these data are crucial to understand the central role of microglia in Alzheimer's Disease damages progression. These studies indicate that microglia recruited to senile plaques might play a part in Aβ-induced neuronal damage in AD. By contrast, there is also increasing in vivo evidence indicating that accumulation of microglia in AD might be protective and promote Aβ clearance. It was found that accumulation of blood-derived microglia before formation of visible Aβ deposits promotes Aβ clearance. Indeed, abolishing such accumulation, as it occurs in mice model deficient in the chemokine receptor CCR2, leads to development of early visible Aβ deposits, specifically around blood vessels, and it has been associated with increased mortality in these mice, indicating that CCR2 function on circulating monocytes or microglia is required for prevention or clearance of perivascular Aβ deposits [292]. In support of this protective role for microglia, it was also found that bone-marrow-derived microglia have an important role in restricting plaque formation in irradiated mice also by promoting phagocytosis and clearance of Aβ [292]. This concept was further supported when IL-1β was overexpressed in the brain of PS1-APP mice. These mice had dramatical induced CCL2 expression in their brains, increased microglia accumulation and activation and reduced AD-like pathology [226]. Similarly, enhancing microglial accumulation by increasing mononuclear phagocyte recruitment from the blood into the brain, as in AD mice deficient in peripheral mononuclear phagocyte transforming growth factor-b signaling, significantly reduced AD-like pathology [293]. Another approach that showed the importance of microglia for plaque removal used intraventricular transplantation of exogenous microglia, which migrate into the parenchyma and increase the clearance of amyloid plaques [294].

This approach provides a basis for the concept that microglia might

play different roles at different time points in the progression of AD pathogenesis. Early microglia accumulation, which begins before formation of visible Aβ deposits, might be beneficial. Microglia recruited at early stages of AD phagocytose and clear Aβ and, hence, protect the brain from the toxic effects of Aβ. As the disease progresses, however, and with persistent production of pro-inflammatory cytokines such as TNFα and IL-1β, microglia lose their protective phenotype and their ability to keep up with Aβ deposition and become dysfunctional and unable to clear all of the Aβ [269], hence promoting plaque formation. In support of these findings, it was recently shown that plaque-associated microglia seemed healthy, maintaining their ability for rapid process and membrane movement, but the plaques continued to grow despite additional microglia accumulation [295] [296]. The inability of microglia to phagocytose and clear Aβ in these plaques might lead to a process called *frustrated phagocytosis*, which results in the persistent release of pro-inflammatory enzymes, reactive oxygen species, cytokines and chemokines [297]. In addition to downregulating Aβ clearance, these products of activated microglia are neurotoxic and, hence, promote neuronal degeneration.

Microglia immune activity is restrained by dedicated immune inhibitory pathways that suppress unwanted inflammatory responses and tissue destruction that are often associated with immune activation [51]. These checkpoint mechanisms include direct inhibitory interactions of microglia with neurons through the receptor-ligand pairs, soluble molecules present in the CNS (e.g. TGF-β) and intracellular regulators such as the transcription factor MafB [298] [299] [300] [301]. As discussed before, numerous studies reported conflicting results regarding the contribution of systemic immunity, recruited monocytes, and tissue-resident microglia to AD onset and disease progression. Some reports show that under such conditions, microglia acquire pro-inflammatory activity, which has been associated with disease escalation [76] [302] [303] [304].

Recently single cell genomic technologies enable unbiased characterization of immune cell types and states, transitions from normal to disease and response to therapies; single-cell analysis can further identify potential markers, pathways, and regulatory factors, promoting testable hypotheses to elucidate molecular mechanisms of immune regulation in AD [305]. [301] Taken together, it is still not clear whether microglial function in neurodegenerative diseases is beneficial but insufficient, or whether these cells are effective at early disease stages but lose their efficacy or even become detrimental later on. In particular, the pathways and molecular mechanisms of microglia activity at the different stages of AD thereby remain controversial. Hadas Keren-Shaul and colleagues in their last 2017 paper combine massively parallel single-cell analysis with chromatin profiling, single-molecule FISH (smFISH), immunohistochemistry, genetic perturbation, and computational modeling to comprehensively characterize de novo the involvement of the immune system in AD progression. Using a mouse model of AD that expresses five human familial AD gene mutations (5XFAD), they uncover a disease-associated microglia (DAM) subtype [8]. Their results also depict the pathways activated in DAM, which have been associated with known AD risk factors, but not specifically attributed to microglia. Using sub-tissue-focused single-cell RNA-seq and smFISH, they identified the spatial location of DAM in proximity to Aβ plaques. Immunohistochemical staining showed an increased number of microglia that are positively stained for intracellular Aβ particles and DAM-markers in both 5XFAD mice and in human AD postmortem brains. From comparison of triggering receptor expressed on myeloid cells 2 (TREM2)/5XFAD with TREM2+/+5XFAD mice revealed that initial DAM activation, that includes upregulation of Tyrobp (TREM2 receptor) Apoe and downregulation of microglia checkpoint genes (e.g., Cx3cr1), is TREM2-independent but full activation of the DAM program including phagocytic and lipid metabolism activity is a consequential event that is TREM2-dependent, see figure

1.22.

Overall, these study identifies a potential protective microglia type associated with neurodegeneration and outlines a general framework for studying the involvement of the immune system in AD pathology.

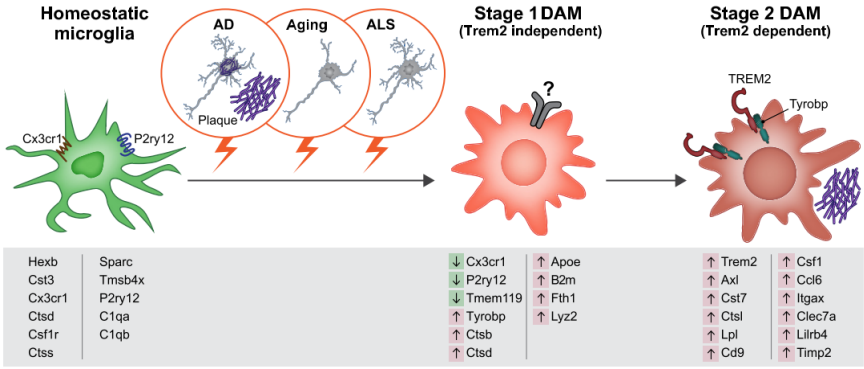

Figure 1.22: DAM are Regulated through a Two-Step Activation Mechanism Schematic illustration showing microglia switching from homeostatic to stage 1 DAM (Trem2-independent) and stage 2 DAM (Trem2-dependent) following signals such as those associated with AD pathology, aging, and ALS pathology. Key genes involved in each stage are shown below each condition. Arrows indicate up (red) or down (green) regulation of the gene in the specific stage [8].

3 Microglia and Neurotrophins

In the previous chapters the physiological functions of microglia were discussed with special attention to its role during pathological conditions which can be protective or toxic because of their pro-inflammatory iper-activation.

As for the potential protective role played by microglia, it is important to describe their capability to produce neurotrophins, which are known to have protective (beneficial) and neurotrophic effects on neurons.

Little is known about microglia and neurotrophins. In particular, it is not well established which functions neurotrophins may have on microglia activity.

Indeed, it exists a sort of neurotrophin theoretical paradigm which states that each neurotrophin is neuroprotective on its corresponding set of target neurons. The correspondence between neurotrophin and its target neurons determines the neuroprotective spectrum of a target neuron. For NGF this paradigm claims that its target cells are basal forebrain cholinergic neurons [15].

The neurotrophins are a family of protein factors composed of at least five structurally related members: nerve growth factor (NGF) [306] [307] [308], brain-derived neurotrophic factor (BDNF) [309] [310], neurotrophin-3 (NT-3) [311] [312] [313] [314] [315], neurotrophin-4/5 (NT-4/5) [316] [317], and NT-6 [318]. These proteins regulate the neuronal function and also support the survival, enhancing the growth of various types of neurons in both the peripheral nervous system (PNS) and the central nervous system [319] [310] [320] [321] [322]. Neurotrophins participate in multiple developmental processes ranging from survival of neurons [323] [324] to proliferation of oligodendrocyte precursors [325] and axon growth [326]. Microglia are sources of trophic factors known to support development and normal function of CNS cells and microglia are responsive to neurotrophins: brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) induce proliferation and phagocytic activity of microglia in vitro [327] [9].

Secretion of nerve growth factor (NGF) from activated brain macrophages has also been reported [328]. Some studies have indicated that microglial conditioned medium promotes survival and development of mesencephalic neurons and stimulates myelination in vitro [329]. Elk-

abes and colleagues in 1996 were the pioneers that investigated the role of microglia and neurtrophins in vivo and in vitro [9]. They focused on these trophic factors because their involvement in the development and growth of the CNS had been well documented.

The finding that removal of neurotrophins from animals by specific antibody or by gene knock out results in a variety of nervous system abnormalities [330] [331] [332] [333] [334] [335] [18] [17] also supports the claim that neurotrophins are functionally prerequisite factors in the nervous system. Neurotrophins such as NGF, BDNF, and NT-3 were originally shown to be produced in target neurons or tissues in both the PNS and the CNS [336] [311] [315], but later they were reported to be produced in glial cells as well. Astrocytes have been found to express mRNAs and proteins of the neurotrophin family, including NGF [337] [338], BDNF [339], NT-3 [340], and NT-4/5 [341] in vitro, and to express NGF [342] [343] [344] in vivo. Microglia have been also found to have the ability to produce NGF in vitro [345] [328], and more recently they have been reported to produce BDNF and NT-3 both in vitro and in vivo [9]. These findings further suggest that the neurotrophins produced by glial cells act on glial cells in an autocrine and/or paracrine fashion in addition to neurons. However, information on the significance of neurotrophins in the regulation of glial function has been quite limited. In 1998 Nakajima and colleagues, for the first time, tested the hypothesis that neurotrophins regulate microglial function in the CNS, demonstrating biochemically the ability of four different neurotrophins to regulate secretory products and characteristic enzyme activities of cultured microglia [346]. They demonstrated how NGF, BDNF, NT-3 and NT-4 does not influence microglia proliferation but enhance, in dose dependent manner, the PGn (Plasminoge) and PA (Plasminogen Activator) release, while decreasing the amount of NO (Nitric oxide). The effect shown by BDNF was stronger than the other neurotrophins. It was also demonstrated in colture microglia the trkA, trkB, trkC and p75NTR receptors mRNA

and protein expression. They demonstrate the Trks activity mediated by their phosphorilation. They tested also p75NTR activity, cultured microglia were treated with each neurotrophin (200 ng/ml) for various time and then analyzed for NF-kappaB and IkB proteins by Western blotting. The exposure of microglia to each neurotrophin led to the time-dependent degradation of both IkappaBα and IkappaBβ but no change was observed in total amount of NF-kappaB (p65) at any point. They have examined immunocytochemically the localization of NF-kappaB by using an antibody to p65 subunit of NF-kappaB. In the non-stimulated microglia, NF-kappaB was found mainly in the cytoplasm. However, when microglia were treated with NGF (200 ng/ml) for 2 hours, p65 was found to be translocated to the nucleus in many cells. BDNF and NT-3 were also effective with similar degree on the translocation of p65 to nucleus as far as microglia are stimulated with a concentration of 200 ng/ml. The degradation of IkappaB and nuclear translocation of NF-kappaB by relatively high dose of neurotrophins strongly suggest the involvement of p75NTR in the microglial responses [346]. The same year, Hesse and colleagues demonstrated that the cytokines interleukin-1β and tumor necrosis factor-α synergistically stimulate microglial NGF transcription and protein release. Moreover, exposure of microglial cells to complement factor C3a induces NGF expression. To assess the role of the transcription factor NF-kappaB in inflammatory mediator-induced microglial NGF expression, the effect of the NF-kappaB inhibitor pyrrolidine dithiocarbamate (PDTC) was analyzed. In the presence of PDTC, a dose-dependent inhibition of cytokine-activated NGF expression occurred. In contrast, the C3a-dependent stimulation of NGF synthesis was not influenced by PDTC. In addition, microglial neurotoxicity-mediating beta-amyloid peptides Aβ(1-40) and Aβ(1-42) failed to alter NGF synthesis, whereas Aβ(25-35) specifically induced NF-kappaB-dependent microglial NGF expression. They concluded that inflammatory signals (cytokines and complement factors), as well as Aβ(25-35), are

potent stimulators of human microglial NGF synthesis involving NF-kappaB-dependent and independent mechanisms. Microglial secretion of neurotrophins appears to be involved in early processes of neuronal regeneration [10].

Along the same lines of interests, Jose Maria Frade and Yves-Alain Barde, published very interesting results about the effect of NGF secrete microglia, demonstrating a new particular role carried out by NGF. In fact it was demonstrated that NGF can also cause cell death during development by activating the neurotrophin receptor p75NTR; they also identified microglial cells as the source of NGF as a killing agent in the developing eye: when the retina was separated from surrounding tissue before the colonization of microglial cells, no NGF can be detected and cell death is dramatically reduced, an the effect is restored by the addition of microglia cells, but these effects was blocked by NGF antibodies. These results indicate an active role form microglia in neuronal death during the developement, mediated by NGF [147].

Around 2001 Nakajima and collegues demonstrated microglia neurotrophin secretion. They observed that BDNF is constitutively secrete while NGF only after LPS stimulation [347].

Later in two different microglia cell lines, BV2 and N9, neurotrophins mitotic activity was tested. The authors demonstrated that BV2 was more sensible to NGF than N9 line. They also demonstrated that NGF enhances cell proliferation in cell lines on the contrary on primary microglia, while NT-3 and NT-4 had no effect on cell proliferation. The response of these cells to neurotrophins was blocked by K252a, a tyrosine kinase inhibitor, suggesting that actions of neurotrophins were mediated by high-affinity tyrosine kinase receptors (Trk)[348].

It was also tested whether NGF was able to influence microglial motility. It was found that NGF induced chemotaxis of microglial cells through the activation of TrkA receptor. In addition, NGF chemotactic activity was increased in the presence of low concentrations (< or

=0.2 ng/ml) of transforming growth factor-beta (TGFβ), which at this concentration showed chemotactic activity per se. On the contrary, NGF-induced microglial migration was reduced in the presence of chemokinetic concentration of TGFβ (> or =2 ng/ml). These observations suggest that both NGF and TGF-beta contribute to microglial recruitment [12].

More recently a milestone paper was published by Parkhurst and colleagues on the role of microglia BDNF during brain development. They studied the physiological roles and function of microglia in brain plasticity using mice expressing tamoxifen-inducible Cre recombinase, finding that microglia could be specifically depleted from the brain upon diphtheria toxin administration. Mice depleted of microglia show deficits in multiple learning tasks and a significant reduction in motor learning-dependent synapse formation. Furthermore, Cre-dependent removal of brain-derived neurotrophic factor (BDNF) from microglia largely recapitulated the effects of microglia depletion. Microglial BDNF increases neuronal TrkB phosphorylation, a key mediator of synaptic plasticity. Together, these findings reveal important physiological functions of microglia in learning and memory by promoting learning-related synapse formation through BDNF signaling [1].

In the end it's quite clear how the neurotrophins are really important modulators of microglia functions but more in general how they are important to modulate brain physiology through microglia activation. However, it is not so well established the direct and accurate role of NGF on microglia and the potential activity conducted by NGF on these cells, and so I decided to investigate more.

Chapter 2

Results

1 Microglia phenotype in AD11 mice

As described in the previous chapter telling about Alzheimer's Disease, in our laboratory the mouse model AD11 was developed [17] [18]. This model produces specific antibodies against the mature murine form of NGF, αD11 [243] [244].

The AD11 mouse shows a progressive comprehensive neurodegeneration that eventually shows all major hallmarks of AD neurodegeneration, massive and widespread neuronal loss, amyloid deposits (from the endogenous APP gene), and extensive neurofibrillary pathology. Moreover, these mice exhibited a severe cholinergic deficit in the basal forebrain and a behavioral impairment in retention and transfer of spatial memory tasks as shown in figure 2.1 [17].

The mechanism of this progressive neurodegeneration have been ascribed to an experimentally induced imbalance between proNGF and NGF [21] [19], but the cellular basis of the progressive neurodegeneration induced by anti NGF antibodies has remained uncertain. In order to detect the earliest changes in the progressive neurode-

generation, following the postnatal onset of the anti NGF antibody expression in the brain, was carried out at 1 month of age, well before any other overt phenotypic change, showing a number of mRNA changes.

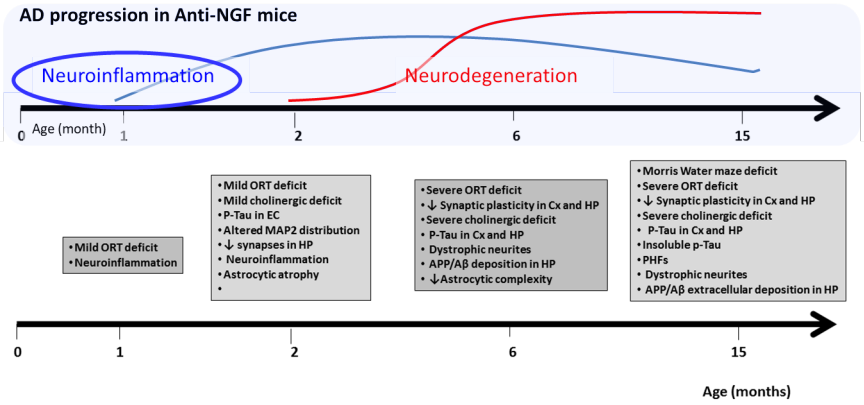

Figure 2.1: Alzheimer's Disease progression in Anti-NGF mice.

Among the most significant mRNA expression changes observed in the hippocampus and cortex of P30 AD11 mice are mRNAs cluster of three families: Wnt signaling, synaptic neurotransmission and inflammation/immune response [20]. Quantitatively speaking the most differentially expressed mRNA are involved in inflammation and this is consistent with what is seen in genetic studies for AD [349].

On this perspective the neurodegenerative phenotype in AD11 mice is more complex compared to what one would on the basis of the simple direct NGF/cholinergic connection. Though the neurodegeneration must be due to the NGF deprivation, since delivery of NGF to the brain induces a complete reversion of the phenotype [350] [351]. The specific mode of neutralization of NGF might be of notice since the Fc constant region of the antibody could entail additional effects

as opposed to a full gene knock out approach. On the other hand, the mere presence of an antibody in the brain is not sufficient to induce the neurodegeneration since other neuroantibody transgenic mice [352] do not develop the same phenotype.

These results suggest us several open questions in AD11 biology, one of them is what cell type mediates the phenotype. So I started to investigate if microglial cells, the inflammatory mediators, were involved in the AD-like phenotype of this peculiar mouse model, focusing in the presympthomatic and early sympthomatic phases (1 and 6 months) proposing a microglial impairment due to NGF deprivation. As at one month changes in the AD11 transcriptome are already detected, I analyzed microglia starting from that time point. The 6 months' time point represents the beginning of the Aβ accumulation and behavioral impairment without the neuronal cell loss associated in AD with the proinflammatory-activated (potentially neurotoxic) microglial phenotype. So it is in this time frame that changes of the homeostasis of microglia might be able to induce the pathological framework of AD, should be detected.

The experiments for AD11 mice were also performed using two groups of controls: wild-type mice (WT) and the corresponding single transgenic controls (VH), homozygous for the anti NGF αD11 heavy chain, expressing only the VH-αD11 heavy chain of the chimeric antibody, αD11, that detect and neutralize NGF. The VH-αD11 heavy chain does not bind NGF.

The microglial phenotype in AD11 mice was evaluated through a classical morphological approach but with specific parameters directed towards a more subtler evaluation of phenotypic changes in microglia. The parameters were valuated in both entorhinal cortex and molecular layer of hippocampus, at AD11 one month age (the earliest pre-symptomatic phase) and at 6 months (the beginning of the Aβ accumulation and behavioral impairment)(Fig.2.2A-B) and Images from AD11, VH and WT mice were acquired by confocal laser scanning mi-

croscope and analized with Bitplane's software Imaris (Fig.2.2D).

We analyzed entorhinal cortex and molecular layer of hippocampus due to their related impairment in AD in general and in AD11 mice specifically. Microglia cells were evaluated measuring the shape and length of the cell processes (filaments) and some other parameters were analized, i.e. filament length per cell, number of branching points per cell, volume of the entire slice, number of cells per slice.

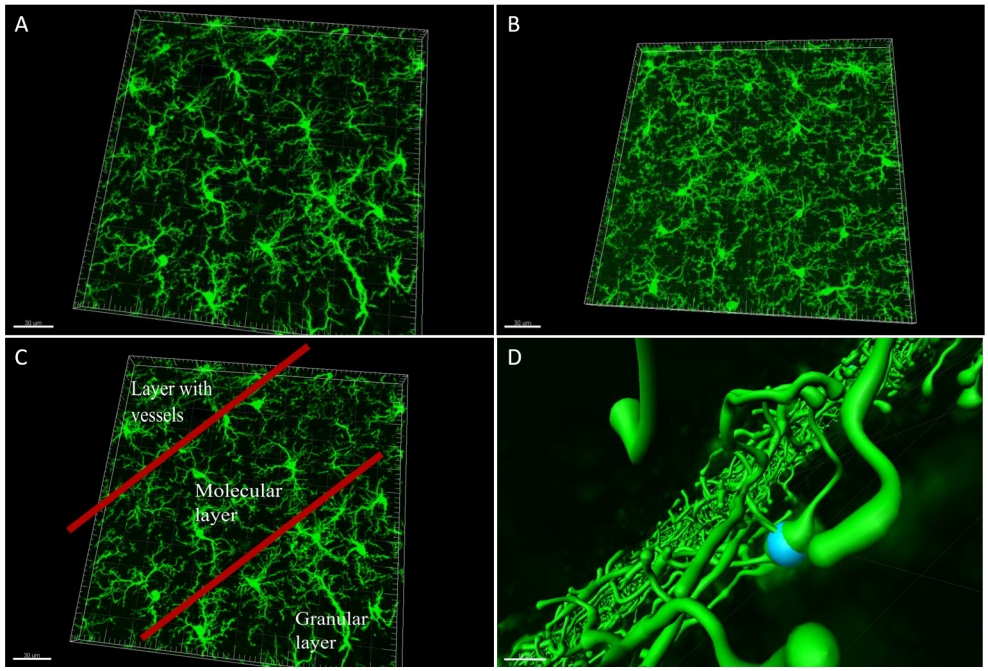

Figure 2.2: Stacks obtained from a WT mice, showing extensive Iba1 positive ramifications all over the hippocampus (A) and the cortex (B). Selection of the ROI in the hippocampus (C). Inside the IHC: Image taken with Imaris (Zurich, Switzerland). Blue spheres are microglial nuclei. Green filaments are the microglial processes (D).

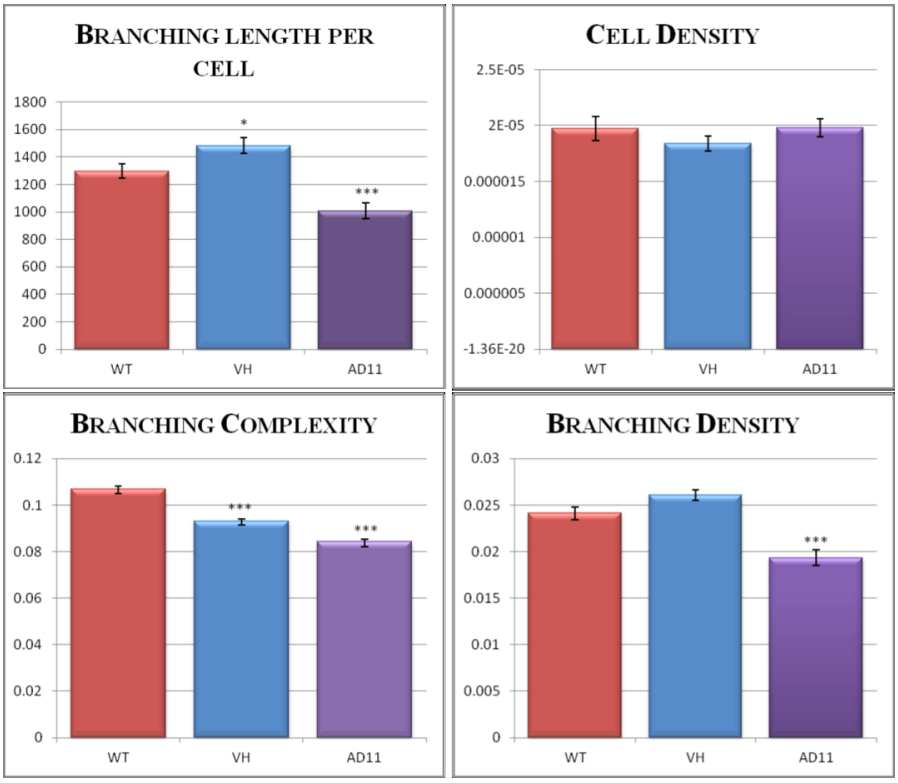

Figure 2.3: These histograms show values on 1 month old animals (enthorinal cortex). Bars are representative of the mean ± S.E.D.

The results shown in Fig.2.3 describe a significant decrease in branching length per cell, branching complexity and branching density of the microglia in AD11 entorhinal cortex compared to controls, indicating that at this early stage of the neurodegeneration there is already a decrease of the microglial branching. Thus a subpathological impairment can be already observed at this age. VH mice seem to show a more subtler decrease in branching complexity that their AD11 counterparts, while also in the Branching length per cell parameters

shows a significant, though barely, increase.

The results of the hippocampus analysis in Fig.2.4,

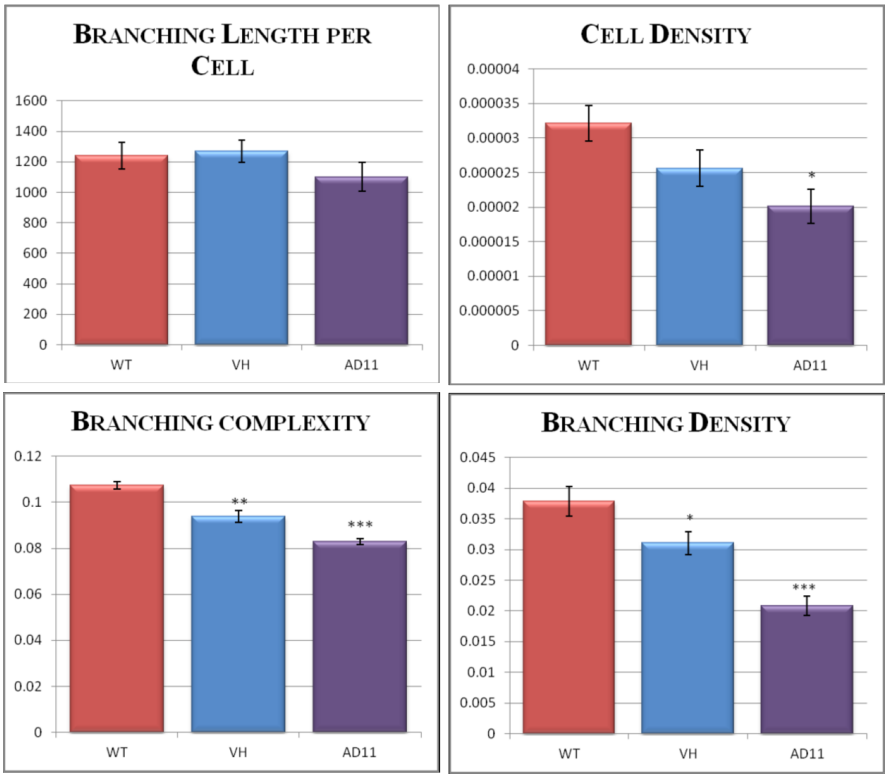

Figure 2.4: These histograms show values on 1 month old animals (hippocampus). Bars are representative of the mean ± S.E.D.

show less pathological changes than in the enthorinal cortex, since only a decrease in branching density and complexity but not branching length per cell can be observed. Interestingly, there is also a slightly significant decrease in cell density in AD11 with respect to controls, as yet another sign of microglia subtle impairment. In many

of the parameters VH show an intermediate phenotype between full on AD11 and WT with decreased branching density and complexity. At 6 months of age, it was shown the same tendency towards a decreased branching in the entorhinal cortex (Fig.2.5),

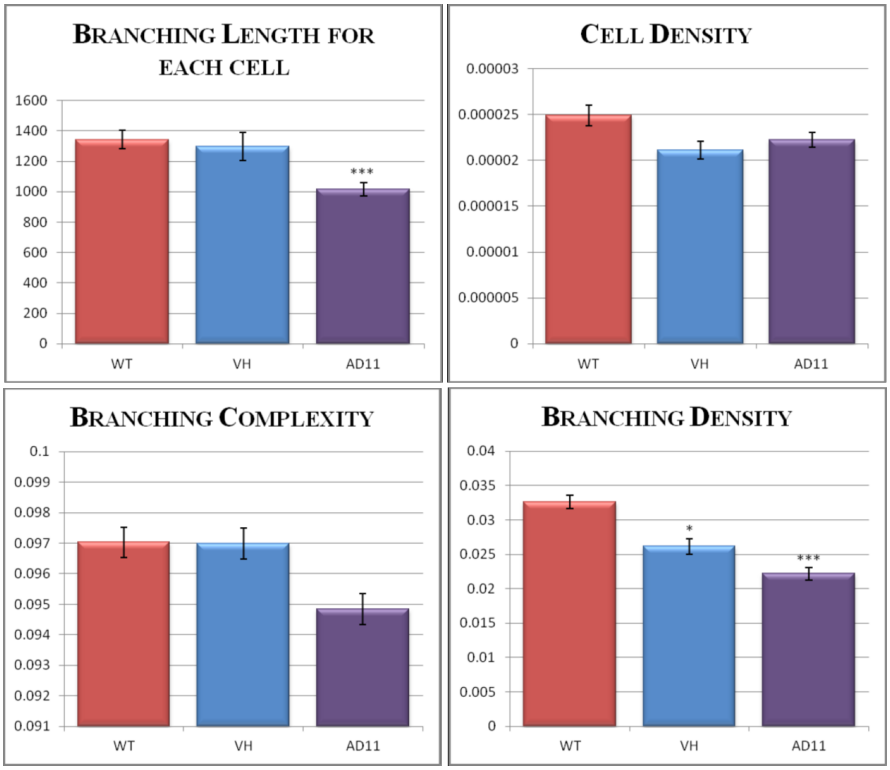

Figure 2.5: These histograms show values on 6 month old animals (enthorinal cortex). Bars are representative of the mean ± S.E.D.

where AD11 mice demonstrate a reduced branching length per cell and branching density. The same result can be observed in the hippocampus (Fig.2.6), where branching length per cell, branching com-

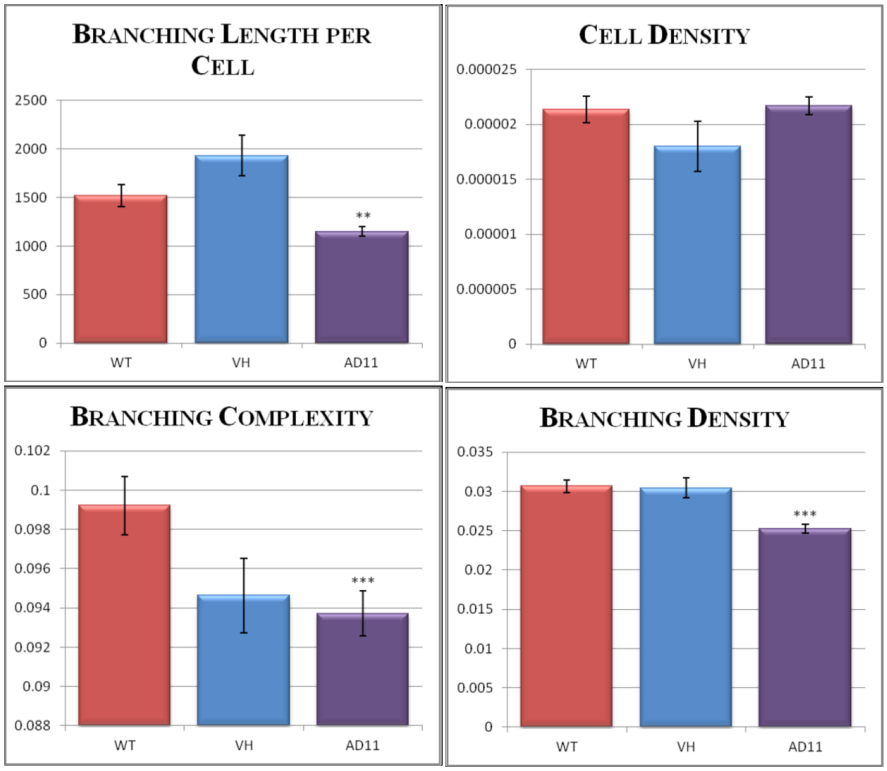

Figure 2.6: These histograms show values on 6 month old animals(hippocampus). Bars are representative of the mean ± S.E.D.

plexity and branching density are all decreased compared to the WT or VH. Yet no difference in cell density was detected. At 6 months there is barely a difference between VH and WT. The microglial impairment evaluated through morphological parameters is therefore present both at 1 and 6 months of age.

Given that microglia ad the inflammatory homeostasis of hippocampus and cortex are affected in anti NGF AD11 mice very early on, as

the earliest observed phenotypic deficit, I postulated that microglia might be a target cell of NGF in the brain and decided to investigate the role of NGF on microglia.

2 Microglia express NGF receptors in vivo and in vitro

The first step to validate microglia as NGF target cells was to ascertain whether they express functional NGF receptors. Experiments were performed using both primary mouse microglia and the immortalized mouse microglial cell line, BV-2 [353]. First, we found by immunofluorescence and FACS (Fig.2.7 A, B) that primary microglia and BV-2 cells express both TrkA and p75NTR. In primary microglia TrkA and p75NTR were expressed respectively by 44.8% and 39.4% of cells (Fig.2.7 B) .

The data obtained by immunofluorescence and FACS were confirmed by western blot (Fig.2.8 C) . To assess the NGF signaling competence of microglial TrkA and p75NTR, cells were grown in serum free conditions for 16 hours and incubated with 100 ng/ml of mouse NGF. We found that while the expression of p75NTR and total TrkA remained constant over time (Fig.2.8 C), NGF determined a significant increase of phosphorylated TrkA (pTrkA) in primary microglia, TrkA exhibited a significant time dependent activation upon treatment with the neurotrophin (measured as the ratio between phosphorylated TrkA and the total amount of TrkA (Fig.1C-D; ($*$ $p \leq 0.05$ at 5 min and $* *$ $p \leq 0.01$ at 15 and 30 min). In BV-2 cells the relative levels of pTrkA remained relatively stable, (Fig.1C-E; $p \leq 0.05$). Concerning the downstream intracellular signaling, the incubation with NGF selectively activated AKT and c-jun (an established proxy for p75NTR activation) in both cell types (Fig.1C-E, $p \leq 0.05$), while the expres-

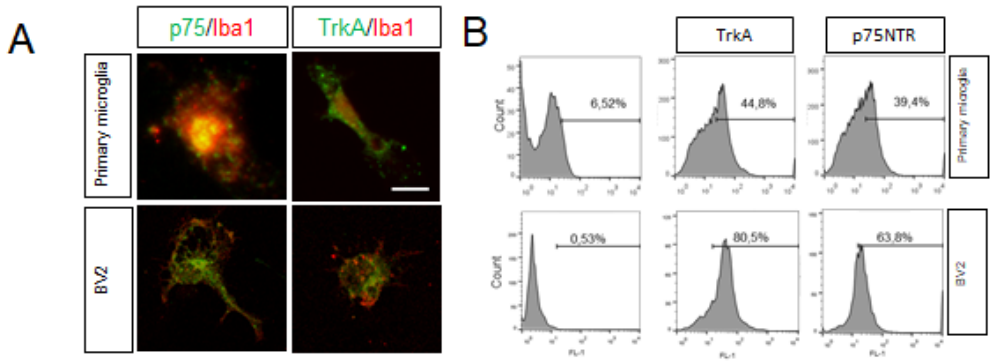

Figure 2.7: Primary microglia and BV2 cells express NGF receptors. (A) Example pictures of NGF receptors staining: TrkA and p75NTR (green) are shown in these images of confocal Immunofluorescence of primary microglia and BV-2 cells (red). (B) Flow cytometry plots shows the over gate cells positive for TrkA and p75NTR receptors in primary microglia and BV2 cells. (Data are mean ± SD all data are representative of three independent experiments, $*$ $p \leq 0.05$, $* *$ $p \leq 0.01$, Student's T-test)

sion of phosphor-Erk remained unchanged (Fig.1C-E; $p \geq 0.05$). Thus, in cultured microglia NGF activates both TrkA and p75NTR receptors, initiating at least some of the signalling cascade specific to these receptors.

We then proceeded to assess TrkA and p75NTR expression in adult ex vivo glial cells. Triple staining was performed to detect the TrkA receptor distribution in sections of mouse cortex. Experiments showed colocalization of TrkA and Iba1 both in the cell bodies and branches of microglial cells (Fig.2.9 A; n=3; 3 experimental replicates) .

On the other hand, astrocytes (GFAP+ cells) showed a sparse overlapping with the anti-TrkA antibody, MNAC13 (Fig.2.9 B: n=3;

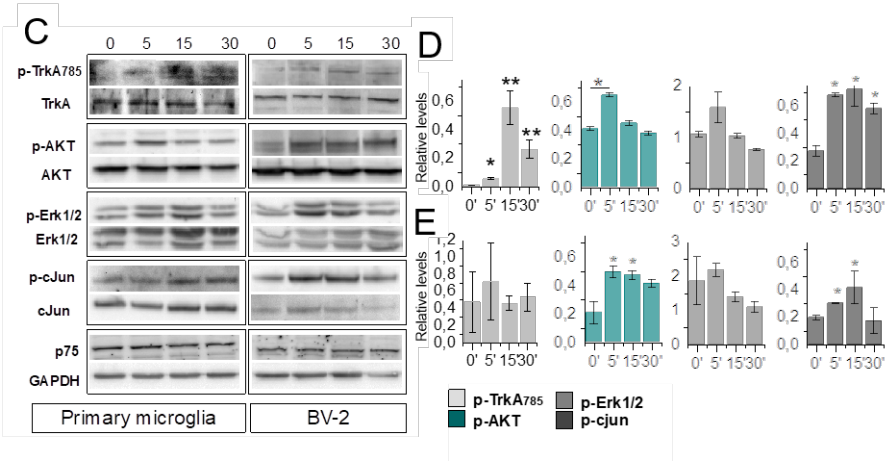

Figure 2.8: Primary microglia and BV2 cells express NGF receptors and activate receptor-mediated intracellular signaling after NGF stimulation. (C) The panel shows western blot signalling results for proteins involved in NGF signalling transduction pathways of TrkA, AKT, Erk and cjun. On the right are shown the histograms quantification of phosphorylated protein normalized on the total protein level for primary microglia (D) and BV2 cells (E). (Data are mean ± SD all data are representative of three independent experiments, $*$ p$\leq$0.05, $**$ p$\leq$0.01, Student's T-test)

3 experimental replicates). Ultimately, labeling of the p75 receptor showed some rare points of colocalization with CD11b+ cells (Fig.2.9 C, n=3/6) while no expression could be detected on astrocyte bodies or branches (n=3/3 data not shown). Thus, we conclude that both in vivo and in vitro microglia possess NGF receptors, and - specifically in cell culture - we could observe normal receptor kinetics in response to the neurotrophin, indicating that these receptors are indeed active.

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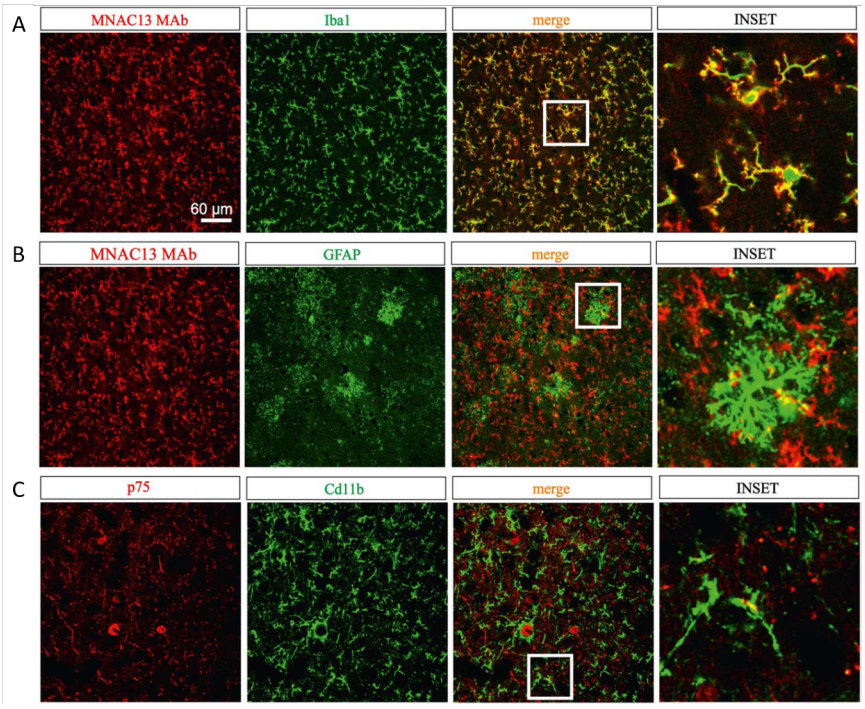

Figure 2.9: Expression pattern of NGF receptors in murine brain slices. (A) The anti-TrkA MAb MNAC13 (red label) stains cortical microglia iba1 positive cells (green label). Merging areas (yellow label) and magnification (inset) show that the labeling involves both cytoplasm and fibers. (B) (middle panels) MNAC13 staining slightly overlapped with the astrocytic marker GFAP (in green). Below, (C) p-75NTR (red label) is slightly expressed in CD11b1 microglia (green label) from cortical sections. Merging areas (yellow label).

3 NGF modulates the expression of genes involved in pathways of cell motility, phagocytosis and protein degradation

To gain insight into the downstream effects of NGF signaling in microglia and on potential functional microglial responses to NGF, mi-

croarray gene expression profiling was performed on primary microglia treated with NGF (100 ng/ml) either for 2, 8 or 24 hours. NGF induced global transcriptomic changes throughout the three time points. At two hours, the majority of differentially expressed genes (DEGs) were downregulated, while at 24 hours there was a reversal, with a trend toward upregulation (Fig.2.10 A).

KEGG gene ontology analysis was performed, to cluster the DEGs into over-represented pathways, thus identifying those primarily modulated by the incubation with NGF. Interestingly, at 2 hours the majority of upregulated genes were linked to focal adhesion and extracellular matrix interactions, while downregulated genes were related to cytoskeleton rearrangement (Fig.2.10 B). At 8 hours, genes of cell adhesion molecules and of the protein digestion and absorption were still upregulated (Fig.2.10 B). At 24 hours, the majority of upregulated genes belonged to the neuro-ligand interaction and to the phagosome pathways, while downregulated genes belonged to endocytosis, focal adhesion, adherens junction, cytokine-cytokine receptor interaction, and chemokine signalling pathways (Fig.2.10 B). Then we focused on the analysis of specific gene clusters highlighted by the KEGG analysis, such as those involved in actin cytoskeleton regulation, endocytosis and chemokine signalling. In addition, we analyzed in more detail the expression of genes involved in protein digestion, neurotrophin signalling and genes linked to Alzheimer's disease. For each of these clusters, we represented the total amount of genes mapped to the specific KEGG category at each time point (Fig.2.11 A)

and the corresponding heat maps, providing a graphical representation of the change in expression of the genes over time (Fig.2.11 B). We found that the major changes occur at 24 hours for all clusters. At this time, NGF induced a significant downregulation of rhoA and rock2, genes involved in actin dynamics [354] [355], while most genes related to endocytosis and lysosomal activity, such as Gm2a [356] , were up-regulated. Concerning the neurotrophin signalling and

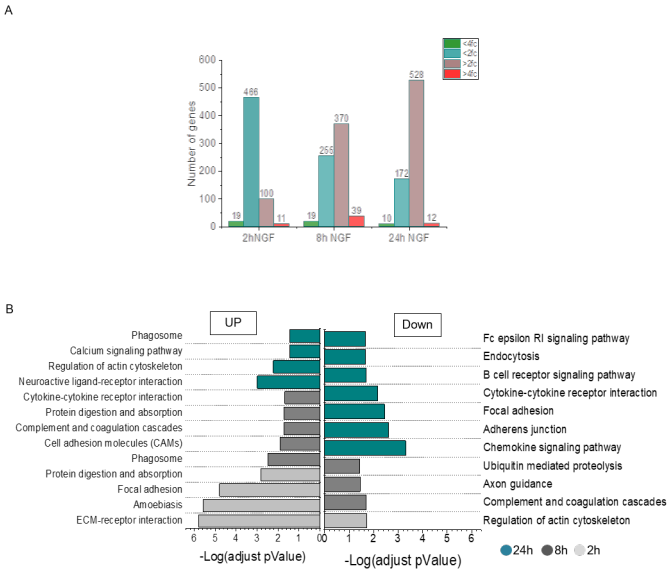

Figure 2.10: NGF modulates microglial gene expression. (A) The bar plot shows the global number of differentially expressed genes, up-and down-regulated by NGF at 2, 8 and 24 hrs. Gene lists were selected using two different thresholds: 2.0 fold-change in linear scale and Limma P-Value$\leq$0.05 (blue for down-regulated and plum for up-regulated); 4.0 fold-change in linear scale and Limma P-Value$\leq$0.05 (green for down-regulated and red for up-regulated). (B) The horizontal bar plot shows the significantly enriched KEGG terms, following NGF treatment. Enriched pathways refer to up-regulated genes (right bars) or down-regulated ones (left bars), at 2, 8 and 24 hrs (green, grey, light grey bars respectively). The analysis was performed on differentially expressed genes selected by two thresholds: corrected P-Value (FDR) $\leq$ 0.05 and 1.0 fold-change in linear scale.

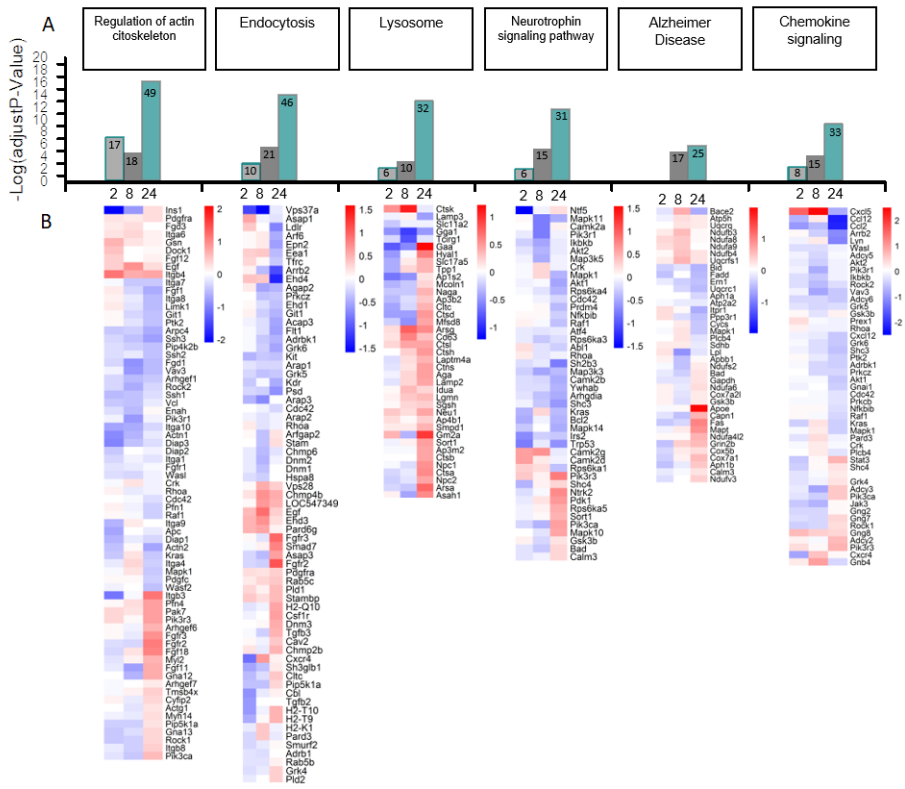

Figure 2.11: NGF modulates different pathways in microglia in time dependent manner. (A) The histograms show the adjusted P-Value (FDR) of selected enriched KEGG pathway at 2, 8 and 24 hrs (green, grey, light grey bars respectively). Each bar contains the number of differential genes mapping to each specific pathway. (B) Heatmaps show the Log2 fold-change ratio of genes mapping to the corresponding modulated pathways on the top.

Alzheimer's disease pathways, we found an upregulation of sort1 and ApoE, both known as risk factors in Alzheimer's disease [357] [358]. Finally, we looked at mRNAs involved in the inflammatory response, whose modulation is a major functional response of microglial cells. Interestingly, this mRNA class was not significantly represented among the mRNAs upregulated by NGF at 24 hours. The largest modulation was actually the downregulated expression of cxcl5, ccl12, ccl2. Overall, these data suggest that NGF might influence the motility, the phagocytic and protein degradation abilities of microglia, without activating them in the classical proinflammatory sense. We therefore looked at these microglial functions in more detail.

4 NGF enhances microglial membrane dynamics, but not their cell speed.

The surveillance activity of microglia cells is mediated by the translocation of their cell body towards sites of injury, where chemoattractant substances are released, and by finer - highly dynamic - movements of cell membrane and processes, resulting in the extension and retraction of their branches in response to either physiological or pathological stimuli [41] [40]. These are accomplished through changes in the structure of their cytoskeleton. The transcriptome analysis demonstrated specific changes regarding cytoskeletal related genes. Thus, we asked whether NGF might induce changes in cell body migration and in the motility of cell membrane and processes. The chemotactic properties of NGF specifically on microglial cells are already documented in the literature [12]. In this work Minghetti and collaborators proved NGF capable of increasing the migratory ability of microglial cells by acting on the high affinity TrkA receptor. Our in vitro approach to assess the effects of NGF on motility - a necessity arisen from transcriptomics

results - was that of operating time-lapse recordings of NGF-treated - freely moving - microglial cells. Primary microglia were monitored for 1h in a culture chamber after treatment. Videos were analyzed by means of a self-made Python-based script capable of extracting and quantifying useful features of the microglial motility behavior. This analysis unveiled that the speed of the cell body of NGF- treated microglial cells was not significantly different from that of untreated microglia (Fig. 4B), meaning there was no overall translocation of the entire cell from one place to another. This result is in line with the work of De Simone et al. [12]: when they eliminated the chemotactic gradient, they could no longer observe the change in migratory behavior induced by NGF thus excluding a chemokinetic effect. We then concentrated on another parameter of cell motility: cell membrane changes. These structural changes occurred on a timescale of minutes and were evaluated as the difference in cell area between two consecutive frames (ΔA) normalized over the cell perimeter p (ΔA/p).

Since this parameter evaluates the change of the cell area, a measurement of its capacity to elongate and retract, this can be thought of as an in vitro measure of exploratory behavior. We found that the treatment with NGF induced a significant increase in this exploratory tendency in microglial cells as in figure 2.12 A, B.

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5 NGF promotes microglial macropinocytosis but not phagocytosis

Microglia are capable of engulfing material through three different mechanisms: phagocytosis, receptor-mediated endocytosis and pinocytosis. Phagocytosis is used to internalize large particles [253], while pinocytosis is typically associated with the uptake of soluble sub-

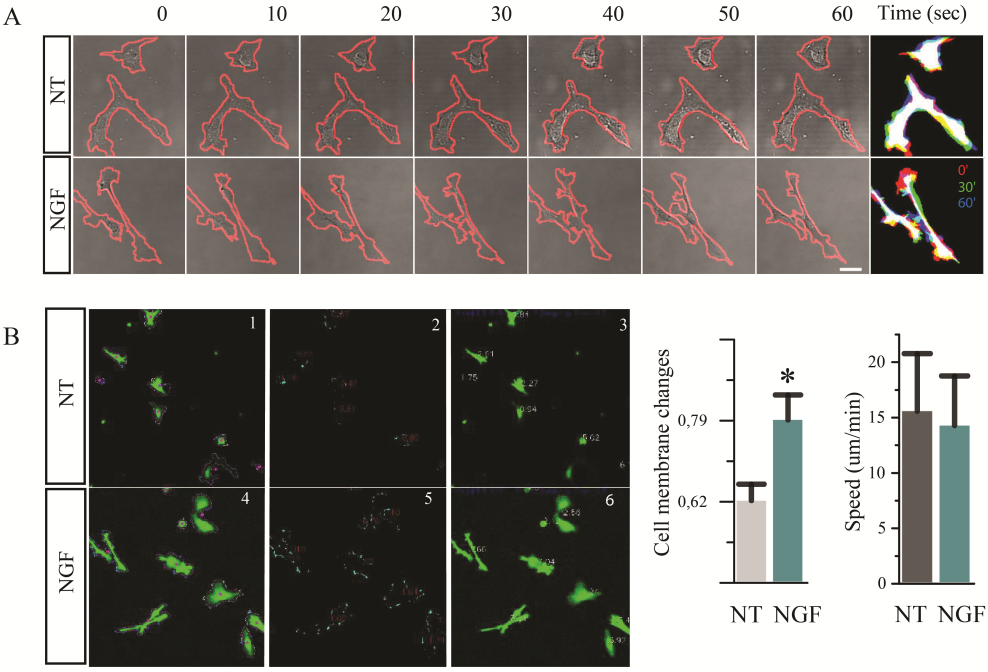


Figure 2.12: NGF modulates microglial motility dynamics. (A) Bright-field image during a time lapse of primary microglia from CX3CR1-GFP mice. (B) In the panel, it is shown the pattern recognition from a homemade Python based script that describes (1-3) naive cells and (4-6) NGF cells. The boxes show in (1, 4) perimeter in violet and cell center in pink, (2, 5) perimeter difference between 2 consecutive frame, (3, 6) cell speed. The histograms show the plotted results of these parameters: cell membrane changes and speed (n=29; data are mean ± SD; all data are representative of three independent experiments $*$ $p \leq 0.05$, Student's T-test).

stances, such as soluble Aβ peptide [257]. In microglia, protrusions of the cell membrane are associated with phagocytic activity. We thus

evaluated whether the NGF-dependent changes in membrane motility might underlie and reflect changes in engulfing processes. To do so, we used an in vitro assay - followed by FACS analysis - where primary microglial cells were incubated with either fluorescent opsonized latex beads or dextran in the presence or absence of NGF. Beads are ingested through a phagocytosis process, while dextran through macropinocytosis [359] and it is possible to discern these processes thanks to known inhibitors and activators of the cellular mechanisms behind these different engulfment processes: for the phagocytosis of big particles (beads) IFN-γ was used as positive control of activation of the process in microglia [360], while PMA and Rac-cdc42 activator I as promoters of macropinocytosis of dextran [359] [361]. NGF was found not to increase the number of latex beads internalized by microglia (Fig.2.13 A) . As regards macropinocytosis, NGF increased the internalization of dextran to a degree comparable to the established activators of the process, PMA and Rac-cdc42 activator-I (Fig.2.13 B). As expected, amiloride and cytochalasinD inhibited the process (Fig.2.13 B). Thus, we conclude that NGF is able to selectively upregulate macropinocytosis while microglial phagocytosis of bigger particles, such as opsonized beads, is not affected by this neurotrophin.

6 NGF activates microglia currents and modulates glutamatergic neurotransmission by acting on microglial cells

An ex vivo correlate to microglial behavior in response to NGF was obtained by performing patch clamp recordings from microglia in acute brain slices. Our data reveal that NGF triggers an outward current (Fig.2.14 A). To study changes in this outward NGF-induced current, we repetitively clamped the membrane from a holding potential of -20

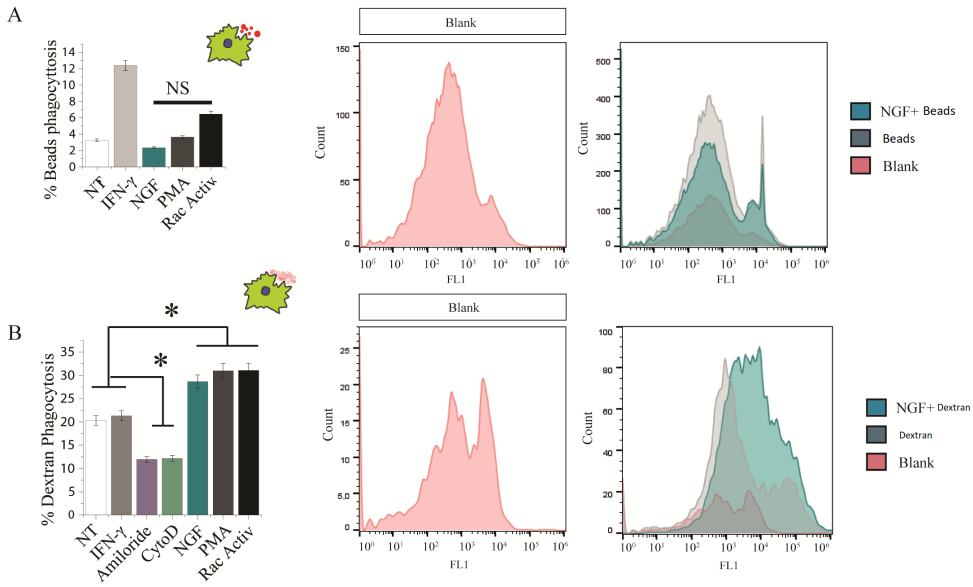


Figure 2.13: NGF enhances macropinocytosis of dextran but not phagocytosis of beads. Flow cytometry (580-650nm absorption) to count the number of cells that phagocyte the differents debries (A) Phagocytosis of beads: Primary microglia from CX3CR1-GFP mice were incubated with 6μm beads and 10 ng/ml IFNγ, 100 ng/ml NGF, 100 nM PMA and 1 μg/ml Rho/Rac/Cdc42 activator I for 3 hrs. (B) Macropinocytosis of dextran: Primary microglia from CX3CR1-GFP mice were incubated with 2.5 mg/ml Dextran and 10 ng/ml IFNγ, 50 μM Amiloride, 5μg/ml Cytochalasin-D, 100 ng/ml NGF, 100 nM PMA and 1μg/ml Rho/Rac/Cdc42 activator I for 3 hrs. (mean $\pm$ SD, $*$ p$\leq$0.05, Student's T-test).

mV to a series of hyperpolarizing and depolarizing voltage steps before and after the application of NGF (Figure2.14 B, left inset). The cur-

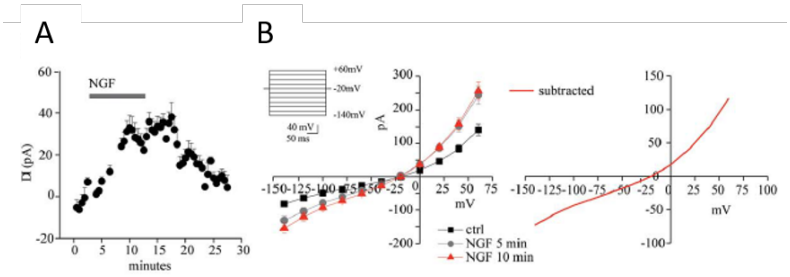


Figure 2.14: NGF affects microglial currents thereby enhancing excitatory neurotransmission. (A) Time plot of the mean current amplitude induced by NGF application recorded from microglial cells (n517). (B) Left, current-voltage relationship of the NGF-induced current by application of NGF (20 γ) in a microglial cell from acute cortical slice of CX3CR11/GFP mouse before (black curve) and after 5 (light gray curve) and 10 min (red curve) NGF application. Right, NGF induces an outward rectifying current with reversal potential at about 15 mV at a holding potential 5220 mV (results obtained by subtracting the current before and after the NGF application).

rentvoltage clamp curve of the response to NGF was outward slightly rectifying and reversed at 15 mV (n=17, p$\leq$.05, Fig.2.14 B). At a holding potential of -70 mV, NGF induced a current that reverses at 25 mV (n=17, data not shown). These data reveal that NGF modulates microglial currents and as such can be considered functionally active on microglia in an ex vivo setting. Emerging evidence is showing that stimulation of microglia by activation of glial receptors affects neurotransmission [362] [363]. Therefore, we hypothesized that also NGF may indirectly modulate glutamatergic neurotransmission by acting on microglial cells. To test this possibility, we first investigated the action of NGF on miniature excitatory postsynaptic currents (mEPSCs) recorded from pyramidal neurons. Bath application of NGF (2

$1mu g/\mu l$) for ten minutes significantly increased both the amplitude and frequency of mEPSCs (from 20.45±0.97 to 22.90±1.00 pA and from 5.50±0.71 to 7.43±1.14, n=22; p≤.01 and p≤.05 respectively; Fig.2.15 A).

These enhancements were, at least partly, due to TrkA receptor activation, since anti-TrkA mAb MNAC13 counteracted the increase of both amplitude and frequency by NGF (from 25.85 ± 1.188053 to 26.28981 ± 1.621783) and frequency (from 6.30 ± 1.249805 to 7.10 ± 1.535248, n=15; p=.12 and p=.07 respectively; Fig.2.15 B). Then, we carried out experiments in the presence of minocycline, which prevents microglia activation [364]. Minocycline (100 nM) inhibited the NGF-induced increase of mEPSC frequency, without affecting the rise in amplitude (from 2.98 ± 0.58 to 3.43 ± 0.55 Hz and from 18.21 ± 1.25 to 20.01 ± 1.23, p=.5 and p≤.05 respectively). Altogether, these data strongly suggest that NGF acts on microglia to modulate glutamatergic neurotransmission.

7 NGF and microglia in the pathological condition: Alzheimer's Disease

We then decided to assess the effect of NGF on microglia in the context of pathology, specifically one marked by protein aggregation such as Alzheimer's disease. Microglia are important players in the pathogenesis of neurodegenerative disorders and they are being studied either as promoters of disease or physiological tools to be exploited to help with disease outcome. In vitro, we assessed the inflammatory profile of cells exposed to the proinflammatory amyloid peptide when NGF was in the culture medium, and finally we studied Aβ pathology in neuron-microglia cocultures and the effect of NGF on this in vitro neuroimmune interface. We tested the phagocytic and degrading ac-

tivity of microglia towards Aβ after NGF treatment in vivo. The pro-phagocytic activity was then confirmed an ex vivo assay on acute slices treated with NGF. Then, we found that the treatment with NGF protects neuronal spines from Aβ-induced toxicity.

8 NGF counteracts Aβ proinflammatory effect on microglia.

Aβ provides an inflammatory stimulus to microglial cells [365]. Given the above-mentioned effects of NGF on microglial cells, it was of interest to ask whether and how NGF can modulate the Aβ-induced inflammatory profile in microglia. To this aim, we profiled the expression of inflammatory cytokines and chemokines in primary microglia in response to NGF, Aβ and Aβ with NGF, with an inflammation antibody array. Looking at the heatmaps (Fig.2.16 B) , we can macroscopically see the inflammatory activity of Aβ by the prevailing of the red bars (increased quantity of cytokines). NGF seems to carry out the opposite effect: not only it is intrinsically anti-inflammatory when administered on his own but, when given in concomitance with Aβ, NGF treatment appears to counteract the inflammatory stimulation by Aβ, returning cytokines to levels of the untreated sample. This effect by NGF was quantified by the PCA analysis (Fig.2.16 A), that shows NGF treated cells to be at opposite sides of the PC1/PC2 plane, with untreated cells having an intermediate position - closer to the NGF groups - and Aβ treated cells clustering elsewhere Thus, NGF is very effective in reverting the pro-inflammatory state of microglia induced by Aβ, while it has only a moderate effect on the inflammatory phenotype of naive microglial cells, consistent with the transcriptomic study we presented in Fig.2.10 A-B and Fig.2.11 C-D.

9 NGF promotes the internalization of soluble toxic Aβ oligomers through TrkA signaling.

Microglia play an important role in the engulfment of different forms of the Alzheimer's hallmark Aβ peptide. While microglial cells endocytose fibrillar Aβ by phagocytosis, the soluble forms of the Aβ peptide are engulfed in microglia by macropinocytosis [257]. Thus, we asked whether NGF, which supposedly increases macropinocytosis in our previous experiments, differentially regulates the engulfment of fibrillar Aβ (fAβ) and soluble Aβ (sAβ). To this aim, we incubated primary microglia with either fluorescent fAβ or sAβ and we tested and measured the effect of NGF by FACS, IF and WB. Consistently with our results with dextran and beads, FACS analysis revealed that NGF did not increase the engulfment of fAβ (Fig.2.17 A) , but increased significantly the macropinocytosis of sAβ (Fig.2.17 B). The quantity of sAβ and AbOs - pure oligomers produced in vitro [366] - inside primary microglial cells after NGF treatment (from both B6129 and Cx3Cr1-GFP) was also measured by immunofluorescence confirming an increase in the internalization of the soluble peptide (Fig.2.18 A e B) . To quantify the increase of NGF-induced Aβ engulfment, and to distinguish different Aβ species, we performed western blot analysis for Aβ on cell extracts. We found that NGF determines a two-fold increase of the internalized Aβ dimers and trimers (Fig.2.19 A) .

To discern the involvement of the different NGF receptors in the internalization of Aβ, we interfered with TrkA and p75NTR signalling through specific inhibitors: K252a, which blocks TrkA phosphorylation and signaling, and TAT-pep5, a p75NTR signalling inhibitor. K252a, and not TAT-pep5, was able to block the increase in the uptake of AbOs in response to NGF (Fig.2.19 B). On the other hand, TAT-pep5 does not interfere with NGF-dependent increase in AbOs

internalization (Fig.2.19 B). Thus, we conclude that NGF is able to increase selectively macropinocytosis of soluble Aβ oligomers in microglia by a TrkA-dependent mechanism.

10 The fate of internalized sAβ following NGF treatment

What are the consequences of the increased macropinocytosis of Aβ oligomers induced by NGF? The Aβ engulfed by microglia could be either accumulated inside the cells, expelled through exocytosis/released in exosomes, or digested. Transcriptome analysis revealed a strong modulation by NGF of genes involved in protein digestion, giving us cause to test the hypothesis that the degradation of internalized sAβ might be modulated in response to NGF. We followed the fate of sAβ using lysotracker, a dye that marks lysosomes (Fig.2.20 A) . The sAβ peptides (green) is internalized by BV2 microglial cells following NGF incubation and colocalize with lysotracker (red), suggesting that the engulfed material might go through lysosomal degradation. In order to quantify such degradation and the hypothetical release of Aβ - such as suggested by (Joshi et al., 2014) [367] - we proceeded as follows (Fig.2.20 B): BV2 microglial cells were treated with soluble Aβ for 3hrs, then supernatant was collected and cells were washed to remove the Aβ excess. We then monitored Aβ intracellular and extracellular levels in parallel experiments at 5, 9 and 21 hrs, by WB of cell extracts - reflective of degradation - and ELISA of supernatants - to detect material that was expelled. This experiment reveals that not only NGF-treated cells ingest more Aβ than non-treated cells (Fig.2.20 C-D) - in accordance to our previous results - but also that NGF-treated microglial cells digest a greater amount of Aβ (Fig.2.20 D) and release a smaller fraction of the ingested Aβ peptide into the extracellular

compartment, compared to untreated control cells (Fig.2.20 E).

11 Phagocytosis of Aβ ex vivo

To assess the translatability to a physiologic environment, we also tested phagocytosis ex vivo in acute brain slice preparations from CX3CR1-GFP mice. The acute slice was incubated for 3 hrs with NGF and s555-Aβ, the slice was then fixed, cut to 45 μm thick slices and mounted on glass slides to quantify internalization of fluorescent Aβ by GFP+ cells. We found a significant increase of internalized Aβ in microglial cells from brain slices that were incubated with NGF (Fig.2.21), suggesting indeed that the effect can translate to microglia in vivo.

12 NGF protects against Aβ induced spine toxicity, rescuing spine density and LTP

In the previous sections, we demonstrated the ability of NGF-stimulated microglia to effectively internalize and process Aβ, thus removing toxic soluble oligomers from the medium. It is known that Aβ oligomers decrease spine density both in vitro and in vivo, and impair synaptic long-term potentiation (LTP) [368] [369] [370] [371]. In the healthy brain, an established physiological function of microglia is the regulation of synapse number by a phagocytic process known as synaptic pruning [3] [1] [2] [372]. Therefore, we asked whether NGF might regulate the dendritic spine phagocytosis activity of microglia. To this aim, we performed co-cultures of primary microglia with mature neurons and we quantified spine density following NGF treatment (Fig.2.22 A) . The number of PSD95 positive puncta was lower on neurons cultured with microglia than in control neuronal cultures; this reflects the nor-

mal phagocytic activity of microglia on synapses [373]. On the other hand, NGF treatment of microglia does not determine any further reduction of spine number compared to untreated microglia-neuron co-cultures (Fig.2.22 B): thus, NGF does not modulate the phagocytosis of synapses by microglia. We next asked if NGF-treated microglia could rescue spine loss mediated by sAβ exposure. Surely, in our control experiment, sAβ addition to pure neuronal cultures significantly decreases spine density by 50%, a decrease that could not be rescued by NGF treatment alone; however, in neuron-microglia co-cultures, while Aβ-induced spine loss could still be detected in the untreated samples, concomitant activation of microglia with NGF completely prevented the decrease in spine density, pointing towards microglia as the mediators of NGF neuroprotective action (Fig.2.22 B). We conclude that NGF can prevent Aβ-mediated spine loss in a microglia-dependent manner. The effects of Aβ on spine number is paralleled by its negative effects on synaptic potentiation in plasticity paradigms [374] [375]. We therefore sought to investigate the interplay between NGF, microglia and spines in a plasticity protocol. We quantified spine potentiation measuring the total amount of GluA1 AMPA receptors in neurons under resting conditions or after glycine-induced chemical LTP (GI-LTP) (as in Fortin et al., 2010 and Ahmad et al., 2012) [376] [377]. As previously reported, the staining intensity of synaptic GluA1 AMPA receptors increased in pure neuronal cultures 1 hr after GI-LTP induction (Fig.2.23).

Under our conditions, in microglia-containing cultures, neurons were found to be more sensitive to GI-LTP induction: GI-LTP induced a greater increase of GluA1 synaptic staining (36 % increase with respect non-LTP cultures), when microglia was present, compared to control cultures without microglia (15,76 % with respect to non-LTP cultures) (Fig.2.23). This suggests an enhancement of synaptic potentiation by microglia, an i vitro correlate of the evidence suggesting a role for microglia in spine formation and potentiation in

vivo [141] [1]. SAβ exposure prevented the spine potentiation by GI-LTP, since the levels of synaptic GluA1 were not significantly different between glycine-stimulated and control cultures. The presence of microglia alone was not sufficient to rescue the synaptic GluA1 levels after sAβ incubation in sister cultures containing microglia (Fig.2.23). Instead, NGF-stimulated microglia cells were able to rescue the impairment of synaptic potentiation caused by sAβ. In fact, in NGF-treated microglia-neurons co-cultures, synaptic GluA1 levels were significantly higher after GI-LTP, even in the presence of sAβ (Fig.2.23). This was not due to a direct action of NGF alone on neurons, since in pure neuronal cultures NGF exposure was not sufficient, per se, to drive a significant change of synaptic GluA1 levels after GI-LTP in the presence of sAβ (Fig.2.23). From these data we conclude that not only NGF-stimulated microglial cells are able to block spine loss induced by sAβ (Fig.2.22 A-B), but they can also attenuate sAβ impairment of spine potentiation (Fig.2.23).

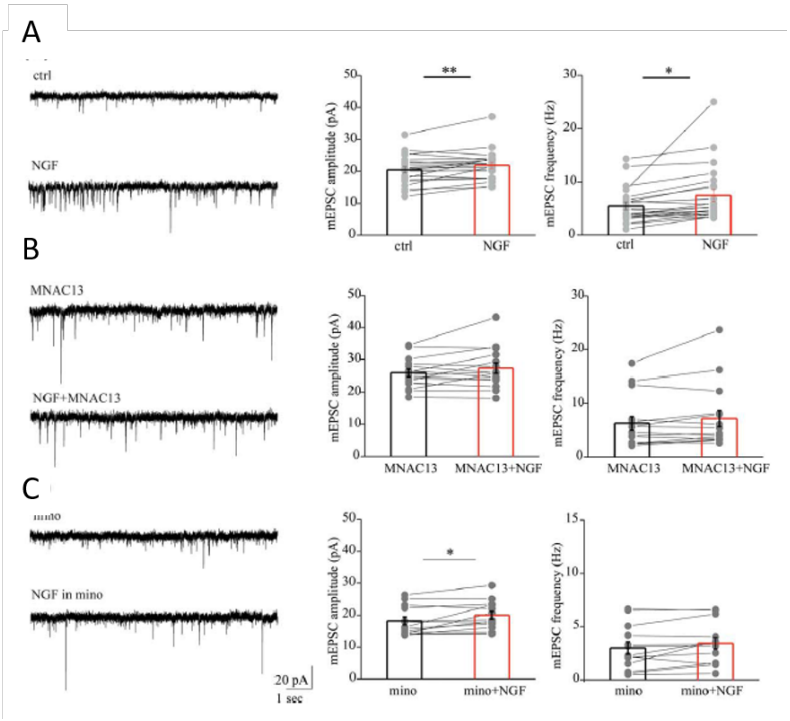


Figure 2.15: NGF affects microglial currents thereby enhancing excitatory neurotransmission. (A) Left, example traces of AMPAR mEPSCs recorded from a pyramidal neuron at 270 mV, in control (ctrl) and after NGF (20 γ), in the presence of picrotoxin (100 mM) and TTX (1μM). Right, bar histograms of group data showing the NGF-mediated increase of mEPSCs amplitude and frequency. (n=22, $*$ p$\leq$0.05, $**$ p$\leq$0.01, paired sample t test. (B) Same as in c but in the presence of the anti-TrkA, MNAC13. Note that 20 γ NGF did not enhance mEPSC amplitude and frequency when TrkA receptors are blocked (n515, p=.012 and p=.7 for amplitude and frequency, respectively; paired sample t test). (C) Left, example recordings of mEPSCs before and during NGF in the presence of 100 nM minocycline (mino). Right, population plots of mEPSC amplitude and frequency in minocycline, before (black bar) and during NGF (red bar), showing that NGF increased selectively the mEPSC frequency but not the amplitude when microglia activation was blocked (n=13, p$\leq$.05 and p=.5, paired sample t test. Data are values from single cells (gray filled circle) and mean$\pm$SEM (bars).

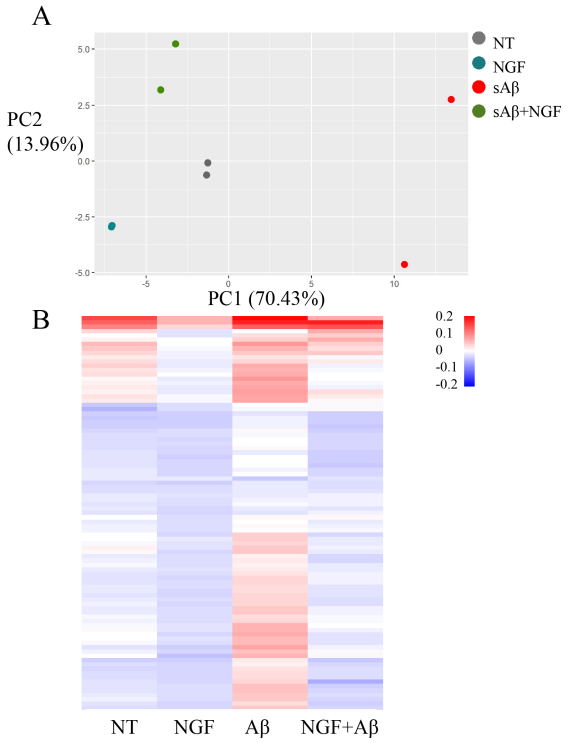


Figure 2.16: Anti-inflammatory effect of NGF on microglia primed with Aβ. (A) PCA analysis of the inflammatory array. In the graph, two biological replicates of four different treatments were plotted. PC1 and PC2 represent the first two principal components, the proportion of variance (POV) held by these components is reported in brackets as percentages. (B) Inflammation array of primary microglia treated with NGF, Aβ, or Aβ and NGF reported as heatmaps, the scale bar represents the minimum and maximum levels of protein mean. Analysis was performed on RStudio (Boston, MA).

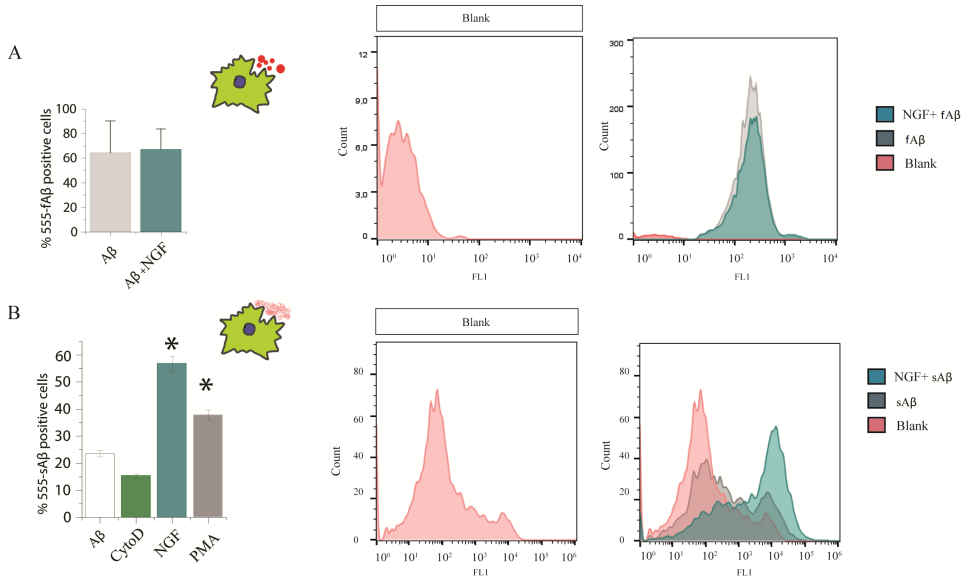


Figure 2.17: NGF increases the macropinocytosis of soluble Aβ but not the phagocytosis of fibrillar Aβ. Flow cytometry (580-650nm absorption) to count the number of cells that phagocyte the differents peptides. Primary microglia from wild type mice were incubated with 1μM of fAβ or sAβ and 100 ng/ml NGF. Uptake was quantified using flow cytometry and compared with control non treated cells (A) Internalization of fAβ is not affected by NGF treatment. (B) Internalization of the soluble peptide is increased after NGF treatment. Controls: NGF has a similar effect to PMA (an activator of macropinocytosis), CytochalasinD: inhibitor of endocytic processes (mean $\pm$ SD, $*$ p$\leq$0.05, Student's T-test).

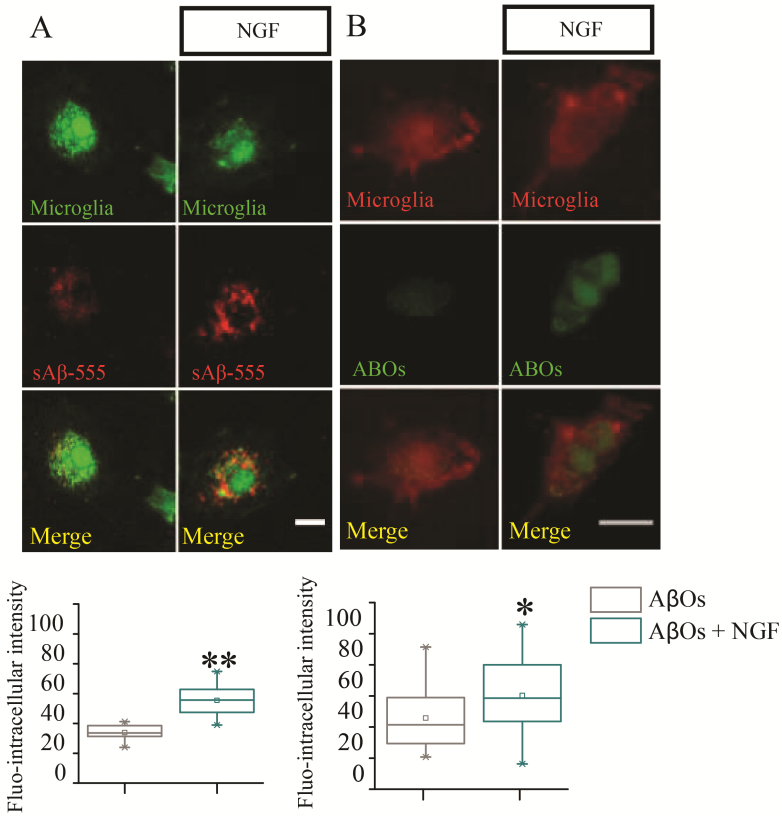


Figure 2.18: NGF increases the engulfment of sAβ peptide and ABOs: Immunofluorescence. Primary microglia from CX3CR1-GFP (A) and wild type mice (B) were incubated respectively with 1 μM of fluo-555 sAβ peptide and ABOs, from 7pA2 supernatant, in presence or absence of 100 ng/ml NGF (10 μm scale bar, 20¡n¡30, $*$ p$\leq$0.05 $**$ p$\leq$0.001, Kolmogorov-Smirnov test).

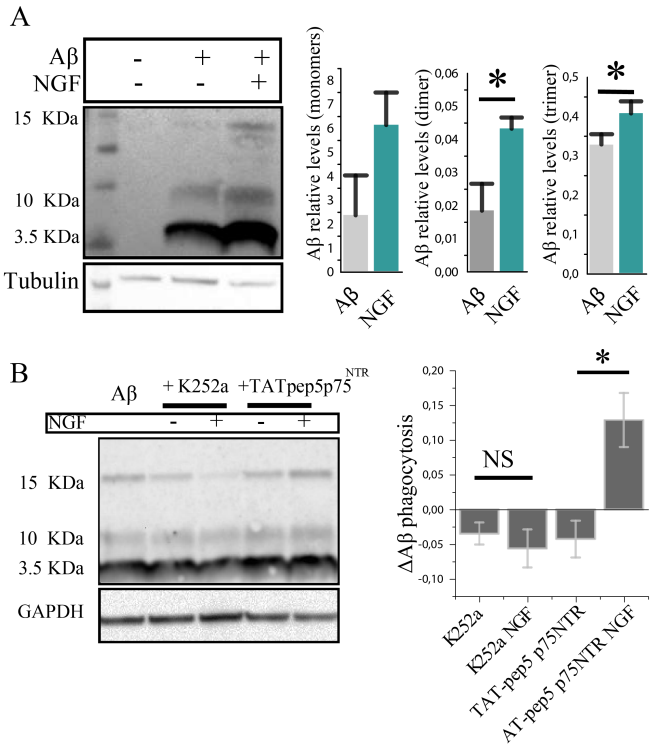


Figure 2.19: The modulation of microglial phagocytic activity is TrkA dependent. (A) Western Blot of primary microglia treated with 1μM of sAβ with or without NGF. Values are expressed as relative levels to controls (mean $\pm$ SD, $*$ p$\leq$0.05, Student's T-test). (B) Primary microglia treated with 200 nM K252a, intracellular TrkA inhibitor and with 1μM TAT-pep5 p75NTR, inhibitor of p75NTR intracellular signaling. Values are normalized to the signal of samples treated with only sAβ (mean $\pm$ SD, $*$ p$\leq$0.05, Student's T-test).

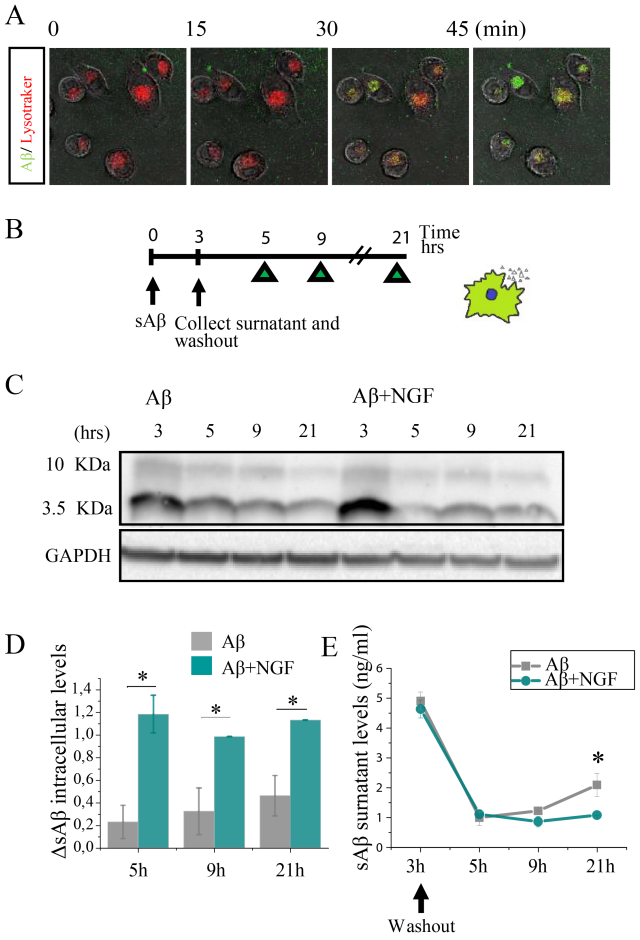


Figure 2.20: sAβ is digested and not released in the extracellular environment in cells treated with NGF in BV2 microglial cells. (A) Soluble Aβ is rapidly trafficked to lysosomes for degradation. Confocal imaging of live BV-2 microglia 45 min of 1 μM soluble Aβ1-42-488 demonstrated localization of Aβ (green) within lysosomes. Lysosomes were stained using LysoTracker (red). (B) Experimental design for the degradation experiment (C) Western blot of cells lysate. (D) The histogram shows the degradation measure as the delta between the protein levels at the n time point and (n+1) time point and thus represents the amount of protein that has been digested from one time point to the other. (E) Extracellular Aβ levels measured by ELISA. The data represent the outcome of three independent experiments (mean $\pm$ SD, $*$ p$\leq$0.05, Student's T-test).

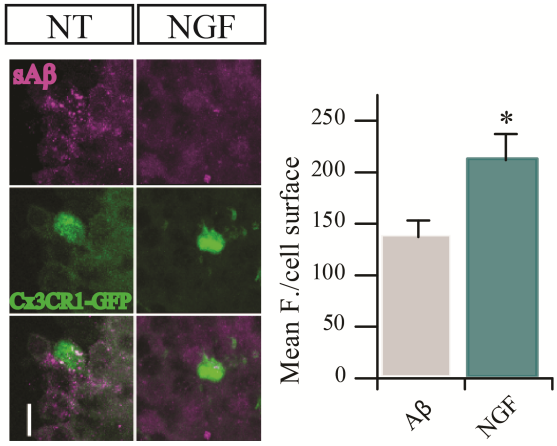


Figure 2.21: NGF increases the engulfment of sAβ ex vivo. Representative images of confocal stack acquisitions from 45 μm Cx3Cr1-GFP slices. Ex vivo 200 μm slices were first treated with 0,1 μM sAβ and with/without of 100 μg/ml NGF then analyzed by IF for Aβ content (mean $\pm$ SD, $*$ p$\leq$0.05, Student's T-test.

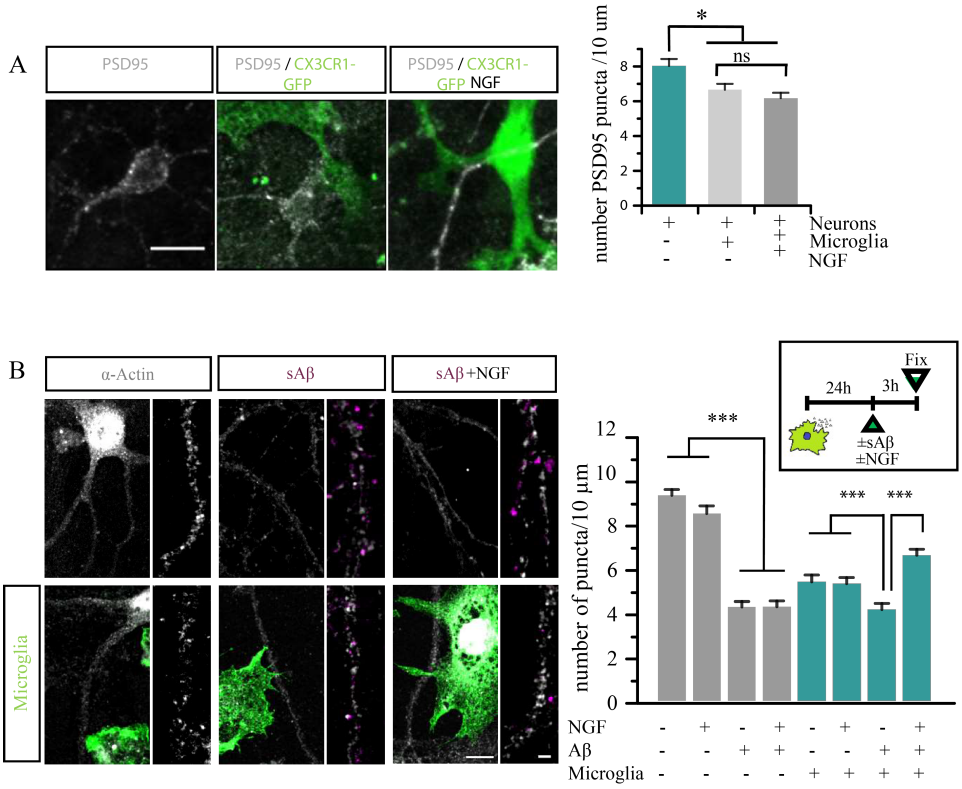


Figure 2.22: NGF protects against Aβ-induced spines toxicity in a microglial dependent fashion. (A) Representative images from confocal acquisition show PSD95 (white) puncta in neurons and in co-cultures +/- NGF. (B) Representative images of confocal acquisition of neuronal spines labeled with actin (white) and the dendrites magnification +/- sAβ (violet) in microglia (green)-neuronal co-cultures; (200¡n spines¡500 for two independent experiments, mean $\pm$ S.E.D $*$ p$\leq$0.05 $* * *$ p$\leq$0.001, one-way ANOVA test, followed by a post-hoc Bonferroni test).

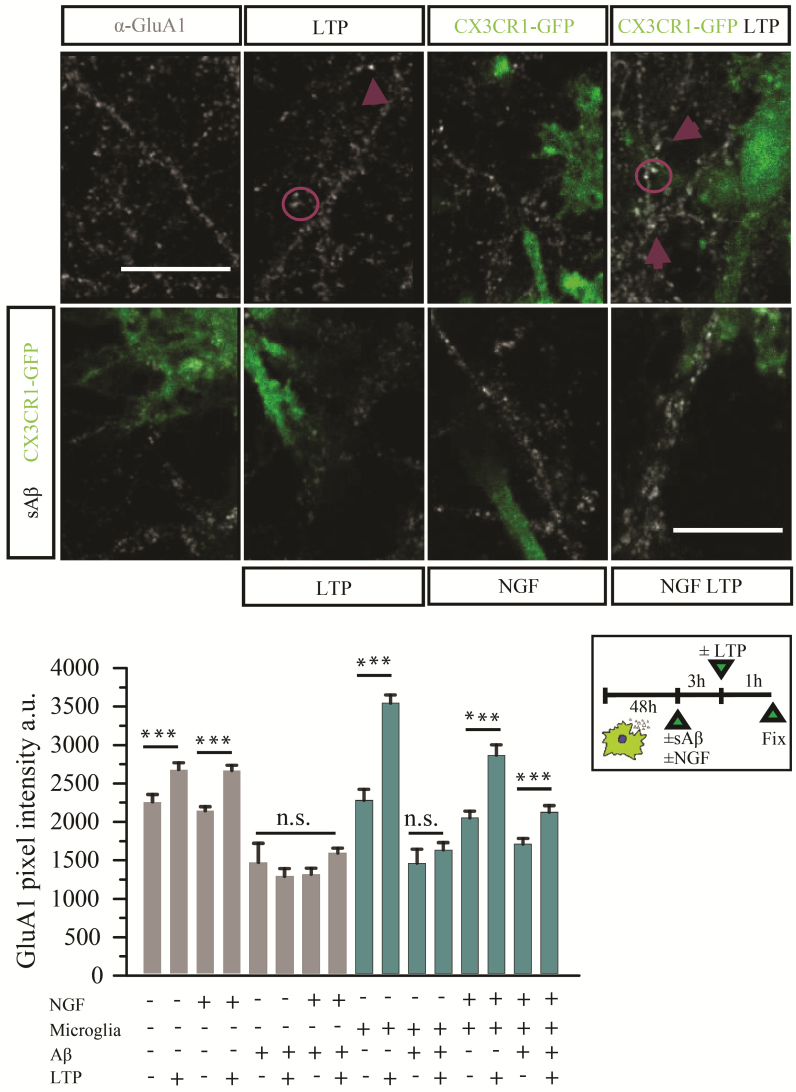
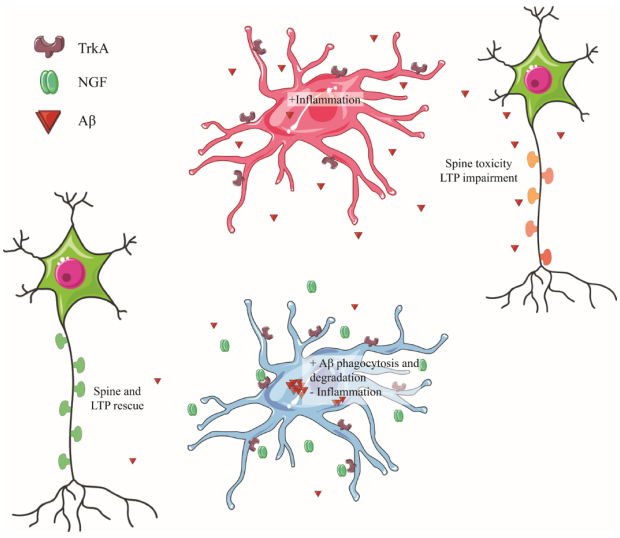


Figure 2.23: NGF protects against Aβ-induced loss of potentiation in neuron-microglia cocultures. (A) Effect of GI- LTP induction on GluA1 receptors (white) in different conditions, +/- microglia (green), +/- NGF, +/- sAβ. Histograms show the values of each experimental condition. (200<n spines<500 for two independent experiments, mean $\pm$ S.E.D $*$ p$\leq$0.05 $*$ $*$ $*$ p$\leq$0.001, one-way ANOVA test, followed by a post-hoc Bonferroni test).

Results highlights

- Microglia are NGF target cells.
- NGF modulates microglial motility and affects neuronal activity and physiology via microglia.
- In the context of Aβ pathology, NGF induces a neuroprotective and anti neuroinflammatory phenotype in microglia.



Chapter 3

Discussion

So far in the literature, despite some scattered evidence [345] [328] [147] [347] [348] [12] no clear systematic evidences about microglia relationship with NGF are reported and nothing is known about a potential neuroprotective role of NGF mediated by microglia.

Indeed, the main cellular targets of the neurotrophin Nerve Growth Factor (NGF) [14] in the central nervous system are considered to be the cholinergic neurons of the basal forebrain (BFCNs) [15], while its sources are mainly cortical and hippocampal neurons [16]. Interfering with trkA-NGF signalling has been related before to mechanisms driving neurodegeneration: in the AD11 transgenic mouse model [17] [18] the expression of anti NGF antibodies selectively neutralizing mature NGF in the adult brain determines a progressive comprehensive neurodegeneration, with neuroinflammation as the earliest observed change, at a presymptomatic phase [19] [20]. A similar progressive neurodegeneration is observed in transgenic mice expressing a neutralizing anti-TrkA antibody in the adult brain [18] Changes in NGF homeostasis in the brain have also been linked to Alzheimer's disease [22] [378]. On the other hand, the loss of NGF-TrkA signaling in the CNS, obtained by conditionally deleting NGF or TrkA genes in CNS

cells derived from nestin-positive cells, has proven not to be sufficient in inducing cognitive impairments in mice [23], suggesting to us that another non neuronal target cell of NGF might be involved in the occurrence of the neurodegeneration phenotype in the AD11 mouse model.

In this thesis I have provided stringent evidence that microglia are target cells for NGF in vitro and ex vivo. My analysis on the mouse cortex and primary microglia demonstrates that microglia express the NGF receptor TrkA. In these cells, NGF is able to elicit the appropriate intracellular signaling, resulting in a modulation of a variety of physiological activities of microglial cells. The activity of microglia is intimately associated with their morphology. Most of the functions of these cells are accomplished through structural changes [5] [40]: from the extension and retraction of their branches in response to physiological stimuli to the migration of the entire cell body to the site of injury. Microglia motility has also been recently correlated with the ability of pruning synapses [2], and therefore represents an important feature when trying to estimate microglial activity in physiological and pathological situations. My gene expression profiling data and time-lapse recordings respectively suggest a modulation of genes involved in cytoskeletal reorganization and variations in membrane dynamics after incubation with NGF. Then I focused on the possible consequences of NGF activity on microglial cells in pathological conditions by using Aβ as a gold standard. Consistently with the hypothesis of neuroinflammation induced by anti-NGF deprivation [20], I found that microglial cells treated with NGF not only respond in a non-inflammatory fashion, but also they seem to be refractory to the inflammatory stimulus of Aβ. Hence, we conclude that NGF is capable of reversing the inflammatory effect brought on by Aβ, while resulting only slightly anti-inflammatory. These protective effect was suggested also by Alexaki et al in their recent research where they demonstrated how the dehydroepiandrosterone (DHEA), the most abundant circulating steroid

hormone in humans, binding to TrkA, exerts its neuroprotective effect reducing the microglia mediated inflammation response through TrkA phosphorilation. In this way they demonstrated how TrkA signaling is a potent regulator of microglia mediated inflammation [379], confirming our results of protective effect of NGF on microglia activity.

In my analysis it was also shown that NGF is capable of enhancing specifically one type of endocytic process in microglial cells, macropinocytosis, which is the mechanism of choice through which microglial cells clear sAβ [257]. Thus, as a further step in demonstrating that NGF neuroprotective activity is mediated by microglia, I demonstrated that, by enhancing macropinocytosis, NGF promotes sAβ clearance in vitro and ex vivo.

Actually increasing the uptake of sAβ is not enough to provide a long term protection over the toxicity of the peptide, since internalized sAβ might be shed again into the medium prior to its degradation. In fact, microglia can release internalized fibrillar Aβ and convert it in neurotoxic forms through the shedding of microvesicles [367]. Moreover, it is still not clear if microglial cells are actually able to digest sAβ efficiently [114] [380][381], due to the evidence suggesting that microglia near plaques is functionally impaired [382]. Here, I showed that NGF not only increases Aβ uptake by microglia but enhances its degradation, consistent with the transcriptome modulation involving lysosome and degradation pathways. Alzheimer's Disease has been described as a synaptopathy, entailing a dysfunction of synaptic function [383] [384] [385]. Synapse loss is indeed an early sign of AD and the process has been directly correlated with Aβ as the most likely culprit. High concentrations of Aβ or Aβ oligomers inhibit the synaptic plasticity responses [386] [387] [375]. Aβ has been proven to be a key player in synaptic plasticity also at physiological concentrations: while short exposures with low concentrations of the peptide actually enhance synaptic plasticity, longer exposures lasting several hours reduce it [388]. This underlines the importance of the homeostasis of

Aβ peptide levels and thus of microglia as an important factor in its clearance and in this homeostatic process. Under physiological conditions microglial cells regulate dendritic spines, either pruning away superfluous spines during development [154] or increasing spine density as observed in the developing somatosensory cortex [140] [141]. We therefore assessed whether NGF is capable of modulating Aβ effect on spine number reduction, in relation to this microglia-neuron communication. In microglia-neuron co-cultures, NGF proves to rescue the spine loss mediated by Aβ, an effect that is specifically dependent on microglia. Plasticity was also studied in vitro by evaluating the efficacy of chemical LTP in the presence of microglia. Interestingly, spine potentiation, measured as AMPAR intensity increase, is stronger on neurons when cultured with microglia. While Aβ causes a dramatic loss of efficacy of chemical LTP in neuron-microglia co-cultures, NGF is able to fully rescue spine potentiation in these conditions. The effect is completely dependent on the presence of microglia in the cultures, since neurons alone do not show the rescue in response to NGF. Intranasal administration of an NGF variant was recently proven to be highly neuroprotective in an AD mouse model. 5xFAD mice chronically treated with the neurotrophin showed a dramatic reduction of the plaque load, with a clear evidence of the involvement of microglial cells in the clearing of Aβ [389]. In that study, the neurotrophin, added to 5xFAD slices (which present LTP deficits), determined a rescue of plasticity, which was dependent on TrkA activity. Our results go into the direction of attributing these events to the action of NGF on microglial cells. Indeed, our electrophysiological recordings on neurons suggest that by affecting microglial physiological activity, NGF is capable of influencing glutamatergic transmission. Indeed, we demonstrate that tampering with NGF-TrkA signaling in microglia affects negatively glutamatergic neurotransmission. Thus, NGF-activated microglia might result neuroprotective in Aβ pathology not only by lowering the amount of circulating Aβ - per se toxic

to neurons - but also by aiding neurons in plasticity tasks. As a message from neurons to immune cells, NGF can serve as a regulator of the activity of the immune system on the nervous system: a mediator of neuroimmune communication. To sum up, these data are pointing towards the myeloid cells of the brain as the culprit for the severe neurodegeneration observed in anti NGF or anti TrkA mice. Indeed, NGF is capable of steering microglia toward a neuroprotective phenotype against Aβ, adding an important element to the rationale for the therapeutic use of NGF in AD.

As a consequence of our results showing the crucial role of NGF in regulating microglia activities and physiological functions, I postulate that the reduced NGF activity might play an early causal role in the onset of the progressive AD11 neurodegeneration phenotype, modifying the morphology of microglia in vivo, and above all modifying the ability of these cells to conduct their physiological protective function in the brain. In order to be able to charge the lack of NGF signaling on microglia as being responsible for the AD phenotype, in future studies it would be interesting to verify directly the role of NGF signaling loss on microglia cells. To identify the specific and direct role of NGF signalling on microglia to the onset of neurodegeneration in the mouse brain, and, more generally, to the onset of AD eziopathology in humans, it would be useful to design a transgenic animal model with a conditional TrkA gene deletion in microglia, and investigate whether this is sufficient to trigger a progressive neurodegeneration as that observed in the AD11 model.

Thus, if the lack of NGF is responsible for the AD phenotype, due to the loss of NGF-TrkA activity in microglia cells, this mouse model should present a progressive AD like phenotype, as in AD11 mice, with changes in microglia morphology and inflammatory activation at the early stages. We cannot exclude that the selective deletion of TrkA gene in microglia cells might on its own not be sufficient to trigger neurodegeneration, whose onset might require a second independent

signal. In any case, these future ongoing experiments would provide a definitive clarification of the early causes and of the effector cells involved in the progressive neurodegeneration observed in the anti NGF AD11 mice and, by extension, in the human pathology. More important, in a therapeutic perspective the results I have obtained in the thesis provide a strong rationale for the use of NGF as a therapeutic neuroprotective agent in the brain for a large class of neurodegenerative disorders, regardless of the particular neuron population involved.

Chapter 4

Materials and Methods

Animals

Adult C57BL/6 (Charles River Laboratories, Como, Italy) mice expressing green fluorescent protein under the fractalkine receptor promoter CX3CR1 (Cx3cr1/GFP+/+ mice) and B6129 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Genotyping of CX3CR1-GFP mice was performed by PCR analysis of tail DNA (IDT 14276: 5'-GTC TTC ACG TTC GGT CTG GT-3', IDT 14277 5'-CCC AGA CAC TCG TTG TCC TT-3', IDT 14278 5'-CTC CCC CTG AAC CTG AAA C-3'). AD11 mice and VH mice control (Ruberti, Capsoni et al. 2000), 1 and 6 months old, were used. Littermates were used as controls for the IHC, since AD11 has a C57BL/6 x SJL background. Genotyping was performed by PCR analysis of tail DNA. All experiments with mice were performed according to the national and international laws for laboratory animal welfare and experimentation (EU directive n. 2010/63/EU and Italian DL n. 26 04/03/2014). Mice were kept under a 12-h dark to light cycle, with food and water ad libitum.

Cell cultures

The immortalized BV-2 murine microglial cell line (Blasi, Barluzzi,

Bocchini, Mazzolla, Bistoni, 1990) was grown and maintained in RPMI (Thermo Fisher Scientific, MA, USA #11835-063) medium containing 1% penicillin/streptomycin (Euroclone, MI, Italy #ECB3001D), 1% Glutamax (Thermo Fisher Scientific, MA, USA; #35050-038) and 10% fetal bovine serum (FBS) (Euroclone, MI, Italy #ECS0180l) in 5% CO2 at 37°C. Primary microglial cells were derived from the brains of B6129 or Cx3cr1/GFP+/+ mice at postnatal day 3-4 as previously described (Butovsky et al., 2014). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM/F12) (Thermo Fisher Scientific, MA, USA #21331-020) containing 1% penicillin/streptomycin, 1% Glutamax and 10% FBS in 5% CO2 pH 7.4 at 37 °C. Microglia were separated from the mixed primary glial cultures by mild shaking, they were re-suspended in DMEM/F12 with 1% penicillin/streptomycin, 1% Glutamax and 10% FBS - this is the standard culture medium unless otherwise stated - and plated on the appropriate support 18hrs before the experiments to allow cells to attach to the substrate. Primary cortical and hippocampal neurons were prepared at postnatal day 0 as described (Beaudoin et al., 2012). Briefly, animals were decapitated, the brain was rapidly excised and placed into ice-cold Hanks Buffered Saline Solution (HBSS) (Thermo Fisher Scientific, MA, USA; #14180046). Hippocampi and cortex were removed and digested for 15 min at 37°C in DMEM-F12 containing 0.1% of trypsin (Thermo Fisher Scientific, MA, USA). Thereafter, tissue was transferred in culture medium containing 10% FBS and gently disrupted using a flame-polished Pasteur pipette. Following centrifugation at 4°C for 8 min at 800 r.p.m. cells were resuspended in fresh DMEM containing 1% Glutamax, 10% FBS, 2% B27 supplement (Gibco, MA, USA, #17504-044), 6 mg/ml Glucose, 12,5 μM Glutamate, 10μg/ml Gentamicin (Gibco, MA, USA, #15710-049) and plated at a density of approx. 150,000 cells per coverslip after proper poly-D-lysine coating (Sigma Aldrich, MO, USA, #P1024). Cells were kept at 37°C in 5% CO2. After 12-24 hours, medium was replaced with pre-warmed Neurobasal A

medium (Thermo Fisher Scientific, MA, USA #10888-022) containing 2% of B27 supplement, 2.5 μM Glutamax, and 10 μg/ml Gentamicin. The second day 2.5 μM AraC (Sigma, Aldrich, MO, USA; #C1768) was added at the medium. The experiments were performed at DIV 17-19.

Immunoblot analysis

NGF signaling: Primary B6129 microglia were plated in 6 well plates at a density of 5X10^5/well in culture medium. After 18 hrs, cells were serum-starved for 16 hrs before the start of the treatments. Cells were treated for 0, 5, 15, 30 min with NGF 100 ng/ml and sequentially collected and lysed in ice-cold RIPA buffer (50 mm Tris-HCl, pH 7.6, 150 mm NaCl, 1% Igepal, 1 mm EDTA, 1% SDS, 0,5% sodium deoxycholate, 1x protease and phosphatase inhibitor cocktails (Roche, Basel; CH)). After sonication, cells were collected by centrifugation for 15 min at 4 °C (13,000 r.p.m.). Protein concentrations of the cell lysates were measured using the Bradford method. Lysates (20 *micron*g) were then separated on a 10% SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blotting. Phagocytosis of Aβ: Primary B6129 microglia were first plated in 6 well plates at a density of 5X105/well in culture medium. After 18 hrs, they were treated with 1 μM soluble Aβ for 3 hrs with or without 100 ng/ml NGF. After collection, they were lysed in ice-cold RIPA buffer and electrophoresed on a 4-12% NuPAGE Bis-Tris precast gel (Thermo Fisher Scientific, MA, USA; #WG1401BX10). After transfer in nitrocellulose, the membrane was boiled in PBS for 10 min, blocked for 1h and incubated with the appropriate primary antibodies. Inhibitors of NGF-receptors used: 200 nM K252a (Abcam, Cambridge, UK; #ab120419), TAT-pep5 p75NTR (Millipore, CA, USA; #506181) (Yamashita Tohyama, 2003), were added 30 min before Aβ and NGF. The following primary antibodies were used: anti-Aβ 1-16 1:1000 (clone 6E10 #SIG-39320); anti-TrkA 1:1000 (Millipore, CA, USA; #06-574), anti-pTrkA 1:1000 (Y794) (Rajagopal, Chen, Lee, Chao, 2004) kindly provided by M. V.

Chao (New York University School of Medicine, New York, USA) anti Akt 1:1000 (Cell Signaling Technology, MA, USA; #C67E7) , anti p-Akt 1:1000 (Cell Signaling Technology #130386), ant-Erk 1:1000 (Promega, WI, USA; #V114A), anti-pErk 1:1000 (Cell Signaling Technology, MA, USA; #4370S), anti-c-Jun 1:1000 (Cell Signaling Technology, MA, USA; #60A8) , anti-phospho-c-Jun 1:1000 (Cell Signaling Technology, MA, USA; #9261), anti-p75 1:1000 (Millipore, CA, USA; AB1554), anti-GAPDH 1:20000 (Fitzgerald, MA, USA; #10R-G109a), anti-tubulin 1:20000 (Sigma Aldrich, MO, USA; #T5168). After incubation with the appropriate HRP-conjugated secondary antibody (Santa Cruz, TX, USA; anti-mouse #sc-2005, anti-rabbit #sc-2004), membranes were developed using ECL-enhanced chemiluminescence kit (Bio-Rad, CA, USA). Densitometric analyses were performed using the NIH ImageJ 1.44p software.

Immunocytochemistry

Immunofluorescence for NGF receptors: Primary microglia were plated on coverslips in 24-well plates coated with poly-D-lysine ($1x10^5$ cells/well) in culture medium. After 18 hrs, cells were fixed with 2% PFA, and blocked for 1 hr at room temperature. Coverslips were incubated overnight at 4°C in primary antibody: anti-Iba1 1:500 (WAKO, Osaka, Japan; #019-19741) or anti-Iba1 1:500 (Abcam, Cambridge, UK; #Ab107159), anti-TrkA 1:100 (MNAC13 (Covaceuszach, Cattaneo, Lamba, 2005)), anti-P75 1:500 (Millipore, CA, USA; AB1554). Immunofluorescence for Aβ uptake: Primary microglia were plated on coverslips in 24-well plates at a density of 1x105 cells/well in culture medium. Then they were treated with 1 μM soluble 555-labeled Aβ (s555-Aβ) (Anaspec, Fremont, CA, USA; #As-60480) or AβOs (Meli et al., 2014) for 3 hrs in either presence or absence of 100 ng/ml NGF. Cells were washed three times with cold PBS, fixed in 2% PFA and permeabilized with 0.1% Triton X-100. Cells were blocked for 1 hr and stained with primary antibodies anti-Iba1 1:500 (WAKO, Osaka, Japan; #019-19741;) and with anti Aβ antibody A13 1:1000 (Meli,

Visintin, Cannistraci, Cattaneo, 2009), then they were washed three times with PBS and incubated with mouse antibody anti-epitope V5 (Sigma Aldrich, MO, USA #V8137;1:5000). After three washings, cells were incubated for 1 hr with appropriate secondary antibodies (1:500) (anti-rabbit Alexa-Fluor 555, anti-mouse Alexa-Fluor 488, Thermo Fisher Scientific, MA, USA; A-21428;A-21201). Coverslips were mounted on glass slides in Fluoromount (Sigma Aldrich, MO, USA #F6057) and confocal images were acquired using a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Immunofluorescence (IF) on slice

IF for NGF receptors/microglia/astrocytes detection: Adult (P80-90) C57BL6J mice were sacrificed with a lethal dose of carbon dioxide and immediately underwent a perfusion procedure. Blood was firstly transcardially washed out with cold phosphate buffer saline solution (PBS), then tissues were fixed with cold 4% paraformaldehyde in 0.1 M pH 7.4 phosphate buffer (PBS) with a peristaltic pump. Brains were dissected, postfixed for 18-22 hrs at 4°C, washed from paraformaldehyde with PBS and cryoprotected in 30% sucrose/PBS at 4°C overnight. Dry ice frozen brains were cut into 40 μm coronal sections with a cryostat microtome (Leica Microsystems, Wetzlar, Germany) at -20°C, including neocortex. Sections were rinsed for three times in PBS and incubated with a mix of primary antibodies in PBS 0.3% Triton X-100 (Applichem, BioChemica, Darmstadt, Germany) overnight at room temperature. Microglia was stained with rabbit anti-Iba1 (Wako, Osaka, Japan, 019-19741, 1:800) or rat anti-CD11b (Serotec; Kidlington, UK, MCA711, 1:300) depending on the second primary antibody used. Astrocytes were stained with rabbit Anti-Glial Fibrillary Acidic Protein (Dako, Cytomation, Glostrup, Denmark, Z0334, 1: 500) or goat Anti-Glial Fibrillary Acidic Protein (Santa Cruz Biotechnology, California, USA, sc-6170, 1:300). NGF receptors were identified by anti-TrkA (clone MNAC13, 1:300, (Cattaneo et al., 1999)) and anti-P75 (Promega Corporation, Madison, WI, USA,

G3231, 1:300). After three 10 min rinses in PBS at RT, sections were incubated for 2 hrs at RT in a mix of the appropriate secondary antibodies - anti-mouse/rabbit/goat/rat Alexa-Fluor 488/555/649 conjugated (Thermo Fisher Scientific, MA, USA; A-21428 diluted 1:500) - followed by three 10 min rinses in PBS. DAPI was applied for 5 min in the second rinse, dissolved in the PBS solution. To exclude non-specific signals of secondary antibodies, sections from each group of animals have also been stained with secondary antibody alone, following the same experimental procedure but omitting the primary antibodies.

Flow cytometry for phagocytosis analysis of beads, dextran or Aβ

Sample preparation: Primary microglia were plated in 6 well plates at a density of $5x10^2$ cells/well. The fluorescent material to be phagocytosed - was placed in the culture medium 3 hrs after treatment with NGF 100 ng/ml. Beads were first opsonized in 50% FBS and PBS for 1 hr RT (Polybead DyedRed 6 μm, Polyscience; PA, USA; Cat#15714), counted with the Burker chamber and given to cells at a concentration of roughly 3 beads/cell. Dextran was used at 2.5 mg/ml (Thermo Fisher Scientific, MA; USA, #D1841 RhodamineB 70,000 MW) while HiLyte Fluor 555 Aβ42 (Anaspec, Fremont, CA, USA; #As-60480) was used 1 μM. Cells were exposed to the material for 1 hr, then they were washed extensively with PBS, and fixed with 2% paraformaldehyde for 7 min. Cells were washed again with PBS and collected for analysis. Data acquisition: A Sorter S3 (BioRad, CA, USA) with a single 488 nm (100 mW) excitation laser was used. The gating strategy was decided on the FSC and SCC scatter plots, in order to gate out debris. Filters are based on the emission spectra of the fluorochromes: RhodamineB for dextran, DyeRed for beads, HiLyte Fluor 555 for Aβ42 - 580-650nm (red channel). The total amount of beads, dextran or Aβ internalized by cells was determined by analyzing the population positive for the fluorescent marker conjugated with the material.

The analysis was performed using the FlowJo software (FlowJo, LLC, Ashland, Ore., USA). List of concentrations and time of treatment for inhibitors and activators of macropinocytosis and phagocytosis used to determine the specific process of internalization activated by NGF administration: IFNγ 10 ng/ml (RD, Minneapolis MN, USA Cat. Number 485-MI), Amiloride 50 μM (Sigma Aldrich, MO, USA; #A3085), CytochalasinD 10 μg/ml (Sigma Aldrich, MO, USA; #C827). These were added 20h before phagocytosis assay. Rho/Rac/cdc42 Activator I (Cytoskeleton, CO, USA; cat. #CN04) was added after a 2 hrs FBS starvation period and 1 hr and 30min before the beginning of the experiment with beads and dextran. Phorbol 12-myristate 13-acetate (PMA) 100 nM (Sigma Aldrich, MO, USA #P8139) was added 3 hrs before the assay.

Microarray transcriptome analysis

Primary microglia were treated with 100 ng/ml NGF for 2 hrs, 8 hrs or 24 hrs. RNA isolation, amplification, and labeling was performed using an RNeasy mini kit according to manufacturer's protocol (Qiagen). Total RNA was isolated from these cells using Trizol (Invitrogen) and DNAse treated by Qiagen columns. Quality and integrity of each sample was checked using the Agilent BioAnalyzer 2100 (Agilent RNA 6000 nano kit): samples with a RNA Integrity Number (RIN) index lower than 8.0 were discarded. All the experimental steps involving the labeling, hybridization, and washing of the samples were done following the standard one-color microAgilent protocol. The gene expression profiling was performed using the Microarray Agilent Platform. 200 ng of RNA was labeled with Low Input Quick Amp Labeling Kit One-Color (Agilent Technologies), purified and hybridized overnight onto the Agilent 8X60K whole mouse genome oligonucleotide microarrays (Grid ID 028005) according to the manufacturer's instructions for one-color protocol. The Agilent DNA microarray scanner (model G2505C) was used for slide acquisition and spot analysis was performed with Feature Extraction software ver 10.7 (Agilent Technologies). Data

filtering and analysis were performed using R-Bioconductor and Microsoft Excel. All the features with the flag gIsWellAboveBG=0 (too close to background) were filtered out and excluded from the following analysis. Filtered data were normalized by aligning samples to the 75th percentile. Differentially expressed genes were selected by a combination of fold change and moderated T-test thresholds (R Limma test p≤0.05; —Log2 fold-change—¿1.0). Principal Component Analysis, Multi-Dimensional Scaling, Hierarchical Clustering of samples and volcano plots were computed using the open source R Studio (Boston, MA,USA).

Live cell imaging

To study microglial motility, primary microglia were plated ($3x10^4$ cells) on Glass Bottom Microwell Dishes (35mm), coated with poly-D-lysine, and left overnight to rest. Then cells were treated with 100 ng/ml NGF for 24 hrs. Cells were imaged for 1 hr through a 40X objective with a Leica SP2 confocal microscope. Cell dynamics was analyzed using a homemade Python script (number of cells per experiment = 29). The parameters taken into consideration were: Morphing speed measures how many times, during the acquisition, cells change their morphology. Two extremes were fixed as opposite morphological endpoints: roundish and polarized (with at least two ramification). We measured how many times cells shift between these two cell configurations. The parameter was used to classify the speed of changes in morphology. Cell membrane changes describes how cells change their Area (A) normalized on cell perimeter (p), in particular we measured $\Delta A/p$ between two consecutive frames (1 frame/min), giving us an intermediary to monitor membrane motility. For the experiment of Aβ lysosome colocalization, microglial BV-2 cells were plated overnight in RPMI containing 2μ FBS on precoated culture plates. Cells were incubated with 1 μg/ml Aβ-488 and 100 nmol/l Lysotracker-Red (Thermo Fisher Scientific, MA, USA; #L12492) and imaged using a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) for 1 hr

with a 63x/1.4NA HCX PL APO objective. We used BV-2 cells instead of primary since live imaging requires long hours and it is too damaging for primary cultures in our laboratory conditions.

Intracellular Aβ Clearance and degradation

BV2 cells were incubated in culture medium with 1 μM soluble Aβ42 (Anaspec, Fremont, CA, USA; #As-64129) for 3 hrs in the presence or absence of 100 ng/ml of NGF. Cells were then either collected (the 3 hrs time point) or the medium was changed after extensive washes with PBS to ensure the removal of Aβ42 in the supernatant. After this step, these cells were collected and lysed in ice-cold RIPA buffer (SDS 1%) after either 5 hrs, 9 hrs or 21 hrs of washout, in order to allow the measurement of the phagocytosed Aβ which could be either digested (and detected in the cell extracts) or expelled (and detected in the supernatant) at each time point - time points are not longitudinal, they are parallel experiments. After brief sonication, they were collected by centrifugation at 13,000 r.p.m. at 4°C for 15 min. The supernatant at each timepoint was also collected. Aβ42 levels in the cell lysates were determined by immunoblotting with the anti-Aβ antibody 6E10 (clone 6E10 #SIG-39320; 1:1000, Covance; NJ, USA). The samples were resolved with 4%-15% bis-tris SDS-PAGE. Aβ levels were measured and normalized on the housekeeping GAPDH total protein levels. The Aβ supernatant levels were measured using ELISA Kit (Human Aβ42 Invitrogen KHB3441). Optical density was read at 450 nm on a Bio-Rad plate reader. BV-2 cells - as opposed to primary microglia - were used because of the high number of cells needed for this experiment (RRR rule).

Aβ phagocytosis in ex vivo hippocampal slices

To prepare hippocampal slices, Cx3Cr1-GFP mice were deeply anesthetized (20% urethane solution, 0.1 ml/100g body weight) via intraperitoneal injection and decapitated after tail pinch reflex disappearance in order to perform the immediate dissection of brain tissue. Horizontal slices containing the hippocampal area (200 μm thick) were

obtained by a vibratome (Leica VT1200S). All of the above steps were performed in ice-cold ACSF solution (artificial cerebrospinal fluid, in mM: NaCl, 119; KCl, 2.5; CaCl2, 2; MgSO4, 1.2; NaH2PO4, 1; NaHCO3, 26.2; glucose, 10) bubbled with 95% O2 /5% CO2. Slices were stored in a recovery chamber containing oxygenated ACSF at room temperature, for at least 30 min prior to the addition of 100 nM s555-Aβ with or without 100 ng/ml of NGF. After 3 hrs, slices were fixed in 4% PFA for 18 hrs at 4°C. Slices were put in 30% sucrose, then they were sectioned into 45 μm thinner slices using a Leica microtome. Brain slices were mounted on glass slides and imaged using a 40X objective with a SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Electrophysiological recordings from neurons

Adult C57BL6 male mice were deeply anesthetized with isoflurane inhalation, decapitated, and brains removed and immersed in cold cutting solution (4°C) containing (in mM): 126 choline, 11 glucose, 26 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 10 MgSO4, 0.5 CaCl2 equilibrated with 95% O2 and 5% CO2. Coronal slices (300 μm) were cut with a vibratome (Leica) and then incubated in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 26 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 2 MgSO4, 2 CaCl2 and 10 glucose, pH 7.4; initially at 32°C for 1h, and subsequently at room temperature, before being transferred to the recording chamber and maintained at 32°C. Recordings were obtained from visually identified pyramidal neurons in layer 2/3, easily distinguished by the presence of an emerging apical dendrite. Experiments were performed in the whole-cell configuration of the patch-clamp technique. Electrodes (tip resistance = 3-4 MΩ) were filled with an intracellular solution containing (in mM): K-gluconate 135, KCl 4, NaCl 2, HEPES 10, EGTA 4, MgATP 4 NaGTP 2; pH adjusted to 7.3 with KOH; 290 mOsm. Whole-cell voltage-clamp recordings (-70 mV holding potential) were obtained using a Muticlamp 700B (Axon CNS, Molecular Device). Action potential in-

dependent spontaneous excitatory postsynaptic currents (mEPSCs), recorded in the presence of tetrodotoxin (TTX) 1 μM and the GABAA receptor antagonist picrotoxin (100 μM), were filtered at 1 kHz, digitized at 10 kHz, and recorded on computer using Digidata1440A and pClamp10 software (Molecular Device). Series resistances were not compensated to maintain the highest possible signal-to noise and were monitored throughout the experiment. Recordings were discarded if Rs changed 25% of its initial value. Spontaneous events were detected and analyzed with Clampfit 10.4 using amplitude and area thresholds set as a multiple (3-4X) of the SD of the noise. Each event was also visually inspected to prevent noise disturbance of the analysis. Each slice received only a single exposure to NGF (20 g).

Electrophysiological recordings from microglia cells

Acute cortical slices (250 μm) were obtained from CX3CR1+/GFP male mice (P18-P30) using the identical experimental procedures described in the above paragraph (recordings from neurons). After recovering for at least 1h at RT, each slice was transferred in the recording chamber under the microscope and perfused (2 ml/min) with warmed ACSF (32°C). Visually identified GFP-expressing cortical microglial cells were patched in whole-cell configuration. Micropipettes (5-6 MΩ) were filled with solution containing the following composition (in mM): KCl 140, EGTA 0.5, MgCl2 2, HEPES 10, and Mg-ATP 2 (pH 7.3 adjusted with KOH, osmolarity 290 mOsm; Sigma Aldrich). Voltage-clamp recordings were performed using a Muticlamp 700B (Axon CNS, Molecular Device). Currents were filtered at 2 kHz, digitized (10 kHz) and collected using Clampex 10 (Molecular Devices); the analysis was performed offline using Clampfit 10 (Molecular Devices). Slicing procedure might activate microglial cells especially near the surface of the slice, therefore recordings were performed on deep cells. Cells were clamped to a holding potential of -20mV. The current/voltage (I/V) relationship of each cell was determined applying voltage steps from -140 to +60 mV (DVm 20mV) of 250 ms duration with interval

of 5 seconds after whole-cell configuration was achieved (HP = -20 mV between steps). Current values for each given voltage step were measured in the last two-thirds to avoid contamination of capacitance artefacts. Resting membrane potential and membrane capacitance were measured at start of recording. NGF was applied in bath for 10 minutes. One to four cells per mice were recorded. At least four animals per group were used.

Neuron/microglia co-cultures

For neuron/microglia co-cultures, at DIV 17-19 for neurons, primary microglia were seeded onto cultured hippocampal neurons ($1x10^5$ cells/well). The culture was maintained in Neurobasal-A supplemented with 2% B27, 2mM L-Glutamine and 10 ug/ml gentamicin and used after 24 hrs for experiments. Co-cultures were treated with soluble Aβ-555 (100 nM), and 100 ng/ml NGF for 3hrs, fixed in 2% PFA and 5% sucrose for 10 min, washed in PBS and blocked for 1 hr at room temperature in BSA 1%. Incubation with primary antibody was performed at the following concentrations: anti-PSD95 1:500 (Abcam Cambridge, UK; ab9909), anti-actin 1:500 (Sigma Aldrich, MO, USA A-3853;), anti GluA1 1:100 (Millipore, CA, USA; #AB1504;). For image acquisition, coverslips were mounted on glass slides in Fluoromount (Sigma Aldrich, MO, USA, F4680-25ML).

Chemical LTP

Cx3Cr1-GFP microglia ($2x10^4$ cells/well) were added to DIV 17 cultured hippocampal neurons. After 48hrs, the cultures were treated with soluble Aβ-555 (100 nM), with or without 100 ng/ml NGF for 3hrs. GI-LTP was induced as reported in the literature (Ahmad et al., 2012). Briefly, cultures were incubated for 15 min at room temperature in standard ACSF (in mM : 125 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2) with 0.02 mM Bicuculline and 0.001 mM TTX, then washed with Mg-free ACSF and treated for 7 min with Mg-free ACSF supplemented with 0.2 mM Glycine, 0.02 mM Bicuculline. After 7 min of stimulation, cultures were washed once in ACSF and left in culture

medium for 1h and fixed in 2% PFA for 10 min.

Measurement of inflammatory markers
Simultaneous detection of multiple cytokines was obtained using the Mouse Inflammation Antibody Array (Raybiotech, USA, Canada; AAM-CYT-6). Primary microglia from B6129 mice were plated in a 6-well at the concentration of $6.5x10^5$ cells/well in culture medium. After 18 hrs, cells were serum starved for 4 hrs, and later treated with Aβ 1 μM or 100 ng/ml NGF or Aβ and NGF simultaneously. Cells were lysed in ice-cold RIPA buffer (50 mM Tris/HCl, 150 mM NaCl, 1mM EDTA, 1% Igepal, 0.5% Sodium Deoxycholate, 0.1% SDS, Protease Cocktail inhibitor) and sonicated briefly, and then collected by centrifugation at 13,000 r.p.m. at 4°C for 15 min. Arrays were incubated with the appropriate blocking buffer for 2hrs. Eighty μg of protein extract were diluted in blocking buffer and incubated with the array overnight at 4°C. Then, arrays were washed accordingly and incubated for 3hrs at room temperature with the Biotinylated Antibody Cocktail solution. After washing, arrays were incubated with HRP-streptavidin for 2hrs and detected using the Detection Buffer. Images were captured using the Chemidoc detection system (Bio-Rad).

Image Analysis Images from AD11, VH and WT mice were acquired with a confocal laser scanning microscope (TCS SP2; Leica Microsystem, Wetzlar, Germany). 10 stacks per region per mice were obtained. On the Z axis, images were taken every 0.4 μm. Regions of interest for morphological changes in the microglia were the entorhinal cortex and the hippocampus(specifically the molecular layer). Image elaboration and analysis was conducted using Bitplane?s software Imaris (Zurich, Switzerland). Experiments in Fig. 4.6A, 4.17: 512x512 pixel images were acquired with a confocal microscope (Leica TCS SP2) using an oil objective: HCX PL APO 63.0X OIL (NA=1.40), and pinhole was set to 1 AU. Sequential illumination with Ar 561 and Ar 488 laser lines was used to detect, sAβ-555, ABOs, IBA1, TrkA, p75 immunofluorescence. Experiments in Fig. 4.8 were acquired in

2048x2048 pixel images with a confocal microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany) equipped with four laser lines: violet diode emitting at 405 nm, argon emitting at 488 nm, and helium/neon emitting at 543 and 633 nm using a HCX PL APO 40X OIL objective, 1 zoom factor , pinhole 1 AU. Points of colocalization were supposed when a merging area in the same cell was evident, showing a yellow resulting color from the overlap of two green-red signals, and they were verified by analysis on the z-axes with 1 μm-stacks. Experiments in Fig.4.21A-B and 4.22: 512x512 pixel images were acquired with a confocal microscope (Leica TCS SP5 on DM6000, equipped with MSD module) using an oil objective HCX PL APO CS 40.0X (NA=1.25), digital and pinhole was set to 1.5 AU. Sequential illumination with HeNe 633, DPSS 561 and Ar 488 laser lines was used to detect Alexa647 (used for PSD95, actin and GluA1 immunofluorescence), sβ-555, and GFP or IBA1 immunofluorescence, respectively. The Aβ intracellular levels was quantified by measuring the mean 555 fluorescence intensity in the area circumscribed by microglial cell perimeter using the segmented line tool in ImageJ. Dendritic spines were counted using ImageJ software. For this analysis, all dendritic protrusions with a clearly recognizable stalk were counted as spines. Spine number was divided by the length of the dendritic segment to generate dendritic spine density, expressed as number per micrometer. Chemical LTP was measured by quantifying the integral GluA1 fluorescence intensity of each spine.

Data Analyses and statistics

Data are presented as means $\pm$ s.d. unless otherwise noted, using Origin (OriginLab Corporation, MA, USA). Means were compared using the unpaired or paired t-test as indicated. Multiple comparisons were made using one-way ANOVA test, followed by a post-hoc Bonferroni test. The variance of each dataset was measured with an F test; $*$ $p \leq 0.05$, $**$ $p \leq 0.01$ and $***$ $p \leq 0.001$.

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