PhD Thesis in Neuroscience

A glial side to the neurotrophin field: studying the effects of neurotrophins on glial cells in the CNS.

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Foreword

"Before you know, you must imagine."

Richard Axel, Nobel Laureate

Glial cells are the support and sentinels of the central nervous system (CNS). If the neurotrophin field has been dominated by the neurocentric view that the primary target of these molecules must be neurons, the ever increasing evidence that glial cells are integral part of neuronal computation calls for a closer introspection as to whether glial cells themselves are capable of responding directly to neurotrophins.

A first part of my PhD has been spent studying, in vitro, the effect of the historically oldest neurotrophin, the Nerve Growth Factor (NGF), on one of the newest interests in neuroscience, **glia**. In particular, in the lab of Antonino Cattaneo and Simona Capsoni, we were interested in two very different glial cells of the CNS: the only immune cell of the brain parenchyma, the microglia, and the support cell by definition, the astrocyte.

Some interest in the effects of neurotrophins on glia had already arisen in the scientific landscape – and this was particularly true for the most intensively studied of them all, the brain derived neurotrophic factor (BDNF). First astrocytes, and most recently microglia, have found themselves in the middle of new relevant circuits, in which BDNF acts on or through glial cells to change how neurons respond and, ultimately, behavior (Parkhurst et al., 2013; Rose et al., 2003; Vignoli et al., 2016).

Our work – culminated in a co-first authorship paper along with Caterina Rizzi (Rizzi et al., 2018) – was though the first, to our knowledge, to address a relationship between the Levi-Montalcini Neurotrophin, NGF, and the CNS very own myeloid cell, microglia.

It was then in the lab of Dr. Wenbiao Gan (NYU Langone Medical Center), a world leader of 2-photon microscopy, that I continued my research, delving into the issues of translating our *in vitro* data into *in vivo* analyses of the effect of neurotrophins on glial cells. With the wider horizon of the complex system that is the awake behaving animal, I tried my best to explore the different sides of the matter of glial physiology – now both microglia and astrocytes – in response to different neurotrophins – NGF and BDNF. Finally, I also had the chance to evaluate neuronal responses by imaging pyramidal neurons and interneurons under the activity of neurotrophins.

My PhD at Scuola Normale Superiore has been a cradle for conciliation of the old and the new, one where advanced techniques were used to answer old questions, a brushing anew of the neurotrophin field.

I cannot help but feel bewildered by having had the chance to see a seemingly impossible link getting formed. I will always remember the first time that I have seen an astrocyte respond with calcium transients to my treatments. That is the magic of glia, a brand new world waiting to be uncovered.

Introduction

The Central Nervous System (CNS) is home to a myriad of cell types, different in ontogenesis, form and function. If the defining feature of a neuron is that of its electrogenic properties, for glia it was precisely the lack of an action potential that first brought scientist to categorize them as “glue”.

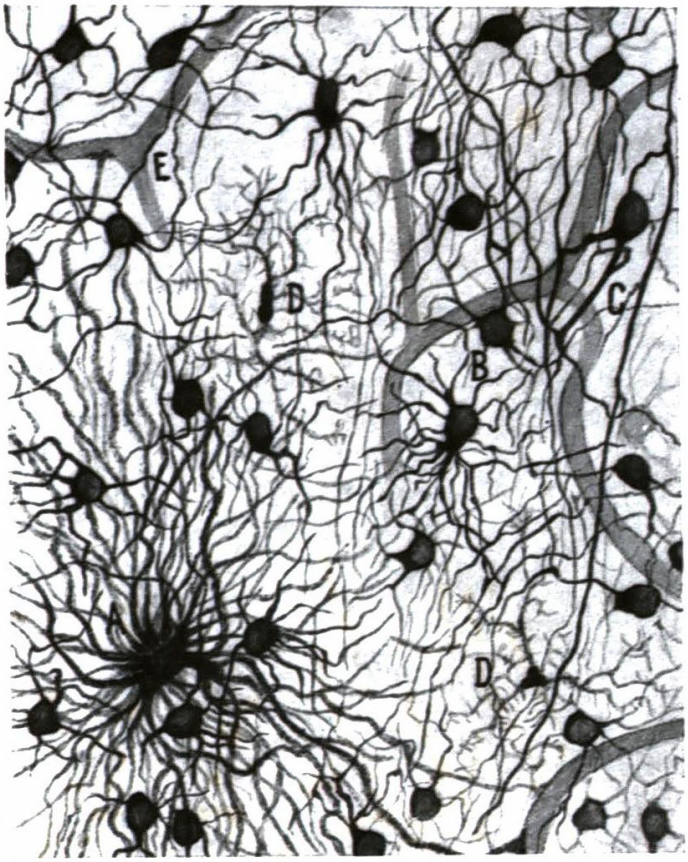

Figure 1. One of the first representation of glia in the brain of the cat cortex from Pio del Rio Hortega in 1922. (A) Fibrous astrocyte; (B) Oligodendrocyte type 1; (C) Oligodendrocyte type 2; (D) Microglia; (E) Blood vessels.

Neuroglia was first labeled by Rudolf Virchow in 1856, who described it as connective tissue containing cell bodies. However, before the concept of neuroglia, Robert Remak had already described in 1838 the cells surrounding nerve fibers, later on called Schwann Cells. Though important work in the glia field continued through the rest of the 19th century (like the historic literature by Heinrich Müller, Otto Deiters, Friedrich Merkel), it was then Pio del Rìo Hortega, a student of Cajal, that finally succeeded in defining and characterizing the *three glial cell types* in the CNS. In a series of seminal

papers from 1919-1928, he was able to properly distinguish *microglial cells* and *oligodendrocytes* from *astrocytes* (**Fig. 1**). The late glial neuroscience luminary, Ben Barres, in a splendid perspective back in 2008 published in Neuron, starts by noting that after a hundred years of glial biology we are still trying to answer the same fundamental questions posed since their discovery (Barres, 2008). And indeed many are the still open debates when it comes to glia, as there is still no definitive answer as to their function in physiology and disease.

What is particularly striking is that the more we find out about these supporting cells and their role in connections to neurons, the more the barrier that had always characterized the distinction between glia and neurons thins away. Thanks to the development of techniques that enable the monitoring of the activity of glial cells and glia specific genetic manipulations, if behavior has always been associated with neuronal firing and activity in general, now evidence is surfacing on how also glia can participate in complex plasticity paradigms and learning. Moreover, molecules once thought to be mostly neurotropic and neurotrophic are now emerging as important factors for glial physiology as well as for that of the neuron.

Since during my PhD I had the pleasure of working mostly on microglia and astrocytes, this introductory analysis of the existing literature will focus entirely on these two so different yet similar glial cells, throwing in there neurotrophins now and then to lay the groundwork of the rationale for a glial function of neurotrophins.

MICROGLIA

Microglia are the sentinels of the brain: they are the only immune cell population present in the brain parenchyma. This vantage position in the CNS enables these myeloid cells to perform the most disparate of tasks: from the classical immune functions of

fighting infections and surveilling the extracellular space for pathogens and damage, to sculpting the neuronal circuitry by pruning unnecessary synapses, to assist neurons in spine formation, aiding in the maintenance of brain homeostasis. As foreigners for ontogenesis from their surrounding neighboring cells, microglia constitute a wonder of neuroscience: a cell without possibility of electrogenesis taking part in processes of development, learning and memory in our fascinating brain.

Last to be properly identified in the glia family – by Pio del Rio Hortega in 1919 –, microglia stayed for decades at the fringes of neuroscience until Georg Kreutzberg's group rekindled interest in these cells in 1968. Their seminal work identified a peculiar activity carried out by activated microglia after facial nerve injury, the displacement of synaptic terminals from regenerating motor neurons (Blinzinger, K; Kreutzberg, 1968).

Microglia are surprisingly very heterogeneous cells: several papers of transcriptomic characterization came out last year, 2019, shedding light on the "various shades of gray, which mirror different topological locations, developmental stages, and possibly, divergent function" of microglia (Böttcher et al., 2019; Brioschi, Peng, & Colonna, 2019; Hammond et al., 2019; Q. Li et al., 2019).

If the molecular signatures of microglia are now resolved and defined in space and time, the functional consequence of such different expression profiles is not yet understood, though, one could certainly pose that the existence of these different subgroups indicates that microglia can differentially be involved, depending on brain region and state, in matrix remodeling, synaptic turnover, neurogenesis, removal of myelin debris, or maintenance of vessels integrity.

Origins

The true origin of microglia has remained uncertain until very recently. The controversy started with Del Rìo Hortega himself. On one hand, he reported that microglial

progenitors invaded the brain very early during brain development – arising from mesodermal cells in the pia mater. On the other hand, he also simultaneously proposed that "microglia may eventually arise from other related elements, chiefly the blood mononuclears," due to the functional and morphological similarities that microglia and their peripheral cousins share (Rio-Hortega, 1939). It has now been conclusively proved that microglia arise from the yolk sac (YS) – an extraembryonic mesoderm site of hematopoiesis – and enter the brain as amoeboid primitive macrophages prenatally, persisting and proliferating in the CNS into adulthood (**Fig. 2**) (Davalos et al., 2005; Ginhoux et al., 2010; Ginhoux & Prinz, 2015; Nimmerjahn, Kirchhoff, & Helmchen, 2005). In the YS, the earliest primitive wave of hematopoiesis occurs at embryonic day (E) 7.5 in mice, and this generates nucleated red blood cells and macrophages that go on to colonize the whole organism. These myeloid cells start invading the neuroectoderm at E9 before the closure of the blood brain barrier (BBB) that will restrict any further access to the brain parenchyma. At these early stages of development, in the brain, neural progenitors are giving rise to the first neuronal cells, and only later on they will generate oligodendrocyte and astrocytes, making microglia the main glial population during a good part of the life of the embryo. Outside of the CNS, YS-derived macrophages are gradually replaced by circulating monocytes coming from the later fetal and definitive bone-marrow hematopoiesis (Q. Li & Barres, 2018; Morgane S. Thion, Ginhoux, & Garel, 2018). Conversely, microglia, which are closed off from the circulation by the BBB, will act as a

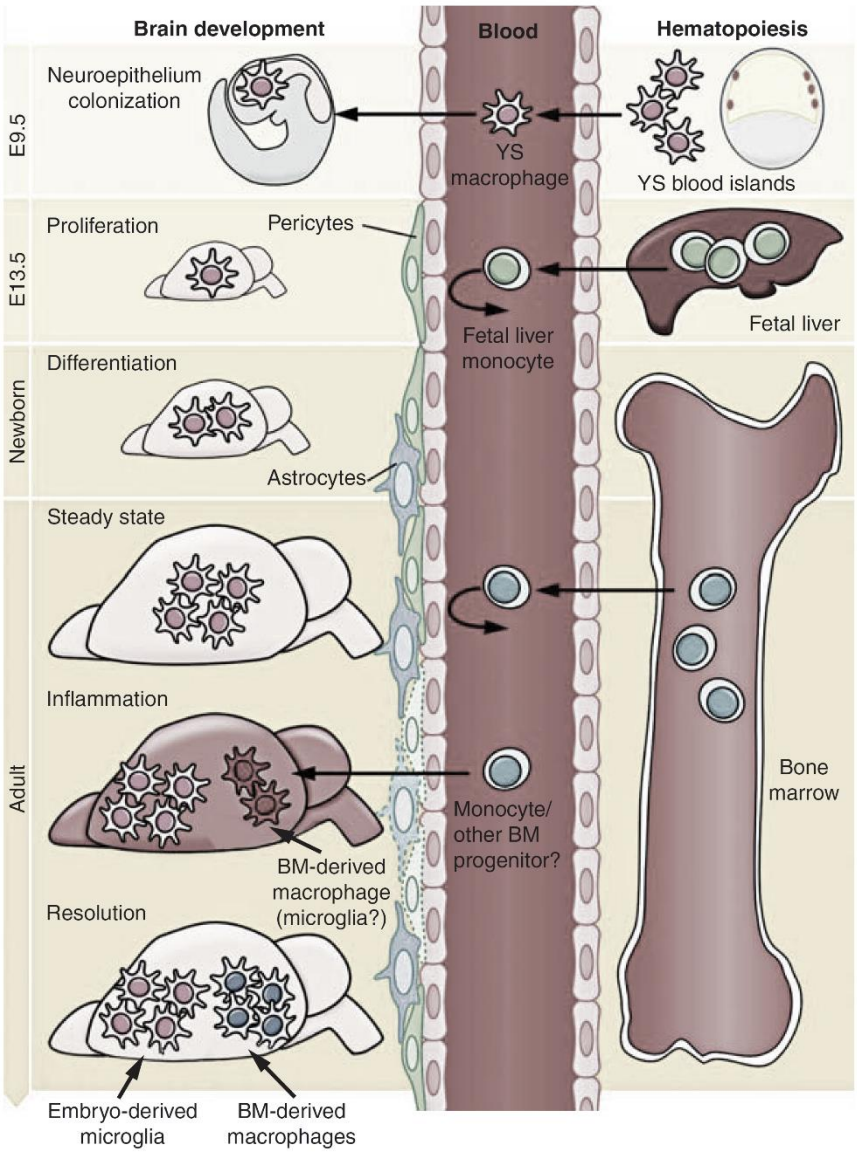

Figure 2. Brain development and microglial ontogeny. Primitive macrophages generated in the yolk sac (YS) blood islands around E8.0 spread into the embryos at the onset of blood circulation established around E8.5 and colonize the neuroepithelium from E9.0/E9.5, giving rise to embryonic microglia. In parallel, definitive hematopoiesis arises in the aorta-gonad mesonephros and gives rise to progenitors that colonize the fetal liver (FL) from E10.5. The blood– brain barrier (BBB) starts to form from E13.5 and may isolate the developing brain from the contribution of FL and, later, of bone marrow (BM) hematopoiesis. Embryonic microglial cells expand, colonize the whole CNS, and will maintain themselves until adulthood via local proliferation during late gestation and postnatal development, as well as in the injured adult brain in reaction to inflammation. Nevertheless, under certain inflammatory conditions found, for example, after BM transplantation, the recruitment of BM-derived progenitors can supplement the microglial population to some extent. Modified from Ginhoux & Prinz, 2015.

standalone population, and will only grow in numbers by self-renewal, at least under steady-state conditions.

Because of their peculiar mesodermal origin, microglia are known to share many of the features of tissue-resident macrophages: they express a similar molecular profile, including the myeloid marker colony-stimulating factor -1 receptor (CSF-1R), the integrin CD11b, the surface glycoproteins F4/80, the inhibitory immune receptor CD200R, the surface enzyme tyrosine-protein phosphatase nonreceptor type substrate or CD172a, the fractalkine receptor CX3CR1, and the calcium-binding protein lba-1 (Prinz & Mildner, 2011). There are, however, some markers, which are specific to microglia and help us set these two populations apart. Transcriptome-wise, microglia have a unique signature, which distinguishes them from other CNS cells and peripheral macrophages or monocytes (Gautier et al., 2012; Keren-Shaul et al., 2017), and express a unique cluster of transcripts encoding proteins for sensing endogenous ligands and microbes, referred to as the "sensome" (Hickman et al., 2013).

It is indeed remarkable to notice that early embryonic brain colonization by microglia is highly conserved across vertebrate species, suggesting that this process is essential for early brain development (Herbomel, Thisse, & Thisse, 2001; Swinnen et al., 2013; Verney, Monier, Fallet-Bianco, & Gressens, 2010). This has been yet again confirmed in 2019 with the publication of two papers reporting 9 human cases of homozygous mutation of the CSFR1: patients where microglia fails to develop and are thus completely devoid of these myeloid cells in the brain (Guo et al., 2019; Oosterhof et al., 2019). In the particular case of an infant, he was born with multiple congenital brain abnormalities, including complete absence of a corpus callosum, enlarged ventricles, and a cerebellar defect called a Dandy-Walker malformation, in which parts of the cerebellum fail to develop. He had low blood calcium and dense bones, a condition the researchers diagnosed as osteopetrosis. During his short life, he had trouble breathing, was unable to eat properly, and had epilepsy. He died from a bacterial infection at 10 months of age. The aftermath of living without microglia is indeed severe for the brain and, undoubtedly,

microglia can no longer be considered passive bystanders in the CNS, only acting as immunity when needed, but have to be viewed as instructive players when it comes to brain development.

The question of the origins of microglia, their fate in adulthood in homeostatic conditions or disease, and the contribution to this brain myeloid population from peripheral macrophages rather than cells proliferated in loco, is vast and complex and I refer to the proper literature for a comprehensive explanation of such multifaceted matter, which is not of primary interest in my thesis (Ginhoux & Prinz, 2015; Q. Li & Barres, 2018; Morgane S. Thion et al., 2018).

Functions in the CNS

Two are the key functional features that define microglia and they mirror their duplicitous nature as immune cells and as cells of the CNS: immune defense and maintenance of CNS homeostasis. As for their immunological functions, microglia constantly sample their environment, scanning and surveying for signals of external danger (Davalos et al., 2005; Nimmerjahn et al., 2005), such as those from invading pathogens, or of internal danger generated locally by damaged or dying cells (Hanisch & Kettenmann, 2007). Detection of such signals initiates a program of microglial responses that aim to resolve the injury, protect the CNS from the effects of the inflammation, and support tissue repair and remodeling (Minghetti & Levi, 1998).

On the other hand, microglia are also molded and shaped by the CNS environment and behave as crucial contributors to brain homeostasis (Blank & Prinz, 2013).

Microglia during Development

After the engraftment in the brain parenchyma, microglial cells progressively acquire a more ramified morphology and reach the adult pattern of homogeneous tiling

during the second postnatal week (Q. Li & Barres, 2018). During this maturation process, microglia undergo different phases of differentiation (Amit, Winter, & Jung, 2016; Matcovitch-Natan et al., 2016; Morgane Sonia Thion et al., 2017) that rely on signals derived from the maturing CNS, the gut microbiome, sexual identity and inflammatory molecules.

Transcriptomic studies have highlighted different signatures for microglia from female and male mice, a sexual dimorphism that appears to be a long lasting reprogramming, conserved after grafting (Hanamsagar et al., 2017; Morgane Sonia Thion et al., 2017; Villa et al., 2018).

In the sequential waves that characterize microglial invasion of the CNS, these cells undergo heterogeneous and stereotypical patterns of localization: for instance, as they invade the deep layers of the neocortex, they associate with specific axonal tracts and populate definite regions of the developing brain containing neuronal progenitors where they control the size of the precursor cell pool (Cunningham, Martinez-Cerdeno, & Noctor, 2013; Swinnen et al., 2013). For example, tampering with such developmental processes affects the outgrowth of dopaminergic axons in the forebrain and the laminar positioning of subsets of neocortical interneurons (Squarzoni et al., 2014).

Microglial cells are the professional phagocytes of the brain and as such, they are capable of eliminating entire cells or cellular substructures, in particular synapses (Hong & Stevens, 2016). During development, there is a high level of turnover, where neurons and glial cells such as oligodendrocytes are first generated and subsequently eliminated, shaping the neuronal circuitry as we know it. As immune cells, microglia not only retain the ability to recognize programmed cell death and engulf dying or dead cells (Bessis, Béchade, Bernard, & Roumier, 2007; Ferrer, Bernet, Soriano, Del Rio, & Fonseca, 1990), but there is multiple evidence that they can themselves actively initiate a cell death

program. Some of the most direct evidence for a more active role came from in vitro studies in chick retina where microglia were proved to induce retinal cell death via microglial-derived nerve growth factor (NGF) (Frade & Barde, 1998). Moreover, in cerebellar slices, microglia induce apoptosis in Purkinje neurons by releasing superoxide ions (Marín-Teva et al., 2004). Another signaling cascade by which microglial cells interact with neurons to induce cell death is mediated by tumor necrosis factor alpha (TNF-α) (Neniskyte, Vilalta, & Brown, 2014). On the other hand, microglial cells can also positively influence development by promoting neural precursor cell proliferation and survival (Choi, Cho, Hoyt, Naegele, & Obrietan, 2008; Vukovic, Colditz, Blackmore, Ruitenberg, & Bartlett, 2012).

Another well-known process during development is synaptic pruning, a term that indicates the elimination of excess synaptic connections.

A first evidence that microglia might be involved in such activity comes from a study done in the mouse hippocampus during postnatal development. Paolicelli et al. indeed shows that microglia actively engulf synaptic material via the interaction of the fractalkine receptor Cx3CR1 with the chemokine fractalkine expressed in neurons.

In the visual system, microglia eliminate the presynaptic inputs from the retinal ganglion cells into the dorsal lateral geniculate nucleus (Schafer et al., 2012). The “eat me” signals proposed by the authors of the paper that supposedly tag the synapses to be engulfed are the complement proteins C1q and C3, which possess similar opsonizing properties in the peripheral immune system. Conversely, CD47 has been identified as one of the “don’t eat me” signals, protecting synapses from excess microglia mediated pruning (Lehrman et al., 2018).

If microglia are then involved in spine elimination, recently, it was also shown that in the somatosensory cortex of a P8 mouse, microglia contact onto dendrites can induce

filopodia formation (Miyamoto et al., 2016). This facilitatory role of microglia in synaptic circuit remodeling and maturation was further confirmed in organotypic hippocampal cultures, where dynamic microglia-synapse interactions induced presynaptic partial phagocytosis (trogocytosis) and formation of post synaptic spine head filopodia by microglial contact (Weinhard et al., 2018). One mediator of such activity of microglia on spine formation has been found in interleukin 10 (IL-10). Apparently, neutralizing antibodies of interleukin 10 receptors attenuated the induction of the synaptic formation by microglia. Moreover, pretreatment with lipopolysaccharide inhibited microglia from inducing synaptic formation, and IL 1β antagonized the induction of synaptic formation by IL-10 (S. H. Lim et al., 2013).

An additional mechanism mediating the communication between neurons and microglia during development is the duo composed by fractalkine (CX3CL1) – a chemokine produced by neurons – and its receptor CX3CR1 – specifically expressed in microglia in the CNS. In a paper from the Gross lab (Zhan et al., 2014), the authors show that, though microglia deficient in CX3CR1 still eliminated apoptotic neurons, they did not offer neurotrophic support to surrounding neurons. CX3CR1-deficient mice showed a reduced connectivity between cortex and hippocampus, which also had an impact on mouse behavior.

Microglia-Synapse Interaction in the Adult Brain

If the involvement of microglia in synaptic pruning during development is now an established fact, there is also increasing evidence that these cells have a deep connection to synapses in the adult healthy brain in the framework of neuronal plasticity. Microglia are highly motile cells and continuously scan the environment with their ever moving processes (Davalos et al., 2005; Nimmerjahn et al., 2005). By using in vivo two-photon imaging, it was shown that microglial processes make direct contact with synapses at a

frequency of about once per hour. Contacts were activity-dependent and decreased in frequency upon reductions of neuronal activity (Wake, Moorhouse, Jinno, Kohsaka, & Nabekura, 2009). In the visual cortex, it was demonstrated that microglia interact with axonal terminals and dendritic spines, and this interaction depends on changes in neuronal activity (Tremblay, Lowery, & Majewska, 2010). Light deprivation induced microglia to become less motile and changed their preference of localization to the vicinity of a subset of larger dendritic spines that persistently shrank, while light re-exposure reversed these behaviors. Moreover, it was shown that microglia contact on spines increases synaptic activity, thus enhancing the synchronization of neuronal populations (Ono et al., 2018). Another report shows how microglia are necessary for ocular dominance plasticity in the visual cortex and individuate P2Y12R as the key molecule for the neuroimmune communication during this paradigm (Sipe et al., 2016).

Interestingly, adenosine triphosphate (ATP), a known important purinergic signal molecule for peripheral immune cells, is released during high neuronal activity to promote enhanced surveillance by microglial processes in an NMDA dependent manner (Dissing-Olesen et al., 2014). In the zebrafish larvae, neuronal activity was reduced by microglia contact while conversely, preventing microglial processes from contacting spontaneously active neurons significantly enhanced neuronal activity (Y. Li, Du, Liu, Wen, & Du, 2012).

These observations imply an active rather than passive form of surveillance and suggest that neuronal activity itself can be altered by microglial contact. This is further supported by the observation that preventing ATP-mediated microglial process outgrowth exacerbated evoked-seizure activity and mortality (Eyo et al., 2014). Taken together, ATP-mediated communication between highly active neurons and the resident immune cells of the brain might provide an important but still to be defined feedback to regulate neuronal activity.

Recently, two new studies demonstrated that microglial dynamics are deeply influenced by norepinergic signaling: noradrenergic tone seems to be suppressing microglia process surveillance during wakeful state (Liu et al., 2019; Stowell et al., 2019). A major question for future studies is whether or how this form of microglia surveillance impacts synaptic function and plasticity.

The activity-dependent modulation of synaptic strength in the adult brain is thought to underlie memory, learning, and widespread aspects of adaptive behavior. Both strengthening and weakening of synapses can result from changes in neuronal activity. Emerging research demonstrate changes in synaptic strength and behavior can arise from signaling in immune- related pathways, particularly those governed by microglia.

The interaction between the microglia chemokine receptor CX3CR1 and its CNS ligand, neuronal CX3CL1, allows for precise communication between neurons and microglia and can result in changes in neuronal activity. Application of CX3CL1 to acutely stimulate microglia in brain slices caused a dose-dependent depression of synaptic transmission that was not observed in the CX3CR1-deficient mouse (Ragozzino et al., 2006). This synaptic depression was mediated by the adenosine A3 receptor, as the depression was blocked by A3 (but not A1 or A2) receptor antagonists and was not observed in A3-deficient mice. Thus, a chemokine expressed by neurons acts on microglia to reduce the activity of neurons via a feedback pathway. The mechanisms underlying this feedback loop still remain to be uncovered.

In addition to the acute action of CX3CL1–CX3CR1 signaling, chronic reduction of this signaling pathway by permanent deletion or reduction of CX3CR1 leads to elevated levels of the inflammatory cytokine interleukin (IL)-1β, leading to a reduction of long-term potentiation (LTP) in the brain. Ventricular infusion of an IL-1β receptor antagonist for 4 weeks via osmotic mini pumps reversed the impaired LTP, while infusion of an

inactivated antagonist did not (Rogers et al., 2011). Intact chemokine signaling between neurons and microglia and appropriate levels of CNS cytokines are therefore crucial for maintenance of normal plasticity mechanisms.

Interestingly, not all neural–immune pathways implicated in neuronal plasticity involve canonical immune molecules. Recent work has identified a potential role for microglial brain-derived neurotrophic factor (BDNF): selective deletion of microglia or genetic removal of microglial-derived BDNF in mice at postnatal day 30 (P30) caused deficits in multiple learning tasks and a significant reduction in motor-learning-dependent synapse formation (Parkhurst et al., 2013).

Microglia, Friend or foe?

Microglial activation has always been considered a negative event. Ramified microglia retract their fine processes, and acquire a morphology which resembles that of phagocytic macrophages, a transition originally described by Rio-Hortega. This morphological reaction often is correlated with a migratory behavior and/or proliferation of the cells and profound changes in the catalogue of surface markers, such as CD14, major histocompatibility complex (MHC) molecules, chemokine receptors (Block, Zecca, & Hong, 2007). This stereotypic response, though, hides a wide complexity in the response spectrum of these cells: “resting” microglia are by no means inactive, and activation can underlie a manifold of different phenotypes that do not necessarily lead to neurotoxicity. The first evidence of a nefarious activity of activated microglia was reported ~ 30 years ago, when researchers found that microglia stimulated with high concentrations of proinflammatory cytokines (or LPS) (or their resulting supernatant) were highly toxic to neurons in co-culture (Boje & Arora, 1992). More recent data, however, indicate that microglial cells can also exhibit important neuroprotective activity even in an activated state (Biber, Owens, & Boddeke, 2014; Z. Chen & Trapp, 2016). For example, microglial

cells can remove inhibitory axosomatic synaptic inputs that are no longer functional in the facial nucleus in a processes termed synaptic stripping (Blinzinger, K; Kreutzberg, 1968).

Since these immune cells possess a variety of neurotransmitter receptors - including those for GABA and glutamate – it is safe to assume that these cells can sense changes in circuitry activity and thereby modulate their behavior in response to it (H. Kettenmann, Hanisch, Noda, & Verkhratsky, 2011). There is increasing evidence that microglial cells can suppress neuroinflammation and thereby protect nerve tissue by the release of anti-inflammatory mediators. In experimental meningitis, microglial cells initially produce proinflammatory IL-1β, but subsequently, they produce anti-inflammatory IL-10 as a negative feedback loop (Henry, Huang, Wynne, & Godbout, 2009). Another factor mediating anti-inflammatory behavior in microglia is TGF-β (Qin et al., 2018).

Microglia in Neurodegeneration

As I have highlighted in the previous chapters, the neuroimmune system is profoundly involved in development and normal functioning of the Central Nervous System. Such a primary role in the homeostatic capacity of the brain entails that any perturbation to microglia has the ability to lead to grave consequences for the CNS, due both to the disruption of their sentinel and housekeeping activities and the gain of neuroinflammatory properties of these immune cells. Initiation or exacerbation of neurodegeneration might arise from an imbalance between these microglial functions and correcting such imbalance may be a potential approach for therapy (Ardura-Fabregat et al., 2017).

Neurodegenerative disorders are usually characterized by an age-related deposition of debris, aggregated and misfolded proteins, and neuronal death. In these conditions, microglia react to these misfolded proteins, aggregates, and cellular debris via a dedicated and specific set of receptors: the pattern recognition receptors (PPRs) that sense conserved

pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Santoni et al., 2015). Molecules associated with neurodegeneration, such as aggregated β-amyloid (Aβ), α-synuclein, mutant huntingtin, superoxide dismutase 1 (SOD1), and chromogranin, act as DAMPs and activate PPRs, leading to the sustained release of neuroinflammatory factors, which causes cell death, promotes pathology, and contributes to neurodegeneration and disease progression.

Microglia in Alzheimer's Disease

Alzheimer's disease (AD) is hallmarked by formation of Aβ containing plaques, neurofibrillary tangles of intracellular hyperphosphorylated tau protein, and neuronal loss (Selkoe & Hardy, 2016). The sequence of events that has been uncovered by the recent literature sees a first accumulation of Aβ leading to a microglial response, which then promotes tau hyperphosphorylation and formation of neurofibrillary tangles, neurodegeneration and cognitive impairment. In the brain parenchyma of AD patients and animal models, microglia accumulate around senile plaques (Frautschy et al., 1998). These cells show profound morphological activation, have increased proinflammatory markers and show intracellular accumulation of Aβ, suggesting phagocytosis (D'Andrea, Cole, & Ard, 2004).

Evidence for a direct microglial role in AD came first from genome-wide association studies. Mutations in triggering receptor expressed on myeloid cells 2 (Trem2) were associated with a 3.0- to 4.5-fold increased AD risk, almost as high as that associated with ApoE ε4. Mutations in other microglial genes, such as CR1, HLA–DRB1, CD33, MS4A6A, and BIN1, were associated with more modest AD risks. Since these genes regulate key microglial functions, understanding how they affect AD will impact on all AD patients whether they have these mutations or not. Interestingly, these proteins are all involved in signaling pathways leading to phagocytosis and cytokine production. Indeed,

TREM2 mutations have been linked to deficits in Aβ phagocytosis and clearance (Y. Wang et al., 2015). Possible involvement of microglia phagocytosis and immune responses in AD was corroborated by a recent large transcriptome study by Zhang et al. (B. Zhang et al., 2013), who defined transcriptome networks in postmortem brain tissues from late-onset AD patients. The study revealed an immune- and microglia-specific module strongly associated with AD pathology, which contained the TREM2-associated protein TYROBP as a key regulator.

As the scavengers of the CNS, microglia have the unique ability to deal with aggregates. One very discussed point in the literature is indeed the microglial role in Aβ clearance. Aβ deposition is regulated by the equilibrium between Aβ production and clearance. Small changes in this equilibrium result in abnormal accumulation. Aβ clearance involves, in part phagocytosis and endocytosis via microglial scavenger receptors (SRs) and extracellular degradation by Aβ-degrading enzymes (Ries & Sastre, 2016). Decreased clearance contributes to Aβ accumulation in late-onset AD. In support of this concept, microglia from a mouse model of Aβ deposition have reduced expression of Aβ-phagocytic receptors and Aβ-degrading enzymes, but their ability to produce proinflammatory cytokines was maintained (Hickman, Allison, & El Khoury, 2008). These results suggest that Aβ accumulation is in part due to failure of microglia to clear this toxic peptide.

Microglia–Aβ interactions have been shown as a causative mechanism leading to the early synapse loss observed in AD mouse models (Hong et al., 2016), a process going through the complement protein C1q. Moreover, as a proinflammatory stimulus, Aβ causes in microglia a production of neurotoxic reactive oxygen and nitrogen species (ROS and RNS), NLRP3 (NLR family pyrin domain containing 3) inflammasome activation, and production of proinflammatory cytokines and TNFα (Hickman & El Khoury, 2012).

Based on these findings, microglial–Aβ interaction is a double-edged sword. While monitoring the brain environment, microglial sensing of Aβ peptides results in Aβ clearance and removal of the injurious agent. However, persistent production of Aβ and its chronic interaction with microglia drive further amyloid deposition. Indeed, Aβ-induced proinflammatory cytokines reduce microglial Aβ clearance ability, and NLRP3 activation releases microglial apoptosis-associated speck-like protein containing a CARD (ASC) which binds to Aβ, causing its aggregation and leading to further amyloid 'seeding' and spreading of amyloid pathology (Venegas et al., 2017). Similarly, Aβ-induced cytokines promote tau hyperphosphorylation and pathology, thus initiating a self-perpetuating loop that culminates in worsening of the disease (Oddo, 2003).

The double-edged sword metaphor refers to various stages of a single microglia in AD. At a certain timepoint during disease progression, microglia assume a useful role, then progress into a dysfunctional cell which ultimately becomes deleterious. In support of this concept, recent transcriptomic studies of microglia in normal and AD mice identified subpopulations defined as disease-associated microglia (DAM) (Keren-Shaul et al., 2017; Krasemann et al., 2017). DAMs are located around Aβ plaques and have dysregulated expression of sensing, housekeeping, and host- defense genes. It is not clear how DAMs differ from 'dark microglia' associated with Aβ deposits, which exhibit condensed cytoplasm and nucleoplasm and express high levels of CD11b and Trem2 (Bisht et al., 2016). These findings support a direct link between aberrant microglial functions and AD and suggest that a subset of microglia transition from a homeostatic to DAMs in AD. It is then imperative to understand factors that might influence positively these cells and modulate their phenotype in order to harness their neuroprotective activities in the CNS.

ASTROCYTES

Astrocytes have a unique morphology that intimately reflects their function in the CNS: with their complex arborization and anatomical specializations, they possess an ideal arrangement to appreciate changes in their microenvironment (B. Zhou, Zuo, & Jiang, 2019). They form highly organized domains with little overlap between neighboring cells and are closely connected via gap junctions into functional networks. Some astrocyte processes closely ensheath synapses – allowing for a direct modulation of the contents of the synaptic cleft - whereas others are in close contact with blood vessels via specialized processes called endfeets – a direct morphological correlate of their primary role in neurovascular coupling. Indeed, cerebral blood flow needs to accommodate changes in energy demand required by the local activity: neurons can indeed release vasoactive substances onto astrocytes that consequently modulate the diameter of nearby vessels, in order to grant the proper metabolic support to neuronal activity (Gordon, Mulligan, & MacVicar, 2007; Iadecola & Nedergaard, 2007).

Depending on the specie and brain region, astrocytes account for almost 20% ~ 40% of the total number of cells in the CNS. The glia-to-neuron ratio (here glia refers to all glial types together) varies widely within the evolutionary tree (Herculano-Houzel, 2014). If the worm, *C. elegans*, counts 302 neurons and 56 glial cells, for a ratio of 0.18 (Oikonomou & Shaham, 2011), in the rat cerebral cortex this ratio goes to 0.4 (Bass et al. 1971) and it reaches up to 1.4 in the human cerebral cortex (Pelvig, Pakkenberg, Stark, & Pakkenberg, 2008).

Despite their large number, astrocytes have been considered mere supporting cells since their discovery. If their structural, metabolic and homeostatic capacities have been reiterated and redefined over the years – and I refer to the proper literature for these functions which are not a necessary introduction to my data (Abbott, Rönnbäck, &

Hansson, 2006; Allaman, Bélanger, & Magistretti, 2011) – in later years astrocytes have gained an added layer of functional complexity: they are capable of directly affecting neuronal activity, and thus the whole brain circuitry, contributing to behavior.

Astrocytes as active partners in synaptic transmission

Astrocytes can contribute to synaptic transmission and plasticity by means of a binary approach: with their direct access to the synapse they can undergo morphological changes to influence the synaptic environment (morphological/structural plasticity). Moreover, they can release factors in the synaptic cleft to affect both pre- and post-synaptic terminals (bidirectional communication) (Santello, Toni, & Volterra, 2019). These particular features gave rise to the concept of the 'tripartite synapse' (Araque, Parpura, Sanzgiri, & Haydon, 1999), a term suggesting an active role of astrocytes in synaptic processing.

Structural Plasticity in Astrocytes

The former type of mechanism relies on perisynaptic astrocytic processes (PAPs), thin lamellae enwrapping synapses, which express essential membrane proteins such as glutamate transporters – needed to terminate synaptic transmission via glutamate uptake – and contain the appropriate equipment to release fast acting transmitters (Araque et al., 2014). The extent of PAP ensheathment of dendritic spine is highly variable, depending on the brain region, cell types involved, and activity of the circuit (Ghézali, Dallérac, & Rouach, 2016). For example, in the mouse somatosensory cortex, whisker stimulation increases PAP coverage, a phenomenon to be expected if one considers the high levels of glutamate released by neurons during high intensity stimulation and, therefore, the need to take up such neurotransmitters (Genoud et al., 2006). Conversely, in the supraoptic nucleus during lactation, astrocytic coverage of synaptic elements decreases bringing

about diminished D-serine concentrations in the synaptic cleft and, therefore, a reduction of NMDAR activity (Panatier et al., 2006).

PAPs morphological modifications are thus not merely structural correlates of plasticity, but they directly contribute to those plasticity mechanisms that ultimately determine learning and memory. For instance, mice lacking connexin-30 – which constitutes gap junctions in astrocytes – or neuronal ephrin A3 or its astrocytic ligand, show an increase in PAP coverage and this causes a rise in glutamate uptake and LTP impairment (Filosa et al., 2009; Pannasch et al., 2014).

Astrocyte-synapse communication

With their direct access to the synaptic cleft, astrocytes have the positional and molecular machinery to influence synaptic transmission via the release of fast acting transmitters.

Astrocytes have been shown to participate in all the three major form of synaptic plasticity: *short-term plasticity* – a rapid, activity dependent, change in synaptic efficacy, *long-term plasticity* – a long lasting change in synaptic transmission, and *homeostatic plasticity* – defined as a compensatory mechanism against the saturation of the system, one that ensures that the relative strengths of synapses are maintained.

Astrocytes have been shown capable of influencing short-term plasticity by affecting the probability of neurotransmitter secretion from the presynaptic terminal via the release of ATP – converted into adenosine in the extracellular space. Depending on the synapse and on the receptor expressed on it, adenosine can either depress activity or enhance it. Binding with the presynaptic A1 receptor causes a decrease in release probability and thus weakens synaptic transmission, whereas the activation of A2A receptor does the opposite, increasing presynaptic release probability (Araque et al., 2014; Panatier et al., 2011; Pascual, 2005).

Another mechanism of presynaptic facilitation involves the endocannabinoid system: activity-dependent endocannabinoid release from CA1 pyramidal neurons activates astrocytic CB1Rs, inducing astrocytic Ca^{2+} elevation and glutamate release and resulting in NMDAR-dependent postsynaptic slow excitatory currents and mGluR1-dependent heterosynaptic facilitation of presynaptic glutamate release (Navarrete & Araque, 2010).

Regarding long term plasticity mechanisms, a classic astrocytic intervention is the modulation of NMDAR activation via the release of the NMDAR co-agonist for the glycine-binding site, D-serine (Y. Yang et al., 2003). In the hippocampus, clamping internal Ca^{2+} in individual CA1 astrocytes blocks LTP induction at nearby excitatory synapses by decreasing the occupancy of the NMDAR co-agonist sites (Henneberger, Papouin, Oliet, & Rusakov, 2010).

Another astrocytic molecule involved in LTP in the hippocampus is L-lactate. This metabolic byproduct is produced by astrocytes during periods of intense activity via glycogenolysis and it is shuttled to neurons. Disrupting glycogenolysis in astrocytes impairs hippocampal LTP and memory, and can be rescued by L-lactate but not equicaloric glucose (Suzuki et al., 2011).

Astrocyte CB1R signaling is also important for long-term synaptic plasticity. Indeed, activation of astrocytic CB1Rs is necessary for induction of spike-timing-dependent long-term depression (LTD) at layer (L) 4–L2/3 cortical synapses, via glutamate release and activation of presynaptic NMDARs that permanently reduce synaptic transmission (Min & Nevian, 2012). Moreover, in CA1 hippocampal synapses, astrocytic CB1Rs are required for LTP, as they determine the availability of D-serine in the synaptic cleft. Exogenous administration of D-serine fully rescued the LTP impairment in the GFAP-CB_1-KO (Robin et al., 2018).

Astrocytes have also an important role in homeostatic synaptic scaling – a type of plasticity that occurs when the activity of the whole neuronal circuit is altered and there is a need for a population wide resetting of synaptic strength: depending on the circuit at hand, TNFα released by astrocytes can either favor AMPA internalization or insertion at excitatory synapses (Lewitus, Pribiag, Duseja, St-Hilaire, & Stellwagen, 2014; Santello & Volterra, 2012; Stellwagen & Malenka, 2006).

Overall, astrocytes contribute to the establishment of numerous types of synaptic plasticity both in the context of hippocampal and cortical circuits.

Calcium events in astrocytes

By definition, neurons are the electrically excitable cells of the CNS. And excitability is precisely what gives neurons their capacity to integrate inputs and compute proper outputs, a feature that is at the basis of behavior.

In the landscape of astrocyte research, an observation that provoked a shift in perspective in the glial field was that these cells possess themselves a form of excitability, specifically calcium excitability (Cornell-Bell, Finkbeiner, Cooper, & Smith, 1990). In these cells, a variety of stimuli can induce calcium transients that evolve as complicated patterns throughout a single cell – as multiple microdomains can be involved – and can also travel from one cell to the next and over long distances. As it is for neurons, this property of astrocytes makes them a suitable substrate for computation of brain activity in general, as activity that starts locally in astrocytic processes can integrate in a single cell or multi cell calcium wave (Khakh & McCarthy, 2015; Perea & Araque, 2005).

Ca^{2+} activity in astrocytes seems to be dependent on neuronal activity both *in vitro* and *in vivo*. This was initially observed in acutely isolated hippocampal slices where electrical stimulation of the Schaffer collateral pathway led to increases in astrocytic Ca^{2+} via the activation of Gq-coupled GPCRs (Porter & McCarthy, 1996). With the

development of 2-photon microscopy it was then possible to evaluate the relevance of such calcium responses *in vivo* and monitor astrocyte responses to sensory stimulation while relating it to neuronal activity in a correlative manner. Interestingly, Ca^{2+} responses to stimulation are variable depending on brain region, circuit, and experimental conditions. For example, cortical astrocytes respond to whisker stimulation with Ca^{2+} responses restricted to their processes with little evidence of widespread Ca^{2+} waves (X. Wang et al., 2006). These calcium responses in processes and endfeet rapidly followed neuronal events (~120 ms after) and did not seem to be dependent on IP3R2 signaling or neuromodulators such as acetylcholine, serotonin and norepinephrine (Stobart et al., 2018) suggesting an origin from local synaptic activity. On the other hand, during startle response, astrocytes show widespread wave-like Ca^{2+} signals among astrocytes in response to noradrenergic input form the locus coeruleus (LC) (Ding et al., 2013). Locomotion has been shown to induce Ca^{2+} transients in cerebellar Bergmann glia (Nimmerjahn, Mukamel, & Schnitzer, 2009) and in astrocytes throughout the cortex (Paukert et al., 2014), via α1-adrenergic receptor signaling in response to noradrenergic input.

These calcium elevations in astrocytes have been linked to multiple astrocytic functions: gliotransmission, modulation of PAP ensheathment of synapses, release of D-serine, modulation of the levels of neurotransmitters in the synaptic cleft (Tanaka et al., 2013). Moreover, activating astrocytes via Gq-coupled receptor hM3Dq in CA1 enhanced memory allocation; i.e., it increased neuronal activity in a task-specific way only when coupled with learning (Adamsky et al., 2018).

Astrocytes as substrates of brain state activity integration

Essentially, behavior emerges from coordinated activity of ensembles of neurons and synaptic circuits. Neuronal network responses to external stimuli are regulated by brain states – such as those correlated with wakefulness or arousal – and state-dependent

excitability of neuronal networks is associated with specific cognitive functions (Buzsaki, 2004). Neuromodulators, which produce spatially diffuse and slower effects than transmitters at fast excitatory or inhibitory synapses, are implicated in the generation of said brain states.

Multiple evidence now points at astrocytes as intermediaries between neuromodulators and neurons, relaying information about the state of the circuit. For instance, arousal, which is associated with activation of the LC and widespread noradrenaline release, activates astrocytes and increases their responsiveness to local cortical activity (Paukert et al., 2014), suggesting that arousal increases the gain of such networks to perceive external stimuli and modulate neuronal function. In zebrafish larvae, radial astrocytes accumulate evidence of futile behavior integrating noradrenergic stimulations in the form of rampant increases of intracellular calcium, inducing passive behavior via inhibition of excitatory neurons. This phenomenon seems to be mediated by astrocytic activation of GABAergic interneurons (Mu et al., 2019).

Acetylcholine, a neurotransmitter released during wakefulness by long-range cholinergic fibers from the basal forebrain, also activates astrocyte networks and promotes neuronal modulation (Araque, Martín, Perea, Arellano, & Buño, 2002; Navarrete et al., 2012; Papouin, Dunphy, Tolman, Dineley, & Haydon, 2017; Takata et al., 2011). Specifically, cholinergic input modulates astrocytic release of D-serine at excitatory synapses, including at CA3–CA1 hippocampal synapses (Papouin et al., 2017) and somatosensory synapses (Takata et al., 2011), to enhance NMDAR activity and NMDAR-dependent functions. Alternatively, they can release glutamate, which excites CA1 pyramidal neurons (Perea & Araque, 2005) and inhibits dentate granule cells via intermediary hilar GABAergic interneurons (Pabst et al., 2016). Moreover, it was shown that stimulation of cholinergic fibers from the nucleus basalis into the visual cortex induces

calcium activity in cortical astrocytes via muscarinic receptors and that this mechanism is important for potentiation of visual responses in excitatory neurons (N. Chen et al., 2012).

Another example of astrocytic integration of neural signals has been shown for neuropeptide Y positive (NPY+) interneurons in the hippocampus. These cells have a specific firing property where high-frequency barrages of action potentials outlast stimulation by tens of seconds, called barrage firing. It was shown that this particular feature is dependent on depolarization of astrocytes, which in turn increase their internal calcium, and release glutamate, which acts on metabotropic glutamate receptors in axon terminals of interneurons to initiate barrage firing (Deemyad, Lüthi, & Spruston, 2018).

Conclusively, astrocytes can integrate neural signals on spatial and temporal scales that are different from, but complementary to, the function of neurons and produce persistent changes in neural activity.

NEUROTROPHINS

The era of neurotrophin (NT) began more than sixty years ago with the discovery of Nerve Growth Factor (NGF). Since then, the momentum associated with this important family of neuroactive molecules has never subsided because of their incredible complexity in mechanism of action and pleiotropic effects that still provide wonder and insight into the workings of the CNS.

The neurotrophin system is ancient, as orthologues of neurotrophins, p75NTR and Trk receptors are found in invertebrates as diverse as sea urchins, mollusks and round worms (Bothwell, 2006). Therefore, this family has had half a billion years of evolution to develop extraordinary complexity in function. Still, the fact that these molecules cannot be found in organisms such as Drosophila melanogaster or Caenorhabditis elegans – and are thus not strictly necessary to develop a simple nervous system – implies that neurotrophins are most likely involved in "higher functions" of the CNS (Chao, 2003; Houlton, Abumaria, Hinkley, & Clarkson, 2019).

Neurotrophins exert a plethora of effects, influencing multiple aspects of brain biology, from developmental neurobiology to neurodegenerative and psychiatric disorders. In addition to their classic effects on neuronal cell survival, neurotrophins can also regulate axonal and dendritic growth and guidance, synaptic structure and connections, neurotransmitter release, LTP and synaptic plasticity (McAllister, Katz, & Lo, 1999; Poo, 2001).

The four classic neurotrophins in mammals – NGF, BDNF, NT-3, NT-4 – rely on a two-receptor system for their signaling, comprised of the tyrosine kinase receptors – TrkA, TrkB, TrkC – and the p75 neurotrophin receptor – a member of the TNF receptor superfamily (Chao & Hempstead, 1995). Neurotrophins are synthesized as precursors (pro-

neurotrophins, proNTs) that are proteolytically cleaved − intracellularly by furin or proconvertases, or extracellularly by metalloproteases and plasmin − to form stable mature neurotrophins. Both mature NTs and proNTs are biologically active, with somewhat

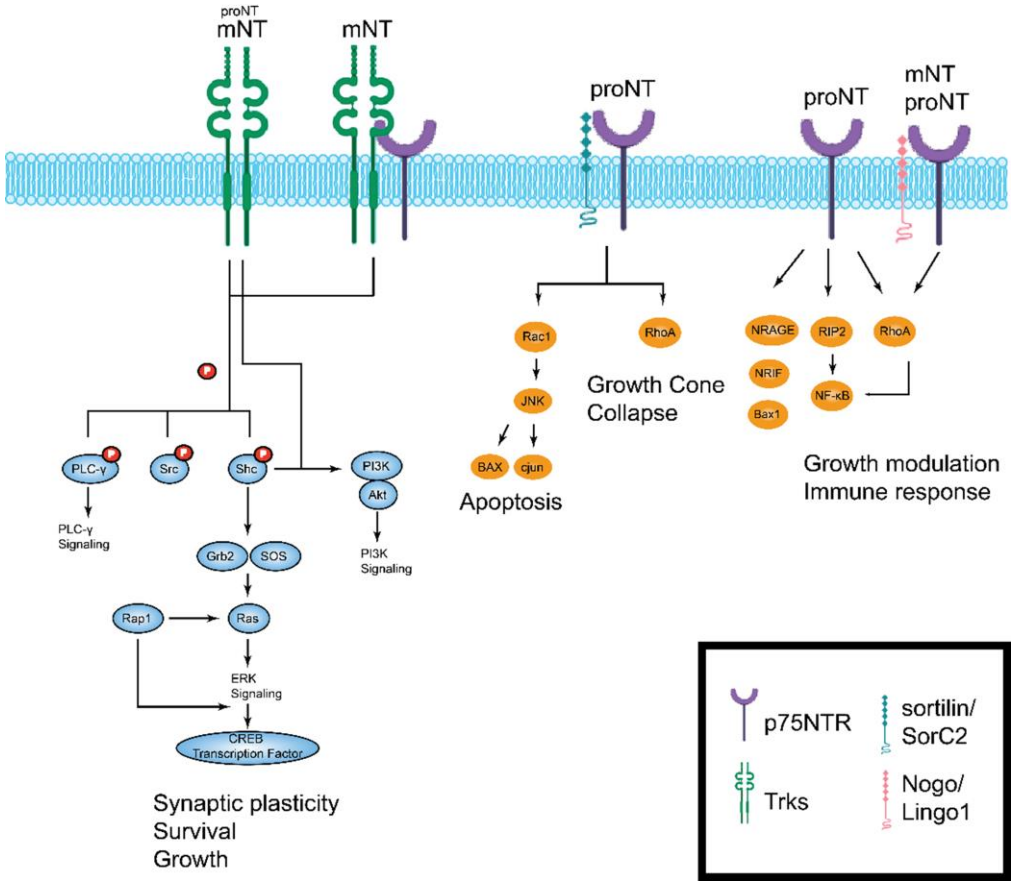

Figure 3. Neurotrophin signaling through the p75NTR and Trk receptors. This diagram depicts the major intracellular signaling pathways associated through each neurotrophin receptor. Each Trk receptor isoform binds mature neurotrophins (and in minor part to proNTs (Fanhestock et al., 2004)) and acts through three predominant pathways. Activation of PLC-γ results in PKC (protein kinase C)-mediated promotion of *synaptic plasticity*. Activation of Ras initiates MAPK (ERK)-mediated promotion of neuronal regeneration and growth. Activation of PI3-K results in activation of Akt and promotion of NF-kB-mediated cell survival. Each of these pathways are also known to regulate genetic transcription via CREB, further promoting pro-survival, and regenerative gene expression. Because of its lack of intrinsic catalytic activity, p75NTR can either function alone by recruiting intracellular components (like NRAGE, NRIF and Bex1) or it can partner with co-receptors including Trks, Sortilin and related protein such as SorC2, myelin associated proteins such as Nogo/Lingo-1 and MAG. When a mature neurotrophin binds to an isolated p75NTR NF-kB-mediated cell survival is promoted. If the p75NTR is co-expressed with the sortilin receptor, pro-neurotrophins can bind, and cause activation of JNK-c-Jun mediated cell death and degeneration. A receptor complex consisting of Nogo, p75NTR, and Lingo1 can bind both pro- and mature-neurotrophins to alter neurite outgrowth in a RhoA-dependent manner; mNT, mature neurotrophin; ProNT, proneurotrophin; p75NTR, pan neurotrophin receptor 75; PLC γ, phospholipase C gamma; MAPK, mitogen-activated protein kinase; TFs, transcription factors; PI3-K, phosphoinositide 3-kinase; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; JNK, c-Jun N-terminal kinase, NRAGE, neurotrophin receptor-interacting MAGE homolog.

antagonist roles that reflect divergent receptor affinities: proNTs display higher affinities for $p75^{NTR}$, whereas mature NTs have greater affinities for Trk receptors – for more details see Fig. 3 legend (Bothwell, 2016; Chao & Bothwell, 2002).

TrkA is highly selective for NGF and, to a lesser extent, NT-3, whereas TrkC binds NT-3 and TrkB BDNF and NT-4-5. In contrast, $p75^{NTR}$ is promiscuous, binding all neurotrophins at low affinity. The interaction and crosstalk between these two signaling machineries – each of which displays different binding sites and affinities to particular neurotrophins as well as different expression levels – determines the specific response to each individual neurotrophin. For example, proNGF can have neurotrophic activities, though to a lesser extent than mature NGF, and drive neuronal survival, as opposed to apoptosis, during its binding to p75NTR, depending on the expression levels of TrkA (Fahnestock et al., 2004; Ioannou & Fahnestock, 2017).

The major signaling pathways activated by the Trk receptors are Ras-mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) -Akt, and Phospholipase C (PLC) -γ (Chao, Rajagopal, & Lee, 2006). On the other hand, p75NTR is by itself functional through a transduction pathway involving NF-kB and c-Jun kinase (JNK) activities. The signaling dynamics of neurotrophins are not entirely straightforward: p75 is able to form heterodimers with auxiliary receptors – like sortilin, SorCS2 and NOGO, and Trk receptors are known to transactivate by means of G-protein coupled receptors without the involvement of neurotrophins. For a more thorough analysis of the complex signaling pathways initiated by neurotrophins, I refer to the proper literature (Chao, 2003; Deinhardt et al., 2011).

Location is an important factor to keep in mind when assessing the effect of neurotrophins. During development, neurotrophins travel long distances: they are produced and released from target cells and then internalized by neurons into vesicles to

be transported retrogradely to the cell body, where they can modulate gene expression (e.g. via the cyclic AMP-response element binding (CREB) transcription factor) (Hendry, Sto¨ckel, Thoenen, & Iversen, 1974; Lonze & Ginty, 2002). Additionally to this long term, long distance outcomes, neurotrophins carry out also local and immediate activities through synaptic release where they are known to affect synaptic strength and plasticity (Park & Poo, 2013).

Since my thesis will focus on the effects of NGF and BDNF, this introduction will now only present literature related to these two molecules.

Nerve growth factor (NGF) was discovered in the early 1950s, when Rita Levi-Montalcini in the lab of Viktor Hamburger lab at Washington University first observed the ability of this neurotrophin to promote growth and differentiation of peripheral sensory and sympathetic neurons, ability to which it owes its name (Levi-Montalcini & Hamburger, 1951).

By implanting a mouse sarcoma into the body wall of a chick embryo, Viktor and Rita described the presence of a powerful growth promoting agent for sensory neurons delivered retrogradely by axons to neuronal cell bodies which they later identified as the diffusible neurotrophin NGF (Levi-Montalcini & Hamburger, 1953). First of its family, NGF has been extensively studied for its pleiotropic action on the peripheral (PNS) and central nervous systems.

In the PNS, NGF dynamically controls neurotransmitter and neuropeptide synthesis. In sympathetic neurons the production of norepinephrine is regulated by NGF through selective induction of tyrosine hydroxylase (TH) (Otten, Schwab, Gagnon, & Thoenen, 1977). In dorsal root ganglia (DRG), the expression of neuropeptides by primary sensory neurons is under the control of NGF (Mearowa & Kril, 1995) such that in vivo deprivation of NGF, as result of nerve transection or anti-NGF treatment, causes a marked decrease in

Substance P (SP) and Calcitonin Gene-Related Peptide (CGRP) synthesis (Verge, Richardson, Wiesenfeld-Hallin, & Hokfelt, 1995). In the periphery, NGF signaling is also involved in nociception: NGF injected into healthy human skin can produce localized pain and hyperalgesia that develops within minutes (Petty et al., 1994). Intravenous injections of low doses of NGF (1 μg/kg) results in myalgia—a widespread, deep musculoskeletal pain affecting proximal body regions and reminiscent of the sensory disturbances observed frequently with mild infections. Mechanisms associated with NGF-induced pain and hyperalgesia include sensitization of nociceptive terminals, altered nociceptor transcription and sprouting of nociceptors (Denk, Bennett, & McMahon, 2017). NGF is indeed a very promising target in the development of new drugs for patients suffering from chronic pain, one of the few cases where preclinical work offered a solid lead into therapy.

In the CNS, NGF is required for the survival and function of the basal forebrain cholinergic neurons (BFCN) (Hefti, 1986), to an extent that disruption of a single allele of the NGF gene results in atrophy of cholinergic neurons accompanied by memory and learning deficits (K. S. Chen et al., 1997). Consistently, interfering with NGF signaling in the adult brain leads to deficits of the cholinergic system (Capsoni et al., 2000; Nagahara et al., 2009; Ruberti et al., 2000). The expression of anti-NGF antibodies selectively neutralizing mature NGF (Capsoni et al., 2000; Ruberti et al., 2000) or of antibodies neutralizing TrkA (Capsoni, Tiveron, Amato, Vignone, & Cattaneo, 2010; Capsoni et al., 2000) in the adult brain of transgenic mice, determines a progressive comprehensive neurodegeneration, synaptic and behavioral deficits. 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 (Cattaneo & Calissano, 2012). The overall neurodegenerative picture induced by anti-NGF or anti-TrkA antibodies in those transgenic models is,

however, much broader than what one would expect on the basis of an action of the antibodies exclusively on the BFCNs.

In the brain, the greatest amount of NGF is produced by the neocortex and the hippocampus – target areas of these cholinergic neurons (Korsching, 1986), primarily by GABAergic interneurons (Biane, Conner, & Tuszynski, 2014); from there, NGF is retrogradely transported to the soma of cholinergic neurons in the basal forebrain (Isaev, Stelmashook, & Genrikhs, 2017; Seiler & Schwab, 1984).

Apart from its neurotrophic properties, NGF also behaves as a neuromodulator of cholinergic activity. Specifically, NGF can enhance acetylcholine (ACh) release from BF (Auld, Mennicken, & Quirion, 2001), medial septum and diagonal band of Broca (MS-DBB) cholinergic neurons (Huh, Danik, Manseau, Trudeau, & Williams, 2008) in a $p75^{NTR}$ dependent manner.

Multiple evidence shows that acetylcholine (ACh) is involved in cognitive processes, including memory and attention (Ballinger, Ananth, Talmage, & Role, 2016). For instance, in a top-down attention mechanism, Gritton et al. (2016) showed that enhancing cue-associated cholinergic transients with optogenetic stimulation improved cue detection and that stimulation of a cholinergic transient during a non-cued trial led to a “false positive” behavioral response from the animal, presumably due to a mistaken detection of a cue. Conversely, inhibiting optogenetically BFCN caused the animals to “miss” many of the cues, consistent with the idea that ACh release is essential for cue detection (Gritton et al., 2016). In sensory cortices (S1, A1, or V1), ACh seems to be mediating the decorrelation of neuronal activity – a feature that increases the signal-to-noise ratio and allows the emergence of the task-relevant activity. In Chen et al. (2015), optogenetic stimulation of cholinergic input in V1 stimulates SOM+ interneurons which in turn inhibit PV+ cells: cholinergic activation of SOM+ interneuron activity appears to be both necessary and sufficient to mediate desynchronization (N. Chen, Sugihara, & Sur,

2015). Similarly, optogenetic basal forebrain inactivation decreased behavioral performance, synchronized cortical activity and impaired visual responses, indicating the importance of cholinergic activity in normal visual processing (Pinto et al., 2013). Thus, NGF modulation of cholinergic activity has in theory the ability to deeply affect cognitive function, though there is a complete lack of data in vivo on the activity of NGF in cortical circuits.

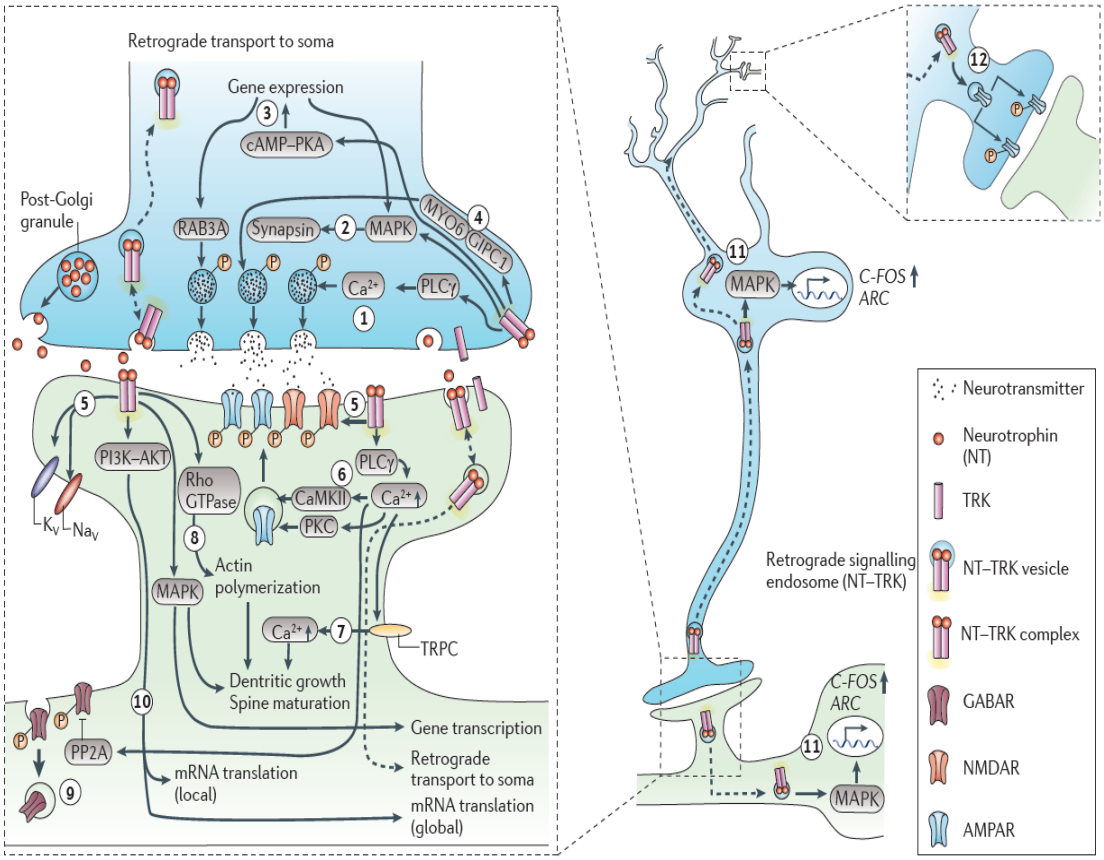

Figure 4. Modulation of synaptic functions by NTs. NTs have diverse pre- and postsynaptic modulatory effects on mature glutamatergic synapses. On the presynaptic side, Trk signaling could activate the phospholipase Cγ (PLCγ) pathway to increase the intracellular Ca2+ concentration ([Ca2+]i) (1), MAPK phosphorylation of synapsin (2), and RAB3 expression (3), leading to increased presynaptic neurotransmitter release. TRKB-mediated activation of the motor myosin VI (MYO6)–adaptor protein GIPC1 complex also contributes to the increased presynaptic release (4). On the postsynaptic side, TRK signaling causes phosphorylation of voltage-gated Na+ (NaV) and voltage-gated K+ (KV) channels and NMDA-type glutamate receptor (NMDAR) subunits, modifying the conductance of all these channels (5). TRK signaling also induces activation of Ca2+/ calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC), which phosphorylate AMPA-type glutamate receptor (AMPAR) subunits and increase their synaptic delivery (6). BDNF-induced store-operated activation of transient receptor potential cation channel subfamily C (TRPC) leads to local [Ca2+]i increase, promoting dendritic growth and spine maturation (7). TRK signaling also activates small Rho GTPases that modulate actin cytoskeleton (8). Postsynaptic GABAergic synapses are suppressed by TRK-dependent activation of protein phosphatase 2A (PP2A) that reduces the surface expression of type A GABA receptors in the dendrite (9). TRK-induced phosphoinositide 3-kinase (PI3K)–AKT signaling enhances both local and global mRNA translation (10). Binding of secreted NTs to their appropriate TRKs leads to endocytic uptake of NT–TRK complexes into ‘signaling endosomes’, which might be transported retrogradely to the soma of both pre- and postsynaptic neurons, triggering MAPK-dependent transcription of activity-related genes such as *C-FOS* (also known as *FOS*) and activity-regulated cytoskeleton-associated protein (*ARC*), and genes required for the establishment of long-term synaptic plasticity (11). Long-range transport of signaling endosomes from the axon terminal to the dendrite could potentiate upstream synapses by increasing postsynaptic AMPAR density and conductance (12). Figure and legend from Park & Poo, 2013.

Isolated in the early ’80 (Barde, Edgar, & Thoenen, 1982; Leibrock et al., 1989), brain-derived neurotrophic factor (BDNF) is the most widely expressed and well-characterized member of the neurotrophin family in the mammalian brain. BDNF exists as different splicing variants. Different exons underlie different functions and spatial segregation in distinct tissues and cellular compartments: most notably, exon IV is expressed in an activity dependent (Ca^{2+} dependent) manner in neurons (Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998; Zheng et al., 2011) and it is preferentially localized in the soma and proximal dendrites, while exon II and VI mRNA are situated in distal dendrites (Baj, Leone, Chao, & Tongiorgi, 2011).

BDNF is significantly involved in all stages of CNS development (Park & Poo, 2013): from survival and proliferation of progenitors (Islam, Loo, & Heese, 2009), neurogenesis and neuronal differentiation (Leschik et al., 2013), neuronal polarization and guidance, branching and survival of differentiated neurons, to formation and maturation of spines and synapses (Cohen-Cory, Kidane, Shirkey, & Marshak, 2010). Unlike NGF, BDNF is not required for the survival of CNS neurons, but works more as a differentiation and plasticity factor in the brain. It is precisely because of its localization in spines and its activity on their function that BDNF has greatly piqued the interest of researchers since its discovery. Its involvement has been studied and identified in nearly every paradigm of neuroscience, from the classical ocular dominance plasticity paradigm of Hubel and Wiesel, to the literature on environmental enrichment, and to the more recent fashions of neuroscience like microglia or the effects of the microbiota in the brain.

In mature circuits, BDNF has many regulatory functions at the level of the synapse and greatly impacts circuit function and, consequently, behavior. BDNF secreted at the synapse can alter synaptic efficacy and modulate LTP/LTD (**Fig. 4**). Changes in synaptic efficacy can either come from a modulation of presynaptic neurotransmitter release or of the degree of the postsynaptic response (Levine, Dreyfus, Black, & Plummer, 1995). For

instance, BDNF can increase glutamate release via a TrkB-dependent increase in MAPK phosphorylation of synapsin (Jovanovic, Czernik, Fienberg, Greengard, & Sihra, 2000). Alternatively, TrkB-BDNF signaling can induce membrane insertion of GluR1 subunits in the postsynaptic compartment (Caldeira et al., 2007).

If BDNF effect on excitatory synapses appears facilitatory, BDNF can attenuate inhibitory transmission via downregulation of surface expression of $GABA_A$ receptors (Jovanovic, 2004; Lu, Cheng, Lim, Khoshnevisrad, & Poo, 2010) or postsynaptic Cl- transport (Wardle & Poo, 2003).

BDNF has also been shown to modulate LTP. In young hippocampal slices, exogenous BDNF facilitated the induction of LTP at CA3–CA1 synapses by enhancing the efficacy of presynaptic transmitter secretion during tetanic stimulation (Figurov, Pozzo-Miller, Olafsson, Wang, & Lu, 1996). Moreover, by imaging granule cells in the hippocampus, BDNF evoked Ca^{2+} transients and caused a robust induction of LTP in a NMDAR dependent manner (Kovalchuk, 2002). Furthermore, BDNF can alter intrinsic neuronal excitability via modulation of expression and function of voltage-gated ion channels (Lesser, Sherwood, & Lo, 1997).

Structural plasticity at the level of the synapse is also dependent on BDNF. Indeed, changes in spine volume in hippocampal neurons have been observed with the TRKB-dependent activation of the RAS–MAPK pathway (Alonso, Medina, & Pozzo-Miller, 2004), transient receptor-potential cation channel subfamily C (TRPC) (Amaral & Pozzo-Miller, 2007) and the phosphoinositide3-kinase (PI3K)–AKT–mTOR pathway (Kumar, Zhang, Swank, Kunz, & Wu, 2005) .

It is then transparent – or I hope it is – after this introductory overview, the pivotal role that neurotrophins hold in neuronal functioning. If such is their importance in physiology, it is then worth to take a quick look to their involvement in pathological

situations – I will focus on Alzheimer's disease, since it is of particular importance for the data presented later in the result section.

Neurotrophins in Alzheimer's Disease

Alzheimer's disease is characterized by progressive loss of neurons and concomitant behavioral deficits such as memory impairment. Neurotrophins have been extensively studied in relation to this disease because AD patients show early deficits in neurotrophin-related signaling pathways and functions to a degree that NTs have been looked at as both causative players and putative therapeutic strategies.

A major and early loss of cholinergic neurons from the basal forebrain (BF) is one of the hallmarks of AD (Hampel et al., 2018). Cholinesterase inhibitors - that increase the availability of acetylcholine at synapses in the brain - are indeed one of the few drug therapies that have been proven clinically useful in the treatment of Alzheimer's disease. Because of its neurotrophic action on these nuclei, NGF has always been connected to AD and indicated as a potential therapy for this neurodegenerative disorder.

Though there does not seem to be a reduction of NGF mRNA levels in AD (Fahnestock, Scott, Jetté, Weingartner, & Crutcher, 1996), several reports indicate that there is decrease in the expression of TrkA in AD brains, which might underlie a reduced TrkA-dependent retrograde transport of NGF and thus loss of neurotrophic support (Counts et al., 2004; Mufson et al., 2000). Other reports reveal that there might be an increase of pro-NGF rather than mature NGF in AD parietal cortex (Fahnestock, Michalski, Xu, & Coughlin, 2001). Thus, the balance between pro-NGF and mature NGF might play a role in AD pathogenesis (Capsoni & Cattaneo, 2006; Tiveron et al., 2013). On the other hand, BDNF mRNA levels are decreased in hippocampi from AD patients (Phillips et al., 1991). Moreover, multiple reports show that BDNF protein levels are

reduced in entorhinal cortex, hippocampus, temporal, frontal and parietal cortex (Connor et al., 1997; Narisawa-Saito, Wakabayashi, Tsuji, Takahashi, & Nawa, 1996). Also TrkB levels seem to be modulated in neurodegeneration: specifically, full length TrkB seems to be reduced in neurons, while the truncated form of TrkB (TrkB.T1) appears to be upregulated in both neurons and glial cells (Ferrer et al., 1999).

Conclusively, there seems to be a loss of neurotrophic support in AD, resulting from changes in neurotrophin levels or their receptors.

Due to its important function during development, disruption of the NGF-TrkA signaling via KO of either gene results in early postnatal lethality in homozygous mice , which has made it difficult to study their effect during neurodegenerative diseases (Crowley et al., 1994). Heterozygous KO mice for NGF present significant deficits in memory acquisition and retention (K. S. Chen et al., 1997). Conditional deletion of NGF/TrkA in the neuronal subpopulation has yielded conflicting results as to their role in neurodegeneration and memory (Müller et al., 2012; Sanchez-Ortiz et al., 2012).

Therefore, the lab of Cattaneo proposed a new approach to neutralize NGF in vivo by expressing an antibody selectively neutralizing NGF. These antibodies are expressed postnatally, obviating the developmental problems associated with the NGFKO. Surprisingly, these mice – named AD11 - developed a pathology reminiscent of AD as they develop Aβ plaques, hyperphosphorylated tau and extensive neuronal loss (Capsoni et al., 2000). These results suggest that a deficit in NGF signaling can directly lead to neurodegeneration.

A glial function for neurotrophins: predictions of the unpredictable

That neurotrophins could have effects outside their canonical activity on neurons has been known for quite some time. Rita Levi-Montalcini herself, in her Nobel Lecture, wonders about the intricate and unpredictable nature that has been intrinsic to the study of NGF (Levi-Montalcini, 1987):

> *"Predictions of the unpredictable are encouraged by the same history of NGF, which may be defined as a long sequence of unanticipated events, which each time resulted in a new turn in the NGF unchartered route and opened new vistas on an ever-changing panorama. This trend, which became manifest from the very beginning and in fact alerted me to the existence of NGF, is perhaps the most attractive, even though elusive, trait of this 35-year-long adventure. One can at present only predict where future developments are most likely to occur. The main causes of unpredictability of the findings reside in the intricacy of the new surroundings where NGF is moving - the CNS and the immune system - rather than in NGF itself. The enormous complexity of these two networks, which on the basis of recent findings are closely interrelated and influence each other through bidirectional signals, opens endless possibilities for NGF activation of distinct repertoires of cells belonging to one or the other system."*

She thus immediately guessed and pursued the idea that neurotrophins could act as means of communication between very different systems – in her case the nervous and the immune system – and that in this thesis I am keen to broaden to the entire glia world.

Neurotrophins and Microglia

A first clue that NGF might be involved in immune processes comes from the fact that its levels are elevated in numerous inflammatory and autoimmune states such as multiple sclerosis, chronic arthritis, systemic lupus erythematosus and mastocytosis (Aloe, Bracci-Laudiero, Bonini, & Manni, 1997; Aloe & Tuveri, 1997). If the neuronal response to such increase is pain sensitization, the immune system as well has the proper equipment – i.e. the receptors – to detect and react to such changes in the environment. For instance, mast cells have been shown to both secrete and respond to NGF (Aloe & Levi-Montalcini, 1977; Leon et al., 1994). The NGF/TrkA system serves as a proliferation factor for B and T lymphocytes and modulates immunoglobulin (Ig) production (Brodie & Gelfand, 1994; Coppola et al., 2004; Otten, Ehrhard, & Peck, 1989). Moreover, NGF and proNGF differentially regulate macrophage phenotype. A report shows that macrophages respond to NGF by secreting TNF-α (Barouch, Kazimirsky, Appel, & Brodie, 2001). Another, describes NGF as an autocrine factor essential for the survival of macrophages infected with HIV (Garaci et al., 1999). Moreover, in a more recent paper, NGF stimulation of macrophages increased membrane ruffling, calcium spiking, phagocytosis and growth factor secretion. In contrast, proNGF induced podosome formation, increased migration, suppressed calcium spikes and increased neurotoxin secretion (Williams, Killebrew, Clary, Seawell, & Meeker, 2015).

This Janus-faced activity of NGF on nervous and immune cells should not come as a surprise. The nervous and immune systems share indeed similar functions: they are both in charge of maintaining homeostasis and they both react to and translate external stimuli (Kioussis & Pachnis, 2009). To achieve such harmony in integrated responses, neuroimmune communication is indeed essential and this requires close anatomical connection and functional interaction based on shared receptors and common pathways (Madden & Felten, 1995; Xanthos & Sandkühler, 2014). Considering NGF activity on

neurons and the fact that NGF receptors are expressed in immune cells and modulates their activity, it is intuitive to think of NGF as precisely a molecule mediating the orchestra of immune and nervous systems (Skaper, 2017).

Again, Rita Levi Montalcini foretold this conceptual shift by redefining NGF as a neurokine – a chemokine with a neuronal tropism (Levi-Montalcini, Skaper, Dal Toso, Petrelli, & Leon, 1996).

As the immune system of the CNS, it stands to reason that microglial cells are also a target of NGF. Some papers in the literature have already strived to make that connection: NGF has indeed been shown to promote microglial migration via TrkA (De Simone, Ambrosini, Carnevale, Ajmone-Cat, & Minghetti, 2007) and to increase proliferation in vitro (J. Zhang et al., 2003).

Moreover, microglia-derived NGF also seems to carry out important functions: this was determined as the cause of cell death in the developing retina via a mechanism involving $p75^{NTR}$ (Frade & Barde, 1998) and in vitro, was shown to greatly increase ChAT activity in cultured basal forebrain neurons (Jonakait, Pratt, Acevedo, & Ni, 2012).

Previous literature from our lab had also suggested that NGF might be important for immunity in the CNS. In particular, the AD11 mouse constitutively expressing an NGF antibody develops a neuroinflammatory phenotype already at 1 month of age (D'Onofrio et al., 2011), suggesting that a lack of NGF in the brain negatively affects the CNS immune system. Moreover, it was shown that treatment of AD mice with NGF in its "painless" version rescues some of the histological and behavioral deficits via its action on glial cells (Capsoni et al., 2017).

Similarly, BDNF and its receptors can be found in essentially all major cell types of the human peripheral immune system, including CD4 and CD8 positive T lymphocytes, B

lymphocytes, and monocytes in vitro (Kerschensteiner et al., 1999). BDNF is also highly expressed in infiltrating immune cells in MS lesions (Stadelmann et al., 2002).

Recent data now highlight a specific function for microglial-derived BDNF in the CNS. In the spinal cord, microglial BDNF appears to be involved in the induction of neuropathic pain: in particular, ATP-stimulated microglia release BDNF which in turn affects lamina I neurons, causing a collapse of their transmembrane anion gradient (Coull et al., 2005). Moreover, in the spinal dorsal horn, it was shown that microglial- BDNF - released via CSF-1 signaling - is important for high frequency stimulation (HFS) LTP and chronic pain (L. Zhou et al., 2019). Microglial BDNF has also an important role for cortical synaptic plasticity. Indeed, mice with a microglial specific BDNF KO show deficits in multiple learning tasks and a significant reduction in motor-learning-dependent synapse formation (Parkhurst et al., 2013).

Conclusively, neurotrophins are important signaling molecules between neurons and microglia in the CNS and can represent a powerful tool to modulate their activity.

Neurotrophins and Astrocytes

Astrocytes take part in the computation at the level of the synapse and – thanks to their direct access to the synaptic cleft, they sense and take up molecules released by neurons while being able to secrete molecules themselves. Multiple reports show how astrocytes are able to both secrete and respond to neurotrophins.

There are numerous reports of the expression of NGF in astrocytes both in vitro (Friedman, Thakur, Seidman, & Rabson, 1996; Yamakuni et al., 1987) and in vivo (García-Mauriño, Boya, López-Muñoz, & Calvo, 1992). and the same goes for its receptors TrkA (Aguado, Ballabriga, Pozas, & Ferrer, 1998; Hutton, DeVellis, & Perez-Polo, 1992) and p75 (Cragnolini, Huang, Gokina, & Friedman, 2009; Hutton et al., 1992).

The interaction between NGF and astrocytes has been studied mainly in relation to pathological situations. In vitro, NGF expression by astrocytes can be increased by several inflammatory stimuli such as IL-1β, IL-5, IL-6 and Aβ (Awatsuji et al., 1993; Čarman-Krž & Wise, 1993; Jauneau et al., 2006; Schulte-Herbrüggen et al., 2007). Catecholamine and dopamine agonists can also positively affect the levels of NGF in cultured astrocytes (Ohta et al., 2003; Schwartz & Mishler, 1990).

Moreover, it was shown that, in vitro, proNGF secreted by astrocytes induces cell death in motor neurons via $p75^{NTR}$ (Domeniconi, Hempstead, & Chao, 2007). Additionally, NGF can also act in an autocrine manner on astrocytic $p75^{NTR}$, decreasing their number without inducing cell death (Cragnolini et al., 2009). This mechanism seems to be mediated by NGF inhibition of cyclins and their association with specific cyclin-dependent kinases, preventing the progression through the G1 phase of the cell cycle. (Cragnolini, Volosin, Huang, & Friedman, 2012).

In vivo, the activity of NGF might be important during development or after injury where the levels of p75 and /or NGF are known to be particularly high. Indeed, NGF seems to be expressed in astrocytes prenatally and early development and during inflammatory conditions (Oderfeld-Nowak & Bacia, 1994), after seizure (Cragnolini et al., 2009), and following traumatic brain injury (Goss et al., 1998; Saadipour et al., 2019) in what is likely a form of neuroprotection. In healthy conditions, levels of NGF and its receptors are low and the main source of NGF are supposedly neuronal targets (Bacia et al., 1992; Calatozzolo et al., 2007). There is no data though on what is the role of NGF on astrocyte physiology in the healthy brain and how this contributes to the proper functioning of the neuronal circuit.

The role of BDNF in astrocyte-neuron communication has been much more intensively addressed in the literature. A key paper from the lab of Marco Canossa sees

BDNF at the center of a recycling mechanism, where astrocytes take up proBDNF secreted by neurons at the synapse via $p75^{NTR}$ receptor, and put it back in the synaptic cleft in the cleaved mature form where it locally phosphorylates neuronal TrkB receptor and allows LTP maintenance and memory consolidation (Vignoli et al., 2016). This phenomenon depends on glioactive ATP (Vignoli & Canossa, 2017). Thus, BDNF from astrocytes seems to be required for long term memory consolidation. Another group, using primary hippocampal co-cultures of astrocytes and neurons, differently reported that astrocytes are the primary recipients of BDNF released in its mature form from neurons, and that this BDNF was an important modulator of the extent of the astrocytic morphology and territory (Stahlberg, Kügler, & Dean, 2018). Moreover, astrocytes in vitro and ex vivo can directly respond to BDNF with calcium transients via TrkB.T1 (Rose et al., 2003). There is still, to my knowledge, no direct evidence that this process is present in the intact brain.

Another important clue into the role of glial derived BDNF comes from pathology. Increasing glial derived BDNF in a 5xFAD model of Alzheimer's disease, rescues structural and functional plasticity, and consequently causes a significant improvement in cognitive tasks (De Pins et al., 2019), though it does not affect β-amyloid load. This suggest that glial derived BDNF is an important factor in the maintenance of brain homeostasis.

It wouldn't come as a surprise if neurotrophins were able to modulate calcium activity in astrocytes in vivo – directly or indirectly – and in turn modulating neuronal activity. Indeed, for IGF-I (Insulin-like growth factor-I), a trophic factor of another family, this was reported in a recent preprint on BioRxiv. Specifically, it was shown that IGF-I induces Ca^{2+} signaling in astrocytes which then release ATP/adenosine activating A2A adenosine receptors at presynaptic inhibitory terminals. Disrupting this process via KO of IGF-IR in cortical astrocytes (IGF-IR-/-) impaired the behavioral performance in a whisker discrimination task (Noriega-prieto et al., 2020).

Aim of the thesis

In this introduction, I explored the vast literature regarding two very different glial cells (1) the immune cell of the CNS, the microglia (2) and the classical support cell for neurons, the astrocyte. What they do have in common is the shared environment with the neuronal components, the molecules they secrete and the overall activity state of the circuits. As I explained earlier, both microglia and astrocytes can sense neuronal activity and respond to it, they are affected by all those neuromodulatory systems – dopaminergic, noradrenergic, cholinergic – that determine how the neuronal system works and cooperate in harmony with them to ensure the proper functioning of the network.

Some fundamental notions prompted us to look for connections between these cells and *neurotrophins* – molecules classically thought to display mainly trophic properties for neurons.

Let's start with what concerns the microglia – an immune foreigner in the brain parenchyma:

- Microglia have been shown to be key players in the pathogenesis and progression of many neurodegenerative disorders. 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 (Block et al., 2007). In contrast, the modulation of 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 (Ardura-Fabregat et al., 2017). Therefore, there is a compelling urgency to find ways to selectively target microglia neuroprotective activities, sparing, or even inhibiting, those features

known to be pathological mediators. The idea of harnessing the CNS immune system—the natural scavengers of the brain—to boost neuroprotection in the brain is intriguing, especially when tackling diseases marked by loss of proteostasis such as Alzheimer's disease (AD).

- In the search of neuroprotective agents against neurodegeneration, neurotrophins have been historically considered as potential therapeutic candidates, mostly due to their actions on neuronal targets.
- Rita Levi Montalcini herself defined the Nerve Growth Factor (NGF) as a neurokine – a combination between a neurotrophin and a chemokine, caught in the middle between the nervous and the immune system. This neuroimmune communication via NGF was studied at length by her and many other groups, finding important modulatory properties of NGF on mast cells, macrophages and lymphocytes in the periphery. Microglia would thus be an obvious target in the CNS, it being the only immune cell in the brain tissue (Levi-Montalcini et al., 1996; Williams et al., 2015).
- Some reports had already drawn the first line – the one from microglia to neurons, describing functions for a *microglial-derived NGF* (Frade & Barde, 1998; Jonakait et al., 2012; Srinivasan, Roque, Hempstead, Al-Ubaidi, & Roque, 2004).
- Other groups drafted a first connection into the direction of an NGF effect on microglia, specifically describing an increase in proliferation and migration in vitro (De Simone et al., 2007; J. Zhang et al., 2003).
- 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) (Capsoni, Covaceuszach, Ugolini, et al., 2011; D'Onofrio et al., 2011)

To what extent NGF might affect physiological microglial functions—and how alterations in this modulation might come into play in neurodegenerative disorders— has not been systematically investigated yet. Thus, the first aim of this thesis is properly address, in vitro and ex vivo, how microglia respond to NGF (CHAPTER I).

As immortalized cell lines are scarcely representative of primary microglia (Das et al., 2016), primary microglia hold substantial differences with microglia in its natural environment - the brain parenchyma. Indeed, microglial specification requires continuous signaling from the CNS, so much so that macrophage precursors treated with astrocyte conditioned medium differentiate into microglial-like cells (Amit et al., 2016; Bohlen et al., 2017; Hinze & Stolzing, 2011). We thus aimed to use available tools such as 2-photon microscopy to study microglial responses to NGF in vivo (CHAPTER III – part I) to further validate the translatability of our results.

Moving to our second glial cell of interest, let's now bring to the surface the emerging questions regarding the activity of neurotrophins on astrocytes. In this introduction we highlighted that, as regards NGF:

- Astrocytes seem to possess, at least in vitro, the machinery to respond to NGF. Though most of the data in vivo highlights a role for NGF in pathological conditions, NGF is also expressed in physiological condition by neurons and thus is present at the synaptic cleft where astrocytes can sense it.
- It is now recognized that astrocytes can influence the neuronal circuit and their calcium excitability is a new and interesting parameter to evaluate their activity.

An aim of this thesis is then to look at astrocyte calcium to understand first in vitro (CHAPTER II) and then in vivo (CHAPTER III), if these glial cells can respond to NGF.

For what concerns the most expressed neurotrophin in the cortex, BDNF:

- Its role in astrocyte physiology has already been address: BDNF in vitro increases calcium activity in astrocytes (Rose et al., 2003), but it is not known if this activity is present in the intact brain.

It is then an aim of this thesis to test this neurotrophin in the intact brain, to confirm that these cells are able to respond to BDNF via calcium transient in vivo.

Chapter I: *In vitro effects of NGF on microglia*

The first chapter of this thesis was published in a recent paper from our lab and was done under the direction of a fellow PhD student, Caterina Rizzi, during the early days of my PhD (Rizzi et al., 2018). In this project, we provide now stringent evidence that microglia are target cells for NGF - both in vitro and ex vivo - and that the activity carried out by this neurotrophin on microglial cells might be neuroprotective and anti-inflammatory in the context of Alzheimer's disease.

MATERIAL AND METHODS I

ANIMALS

Adult C57BL/6, 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: 50-GTC TTC ACG TTC GGT CTG GT-30, IDT 14277 50-CCC AGA CAC TCG TTG TCC TT-30, IDT 14278 50-CTC CCC CTG AAC CTG AAA C-30). 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-hrs dark to light cycle, with food and water ad libitum.

CELL CULTURES

The immortalized BV-2 murine microglial cell line was maintained in RPMI (Thermo Fisher Scientific, MA; #11835-063) medium containing 1% penicillin/streptomycin (Euroclone, MI, Italy; #ECB3001D), 1% Glutamax (Thermo Fisher Scientific; #35050-038) and 10% fetal bovine serum (FBS; Euroclone; #ECS0180l) in 5% CO2 at 37°C. Primary microglial cells were derived from the brains of B6129 or Cx3cr1-GFP $^{+/+}$ mice at P3–4 as previously described (Butovsky et al., 2014). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM/F12; Thermo Fisher Scientific; #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 18 hrs before the experiments. Primary cortical and hippocampal neurons were prepared at P0 as described (Gobbo et al., 2017). Briefly,

animals were decapitated, the brain was rapidly excised and placed into ice-cold Hanks Buffered Saline Solution (HBSS; Thermo Fisher Scientific, Waltham, MA; #14180046). Hippocampi and cortex were removed and digested for 15 min at 37°C in DMEM-F12 containing 0.1% trypsin (Thermo Fisher Scientific). 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 rpm, cells were resuspended in fresh DMEM containing 1% Glutamax, 10% FBS, 2% B27 supplement (Gibco, Waltham, MA; #17504-044), 6 mg/ml Glucose, 12.5 mM Glutamate, 10 mg/ml Gentamicin (Gibco; #15710-049) and plated (150,000 cells/coverslip) after proper poly-D-lysine coating (Sigma-Aldrich, St. Louis, MO; #P1024). Cells were kept at 37°Cin 5% CO2. After 12–24 hrs, medium was replaced with Neurobasal A medium (Thermo Fisher Scientific; #10888-022) containing 2% of B27 supplement, 2.5 mM Glutamax, and 10 mg/ml Gentamicin. The second day 2.5 mM AraC (Sigma-Aldrich; #C1768) was added to the medium. The experiments were performed at DIV (days in vitro) 17–19.

IMMUNOBLOT ANALYSIS

NGF signaling: Primary B6129 microglia were plated in six-well plates (5 x 10^5cells/well) in culture medium. Cells were serum-starved for 16 hr before the start of the treatments, then they were treated for 0, 5, 15, and 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% Ige-pal, 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 rpm). Protein concentrations of the cell lysates were measured using the Bradford method. Lysates (20 mg) 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 six-well plates (5 x 10^5 cells/well) in culture medium. 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; #WG1401BX10). After transfer in nitrocellulose, the membrane was boiled in PBS for 10 min, blocked for 1 hr and incubated with the appropriate primary antibodies. *Inhibitors of NGF-receptors used:* 200 nM K252a (Abcam, Cambridge, UK; #ab120419), 1 μM TAT-pep5 p75NTR (Millipore, Temecula, CA; # 506181; Yamashita & Tohyama, 2003), were added 30 min before Aβ and NGF. A concentration of 100 ng/ml was chosen because of previous literature indicating this as the recommended dose to elicit a response in microglia in different assays (De Simone et al., 2007).

The following primary antibodies were used: anti-Aβ 1–16 1:1000 (clone 6E10 #SIG-39320); anti-TrkA 1:1000 (Millipore; #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, NY) anti-Akt 1:1000 (Cell Signaling Technology, Danvers, MA; #C67E7), anti p-Akt 1:1000 (Cell Signaling Technology; #130386), ant- Erk 1:1000 (Promega, Fitchburg, WI; #V114A), anti-pErk 1:1000 (Cell Signaling Technology; #4370S), anti-c-Jun 1:1000 (Cell Signaling Technology; #60A8), anti-phospho-c-Jun 1:1000 (Cell Signaling Technology; #9261), anti-p75 1:1000 (Millipore; AB1554), anti-GAPDH 1:20000 (Fitzgerald, Acton, MA; #10R-G109a), anti-tubulin 1:20000 (Sigma-Aldrich; #T5168). After incubation with the appropriate HRP- conjugated secondary antibody (Santa Cruz, Dallas, TX; anti-mouse #sc-2005, anti-rabbit #sc-2004), membranes were developed using ECL-enhanced chemiluminescence kit (Bio-Rad, Hercules, CA). 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 (1 x 10^5 cells/ well) in culture medium. Cells

were fixed with 2% PFA, and blocked for 1 hr at room temperature. Primary antibodies (O.N. – 4°C): anti-Iba1 1:500 (WAKO, Osaka, Japan; #019–19741) or anti-Iba1 1:500 (Abcam; #Ab107159), anti-TrkA 1:100 (MNAC13 from (Cattaneo et al., 1999)), anti-P75 1:500 (Millipore; AB1554).

Immunofluorescence for Aβ uptake: Primary microglia were plated on coverslips in 24-well (1 x 10^5 cells/well) in culture medium. They were treated with 1 µM soluble 555-labeled Aβ (s555-Aβ; Anaspec, Fremont, CA; #As-60480) or AbOs (Meli et al., 2014) and 100 ng/ml NGF for 3 hr. After fixation and permeabilization, cells were blocked for 1 hr and stained with primary antibodies anti-Iba1 1:500 (WAKO, Osaka, Japan; #019–19741;) and with anti-Aβ oligomers A13 1:1000 (Meli, Visintin, Cannistraci, & Cattaneo, 2009), then incubated with mouse antibody anti-epitope V5 (Sigma-Aldrich; #V8137;1:5000). Appropriate secondary antibodies were used (1:500) (anti-rabbit Alexa- Fluor 555, anti-mouse Alexa-Fluor 488, Thermo Fisher Scientific; A- 21428; A-21201).

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. Dry ice frozen brains were cut into 40 µm coronal sections with a cryostat microtome (Leica Microsystems, Wetzlar, Germany) at – 22°C, including neocortex. Sections were with a mix of primary antibodies in PBS 0.3% Triton X-100 (Applichem, BioChemica, Darmstadt, Germany) overnight at room temperature. Microglia were stained with either rabbit anti-Iba1 1:800 (Wako, Osaka, Japan, 019–19741) or rat anti-CD11b 1:300 (Serotec; Kidlington, UK, MCA711). Astrocytes were stained with rabbit Anti- Glial Fibrillary Acidic Protein 1: 500 (Dako, Cytomation, Glostrup, Den- mark, Z0334) or goat Anti-Glial Fibrillary Acidic Protein 1:300 (Santa Cruz Biotechnology; sc-6170). NGF receptors were identified by anti- TrkA 1:300 (clone MNAC13; Cattaneo et

al., 1999) and anti-P75 1:300 (Promega Corporation, Madison, WI, G3231). 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; A-21428 diluted 1:500). DAPI was applied for 5 min in the second rinse.

FLOW CYTOMETRY FOR PHAGOCYTOSIS ANALYSIS OF BEADS, DEXTRAN OR Aβ

Sample preparation: Primary microglia were plated in six-well plates at a density of 5 x 10^2 cells/well. The fluorescent material to be phagocytosed was placed in the culture medium 3 hr after treatment with NGF 100 ng/ml. Beads were first opsonized in 50% FBS and PBS for 1 hr RT (Polybead DyedRed 6 mm, Polyscience, Warrington, PA; Cat#15714), counted with the Burker chamber and given to cells at a concentration of roughly three beads/cell. Dextran was used at 2.5 mg/ml (Thermo Fisher Scientific; #D1841 RhodamineB 70,000 MW) while HiLyte Fluor 555 Aβ42 (Anaspec, Fremont, CA; #As-60480) was used at 1 μM. Fibrillar Aβ was prepared from fluorescent sAβ as previously published in (Lorenzo & Yankner, 1994). Cells were exposed to the material for 1 hr, then they were washed extensively with PBS, and fixed with 2% para-formaldehyde for 7 min. Cells were washed again with PBS and collected for analysis. Data acquisition: A Sorter S3 (BioRad) 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 were based on the emission spectra of the fluorochromes: RhodamineB for dextran, DyeRed for beads, HiLyte Fluor 555 for Aβ42— 580–650 nm (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, OR).

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: IFNg 10 ng/ ml (R&D, Minneapolis MN, USA Cat. Number 485-MI), Amiloride 50 µM (Sigma-Aldrich; #A3085), CytochalasinD 10 µg/ml (Sigma-Aldrich; #C827). These were added 20 hr before phagocytosis assay. Rho/Rac/ cdc42 Activator I (Cytoskeleton, Denver, CO; cat. #CN04) was added after a 2 hr FBS starvation period and 1 hr and 30 min before the beginning of the experiment with beads and dextran. Phorbol 12- myristate 13-acetate (PMA) 100 nM (Sigma-Aldrich; #P8139) was added 3 hr before the assay.

MICROARRAY TRANSCRIPTOME ANALYSIS

Primary microglia were treated with 100 ng/ml NGF for 2, 8, or 24 hr. RNA isolation, amplification, and labeling was performed using an RNeasy mini kit according to manufacturer's protocol (Qiagen, Venlo, The Netherlands). Total RNA was isolated from these cells using Trizol (Invitrogen, Carlsbad, CA) 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, Santa Clara, CA), 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 gIsWellAboveBG50 (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 value <.05; | Log2 fold-change|>1.0). Principal Component Analysis, Multidimensional Scaling, Hierarchical Clustering of samples and volcano plots were computed using the open source RStudio (Boston, MA).

LIVE CELL IMAGING

Primary microglia were plated (3 x 10^4 cells) on Glass Bottom Microwell Dishes (35 mm), coated with poly-D-lysine, and left overnight to rest. Cells were treated with 100 ng/ml NGF for 24 hr. Cells were imaged for 1 hr through a 40x objective with a Leica SP2 confocal microscope (1 frame each 30 s). Cell dynamics were analyzed using a homemade Python script (number of cells imaged per experiment=29). Parameters: 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 ramifications). We measured how many times cells shifted 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 to 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 2% FBS on pre-coated culture plates. Cells were incubated with 1 µg/ml Aβ-488 and 100 nmol/l Lysotracker-Red (Thermo Fisher Scientific; #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 cultures

of microglia since live imaging requires long hours and it is too damaging for primary cultures.

INTRACELLULAR AB CLEARANCE AND DEGRADATION

BV2 cells were incubated in culture medium with 1 μM soluble Aβ42 (Anaspec, Fremont, CA; #As-64129) and 100 ng/ml of NGF for 3 hr. Cells were then either collected (the 3 hr time point) or the medium was changed after extensive washes with PBS to ensure the removal of Aβ42 in the supernatant. Cells were collected and lysed in ice-cold RIPA buffer (SDS 1%) after either 5, 9, or 21 hr 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. After brief sonication, protein extracts underwent centrifugation at 13,000 rpm at 4°C for 15 min. The supernatant at each time point 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, Princeton, NJ). The samples were resolved with 4%–15% bis-tris SDS-PAGE. Aβ levels were measured and normalized to 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 —each time point is indeed a parallel experiment.

Aβ PHAGOCYTOSIS IN EX VIVO HIPPOCAMPAL SLICES

Cx3Cr1-GFP mice were deeply anesthetized (20% urethane solution, 0.1 ml/100g body weight) via i.p. and decapitated 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 hr, slices were fixed in 4% PFA for 18 hr at 4°C. Slices were put in 30% sucrose/PBS, then they were sectioned into 45 µm slices using a Leica microtome.

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°Cfor 1 hr, 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=3–4MΩ) 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 independent 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 1 hr 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 Multiclamp 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 ~20 mV. The current/voltage (I/V) relationship of each cell was determined applying voltage steps from -140 to +60 mV (DVm 20 mV) of 250 ms duration with interval of 5 s 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 mouse were recorded. At least four animals per group were used.

NEURON/MICROGLIA CO-CULTURES

At DIV 17–19 for neurons, primary microglia were seeded onto cultured hippocampal neurons (1 x 10^5 cells/well). The culture was maintained in Neurobasal-A supplemented with 2% B27, 2mM L-glutamine and 10 µg/ml gentamicin and used after 24 hr for experiments. Co-cultures were treated with soluble Aβ-555 (100 nM), and 100 ng/ml NGF for 3 hr, 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; ab9909), anti-actin 1:500 (Sigma-Aldrich; A-3853;), anti GluA1 1:100 (Millipore; #AB1504;).

CHEMICAL LTP

Cx3Cr1-GFP microglia (2 x 10^4 cells/well) were added to DIV 17 cultured hippocampal neurons. After 48 hr, the cultures were treated with soluble Aβ-555 (100 nM), with or without 100 ng/ml NGF for 3 hr. GI-LTP (a form of chemical 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 MgCl2,2CaCl2) 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 1 hr 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, Norcross, GA; Canada; AAM-CYT-6). Primary microglia from B6129 mice were plated in a 6-well plate at the concentration of 6.5 x 10^5 cells/well in culture medium. After 18 hr, cells were serum starved for 4 hr, 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, 1 mM EDTA, 1% Igepal, 0.5% Sodium Deoxycholate, 0.1% SDS, Protease Cocktail inhibitor) and sonicated briefly, and then collected by centrifugation at 13,000 rpm at 48°C for 15 min. Arrays were incubated with the appropriate blocking buffer for 2 hr. 80 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 3 hr at room temperature with the Biotinylated Antibody Cocktail solution. After washing, arrays were incubated with HRP-streptavidin for 2 hr and detected using the Detection Buffer. Images were captured using the Chemidoc detection system (Bio-Rad).

IMAGE ANALYSIS

Experiments in Figures 1a and 9: 512 x 512 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 Figure 2: 2,048 x 2,048 pixel images were acquired 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 Figures 12–13 and 14: 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 40.03 (NA51.25), 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), sAβ-555, and GFP or IBA1 immunofluorescence,

respectively. The Aβ intracellular levels were 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 ± SD unless otherwise noted, using Origin (OriginLab Corporation, Northampton, MA). 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<.05, **p<.01 and ***p<.001.

RESULTS I

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. By immunocytochemistry, we could detect the expression of both TrkA and p75 in primary microglia (**Fig. 1a, b**). To assess the responsiveness of such receptors, primary microglia were treated with NGF and analyzed by WB (**Fig. 1c**) at different time points. TrkA exhibited a significant time dependent

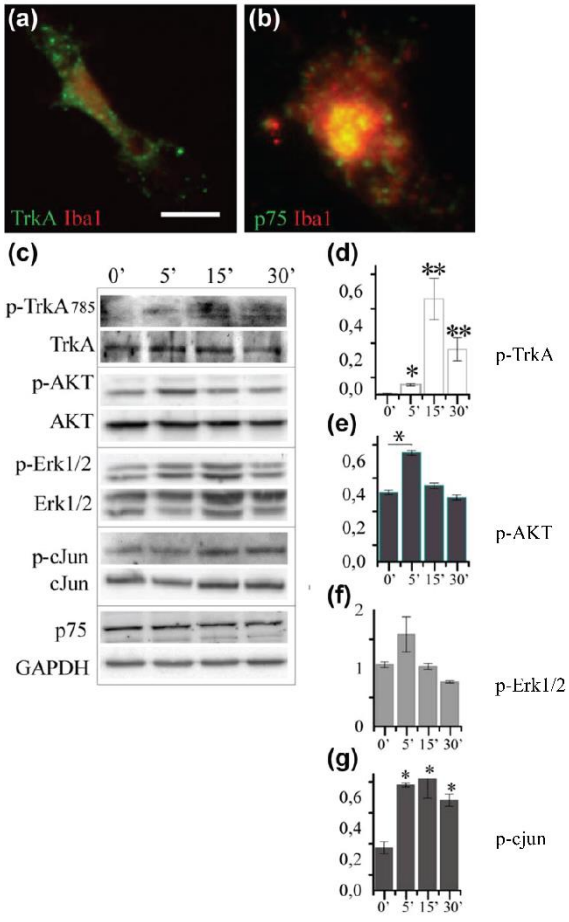

Figure 1. Primary microglia express NGF receptors and activate receptor-mediated intracellular signaling after NGF stimulation. (a) TrkA and (b)p75NTR (green) staining in primary microglia (red). (c-g) Western blots for proteins involved in NGF signaling transduction pathways: TrkA, AKT, Erk, and c-jun. On the right, the histograms of the quantification of phosphorylated protein normalized on the total protein level (Data are mean±SD; all data are representative of three independent experiments, *p<0.05, **p<0.01, One-way ANOVA).

activation upon treatment with the neurotrophin (measured as the ratio between phosphorylated TrkA and the total amount of TrkA; **Fig. 1d**; *p<0.05 at 5 in and **p<0.01 at 15 and 30 min). Concerning the downstream intracellular signaling pathways to TrkA activated by NGF in primary microglia, we could detect the activation of AKT but not ERK suggesting that AKT signaling pathway is more important in microglial cells for NGF mediated responses (**Fig. 1e, f**), Downstream to p75NTR, c-jun also exhibited NGF dependent activation, suggesting that p75NTR might also be involved in mediating NGF activity on microglial cells (**Fig. 1g**).

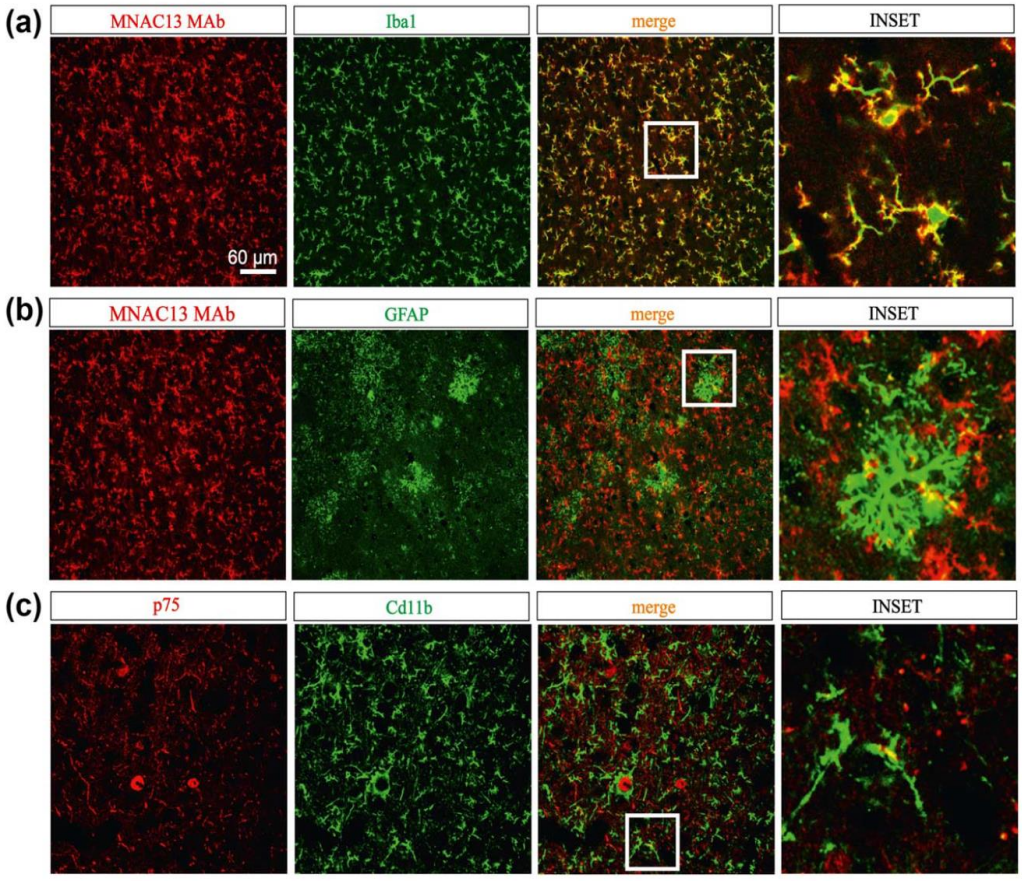

Figure 2. Expression pattern of NGF receptors in murine brain slices. (a) The anti-TrkA MAb MNAC13 (red label) stains cortical microglia iba1 positive cells (green label). Merging areas (yellow label) and magnification (inset) show that the labeling involves both cytoplasm and fibers. (b) (middle panels) 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).

We then proceeded to assess TrkA and p75NTR expression in adult ex vivo glial cells. In sections of mouse cortex, we detected colocalization of TrkA and Iba1 both in the cell bodies and on branches of microglial cells (**Fig. 2a**). In contrast, astrocytes (GFAP1 cells) showed a sparse overlapping with TrkA (**Fig. 2b**). Labeling of the p75 receptor showed some rare points of colocalization with CD11b1 cells (**Figure 2c**) while no expression could be detected on astrocytes' bodies or branches (data not shown).

Thus, we conclude that both in vivo (P80-P90) and in vitro (primary from neonatal tissue) microglia possess NGF receptors, and — specifically in cell culture — we could observe standard receptor signaling in response to the neurotrophin, indicating that these receptors are indeed active.

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

To gain insight into potential functional microglial responses to NGF, gene expression profiling was performed on primary microglia treated with NGF (100 ng/ml) either for 2, 8, or 24 hrs. 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 hrs there was a reversal, with a trend toward upregulation (**Fig. 3a**). KEGG gene ontology analysis was performed to cluster DEGs into pathways, thus identifying those primarily modulated by NGF. At 2 hrs the majority of upregulated genes were linked to focal adhesion and extracellular matrix interactions, while downregulated genes were related to cytoskeletal rearrangements (**Fig. 3b**). At 8 hrs, genes of cell adhesion molecules and of the protein digestion and absorption pathways were still upregulated (**Fig. 3b**).

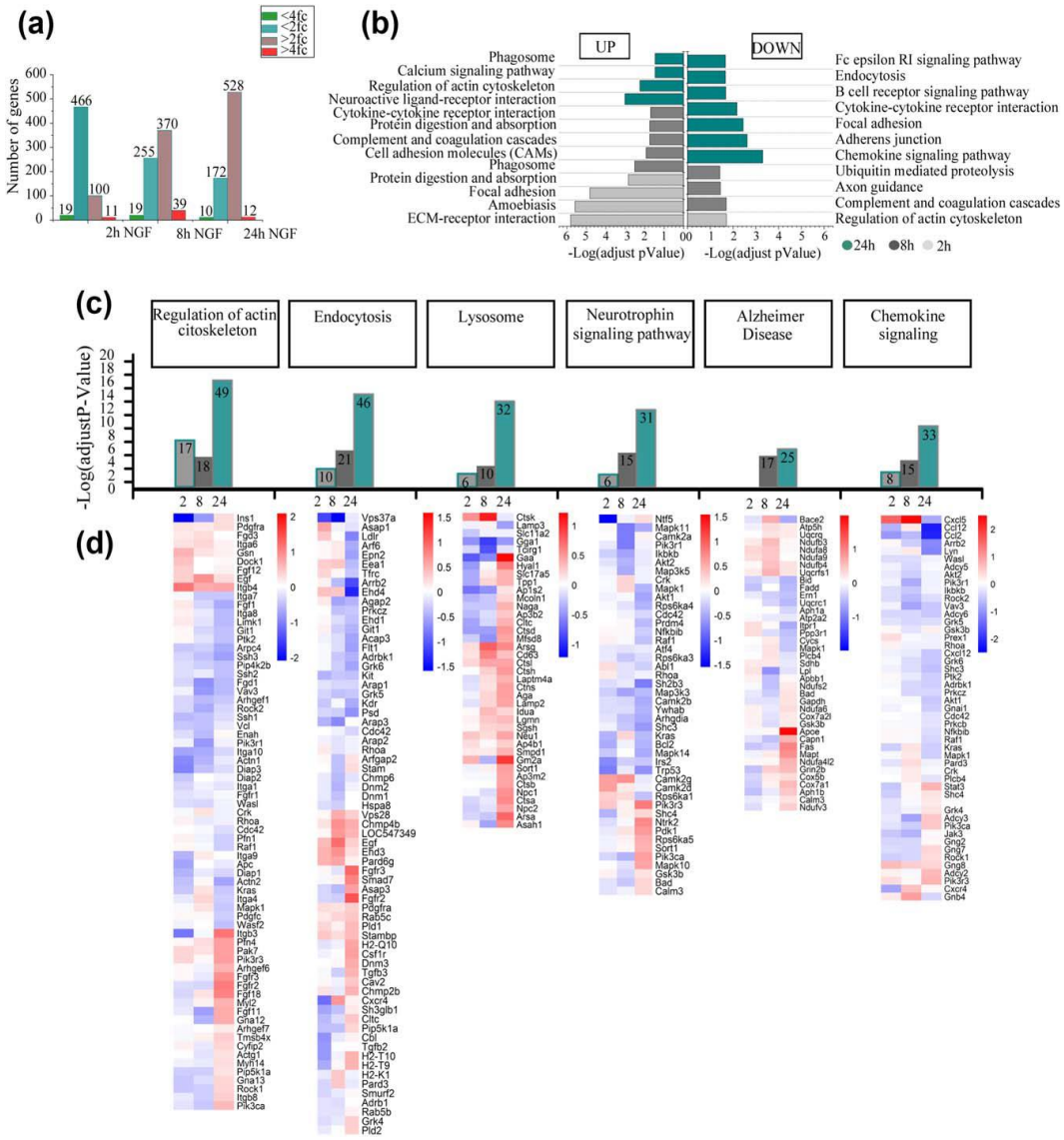

Figure 3. NGF modulates microglial gene expression. (a) The bar plot shows the global number of differentially expressed genes, up-and downregulated by NGF at 2, 8, and 24 hr. Gene lists were selected using two different thresholds: 2.0 fold-change in linear scale and Limma p value <.05 (blue for down-regulated and plum for up-regulated); 4.0 fold-change in linear scale and Limma p value <.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 hr (green, grey, light grey bars respectively). The analysis was performed on differentially expressed genes selected by two thresholds: corrected p value (FDR) <.05 and 1.0 fold-change in linear scale. (c) The histograms show the adjusted p value (FDR) of selected enriched KEGG pathway at 2, 8, and 24 hr (green, gray, light gray bars respectively). Each bar contains the number of differential genes mapping to each specific pathway. Heatmaps show the Log2 fold-change ratio of genes mapping to the corresponding modulated pathways on the top.

At 24 hrs, the majority of upregulated genes were associated with the regulation of actin cytoskeleton and to the phagosome pathways, while downregulated genes belonged to endocytosis, focal adhesion, adherens junction, cytokine-cytokine receptor interaction,

and chemokine signaling pathways (**Fig. 3b**). We then focused on the analysis of specific gene clusters highlighted by the KEGG analysis — actin cytoskeleton regulation, endocytosis, chemokine signaling, protein digestion, neurotrophin signaling and genes linked to Alzheimer's disease (AD)—and represented the total amount of genes mapped to the specific KEGG category at each time point (**Fig. 3c**) and the corresponding heat maps (**Fig. 3d**). We found that, for each of these clusters, most changes occurred at 24 hrs. At this time, NGF induced a significant downregulation of rhoA and rock2, genes involved in actin dynamics (Sackmann, 2015), while most genes related to endocytosis and lysosomal activity, such as Gm2a (Sandhoff & Kolter, 1998), were up-regulated. Concerning neurotrophins and AD pathways, we found an upregulation of sort1 and ApoE, respectively. Finally, we looked at mRNAs involved in the inflammatory response, whose modulation is a major functional response of microglia. Interestingly, this mRNA class was not significantly represented among those upregulated by NGF.

The largest modulation was actually the downregulated expression of cxcl5, ccl12, ccl2. Overall, our data suggest that NGF might influence the motility, the phagocytic and protein degradation abilities of microglia, without activating them in the classical proinflammatory sense.

NGF enhances microglial membrane dynamics, but not their cell speed

The surveillance activity of microglia can be mediated either by the translocation of their cell body toward sites of injury, where chemoattractant substances are released, or by finer movements of their branches—by extension and retraction—in response to either physiological or pathological stimuli (Davalos et al., 2005; Stence, Waite, & Dailey, 2001). Since transcriptome analysis revealed changes in cytoskeletal related genes, we asked whether NGF might induce changes in cell body migration and/or in the motility of cell membrane and processes. The chemotactic properties of NGF specifically on rat

microglial cells have been previously documented (De Simone et al., 2007). Our in vitro approach to assess the effects of NGF on motility—inspired from transcriptomic results—was that of operating time-lapse recordings of NGF-treated—freely moving – microglia. Primary microglia were monitored for 1h in a culture chamber after treatment. Videos were analyzed by means of a Python 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 from one place to another.

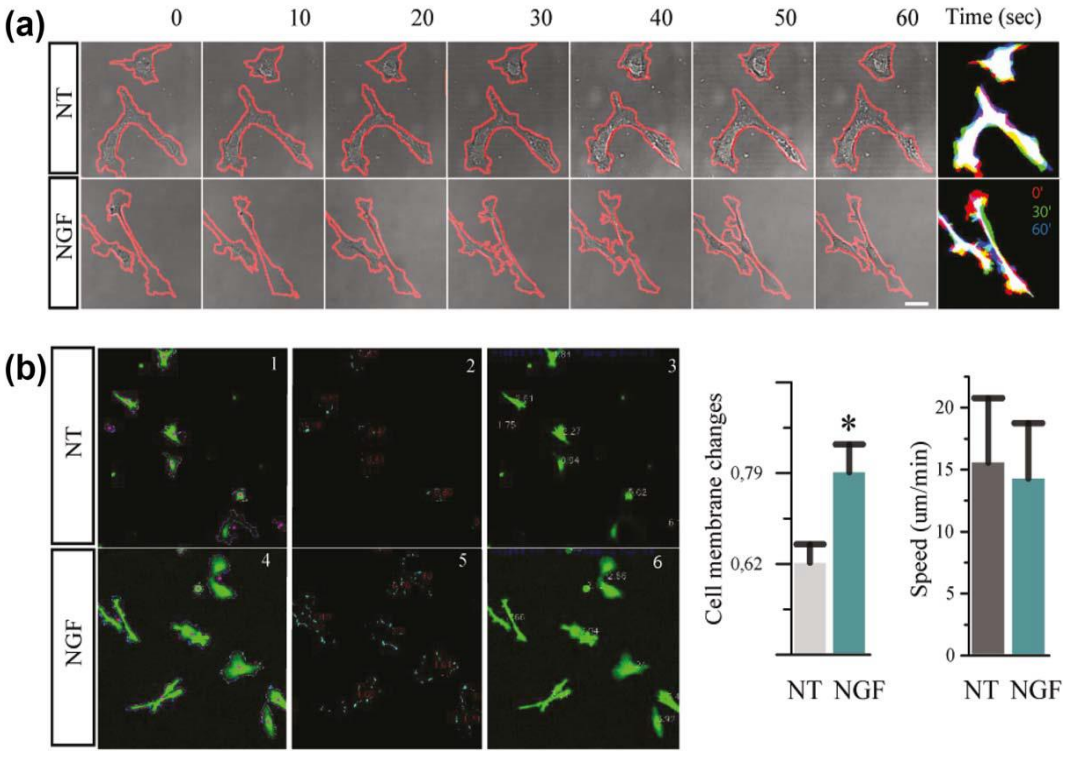

Figure 4. NGF modulates microglial motility dynamics. (a) Bright-field image during a time lapse of primary microglia from CX3CR1-GFP mice. (b) In the panel, it is shown the pattern recognition from a Python based script that describes (1–3) naive cells and (4–6) NGF cells. The boxes show in (1, 4) perimeter in violet and cell center in pink, (2, 5) perimeter difference between two 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<.05, Student's t test).

We thus 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 (DA) normalized over the cell perimeter (p) (DA/p). Since this parameter evaluates the rate of change in cell area—a measure of its ability to elongate and retract—this can be thought of as an in vitro measure of exploratory behavior. We found that NGF treatment induced a significant increase in exploratory tendency in microglia (**Fig. 4b**).

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

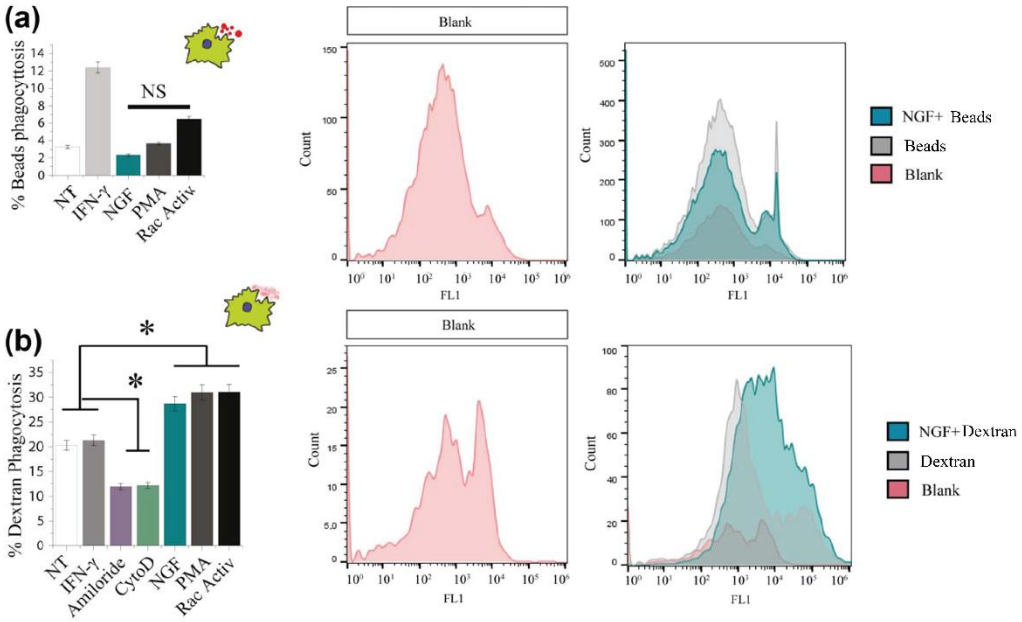

Figure 5. NGF enhances macropinocytosis of dextran but not phagocytosis of beads. (a) phagocytosis of beads: Primary microglia from CX3CR1-GFP mice were incubated with 6 mm beads and 10 ng/ml IFNg, 100 ng/ml NGF, 100 nM PMA, and 1 mg/ml Rho/Rac/Cdc42 activator I for 3 hr. (b) Macropinocytosis of dextran: Primary microglia from CX3CR1-GFP mice were incubated with 2.5 mg/ml Dextran and 10 ng/ml IFNg, 50 mM Amiloride, 5 mg/ml Cytochalasin-D, 100 ng/ml NGF, 100 nM PMA, and 1 mg/ml Rho/Rac/Cdc42 activator I for 3 hr. (mean±SD, *p<.05, Oneway ANOVA).

internalize large particles (Stuart & Ezekowitz, 2005), while pinocytosis is typically associated with the uptake of soluble substances, such as, for instance, Aβ peptide (Mandrekar et al., 2009). We evaluated whether the observed NGF dependent changes in membrane motility might underlie changes in engulfing processes. To investigate this, we used an in vitro assay—followed by FACS analysis—where primary microglia 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 micropinocytosis (BoseDasgupta & Pieters, 2014), different engulfment processes that can be distinguished by means of known inhibitors and activators of the cellular mechanisms subserving them. IFN-γ was used as a positive activation control for phagocytosis, while PMA and Rac-cdc42 activator-I were used as positive activation controls of dextran micropinocytosis (BoseDasgupta & Pieters, 2014). NGF was found not to increase the number of latex beads internalized by microglia (**Fig. 5a**) but to selectively upregulate the internalization of dextran (**Fig. 5b**). Thus, we conclude that NGF positively affects micropinocytosis though sparing other phagocytosis processes in primary microglia.

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. 6a**). To study changes in this outward NGF-

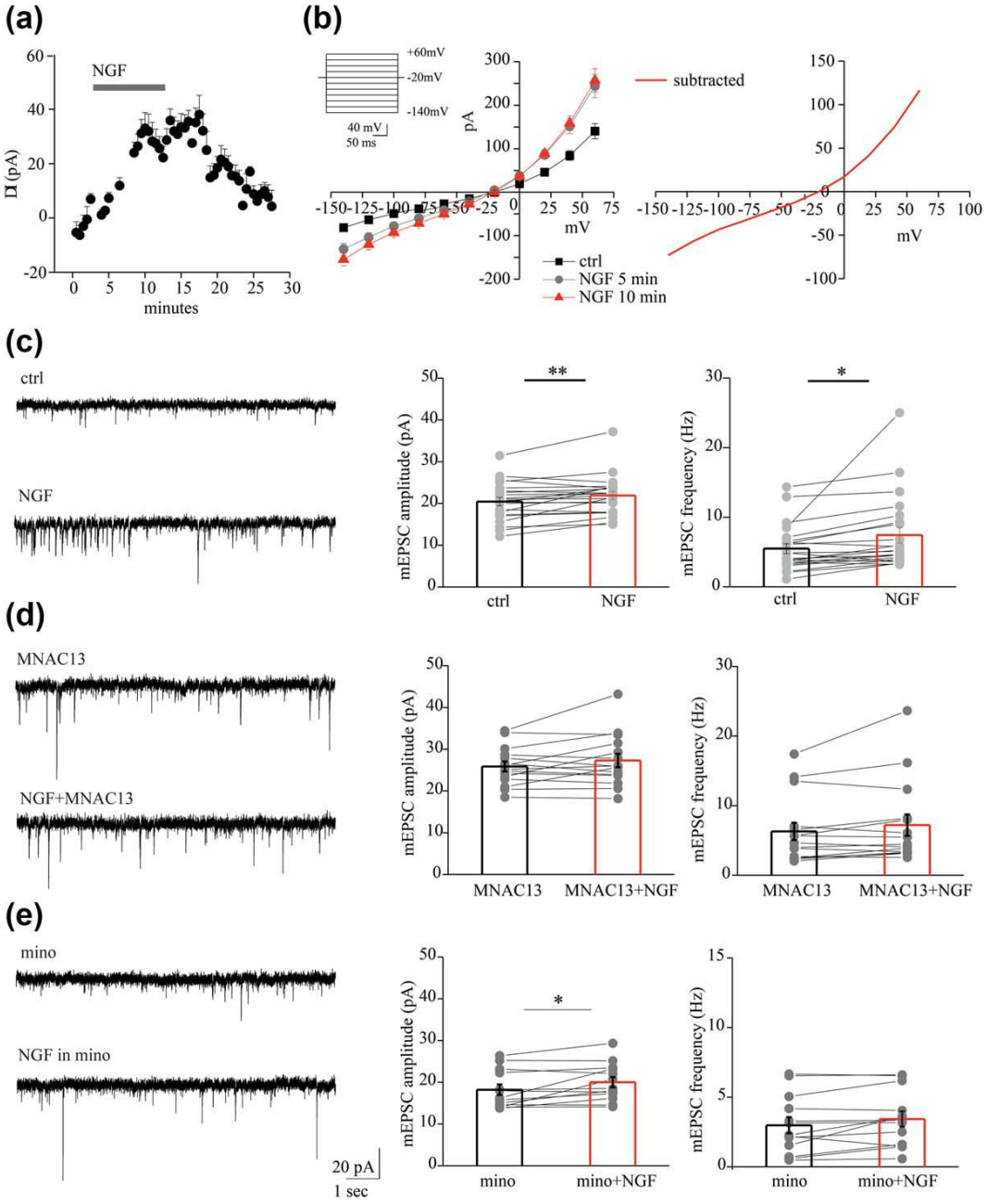

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Figure 6. NGF affects microglial currents thereby enhancing excitatory neurotransmission. (a) Time plot of the mean current amplitude induced by NGF application recorded from microglial cells (n517). (b) Left, current-voltage relationship of the NGF-induced current by application of NGF (20 g) 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 of 220 mV (results obtained by subtracting the current before and after the NGF application). (c) Left, example traces of AMPAR mEPSCs recorded from a pyramidal neuron at 270 mV, in control (ctrl) and after NGF (20 g), in the presence of picrotoxin (100 mM) and TTX (1 mM). Right, bar histograms of group data showing the NGF-mediated increase of mEPSCs amplitude and frequency. (n=22, *p<.05, **p<.01, paired sample t test. (d) Same as in c but in the presence of the anti-TrkA, MNAC13. Note that 20 g NGF did not enhance mEPSC amplitude and frequency when TrkA receptors are blocked (n=15, p=.012 and p=.7 for amplitude and frequency, respectively; paired sample t test). (e) 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<.05 and p5.5, paired sample t test. Data are values from single cells (gray filled circle) and mean±SEM (bars).

induced current, we repetitively clamped the membrane from a holding potential of 220 mV to a series of hyperpolarizing and depolarizing voltage steps before and after the application of NGF (**Fig. 6b**, left inset). The current voltage clamp curve of the response to NGF was outward slightly rectifying and reversed at 15 mV (n=17, p<.05, Figure 6b). At a holding potential of 270 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 (Marrone et al., 2017; Riazi et al., 2015).

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 µg/ml) 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;

Figure 6c). 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; Figure 6d). Then, we carried out experiments in the presence of minocycline, which prevents microglia activation (Plane, Shen, Pleasure, & Deng, 2010). 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=0.5 and $p<0.05$ respectively). Altogether, these data strongly suggest that NGF acts on microglia to modulate glutamatergic neurotransmission.

NGF and microglia in pathological conditions: Alzheimer's disease

Having established that NGF modulates microglial activity in physiological conditions, we then assessed the effect of NGF on microglia in a pathology-related context, 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.

NGF counteracts Aβ proinflammatory effect on microglia

The amyloid-β peptide provides an inflammatory stimulus to microglia (Combs, Karlo, Kao, & Landreth, 2001). Given the above-mentioned effects of NGF on microglial cells, it was of interest to ask whether and how NGF can modulate their Aβ-induced inflammatory profile. To this aim, we investigated the expression of inflammatory

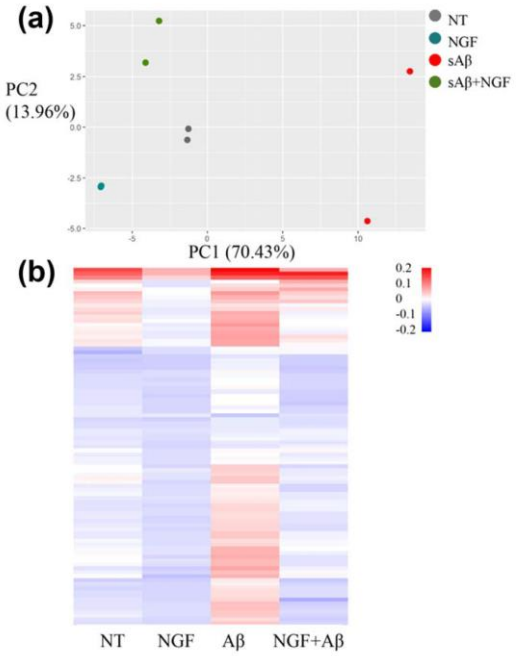


Figure 7. Anti-inflammatory effect of NGF on microglia primed with Aβ. (a) PCA analysis of the inflammatory array. In the graph, two biological replicates of four different treatments were plotted. PC1 and PC2 represent the first two principal components, the proportion of variance (POV) held by these 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).

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. 7b**), we can macroscopically see the pro-inflammatory activity of Aβ by the prevalence of the red bars (increased quantity of cytokines).

It is apparent that NGF carries out the opposite effect: not only it is intrinsically anti-inflammatory when administered on his own but, given in concomitance with Aβ, NGF treatment effectively counteracts Aβ-induced pro-inflammation, returning cytokines

to levels of untreated microglia. This effect was quantified by the PCA analysis (**Fig. 7a**), 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 (the list of values with each specific cytokine is provided as Supporting Information). Thus, NGF is very effective in reverting the microglial proinflammatory state induced by Aβ, while it has a moderate effect on the inflammatory phenotype of naive cells, consistent with the transcriptomic study we presented in Figure 3.

NGF promotes the internalization of soluble 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 by macropinocytosis

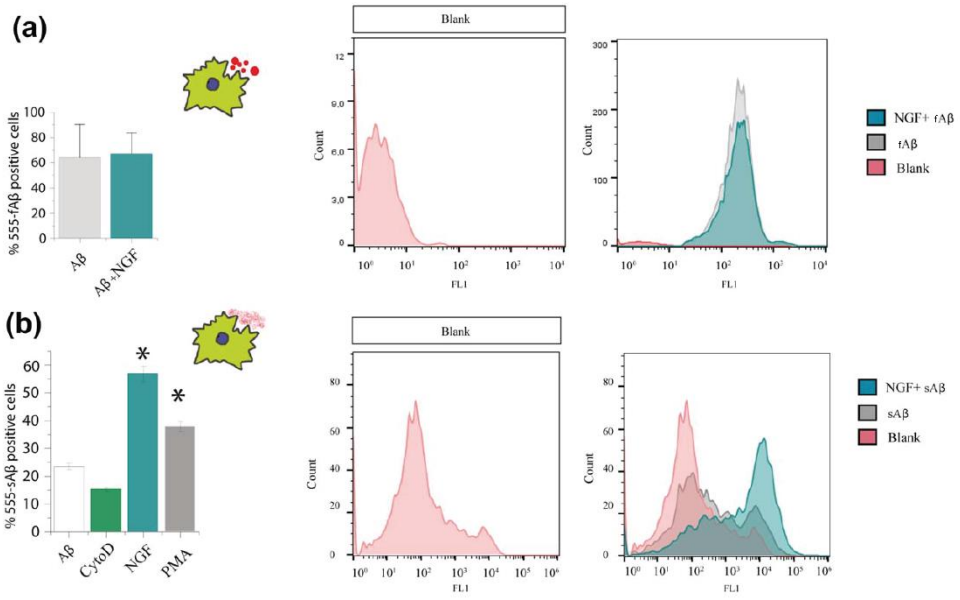


Figure 8 NGF increases the macropinocytosis of soluble Ab but not the phagocytosis of fibrillar Aβ. Primary microglia from wild type mice were incubated with 1 µM of fAβ or sAβ and 100 ng/ml NGF. Uptake was quantified using flow cytometry and compared with control non treated cells (a) Internalization 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), Cytochalasin D: inhibitor of endocytic processes (mean±SD, *p<.05, Student's t test).

(Mandrekar et al., 2009). Thus, we asked whether NGF, which our previous experiments have shown to increase macropinocytosis, differentially regulates the engulfment of fibrillary (fAβ) and soluble Aβ (sAβ). To this aim, we incubated primary microglia with either fluorescent fAβ or sAβ and we tested the effect of NGF by FACS, IF and WB.

Consistent with our previous results (**Fig. 5**), FACS analysis revealed that NGF did not increase the engulfment of fAβ (**Fig. 8a**) but increased significantly the

macropinocytosis of sAβ (**Fig. 8b**). The levels of sAβ and AβO—pure oligomers produced in vitro (Walsh et al., 2002)—inside primary microglial cells (from both B6129 and Cx3Cr1-GFP mice) was also measured by immunofluorescence (for AβO the specific anti-oligomer scFv A13 antibody was used (Meli et al., 2009)) confirming an increase in the internalization of the soluble peptide after NGF treatment (**Fig. 9a,b**).

To distinguish different Aβ species, we performed western blot analysis for Aβ on cell extracts. We found that NGF determines a twofold increase of the internalized Aβ dimers and trimers (**Fig. 10a**). To discern the involvement of the different NGF receptors in the internalization of Aβ, we interfered with TrkA and p75NTR signaling through specific inhibitors: K252a, which blocks TrkA phosphorylation and signaling, and TAT-pep5, a p75NTR signaling inhibitor. K252a, and not TAT-pep5, was able to block the

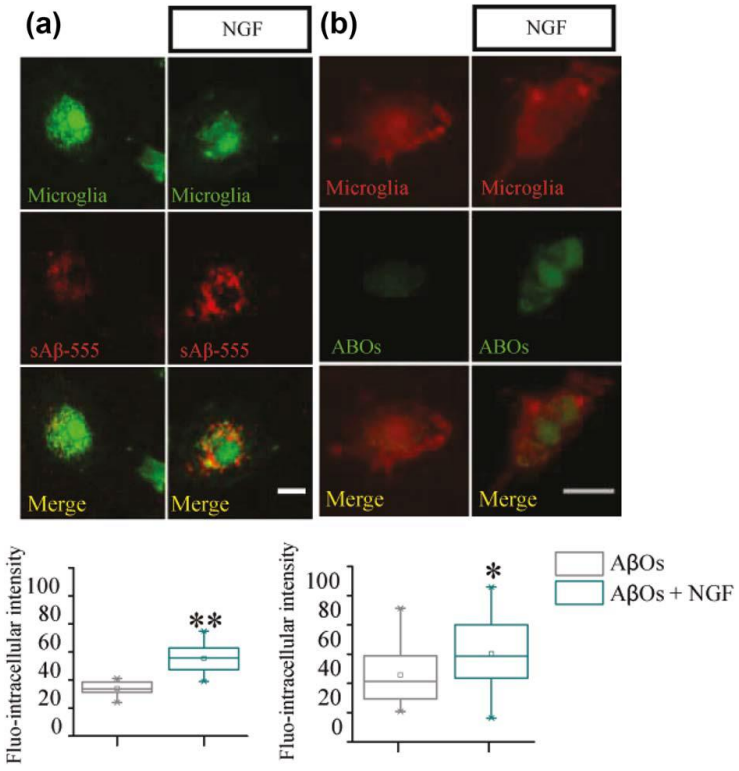


Figure 9. NGF increases the engulfment of sAβ peptide and ABOs: Immunofluorescence. Primary microglia from CX3CR1-GFP (a) and wild-type mice (b) were incubated respectively with 1 mM of fluo-555 sAβ peptide and ABOs, from 7pA2 supernatant, in presence or absence of 100 ng/ml NGF (10 μm scale bar, 20<n<30, *p<.05 **p<.001, Kolmogorov-Smirnov test).

increase in the uptake of AβOs in response to NGF (**Fig. 10b**). Thus, we conclude that NGF is able to increase selectively macropinocytosis of soluble Aβ oligomers in primary microglia by a TrkA-dependent mechanism.

The fate of internalized sAB following NGF treatment

What are the consequences of the increased macropinocytosis of Aβ oligomers induced by NGF? The Aβ engulfed 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, in addition to sAβ internalization, also the degradation of internalized

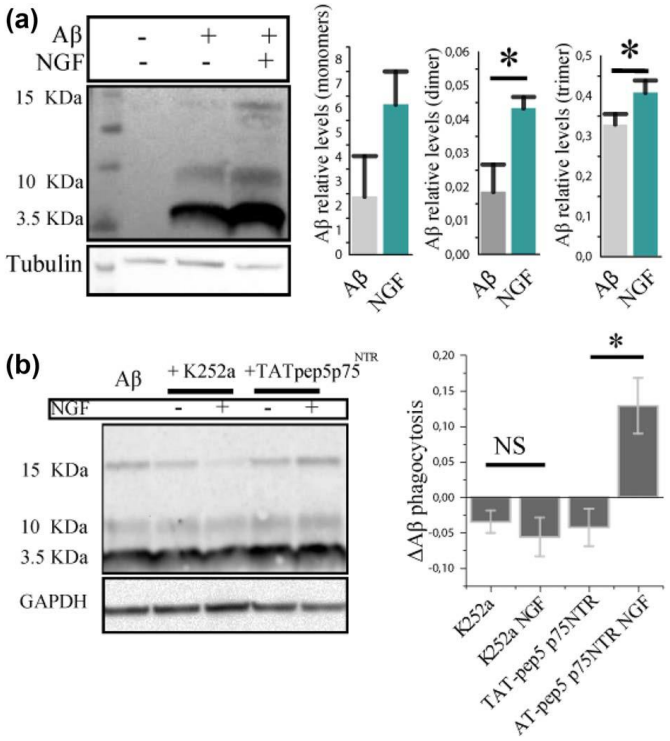


Figure 10. The modulation of microglial phagocytic activity is TrkA dependent. (a)Western blot of primary microglia treated with 1 mM of sAβ with or without NGF. Values are expressed as relative levels to controls (mean±SD, *p<.05, Student's t test). (b) Primary microglia treated with 200 nM K252a, intracellular TrkA inhibitor and with 1 µM TAT-pep5 p75NTR, inhibitor of p75NTR intracellular signaling. Values are normalized to the signal of samples treated with only sAβ (mean±SD, *p<.05, Student's t test).

sAβ might be modulated in response to NGF. We followed the fate of sAβ using lysotracker, a dye that marks lysosomes. We used, for this experiment, microglia BV2 cells, that also display functional TrkA and p75 receptors (Supporting Information). The quantity of sAβ peptides (green) internalized by BV2 microglial cells following NGF incubation is increased with respect to control. Moreover, the internalized Aβ colocalizes

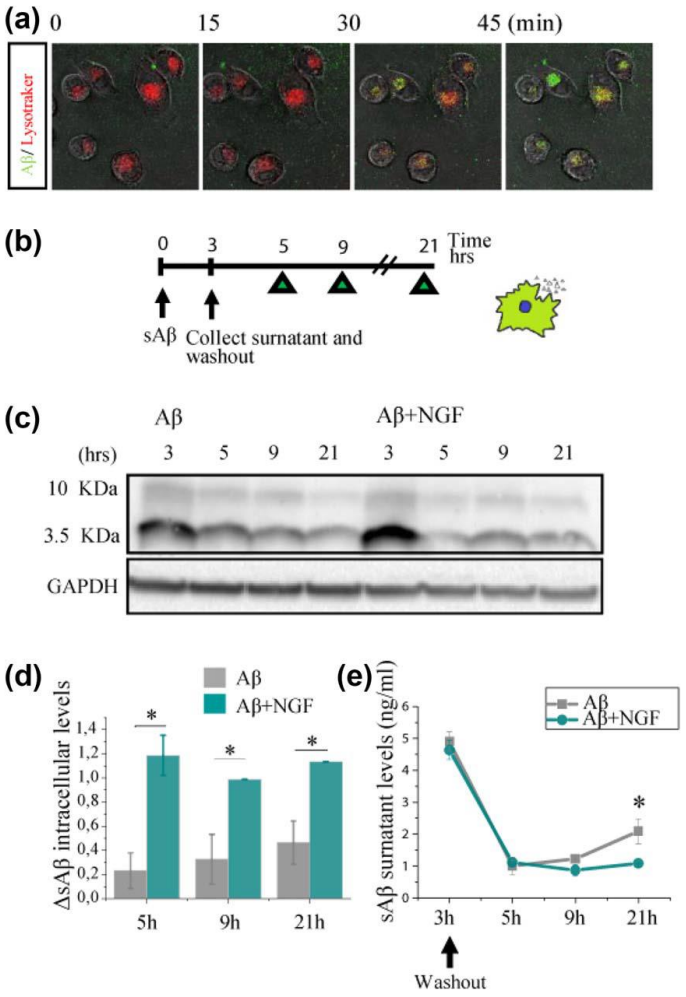


Figure 11. sAβ is digested and not released in the extracellular environment in cells treated with NGF in BV2 microglial cells. (a) Soluble Ab is rapidly trafficked to lysosomes for degradation. Confocal imaging of live BV-2 microglia 45 min of 1 mM soluble Aβ1–42-488 demonstrated localization of Aβ (green) within lysosomes. Lysosomes were stained using LysoTracker (red). (b) Experimental design for the degradation experiment (c) Western blot of cell lysates. (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 timepoint to the other. (e) Extracellular Ab levels measured by ELISA. The data represent the outcome of three independent experiments (mean±SD, *p<.05)

with lysotracker (red), suggesting that the engulfed material might go through lysosomal degradation (**Fig. 11a**).

In order to quantify such degradation and the hypothetical release of Aβ—such as suggested by (Joshi et al., 2014)—we proceeded as follows (**Fig. 11b**): BV2 microglial cells were treated with soluble Aβ for 3 hr, 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 hr, 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. 11c,d**)—as expected—but also that NGF-treated BV2 microglial cells digest a greater amount of Aβ (**Fig. 11d**) and release a smaller fraction of it into the extracellular compartment, compared with untreated control cells (**Fig. 11e**).

NGF increases internalization of sAβ by microglia in ex vivo brain slices

A big question was then to assess whether NGF effect on Aβ internalization might also be active on microglial cells integrated in the physiological circuit of the brain. To this aim, Aβ phagocytosis was tested in an ex vivo setting, in acute brain slice preparations

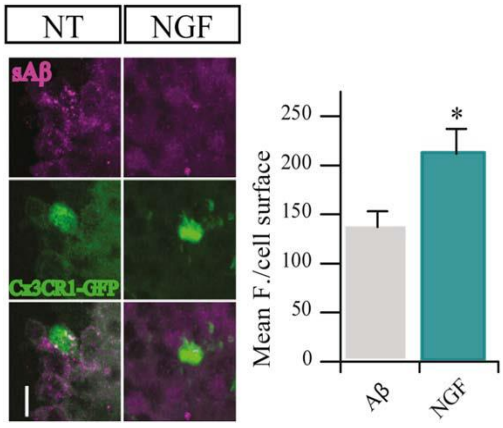


Figure 12. NGF increases the engulfment of sAβ ex vivo. Representative images of confocal stack acquisitions from 45 µm Cx3Cr1-GFP slices. Ex vivo 200 µm slices were first treated with 0.1 mM sAβ and with/without of 100 µg/ml NGF then analyzed by IF for Aβ content (mean±SD, *p<.05, Student's t test).

from CX3CR1-GFP adult mice. The acute slice was incubated for 3 hrs with NGF and fluorescent 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. 12**), demonstrating that, indeed, the modulatory effect by NGF can translate to microglia in vivo.

NGF protects against Aβ-induced spine toxicity and rescues spine density and LTP deficit, in a microglia-dependent way

It is known that Aβ oligomers decrease spine density both in vitro and in vivo, and impair synaptic long-term potentiation (LTP) (Palop & Mucke, 2010; Wei et al., 2010). Moreover, in the healthy developing and adult brain, an established physiological function of microglia is precisely the regulation of synapse number—synaptic pruning (Paolicelli et al., 2011; Parkhurst et al., 2013; Sipe et al., 2016; Zhan et al., 2014). Therefore, we asked whether NGF might regulate the activity of microglia on spines. To this aim, we

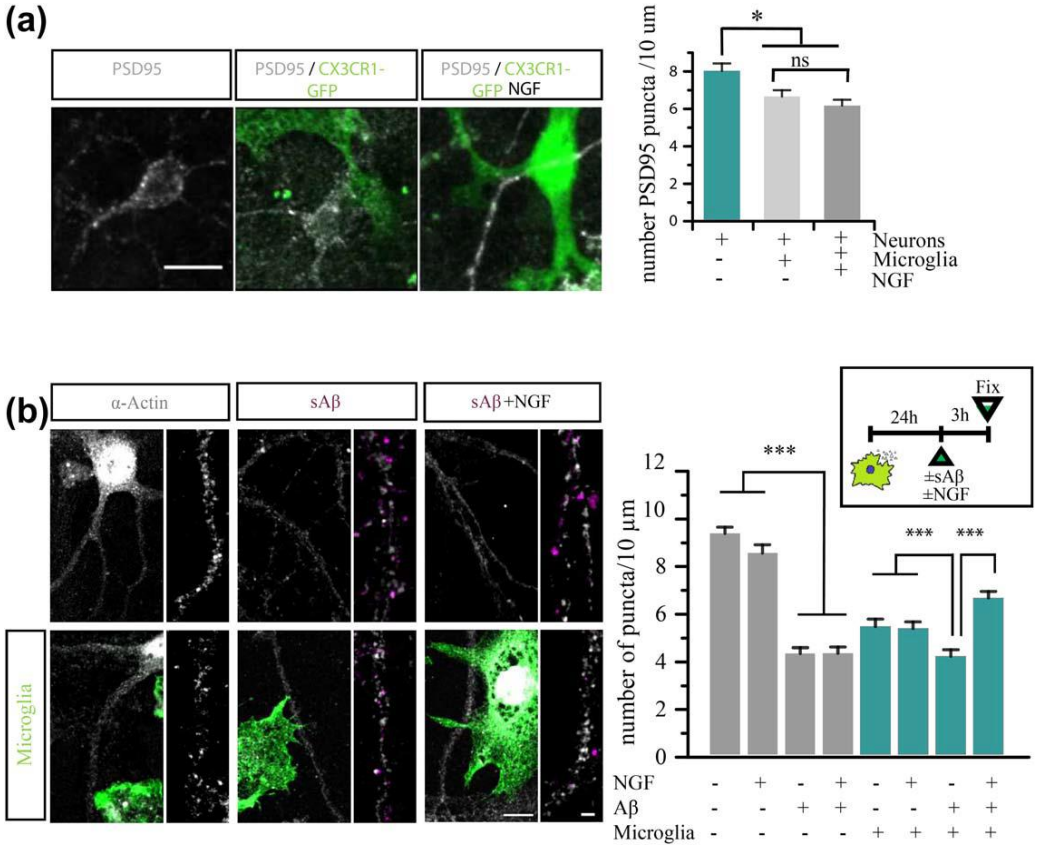


Figure 13. NGF protects against Aβ-induced spine toxicity in a microglial dependent fashion. (a) Representative images from confocal acquisition show PSD95 (white) puncta in neurons and in co-cultures ±NGF. (b) Representative images of confocal acquisition of neuronal spines labeled with actin (white) and the dendrites magnification ± sAβ (violet) in microglia (green)-neuronal co-cultures; (200<n spines <500 for two independent experiments, mean±SED *p<.05 ***p<.001, one-way ANOVA test, followed by a post-hoc Bonferroni test).

performed co-cultures of primary microglia with mature neurons and we quantified spine density following NGF treatment (**Fig. 13a**).

After 24 hrs of co-culture, the number of PSD95 positive puncta was lower on neurons cultured with microglia than in control neuronal cultures; this reflects the normal phagocytic activity of microglia on synapses (Ji, Akgul, Wollmuth, & Tsirka, 2013). In contrast, NGF treatment of microglia does not determine any further reduction—nor increase—of spine number compared with untreated microglia-neuron co-cultures (**Fig. 13b**). 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 in pure neuronal cultures, sAβ significantly decreases spine density by 50%, a decrease that could not be rescued by NGF treatment; however, in neuron-microglia co-cultures, where Aβ-induced spine loss could still be detected, concomitant treatment with NGF completely prevented the decrease in spine density, demonstrating microglia as the mediator of NGF neuroprotective activity (**Fig. 13b**). We conclude that NGF can prevent Aβ-mediated spine loss in a microglia-dependent manner. The effect of Aβ on spine number is paralleled by its negative effects on synaptic potentiation in plasticity paradigms (Q. S. Chen, Kagan, Hirakura, & Xie, 2000; Walsh et al., 2002). We therefore sought to investigate the interplay between NGF, microglia and spines in a plasticity protocol. We quantified synaptic 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)). As previously reported, the staining intensity of synaptic GluA1 AMPA receptors increased in pure neuronal cultures 1 hr after GI-LTP induction (**Fig. 14**). 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 were present, compared with control cultures without microglia (15.76% with respect to non-LTP cultures; **Fig. 14**).

This suggests an enhancement of synaptic potentiation by microglia, an in vitro correlate of the evidence suggesting a role for microglia in spine formation and potentiation in vivo (Miyamoto et al., 2016; Parkhurst et al., 2013). sAβ exposure

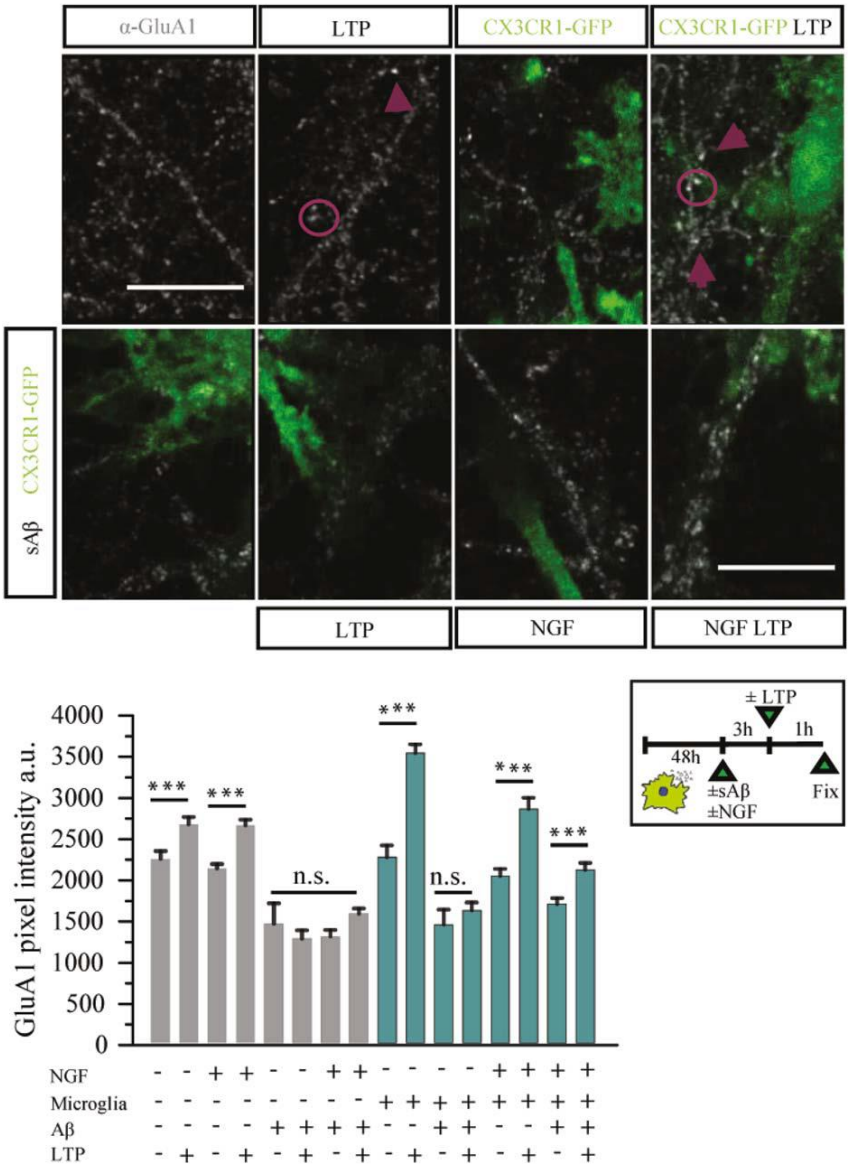


Figure 14. NGF protects against Aβ-induced loss of potentiation in neuron-microglia cocultures. (a) Effect of GI- LTP induction on GluA1 receptors (white) in different conditions, 1/2 microglia (green), ±NGF, ±sAβ. Histograms show the values of each experimental condition. (200<n spines<500 for two independent experiments, mean±SED *p<.05 ***p<.001, one-way ANOVA test, followed by a post-hoc Bonferroni test).

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 sAβ and microglia (**Fig. 14**). Instead, NGF-stimulated microglia cells were able to fully rescue the impairment of synaptic potentiation caused by sAβ. In fact, in NGF-treated microglia-neuron co-cultures, synaptic GluA1 levels were significantly higher after GI-LTP, even in the presence of sAβ (**Fig. 14**). 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. 14**). From these data, we conclude that—in our in vitro model of neuroimmune interface—not only NGF-stimulated microglial cells are able to block spine loss induced by sAβ (**Fig. 13a ,b**), but they can also attenuate the sAβ-mediated impairment of spine potentiation (**Fig. 14**), most likely by sAβ removal from the neuron surroundings.

IN BRIEF I

Microglia are the sentinels of the brain but a clear understanding of the factors that modulate their activation in physiological and pathological conditions is still lacking. In this first chapter, we demonstrate that Nerve Growth Factor (NGF) acts on microglia by steering them toward a neuroprotective and anti- inflammatory phenotype. We show that microglial cells express functional NGF receptors in vitro and ex vivo. Our transcriptomic analysis reveals how, in primary microglia, NGF treatment leads to a modulation of motility, phagocytosis and degradation pathways. At the functional level, NGF induces an increase in membrane dynamics and macropinocytosis and, ex vivo, it activates an out-ward rectifying current that appears to modulate glutamatergic neurotransmission in nearby neurons. Since microglia are supposed to be a major player in Aβ peptide clearance in the brain, we tested the effects of NGF on its phagocytosis. NGF was shown to promote TrkA-mediated engulfment of Aβ by microglia, and to enhance its degradation. Additionally, the proinflammatory activation induced by Aβ treatment is counteracted by the concomitant administration of NGF. Moreover, by acting specifically on microglia, NGF protects neurons from the Aβ-induced loss of dendritic spines and inhibition of long term potentiation. Finally, in an ex-vivo setup of acute brain slices, we observed a similar increase in Aβ engulfment by microglial cells under the influence of NGF. Our work substantiates a role for NGF in the regulation of microglial homeostatic activities and points toward this neurotrophin as a neuroprotective agent in Aβ accumulation pathologies, via its anti-inflammatory activity on microglia.

Chapter II: *NGF depletion affects astrocytes in vitro*

In the second chapter of this thesis, I briefly present some of the work done under the lead of one of the members of the lab, Nicola Maria Carucci. We investigated the relationship between NGF and astrocytes in cultured primary astrocytes. Specifically, I am going to present just a few of the key experiments in vitro that then prompted what is presented in the last chapter of this thesis.

MATERIAL AND METHODS II

PRIMARY CULTURES

All primary cell cultures were grown and maintained in DMEM/F12 or 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% CO^2 at 37°C.

Primary astrocyte cells were derived from the hippocampal tissue of B6129 at postnatal day 3-4 as described by Butovsky et al., 2014. Astrocytes 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 eliminated from the mixed primary glial cultures by mild shaking. Astrocytes, after 14 days in culture, were detached by mild trypsinization (10 min, 0.01% of trypsin (Thermo Fisher Scientific, MA, USA), then collected and re-suspended in DMEM/F12 with 1% penicillin/streptomycin, 1% Glutamax and 10% FBS and plated on the appropriate support 24 hrs before the experiments to allow cells to attach to the substrate.

IMMUNOCYTOCHEMISTRY

Primary astrocytes 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: 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) 1:500 anti-TrkA 1:100 (MNAC13

(Covaceuszach, Cattaneo, Lamba, 2005)), anti-TrkA 1:100 (Millipore, CA, USA; #06-574), anti-p75 1:500 (Millipore, CA, USA; AB1554).

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).

FLOW CYTOMETRY

Primary astrocytes were plated in 6 well plates at a density of $5x10^2$ cells/well. Cells were washed 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 or DyeRed. The analysis was performed using the FlowJo software (FlowJo, LLC, Ashland, Ore., USA).

WESTERN BLOTTING

Primary astrocytes were plated in 6 well plates at a density of 5x105/well in culture medium. Cells were 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) containing 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 concentration of the cell lysates were measured using the Bradford method. Lysates (20 to 50 µg) were then separated by a 10% SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blotting.

After transfer to nitrocellulose, the membrane was blocked for 1h and incubated with the appropriate primary antibodies. The following primary antibodies were used: anti-TrkA 1:1000 (Millipore, CA, USA; #06-574), anti-p75 1:1000 (Millipore, CA, USA; AB1554), anti-H3 1:5000 (Abcam, ab1791). 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.

ELISA FOR NGF

An ELISA kit (ab99986; Abcam) was used to measure the NGF levels in 100 µl of the astrocyte primary culture supernatant at different DIV. According to the manufacturer, this kit detects the pro- and mature forms of NGF. Recombinant NGF was used to create the standard curve. Assays were performed in triplicate.

CALCIUM IMAGING IN ASTROCYTES IN VITRO

Oregon Green™ 488 BAPTA-2 (O6809, Thermofisher) (1 µM) was used as the calcium indicator to detect calcium transients. Astrocytes plated in an imaging well were treated with the indicator for 20 min and then washed carefully as specified by the datasheet.

We registered a very good and constant basal activity and we express the results, due to the non-ratiometric intrinsic nature of the Oregon Green™ 488 BAPTA-2, as the percentage of responding cells for each frame. More than 60 cells per frame were oscillating. We considered active a cell if the increase of the signal was 5% higher than the average brightness of the same cell in the previous 15 and subsequent 15 frames.

After 250 frames (20 minutes) of baseline registration, we added anti-V5 antibody (800 ng/ml), αD11 (800ng/ml) or murine NGF (100ng/ml); Thapsigargin was used at a

concentration of 1µM 30 minutes before the beginning of the imaging session. The total volume in the registration chamber was 3 ml; the compounds were diluted in 20 µl of KREBS salt solution and slowly added in the Petri dish with a small tip, far from the focused registration area. Each registration session consisted of 1000 single frames acquisition taken every 5 seconds, for a total duration of 1 hour. The registration wasconducted under still medium condition (no perfusion was running) due to the astrocytes mechanosensor that could modulate the calcium waves (Shibasaki, Ishizaki, & Mandadi, 2013). For the experiments with the drugable TrkA from Ntrk1^{tm1Ddg}/J mice, 1NMPP1 was used at a concentration of 10 µM.

RESULTS II

Astrocytes express NGF receptors and secrete NGF in vitro

To address the question of the activity of NGF on cultured astrocytes, we first went to look for the receptors of this neurotrophin. In fact, the presence of NGF high affinity (NTRK1) and low affinity ($p75^{NTR}$) receptors on astrocytes *in vivo* and *in vitro* is still debated (Cragnolini et al., 2009; Hutton et al., 1992). Here we demonstrate that, in our culture conditions, astrocytes possess both p75 and TrkA. First of all, we performed ICC in detergent free conditions to determine membrane immunoreactivity of the receptors. In this experiment, we used both a polyclonal antibody and the monoclonal antibody MNAC13 to detect TrkA, yielding similar results (**Fig. 1a**). The abundance of the receptor was also measured by FACS, sorting astrocytes labelled with anti-p75 and MNAC13 antibodies and detecting, respectively, an enrichment of 75.17% and 86.32% in the

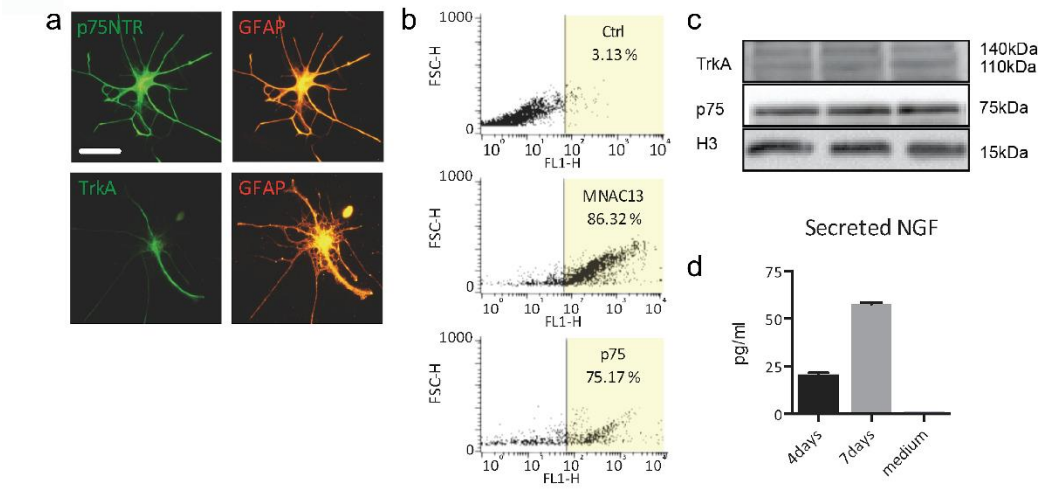


Figure 1: Expression of P75NTR and TrkA in cultured $GFAP^{+}$ astrocytes. (a) Representative images of the staining of TrkA and P75 antibodies in cultured astrocytes. (b) FACS analysis using antibodies for NGF receptors (d) Western blot showing the expression of p75NTR and TrkA in three independent samples (e) Quantification of the amount of NGF in astrocytic culture medium (One-Way ANOVA, medium vs 4 days, $p<0.001$, medium vs 7 days, $p<0.001$, n=3).

GFAP+ fraction (**Fig. 1b**). Western blot analysis of protein extracts from cultured astrocytes also show the expression of both receptors at the appropriate molecular weight ($p75^{NTR}$=75kDa; TrkA~110-140kDa) (**Fig.1c**). In addition, we also demonstrated the capability of cultured astrocytes to produce NGF, by measuring the progressive accumulation of the neurotrophin in culture medium via ELISA (**Fig. 1d**). Conclusively, cultured astrocytes express both receptors for NGF and can secrete themselves the neurotrophin.

Astrocytes respond to the lack of NGF via calcium transients

One of the most interesting responses in the spectrum of astrocyte activation is the generation of calcium transients. These calcium elevations have been connected to modulations at the synaptic level, changes to the neuronal circuitry and ultimately to alterations of behavior (Guerra-Gomes, Sousa, Pinto, & Oliveira, 2018). To determine astrocytic responses to the levels of NGF in vivo, we imaged cells previously stained with a calcium indicator, Oregon Green™ 488 BAPTA-2. After 20 min of baseline imaging, we added NGF or a neutralizing antibody against the neurotrophin (αD11) to adequately decrease NGF levels (Cattaneo, Rapposelli, & Calissano, 1988). αD11 has a very high affinity antibody for mature NGF, with a rapid association (k_a = 1.2 x 10^6 $M^{-1}s^{-1}$) and a very slow dissociation ($k_d < 10^{-6}$ s_{-1}) constants. On the other hand, the binding kinetics of proNGF showed a slower association (k_a = 5.9 x $10^5 M^{-1}s^{-1}$) and a faster dissociation (k_d = 6.9 x10^{-4} s^{-1}) constants when compared with mature NGF (Paoletti et al., 2009). We could observe no change in activity when NGF was added to the medium, but interestingly, astrocytes responded to a decrease in the levels of NGF, within 5 min from αD11 treatment (**Fig. 2a, b**).

Calcium events can either be generated from Ca^{2+} release from the IP3 receptors (IP3Rs) located in the endoplasmic reticulum (ER), or via transmembrane Ca^{2+} channels

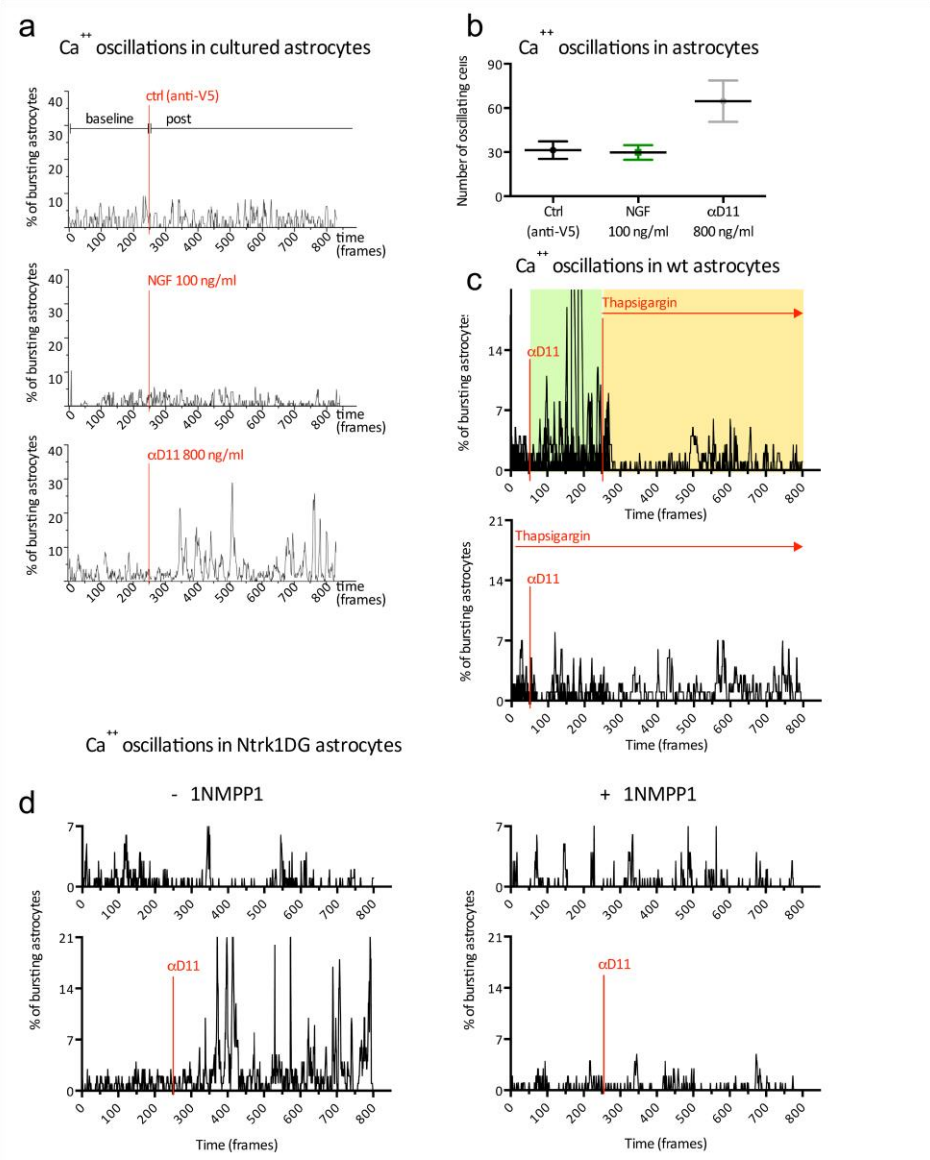


Figure 2: Calcium response of cultured astrocytes to NGF, a neutralizing antibody for NGF (αD11) and a control antibody (αV5). (a) Traces (b) and means of the percentages of responsive astrocytes during different treatments (One-Way ANOVA, ctrl vs NGF n.s., ctrl vs αD11 $p<0.001$). (c) Percentages of responding astrocytes treated with αD11 and Thapsigargin. Thapsigargin was added either after (top panel) or before αD11 (bottom panel). (d) Calcium imaging after αD11 treatment on astrocytes from Ntrk1$_{tm1Ddg}$/J mice in response to the absence (left panel) or presence (right panel), of the TrkA phosphorylation inhibitor. Top graphs on both sides represent baseline activity.

like TRPA1 (Khakh & McCarthy, 2015). To determine the origin of the Ca^{2+} involved in αD11 mediated calcium transients, we thus treated astrocytes with thapsigargin, an

inhibitor of SERCA channels that transport Ca^{2+} from the cytosol to the ER, in order to deplete intracellular stores of calcium. Astrocyte calcium responses to αD11 dropped in the presence of thapsigargin, either if this drug was applied before αD11 or after (**Fig. 2c**).

Thus, it appears that the lack of NGF induces calcium release from intracellular stores of calcium.

To further characterize the NGF-deprivation-induced calcium waves in astrocytes, we then performed the same experiment in primary astrocytes derived from a TrkA mutant mouse line, $Ntrk1^{tm1Ddg}$/J mice, carrying a mutation in the TrkA gene that renders Trk phosphorylation sensitive to inhibition by a small, membrane permeable molecule, 1NMPP1 (X. Chen et al., 2005). Thus, treating primary astrocytes derived from $Ntrk1^{tm1Ddg}$/J mice with 1NMPP1 virtually abolishes TrkA activity in astrocytes.

As expected, in the absence of 1NMPP1, the treatment with αD11 induced a fast and durable increase of calcium oscillations in astrocytes from $Ntrk1^{tm1Ddg}$/J mice, as was the case for wt astrocytes. Interestingly though, inhibiting TrkA signaling via 1NMPP1 treatment completely blocked the αD11-induced calcium increase. (**Fig. 2d**). Overall, we conclude that astrocytes respond to the decreases in the levels of NGF in the extracellular medium and do so in vitro by increasing their calcium activity.

IN BRIEF II

In this brief chapter, we presented some of our data on the effects of the modulation of NGF levels on astrocyte physiology. First, we showed how astrocytes not only possess the receptors for NGF, but also retain the ability to secrete the neurotrophin. We then performed Ca^{2+} imaging in vitro to determine the response to the neurotrophin and found out that, while NGF did little to change Ca^{2+} activity, taking out NGF via the sequestering antibody αD11 increased Ca^{2+} activity in cultured astrocytes. This increase in calcium seemed to be dependent on ER Ca^{2+} stores and on functional TrkA receptors.

Overall, this chapter identifies astrocytes as sensors of NGF levels and highlights the existence of a feedback loop, where levels of NGF are needed to maintain astrocytes in a physiological condition.

Chapter III: *Glial activity in vivo is modulated by neurotrophins*

In the first two chapters of this thesis, I presented data, mostly in vitro, regarding the effects of NGF on two very different glial cells, astrocytes and microglia.

If these two cells are indeed so different, they at least have in common the fact that their phenotype is tightly connected with the neuronal environment, so much so that in vitro their appearance and their whole transcriptome is a whole lot different than what is found in an intact brain. It is then the last aim of this thesis to address some key points of what has been presented before and translate to in vivo some of the new findings that were uncovered in the first two sections. To do so, I employed 2-photon microscopy, a technique that enables the scientist to look inside the brain of an intact behaving animal, and imaged – first microglia and then astrocytes – under the influence of NGF.

For what concerns microglia, I used a mouse expressing GFP under the fractalkine receptor – thus myeloid cells are GFP^+, to study microglial motility in vivo. Indeed, by taking consecutive z-stacks, it is then possible to analyze how these cells move in the CNS environment, and this can give a hint on the state of the cells. This is called *structural* imaging, implying that the technique will allow to study structural changes in the cells in response to a supposed treatment in the order of minutes and hours – if such is the nature of the structural changes which are typically slow. Structural changes and changes in the motility of microglial cells have been previously connected to function (Sipe et al., 2016; Wake et al., 2009) and it is thus of interest to monitor this feature of microglia.

On the other hand, astrocytes possess calcium excitability which can be studied using GECI (Genetically Encoded Calcium Indicators). In our case, I used an adeno

associated virus (AAV) to induce the expression of Gcamp6 in GFAP+ cells – that's to say – astrocytes. This *functional* imaging allows to study actual responses of these cells, in the order of fractions of seconds, as calcium transients in astrocytes are typically lasting a few seconds. Though the exact relationship between calcium and function has not been totally discovered – and on that matter I refer to my introduction chapter on calcium transients in astrocytes – it is now recognized that astrocytes exert many functions via their calcium activity, and it is thus of interest to study this property under the influence of our neurotrophin.

Let's then dig into the data!

MATERIAL AND METHODS III

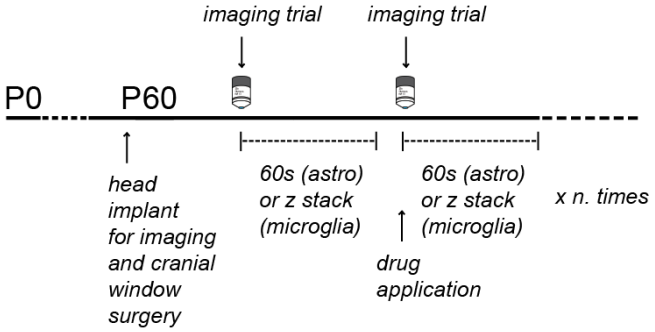
TRANSGENIC MICE

Heterozygous Cx3cr1GFP/+ (5xFAD x Cx3cr1GFP/+, TREM2KO x Cx3cr1GFP/+) mice were used for all experiments regarding microglia. Cx3cr1 (encoding fractalkine receptor) is expressed in microglia, some monocytes and dendritic cells. No obvious defects of parenchymal microglia were found in homozygous mutant Cx3cr1GFP/GFP mice, and the rapid microglial response to laser-induced injury was also observed in these mice (Davalos et al., 2005).

Wild-type mice (C57J\B) were used for viral injection at 1-1.5 month of age to achieve expression of GCamp6f in astrocytes, Gcamp7 in SST and the AchSensor (Jing et al., 2018). Transgenic mice expressing GCaMP6s in pyramidal neurons (Thy1.2-GCaMP6s Line 1), Cre recombinase in SST INs (SST-IRES-Cre), CX3CR1CreER, TrkB.T1, BDNFflox and 5xFAD mice were group-housed (5 per cage) in the Science Building Animal Facility at New York University Medical Center. All BDNF flox mice were given tamoxifen at P26 and P28 and analyzed starting at P30. Tamoxifen (Sigma) was given as a solution in corn oil (Sigma) to adult mice by gavage. Animals received two doses of 10mg of tamoxifen with a separation of 48 hours between doses. Mice were maintained at 22 C± 2°C with a 12-h light: dark cycle. All experiments were conducted during the light cycle between 08:00-18:00. Food and water were available at libitum. 1-1.5 month-old animals of both sexes were used for all the experiments in accordance with ethical regulations and the New York University Medical Center (NYUMC) guidelines, and approved by the Institutional Animal Care and Use Committee (IACUC) at NYUMC. No association was found between animals' sex and experimental results.

IN VIVO IMAGING WITH TWO-PHOTON MICROSCOPY IN THE ANESTHETIZED MOUSE.

GFP-labeled microglia and GCamp6f astrocytes were imaged by two-photon microscopy, through a small craniotomy (Grutzendler, Kasthuri, & Gan, 2002). Briefly, adult transgenic mice were anesthetized intraperitoneally with ketamine (200 mg/kg body weight) and xylazine (30 mg/kg body weight) in 0.9% NaCl solution. For imaging through a window in the skull, a thinned region (1–2 mm in diameter) was opened either with a needle or forceps. A drop (200 µl) of artificial mouse cerebrospinal fluid (ACSF, 125 mM NaCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 25 mM glucose; Bubble 95% O_2 and 5% CO_2 through the solution) was applied on the exposed region for the duration of the experiment. The skull surrounding either the thinned region or the open skull window was attached to a custom-made steel plate to reduce respiratory-induced movement. The animal was placed under either a Bio-Rad multi-photon microscope (Radiance 2001) or a custom-made two-photon microscope described previously. The Ti-sapphire laser was tuned to the excitation wavelength for GFP (920 nm). A stack of image planes with a step size of 0.75–2 µm was acquired using a water-immersion objective (Olympus 60x, 0.9 N.A.; Olympus 25X, N.A. 1.1) at zoom of 1.0–3.0. The maximum imaging depth was ~200 µm from the pial surface.



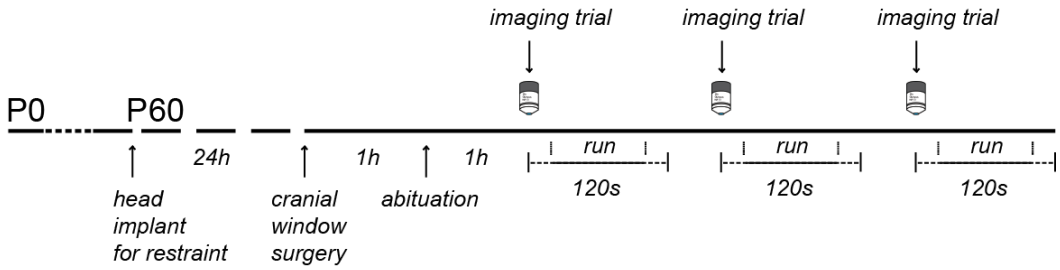
TWO-PHOTON LASER ABLATION INSIDE THE CORTEX.

A highly localized injury was achieved by focusing a two-photon laser beam (1 µm in size) in the superficial layer of the cortex either through a thinned, intact skull or with a small piece of the skull removed. The wavelength of the two-photon laser was set at 780 nm and the laser power was 60–80 mW at the sample. The beam was parked at the desired position for approximately 1–3 s to create a small injury site as indicated by a bright autofluorescent sphere (15 µm in diameter) around the focal point of the beam (**Fig. 1c**). The injury was confined to the area 15–20 µm in diameter around the laser focal point, because microglia within this area lost their GFP immediately after laser ablation, whereas those ~30 µm from the injury site still responded to the ablation. The laser-induced focal ablation is a useful injury model, as the site and degree of injury are easy to control, and the response of microglia toward the injury is highly reproducible (Davalos et al., 2005).

SURGICAL PREPARATION FOR IMAGING AWAKE, HEAD-RESTRAINED MICE.

We performed imaging in awake, head restrained mice through a cranial window. 24 h prior to imaging, mice underwent surgery to attach a head holder (G. Yang, Pan, Chang, Gooden, & Gan, 2013). Specifically, mice were deeply anesthetized with an intraperitoneal injection of ketamine (100 mg/g) and xylazine (10 mg/g). The mouse head was shaved and the skull surface was exposed with a midline scalp incision. The periosteum tissue over the skull surface was removed without damaging the temporal and occipital muscles. Two parallel micro-metal bars were attached to the animal's skull to serve as the head holder to help restrain the animal's head and reduce motion-induced artifact during imaging. A skull region of 0.2 mm in diameter was located over the primary motor cortex based on stereotactic coordinates (at 0.2 mm anterior to bregma and 1.2 mm lateral to midline) and marked with a pen. Next the head holder was mounted with dental acrylic cement (Lang Dental Manufacturing Co., IL, USA) such that the marked skull

region was exposed between the two bars. The marked region for imaging was kept exposed, uncovered with dental acrylic cement. The next day, we created the cranial window over the marked region. Briefly, the head holder was screwed to two metal cubes that were attached to a solid metal base. A high-speed drill was used to carefully reduce the skull thickness under a dissecting microscope (skull thickness 20µm). The skull was immersed in artificial cerebrospinal fluid (ACSF) during drilling. For open skull preparation a small circular craniotomy (1.0-1.5 mm diameter) was made and covered with a round glass coverslip (World Precision Instruments, coverslips, 5mm diameter) custom-madeto the size of the bone removed. The coverslip was glued to the skull to reduce motion



of the exposed brain. Before imaging, mice were given 1 day to recover from the surgery related anesthesia and habituated for a few times (10 min each) in the imaging apparatus to minimize potential stress effects due to head restraining and awake imaging.

DRUG APPLICATION THROUGH AN OPEN WINDOW IN THE SKULL.

Various reagents were dissolved in ACSF (artificial cerebrospinal fluid), and a small drop of the solution (200 µl) containing the compound of interest was applied directly onto the cortex through the open window after removing the dura. Imaging started either right away for anesthetized experiments or 10 min after drug application for awake head restrained experiments. Drugs: NGF (200-1000ng/ml), proNGF (400-1000 ng/ml), painlessNGF (200-1000 ng/ml), BDNF (200-1000 ng/ml), Phenilephrine (Sigma, 100 nM), anti-NGF clone αD11 (8µg/ml), anti-TrkA clone MNAC13 (8µg/ml), TrkAFc

(1µg/ml, R&D Systems). The NT condition in all in vivo experiments stands for "ACSF only".

CALCIUM IMAGING OF LAYER 1 DENDRITES, L2\3 SST SOMAS AND ASTROCYTES EXPRESSING GCAMP

Genetically-encoded Ca2+ indicator, GCaMP6 was used for Ca2+ imaging of somas of Layer 2/3 (L2/3) SST, dendrites from Layer 5 (L5) pyramidal neurons (pyramidal neurons), and astrocytes in Layer 1 (L1) in the primary motor cortex. Transgenic mice expressing GCaMP6s under the control of Thy1 promoter were used for imaging dendrites from L5 PN. In all other experiments GCaMP6 was expressed with recombinant adeno-associated viruses (AAVs). In experiments in which we imaged astrocytes, GCaMP6s was expressed with recombinant AAV under the human Glial Fibrillary Acidic Protein promoter (gfaABC1D-cyto-GCaMP6f, Addgene). 0.2 µl of AAV virus was injected (Picospritzer III; 20 p.s.i., 20 ms, 0.2 Hz) into L1 (depth of 50 µm) of the primary motor cortex using a glass microelectrode around the coordinate of 0.3 cm anterior and 1.5 cm lateral to bregma (Tennant et al., 2011). The glass microelectrode was inserted into the brain using a micromanipulator (M3301 World Precision Instruments), at a 60 degree angle relative to the skull surface. Mice were injected at post-natal day 21 and imaged 30 days later. In experiments in which we imaged somas of L2/3 SST-expressing INs, a Cre-dependent AAV-GCaMP7 (AAV-CAG-Flex-GCaMP7) was injected into SST-IRES-Cre mice which expressed Cre recombinase exclusively in SSTs. Mice were injected at post-natal day 21 and imaged 14 days later. In vivo two-photon Ca2+ imaging of motor cortex was done at the depth of 200-350 mm below the pial surface for detecting L2/3 somas of PNs and SST-expressing cells and at the depth of 20-70 mm below the pial surface for detecting apical dendrites and spines of L5 PNs. In each animal, time-lapse imaging was performed at one focal plane for PNs, astrocytes and SST INs. Imaging was performed with an Olympus Fluoview 1000 two-photon system (tuned to 920 nm) equipped with a

Ti:Sapphire laser (MaiTai DeepSee, Spectra Physics). The average laser power on the tissue sample was ~5–15 and 20-30 mW for imaging in L1 and L2/3 of the cortex respectively. All experiments were performed using a 25X objective (N.A. 1.1) immersed in ACSF solution and with a 2X (soma), 5X (dendrites), 1X (astrocytes) digital zoom. All images were acquired at frame rates of 2 Hz (2-ms pixel dwell time). The duration of imaging and number of repetitions were kept to a minimum that enabled us to perform the experiments from one hand but did not cause photo damage on the other (Hopt & Neher, 2001). Image acquisition was performed using FV10-ASW v.2.0 software and analyzed post hoc using NIH ImageJ software.

IMAGE PROCESSING AND QUANTIFICATION OF MICROGLIAL MOTILITY AND LASER LESION EXPERIMENT.

All image processing was done using US National Institutes of Health Image J software. All z-stacks of images were projected along the z-axis to recreate a two-dimensional (2D) representation of 3D structures. Time-lapse movies were generated by z-projections of stacks of images taken sequentially over time. 3-4 cell were analyzed per mouse. In making both the movies and figures, we made sure that the 3D image stacks at all timepoints passed above and below the processes studied. The laser ablation appeared as a small autofluorescent sphere ~15 μm in diameter. Following the ablation, the processes of neighboring microglia invaded the area around the site of injury and eventually reached the sphere. To quantify the extent and speed of microglial responses to laser-induced injury, we measured the number of microglial processes entering from the outer area Y (70 μm in radius) into the inner area X (35 μm in radius) surrounding the ablation site as a function of time. To account for signal intensity differences among different experiments, we thresholded every image so that all processes had the maximum value (255), and all background was set to 0. We then counted the number of white pixels in area X over time (Rx(t)) and compared it with the first picture taken immediately after

the ablation (Rx(0)). The number of white pixels corresponds to the region covered by processes within the area X, and its increase over time provides a measure of the microglial response. To account for the variability in the number of microglia located in the outer area Y in different experiments, we calculated the microglial response relative to the number of processes in the outer area Y immediately after the ablation (Ry(0)). The microglial response at any time point (R(t)) is therefore given by R(t) ¼ (Rx(t)– Rx(0))/Ry(0) (Davalos et al., 2005).

IMAGING DATA ANALYSIS OF GCAMP.

ROIs corresponding to visually identifiable, apical tuft dendrites or somata were selected for quantification. During running trials, the lateral movement of the images was typically less than 1 μm. Vertical movements were infrequent and minimized owing to flexible belt design, two micro-metal bars attached to the animal's skull (described above) by dental acrylic, and a custom-built body support to minimize spinal cord movements generated by the hindlimbs. All imaging stacks were registered using ImageJ plugin StackReg. Dendrites and soma neurons that could be identified in all imaged sessions were included in the data set. The fluorescence time course of each dendritic segment or cell body was measured with ImageJ software by averaging all pixels within the ROIs covering the dendrite or somata. The ΔF/F0 value was calculated as $\Delta F/F0 = (F - F0)/F0 \times 100\%$, in which F0 is the baseline fluorescence signal averaged over a 2-s period before the onset of the motor task. In all imaging sessions, resting awake images were collected before running trials.

Dendritic Ca2+ spikes were defined as the events when changes of fluorescence (ΔF/F0) observed in both dendritic spines and shaft (or dendritic branch) were >35% for GCaMP6s during the 120 sec imaging sessions. F0 is the fluorescence intensity in dendritic segments after background subtraction. Fluorescence background was measured

from a region adjacent to the dendrite segment. The threshold for detecting dendritic spikes was more than three times the standard deviation of baseline fluorescence noise for GCaMP6s. The $Ca2+$ spike frequency was determined by recording the number of $Ca2+$ spikes over 120 sec from the same motor region under various conditions.

STATISTICAL ANALYSES

All imaging data were presented as mean ± s.e.m. Student's t-test (two-tailed) was used to test for differences between groups whose distributions passed tests for normality (Kolmogorov–Smirnov). Wilcoxon matched-pairs signed rank test and Mann–Whitney U test were used to analyze those groups whose distributions did not pass tests for normality. 1way ANOVA was used when confronting groups $n>2$. 2way ANOVA was used when confronting traces to take into account time, treatment and replicates. Significant levels were set at $P \leq 0.05$. All statistical analyses were performed using the GraphPad Prism.

RESULTS III

Part I: Microglia and NGF

NGF increases microglial motility in vivo

Microglial cells are highly motile elements in the central nervous system (CNS) (Davalos et al., 2005; Nimmerjahn et al., 2005). Their ever moving processes continuously scan the environment and such movements can be followed and measured in vivo via 2-photon imaging. In chapter I, we showed how microglial cells do possess TrkA receptors in vivo (**Fig. 2, Chapter I**) and respond ex vivo to NGF treatments (**Fig. 6, Chapter I**). To test whether the Nerve Growth Factor (NGF) is able to affect microglia in the intact brain, I performed in vivo two-photon microscopy on transgenic mice expressing GFP in microglial cells ($Cx3cr1^{GFP/+}$). In an anesthetized mouse, I applied NGF (1µg/ml) on top of a cranial window over the motor cortex and started imaging around 10 min from treatment. I first quantified the resting microglial motility by taking consecutive z-stacks of an entire cell (**Fig. 1a**). In this experimental setup, NGF significantly increased microglial motility (**Fig. 1b**). Since microglial cells have the ability of responding to injury, such as that of a laser lesion, by extending their processes toward the injury site (Davalos et al., 2005) in a manner that is both quantifiable and reliable, I measured the response to laser lesion in animals before and after the local administration of NGF. Again, the neurotrophin was capable of increasing the motility of microglia in these conditions (**Fig. 1c, d**). To figure out whether the changes carried out by NGF are reversible, I performed three consecutive laser lesions, one in non-treated conditions, one with NGF and the last one with a sequestering antibody for NGF (αD11). αD11 was able to counteract the effect of the exogenously administered NGF (**Fig. 1f**) suggesting that the mechanism by which NGF acts is reversible in the time window of a lesion experiment (45 min).

The classical receptors for NGF in the CNS are the low-affinity nerve growth, p75NTR, and the high affinity tropomyosin receptor kinase A (TrkA).To determine whether TrkA might be involved in this phenomenon, in the same laser lesion assay, I applied a TrkA receptor antagonist (MNAC13 antibody) (Ugolini, Marinelli, Covaceuszach, Cattaneo, & Pavone, 2007). This was able to reduce microglial motility (**Fig. 1e**). To further investigate the matter of receptors, I applied both a mutant NGF (painless NGF) (Capsoni, Covaceuszach, Marinelli, et al., 2011) presenting a high affinity for TrkA and a lower affinity to p75 compared to wtNGF (Capsoni, Covaceuszach, Marinelli, et al., 2011; Covaceuszach et al., 2010) and proNGF which is more likely to bind p75 (R. Lee, Kermani, Teng, & Hempstead, 2001). I thus quantified resting microglial motility under these two neurotrophins (1 µg/ml each) (**Fig. 1g**) and found that both increased microglial motility unveiling complexity as to what receptor is relaying the effect of NGF in the cortical circuit. Indeed, if the effect on microglial motility had been in its entirety due to TrkA, NGFp should have brought on the same results as wtNGF. These data all together show that NGF increases microglial motility both in resting and injury conditions. Moreover, the data suggest that the mechanism might involve both the classical receptors for the neurotrophin in the CNS.

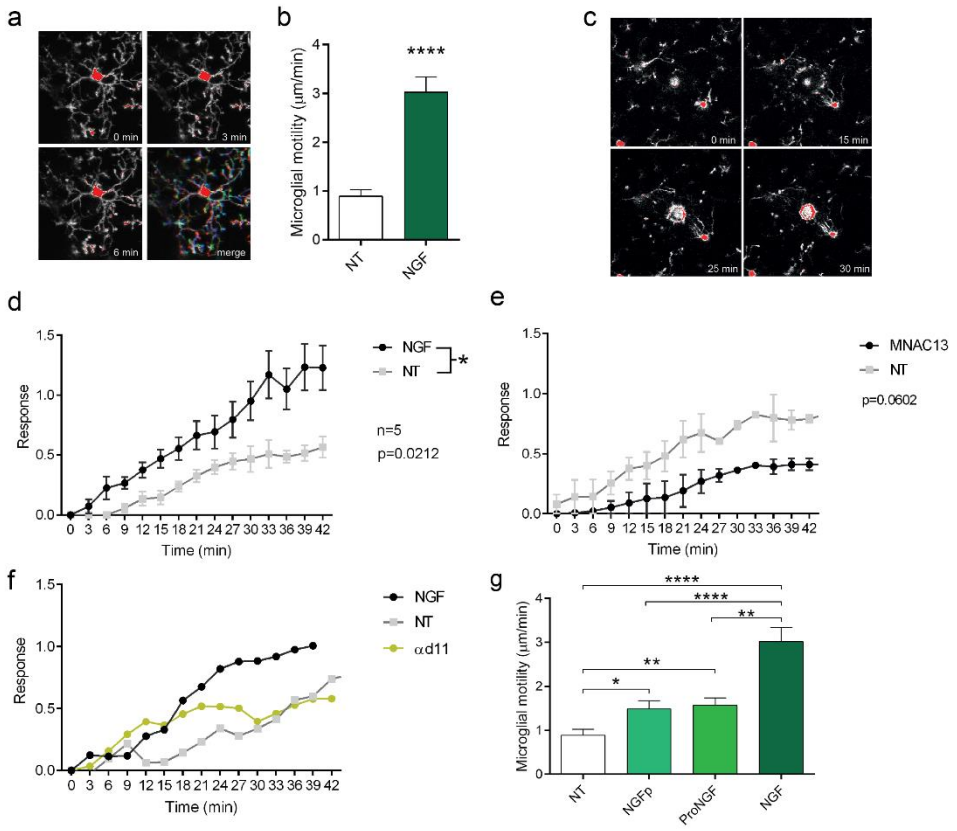


Figure 1: NGF activity on microglia in vivo: 2-photon imaging of microglia in the motor cortex of anesthetized mice. Cx3cr1 $^{GFP/+}$ mice were imaged, creating consecutive z-stacks of ~60µm with a 1µm z-step, three minutes apart. (a) Time-lapse imaging of the same microglial cells to determine the dynamics of their processes. (b) Quantification of the motility of microglia in non-treated (NT) and NGF treated conditions reported as µm/min (Two- tailed t-test, $p<0.0001$, n=4, 4 cells per mouse). (c) Time-lapse imaging of microglial processes after laser lesion. (d) **Laser injury under NGF:** Response of microglial processes in the NT condition and after NGF (1 µg/ml) (2-WAY ANOVA, p=0.0212, n=5, 3 trials per mouse). The Y axis represent the normalized mean fluorescence over time of the region surrounding the lesion which increases as microglial processes enter such region, as specified in the M&M. (e) **Inhibition of endogenous NGF:** to inhibit TrkA signaling due to endogenous NGF activity, we used an antibody against TrkA, reported to neutralize the interaction between the neurotrophin and its receptor (Covaceuszach, Cattaneo, & Lamba, 2005). (e) Quantification of the response to laser injury in NT and MNAC13 (8µg/ml) conditions (Two-Way ANOVA, p=0.0602, n=2, 3 trials per mouse). (f) **Chelation experiment:** microglial response to laser injury was first assessed in the NT condition, then NGF was applied to the tissue and another lesion was performed. Lastly, an antibody chelating NGF, αD11, was added to inhibit the exogenous NGF. (f) Quantification of the microglial response in the three different phases of the chelation experiment. (g) **Other neurotrophins:** the resting motility of microglia was also quantified in response to different forms of NGF such as its precursor proNGF (1µg/ml) and the mutated "painless version of NGF" NGFp (1µg/ml) (Capsoni, Covaceuszach, Marinelli, et al., 2011) (One-way ANOVA, NT vs NGFp: p= 0,0207; NT vs proNGF:p=0.0029; NT vs. NGF: $p<0.0001$; proNGF vs NGF: p=0.0072; NGF vs NGFp: $p<0.0001$; n=3, 4 cells per mouse).

NGF increases microglial motility in the awake behaving animal

Next, I evaluated the activity of NGF in the cortical circuit in the awake animal, head restrained under the microscope, placed on top of a treadmill (**Fig. 2a**). It was recently

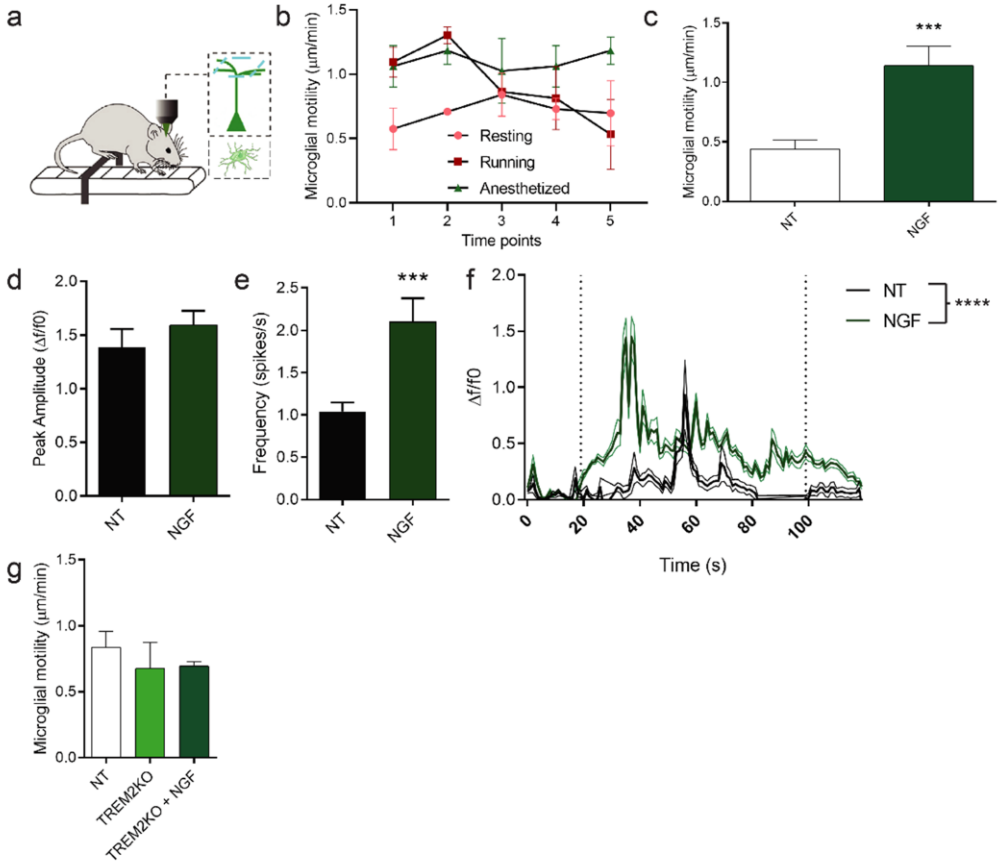


Figure 2. NGF activity in the awake resting mouse and in TREM2 KO mice. (a) We imaged either microglia in the Cx3Cr1-GFP/+ mice or dendrites in layer I from Thy1-Gcamp6 pyramidal neurons. (b) We measured microglial motility in 3 different conditions, awake resting, awake after a running trial, and anesthetized; a z-stack every 5 min (Two-Way ANOVA, Interaction; p=0.05; Resting vs Running p=0.018, Resting vs Anesthetized p=0.027, Running vs Anesthetized n.s). (c) We tested the effect of NGF in the awake behaving animal, head restrained and positioned on a treadmill under the 2-photon scope but kept resting. NGF increases microglial motility also in the awake behaving animal. Motility was quantified while the animal was resting (Two tailed, unpaired t-test p= 0.0022, n=3 per group). (d, e, f) GCamP6 response in dendrites of pyramidal neurons during running. Imaging of layer I dendrites under the influence of NGF in the cranial window. (d) ΔF/F0 amplitudes (e) frequency of firing (f) and traces of the responses (Run between 20-100 seconds) (Two-Way ANOVA, p<0.0001). (g) Microglial motility in the anesthetized TREM2 KO mice. NGF has no effect on the microglial motility of KO mice for TREM2 (One-Way ANOVA, n=3 per group).

reported that microglial motility is decreased in the wakeful state compared to anesthetized animals because of norepinephrine signaling (Liu et al., 2019; Stowell et al., 2019). I thus first quantified if in our data the same results could be observed and indeed microglial motility in the awake resting animals was reduced compared to anesthetized (**Fig. 2b**). In this setup, we then measured microglial motility to find that NGF exerts the same effects as in the anesthetized mouse increasing the surveilling activity of these myeloid cells (**Fig. 2c**).

Moreover, in the awake behaving animal, I assessed the neuronal response to NGF by imaging dendrites in mice expressing Gcamp6 in layer V pyramidal neurons during a running trial. NGF consistently increased the neuronal response to running (**Fig. 2d, e, f**).

TREM2 could mediate NGF activity on microglial cells

I then decided to test the neurotrophin on the knock out mouse of a key factor for microglial phagocytosis: The Triggering Receptor Expressed on Myeloid Cells 2 (TREM2).

As the name suggests, the receptor, though still orphan for a ligand, is solely expressed on myeloid cells, which for the healthy brain parenchyma means microglia. Hypomorphic variants of TREM2 are associated with an elevated risk of developing Alzheimer's disease (Benitez et al., 2013; Jonsson et al., 2013) and this has been linked to the role of the receptor in homeostatic microglial functions such as proliferation, phagocytosis and metabolic fitness (Kleinberger et al., 2017; Ulland et al., 2017). Most notably, TREM2 has been shown capable of directly binding Aβ and its deficiency has been linked to deficit in phagocytosis of amyloid plaques (Y. Wang et al., 2015; Zhao et al., 2018). Moreover, literature shows that microglia in the TREM2KO mice have deficits in microglial motility in response to injury at old ages (9 months), but not in the young adult mouse (2 months) (Sayed et al., 2018).

We have previously demonstrated in our in vitro data that NGF is able to affect, besides motility, the phagocytic behavior of microglia towards Aβ (Chapter I – Figure 5 and 8). I thus wanted to test the neurotrophin in the young adult TREM2KO mouse, to see whether microglial cells are capable of responding to the neurotrophin or are indeed already deficient. In the anesthetized mouse, TREM2KO mice did not show any significant difference in microglial motility compared to WT mice, suggesting that there is no deficit in motility due to the mutation. Interestingly though, NGF did not affect microglial motility in the TREM2KO (**Fig. 2f**), which could imply that the receptor is important to carry out the effects of NGF on microglia. Moreover, this should be kept in mind since an NGF based therapy for patients carrying mutation in TREM2 could not bring about the desired positive effects.

NGF modulates microglial motility in an Alzheimer's mouse model

There has been an increase in interest in the relationship between microglial cells and neurodegenerative diseases. In the particular case of Alzheimer's disease, this correlation is indeed the strongest: a number of risk factors for the disease come from genes related to immunity (Hollingworth et al., 2011; Ulland & Colonna, 2018), one but not the only, TREM2, that was introduced in the last paragraph.

Microglia isolated from transgenic AD mice show reduced phagocytic abilities and increased proinflammatory responses (Hickman et al., 2008). In the first Chapter of this thesis, we showed how NGF was able to modulate microglia to make them neuroprotective in an Aβ ridden context, increasing phagocytosis of the toxic peptide and increasing motility.

NGF has been looked at as a putative therapeutic strategy, due to its trophic action toward cholinergic neurons, that undergo degeneration early in the disease (Castle et al.,

2020; Rafii et al., 2018; Tuszynski, 2000; Tuszynski et al., 2005). But the functional significance of an NGF based therapy on microglial cells in vivo has not been established yet.

To investigate in vivo the effect of NGF on microglia in a neurodegenerative environment, I evaluated microglia motility in the 5xFAD mouse, a model for AD, expressing 5 mutations linked to familial AD cases in humans (Oakley et al., 2006). I thus performed two-photon microscopy in awake resting 5xFADxCX3CR1$^{GFP/+}$ mice,

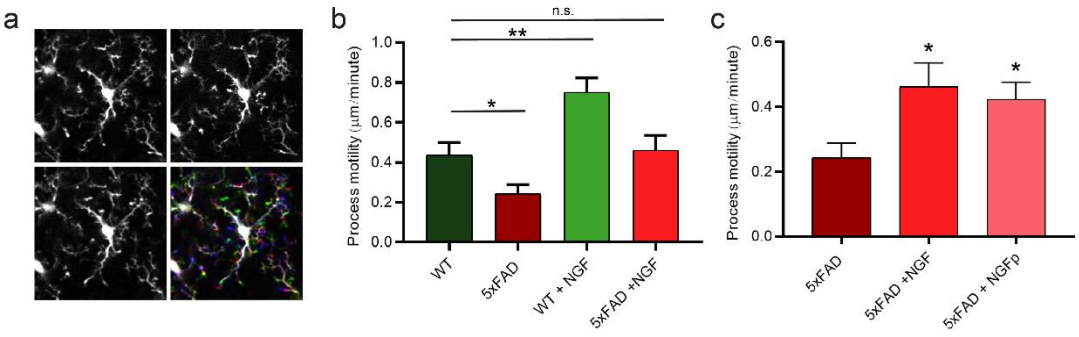


Figure 3. NGF rescues deficits in microglial motility in the 5xFAD mice: (a) Representative two-photon images of microglia at the 2-photon microscope. (b) quantification of microglial motility in 5xFAD and control littermates in NT or NGF (1µg/ml) (1way ANOVA, WT vs. 5XFAD, p=0.00411; WT vs. WT + NGF, p=0.00105; 5xFAD vs 5xFAD +NGF, p= 0.0156; WT vs. 5xFAD +NGF, n.s.; n=3 per group, ~ 5 cells per animal). (c) comparison of the effect of NGFwt (1µg/ml) and NGFp (1µg/ml) (1way ANOVA, 5xFAD vs. 5xFAD +NGF, p=0,0002847; 5xFAD vs. 5xFAD + NGFp, p=0.0002226, n= 3 per group, ~ 5 cells per animal).

expressing GFP in microglial cells to follow their motility behavior.

In 4 months old mutant and control mice, I quantified microglial motility under non treated and NGF treated conditions (**Fig. 3a**). I first observed that microglia in the 5xFAD have a deficit in process motility at this age (**Fig. 3b**). Interestingly, as it was for the WT, microglia in the 5xFAD increased their baseline motility in response to NGF, thus rescuing the deficit previously reported (**Fig. 3b**).

NGF-based therapies have an important caveat: since the neurotrophin is involved in pain-related signaling pathways, systemic NGF treatment is thus unfeasible. Our laboratory exploited a mutant form of NGF, "NGF painless" (NGFp), carrying the mutation R100, inspired by the human genetic disease HSAN V (Hereditary Sensory Autonomic Neuropathy Type V) that allows high doses of NGF without triggering pain (Capsoni, Covaceuszach, Marinelli, et al., 2011). It has been previously demonstrated that NGFp has potent neuroprotective and antiamyloidogenic properties in AD mouse models (Capsoni et al., 2012). Moreover, it has been shown that NGFp might be acting on glial cells, modulating inflammatory proteins such as TNFαRII and the chemokine CXCL12 (Capsoni et al., 2017).

I thus compared the effect of NGFwt and NGFp on microglial motility in the 5xFAD, to find that these two neurotrophins exerted a similar effect in increasing microglial motility in the AD mouse model (**Fig. 3c**).

In conclusion, these data support a role for microglial cells in mediating the neuroprotective effects of NGF painless in mouse models of AD, as stated in Capsoni et al., 2017.

Part II: Astrocytes and neurotrophins

Astrocytes and NGF

As the most numerous population of the CNS, astrocytes represent scaffold and support to neurons and their connections. Their ability to respond to inflammatory stimuli, to participate in synaptic computation with their direct access to the synapse, and to elicit rapid calcium signals in retort to a variety of stimuli makes them interesting putative targets of neurotrophic factors. In Chapter II, we presented data suggesting that primary cultured astrocytes might somehow be able to sense the extracellular levels of NGF and respond to NGF deprivation via calcium transients. Due to the intrinsic nature of in vitro work, which is biased by differences in the activation state of primary astrocytes (Guttenplan & Liddelow, 2019), it was then of interest to study how modulations of NGF levels affect astrocytes in their natural environment – the brain parenchyma. As anticipated in the introduction to this chapter, to do so, I exploited adeno-associated viruses to induce the expression of a calcium reporter, Gcamp6, in astrocytes in the motor cortex of WT mice and measured the acute response of astrocytes to modulations of NGF levels.

NGF levels modulate astrocyte calcium activity in vivo

I first sought to investigate whether astrocytes could respond to changes in levels of NGF by applying the neurotrophin exogenously or by decreasing its levels via a neutralizing antibody, αD11. In anesthetized mice, which were infected with AAV5-GfapABC1D-cyto-GCaMP6f-SV40 virus (GFAP.GCaMP6.cyto) to drive GCaMP6 expression in astrocytes, I imaged astrocyte calcium levels both as baseline activity and in response to an α1-receptor agonist phenylephrine (PE), which is capable of instantly inducing a strong calcium wave in astrocytes (Ding et al., 2013). PE is used to activate astrocytes that otherwise, in the anesthetized mouse, are more or less silent, in order to

detect the global activation capacity of these glial cells. Treatments were diluted in ACSF (NGF 1µg/ml, αD11 8µg/ml, PE 100 µM) and applied directly onto the cortical surface through a small cranial window over the motor cortex. Imaging began right after the application of the drugs.

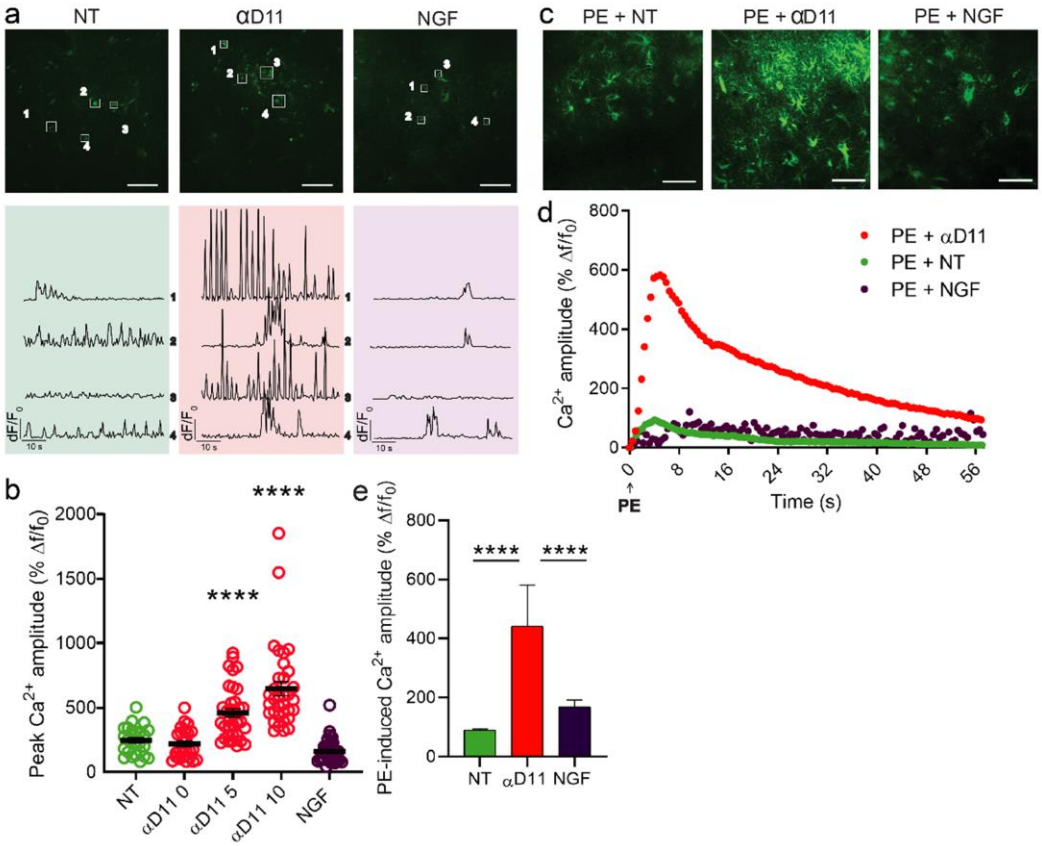


Figure 4. Lack of NGF increases astrocyte calcium. (a) Representative two-photon images and traces of Ca^{2+} response in astrocytic processes expressing GCaMP6s before (NT) and after NGF (1µg/ml) or αD11 (8µg/ml) application in the motor cortex of an anesthetized mouse. (b) Quantification of the peak amplitude of Ca^{2+} transients in astrocytic processes during baseline (NT), right after (αD11 t0), after 5 min (αD11 t5) and 10 min (αD11 t10) of αD11 (8µg/ml) application (One-way ANOVA, NT vs. αD11 NF t5 $p < 0.0001$; NT vs. αD11 t10 $p<0.0001$, n=4 per group, ~20 processes per animal). (c) Representative images of non-treated (NT), αD11 and NGF treated astrocytic response to phenylephrine (PE) 100 µM. (d) Representative traces of the bulk astrocytic response to PE administered at t0. (e) Quantification of the astrocytic response to PE 100 µM (One-way ANOVA, NT vs αD11, p=0.0001; NT vs NGF, n.s.; NT αD11 vs NGF, p=0.0001; n=3, 10 cells per animal).

Astrocytic Ca^{2+} responses were first measured in the fine processes of the astrocytes during baseline activity (**Fig. 4a**). The effect of treatments on Ca^{2+} activity of astrocytes was monitored right after their application (t0), then after 5 (t5) and 10 minutes (t10). At any time point, NGF did not induce any change in the Ca^{2+} baseline activity of astrocytes – data was thus pooled into a single bar (**Fig. 4b**). Interestingly though, decreasing the levels of NGF via αD11 treatment significantly increased astrocytic Ca^{2+} responses in fine processes (**Fig. 4b**). I then measured the bulk activation response of astrocytes, pretreated with NGF or αD11 10 for 10 minutes, to the adrenergic agonist PE (**Fig. 4c**). I found that PE treatment induced a much larger astrocytic Ca^{2+} response in αD11 treated mice as compared with non-treated controls (**Fig. 4d, e**), while no difference was detected in the NGF treated cohort.

Reducing NGF levels increases astrocyte calcium in the awake behaving animal

Since the lack of NGF in cortical tissue relayed such a powerful response from astrocytes I decided to focus on this treatment, switching to the more complex system of the awake behaving animal. I performed 2-photon imaging in mice previously injected

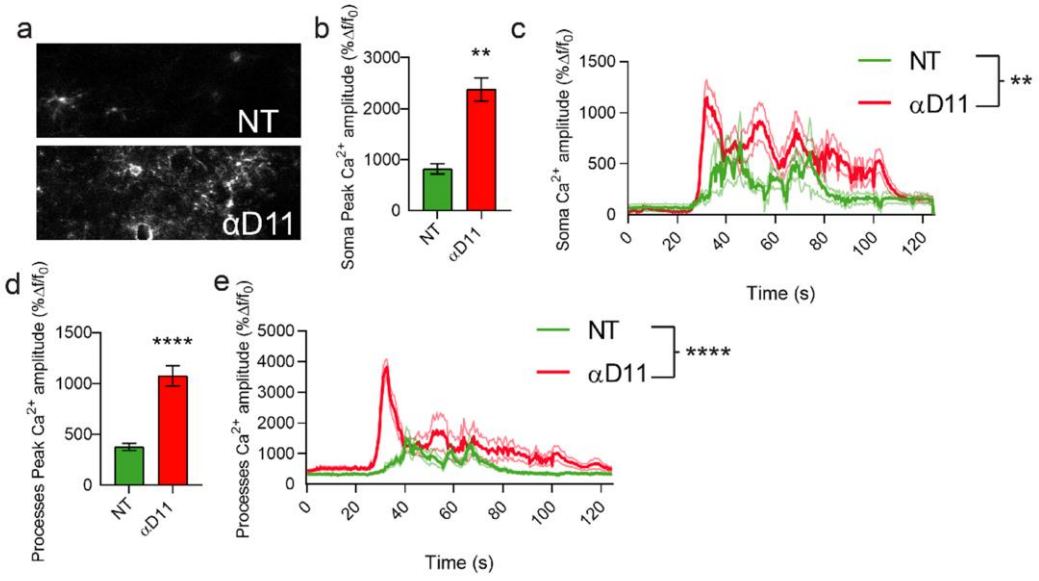


Figure 5: Lack of NGF increases astrocytic activity in the awake behaving animal. (a) Representative images of the astrocytic responses. (b) Soma peak amplitude (2tailed t-test, p=0.0012) and (c) traces (2way ANOVA, p<0.0001) to running in NT and αD11 treated conditions. (d) Process peak amplitude (2tailed t-test, p<0.0001) and (e) traces (2way ANOVA, p<0.0001) to running in NT and αD11 treated conditions (n=4 per group, ~10 cells per animal).

with AAV5-GfapABC1D-cyto-GCaMP6f-SV40 virus (GFAP.GCaMP6.cyto) to drive Gcamp6f expression in astrocytes. I then imaged the astrocytic calcium response in the mouse motor cortex during a running task (120 seconds total, running 20-100). It has already been reported that astrocytes can respond to running with a wide and global calcium response that interests both soma and fine processes. I thus quantified the response to such task under the influence of αD11 or NT conditions. In a similar manner to

anesthetized conditions, in the awake behaving animal, lack of NGF increases astrocytic activity both in the soma (**Fig. 5b, c**) and processes (**Fig. 5d, e**).

Next, I used another NGF inhibitor, TrkAFc, to further confirm the effect of the lack of NGF onto astrocyte calcium levels. As for αD11, TrkAFc was able to consistently increase astrocyte calcium in their processes (**Fig. 6d, e**).

In conclusion, using two different ways to decrease NGF levels in cortical tissue, I

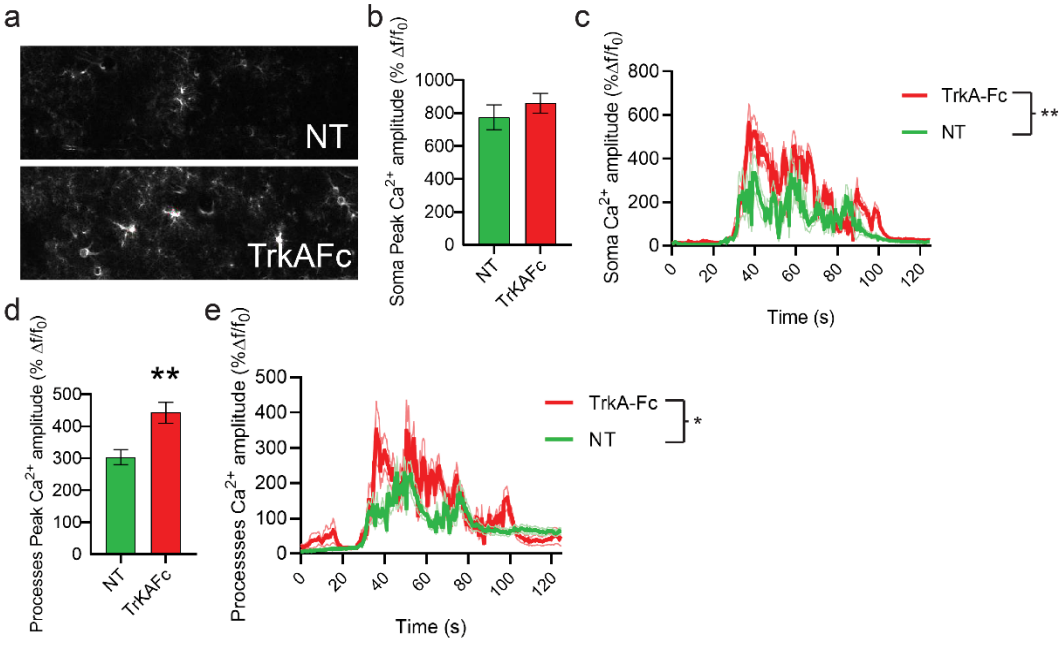


Figure 6: Inhibition of NGF via TrkAFc increases astrocytic calcium activity. (a) Representative images of the astrocytic response to TrkAFc. Soma peak amplitudes (2tailed t test, n.s.) (b) and traces (c) (2way ANOVA, p= 0.0016) of the astrocytic Ca^{2+}. Process peak amplitudes (d) (2tailed t test, p=0.001) and traces (e) (2way ANOVA, p= 0.0103) (n=3 per group, ,~10 cells per animal).

report that lack of NGF can increase acutely astrocyte calcium in vivo.

Next, I further defined the effect of NGF deprivation by measuring the layer specific astrocytic responses to αD11 treatment (**Fig. 7a**). I thus quantified these responses in different layers of the cortex (layer I vs layer II/III): I observed that layer I astrocytes were

much more affected by the lack of NGF compared to layer II/III astrocytes, suggesting a layer specific effect of NGF (**Fig. 7b, c, d, e**).

Conclusively, there seems to be a layer specific astrocytic response to levels of NGF in the motor cortex of the mouse.

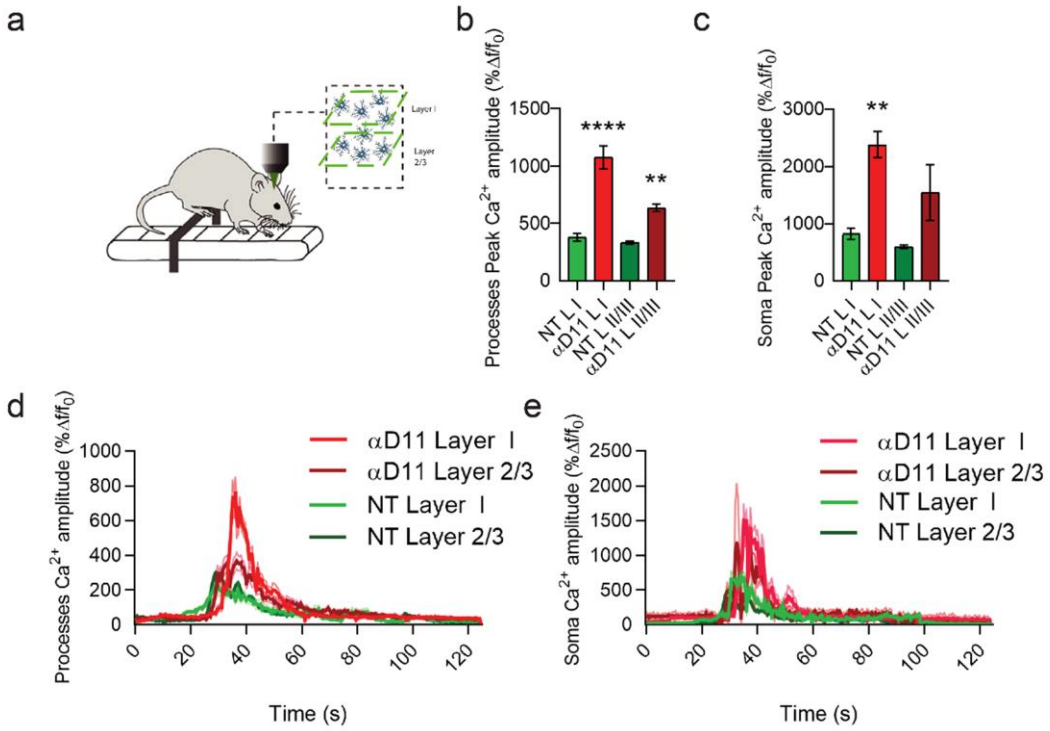


Figure 7: Lack of NGF increases astrocytic activity specifically in Layer I astrocytes in the awake behaving animal. (a) Head fixed animal under the 2P microscope over a treadmill. *Layer specific responses*: Peak amplitudes of processes (b) and soma (c) (One-way ANOVA, NT L I vs. αD11 L I, $p<0.0001$; NT L I vs. αD11 L II/III, $p=0.0087$) of astrocyte calcium responses and their respective traces (d, e) in Layer I and Layer II/III of the motor cortex (n=2).

NGF deprivation decreases dendritic neuronal activity

To understand the global effects of NGF deprivation on neuronal circuits, I then evaluated the effect of the lack of NGF on the neuronal dendritic compartment. Specifically, I imaged dendrites in layer I coming from Layer V pyramidal neurons in thy1-gcamp6 (SL1) mice placed on a treadmill during running trials of 120 seconds. Interestingly, αD11 was able to determine a decrease in the neuronal response to running (**Fig. 8**).

Thus, we can conclude that, in layer I, the lack of NGF increases astrocyte calcium specifically and, at the same time – though we cannot infer causality, it also decreases neuronal firing.

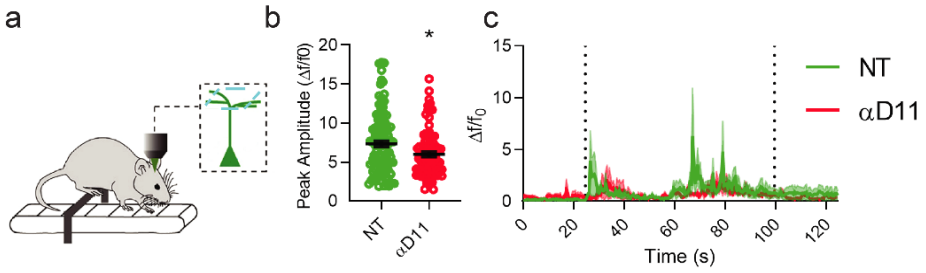


Figure 8: Lack of NGF reduces dendritic activity of Layer V pyramidal neurons. (a) thy1-Gcamp6 (SL1) head-fixed mouse under the 2P microscope, placed over a treadmill for running trials of 120 seconds. Imaging was performed on Layer I dendrites of the motor cortex. Peak amplitude responses (b) (2tailed t test, p=0.01) and traces (c) of the dendritic compartments under the influence of αD11 or in non-treated (NT) conditions (running between 25-100s – as indicated by the vertical dotted lines, n=3).

NGF deprivation modulates ACh levels and SST interneurons activity

NGF is a known modulator of cholinergic neuron activity: it has indeed been shown that NGF treatment can increase acetylcholine (ACh) release in cultured basal forebrain neurons (Auld et al., 2001; Huh et al., 2008). I thus aimed at understanding whether there was this sort of modulation also in vivo. To evaluate changes in cholinergic input in the

cortex in response to fluctuations in NGF levels, I used 2-photon imaging on a mouse expressing an acetylcholine fluorescent indicator (Jing et al., 2018) (**Fig. 9a**), that increases fluorescence in response to binding to ACh, such that total fluorescence correlates to ACh levels. Imaging was performed in layer I of the motor cortex in an awake head restrained animal (resting) and ACh levels were monitored before and after an

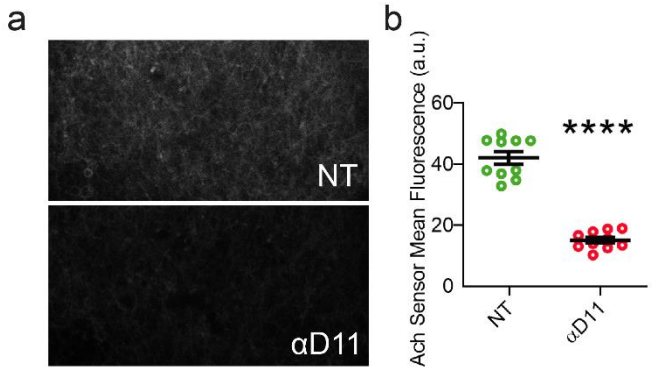


Figure 9: Decreasing NGF levels diminishes ACh input in the motor cortex. (a) Representative images of the ACh Sensor in NT or αD11 treated conditions. (b) Histogram showing the mean fluorescence of the signal of the ACh Sensor before (NT) and after 10 min from the application of αD11 8 µg/ml (Two-tailed t-test, $p<0.0001$, $n=2$).

application of αD11 (8 µg/ml). In these conditions, αD11 consistently decreased the ACh levels in the cortex after a 10 minutes' application (**Fig. 9b**). This suggests that cholinergic activity in the cortex might be dependent on NGF levels as previously reported in vitro.

In turn, acetylcholine (ACh) levels are capable of driving somatostatin (SST) interneuron activity in the cortex (Dasgupta, Seibt, & Beierlein, 2018; Muñoz & Rudy, 2014) but the effect of NGF levels on SST interneurons has never been assessed in vivo. I thus imaged awake restrained mice expressing GCAMP7 in SST interneurons during running trials (as always trials of 120 seconds running between 25 to 100 seconds) (**Fig. 10a**): SST activity consistently increased when NGF was administered to the cortical area

(**Fig. 10d, e**), while it decreases when NGF levels were suppressed via TrkAFc or αD11 (**Fig. 10b, c**).

Thus, NGF seems to be a strong and previously overlooked modulator of cortical circuitry and activity; further experiments are needed to determine the precise actors in play.

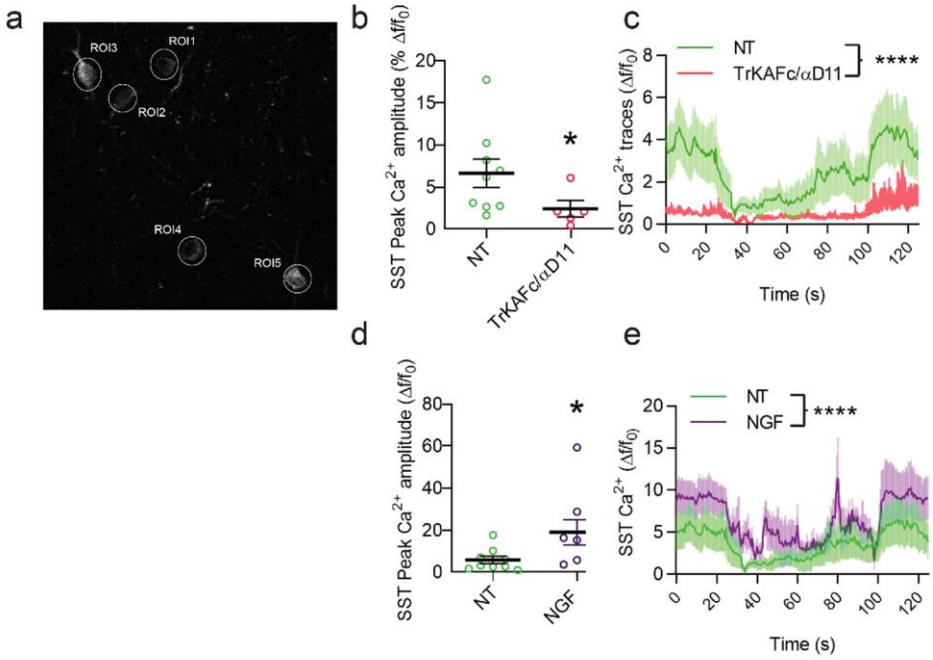


Figure 10: Somatostatin(SST) interneuron activity in the motor cortex in the presence of increased or decreased levels of NGF. In SST-Gcamp7 recording (a) ROI were drawn on top of SST somata. SST (b) peak amplitude and (c) traces in response to TrkAFc/αD11 (Mann-Whitney, p=0.029, Two-way ANOVA, p<0.0001; n=4). SST (d) peak amplitude and (e) traces in response to NGF (Mann-Whitney, p=0.0315, Two-way ANOVA, p<0.0001; n=2 per group, ~10 cells per animal).

Astrocyte and NGF in neurodegeneration

Astrocytes are an integral part of normal brain function and astrocyte reactivity is an early feature of AD (Carter et al., 2019). Indeed, reports show that Aβ oligomers can drive Ca^{2+} waves in cultured astrocytes (Alberdi et al., 2013). Moreover, calcium transients

are increased in synchrony and intensity in reactive astrocytes from APP/PS1 mice (Delekate et al., 2014; Kuchibhotla, Lattarulo, Hyman, & Bacskai, 2009).

NGF has been proposed as a putative therapeutic when it comes to AD both for its beneficial effect on cholinergic neurons and its anti-inflammatory properties (Capsoni et al., 2017). I thus decided to evaluate the effect of NGF on astrocyte calcium responses in vivo in the 5xFAD mouse.

As previously reported for other AD models, I also observed an increase in the astrocytic calcium response in the 5xFAD at 4 months of age (**Fig. 11a, b**). Interestingly when I applied NGF, the neurotrophin was able to reduce astrocyte calcium though not to the level of the WT (**Fig. 11a, b**).

In summary, our data shows how neurotrophins can be harnessed to modulate glial cell physiology also in a condition of neurodegeneration such as AD.

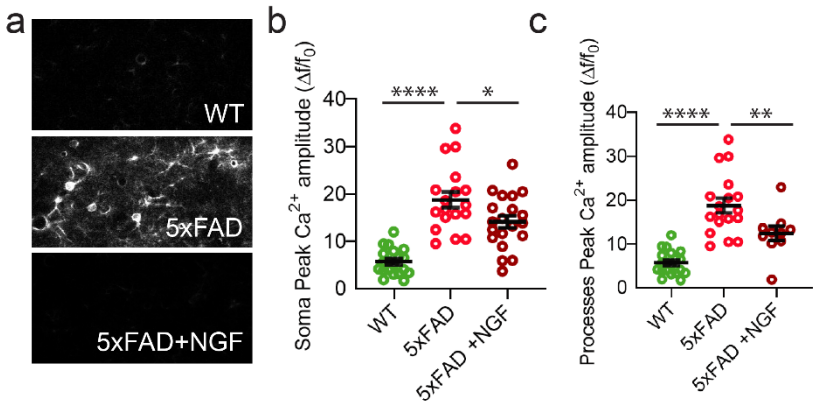


Figure 11. NGF effect on astrocyte calcium in the 5xFAD: (a) Representative two-photon images of astrocytic activity in WT and 5xFAD before and after NGF administration. (b) Soma peak amplitudes (1way ANOVA, WT vs. 5xFAD, $p<0.0001$; WT vs. 5xFAD +NGF, $p<0.0001$; 5xFAD vs. 5xFAD +NGF, $p=0.0262$) of the astrocytic Ca2+. (c) Processes peak amplitudes (1way ANOVA, WT vs. 5xFAD, $p<0.0001$; WT vs. 5xFAD +NGF, $p=0.0044$; 5xFAD vs. 5xFAD +NGF, $p=0.0083$) of the astrocytic Ca^{2+} (n=3).

Astrocytes and BDNF

Brain Derived Neurotrophic Factor (BDNF) was previously reported to have an effect on Ca^{2+} signaling in astrocytes *in vitro* through the truncated TrkB receptor (Rose et al., 2003), but there is a lack of evidence for its activity in vivo. In order to investigate

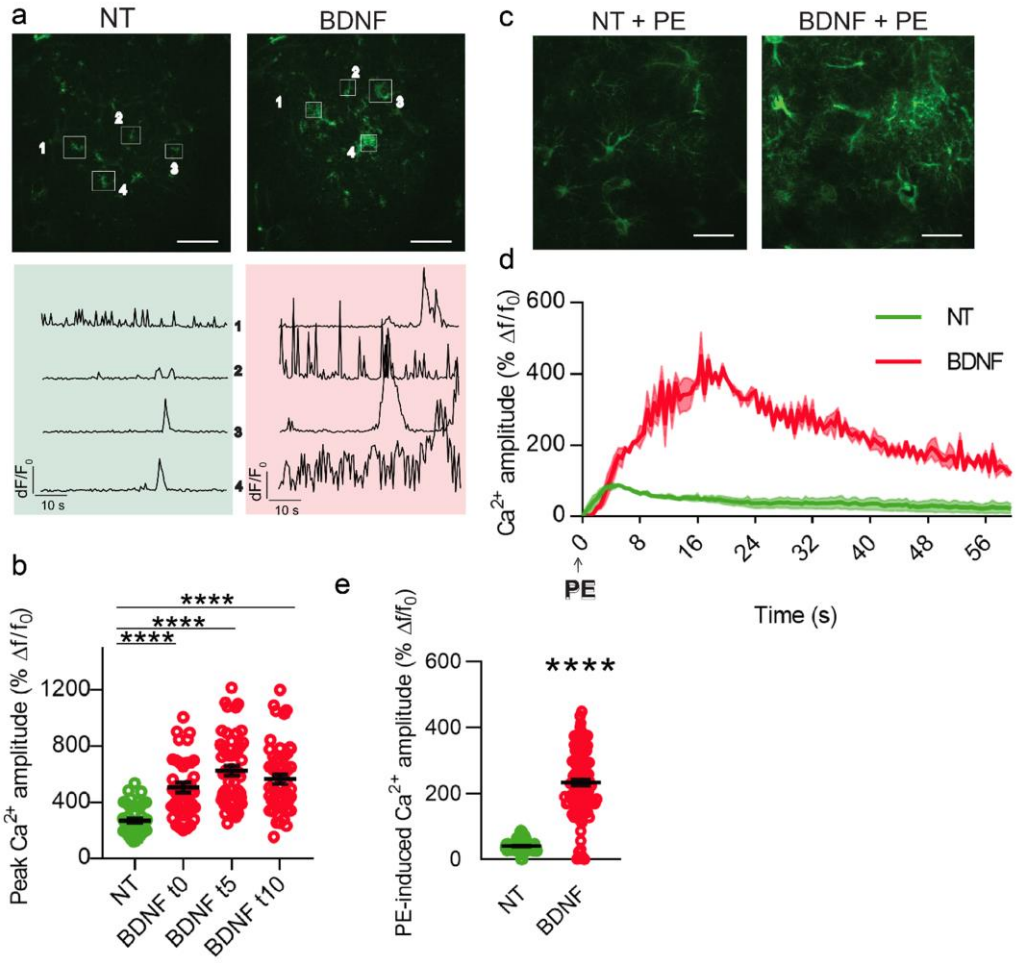


Figure 12. BDNF increases astrocytic Ca^{2+}: (a) Representative two-photon images and traces of Ca^{2+} response in astrocytic processes expressing GCaMP6s before (NT) and after BDNF (1µg/ml) application in the motor cortex of an anesthetized mouse. (b) Quantification of the peak amplitude of Ca^{2+} transients in astrocytic processes during baseline (NT), right after (BDNF t0), after 5 min (BDNF t5) and 10 min (BDNF t10) of BDNF (1µg/ml) application (One-way ANOVA, NT vs. BDNF t0 p= 0.0001; NT vs. BDNF t5 p = 0.0001; NT vs. BDNF t10 p=0.0001; n=3). (c) Representative images of non-treated (NT) and BDNF treated astrocytic response to phenylephrine (PE) 100 µM. (d) Representative traces of the bulk astrocytic response to PE administered at t0. (e) Quantification of the astrocytic response to PE 100 µM (T-test, p=0.0001) (n=4).

the effect of BDNF on astrocytic activity in vivo, I performed in vivo two-photon imaging of astrocytic Ca^{2+} before and after BDNF treatments in mice, which were infected with AAV5-GfapABC1D-cyto-GCaMP6f-SV40 virus (GFAP.GCaMP6.cyto) to drive GCaMP6 expression in astrocytes. BDNF was diluted in ACSF (1μg/ml) and applied directly onto the cortical surface through a small cranial window over the motor cortex of anesthetized mice. Astrocytic Ca^{2+} responses to BDNF and during non-treated (NT) conditions were measured in the fine processes of the astrocytes (**Fig. 12a**).

The effect of BDNF on Ca^{2+} activity of astrocytes was monitored right after its application (t0), then after 5 (t5) and 10 minutes (t10). At both t0 and t5 and t10, BDNF significantly increased astrocytic Ca^{2+} responses in fine processes (**Fig. 12b**). Astrocytes are known to respond strongly to adrenergic signaling, specifically through α_1 -adrenergic receptors. When I used a α_1 -adrenergic agonist, phenylephrine (PE), to elicit a strong astrocytic Ca^{2+} response in non-treated or BDNF treated mice expressing GCAMP6f in astrocytes (**Fig. 12c**), I found PE treatment induced a much larger astrocytic Ca^{2+} response in BDNF treated mice as compared with non-treated controls (**Fig. 12d, e**).

BDNF activity on astrocytes in the awake behaving animal

I then switched to the awake behaving animal to study the contribution of neuronal activity to the circuitry. I evaluated the effect of BDNF on a mouse previously injected with a AAV5-GfapABC1D-cyto-GCaMP6f-SV40 virus expressing GCAmp6f in astrocytes, head fixed under the two-photon microscope during a running trial (**Fig. 13a**). In this experimental setup BDNF was able to increase astrocyte calcium both in the soma (**Fig. 13b, c**) and in the fine processes (**Fig. 13d, e**)

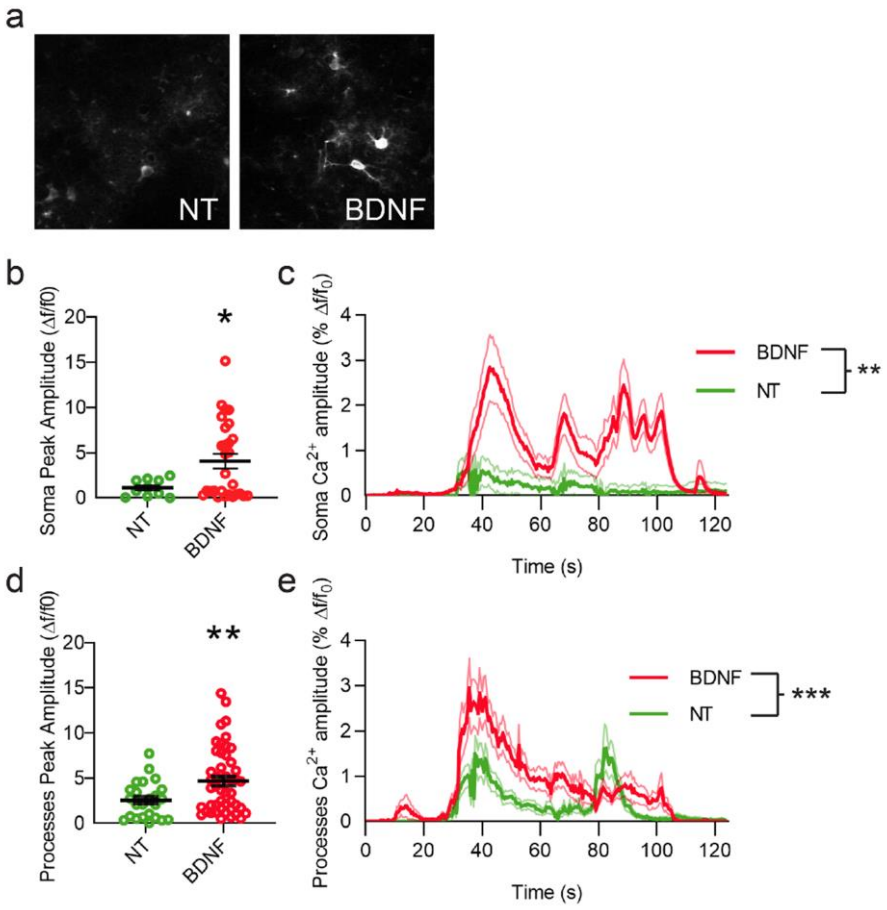


Figure 13. BDNF increases astrocytic Ca^{2+} in awake behaving animals: (a) Representative images of the astrocytic response to BDNF. Soma peak amplitudes (2tailed t test, p=0.0366) (b) and traces (c) (2way ANOVA, p= 0.0018) of the astrocytic Ca^{2+}. Process peak amplitudes (d) (2tailed t test, p=0.0087) and traces (e) (2way ANOVA, p= 0.0006) (n=4, , ~20 cells per animal).

BDNF activity on Neurons in the awake behaving animal

I then evaluated the effect of BDNF on neurons. Specifically, I imaged dendrites in layer I coming from Layer V pyramidal neurons in thy1-gcamp6 (SL1) mice placed on a treadmill during running trials of 120 seconds (RUN 25-100). In the BDNF treated animals, dendritic activity was significantly reduced in response to running (**Fig. 14b, c**).

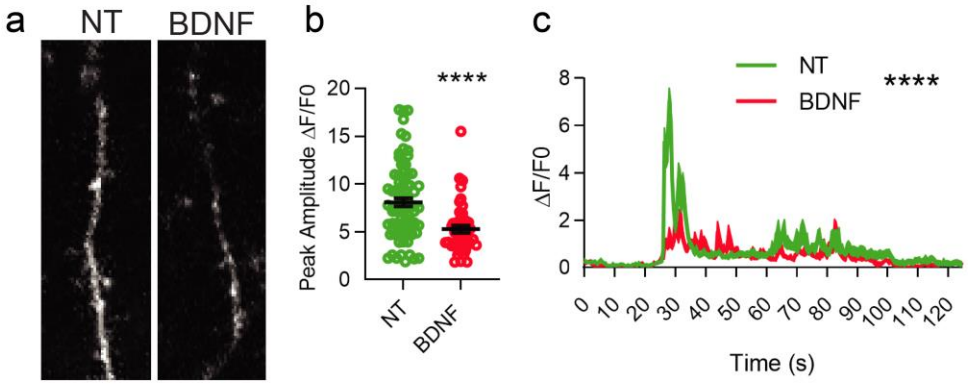


Figure 14. BDNF decreases dendritic Ca^{2+} in awake behaving animals: (a) Representative two-photon images of dendrites**.** (b) Peak amplitude responses (c) (2tailed t test, $p<0.0001$) and traces (c) of the dendritic compartments under the influence of BDNF (Two-Way ANOVA, $p<0.0001$; n=2).

Astrocyte calcium in Microglial BDNF deletion mice and TrkB.T1 KO mice

To investigate whether and how microglial BDNF affects astrocytic Ca^{2+} signaling, I performed two-photon imaging of astrocytic Ca^{2+} in the motor cortex of awake $CX3CR1^{CreERT2/+}$:$BDNF^{flox/+}$ and $CX3CR1^{CreERT2/+}$: $BDNF^{flox/floxed}$ mice that run on a treadmill under a two-photon microscope, generated as defined in previous literature (Parkhurst et al., 2013). Mice were given tamoxifen 5 days prior to two-photon in vivo imaging to remove one copy of BDNF from controls and two copies from microglial BDNF deletion mice. We found that treadmill running in awake, head-fixed controls triggered a large increase in Ca^{2+} transients in nearly all the astrocytes within the field of view. Running- induced Ca^{2+} transients peaked ~10s after the running onset and gradually declined over tens of seconds (**Fig. 15a, b**). In microglial BDNF deletion mice, Ca^{2+} transients returned to baseline levels much more quickly (**Fig. 15a, b**) and the total

integrated astrocyte Ca^{2+} activity was significantly reduced as compared to the controls (**Fig. 15c**).

Similar experiments were performed in global and astrocyte-specific TrkB.T1 deletion mice and control wild type mice on the same C57BL6/J background. Compared to WT, both global and astrocyte-specific TrkB.T1 deletion had significantly decreased astrocytic Ca^{2+} activity during running (**Fig. 15d, e**) and significantly reduced total

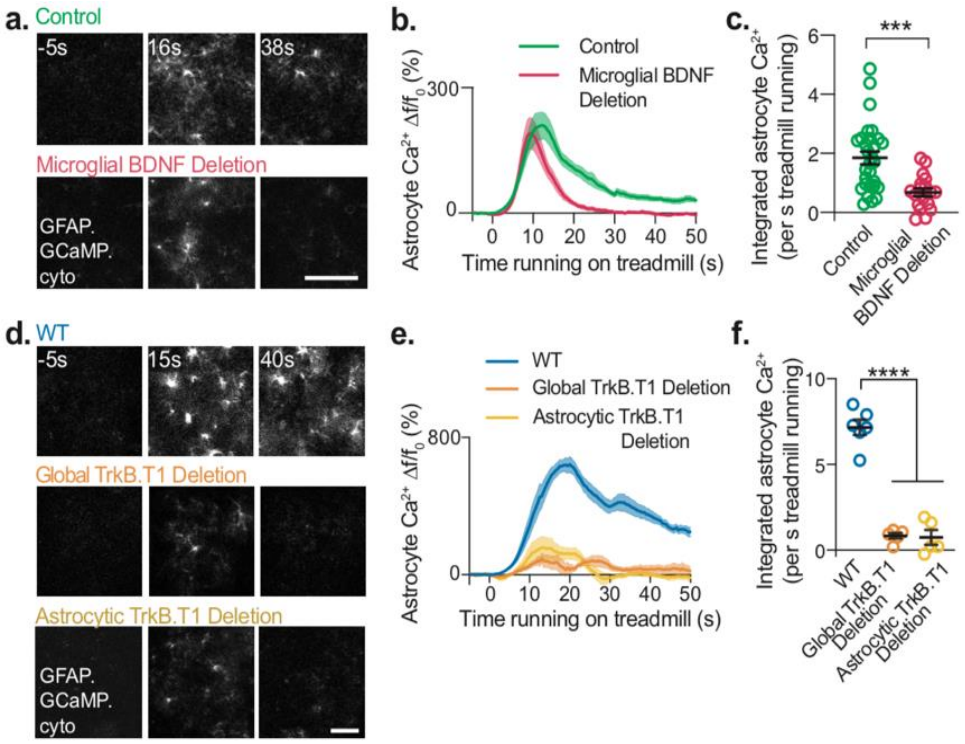


Figure 15. Deletion of microglial BDNF and TrkB.T1 reduces running-evoked astrocytic Ca^{2+}. (a) Representative images of forelimb primary motor cortex astrocyte Ca^{2+} with thin skull cranial window in control (top row, green) or microglial BDNF deletion (bottom row, red) mice before (left panel), at (middle), and after (right) the peak activity induced by treadmill running in awake, head-fixed mice; scale bar, 50um. (b) Mean ± SEM traces of field-of-view (FOV) astrocyte Ca^{2+} during treadmill running in control (green, n=4 mice, 27 trials) and microglial BDNF deletion (red, n=3 mice, n=18 trials) mice. (d) Quantification of total integrated FOV Ca^{2+} activity per second of treadmill running in control (green, n=4 mice, 27 trials) and microglial BDNF deletion mice (red, n=3 mice, 18 trials); $p<0.001$. (d-f) Same as (a-c) but imaging was performed with open skull cranial window in WT (top row, blue, 6 trials, 2 mice), global TrkB.T1 knockout (middle row, orange, 5 trials, 3 mice) mice, and astrocytic TrkB.T1 deletion (bottom row, yellow, 5 trials) mice; ****$p<0.001$.

integrated Ca^{2+} activity (**Fig. 15f**). Together, these results suggest that microglial BDNF may increase running-evoked astrocyte Ca^{2+} activity via astrocytic TrkB.T1.

In conclusion, as previously reported in the literature (Prakash, Cohen-Cory, & Frostig, 1996), it seems that NGF and BDNF might have opposite effects on the CNS: BDNF seems to be primarily inhibiting, possibly through astrocytes, while NGF seems to be activating cortical circuits (via modulation of acetylcholine levels or direct effects on glial cells/ neurons).

IN BRIEF III

In this third and last chapter of my thesis, we have tackled the in vivo translatability of the data from the first two chapters by using in vivo 2-photon microscopy.

In a first part of chapter II we analyzed the effect of NGF on microglia in the environment of the intact brain. Using 2-photon microscopy on mice expressing GFP in microglia, we discovered that NGF can increase microglia baseline motility in resting conditions and during injury, first in the anesthetized mouse and then in the awake behaving animal. The effect of NGF on microglia seemed to be dependent on TREM2 expression in microglia, since knocking out the receptor abolished the increase in motility. To get a handle on the activity changes of the cortical circuit, we then imaged dendrites in layer I under the effects of exogenous NGF, to discover that this exerted an increase in neuronal activity.

A last experiment of the section was then to address the matter of the therapeutical validity of NGF in the context of Alzheimer's disease. In a murine model of AD, the 5xFAD, we demonstrate that microglia have motility deficits that can be rescued by NGF/NGFp local administration.

In a second part of Chapter III, we move to the effects of NGF on astrocyte physiology, by monitoring the changes in calcium activity of these cells during modulations of NGF levels in the extracellular environment. We discover that, while NGF exerted no effect, a lack of the neurotrophin increased astrocyte calcium activity in the anesthetized mouse and in the awake behaving animal. Moreover, the effect of NGF chelating antibodies seemed to be more significant in layer I, suggesting a layer specific effect.

We then monitored ACh levels using an in vivo fluorescent sensor (Jing et al., 2018) to find out that decreasing NGF levels causes a decrease in the cholinergic tone in the cortex. Moreover, looking at SST activity via calcium imaging, we show that these interneurons decreased their activity when NGF was chelated from the extracellular space, while they increased it when NGF was applied locally.

As a mirror to the microglia section, we also measured calcium activity in the AD mouse model, 5xFAD. In our data, astrocytes in the motor cortex of 4 months old 5xFAD indeed show an increase in calcium activity, as was already reported for other AD mouse models. Interestingly though, administration of NGF lowers astrocyte calcium.

In the last section of Chapter III, we analyze a more classical neurotrophin when it comes to the CNS, BDNF – and its activity on astrocytes.

In our data, BDNF increased calcium responses in astrocytes both in the anesthetized and in the awake behaving animal. This was accompanied by a slight decrease in neuronal dendritic activity. This effect seemed to be cell autonomous since tampering with BDNF signaling – either knocking out BDNF in microglia, or by knocking out T1-TrkB in astrocytes, decreased astrocytic calcium responses.

In conclusion, the chapter brings forward neurotrophins as potent modulators of glial physiology in vivo.

Discussion

My thesis poses the question of whether neurotrophins can influence glial physiology. In particular, I have studied two cell types which are as important for brain homeostasis as they are different among themselves. Microglia represent the immune system of the CNS: they are of hematopoietic origin and they invade the neural tissue both pre and postnatally. Astrocytes share the ontogeny of the whole nervous system, being originated from the neuroectoderm. Once thought to be mere support to its neuronal neighbors, it is now established that these cells hide much more important roles in the CNS.

It is no surprise, then, that glial cells, so often treated as a whole, are of the utmost heterogeneity. What they do have in common though, is that they share the same environment, they have access to the same types of molecules, they come in contact with the same populations of cells, they work in unison to enable the proper functioning of the neuronal circuitry. As part of the aforementioned molecular landscape of the brain, neurotrophins are important factors for neurophysiology. Having been discovered more than 70 years ago, neurotrophins are part of the history of neuroscience: they were the first neuroactive molecules ever to be studied and they take part in many classical paradigms of neuroscience. Here, we develop and explore one of the roads less traveled for these interesting molecules – *a glial function for neurotrophins*.

The first two chapters of this thesis address the aforementioned issue mostly in vitro, while the last chapter bears some of the data that I collected in the lab of Dr. Wenbiao Gan, a world expert in two photon microscopy *in vivo*, in New York City during the last year of my PhD, where I took stock of what I had learned until that point and tried to approach the

matter at hand with a tool that enables the direct in vivo monitoring of the activity of a myriad of cells.

In the first chapter of this thesis, we have tackled – mostly through *in vitro* work – the relationship between NGF and microglia to discover how NGF affects microglial physiology by rendering them neuroprotective, particularly in an Alzheimer-like context.

Indeed, recent genetic studies have underscored the emerging role of microglia in Alzheimer's disease pathogenesis (Keren-Shaul et al., 2017). Microglia lose their amyloid-β-clearing capabilities with age and as AD progresses (Galatro et al., 2017; Krabbe et al., 2013). Therefore, affecting microglial homeostatic activities offers a potentially promising therapeutic avenue for AD pathology (Ardura-Fabregat et al., 2017). However, approaches currently pursued to stimulate innate immunity via the Toll-like receptor (TLR) pathway, such as the use of class B CpG (cytosine-phosphate-guanine) oligodeoxynucleotides (ODNs) (Scholtzova et al., 2014, 2009), suffer from the problem that TLR ligands need to be very carefully titrated, to avoid excessive microglial stimulation. Indeed, while stimulation of innate immunity via TLR signaling pathways has been shown to be sometimes beneficial in modulating AD pathology (Richard, Filali, Prefontaine, & Rivest, 2008; Su, Bai, Zhou, & Zhang, 2016), it can also exert adverse effects in AD models (J. W. Lee et al., 2008; Su et al., 2016). In this respect, according to the results presented in chapter I, NGF appears to be able to stimulate an anti-inflammatory response in microglia and to steer them to a fully neuroprotective phenotype, at many different levels, including cytokine and chemokine profile, motility, electrophysiological properties, engulfment of extracellular material, interactions with neurons and dendritic spines. Most notably, the inflammation-modulating actions of NGF, such as for instance the transcriptomic changes, cytokine profile and the dendritic spine engulfment, are much more pronounced on Aβ treated microglia than on naive microglia.

These properties of NGF could be exploited to harness the brain innate immunity as a safer in loco neuroprotective agent. Our analysis shows that the receptor-mediated signaling activated by NGF in microglia regulates a number of physiological activities of these cells, even triggering an outward rectifying membrane current. Although the identity and function of this current remains to be determined, the value of its reversal potential leads to propose that this NGF-induced current may be subserved by chloride channels (Murana et al., 2017).

Microglial activity is intimately associated with morphological changes (Davalos et al., 2005; Nimmerjahn et al., 2005)—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 (Sipe et al., 2016). Therefore, motility represents an important feature to keep into account when trying to estimate microglial activity in physiological and pathological situations. Our gene expression profiling data and time-lapse recordings respectively suggest a modulation by NGF of genes involved in cytoskeletal reorganization and of membrane dynamics.

We then focused on the possible consequences of NGF activity on microglial cells in pathological conditions, i.e. by challenging them with the Aβ peptide, a well-established proinflammatory, neurodegenerative insult. We found that microglia treated with NGF become refractory to the potent inflammatory stimulus of Aβ, while naive microglia do not significantly change their inflammatory profile. We show that NGF is capable of enhancing specifically one type of endocytic process in microglial cells, macropinocytosis, the mechanism of choice through which microglial cells clear sAβ (Mandrekar et al., 2009). Thus, as a further step, we demonstrated that, by enhancing macropinocytosis, NGF promotes sAβ clearance *in vitro* and, most remarkably, *ex vivo*. Increasing the uptake of sAβ, however, might not actually provide a long term protection over the toxicity of the

peptide, since internalized sAβ could be shed again into the extracellular space. In fact, microglia can release internalized Aβ and convert it in neurotoxic forms through the shedding of microvesicles (Joshi et al., 2014). Moreover, it is still not clear if microglial cells are actually able to digest sAβ efficiently (Mandrekar-Colucci & Landreth, 2010), due to evidence suggesting that microglia near plaques are functionally impaired (Krabbe et al., 2013). Here, we showed that, in BV2 microglia, NGF not only increases Aβ uptake but enhances its degradation.

Alzheimer's disease has been described as a synaptopathy, entailing a dysfunction of synaptic function (Haass & Selkoe, 2007; Mucke & Selkoe, 2012). Synapse loss is indeed an early sign of AD and the process has been directly correlated with Aβ as the most likely culprit (Hong et al., 2016). High concentrations of Aβ or Aβ oligomers inhibit synaptic plasticity processes (Selkoe, 2008; Shankar et al., 2007; Walsh et al., 2002). Aβ has proven to be a key player in synaptic plasticity also at physiological concentrations: while short exposure with low concentrations of the peptide actually enhance synaptic plasticity, longer exposures lasting several hours reduce it (Koppensteiner et al., 2016).

This underlines the importance of the homeostasis of Aβ levels and its processing in the brain, and thus of microglia themselves as an important factor in its clearance. On their part, under physiological conditions, microglial cells regulate dendritic spines, either by pruning away superfluous spines during development (Schafer et al., 2012) or promoting spine formation, as observed in the developing somatosensory cortex (Helmut Kettenmann, Kirchhoff, & Verkhratsky, 2013; Miyamoto et al., 2016). In relation to this microglia-neuron communication, we demonstrate that NGF rescues the spine loss mediated by Aβ, an effect that is strictly 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 in neurons 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 the Aβ-induced LTP deficit is not rescued by NGF, when neurons are cultured in the absence of microglia. Thus, in this assay, NGF exerts its neuroprotective effects on neurons via microglia.

A further demonstration of NGF as a modulator of the microglia to neuron communication is provided by the observed stimulation by NGF of glutamatergic transmission in a microglia-dependent manner. This demonstration adds one more line of evidence to the emerging theme of microglia as a modulator of neurotransmission (Béchade, Cantaut-Belarif, & Bessis, 2013; Marrone et al., 2017).

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 clearance of Aβ (Capsoni et al., 2017). In that study, the neurotrophin, added to 5xFAD slices (which present synaptic transmission and LTP deficits), demonstrated a TrkA-dependent rescue of both synaptic transmission and synaptic plasticity deficits. Our results go in the direction of attributing those events to the action of NGF on microglial cells. By affecting microglial physiological activity, NGF is capable of influencing glutamatergic transmission. Indeed, along with TrkA expression in microglia, we found that tampering with NGF-TrkA signaling reduces glutamatergic neurotransmission. Thus, NGF-activated microglia might be neuroprotective in Aβ pathology not only by lowering the amount of circulating Aβ—per se toxic to synapses and neurons—but also by aiding neurons in synaptic plasticity tasks.

In an in vivo context, it has to be taken into account that microglia can also interact with astrocytes. Indeed, we can speculate that if we put astrocytes in the midst the

neuroprotective effect of NGF could be even stronger, as astrocytes can themselves phagocyte Aβ and might positively react to the anti-inflammatory phenotype of microglia. Indeed, it is known that microglia can influence astrocytes via cytokine secretion, and that proinflammatory cytokines by microglia make astrocytes neurotoxic (Liddelow et al., 2017).

Our data point toward these myeloid cells of the brain as the culprit for the severe neurodegeneration observed in anti-NGF or anti- TrkA mice (Capsoni, Tiveron, Vignone, Amato, & Cattaneo, 2010; Capsoni et al., 2000), a conclusion that might be relevant also for human brain pathologies. Moreover, our results add an important element to the rationale for the therapeutic use of NGF in AD (Cattaneo & Calissano, 2012; Cattaneo, Capsoni, & Paoletti, 2008; Eyjolfsdottir et al., 2016; Tuszynski et al., 2005). These broadly neuroprotective actions of NGF via microglia enlarge the spectrum of neurons that can be considered NGF targets—way beyond BFCN—thus extending the therapeutic potential of NGF and its derivatives (Capsoni et al., 2017). Future studies will be needed to investigate whether there are regional differences in the responsiveness to NGF of microglia from different brain regions and from different ages.

In any case, this demonstration of the broad influence of NGF on microglial cells vindicates the early and visionary view by Rita Levi- Montalcini that considered NGF as a neurokine, a mediator of neuroimmune communication (Levi-montalcini, 1987).

In conclusion, the evidence presented in the first chapter of this thesis corroborates the view that exploiting the innovative immunomodulatory and neuroprotective mechanisms displayed by NGF may be a viable clinical approach to ameliorate all hallmarks of AD pathology and, potentially, a spectrum of other neurodegenerative diseases.

After the publication of our paper – containing the data in chapter I – the immunomodulatory properties of NGF on microglia were confirmed by another group. The authors show that NGF, via its high affinity receptor TrkA, downregulated LPS - induced production of pro-inflammatory cytokines and NO in primary mouse microglia and inhibited TLR4 - mediated activation of the NF-κB and JNK pathways (Fodelianaki et al., 2019).

In the second chapter, we have then discovered how another glial cell of the brain, the astrocyte, responds strongly to negative modulations of the levels of NGF by increasing their calcium activity.

These results are highly significant in the grand scheme that is the understanding of the effect of neurotrophins on astrocyte physiology. (1) The presence of NGF receptors on the astrocyte membrane and the ability of these cells to produce themselves the neurotrophin, somehow suggest the existence of an autocrine loop, in which NGF can act in a cell autonomous manner on astrocytes and modulate their responses. This could be important in situations where astrocytes are known to modulate the expression of the neurotrophin and its receptors like during development, injury or disease. Loss of calcium homeostasis in the absence of NGF in pathological conditions could be correlated with neurotoxicity. It is indeed known that excessive astrocyte calcium can induce excitotoxicity via mechanisms such as gliotransmission (D. Lim, Ronco, Grolla, Verkhratsky, & Genazzani, 2014). If we then look at the existing literature with our data in mind, these excessive calcium responses in astrocytes in AD could then be easily linked to the deficits in NGF-TrkA that have also been detected in AD (Cattaneo & Calissano, 2012).

Moreover, it has been shown that NGF – more precisely a mutant TrkA selective version of the neurotrophin – treatment in the AD mouse model, 5XFAD, rescues some of

the neurodegenerative phenotypes, precisely by acting on glial cells; this and other literature have actually pointed to NGF as a promising therapeutic strategy in various pathological states (Capsoni et al., 2017, 2012; Cattaneo et al., 2008; H. Zhang et al., 2014).

We might then imply that NGF can act positively on astrocytes to keep in check their reactive responses such as excessive calcium.

(2) Another important point is, though, that in physiological situations, when the levels of NGF are lower and mostly secreted by neurons, there might also be an activity, local and acute, at the level of the synapse where the neurotrophin is secreted and astrocytes have the direct access to sense and react to it. This dual mode of action of molecules is not unusual in the CNS. Let's take for example TNFα. This classic inflammatory cytokine is mostly known for its role in regulating the immune system. Expressed by immune cells at high levels during inflammatory events, it is a powerful pro-inflammatory molecule that has been shown to be quite neurotoxic to neurons if produced at high concentrations. TNFα though has been shown to have a most interesting role in the healthy brain. It was indeed shown that this molecule, that can be secreted also at low concentrations by astrocytes in the healthy brain, is a powerful regulator of homeostatic plasticity (Santello & Volterra, 2012; Stellwagen & Malenka, 2006). As I already highlighted in the introduction of this thesis, Rita Levi Montalcini herself define NGF as a neurokine, for the similarities in behavior and function that the neurotrophin shows towards molecules that are known immune mediators, chemokines and cytokines. Comparing NGF and TNFα might not be a farfetched deal then, when one and the other seem to carry very similar functions in a very similar manner.

The aim of the third chapter has been then to use in vivo imaging to confirm some of our *in vitro* data in a physiological environment, and from there to play around with

such tools to broaden our understanding of the effects of neurotrophins on the cortical circuits.

In chapter I, we showed how primary microglia express NGF receptors and respond to NGF by increasing their membrane motility and their phagocytic abilities in a TrkA dependent manner. Ex vivo, we then demonstrated how microglial cells possess TrkA receptors in vivo, while patch clamp recordings from microglia in acute brain slices revealed that NGF triggers an outward current in these cells, suggesting translatability of these data in the intact brain.

In chapter III, we then used 2-photon microscopy on mice expressing GFP in microglia, to discover that NGF can affect microglia baseline motility in resting conditions and during injury, first in the anesthetized mouse and then in the awake behaving animal. When using either pro- or mature NGF, these two were both able to increase the surveilling capacity of microglia. To properly understand whether these responses are due to different receptors (p75 vs TrkA), more experiments are needed (**Future Plans**).

If these data alone cannot account for cell autonomous processes, the data presented in Chapter I call for the existence of cell autonomous processes in vivo.

The partial incongruence in receptor involvement between the in vivo and in vitro data can be explained either as a difference in expression of NGF receptors in these cells in the two different conditions, or by the existence of different mechanisms that can modulate their behavior: one cell autonomous via TrkA – already demonstrated in chapter I - and one non-cell-autonomous where the neurotrophins act on other cell types, changing the environment and consequently changing the response of microglia to said variations in activity – this one has yet to be discovered.

To investigate the potential molecular mechanisms underlying this phenomenon, we then performed NGF treatments on TREM2KO mice, a molecule only expressed on

microglia in the brain. TREM2 is a key factor for microglial physiology: it has been linked to Alzheimer's disease, such that a loss of function mutation found in humans constitutes the most potent risk factor for the development of the disease in its sporadic form. This has been associated with an impairment in the phagocytosis capacity of microglia towards the amyloid peptide, which still represents the best bet as the etiologic factor for AD. Most interestingly, microglia in the TREM2KO mice, though not having an impairment in motility at baseline, were unable to respond to the neurotrophin indicating, first of all, that this molecule is involved in the molecular mechanism through which NGF acts to change microglia motility, and, second of all, suggests that the effect of NGF on microglia is direct, since a single microglial mutation inhibits NGF capacity to change microglial physiology.

Interestingly, NGF treatment entailed changes in neuronal activity – measured via Gcamp6 imaging on layer I dendrites - in our data set. Again, our electrophysiology data ex vivo presented in Chapter I advocate for a direct effect of NGF on microglia for this modulation of neuronal activity. Indeed, just as in vivo, NGF increased excitatory neurotransmission in ex vivo slices, and did so specifically via TrkA signaling in microglia. Our data cannot exclude for additional in vivo mechanisms of action of the neurotrophin through other cell types, and to most certainly understand whether, also in the complex system of the awake behaving animal, NGF acts primarily through microglia to modulate neuronal activity, we will need to repeat our experiments on KO mice for TrkA specifically in microglia (**Future Plans**).

In the context of AD, NGF has been proposed time and again as a putative therapy due to its action on cholinergic neurons, a neuronal population highly affected in patients.

Since NGF in its wild-type form has potent pain-inducing side effects, a painless version of the neurotrophin was developed, taking inspiration from the human genetic

disease hereditary sensory and autonomic neuropathy type V (Capsoni et al., 2012; Covaceuszach et al., 2009; Malerba et al., 2015). hNGFp has identical neurotrophic potency as wild-type human nerve growth factor, but a 10-fold lower pain sensitizing activity. Though clinical trials with wtNGF are already ongoing, it is of primary interest to understand the effects that such treatments bring about in the CNS.

As for NGFp, literature from our lab shows how treatments in murine models of AD have neurotrophic and anti-amyloidogenic activities (Capsoni et al., 2017, 2012). Specifically, it was shown that NGFp acts via glial cells, modulating inflammatory proteins such as the soluble TNFα receptor II and the chemokine CXCL12 in the 5xFAD (Capsoni et al., 2017).

Here we first show that, in vitro, NGF steers microglia toward a neuroprotective phenotype in a condition of amyloid-β overexpression, by increasing these cells capacity to phagocyte Aβ oligomers and modulating microglial transcriptomic profile towards anti-inflammatory behaviors. Then, in a murine model of AD, 5xFAD, we demonstrate that microglia have motility deficits that can be rescued by NGF/NGFp local administration, further confirming the beneficial effect of NGF in the context of neurodegenerative disorders.

In a second part of Chapter III, we have then evaluated the in vivo effects of NGF modulation on astrocyte physiology. In Fig. 2, Chapter I, we showed how astrocytes possess some immunoreactivity for TrkA in cortical slices. The presence of the appropriate response machinery to the neurotrophin opened the possibility, later explored, for an effect of NGF on astrocytes in vitro.

As was the case for in vitro primary astrocytes, a lack of NGF increased astrocyte calcium activity in the anesthetized mouse and in the awake behaving animal, while NGF exerted no effect. Moreover, the effect of NGF chelating antibodies seemed to be more

significant in layer I, suggesting a layer specific effect. This layer specificity can be either due to diverse astrocytic populations in the different layers of the cortex, or to the presence in such layers of other cell types responsible for mediating the effect of the lack of NGF on astrocytes. As for the first hypothesis, astrocytes are already known to be highly heterogeneous in the cortex for morphology, calcium activity and molecular profile, so much that one could speak of functional segregation in the different layers of the neocortex (Lanjakornsiripan et al., 2018; Takata & Hirase, 2008; Zeisel et al., 2015). On the other hand, different layers in the cortex contain different neuronal types and projections from other regions of the CNS. For instance, in the mouse neocortex, L1 is highly innervated by cholinergic projections (Alitto & Dan, 2012) and ACh can exert a number of effects depending on the cell type affected or the state of the neuronal circuit (Colangelo, Shichkova, Keller, Markram, & Ramaswamy, 2019).

To explore this possibility, we decided to monitor ACh levels using an in vivo fluorescent sensor (Jing et al., 2018). If it had already been shown in vitro and ex vivo that NGF can increase acetylcholine release from cholinergic terminals by increasing choline acetyltransferase (ChAT) activity (Auld et al., 2001; Huh et al., 2008; Mobley et al., 1986), there is a lack of data of such activity in vivo especially for the cortex. Here we show that decreasing NGF levels in vivo in the awake behaving animal causes a decrease in the cholinergic tone.

Acetylcholine is a major excitatory drive for SST interneurons (Muñoz, Tremblay, Levenstein, & Rudy, 2017). We thus looked at SST activity under the effect of either NGF or αD11/TrkA-Fc. Consistent with the data on ACh, SST activity was decreased when NGF was chelated, while it increased when NGF was applied locally. These data suggest a previously overlooked circuit, where NGF levels can modulate the cholinergic tone in the cortex and in turn change the cortical circuit activity.

Astrocytes are also intimately connected to Alzheimer's disease. Alterations in glial cells surrounding Aβ plaques in AD brains had already been observed by Alois Alzheimer himself in 1911: "[…] we see a far larger number of fibre producing glial cells lying in the immediate vicinity of the plaque […]" (Tagarelli, Piro, Tagarelli, Lagonia, & Quattrone, 2006). We now know that the receptor profile of reactive astrocytes near Aβ accumulation sites is very much different from that of a physiological condition. Most notably, there is upregulation of α7 adrenergic receptors, which cause abnormal increases in Ca^{2+} rises in astrocytes and excitotoxic glutamate release (Pirttimaki et al., 2013; Talantova et al., 2013; Teaktong et al., 2003). Moreover, mGlu5 receptors (D. Lim et al., 2013) and P2Y1 receptors (Delekate et al., 2014) enhance Gq-IP3-dependent Ca^{2+} signaling and contribute to astrocytic Ca^{2+} alteration in AD. Aβ is known to impair synaptic transmission in a dose dependent manner (Palop & Mucke, 2010). In the APP23xPS45 mouse model of AD, neurons in the vicinity of Aβ plaques are hyperexcitable (Busche et al., 2008), a feature that can be due to Aβ-mediated disruptions of glutamate uptake (S. Li et al., 2009), glutamate release (Fogel et al., 2014) and GABAergic inhibition (Verret et al., 2012). Remarkably, astrocytes neighboring Aβ plaques also display higher resting Ca^{2+} levels, more frequent and larger Ca^{2+} elevations, long-range coordinated Ca^{2+} waves that are never seen in control mice (Delekate et al., 2014; Kuchibhotla et al., 2009; Pirttimaki et al., 2013), and increased gliotransmission (Gómez-Gonzalo et al., 2017). These anomalies of course can either be due to a direct action of Aβ on astrocytes (Abramov, Canevari, & Duchen, 2003) , as well as to an enhanced receptor-mediated Ca^{2+} signaling (see above), and are likely to affect the neuronal network, for example, by extending the areas of functional alteration and abnormal synchronization. Preventing excessive Ca^{2+} increase from astrocytes might therefore represent a promising strategy for AD therapy. Indeed, P2Y1R antagonists or genetic suppression of IP3R2-dependent astrocyte Ca^{2+} signaling

reduce neuron–astrocyte network hyperactivity and ameliorate spatial learning and memory deficits in AD mice (Reichenbach et al., 2018).

Interestingly, the acetylcholinesterase inhibitor donepezil, which is approved for symptomatic AD treatment, appears to reduce astrocyte Ca^{2+} signaling (Makitani, Nakagawa, Izumi, Akaike, & Kume, 2017).

In our data, astrocytes in the motor cortex of 4 months old 5xFAD mice indeed showed an increase in calcium activity, as was already reported for other AD mouse models. Interestingly though, administration of NGF lowered astrocyte calcium, thus further supporting an NGF-based therapy for AD. This is also in line with the literature that finds deficits of neurotrophin signaling in AD (Counts et al., 2004; Mufson et al., 2000). That is to say, our treatment might indeed compensate for existing deficits in neurotrophin signaling in 5xFAD astrocytes, and that would explain why WT astrocytes have no response to NGF – no deficit is present there and we might be looking at a "roof effect. More experiments are though needed to address that.

In the last part of Chapter III, we abandon NGF, which has been the star of this thesis, and approach a different neurotrophin, BDNF.

BDNF activity in the cortex has been more thoroughly assessed when it comes to changes in neuronal and glial physiological responses. Specifically, literature points to BDNF as a molecule mediating the communication between astrocytes and neurons (Vignoli et al., 2016; Vignoli & Canossa, 2017). It has already been reported ex vivo and in vitro that BDNF can mediate Ca^{2+} responses in astrocytes via TrkB-T1 (Rose et al., 2003). The TrkB truncated isoforms lack this intracellular kinase domain (Blum & Konnerth, 2005), thus cannot undergo autophosphorylation and do not function as true tyrosine kinase receptors. However, these authors show that astrocytes express

predominantly the TrkB-T1 isoform and that the BDNF-dependent Ca^{2+} release is mediated by the activation of PLC.

Consistent with the existing literature, in our data, BDNF increased calcium responses in astrocytes both in the anesthetized and in the awake behaving animal. On the other hand, neuronal activity underwent a slight decrease under the effect of. The effect of BDNF on astrocytes has reason to be considered as cell autonomous: decreasing BDNF signaling – either by knocking out BDNF in microglia, or by knocking out T1-TrkB in astrocytes indeed decreased astrocytic calcium responses. As per the decrease in neuronal activity seen with BDNF, we cannot directly assume that it is an effect mediated by the astrocytic response: we can though speculate that (1) in response to BDNF release – which is a known byproduct of neuronal activity even during running - astrocytes increase their calcium activity which is known to activate inhibitory neurons which in turn would decrease the activity in excitatory neurons - a sort of homeostatic loop; or that (2) neurons can themselves respond with changes in calcium to BDNF. To prove either theory one would need to tamper with BDNF receptors in either populations and see the calcium activity. If the "astrocyte" experiment has been shown with the T1-TrkB KO, a mirror experiment on neurons should be performed to exclude direct activities of the neurotrophin on neurons.

Looking at astrocyte activity as a proxy for circuit activity, in our data, it does seem that NGF and BDNF might exert opposite effects in the mouse motor cortex. Interestingly, this phenomenon has already been reported for the mouse somatosensory (barrel) cortex (Prakash et al., 1996). The authors show rapid and opposite effects of BDNF and NGF on the functional organization of the adult cortex via intrinsic optical imaging. In this context, topical application of BDNF resulted in a rapid and long-lasting decrease in the size of a whisker representation, and a decrease in the amplitude of the activity-dependent intrinsic

signal. In contrast, NGF application resulted in a rapid but transient increase in the size of a representation, and an increase in the amplitude of the activity-dependent intrinsic signal.

Similarly, in the data presented here, NGF levels seem to negatively impact astrocyte activity while BDNF increases it. This corresponds to an opposite effect on neuronal activity where there is an increase in neuronal activity for NGF and a decrease for BDNF. This inverse proportionality between astrocyte and neuronal calcium finds strong roots in the existing literature on astrocyte calcium. Multiple reports indeed now highlight the existence of a dynamic communication between inhibitory interneurons and astrocytes whereby glial cells can activate inhibitory circuits and contribute to efficient neuronal coding (Deemyad et al., 2018; Mederos & Perea, 2019; Mu et al., 2019), precisely via their calcium excitability.

A point needs now to be addressed: this last chapter of the thesis suffers the exogenous nature of our neurotrophin treatments, so the question remains, physiologically speaking, of what is the source of neurotrophins in the cortex. NGF and BDNF are distributed in the cortex in a very precise and definite manner: if BDNF is primarily produced by pyramidal – excitatory – neurons of all layers, its cousin NGF seems to be exclusively expressed in inhibitory neurons (Biane et al., 2014) (though this is not the case for the hippocampus where NGF is produced by dentate gyrus granule neurons (Kokaia et al., 1993)). And not any inhibitory neurons. What is really striking if one looks at a single cell RNAseq database – the figure in the next page is taken from the Allen Brain Atlas – is that NGF seems to be stratified primarily in Layer I (with Neuron Derived Neurotrophic Factor (NDNF) interneurons as a primary source) and its levels are nothing if not impressive. If it is true that also VIP and Parvalbumin interneurons express NGF – and will be thus restricted to deeper layers of the cortex, one wonders what is the function of NGF in layer I. Our data suggests that this stratification is related to the presence of cholinergic terminals in layer I. To properly address this point, we would need to study

physiological changes of neurotrophin levels, or genetic murine mutants of these neurotrophins. Conclusively, this last chapter of my thesis seems to put BDNF and NGF at the opposite sides of the E/I balance in the CNS, raising the question – only partially answered – of whether they might have opposite effects on brain physiology.

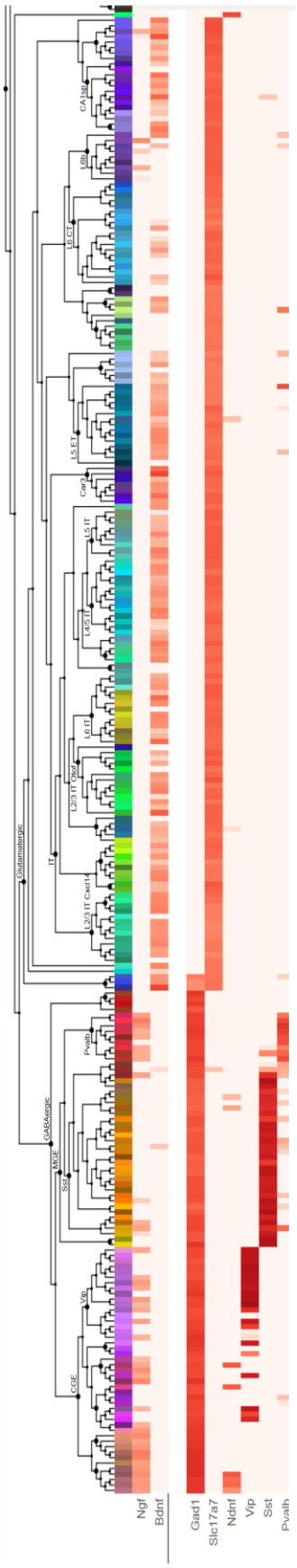


Figure 1 taken from the Allen Brain Atlas transcriptomic Explorer (https://celltypes.brain-map.org/rnaseq/mouse) showing expression data from their single-cell-RNA-Seq in the Whole Cortex + Hippocampus. In particular, BDNF and NGF expression levels are shown on the top, while marker genes identifying the populations are put on the bottom.

Future Plans

In order to give a final answer to the questions posed in this doctoral thesis, there is the need to properly address the matter of cell autonomous versus non cell autonomous processes in vivo. If Chapter I and II gave us proofs on the existence of direct activities of neurotrophins on glial cells in vitro and ex vivo, to properly address the matter in the intact brain, we will likely need the help of transgenic mice where TrkA/TrkB/p75NTR is selectively knocked out in microglia (via Cx3Cr1-CreER crossing) or astrocytes (via Glast-CreERT crossing). Application of neurotrophins in these mice would directly link these cells to responses or lack thereof. Since our data point to TrkA as the receptor of interest for NGF in microglia, in the lab, we already started to cross floxedTrkA mice (Sanchez-Ortiz et al., 2012) with mice expressing CRE conditionally in microglial cells (Cx3CR1-CreERT, https://www.jax.org/strain/021160, The Jackson Laboratory). We thus aim to understand what the lack of TrkA signaling specifically in microglial cells can do in vivo and, by administering NGF, to rule out non cell autonomous processes. The same should be done for Glast-CreERT to selectively KO NGF receptors in astrocytes.

Moreover, we plan to address the issue of an involvement of p75 in our glial responses by using a p75 agonist and looking at both astrocytes and microglia.

Another point to be addressed remains that of studying the effects of physiological changes of NGF levels. Indeed, we could exploit animals where neurotrophin signaling is reduced – e.g. AD11 mice, proNGF mice, NGF KO mice, and record changes in activity in microglia and astrocytes.

What would interest me the most, though, would be trying to expand on the circuit delineated in the last part of chapter III, where SST and ACh activity seemed to work in unison with astrocytes in response to neurotrophin. If the literature would suggest a direct

effect of NGF on cholinergic terminals that go increase ACh levels which then would change SST and astrocytic activity, to properly define the circuit, it should be addressed which population of cells is the first responder and which respond indirectly to ACh. One way to do that would be to manipulate SST activity – either by suppressing it optogenetically or knocking out specific muscarinic receptors on their surface to make them deaf to acetylcholine, to understand if the response to the lack of NGF in astrocytes is dependent on ACh levels or SST activity. Conversely, driving SST activity and recording astrocyte calcium would tell us if glial cells are simply responding to changes in the activity of the circuit. The existing literature would argue for an active role of astrocytes in determining changes in interneuron activity, but both SST interneurons and astrocytes have been shown capable of responding directly to changes in ACh levels, so there is room for this circuit to work one way or the other.

This thesis thus leaves us with new questions to be answered. As RLM would have put it, studying something as intricate as the nervous system and its relationship with all the glial types that reside in the brain parenchyma creates space to make prediction of the unpredictable. Seemingly disparate pieces coming together to create the most difficult and strange puzzle, one where a brain tries to understand itself. The literature has created new interesting routes for the world of neurotrophins that remain yet to be explored, and it falls to us the chance and burden to bring them to light.

Afterword

If this thesis does not intend or manage to give a definitive answer to the burning question of the functions of neurotrophin in the cortex and its circuits, it at least tackles it with methods and techniques that in the neurotrophin field have rarely been used, opening up new questions, new worlds to be discovered.

I first want to dedicate this thesis to the one who started this all, a mentor – though not to me physically but to my mentor, a free mind and a role model in how to do science and maybe in life, Rita Levi Montalcini.

In Scuola Normale, I first have to thank Antonino Cattaneo and Simona Capsoni who created an environment for me to learn all that I possibly could. I surely cannot spare thanks to all and every professor in Scuola Normale that, with their efforts as teachers, gave me the ability to understand science, first in the list, Alessandro Cellerino and Federico Cremisi. In CNR, I want to thank Tommaso Pizzorusso and all the people in his lab especially Elena Putignano and Raffaele Mazziotti, that helped me open my mind when I could not find answers to my scientific problems.

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Publications

(**Research Article**)

Saadipour K., Tiberi A., Lomardo S., Grajales E., Montroull L., Mañucat-Tan N. B., … Chao M. V. (2019). Regulation of BACE1 expression after injury is linked to the p75 neurotrophin receptor. *Molecular and Cellular Neuroscience*, 99, 103395. https://doi.org/10.1016/j.mcn.2019.103395

(**Research Article**)

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(**Review**)

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