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ProNGF is a cell-type specific mitogen for adult hippocampal and for induced neural stem cells

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1. ABSTRACT

ProNGF is a cell-type specific mitogen for adult hippocampal and for induced neural stem cells.

The role of proNGF, the precursor of Nerve Growth Factor (NGF), on the biology of adult neural stem cells (aNSCs) is still unclear. Here I analyzed adult hippocampal neurogenesis in AD11 transgenic mice, in which the constitutive expression of anti-NGF antibody leads to an imbalance of proNGF over mature NGF. I found increased proliferation of progenitors but a reduced neurogenesis in the AD11 DG-hippocampus (HP-DG). Also *in vitro*, AD11 hippocampal neural stem cells (NSCs) proliferated more but were unable to differentiate into morphologically mature neurons. By treating wild-type (WT) hippocampal progenitors with the uncleavable form of proNGF (proNGF-KR) I demonstrated that proNGF acts as mitogen on aNSCs at low concentration. The mitogenic effect of proNGF was specifically addressed to the radial glia-like (RGL) neural stem cells through the induction of cyclin D1 expression. These cells express high level of $p75^{NTR}$, as demonstrated by immunofluorescence analyses performed *ex vivo* on RGL cells isolated from freshly-dissociated HP-DG or selected *in vitro* from NSCs by LIF (leukemia inhibitory factor). Clonogenic assay performed in the absence of mitogens showed that RGLs respond to proNGF-KR by reactivating their proliferation and thus leading to neurospheres formation. The mitogenic effect of proNGF was further exploited in the expansion of mouse induced Neural Stem Cells (iNSCs). Chronic exposure of iNSCs to proNGF-KR increased their proliferation. Altogether, I demonstrated that proNGF acts as mitogen on hippocampal and induced neural stem cells.

2. INTRODUCTION

2.1 Adult Neurogenesis

2.1.a. An overview on adult neurogenesis and its function in adult hippocampus

The process called adult neurogenesis is the object of a modern and continuously expanding field in neuroscience and is defined as the process that leads to the generation of new functional neurons from Neural Stem Cells (NSCs) in the postnatal age, lasting for all lifespan. This process is a very robust form of plasticity of the adult brain, as it adds new elaborating units in a neural circuit, so allowing the formation of new patterns of this circuit (assuming the correct integration of the newborn neurons in the old pattern). The importance of this concept is remarkable as nervous system plasticity was long thought to be involved only in modulating the contacts between preexisting old neurons. At date, such plasticity provided by adult neurogenesis has been investigated until be linked with the high complex question of development of individuality mediated by the cognitive challenges [1]. So, the key function of adult neurogenesis is to shape neural connectivity in the brain according to individual needs.

At a merely functional level, it is noteworthy that newborn neurons have special electrophysiological features for about 1 month after their generation, as they undergo a period of increased excitability and plasticity (although they are unlikely to influence behavior before they integrate in the networks) [2-4]. So, the continuous production of new neurons may serve to maintain a pool of neurons with such special properties.

The concept of neurogenic niche

The Subgranular Zone (SGZ) in the Dentate Gyrus (DG) of the hippocampus and the Subventricular Zone (SVZ) of the lateral ventricles are the two main neurogenic niches that participate in the plasticity of adult brain and have been extensive-

ly investigated in the field of adult neurogenesis; but in the CNS (Central Nervous System) of different species of mammals, as rat, mouse, rabbit and primates, also alternative regions of adult neurogenesis were described. These are paraventricular regions as the hypothalamus [5], circumventricular organs [6] and striatum [7] (phenomenon independent from that occurring in the adjacent SVZ [8]), olfactory epithelium [9], cerebral cortex [7, 10, 11], cerebellum [12-14] and spinal cord [15-23](Fig. 1).

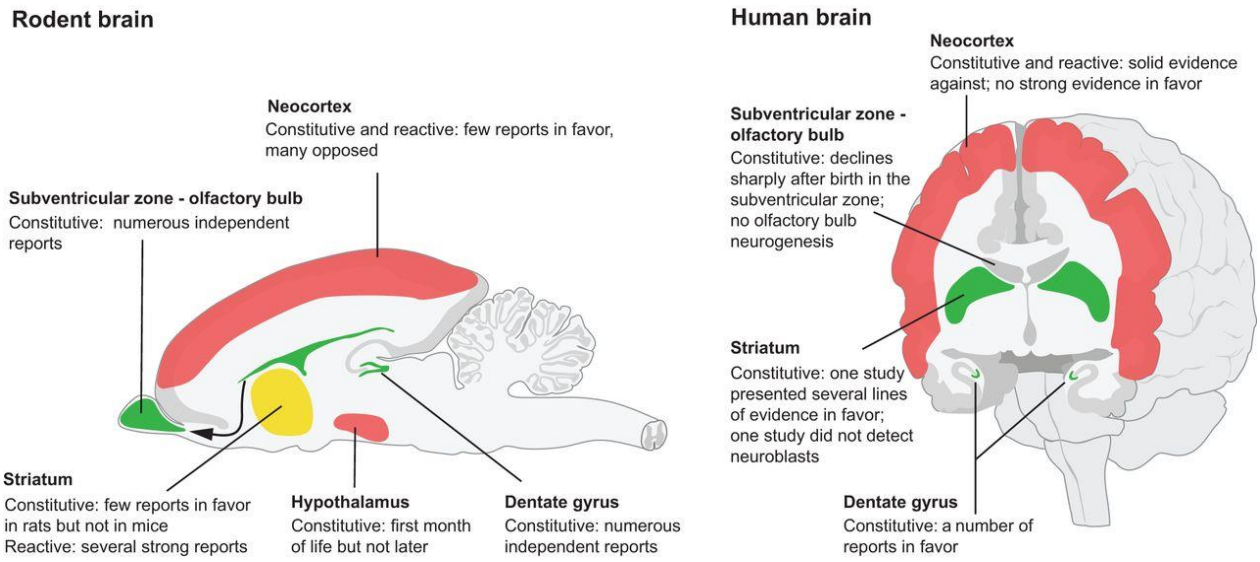


Figure 1. The extent of neurogenesis in different regions of the adult brain of rodents and humans.
In some regions, neurogenesis takes place throughout life (green), in other regions it is mostly in response to injuries (yellow) and in yet other regions, there is no strong evidence that it ever occurs in adulthood (red; figure from Magnusson and Frisén, 2016).

However, when we consider these results we have to pay attention if we are in the presence of a truly neurogenic niche that preserves a population of putative adult neural stem cells, able to sustain a process of adult neurogenesis for all lifespan of organism, or if we are in the presence of a population of long-lived progenitors derived from the end of the CNS development. Moreover, in some cases these cells (stem cells or progenitor of these alternative proliferative regions) could generate only glial cells and not neurons [18-20] (Fig. 2A-B).

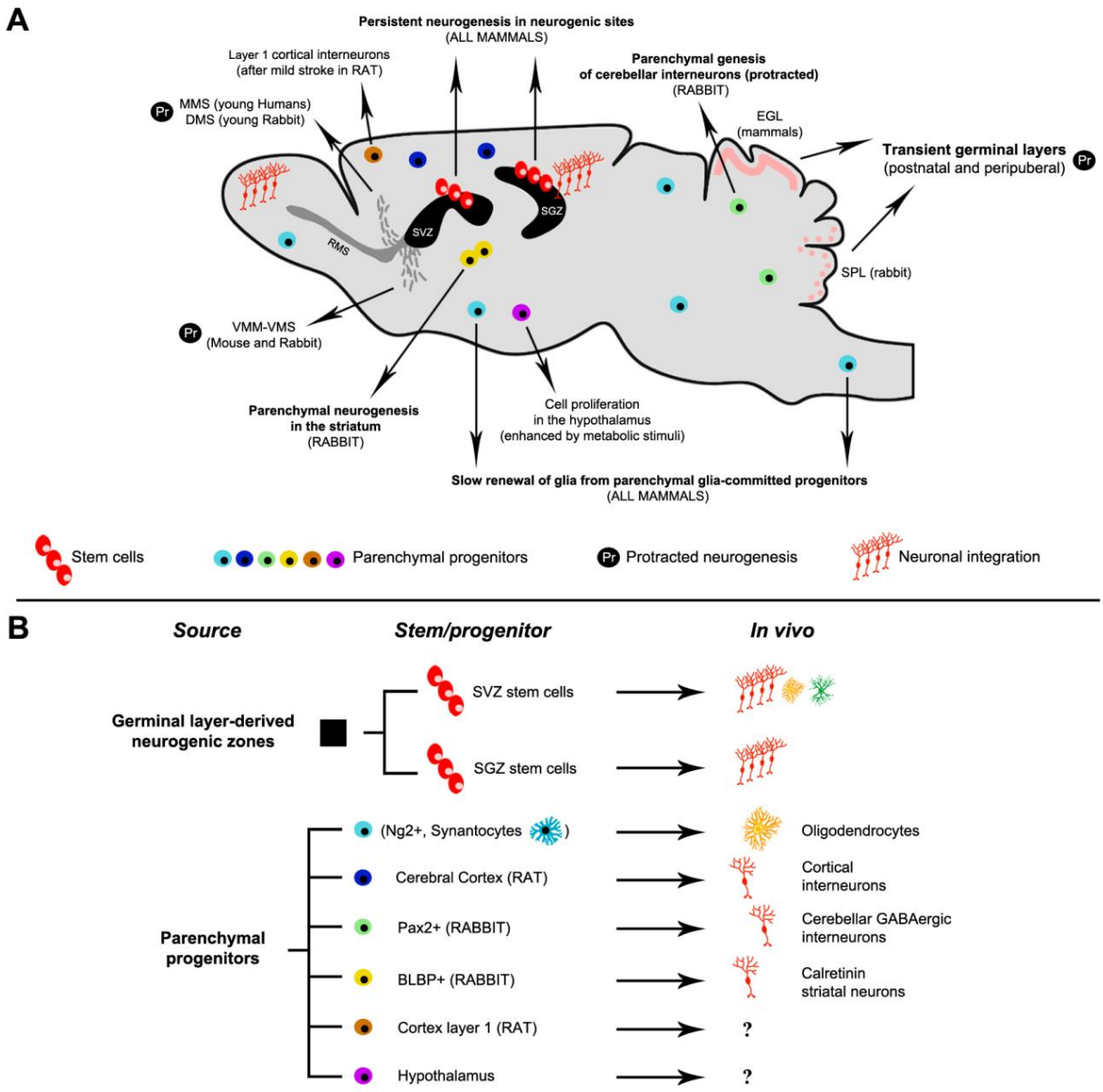


Figure 2. Neurogenic regions in mammal brain: niche stem cells and parenchymal progenitors.
A) Schematic representation of a sagittal section of the rat brain. Neural or glial progenitors are widespread in the parenchyma of adult mammalian brain (parenchymal progenitors), but they do not descend from niche stem cells (red), which reside in SVZ and SGZ neurogenic niches (the only two sites of truly persistent neurogenesis, black). Stem cells of SVZ and SGZ produce new neurons intended to specific sites: the olfactory bulb and the granule layer of hippocampus respectively. Parenchimal progenitors can sustain a protracted neuro- or gliogenesis, in same regions of adult brain (with difference between species), but such phenomenon is limited to a determined phase of life of the organism (postnatal, peripuberal or young life). B) Legend for panel A that indicates the derivation and the differentiation destiny stem cells of from the two niches. The legend also distinguishes the different parenchymal progenitors, for marker they express or region in which they are founded and indicates their relative differentiation destiny (figure from Bonfanti et al., 2013).

Adult neurogenesis in the forebrain is evolutionary conserved across mammals, birds, reptiles, amphibians and fish but in mammals this process is restricted and specialized to SVZ and SGZ. These two neurogenic processes have different biological significance, as described below.

SVZ

Neurogenesis in the SVZ is correlated with the maintenance of the structural and functional integrity of regions of adult forebrain where the neural precursors, product from NSCs in the SVZ, migrate and integrate in the local circuits [24]. While in rodents the region of destination for neural precursors is the Olfactory Bulb (OB), through the Rostral Migratory Stream (RMS), in humans there is an apparent loss of neurogenesis in the adult OB, possibly because humans rely more on the visual system than rodents, and, in parallel, have lost a number of olfactory receptor genes. But intriguingly, in humans, the neural precursors derived from the SVZ migrate into several other brain regions, including the frontal cortex in the infant brain and the striatum in the adult brain [25-27].

SGZ and behavioral consequences of new neurons addition

In the adult hippocampus, instead, neurogenesis is preserved with the same modality in rodents and humans, implying that it plays a significant role in behavior. Interest in adult hippocampal neurogenesis derived from its involvement in the functions of this organ, as learning and memory and the consequential link with several psychiatric and neurological disorders. The DG is an area of the brain characterized by a large, dense population of glutamatergic granule cells with very sparse activity [28-30]. It is the major input region to the hippocampus and is therefore thought to play an essential role in learning, episodic memory and spatial navigation tasks associated with that structure (Fig. 3A-B).

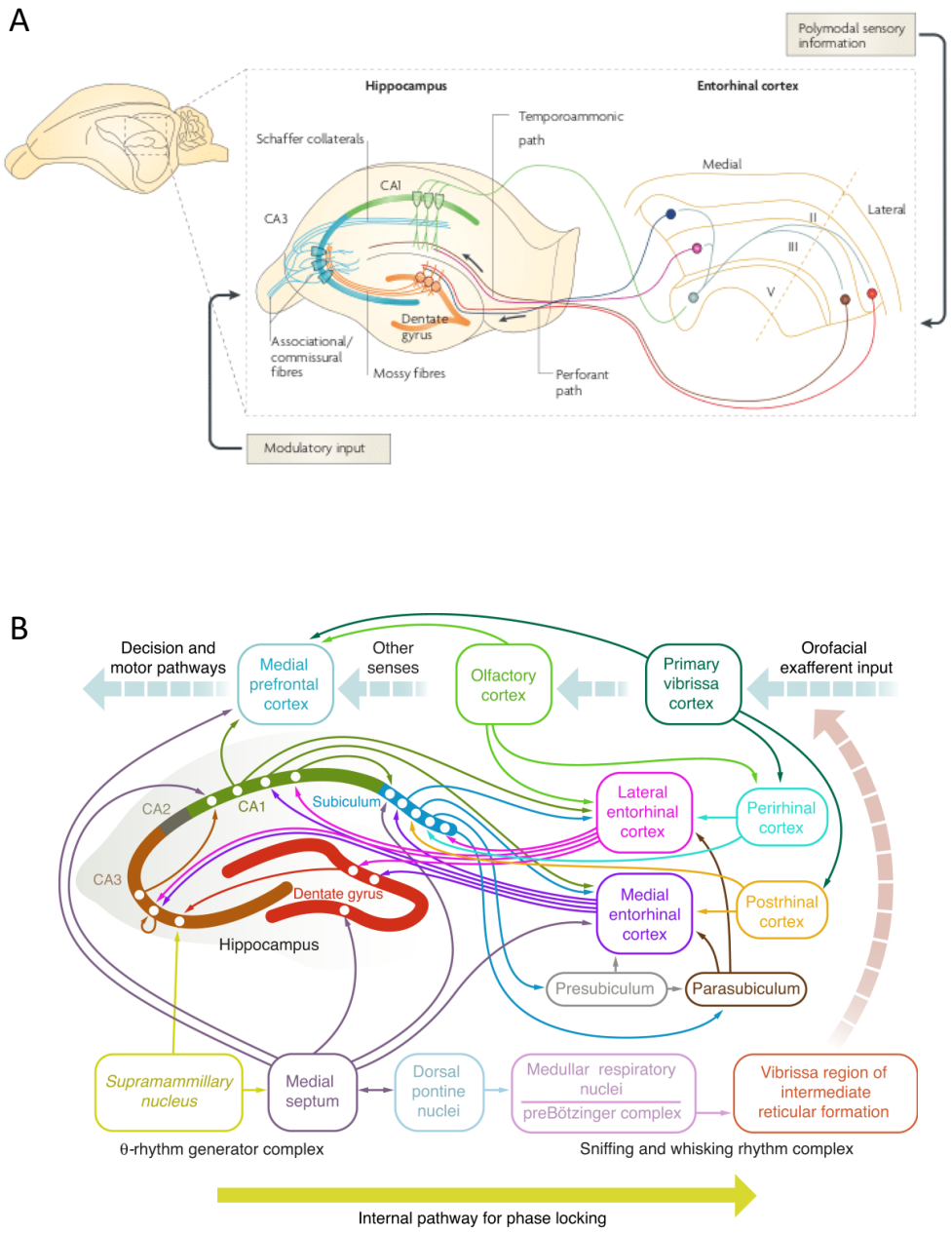


Figure 3. Structure and connectivity of the DG of adult hippocampus.
A) The schematic diagram shows the traditional trisynaptic loop of hippocampus wiring. Neurons project from layer II of the entorhinal cortex to granule cells of the dentate gyrus. These in turn project via mossy fibers to CA3 pyramidal cells. Schaffer collaterals projecting from CA3 to CA1 transmit signals to CA1 subregion. These neurons project back to layer V entorhinal cortex. As CA1 neurons can receive direct input from layer II cells of the entorhinal cortex and project back to layer V, a second, directly interconnected pathway is established. (Figure from Neves et al.,2008). B) Schematic view of the circuitry of the temporal lobe and its connections to other brain areas of relevance (figure from Kleinfeld et al., 2016).

Computational modeling of the effect of adult neurogenesis on hippocampal function has generated different theories for the role of newborn neurons. These include encoding of temporal information into memories [31, 32], avoidance of memory interference and cognitive flexibility during learning of new task [33] and balancing pattern separation/integration [34]. Pattern separation is the ability to discriminate similar experience. At a computational level this process produces distinct outputs from similar inputs, in the case of memories, by reducing the overlap in their representations. So, adult DG neurogenesis has a specific role in this mechanism, as newborn neurons help separate the perception of similar event for storage as distinct memories [35, 36]. This mechanism is critical for adapting to a complex environment (Fig. 4).

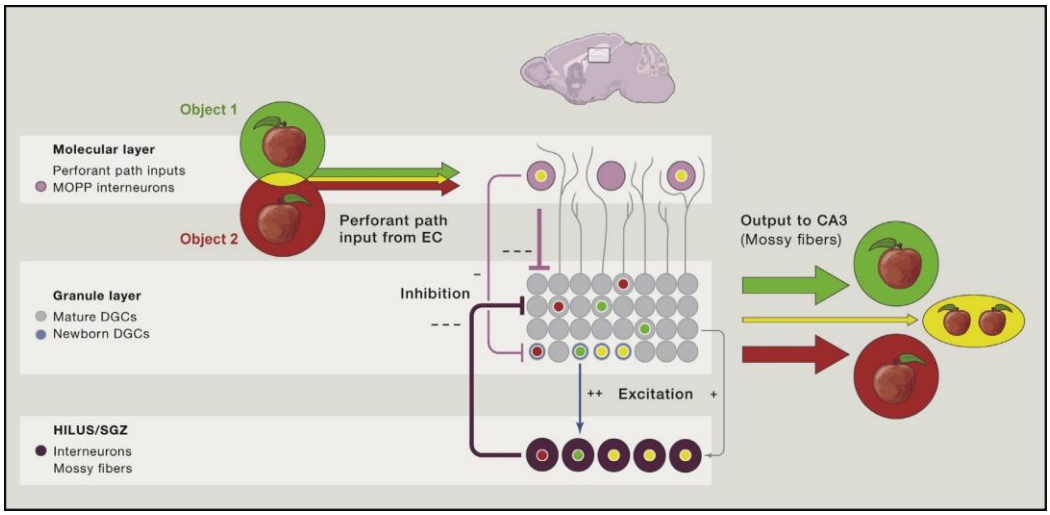


Figure 4. Connectivity of Adult-Born DGCs potentially enhances Pattern Separation trought feedback inhibition. *Memories of similarobjects are thought to ben encoded by separate but partially overlapping population of activated DGCs (red and green, with overlap in yellow), here exemplified by a recall task where subject is asked to identify which of two imeges is novel. In this example, the two apples differ only in their green leaves. The more similar the performant path inputs from the EC, the greater the overlap of their representation in the DG. Mature DGCs (gray) receive strong inhibitory inputs from interneurons (purple) in the hilus, molecular, and subgranular zones (denoted by ---). Immature adult-born DGCs (blue) are more active than mature DGCs (gray) due to their intrinsic properties and reduced inhibitory inputs (denoted by -). However, the firing of immature neurons is also though to strongly enhance feedback inhibition from hilar interneurons, resulting in overall sparser DG responses and, consequently, a decreased overlap of memoryrepresentations. Therefore, although the responses of newborn DGCs are less discriminating, with a large overlap between rapresentations, they are tought to enhance pattern separation by minimizing the overlap between object representations of their mature counterparts. These representations are then relayed to CA3 through the mossy fiber outputs. Most mossy fibers respond to only one of the images (red and green arrows), although some, primarily those of newborn neurons, fire in response to both (yellow arrow; figure from Gonçalves et al., 2016).*

The newborn neurons in the DG perform distinct functions depending on the environmental inputs and cognitive demands present during maturation. Experience during this early maturation period changes the timing of the integration of neurons into hippocampal networks and shape their connectivity [37-39]. The importance of neurogenesis in the maintenance of DG functions is revealed by the direct cognitive defects that occur when the number of newborn cells in DG is reduced. One common strategy used for this type of analysis is to ablate adult-born neurons by antimitotic reagents [40, 41], focal X-ray irradiation and genetic ablation based on transgenic animals using neural progenitor-specific promoters [24, 38, 42-45]. The results of such depletion of neurogenesis are disruption of spatial memory in many instances, in particular long-term memory retention, context-dependent memory and specifically performance in contextual fear conditioning task [37-39]. In a consistent manner, mice with increased neurogenesis, either through behavioral interventions (exercise [46], enrichment environment [47]) or by a genetic enhancement of the survival of new neurons, perform better in task that required optimal pattern separation [36, 48]. The existence of adult neurogenesis in human DG is actually a controversial issue. Indeed, the extent and relevance of a similar process in humans is currently a matter of debate, in terms of proliferation of neural precursors, number of neuroblasts present in the human neurogenic niches and the amount of newly generated neurons in adulthood. In 2013, Spalding and colleagues, by using ^{14}C retrograde analysis in brain post-mortem tissues, demonstrated that 700 new neurons are added in each hippocampus per day[49, 50]. This corresponds to an annual turnover of 1.75% of the neurons within the renewing fraction, with a modest decline during aging [51]. In another study, the analysis of both fetal and adult post-mortem samples (up to 100 years of age) revealed the presence of neural progenitors and NSCs (using neural specific markers) in the granule layer of the hippocampus [52]. Three more recent papers, published between 2018 and 2019, came to opposite conclusions regarding the existence of lifelong neurogenesis in humans [53, 54]. However, even in the case of

low neurogenesis rates under homeostatic conditions in the human brain, the possibility to induce human neural precursor to generate new neurons is an attractive and challenging prospect for cell-replacement therapy in neurological diseases [55].

The final interesting aspect of adult neurogenesis, not only in hippocampus but also in SVZ, is that this process is under control of lifestyle. For example, physical exercise increases the generation of new neurons; in particular, running rescues defective adult neurogenesis, and this effect is at charge of cell cycle length [56, 57]. Moreover, nutritional factors such as high-fat and high sugar diets, or alcohol and opioid addiction, negatively affect adult neurogenesis [58].

2.1.b. Adult Neural Stem Cells biology in the hippocampal neurogenic niche

Adult neurogenesis starts by activation and proliferation of the adult NSCs in their niche, that is the anatomical site where stem cells reside, and represents a microenvironment where many complex signals work for preserving the maintenance of the quiescent population of stem cells and for modulating proliferation, differentiation and migration of their lineage [59, 60].

As the other stem cells of the adult organism, adult NSCs have the two essential properties: self-renewal (generation of an identical daughter cell) and multipotency (generation of all cell-type of the resident tissue: neurons, astrocytes and oligodendrocytes in the case of NSCs). The common maturation path from adult stem cell to mature progeny in many organs implies that stem cells divide relatively infrequently to generate transit-amplifying cells, which in turn divide to rapidly expand their number before generating more mature progeny. This hierarchy of division and differentiation allows the amplification of the number of mature cells that can be derived from a single stem cell, while minimizing the possibility of mutations due to DNA replication in the genome of old long-lived stem cells [61].

The mechanism that determines whether a daughter of a stem cell remains a stem cell or commits to differentiation can depend, in principle, on the inheritance of cell-fate determinants from the mother cell, on environmental factors, or on both [59, 62].

Although stem cells occupy a small percentage of an adult tissue, they have profound biological significance. The basic biological significance of adult stem cells is to act as a reservoir of progenitor cells that can in turn act as a repair system, primarily for that particular tissue, or other tissues of a particular germline. The stem cell is an essential component of a developmental phenomenon, one of the key components of a program fundamental to organogenesis and maintenance of homeostasis throughout life.

In most tissue, stem cells are rare. As a result, stem cells must be identified prospectively and purified carefully in order to study their properties. Although it seem reasonable to propose that each tissue arises from a tissue-specific stem cell, the rigorous identification and isolation of this somatic stem cells has been accomplished only in a few instances. For example, hematopoietic stem cells (HSCs) have been isolated from mice and humans [63-65], and have been shown to be responsible for the generation and regeneration of the blood-forming and immune (hemato-lymphoid) system.

Stemness in adult brain

The principal modality to ascertain in vitro the "stemness" of adult neural stem cells is the neurosphere assay. By this assay, NSCs are often identified as cells that form floating cell aggregates, or neurospheres, when cultured in serum-free medium on a non-adherent surface in the presence of EGF (Epidermal Growth Factor) and bFGF (basic Fibroblast Growth Factor) [66, 67] as mitogens. NSCs within the neurosphere are able to proliferate, self-renew and generate multipotent progeny, so in turn neurons, astrocytes or oligodendrocytes, or some combination of the

three cell types [67, 68]. Another way to conferm stemness of aNSC are the transplantation experiments. When transplanted to new CNS location, these cells can adopt some of the characteristic appropriate to the new environment [69, 70] and, significantly, basic science research has shown that transplanted aNSCs can survive, migrate, differentiate and integrate in the brain of rodent models of several brain injury and pathological conditions like stroke and Huntington's disease [71-73].

The hippocampal neurogenic maturation path

In the DG of hippocampus, based on the combination of specific markers (usually GFAP, Nestin, Mash1 and Dcx), four stages of mitotic cells during the maturation path have been classically distinguished, which are in order: the quiescent Type-1 Radial Glial Like Cell (RGL) ($GFAP^+/Nest^+/Mash^-/Dcx^-$), the Type-2a Transient Amplyfing Progenitor ($GFAP^-/Nest^+/Mash^+/Dcx^-$), the Type-2b Transient Amplyfing Progenitor ($GFAP^-/Nest^+/Mash^-/Dcx^+$), and the neural committed Type-3 Neuroblast ($GFAP^-/Nest^-/Mash^-/Dcx^+$) which closes the mitotic phase of neurogenesis by maturing in the post-mitotic $NeuN^+$ newborn granule neuron (not yet mature granule neuron) [52, 74-77]. Other markers such as Sox2, BLBP, Musashi 1 (Msi1), NeuroD1 (ND1), Neurogenin2 (Ngn2), Tis21 and Prox1 allow to better characterize the specific stage of maturation and the eventual further classification in sub-stages [56, 78-82] (Fig.5).

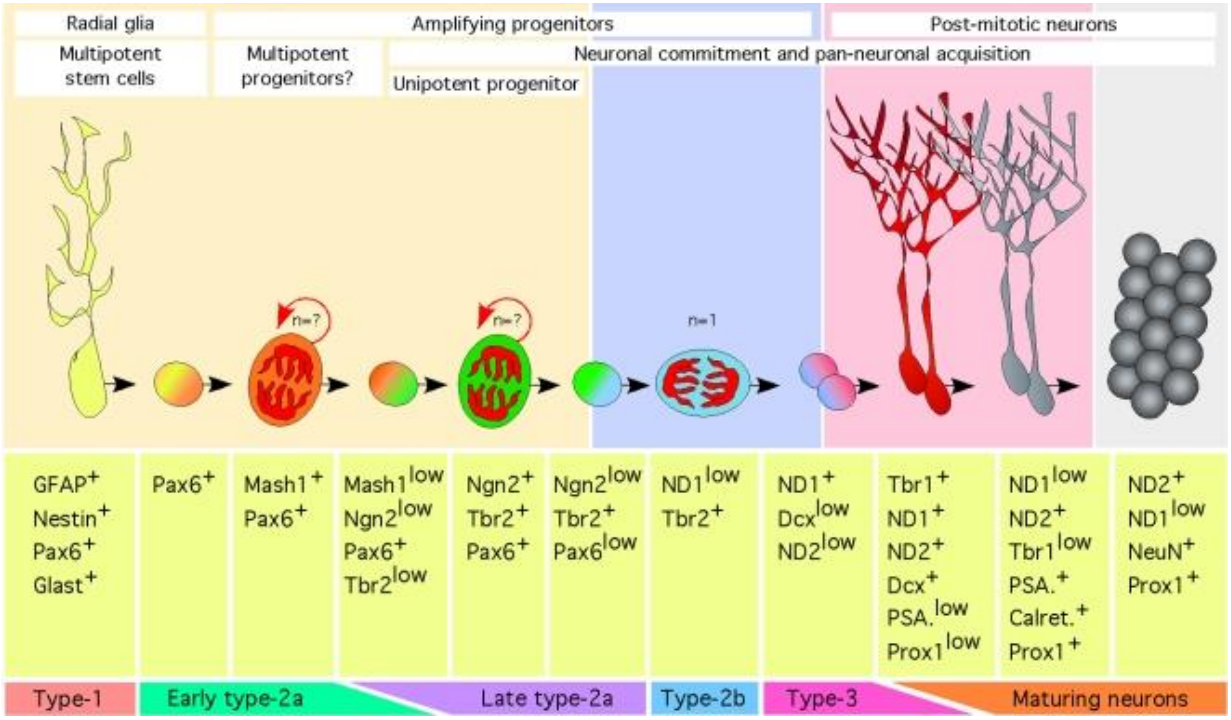


Figura 5. Model of postnatal hippocampal granule neurogenesis.
Multipotent GFAP/Nestin/Pax6/Glast+ radial glia stem cells give rise to multipotent and highly dividing Pax6/Mash1+ progenitors. Ngn2 initiates neuronal commitment of Pax6/Mash1+ progenitors. Ngn2 progenies undergo asymmetric divisions and amplify until they divide symmetrically and express NeuroD1. NeuroD1 stops the amplification phase of Ngn2 progenies and direct neuronal maturation. NeuroD1 progenies undergo maturation through the expression of the transcription factors Tbr1, NeuroD2 and Prox1 and the cellular markers Dcx, PSA-NCAM, Calretinin and NeuN (modified from Roybon et al., 2009).

The NSCs lineage in the hippocampus is responsible for the generation of neurons and glia (astrocytes and, potentially but physiologically repressed, oligodendrocytes [83, 84]), but it is not well known at what maturation level the fate commitment (separation of the lineage in glial and neural) occurs, if at the RGL or the Transit Amplifying Progenitor level. In the hippocampus, clonal analysis showed that RGLs have self-renewal and multipotency [85, 86]. For sure, at the stage of Type-2b Amplifying Progenitors, the neural determination becomes apparent, with overlapping expression of the transcription factors Prox1, NeuroD1, and the structural protein Doublecortin (Dcx)[75, 87-89].

The Type-3 Neuroblasts, generated in the SGZ, start to migrate through the inner Granule Cell Layer (GCL) while they mature and rapidly extend long axonal projections, along the mossy fibers path, that reach their target, the CA3 layer of py-

ramidal neurons, to functionally integrate in the circuitry at day 4th to 10th from division [90-93]. The hippocampal neurogenic niche, *i.e.* the SGZ, is located between the hilus and the Granule Cell Layer (GCL, the layer composed of the mature granular neurons) of the DG. So, the newly generated granule neurons born in the SGZ migrate only for a short distance to reach the granule cell layer.

Astrocytic features of RGLs

Adult NSCs (referring to the staminal stage of RGL) have been defined as astrocytes (so glial cells associated with support functions in the brain) based on their ultrastructural features, the markers they express and their electrophysiological properties. One hypothesis is that stem cells are contained within the astrocyte lineage [94, 95]. During development, radial glia are the *in vivo* primary precursors of neurons and glia [96-101]. Post-natally, radial glia make transition into astrocytes [102-105], some of which are retained as stem cells in adult neurogenic niches [105]. So, the population of astrocytes is likely to undertake a role subdivision: mature astrocytes and stem cells (RGLs).

For what concerns the stem cells role of the astrocytes within the niche, in the hippocampus it has been showed that RGLs have self-renewal and multipotency [85, 86]. According to many scientists, multilineage differentiation and self-renewal may represent a collective property derived from a mixed population of unipotent neural progenitors that are neurogenic or gliogenic under physiological conditions [106]. Another model proposes that some activated RGLs differentiate only into astrocytes after several rounds of division [107]. The two models are not exclusive, and recent evidences suggest that RGLs are a heterogeneous population, differentially responding to the stimuli (physiological and pathological) depending on their subtype [79, 108]. Further investigation of RGLs using single-cell RNA sequencing methods currently under development should help to reveal the nature of the heterogeneity of RGLs [109, 110].

The radial astrocytes, the Type-1 RGLs, have a prominent process that crosses the granule cell layer as well as smaller horizontally oriented processes along the SGZ [111, 112]. Adult neurogenic niches have an instructive role in directing neural production and stem cells maintenance and shield ongoing neurogenesis from possible external inhibitory influences. Neuronal and non-neuronal cell types are key players that mediate this process (reviewed in [113]). Within adult neurogenic niches, in addition to their role as stem cells, astrocytes are uniquely poised to be sensors and regulators of the environment. Their long process envelopes and contacts all cell types and structure of the niche, including blood vessels and the basal lamina [111, 114] (Fig 6). Moreover, astrocytes are often coupled via gap junctions and can form a syncytium, which may allow them to propagate signals locally or throughout the entire niche (reviewed in [115, 116]), thereby regulating activation and differentiation of stem cells themselves. Astrocytes also contribute to the neurogenic niche through contact-mediated cues and by secreting diffusible signals [117-120].

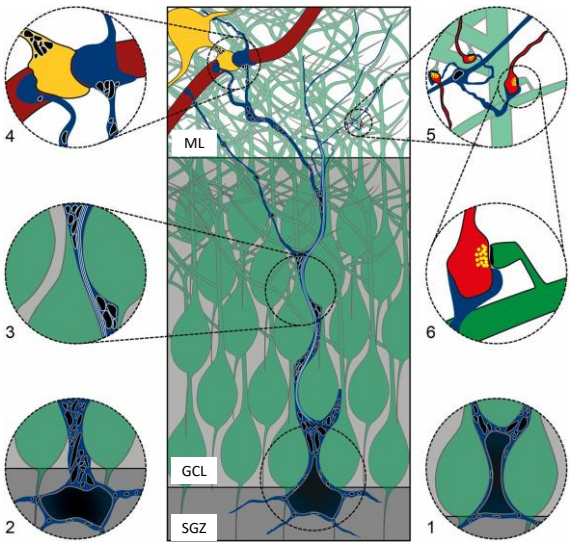


Figure 6. RGLs and astrocytes in the architecture of hippocampal neurogenic niche.
RGL stem cells interact with neuronal, vascular, and glial cells. The soma of the RGL (blue) sits above (1), across (center), or below (2) the border of the SGZ and GCL, and takes different shapes. The primary process of the stem cell extends through the GCL (3), with its path and surface impacted on by granule neurons (green). Mitochondria (black) reside in the thicker parts of the process, but, in thinner regions, there is space only for the filaments (white) to grow through the process (3). Some processes in the ML make small endfeet-like contacts onto blood vessels (dark red) or wrap large thin sheets around them, sometimes continuing beyond the vessel after wrapping it (4). Astrocytic processes (yellow) share the blood vessel surface with the processes of the stem cell, with adhesion points where they meet. Thin processes possess regularly spaced mitochondria- filled varicosities along their length (5). Finer processes extend from these varicosities to approach and/or wrap around local asymmetrical synapses (light red; 5 and 6). (Figure from Moss, 2016).

Non-neuronal component of the niche: endothelial cells and ECM

The SGZ is located next to an extensive vascular niche, so neurogenesis occurs in close proximity of blood vessels, with proliferative clusters containing neural progenitors, glial cells, newborn neurons and endothelial cells, suggesting that factors derived from blood vessels influence the behavior of NSCs in the SGZ, so that neurogenesis and angiogenesis are coordinated processes [121].

Other important components of the niche are the endothelial cells, the extracellular matrix (ECM) and the ECM-associated molecules. Endothelial cells are critical niche cells that regulate stem cell self-renewal and neurogenesis. These cells secrete factors as Leukemia Inhibitory Factor (LIF) [122] and Brain Derived Neurotrophic Factor (BDNF) [123, 124], which are known to influence proliferation and/or differentiation. The Extracellular Matrix (ECM) and the ECM-associated molecules contribute to niche architecture and to create a favorable environment within the niche. They regulate signaling in the niche by providing, storing and compartmentalizing growth factors and cytokines indispensable for cell proliferation and differentiation, as well as by acting as a substrate for anchoring cells. For example, integrins are receptors that provide structural links between the ECM and the cytoskeleton, allowing for oriented cell division. In addition, they cooperate to enhance signal transduction [125].

Overall signaling integration in aNSC biology

At the molecular level, the biology of adult NSCs has not been yet fully understood since their first isolation and characterization from rodents in the 1992 by Reynold and Weiss [67]. Their maintenance, activation, proliferation, surviving and differentiation, are regulated by the convergence of many signals (even kind of activity-dependent [93, 126-129]) that may occur at several levels within, and in proximity to, the signal-receiving cell. The surrounding niche provides the environment for a first level of signal integration. A second and more complex level of integration is the network of signaling components existing within a particular context of the signal-

receiving cell itself: the different expression of receptor and downstream target in space and time may set or alter the threshold for certain signals from the niche by integrating or differentiating incoming information.

Maintenance of the Stem Cell Pool: The Notch signaling

An essential mechanism for the persistence of adult neurogenesis throughout lifespan is what preserves the maintenance of a quiescent stem cells pool while transit-amplifying progenitors are produced. Such essential mechanism has Notch as its central actor. The Notch signal cascade is preserved in adult neurogenesis with the same features of embryonic neurogenesis, when the maintenance of the population of neural progenitor cell until the final stage (while immature neuron or basal progenitor cells are produced) is essential for achieving production of both a proper number of cells and a full diversity of cell types [130]. This mechanism works by the activation of the Notch receptor that causes the release of the intracellular domain and its transfer to the nucleus, activating the transcriptional cascade that maintains the cell in the earlier stage of the neurogenic path, by repression of pro-neural genes such Mash1 and Neurogenin2 [131-133] (Fig.7).

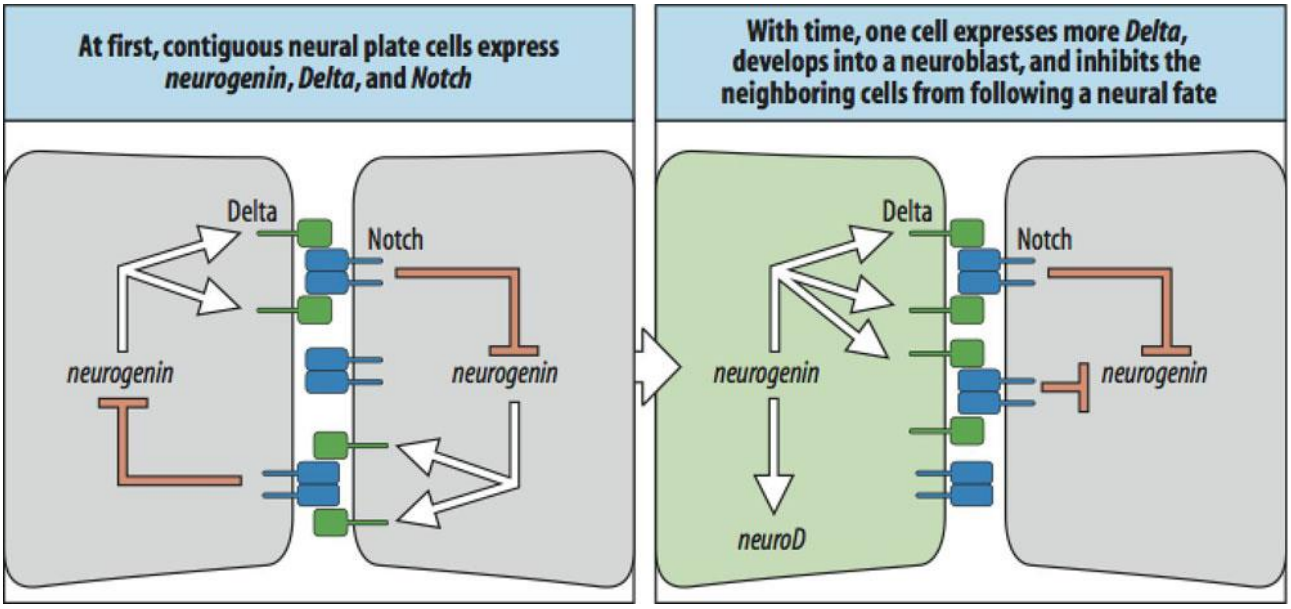


Figure 7. The competitive mechanism of Notch-Delta lateral inhibition.
The cell that acquires neural fate by high neurogenin expression and consequent pro-neural genes expression, like NeuroD (green cell in the right panel) is also able to continue to express delta receptor and so inhibits maturation in the neighboring cell (gray cell in the left panel) by continuing to activate notch in that cell, repressing his pro-neural genes and keep it in an earlier undifferentiated state.

In the embryonic brain, this mechanism prevents the exhaustion of highly proliferating neural progenitors that could prematurely differentiate into post-mitotic neurons; in contrast, in the adult neurogenesis such mechanism prevents the switch of the slowly dividing NSCs in fast dividing transit-amplifying progenitors, preserving the quiescent pool [134]. In the embryonic brain the mechanism is called lateral inhibition, because what activate the Notch receptor are ligands, such as Delta1, expressed by the cells that have not yet undertaken the path of pro-neural genes expression [131, 132]. So, as a result, a differentiating neuron prevents neighboring neural progenitor cell from differentiating, promoting thereby asymmetric division into one neural progenitor cell and one differentiating daughter neuron. In a similar manner, in the adult telencephalon of zebrafish, Notch receptor activation appears predominantly triggered by newly recruited progenitors onto their neighbors, involving binding of Delta or Jagged, suggesting an involvement of Notch in a self-limiting mechanism, once neurogenesis is started; in this way, quiescence is preserved by a feed-

back control arising from active progenitors [135]. For what concerns hippocampal neurogenesis, it was shown that inactivation of Notch1 promotes neuronal differentiation in the adult murine DG [136]. In the hippocampus, Notch1 was found to be required for self-renewal and expansion of nestin-expressing NSCs. In line with these findings, inactivation of the Notch pathway component RBPj led to premature differentiation, which in turn resulted in depletion of the stem cell pool and suppression of adult hippocampal neurogenesis [137].

Others principal signaling cascades in adult DG neurogenesis

Apart from Notch, other important signaling cascades that regulate the basal activity of aNSCs are those of Sonic hedgehog (Shh), Bone Morphogenetic Protein (BMP), and Wnt.

Shh signaling plays an important role in hippocampal neurogenesis. The receptor Patched (Ptc) and the transmembrane protein Smoothened (Smo), mediator of the Shh cascade, are expressed in the adult hippocampus and in progenitors derived from this region [127]. The source of Shh has not yet been clearly identified but studies have revealed Shh signaling activity in DG adult NSCs [138]. The role of this signaling resides clearly in proliferation. Exogenous Shh has been shown to directly promote progenitor proliferation *in vitro*. Overexpression of Shh within the DG, using an adeno-associated viral system, resulted in a marked increase in hippocampal progenitor cells proliferation *in vivo.* Pharmacological inhibition of Shh signaling through cyclopamine, directly delivered into the adult hippocampus, reduced hippocampal proliferation [127]. The fundamental role of Shh in hippocampal neurogenesis emerges since post-natal period. Postnatal progenitors failed to develop after embryonic ablation of Smo in $GFAP^+$ and $Nestin^+$ neural precursor cells [139]. In contrast, the expression of a constitutively active Smo resulted in a marked expansion of DG volume, indicating an important role for Shh signaling in the expansion and establishment of postnatal hippocampal progenitors. Moreover, there is a selective tar-

geting of the Shh-signaling machinery to the primary cilia that is thought to enable RGLs to differentially respond to mitogenic signals, thereby functioning as cellular "antennae" [140].

For what concerns BMPs, they comprise a group of more than 20 ligands that constitute the largest subgroup of the transforming growth factor-beta (TGF-beta) superfamily of cytokines. In the adult neurogenic niche, BMPs can act as short-range morphogens due to a limited spread and thanks to their ability to bind to extracellular matrix component. In the postnatal hippocampus, BMPs are chronically secreted by granule neurons, NSCs, and other niche cells and are essential for regulating the equilibrium between proliferation and quiescence [141-144]. BMPs are not only necessary for maintaining quiescence, but they also play crucial roles in controlling the rate at which DG cells mature [142]. Such a dual role may be explained by a differential expression of the BMP receptors [143]. In the hippocampal neurogenic niche, there are present several BMP inhibitors that adjust locally the BMP signaling. One of these signals, the strong inhibitor Noggin, becomes concentrated in DG in adulthood, controlled by the RNA binding protein FXR2 [145]. BMP signaling has also been shown to be involved in linking the mechanism of voluntary exercise with change in neurogenesis. Moreover, an age-associated increase in BMP signaling has recently been reported and it may partly contribute to the decline of neurogenesis in old animals, suggesting that inhibition of this pathway could potentially allow rescue of this age-related drop [144].

Wnt signaling, canonically fundamental during the development of cortex and hippocampus, induces the differentiation of intermediate progenitors during mid and late neurogenesis. Recent papers suggest an important function for Wnt pathway also in the adult hippocampal neurogenesis, as a factor that induces differentiation toward the neuronal lineage [119, 146]. It was shown that the transcriptional target of Wnt signaling cascade are genes specifically known to be involved in neuronal differentiation, such as Prox1 and NeuroD1 [146-148]. NeuroD1, in particular, is required

for survival and maturation in hippocampus [147]. The overall role of Wnt signaling is complicated by the fact that it can also promote proliferation [149]. The involvement of this signaling in both aspect (progenitor pool maintenance and neuronal cell fate) does not appear contradictory and several studies have shown how aging and neuronal activity dynamically control adult hippocampal neurogenesis through modulation of this central pathway. For example, the secretion of the Wnt antagonist Dkk1 increases whit age in the adult hippocampus and seems to be responsible for the aging-correlated decline in faculties associated with DG neurogenesis, as dorsal hippocampal infusion of Dkk1 resulted in impaired object recognition memory consolidation [150]. Moreover, Dkk1 deletion from granule neurons was sufficient to restore neurogenesis in old mice [151]. As Wnt signaling provides the basis for a wide range of possible interactions, it may seem difficult for this signal to converge in space and time to allow stage-specific regulation. A recent study revealed a transition of Wnt signaling responsiveness from the canonical branch to the non-canonical, in the course of neuronal differentiation. While canonical Wnt signaling progressively faded, the emerging non-canonical branch was required for late stages of maturation, such as dendrite initiation, radial migration and dendritic patterning [152]. So, in DG adult neurogenesis, Wnt signal could be highly stage dependent.

Neuronal activity-dependent regulation

Adult neurogenesis is also regulated by neuronal activity-dependent signals that reach the neurogenic niche by axonal inputs (where niche are richly innerved) of local and distant origin. In the hippocampus, the SGZ receives inputs originating from distant brain regions and locally from interneurons within the hippocampus, which influence neurogenesis directly or indirectly [93, 126-129].

2.2 Neurotrophins, proneurotrophins and adult neurogenesis

2.2.a. An overview on general features and actions of neurotrophins

One of the most interesting signaling pathway involved in aNSC biology is that of neurotrophins family, due to its central role in regulating the development of central nervous system and in balancing the surviving/death response of mature cells. Neurotrophins are important regulators of neural survival, development, function and plasticity. The central concept in neurotrophins biology arise from their now well-established essential role during development, when the targets of innervations secrete limiting amounts of these proteins that function as survival factors to ensure a balance between the size of a target organ and the number of innervating neurons by the intermediating mechanism of programmed cell death of these neurons. After development, neurotrophins play critical roles in maintaining neuronal morphologies and functions and work as well as providing trophic and tropic activities in the neuronal responses to injury [153, 154].

The first and best characterized member of the neurotrophin family is the Nerve Growth Factor (NGF)[155]. NGF was discovered in the 1987 by the Nobel laureate Rita Levi-Montalcini, during a search for survival factor that could explain the deleterious effects of target tissue ablation on the subsequent survival of motor and sensory neurons [156]. This important discovery led to postulate the “neurotrophic factor theory” (reviewed in [157]). According to this theory, neurotrophins are synthesized, at a considerable distance from the cell body, by peripheral tissues or neurons ("targets") that are contacted by axons of the neurotrophin-sensitive neurons. In the periphery, the tissue sources of neurotrophins are typically non-neuronal cells, whereas in the CNS, they are synthesized predominantly by neurons under physiological conditions [158]. During development, a retrograde flow of a neurotrophin is es-

tablished (by a vescicles-mediated, energy and microtuble-dependent mechanism), transporting the protein from the target (Target-derived) into the nerve terminal and up the axon to the cell body [159]. Those neurons that establish this flow survive the period of neuronal cell death, while those that do not, degenerate (Fig.8).

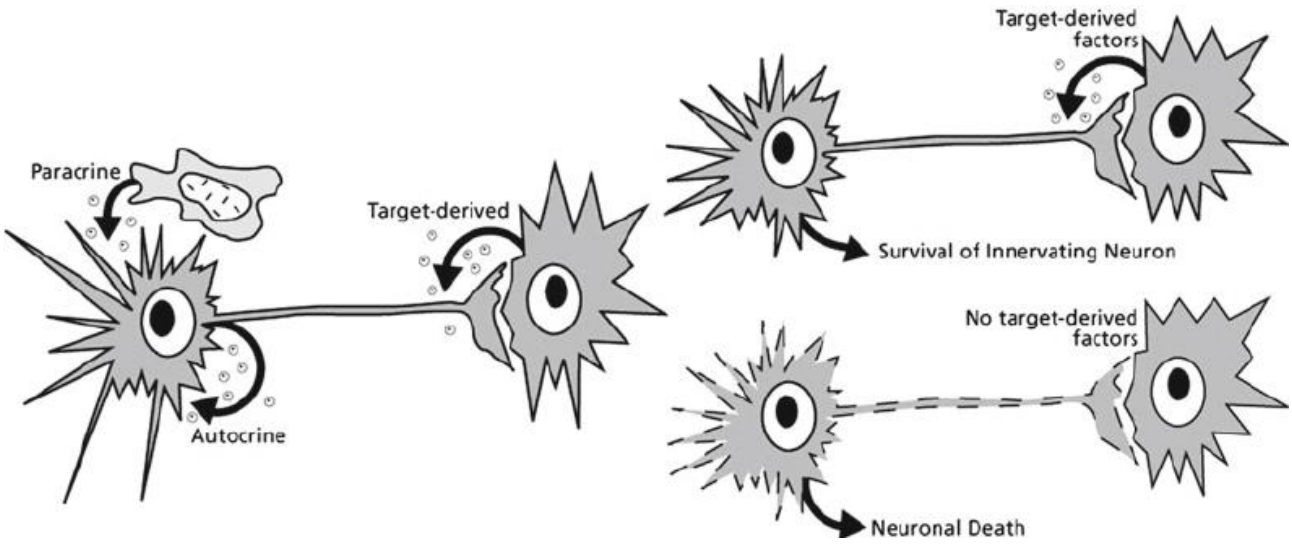


Figure 8. Mode of neurotrophic factor presentation.
Neurotrophic factor presentation can take place in three modes (left): Paracrine, Autocrine or Target-derived. In Target-derived mode, programmed Cell Death occurs in neurons that does not receive sufficient amount of neurotrophic survival factors from target cells (right).

Once the retrograde flow of neurotrophin is established, it must continue for the lifetime of the neuron to maintain the functional differentiated state of the neuron [160]. Studies indicates that, in addition to target-derived neurotrophic factor, other modes of factor presentation, such as autocrine and non-target-derived paracrine, are likely to be important [161]. At date we know that the phenomenon of programmed cell death after target deprivation (axotomy) is a general response, and most of neurons responds to and are regulated by neurotrophic factors, as supposed by Oppenheim in 1991 [162]. The isolation and characterization of the other member of neurotrophin family have validated this concept.

The entire neurotrophin family at date is composed of: the already mentioned NGF, the Brain-Derived Neurotrophic Factor (BDNF), the Neurotrophin-3 (NT3) and the Neurotrophin-4/5 (NT4/5), that have been all characterized in mammals. Neu-

rophin-6 and Neurotrophin-7, cloned from the platy fish and carp [163, 164] and that do not have orthologs in mammals or birds but seem to interact with the same receptors as the mammalian proteins. BDNF, isolated initially from pig brain [165], revealed similarity to NGF [166] and led to the concept of neurotrophin family, while NT-3 and NT-4/5 were isolated subsequently [167]. The term neurotrophin-4/5 resulted from uncertainties about whether the human neurotrophin-5 [168] was a species homolog of the NT-4 found in *Xenopus* [169].

All neurotrophins are structurally related proteins [170]. In their mature form, they are non-covalently associated homodimers. Neurotrophins share a highly homologous structure and are members of a large superfamily of growth factors that contain a tertiary fold and cysteine "knot" (three disulfide bonds that form a true knot of the polypeptide chain). These features are present in Transforming Growth Factor-β (TGF-β), Platelet-Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF) and others. NGF, BDNF, NT-3 and NT 4/5 share approximately 50% identity [171]. Neurotrophin residues are generally divided into two categories, conservable or variable, based on sequence alignments [167]. Amino acid residues implicated in neurotrophin binding that are conserved are likely to represent a common interface to the Tropomyosin-related kinase (Trk) receptors, while the unique ones may represent elements of specificity [172].

2.2.b. Neurotrophin responsiveness and signaling

Neurotrophins exert their biological actions by binding to two different classes of transmembrane receptors, the Trk family of receptors and the Pan-Neurotrophin Receptor p75 ($p75^{NTR}$). Both receptors can trigger downstream signaling pathways to exert biological effects of neurotrophins and other related ligands. One can broadly divide these pathways into Trk-mediated signaling, which is generally growth-promoting and pro-survival, and $p75^{NTR}$ -mediated signaling, which is generally pro-apoptotic and growth-inhibiting (Fig.9).

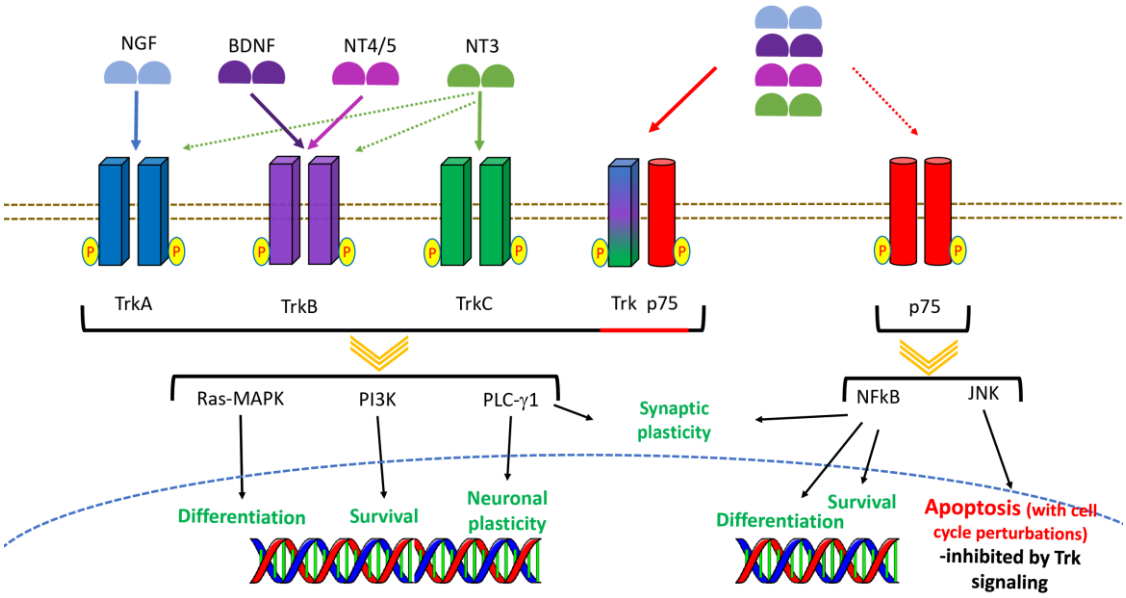


Figure 9. Trks and p75^{NTR} signaling pathways.
Each of the four neurotrophins bind with high affinity only one type of Trk receptor homodimer or Trk/p75 heterodimer, activating Ras-MAPK, PI3K or PLC-γ1 signaling cascade that drives differentiation, survival or plasticity responses. All neurotrophins can bind, although with less affinity, also the p75 homodimer, activating the NFkB or the JNK signaling cascade, the latter inducing apoptotic response.

The Trk proteins, TrkA, TrkB and TrkC, share the greatest degree of homology in their intracellular regions that possess tyrosine kinase activity. The extracellular regions that confer ligand-binding specificity are the most variable [172-174]. Specificity of neurotrophin action is believed to be achieved in part by the selective interaction between members of the Trk family of receptors and the different neurotrophins. Thus, NGF binds to TrkA [175, 176], TrkB binds BDNF and NT-4/5 with high affinity [177, 178], and TrkC binds NT-3 [179]. NT-3 can also interact, albeit with less efficiency, with TrkA and TrkB [178, 180]. Expression of p75^{NTR} appears to allow NT-3 to discriminate its preferred TrkC from the other Trk receptors [181]. These different Trk receptors are expressed in both primary neurons and neuronal cell lines, and targeted mutation of *trkA*, *trkB* and *trkC* genes in mice disrupts neuronal development consistent with a loss of neurotrophin action (reviewed in [182]).

Usually, endogenous expression of a Trk receptor confers responsiveness to the neurotrophins to which it binds, but this generalization is oversemplified. In-

deed, differential splicing of the Trk receptors result in expression of proteins with differences in their extracellular domains that enhances receptor binding to non-preferred ligands [183, 184]. Many of the signaling mechanisms activated by the Trk receptors converge upon the nucleus to alter gene expression programs. With a few exceptions, ectopic expression of a Trk receptor is sufficient to confer a neurotrophin-dependent survival and differentiation response [185, 186].

Neurotrophins have been shown to directly bind to and dimerize Trk receptors, which results in the activation (by trans-phosphorylation) of the tyrosine kinases present in their cytoplasmic domains [187]. The cytoplasmic domain of the Trk receptor contains several additional tyrosines that are also substrates for phosphorylation by each receptor's tyrosine kinase. When phosphorylated, these residues form the cores of binding sites that serve as scaffold for the recruitment of a variety of adaptor proteins and enzymes, that ultimately propagate the neurotrophin signal.

The major pathways activated by this Trk signal transduction are the Ras-MAP kinase, the phosphatidylinositol-3 kinase (PI-3 kinase) and the phospholipase C-γ1 (PLC- γ1) pathways.

Ras-Map kinase pathway activation is required for normal differentiation of PC12 cell (rat pheochromocytoma cell line, canonical model for neuronal function, differentiation and survival) and neurons, and also promotes survival of many neuron subpopulations. Transient versus prolonged activation of this pathway has been closely associated, respectively, with a proliferation-inducing versus a differentiation promoting response to neurotrophin application [188]. The pathway leading to activation of Ras is surprisingly complex. The transcription factors activated by this pathway in turn control the expression of many genes known to be regulated by NGF and other neurotrophins. Among these, CREB regulates genes whose products are essential for prolonged neurotrophin-dependent survival of neurons [189, 190]. A key role of Ras in NGF-dependent neurons is proposed by the observation that sympathetic and sensory neurons cultured from neurofibromin-1 null mice (neurofibromin

inactivates Ras) lose their requirement for NGF [191]. Moreover, introducing activated forms of upstream regulators of MAP kinase, including Ras [192], Raf [193], or MEK [194], mimics NGF by inducing neurites outgrowth in PC12 cells. Conversely, dominant negative forms of Ras [195], Raf [196], and MEK [194], block neurites outgrowth in PC12 cells stimulated by NGF.

The PI-3 kinase pathway mediates neurotrophin survival effects, such as, for example, that of NGF on PC12 cells survival [197]. Pharmacological agents that suppress PI-3 kinase activity block the capacity of BDNF to sustain the survival of cerebellar granule neurons upon growth signal withdrawal [198]. An important protein activated by PI-3 kinase is the serin-threonine Akt (also known as protein kinase B). Akt controls substrates that directly regulate the caspase cascade, such as BAD, a proapoptotic Bcl-2 family member, preventing its proapoptotic action. Akt also regulates the activity of several transcription factors preventing the expression of several proapoptotic genes [199]. Targets of PI-3 kinase activation promote axon growth and pathfinding, and cell differentiation through recruitment of signaling proteins to the membrane [200].

For what concerns the PLC-γ1 signaling pathway, it is required for neurites outgrowth in PC12 cells [201]. The activity of PLC-γ1 has been also implicated in the ability of TrkB receptor to modulate synaptic transmission and long-term potentiation [202, 203].

The $p75^{NTR}$ is the low-affinity neurotrophin receptor and it binds all members of the neurotrophin family with a similar affinity [169, 204, 205]. It is a transmembrane glycoprotein that shares a high degree of homology with member of the Tumor Necrosis Factor (TNF) receptor superfamily [206]. Like the other member of this superfamily, $p75^{NTR}$ has an extracellular domain that includes four cysteine-rich motifs, a single transmembrane domain and a cytoplasmic domain that includes a "death" domain [207, 208]. While this receptor does not contain a catalytic motif, it interacts

with several proteins that relay signals important for regulating neuronal cell survival, differentiation, and synaptic plasticity.

Each of the four cysteine-rich repeats of the $p75^{NTR}$ participates in binding to NGF [208]. The tridimensional structure of the extracellular domain of $p75^{NTR}$ in association with an NGF dimer suggest that binding of NGF to $p75^{NTR}$ may result in dissociation of $p75^{NTR}$ multimers and propose the possibility that Trk and $p75^{NTR}$ monomers simultaneously bind to the same neurotrophin monomer. The $p75^{NTR}$ -related protein, NRH2, lacks the extracellular cysteine-rich repeats present in $p75^{NTR}$ and is unable to bind NGF, but regulates NGF binding to TrkA [209].

Indeed, $p75^{NTR}$ can modulate Trk receptor function on several levels: by promoting ligand binding, by promoting accessibility to neurotrophins through the induction of axonal growth and target innervation, and by promoting endocytosis and retrograde transport to membrane compartments (where internal engagement of neurotrophins with Trk receptors may promote signaling). For example, $p75^{NTR}$ inhibits activation of Trk receptors by non-preferred neurotrophin both *in vivo* and *in vitro* [210, 211].

The presence of $p75^{NTR}$ strengthens the activation of TrkA by suboptimal concentrations of NGF, although it does not appear to reinforce the activation of other Trk receptors similarly to their ligands [212, 213], but it rather cooperates with TrkA to form high affinity binding site for NGF [214]. $p75^{NTR}$ can promote retrograde transport of several neurotrophins [215] and may reduce ligand-induced Trk receptor ubiquitination, thereby delaying Trk internalization and degradation [216]; alternatively, $p75^{NTR}$ can promote Trk receptor endocytosis through polyubiquitination and subsequent internalization to endosomal compartments, resulting in enhanced signaling [217].

These findings suggest a mechanism by which $p75^{NTR}$ may promote axon growth and target innervation *in vivo* and *in vitro* [218, 219]. Sensory and sympathetic deficits are seen in mice lacking $p75^{NTR}$ [220-222].

Beyond this cross-interaction between $p75^{NTR}$ and Trk receptors, ligand engagement of $p75^{NTR}$ can directly induce neuronal death via apoptosis (reviewed in [223, 224, 225]). Analysis of the $p75^{NTR}$ mutant phenotype has demonstrated that the regulation of apoptosis by ligand engagement of $p75^{NTR}$ is important during peripheral nervous system as well as CNS development *in vivo* [226, 227]. Then, for mature neurotrophin interaction with $p75^{NTR}$ opposite cellular effects occur, depending on whether or not a cognate Trk receptor is present. For example, BDNF can promote apoptosis in primary sympathetic neurons that express $p75^{NTR}$ and TrkA but not TrkB [226].

Moreover, the $p75^{NTR}$ proper pathway of ligand engagement (independent from Trk) can promote not only apoptosis but also survival of many cell populations, as embryonic sensory and sympathetic neurons [228, 229]. This pathway of neural survival involves activation of NF*k*B [230].

The pro-apoptotic pathway of $p75^{NTR}$, instead, involves the Jun Kinase signaling [231-233] that leads, at the end, to the activation of p53, which controls cell survival in many cells besides neurons by targeting different pro-apoptotic genes, including Bax.

Another pathway downstream of $p75^{NTR}$ ligand engagement is the activation of acidic sphingomyelinase, which results in generation of ceramide [234]. This latter promotes apoptosis and mitogenic response in different cell types through the control of many signaling pathways, including the ERK, Jun Kinase and NF*k*B cascades. Ceramide inhibits at least two of the survival and differentiation-promoting pathways activated by Trk receptor signaling [235, 236].

Finally, and particularly important for synaptic plasticity, is the capacity of $p75^{NTR}$ to control the cytoskeleton. Indeed, $p75^{NTR}$ ligand engagement directly enhances neurites outgrowth by ciliary neurons in culture and this effect seems to be due to inactivation of RhoA by $p75^{NTR}$ [237]. Sensory and motor neurons extend axons more slowly towards their peripheral targets in mouse embryos lacking $p75^{NTR}$ [218,

[237]. Moreover, the presence of $p75^{NTR}$ has been shown to promote retrograde transport of NGF, BDNF and NT-4 [215, 219]. Reduction in retrograde transport may result in reduced axon growth and neuronal survival.

As a reciprocal effect, also Trk receptors strongly modulate $p75^{NTR}$-dependent signaling. Neurotrophins are much more effective in inducing apoptosis through $p75^{NTR}$ in the absence than in the presence of Trk receptor activation [238, 239]. Therefore, activation of Ras (Trk-mediated signaling) in sympathetic neurons suppress the pro-apoptotic Jun Kinase cascade ($p75^{NTR}$-mediated signaling) [240]. It is notable that the other $p75^{NTR}$-activated cascade, *i.e.* the NF*k*B cascade, is not inhibited by Trk signaling [239]. Thus, in the presence of Trk signaling, activation of NF*k*B cascade makes a synergistic contribution to survival [228, 229]. However, although kinase activity of Trk receptors suppresses the apoptotic signaling pathways mediated by $p75^{NTR}$, such suppression is not invariably and completely efficient. For example, in developing motor neurons, NGF is able to antagonize (through $p75^{NTR}$, highly expressed in this cells) the BDNF- and NT-3- mediated survival signaling, so to induce apoptosis [241]; in PC12 cells, BDNF binding to $p75^{NTR}$ reduces NGF-dependent autophosphorylation of TrkA [242].

The overall picture that emerges from the studies on neurotrophin biology is that the pro-apoptotic signal of $p75^{NTR}$ is largely suppressed by Trk-mediated activation of Ras by neurotrophins. Thus, $p75^{NTR}$ appears to refine the ligand-specificity of Trk receptors and may promote elimination of neurons not exposed to an appropriate neurotrophic factor environment. So, the final response of the neurotrophins signaling is always relative to the cellular system and the physiological context.

2.2.c. Neurotrophins functions on mature cells and therapeutic implication: survival regulation and synaptic plasticity

The general results of the research on neurotrophin biology field have revealed that all neurons depend on trophic support derived from their target for con-

tinued survival, not only during development but also in the adult nervous system. The function of neurotrophins on mature neuronal cells are not limited to a survival regulation, but they also regulate mechanism of synaptic plasticity. Mechanistically, the survival regulation is a systemic or whole cell response that is initiated by retrograde signaling to the cell body and nucleus (far from the source of neurotrophin production/release). Instead, the synaptic plasticity regulation is a local effect that occurs adjacent to point of release of the ligands.

The positive effects of NGF on survival are exerted in PNS (Peripheral Nervous System) by NGF on sympathetic and sensory neurons [171]. In the CNS, NGF supplies trophic support for septal and basal forebrain cholinergic neurons of hippocampus, where there are the highest levels of mature NGF in CNS, through retrograde transport [243]. Studies have highlighted that mouse models in which this retrograde supplying of NGF is reduced display an Alzheimer's disease-like phenotype [244, 245]. These studies have provided support for the notion that exogenous NGF could be used as a treatment for Alzheimer's disease. Also BDNF [160] and NT-4/5 [246] are trophic factors for sensory neurons. NT-3 promotes survival and neurites outgrowth of the large-diameter proprioceptive neurons of the DRG (Dorsal Root Ganglia) that innervate stretch and tension receptors in muscle and joints [247, 248]. NT-3 also has trophic actions on neurons of the nodose ganglia, sympathetic ganglia, Remak's ganglia, ciliary ganglia, trigeminal mesencephalic nucleus and spiral ganglia.

For what concerns the role in synaptic plasticity exerted by local effect, neurotrophins act upon existing macromolecules to alter protein function and cytoskeletal organization. Additionally, the neurotrophins can alter protein levels by directly modulating activity of the protein synthesis machinery that is concentrated near dendritic spines and whitin growth cone of axons. Neurotrophin expression can be regulated by neuronal activity, and increase in neurotrophins levels has been shown to facilitate neurotransmission (reviewed in [249, 250]). The neurotrophins involved in such mechanism are BDNF and NT-3 that can enhance synaptic efficacy trough local

effect at the synapse [251, 252]. Localized activation of postsynaptic and presynaptic protein synthesis has been shown to modulate synaptic efficiency in several different neuronal systems (reviewed [253-255]). BDNF is associated with an important form of synaptic plasticity: Long-Term Potentiation (LTP), corresponding to an increase in synaptic efficacy, important for learning and memory [256]. Exogenous applied BDNF has been shown to facilitate induction of LTP, particularly long-lasting LTP that is protein synthesis dependent [257]. Other studies further implicate BDNF with the cognitive deficits associated with Alzheimer's disease [258, 259]. Synaptic plasticity has been associated also with growth of neuronal processes generating increased dendritic spine complexity. Alterations in dendritic spine complexity have been demonstrated into several human neurodevelopmental disorders such as Rett syndrome (reviewed in [260] and [261, 262]). Thus, activity-regulation of BDNF expression could indeed alter dendritic complexity in human neurodevelopmental disorders.

2.2.d. Neurotrophins: relevance in adult hippocampal neurogenesis

In recent years, neurotrophins and their receptor have emerged as important regulators of adult hippocampal neurogenesis. Neurotrophic factors and their receptors are abundantly expressed in the hippocampus, and it is noteworthy that their expression pattern in the DG niche differs markedly to that of SVZ, the other principal neurogenic niche.

Several comprehensive analyses of adult SGZ neurogenesis have been conducted in relation to neurotrophins, highlighting $p75^{NTR}$ as an important mediator of this process. $p75^{NTR}$ expression is observed in a very narrow time window during the course of SGZ neurogenesis. Retroviral expression tracing experiments indicate that $p75^{NTR}$ expression is mainly confined to newborn cells between 3 and 7 days after retroviral injection [263]; these correspond to cells initiating the growth of the axon and dendritic processes, before axonal fibers reach the CA3 area [4]. At this stage, $p75^{NTR}$ is asymmetrically enriched at the initiation site of the axon fibers and in prox-

imal axon segment. In more mature neurons, $p75^{NTR}$ expression is decreased and the asymmetric distribution of the receptor is lost [263]. Therefore, this profile of expression indicates a $p75^{NTR}$-mediated role of neurotrophins during the integration phase of SGZ neurogenesis.

In $p75^{NTR\text{-}ExIII}$ knockout mice, which lack the full-length receptor but express the short $p75^{NTR}$ isoform, a reduction in the number of neuroblasts and newborn neurons in the DG is paralleled by an increase in the death of newly born cells and impaired performance of hippocampus-dependent behavioral task [264]. However, $p75^{NTR\text{-}ExIV}$ knockout mice, in which both the long and the short isoforms are deleted, show an increase in the number and degree of maturation of Dcx^{+} newborn neurons, togheter with a decrease in cell death [265]. These contradictory findings may be explained by the differential levels of expression of the short isoform between the two mouse models. Another study shows that in $p75^{NTR}$ knockout mice ($p75^{NTR\text{-}/\text{-}}$ exon III deletion) there is a reduction in the number of newborn cells (neuronal and non-neuronal) in the DG, and that expression of $p75^{NTR}$ in DG of wild-type mice is restricted to early stages of proliferation [266].

NT-3 is expressed at very high-level in DG (more than in SVZ) and is expressed in neurons [267-269]. NT-3 facilitates learning and memory, possibly by stimulating neuronal differentiation and/or the survival of newly born cells [269]. Conditional NT-3 knockout mice, in which the gene encoding NT-3 is deleted in the brain throughout development, show normal proliferation in the SGZ, a reduction in the number of newly generated $NeuN^{+}$ granule neurons, and an increase in the proportion of cells that do not express differentiation markers., This data suggest a role for NT-3, and perhaps also for its preferred receptor TrkC, in maturation [269]. Beyond the SGZ neurogenesis, a fundamental role of NT-3 for quiescence and long-term maintenance of NSCs has been also identified in the mouse SVZ [270].

Of clearly overall importance in adult hippocampal neurogenesis is the BDNF/TrkB signaling. BDNF is strongly expressed in DG [271]. Both BDNF mRNA and

protein expression are particularly high, with mossy fiber axons of dentate granule neurons displaying strong BDNF immunoreactivity due to anterograde transport [272]. BDNF is also likely expressed in non-neuronal cells [273]. TrkB appears to be broadly expressed: its expression is high in NSCs with radial morphology and low in proliferating progenitors, while young Dcx^+ neurons and more mature granule neurons reacquire high levels of TrkB expression [274]. Thus, there is a dynamic expression of the neurotrophin receptors during the neurogenic path that could indicate a dynamic role of the specific neurotrophins. Neurogenesis is attenuated by BDNF knockdown in the DG using lentiviral-mediated RNA interference [275], but increases in response to exogenous BDNF injection [276]. Nonetheless, there is less consensus regarding the participation of BDNF/TrkB in certain aspects of neurogenesis, such as the proliferation of progenitor cells and the survival of new neurons. TrkB is required for normal proliferation and neurogenesis in the SGZ, although conflicting results have been reported. Conditional deletion of TrkB in hippocampal NSCs reduces SGZ proliferation in postnatal day 15 (P15) and adults animals, but has no effect on overall cell survival [271]. Animals with impaired TrkB activation (TrkB-T1-overexpressing mice) display an increase in proliferation and a reduction in survival [277, 278]. *In vitro*, BDNF promotes the proliferation of hippocampal neural progenitor cultures in a TrkB-dependent manner [271]. Studies using conditional knockout mice, in which mature hippocampal neurons lack the BDNF gene, have also been inconclusive, with some authors describing increased proliferation of SGZ progenitor cells [279] and others reporting no alteration [280]. These conflicting results have not yet been explained, although it is possible that developmental and/or behavioral differences between the strains used in the aforementioned studies may contribute to the divergent findings.

A greater consensus has been reached, however, regarding the role of BDNF/TrkB signaling in dendrite morphogenesis in newborn SGZ neurons [281, 282]. Dendrite and spine growth is markedly altered in adult-born granule neurons of $TrkB^{lox/lox}$ mice, in which TrkB-FL (TrkB floxated) is deleted in progenitors via Cre expres-

sion [281]. Moreover, a portion of the TrkB deficient, newly generated neurons, die during the transition from immature to more mature stages. BDNF has also been shown to regulate late phases of neuronal differentiation, and dendritic development of adult-generated granule neurons is compromised in BDNF conditional mutants [279]. A recent study showed that dendrite growth is decreased in response to BDNF deletion in adult-born hippocampal neurons and increased by BDNF overexpression [282]. This effect appears to be largely autocrine, as BDNF deletion in newborn neurons only gives rise to dendritic abnormalities similar to those observed in conditional knockout mice in which BDNF is deleted throughout the entire forebrain. Thus, in general, the modulation of adult neurogenesis in DG by BDNF concerns essentially the late phase of integration, and involves the mechanism of maturation and neurites outgrowth. So, it is noteworthy that the function of BDNF in adult neurogenesis is comparable, at the cellular level, with its role in dendritic spine growth in relation to the synaptic plasticity of the mature neurons.

A fascinating aspect of the regulation of adult hippocampal neurogenesis by neurotrophins is the connection between BDNF and the modulation of hippocampal neurogenesis by external stimuli, a topic that has been extensively studied in recent years (reviewed in [283, 284]). Adult neurogenesis in DG is enhanced by voluntary exercise, exposure to an enriched environment, and chronic antidepressant administration. Interestingly, many studies shown that physical exercise increases hippocampal expression of BDNF (and NGF, but apparently not NT-3; [283, 285-288]). This increase correlates with the beneficial effect of exercise. For instance, long-term voluntary running increases BDNF levels while improving spatial memory and hippocampal neurogenesis. [289]. Five weeks of treadmill running increases BDNF and TrkB expression, enhances NSC proliferation, and promotes the maturation and survival of immature neurons [290]. TrkB ablation in adult hippocampal NSCs also blocks the effect of voluntary exercise on proliferation and neurogenesis [271]. Some other evidences suggest a role for BDNF also in mediating the increased hippocampal neurogenesis following

environmental enrichment (EE), but the results are not always consistent. A noteworthy fact is that EE increases hippocampal BDNF levels in long-term paradigms [291, 292]. Similarly, there could be role of mediation of BDNF for what concerns the positive effect of antidepressant on hippocampal neurogenesis [271, 278, 293, 294].

Finally, it is noteworthy to underline that adult NSCs have been found to naturally and constitutively secrete significant quantities of several neurotrophic factors, including the NGF and BDNF [295], indicating a possible autocrine regulation of adult NSCs during the neurogenic path.

While many studies have investigated the role of BDNF in adult neurogenesis, for what concern NGF much less is known (see below).

2.2.e. The pro-/mature form equilibrium of neurotrophins

An important feature of the biology of neurotrophins is that they exist in the CNS as equilibrium between the mature and the immature forms. Like many growth factors, neurotrophins are translated as larger precursors, the proneurotrophins (proNGF, proBDNF, proNT-3, proNT-4/5). Before the cleavage, the amino-terminal prodomain promotes protein folding and direct trafficking to secretory vescicles [296, 297]. At the functional level, proneurotrophins are signaling molecules rather than inactive precursors. So, it will be important in the future to fully determine their distribution in the nervous system. In general, the action of proneurotrophin is antagonist to that of the mature neurotrophin (reviewed in [298]).

The proneurotrophins preferentially bind to $p75^{NTR}$ (on the contrary the mature form prefers Trk), but not all $p75^{NTR}$ expressing cells are sensitive to proneurotrophins; expression of the neurotensin receptor Sortilin is apparently needed for proneurotrophins to induce their biological effects [299]. Binding studies using purified proNGF demonstrate that proNGF interacts with a heteromeric complex composed of $p75^{NTR}$ and Sortilin, wherein the prodomain interacts with Sortilin and the mature domain interacts with $p75^{NTR}$ [299, 300] (Fig.10).

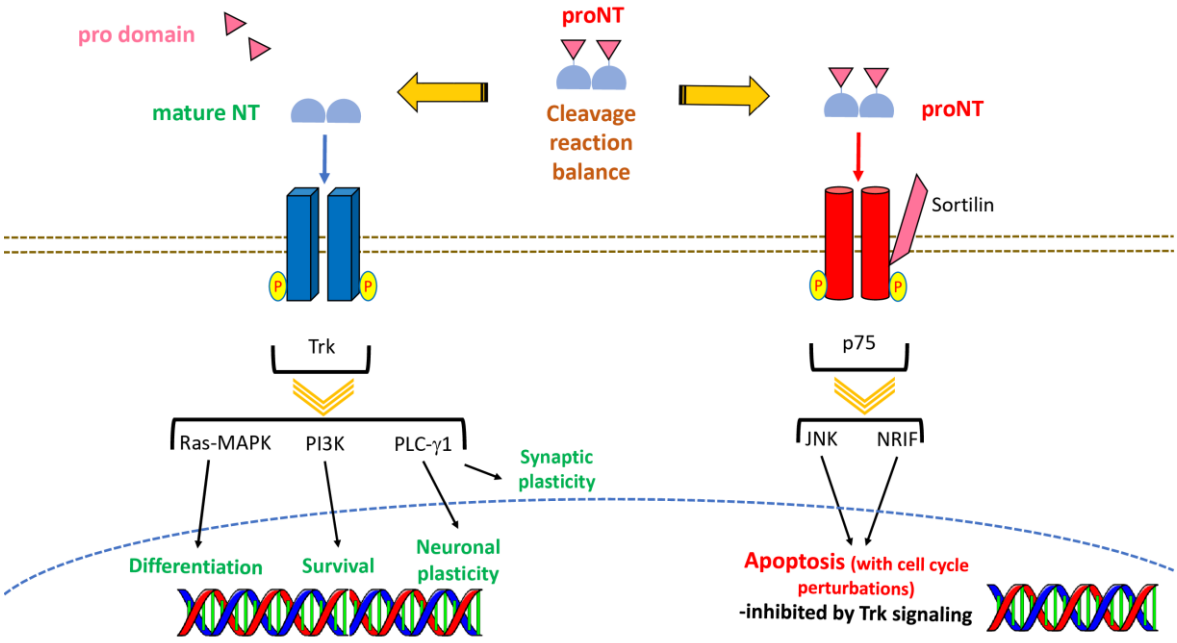


Figure 10. Balance between the opposing effects of the pro- and the mature form of NGF.
The extent of proneurotrophins cleavage determines a balance between the stimulation of the Trk signaling and the stimulation of the p75/sortiling signaling, which, in turn induce opposing response (i.e. survival versus cell death).

Currently available evidences indicate that the antagonist effects of the proneurotrophins have pathophysiological relevance in human disease and injury. For example, proNGF levels increase following axotomy through lesion of the internal capsule (an anatomical structure composed of nerve fibers bundles connecting cortex with diencephalon, brainstem and spinal cord), and this leads to apoptosis of corticospinal neurons through binding to $p75^{NTR}$ [301]. Also proBDNF induces neuronal apoptosis through the $p75^{NTR}$/Sortilin complex [302]. Actions of proneurotrophins are not limited to neurons, indeed they also kills oligodendrocytes [303]. Analysis of rodent and human tissue indicates that proNGF, rather than the mature NGF peptide, is the predominant form in the brain, and proNGF appears to be increased in Alzheimer's disease as well as patient with mild cognitive impairment [304, 305].

The antagonist functions of mature neurotrophins and proneurotrophins extend beyond the regulation of cell death. For example, if BDNF is associated with

LTP (as mentioned above), proBDNF was shown to weaken synaptic strength, resulting in Long-Term Depression (LTD) [306].

Based on the functional features of proneurotrophins, a binary action of neurotrophins has been recently proposed, depending on both the forms of the neurotrophin (pro- versus mature) and the class of receptor that is activated. Accordingly, the proteolytic cleavage of proneurotrophins represents a mechanism that controls the direction of action of neurotrophins, and thus its regulation is of extreme importance. Indeed, there are three ultimate fates for proneurotrophins: intracellular cleavage followed by secretion of the mature forms; secretion followed by extracellular cleavage; or secretion without subsequent cleavage (Fig.11).

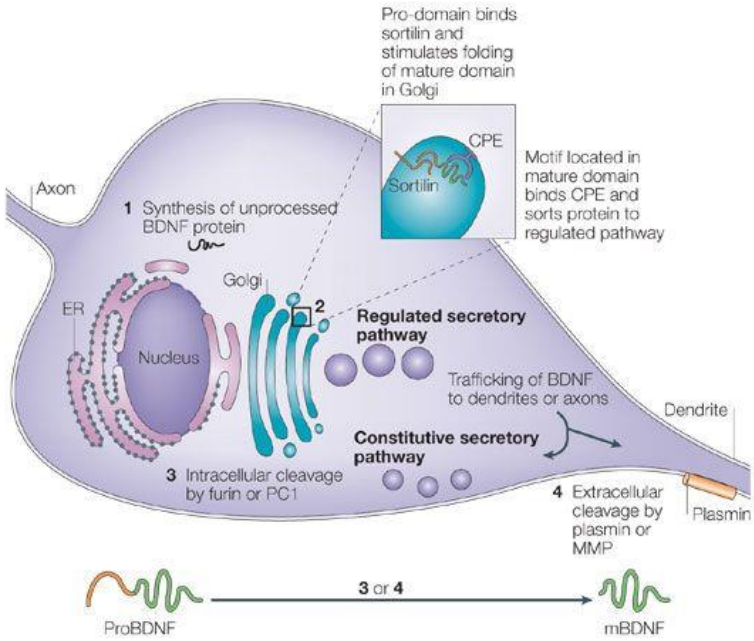


Figure11. The synthesis and sorting of BDNF.
A schematic showing the synthesis and sorting of brain-derived neurotrophic factor (BDNF) in a typical neuron. First synthesized in the endoplasmic reticulum (ER) (1), proBDNF (precursor of BDNF) binds to intracellular sortilin in the Golgi to facilitate proper folding of the mature domain (2). A motif in the mature domain of BDNF binds to carboxypeptidase E (CPE), an interaction that sorts BDNF into large dense core vesicles, which are a component of the regulated secretory pathway. In the absence of this motif, BDNF is sorted into the constitutive pathway. After the binary decision of sorting, BDNF is transported to the appropriate site of release, either in dendrites or in axons. Because, in some cases, the pro-domain is not cleaved intracellularly by furin or protein convertases (such as protein convertase 1, PC1) (3), proBDNF can be released by neurons. Extracellular proteases, such as metalloproteinases and plasmin, can subsequently cleave the pro-region to yield mature BDNF (mBDNF) (4). MMP, matrix metalloproteinase. (Figure from Lu, 2005).

Intracellularly proneurotrophins can be cleaved by the serin protease furin or by the prohormone proconvertases in the trans-Golgi network or in secretory vesicles, respectively [307]. When proneurotrophins are not intracellularly processed, they are secreted in the extracellular space, where several matrix metalloproteinases (MMPs) have been shown to cleave proNGF and proBDNF [300]. So, the extracellular regulation of such MMPs is the mechanism that controls the equilibrium between pro-/mature form of neurotrophins. In cultured cortical neurons, extracellular zinc can activate MMPs and allows the conversion of pro- to mature BDNF [308]. However, the most significant form of control of the pro-/mature form balance is the cleavage by the serin protease plasmin [309]. In the brain, neurons express the inactive form of this enzyme, the plasminogen, and secrete it in the extracellular space, particularly at the synaptic cleft [310]. So, the extracellular space of the nervous tissue has continuously the potential to cleave the proneurotrophins. High neural activity frequency leads to the secretion of the tissue plasminogen activator (tPA) from the axon terminal [311, 312]. So tPA converts plasminogen into plasmin (reviewed in [313]), which in turns activates MMPs. This leads to the extracellular cleavage of proneurotrophins and, at local level, the pro-/mature form equilibrium is pushed to mature form. In this way the neurotrophin signaling is spatially and temporally regulated.

2.2.f. NGF and adult neurogenesis

The role of NGF in adult neurogenesis has not been yet fully clarified. Nevertheless, the involvement of NGF in this process is emerging. A positive proliferative effect of NGF on NSCs exposed to Fibroblast Growth Factor-2 (FGF-2) was reported since 1990, although it concerns neural precursors derived from embryonic brain [314]. Later on, it was demonstrated that a previous exposure to NGF is necessary for TrkA expression [315]. Yet in embryonic brain it has been demonstrated that the promotion of proliferation of NSCs by NGF occurs through phosphorylation of Erk1/2 [316]. NGF, produced by fetal NSCs, has been shown to induce neurite outgrowth on NSCs

themselves [317]. Finally, and yet in embryonic brain, it was also demonstrated that NGF regulates differentiation of NSCs into mature neural phenotypes [315]. Although concerning embryonic NSCs, and performed exclusively *in vitro*, all these studies suggest an important role of NGF in the neurogenic process and in regulating the biology of neural precursors.

A direct *in vivo* analysis of the effect of NGF on hippocampal adult neurogenesis was performed by *Frielingsdorf et al.* [318] By intracerebro-ventricular NGF infusion, NGF was found to increase hippocampal neurogenesis through a positive effect on neural progenitors survival. This effect was found in adult (13-14 week old) but not in aged rats (23 month old). They shows that NGF does not affect proliferation, as the number of new cells in GCL is not affected after 2 hour of NGF infusion, but the positive effect on cell survival increases the final number of newborn Dcx^+ and $NeuN^+$ neurons in the GCL. Likewise, NGF restores hippocampal neurogenesis in a mouse model of Huntington's Disease (in which hippocampal neurogenesis is compromised) [319]. Interestingly, this study shows that, in the same mouse model, NGF increases spatial working memory, one of the functions ascribed to hippocampal neurogenesis.

A role for proNGF in modulating cell cycle of neural/stem cells derived from postnatal hippocampus, through $p75^{NTR}$ and modulation of cyclin E, was recently reported [320]. Inhibition of cell proliferation was demonstrated *in vitro* by analyzing how the global NSCs population is distributed in the different phases of cell cycle. proNGF-treated cells were found blocked in the G0/G1 phase.

Finally, NGF has emerged also a determinant in SVZ adult neurogenesis, were neutralization of this factor led to an impairment of proliferation and differentiation of neural progenitors both *in vivo* and *in vitro* [321].

2.3 The anti-NGF AD11 mice as a model for proNGF/NGF unbalance

The AD11 mice are transgenic for a recombinant version of a neutralizing anti-NGF monoclonal antibody (mAb αD11) [322]. They derived from the crossing between two homozygous lines (VH-αD11 and VK-αD11 mice, respectively), each transgenic for the heavy chain (VH-αD11) and the light chain (VK-αD11) of the mAb αD11. The single transgene in AD11 mice stays in haploid condition (and each one randomly inserted in the genome). This approach allows to limit the developmental consequences deriving from the exposure of fetuses and newborns to anti-NGF antibodies [323, 324].

The anti-NGF monoclonal antibody αD11 neutralizes the biological action of NGF *in vitro* [325] and *in vivo* [326, 327]. The NGF epitope recognized by mAb αD11 includes a loop region (residues 41-49, [328]) which is part of the surface of interaction between NGF and TrkA and distinguishes NGF from the other neurotrophins [167, 329]. Thus, mAb αD11 does not bind to other neurotrophins and does not block their biological activity [328, 330]. Interestingly, mAb αD11 binds mature NGF with a 2000 fold higher affinity than proNGF [331] (see below).

The AD11 mice phenotype displays many features of Alzheimer's disease-like neurodegeneration [332]. In aged AD11 mice, such features are, at anatomical level, ventricle dilatation, cortical and hippocampal atrophy and cholinergic deficit of basal forebrain; at molecular level, amyloid plaques, hyper-phosphorylated tau, dystrophic neurites and neurofibrillary extracellular depositions; and finally, at behavioral level, spatial memory and object recognition impairments. AD11 mice have been also analyzed for what concerns SVZ adult neurogenesis [321]. The results obtained in the group of Prof. Cattaneo demonstrate that AD11 NSCs display a reduced proliferation and are unable to differentiate into βIII-tubulin positive neuron, both *in vitro* and *in vivo.* The AD11 SVZ phenotype was reproduced in vitro by mAb αD11

treatment and rescued in vivo by the intranasal delivery of NGF, demonstrating that NGF neutralization causes an impairment of SVZ neurogenesis [321].

The anti-NGF mAb αD11 binds NGF with an affinity of three orders of magnitude higher than that of proNGF ($K_D = 10^{-12}$ M and 10^{-9} M for NGF and proNGF, respectively). Thus, the preferential binding of mAb αD11 to mature NGF, with respect to proNGF, would determine, under limiting concentrations in the mouse brain, an experimentally-induced, functional imbalance between NGF and proNGF, in whichmature NGF is sequestered, while proNGF is free to act. As proneurotrophins are well established functional signaling molecules that counteract the effect of mature neurotrophins, their concentration in the brain, relatively to the mature form, is of fundamental importance. In this view, AD11 mouse represent a good model for proNGF/NGF unbalance, in favor of proNGF.

3. AIM OF THESIS

The aim of this thesis is to better characterize the role of pro- and mature NGF in regulating adult hippocampal neurogenesis. The possibility to enhance adult neurogenesis, through the modulation of the NGF system, represents a fascinating challenge in the development of new translational approaches for the cure of neurological disorders. In this view, one potential clinical application of neurotrophins could be addressed at the potentiation of the adult neurogenic process to counteract the onset and/or the progression of neurodegeneration in Alzheimer's disease, where alterations of hippocampal neurogenesis have been differently reported [333- 323].

In this study, I analyzed adult hippocampal neurogenesis *in vivo* in the AD11 anti-NGF transgenic mice. This mouse model allowed me to study the role of mature NGF per se in hippocampal neurogenesis and to explore, at the same time, the importance of the proNGF/NGF equilibrium in the same biological context. From this initial analysis I could unravel a new mechanism of regulation of adult hippocampal neurogenesis based on the proNGF/NGF balance. I thus tried to assign a more specific function to the immature and mature form of NGF by several *in vitro* experiments, in which I used an unclevable form of the proNGF, the proNGF-KR, the mAb αD11 and the NGF. Finally, I tried to better characterize the specific function of proNGF in the complexity of the neurogenic path by separating conceptually and experimentally the NSCs population in the different stages: the quiescent stem cells, the early progenitors and the late neural progenitors.

My results shed new light on the overall role of the NGF system in regulating adult hippocampal neurogenesis. In this scenario, proNGF acts as cell-type specific mitogen, while mature NGF acts as "calibrator" of the proNGF effect for a functional neurogenesis in the DG of hippocampus.

4. MATERIALS AND METHODS

4.1 Experimental animals

AD11 mice expressing the functional αD11 anti-NGF antibody were obtained by intercrossing mice homozygous for the αD11 heavy chain transgene (CMV-VH-aD11 mice) and mice homozygous for the αD11 light chain transgene (CMV-VK-aD11 mice), as described [334]. The individual heavy and light chain aD11 transgenes start to be expressed at high levels in the late postnatal period, leading to the formation of functional anti-NGF antibodies at P90 [332].

TgProNGF mice constitutively express the furin-resistant mouse proNGF (proNGF-KR) in a background of normal endogenous proNGF\NGF production [335].

AD11 and ProNGF mice were used at 6 and 3 months of age, respectively. The corresponding wild type littermates were used as control. All experiments with transgenic and control mice were conducted according to national and international laws for laboratory animal welfare and experimentation (EEC council directive 86/609, OJ L 358, 12 December 1987; Dlgs 116/92; authorization n° 1214/2015-PR, 19/11/2015). In detail, mice were grouped in standard cages (hardwoods bedding) in conventional animal facility (12 hour light/dark cycle). Groups included four mice per cage, balanced for genotype and mice were monitored for health and welfare for the whole duration of the experiments. Only mice without stress or discomfort signs (including hair loss, stereotyped behaviors) and weight ranging between 25-30 grams were included in the study.

4.2 In vivo analysis of proliferation and differentiation

4.2.a. Brain dissection and tissue processing

All experiments with transgenic and control mice were conducted according to national and international laws for laboratory animal welfare and experimentation (EEC council directive 86/609, OJ L 358, 12 December 1987). Prior to brain dissection, adult mice were anesthetized with about 1 ml of 2,2,2-tribromoethanol (Sigma-

Aldrich, St. Louis, MO, USA), and intracardially perfused with 4% paraformaldehyde. The whole brain was therefore extracted and the fixation continued in 4% paraformaldehyde overnight at 4°C. After cryoprotection in 30% sucrose, brains were cryosectioned at 40 µm of thickness, and slices encompassing the hippocampus (HP) were analyzed by immunohistochemistry.

4.2.b. In vivo BrdU labeling

BrdU was administered to AD11 and control mice (six-month-old) at 100 mg/Kg by daily intraperitoneal injection for 5 days. Animals were then sacrified either 2 (for proliferation analysis) or 28 (for differentiation analysis) days after the last injection and brains were collected and processed as described before.

4.2.c. Quantification of cell number

Stereological analysis of the number of cells was performed on series of 40-µm free floating coronal sections of the entire dentate gyrus (DG) of the HP, which were analyzed by confocal microscopy to count cells expressing BrdU throughout the rostro-caudal extent of the granule cell layer. To obtain the average number of DG cells per section, the number of positive cells for each DG section was divided by the total number of sections counted. Three animals per group (n=3) were analyzed. Statistical analysis was performed by unpaired Student's *t* test, and all experiments were expressed as mean ± SEM.

4.3 Cell culture

4.3.a. Hippocampal adult neural stem cells

Neural stem cell cultures from single hippocampus were performed as described [321]. Six-month-old mice (wild-type or AD11) were anesthetized as described before and killed by decapitation. Brains were extracted out of the skull and separate in two

hemispheres, then one half of the hippocampal formation were extracted from every hemisphere and break into 5-6 pieces. Hippocampus from each animal were processed separately to obtain cultures from single sample. Cells were isolated by enzymatic digestion (1.33 mg/ml trypsin, 0.7 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid) (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C and mechanical dissociation with small-bore Paster pipette. Cell were plated at 5 X 10^3 cells/cm^2 cells density and cultured in NS-A medium [321] supplemented with B27 (Invitrogen, San Diego, CA, USA), EGF and bFGF (20 and 10 ng/ml, respectively; Peprotech) (NSAC growing medium) in a humidified incubator at 37°C in 5% CO_2 for 3 weeks. Growth factors were replenished weekly. By the end of the 3 weeks, primary neurospheres (≥1 mm in diameter) were subcultured by mechanical dissociation into single cells every 4th day.

4.3.b. Induced Neural Stem Cells

Mouse induced neural stem cells (iNSCs) were derived by reprogramming wild-type mouse embryonic fibroblasts (MEFs) with a SOX2-retroviral vector, as described [336]. Reprogramming of MEFs were performed in 24 mw plate on a feeder of mitomycin-treated STO cells growth on 0,1% gelatin-coated glass. MEFs were plated at 1.25 X 10^5 cells/cm^2 cells density. We used passage 2 STO-MEFs for feeder cells and passage 1 MEFs for reprogramming. After infection, viral medium was replaced with NS-A medium [321] supplemented with B27 (Invitrogen, San Diego, CA, USA), bFGF (20 ng/ml) and 20 ng/ml EGF (Peprotech). Growing medium was completely refreshed every day until obtaining iNSCs colonies, which were subsequently sub-cultured as neurospheres like aNSCs.

4.3.c. Cell proliferation and differentiation

To quantify cell proliferation (Fold Increase analysis) 2X10^3 cells were plated at 1X10^3 cells/cm^2 cell density in growing medium. After 7 days *in vitro* (DIV 7) the total

number of viable cells was counted by Trypan blue exclusion, and again $2X10^3$ viable cells were re-plated under the same conditions. Cell proliferation was expressed as Fold Increase in (F.I.), calculated by dividing the number of cells at DIV 7 by the initial number of seeded cells. For average Fold Increase analysis of DG-aNSCs we grew cells at consecutive passages from p7 to p21, by dissociating and re-plating $2X10^3$ viable cells every 7 days. For Fold Increase analysis in the treatment experiments (purified anti-NGF mAb αD11 [325], proNGF and NGF proteins [331]) $2X10^3$ cells at passage 30 were seeded at $1X10^3$ cells/cm^2 and cultured for 2 weeks. For proliferation curves, we plated $2X10^3$ cells for DG-aNSCs and $1X10^{?}$ cells for iNSCs, at $1X10^3$ cells/cm^2 or at $5X10^3$ cells/cm^2 cell density, respectively. All proliferation curves were repeated three times in independent experiments, using at least three technical replicates. To assess for differentiation, neurospheres were dissociated into single cells and $1X10^5$ cells were transferred onto matrigel-coated glass coverslips (12 mm diameter) in differentiating medium (growth medium without EGF and FGF). Five days after plating, cultures were fixed in 4% paraformaldehyde and processed for immunocytochemistry.

4.3.d. Monolayer cultures for single cell immunofluorescence quantification

In order to analyze the composition of the cell populations by quantifying the immunofluorescence signals at single cell level, cultures of DG-aNSCs and iNSCs were grown as monolayers by dissociating neurospheres into single cells and transferring them onto poly-ornithine/laminin coated glass coverslips (12 mm diameter) at $1X10^3/cm^2$ cells density. Cultures were left to grow until confluence. Coated glasses were prepared as described below. Glass coverslips were coated with 20 μg/ml of poly-ornithine (Sigma-Aldrich, St. Louis, MO, USA) solution in H_2O_{dd} and incubated at 37°C. After 24h the multiwell plate with coverslips was left to reach room temperature out of the incubator, then poly-ornithin was removed and coverslips were

coated with 2,5 µg/ml of laminin (Invitrogen, San Diego, CA, USA) solution in PBS and incubated at 37°C. After 24h multiwell plate was left to reach room temperature and cells were plated. For analyzing population composition and receptors in the different cell types, cultures were fixed with 4% paraformaldehyde when confluence was reached. In proNGF-KR acute treatment experiments 50 ng/ml of proNGF-KR was added to WT cells and cultures were fixed 48h later. Intensity fluorescence quantification was performed using Image-J software. Purified recombinant proNGF-KR was prepared as described [331] and the stock solution (1.7 mg/ml in 50mM Na_3PO_4) was diluted at the experimental concentration in cell culture medium.

4.3.e. Stem cell enrichment and LIF selection method

For putative stem cells selection (RGL/early stage), neurospheres were dissociated and plated at $4X10^4/cm^2$ cell density in NS-A medium supplemented with B27 (Invitrogen, San Diego, CA, USA) and 10 ng/ml of LIF (Human, PHC9484, Thermo Fisher Scientific, Whaltam, MA, USA) (NSAL medium). After 9 days of cultures, selected putative stem cells cells (about 1% of the initial number) were seeded on poly-ornithine/laminin coated glass and after 2 days were fixed in 4% paraformaldehyde to perform cell morphology analysis and marker characterization. Clonogenic assay was performed at DIV 9 by plating cells at $1X10^3/cm^2$ in the following experimental conditions: NSAL and NSAC growing medium (see *Cell Culture* section above) with or without proNGF-KR at the indicated concentration. The total number of neurospheres forming units was counted after 5 days of culture. A minimum of 4 technical replicates was performed for every condition, in order to provide the statistical mean of the number of neurospheres forming units. To expand the self-renewal capacity of DG-aNSCs, cells were grown in NSAL medium for 1 week and then replated in NSAC medium, using LIF to stimulate neural stem cell renewal [337]. In this way we could isolate few clones of transient amplifying progenitors with high self-

renew capacity. This protocol was repeated by culturing LIF-selected cells in NSAL for 3 days every time we observed a slowdown of cell growth, in order to allow a continue expansion of the transient amplifying progenitors.

4.4 Immunocytochemistry on brain section and cultures

Immunohistochemistry of HP was performed on 40 µm serial free-floating sections. Prior to BrdU antibody staining, sections were exposed to 2N HCl for 45 min at 37°C and then washed with 0.1 M sodium borate buffer pH 8.5 for 10 min. Immunostaining on cell cultures was performed after fixation in 4% PFA for 10 min at room temperature. Cells fixed on coverslips were permeabilized in 0.1% Triton X-100 in PBS and then incubated with the antibody of interest, as described in Supplementary Materials. The total number of cells in each field was determined by counterstaining cell nuclei with 4,6-diamine-2-phenylindole dihydrocloride (DAPI; Sigma-Aldrich, St. Louis, MO, USA; 50 mg/ml in PBS for 15 min at RT). Immunostained sections and cells were mounted in Acqua-Poly/Mount (Polysciences, Inc., PA, USA) and analyzed at fluorescent or confocal microscopy, using a TCS SP5 microscope (Leica Microsystem).

4.5 Antibodies used in immunocytochemistry and immunohistochemistry

The following primary antibodies were used: rat monoclonal anti-BrdU (AbD Serotech, Raleigh, NC, USA; MCA2060, 1:400), mouse monoclonal anti-NeuN (Merck Millipore, Germany; MAB377, 1:500), mouse monoclonal anti-Neuronal class III β-Tubulin (Covance, Princeton, NJ, USA; MMS-435P, 1:250), goat polyclonal anti-human IgG (γ-chain specific)-Biotinylated (Sigma–Aldrich, St. Louis, MO, USA; B1140, 1:100), goat polyclonal anti-human kappa light chain-FITC (Sigma–Aldrich, St. Louis, MO, USA; F3761, 1:40), rabbit polyclonal anti-Nerve Growth Factor (Santa Cruz Bio-

technology, Texas, USA; sc-548, 1:50), goat polyclonal anti-GFAP (Santa Cruz Biotechnology, Texas, USA; sc-6170, 1:300), rabbit polyclonal anti-GFAP (DakoCytomation, Denmark; Z0334, 1:250), mouse monoclonal anti-Nestin (Merck Millipore, Germany; MAB353, 1:50), rat monoclonal anti-Nestin (Abcam, UK; ab81462, 1:250), goat polyclonal anti-Sox2 (Santa Cruz Biotechnology,Texas; Sc-17320, 1:400), goat polyclonal anti-Dcx (Santa Cruz Biotechnology Texas; sc-8066, 1:300), mouse monoclonal anti-p75 (Abcam, UK; ab8877, 1:500), rabbit polyclonal anti-p75 (Promega, Wisconsin, USA; G323A, 1:100), rabbit polyclonal anti-TrkA (Abcam, UK; ab8871, 1:500), rabbit monoclonal anti-Cyclin D1 (AbCam, UK; ab134175, 1:50), mouse anti-Musashi1 (Msi1) (LsBio, Seattle, WA, USA; LS-C172587, 1:100), goat polyclonal anti-Ascl1/Mash1 (Novus biological, CO, USA; AF2567, 1:40 of 0.2 mg/ml reconstitution). The following secondary antibody were used: (Thermo Fisher Scientific, Whaltam, MA, USA): goat anti-rat conjugated to Alexa 594, 1:500 (BrdU); donkey anti-rat conjugated to Alexa 488, 1:500 (Nestin); donkey anti-mouse conjugated to Alexa 555, 1:500 (p75) or to Alexa 647, 1:500 (p75, Cyc-D1, Msi1); goat anti-mouse conjugated to Alexa 594, 1:500 (βIII-tubulin) or to Alexa 488, 1:500 (NeuN), donkey anti-rabbit conjugated to Alexa 555, 1:500 (GFAP) or to Alexa 647, 1:500 (p75), goat anti-rabbit conjugated to Alexa 488, 1:500 (GFAP) or to Alexa 594, 1:500 (TrkA), donkey anti-goat conjugated to Alexa 488, 1:500 (Dcx) or 555, 1:500 (Mash1, Dcx). (Sigma-Aldrich, St. Louis, MO, USA): Extravidin conjugated to Alexa-Flour 594 (AD11 γ-chain), 1:500.

4.6 Recombinant proteins

Purified recombinant NGF, proNGF-WT and proNGF-KR proteins were prepared as described [331]. Stock solutions (1-2 mg/ml in 50mM Na_3PO_4) were diluted in cell culture medium at the proper concentration indicated in each experimental condition. Purified recombinant αD11 anti-NGF antibody was prepared as described [331] and the

stock solution (1 mg/ml in PBS) was diluted at 20ng/ml, 100 ng/ml or 1mg/ml in cell culture medium.

4.7 RNA isolation and reverse transcription-PCR

RNA was isolated from 3 different AD11 and 3 different WT neurosphere cultures. Briefly, neurospheres were lysed with Trizol (Invitrogen, San Diego, CA, USA) and DNAse treated by Qiagen columns. RNA quantity was determined on a NanoDrop UV-VIS. Only samples with an absorbance ratio of 1.8,OD260/OD280, 2.0 were processed further. Each sample was then quality checked for integrity using the Agilent BioAnalyzer 2100 (Agilent G2938C, RNA 6000 nano kit): samples with a RNA Integrity Number (RIN) index lower than 8.0 were discarded. The purified RNA was used for qRTPCR. The first strand cDNA template was synthesized from 500 ng of total RNA using random primers and Superscript III reverse transcriptase (Invitrogen, San Diego, CA, USA). All reactions were performed with SYBR Green PCR Master Mix (BioRad) and carried out in the iCycler (BioRad). Primers for Quantitative PCR (QTR-PCR) analysis were designed with the assistance of Universal Probe Library Software (Roche Applied Science). All samples were analysed in triplicates. Relative change of mRNA amount was calculated based DCt method, as described [338].

4.8 Statistical analysis

4.8.a. Animal studies

Power analysis has been conducted to estimate the appropriate sample size by setting the probability of a Type I error (α) at 0.05, power at 0.95, effect size at 0.4. To minimize the effects of subjective bias we used randomization procedures for allocating animals to experimental groups and treatments and blind analysis of results.

4.8.b. In vitro studies

The statistical analyses were conducted by using Mann-Whitney (n≥5) or by unpaired Student's t test (n<5) for repeated measures. Error bars on graphs are SEM. Significance markers on figures are from post hoc analysis (ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001).

4.9 Western Blotting

Protein extraction from neurospheres (about 25 ml of growing medium from a 75 cm2 flask) was performed as described [25] For phosphorylated AKT and phosphorylated c-Jun detection, cells were starved overnight in DMEM/F12 and then challenged with 50 ng/ml of purified recombinant mouse proNGF-KR for 48 hours at 37°C, prior to protein extraction. 50–100 mg of proteins was loaded on SDSPAGE 10% and Western blotting was performed as described [321]. The following primary antibodies were used: rabbit polyclonal anti-phosporylated-AKT (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com, 9271, 1:1,000); rabbit polyclonal anti-phosporylated-c-Jun (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com, 9261, 1:1000); rabbit polyclonal anti-activated Caspase-3 (cleaved caspase-3, Cell Signalling Technology, 9664, 1:1,000); anti-Cyclin D1 (AbCam, UK; ab134175, 1:50); mouse anti-b-actin (Sigma-Aldrich, A5316, 1:10,000). Secondary horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies and ECL reagent (GE Healthcare, Fairfield, CT, https://www.ge.com/) were used. Chemiluminescence's signal was analyzed by Kodak Image Station 2000R and quantified with the Kodak Molecular Imaging Software, as a measure of the protein expression level.

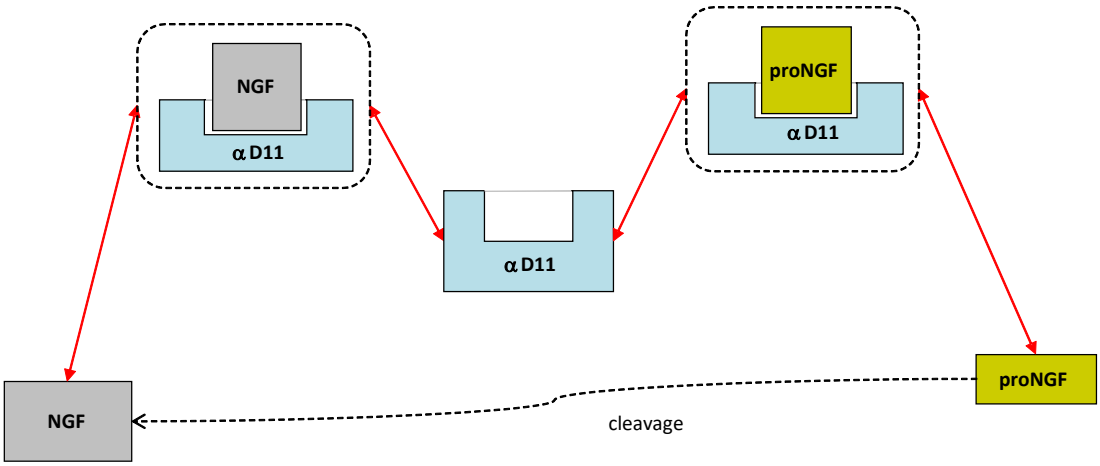
4.10 proNGF processing

The processing of proNGF-KR and proNGF-WT was evaluated by Western blotting a spiking experiment. An amount of the recombinant proteins, sufficiently high to be detected in a direct Western Blot analysis, was spiked into a medium conditioned by hippocampal colinergic neuron, after 12 days in Neuron Chow (Neuron Chow (Neurobasal Medium (Gibco), 2% B27 Supplement (Gibco), 500 μM glutamine (Gibco) and 12.5 μM glutamate). In details, 1 μg of recombinant proNGF-WT or proNGF-KR was spiked into 50 μl of conditioned or fresh medium, and incubated for 72h at 37°C. Also the conditioned medium without any spiking was incubated in the same conditions. All the conditions were repeated twice in independent experiments. 20 μl/sample were run on SDS-PAGE for Western blotting analysis with an anti-NGF antibody (anti NGF M-20 Santa Cruz, 16 hours at 4°C, secondary antibody: Goat Anti-Rabbit, HRP conjugated (Jackson Lab), 1:7000, 1 hour at room temperature). The Image was acquired by using a Kodak Digital Imager, after incubation with ECL advance (GE-Healthcare).

4.11 Modeling of proNGF and NGF molecular interaction with anti-NGF antibody

We studied the kinetics of the NGF-proNGF-anti-NGF antibody interaction using the following protein-protein interaction network model: αD11 anti-NGF antibody and neurotrophins with proNGF to NGF cleavage. Molecular interactions were modeled using mass-action reactions, using experimentally measured kinetic constants. Kinetic parameters were obtained from previous Surface Plasmon Resonance Biacore measurements performed in the lab (Table II in [331]).

Network model: interaction of NGF and proNGF with the anti-NGF mAb αD11.



Mass action reactions:

$\mathrm{proNGF} \rightarrow \mathrm{NGF}$
$\mathrm{\alpha D11} + \mathrm{NGF} \leftrightharpoons \mathrm{C_{\alpha D11,NGF}}$
$\mathrm{\alpha D11} + \mathrm{proNGF} \leftrightharpoons \mathrm{C_{\alpha D11,proNGF}}$

Differential equations:

$$\frac{d[\mathrm{NGF}]}{dt} = k_1\left[\mathrm{C_{\alpha D11,NGF}}\right] - k_2[\mathrm{NGF}][\alpha\mathrm{D11}] + k_5[\mathrm{proNGF}]$$

$$\frac{d[\mathrm{proNGF}]}{dt} = k_3\left[\mathrm{C_{\alpha D11,proNGF}}\right] - k_4[\mathrm{proNGF}][\alpha\mathrm{D11}] - k_5[\mathrm{proNGF}]$$

$$\frac{d[\alpha\mathrm{D11}]}{dt} = k_1\left[\mathrm{C_{\alpha D11,NGF}}\right] + k_2\left[\mathrm{C_{\alpha D11,proNGF}}\right] - k_2[\mathrm{NGF}][\alpha\mathrm{D11}] - k_4[\mathrm{proNGF}][\alpha\mathrm{D11}]$$

$$\frac{d\left[\mathrm{C_{\alpha D11,NGF}}\right]}{dt} = -k_1\left[\mathrm{C_{\alpha D11,NGF}}\right] + k_2[\mathrm{NGF}][\alpha\mathrm{D11}]$$

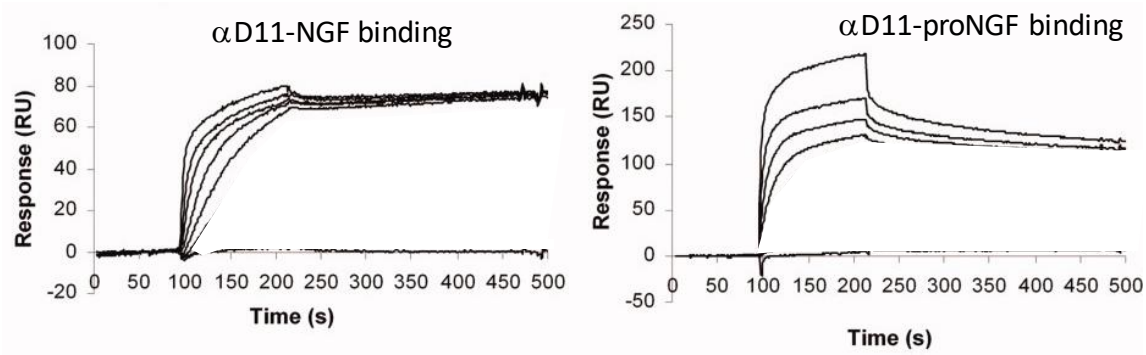
$$\frac{d\left[\mathrm{C_{\alpha D11,proNGF}}\right]}{dt} = -k_3\left[\mathrm{C_{\alpha D11,proNGF}}\right] + k_4[\mathrm{proNGF}][\alpha\mathrm{D11}]$$

Initial conditions (t=0):
$[\mathrm{NGF}]_0$ = 2.22 pM
$[\mathrm{proNGF}]_0$ = 22.2 pM

$[\alpha D11]_0$ = 0.066 … 13.33 nM
$[Complexes]_0$ =0

Parameters:

$k_1=5*10^{-7}\ s^{-1}$
$k_2=1.2*10^{6}\ M^{-1}s^{-1}$
$k_3=6.9*10^{-4}\ s^{-1}$
$k_4=5.9*10^{5}\ M^{-1}s^{-1}$
$k_5=2.3*10^{-7}\ s^{-1}$



For the kinetics proNGF to NGF cleavage, we assumed an irreversible mass-action reaction: proNGF→NGF. This implies an exponential decay of proNGF concentration. The two time-dependent equations for proNGF and proNGF are thus:

$[NGF]= [proNGF]_{t=0} * (1 - e^{-k*t}) + [NGF]_{t=0}$

$[proNGF]= [proNGF]_{t=0} * e^{-k*t}$

If we define [proNGF](t)= C(t), this leads to $C(t) = C_0e^{-k*t} \rightarrow C(t) / C_0 = e^{-k*t} \rightarrow k=-\log_e(C(t) / C_0)/ t$.

At time t=72 h=259200 sec, $C(t) / C_0$ was experimentally estimated in our lab to be about 94.2% in primary hippocampal neurons [unpublished data] leading to k ~ $2.3*10^{-7}\ sec^{-1}$.

This implies that half-life time of proNGF-KR is $t_{1/2} = 1/k*\log_e(2) = 3{,}013{,}683$ sec ~ 34.9 days.

For molecular complexes the initial conditions were set to zero. NGF and proNGF initial concentrations were reasonably estimated from experimental conditions. In models without exogenous neurotrophins treatment, endogenous secreted (free) NGF concentration was set to 60 pg/ml = 2.22 pM based on alphaLisa measurement from neural stem cells medium, while proNGF was set = 22 pM, 10 times larger than mature NGF, based on [304]. In simulations with exogenous proNGF treatment, endogenous NGF and proNGF initial concentrations were set to zero.

The time dynamics was simulated by ODEs using the COPASI software [339].

4.12 Mouse genotyping

Analysis of transgenic mice was performed by PCR on genomic DNA from tail biopsies using the RED Extract-N-Amp-Tissue PCR Kit (Sigma), following the manufacturer's instructions. Genotyping of the VH and VK alleles (for AD11 mice) or the EGFP allele (for proNGF-KR mice) was performed with the following primers:

VH forward: 5'-TGAGGAGACGGTGACCGAAGTTCCTTGACC-3';
VH reverse: 5'-CAGGTGCAGCTGCAGGAATCAGGACCT-3';
VK forward: 5'-CGTCCGAGGATAATGGAAATAGTGCTG-3';
VK reverse: 5'-GACATTCAGCTGACCCAGTCTCCA-3';
EGFP forward: 5'-CTGCTGCCCGACAACCA-3';:
EGFP reverse: 5'-TGTGATCGCGCTTCTCGTT-3'.

PCR conditions for the VH and VK alleles were 30 cycles of 94°C/1 min; 65°C/1 min; 72°C/1 min; for EGFP allele were 30 cycles of 94°C/1 min; 60°C/1 min; 72°C/1 min.

4.13 AlphaLISA measurement of NGF

NGF was measured in the supernatant of WT neurospheres using the AlphaLISA bead-based technology (Perkin Elmer), as described [340]. Briefly, $5X10^6$ WT neurospheres were cultured in growing medium for 72 hours. Conditioned medium (CM) was collected upon cell centrifugation and dialyzed against PBS in order to remove cell medium components of low molecular weight. Dialyzed CM was analyzed neat and 1:2 diluted, following the alphaLISA protocol described in [340]. The plate was read by instrument 'Enspire alpha' (Perkin Elmer).

5. RESULTS

5.1 The anti-NGF AD11 mouse model.

In the adult brain the pro- and mature forms of NGF exist in a well-defined homeostatic equilibrium [304, 341], dependent on synthesis of proNGF precursor, on its cleavage by intracellular and extracellular proteases and on the degradation of mature NGF [300, 308, 309, 342, 343]. In order to study the role of NGF in adult hippocampal neurogenesis I exploited the AD11 anti-NGF transgenic mice, in which the expression of the recombinant anti-NGF antibody mAb αD11 results in a chronic post-natal interference with the activity of endogenous NGF in the brain [334]. Since the anti-NGF antibody binds mature NGF with an affinity three orders of magnitude higher than that for proNGF [331], the AD11 mice, unlike NGF KO mice, is a model for proNGF/NGF unbalance [344] (Fig. 12)

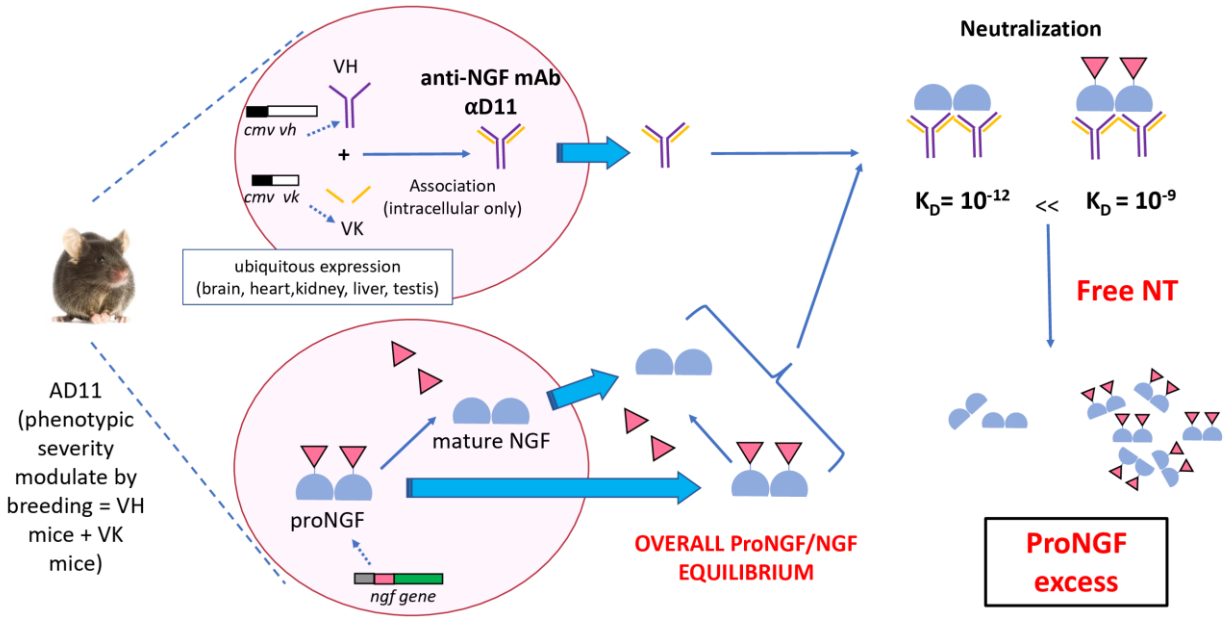


Figure 12. proNGF/NGF unbalance in AD11 mice.
In the AD11 mice, anti-NGF antibody (αD11) is assembled, starting from the expression of the two genes for heavy (VH) and light (VK) chains. Meanwhile, an overall proNGF/NGF equilibrium derives from NGF gene expression, synthesis and cleavage of the protein. The different binding affinity of αD11 for NGF and proNGF determines a strong neutralization of mature NGF and thus an unbalance in favor of proNGF.

AD11 anti-NGF mice represent a comprehensive murine model for AD-like neuro-degeneration, as they progressively develop, from 1.5-2 months onwards, functional and behavioral impairments that encompass several features of human AD (Fig. 13).

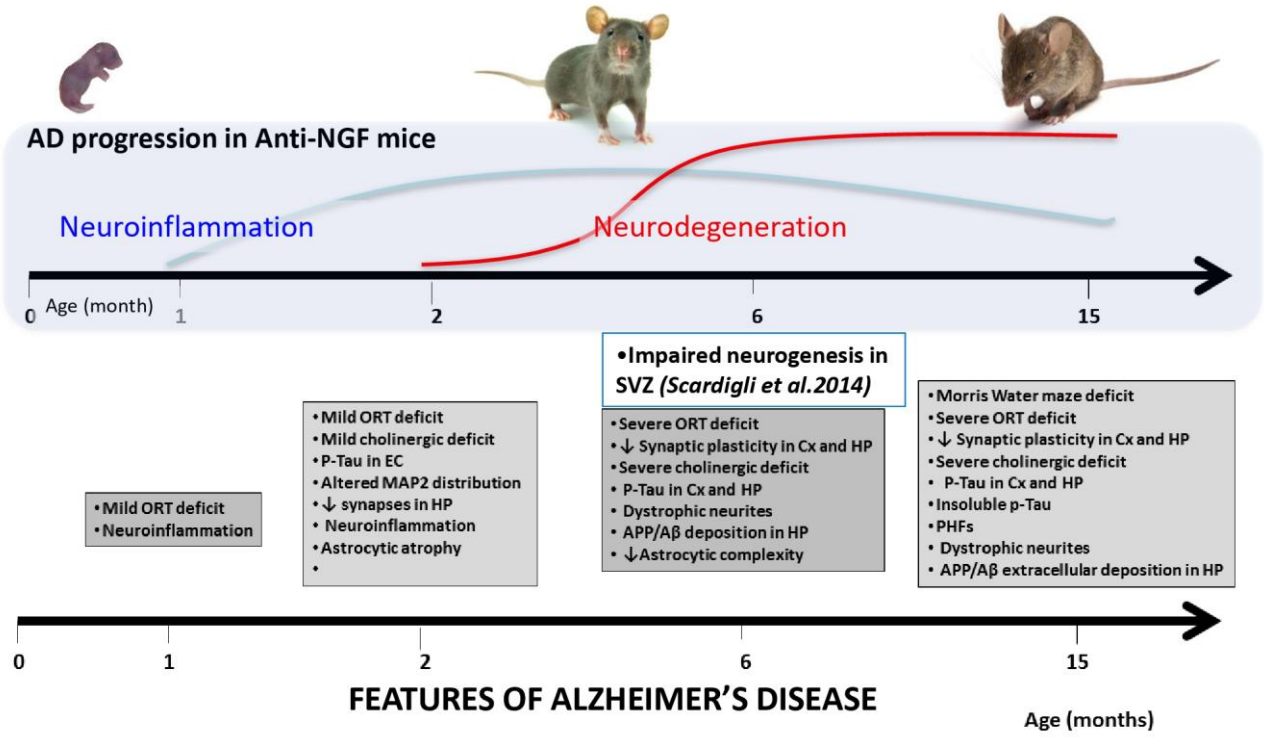


Figura 13. Progression of Alzheimer's Disease-like phenotype in AD11 mice.

5.2 Adult hippocampal neurogenesis is decreased in anti-NGF AD11 mice

An important role of NGF in regulating adult mouse SVZ neurogenesis has been previously demonstrated by our group in AD11 model [321]. In AD11 mice of 6 months of age NGF neutralization led to an impairment of adult subventricular zone (SVZ) neurogenesis, in terms of reduced proliferation of neural progenitors and re-

duced ability to differentiate into βIII-tubulin positive neurons. In order to extend this observation to the hippocampal dentate gyrus (HP-DG), I analyzed the proliferative and differentiative potential of this neurogenic niche in the same animal model. I first measured the proliferative rate of HP-DG adult neural stem cells (aNSCs) by *in vivo* BrdU labeling in 6 months old AD11 and WT mice. BrdU was administered to AD11 and control mice (six-month-old) at 100 mg/Kg by daily intraperitoneal injection for 5 days. Animals were then sacrified either 2 (for proliferation analysis) or 28 (for differentiation analysis) days after the last injection and brains were collected and processed as described before.

Anti-BrdU staining, performed on brain sections encompassing the entire hippocampus, showed that AD11 HP-DG contains 2 folds more BrdU positive cells, compared to control mice (Fig. 14A, left panels), as quantified in Fig. 14B (n° of positive cells/mm^2 DG: AD11 36.27±4.31; WT 18.13±3.7, p=0.005).

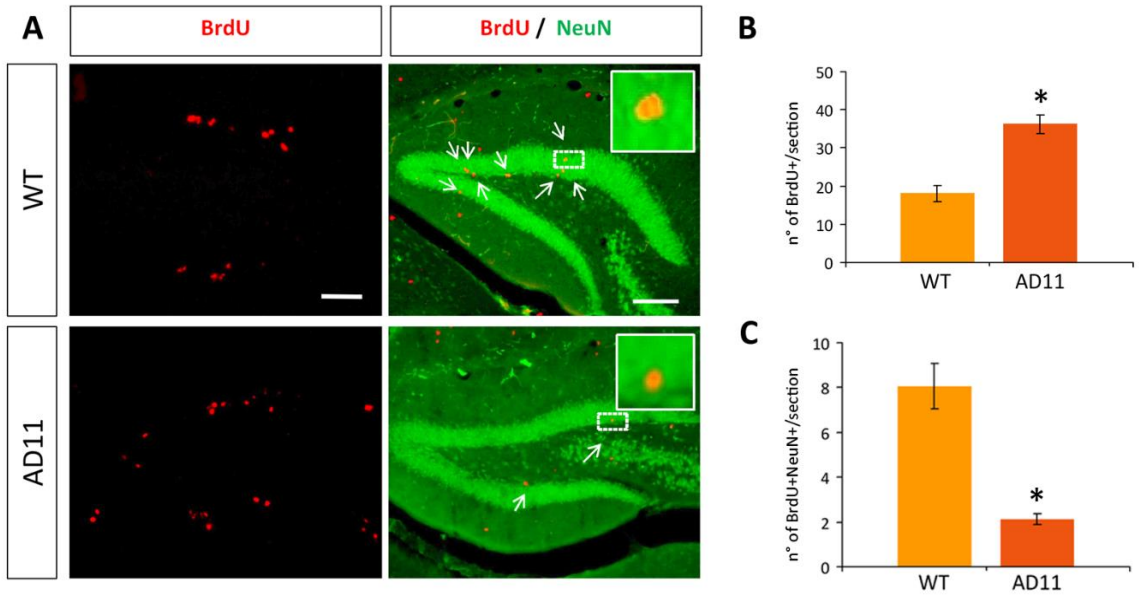


Figure 14. Increased progenitor proliferation but reduced neurogenesis in AD11 DG-hippocampus.
*A) Immunofluorescence staining for BrdU and for BrdU/NeuN (red and green, respectively) in adult DG of AD11 and WT mice. In the hippocampus of AD11 animals there is a higher number of BrdU+ cells (arrows in left panels) but a significant reduction of newborn neurons (arrows in right panels) compared to WT. White-squared boxes represent a 10X magnification of the corresponding cells in the dot-lines insets. Scale bar 100µm, 20X magnification. B-C) Quantification of BrdU (B) or double positive BrdU/NeuN cells (C) in AD11 (red) and control (orange) DG. Data are means ± SEM of three individual animals (n=5) for each experimental group. * p <0.05, significantly different from WT, Student's t-test.*

Despite this increase in proliferation, hippocampal neurogenesis was greatly affected in AD11 mice, with a significant decrease in the number of newborn neurons, as shown by double immunostaining for BrdU and NeuN in Fig. 1A (right panels) and quantified in Fig. 14C (n° of BrdU+/NeuN+ positive cells/mm^2 DG: AD11 2.13±0.42; WT 8.07±1.79, p=0.005).

5.3 Neurogenic defects of AD11 hippocampal progenitors are maintained also in vitro.

I analyzed in detail the biological properties of hippocampal neural stem/progenitor cells of AD11 and WT mice by establishing *in vitro* cultures of aNSCs isolated from 6 months old animals. Differently from other aNSCs culture protocols, based on growing cells at high density, which promotes the rapid expansion of late progenitors with limited proliferative potential, I chose to establish neurosphere cultures from individual mice, plated at low density (5 X 10^3 cells/cm^2), in order to promote the propagation of putative stem cells. With this method, I obtained three long-term (> 70 passages) stable aNSCs samples: two WT (WT1 and WT2) and two AD11 (AD3 and AD4). The average number of primary AD11 neurospheres was about two-fold higher than WT (Fig. 15A, p-value = 0.049) and, AD11 neurospheres formed in half the time of that required for WT neurospheres (Fig. 15B). This result reproduced *in vitro* the major proliferative rate of AD11 versus WT aNSCs observed *in vivo* in the DG niche.

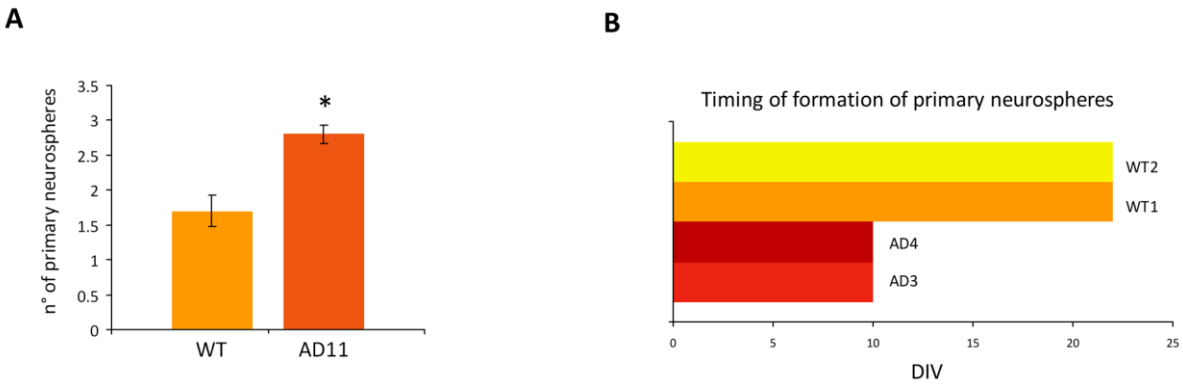


Figure 15. AD11 hippocampal neurogenic niche shows high activation by in vitro neurospheres forming ability
*A) More primary neurspheres are obtained from AD11 DG, and in two folds less time compared to WT (B). Data is expressed as mean ± SEM of five individual animals (n=5, A) or two (n=2, B) for each experimental group. * p <0.05, significantly different from WT, Student's t-test. Data are mean ± SEM of 3 independent experiments.*

To further confirm this observation, I performed growth curves of these cultures at different passages during their expansion *in vitro* (see Materials and Methods for details). As shown in Fig. 16A, AD11 neurospheres cultures proliferated significantly more than the control WT cultures.

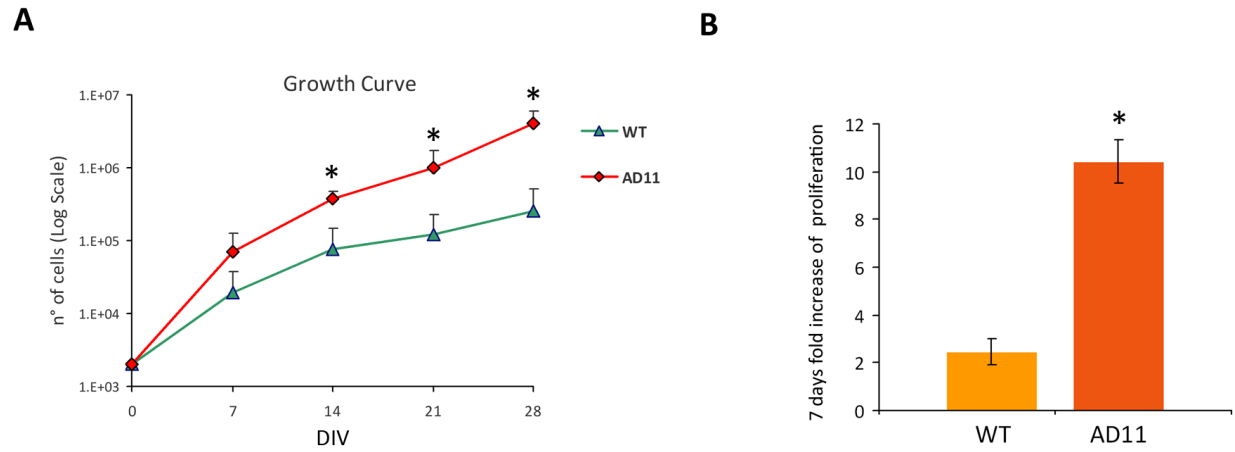


Figure 16. Increased proliferation of AD11 hippocampal progenitors is maintained also in vitro.
*A) Proliferation curve of AD11 and WT neurospheres. AD11 cells (red line) proliferated significantly more than control (green line), as also quantified as average fold increase (B), calculated between p7 and p21. Data are mean ± SEM of 3 independent experiments. * p <0.05 significantly different from WT, Student's t-test.*

This difference in proliferation rate was also quantified as average fold increase of cell proliferation (F.I.), calculated between p7 and p21 (Fig.16B), as described in Materials and Methods.

To assess if this effect on proliferation was due to the persistency *in vitro* of the NGF neutralization occurring *in vivo*, by transgenic anti-NGF antibodies still expressed in the cultures, I verified the expression of the anti-NGF antibody αD11 and of NGF in the expanded aNSCs. Double immunostaining for the human heavy and light chains of the transgenic antibody was performed on AD11 and WT bulk neurospheres, as described [321]. Hu-mAb αD11 expression was confirmed by the concomitant immunoreactivity for the two antibody chains in AD11 and not in WT neurospheres (Fig. 17A).

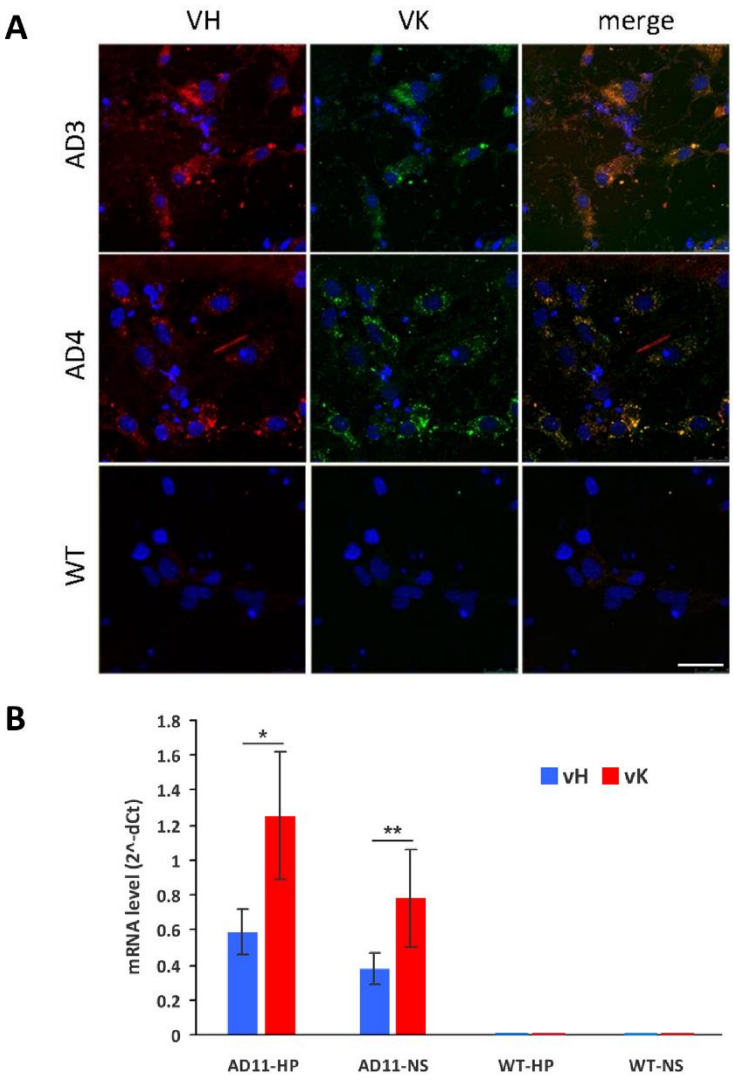


Figure 17. Anti-NGF antibody expression in AD11 aNSC.

Expression of recombinant anti-NGF antibody αD11 in vitro. A) Immunofluorescence staining for the heavy (VH, green) and light (VK, red) chains of mAb anti-NGF (αD11), showing that antibody is expressed in vitro in adult hippocampal neural progenitor cells isolated from AD11 mice (upper and middle panel), but not in those from WT animals (lower panel). DAPI staining on nuclei in blue. Scale bar 10 µm, 40X magnification. B) Real-time polymerase chain reaction for messenger RNA of heavy chain (VH) and light chain (VK) confirms the expression of αD11 antibody in AD11 hippocampus (AD11-HP) and neurospheres (AD11-NS). Data are mean ± SEM of 3 independent experiments.

In addition, αD11 expression was further confirmed by real-time PCR for the heavy and light chains mRNA in both AD3 and AD4 cultures (Fig. 17B). As already reported [321], the expression of the recombinant mAb αD11 results in an overall neutralization of NGF activity, without affecting NGF expression. Immunofluorescence for NGF performed on neurospheres or freshly isolated DG-cells showed that i) endogenous NGF was expressed in all cells of both genotypes at similar levels (Fig. 18A), and ii) NGF was expressed both in Type I progenitors ($GFAP^+/nestin^+$) and neuroblasts ($Dcx^+/nestin^+$) (Fig. 18B).

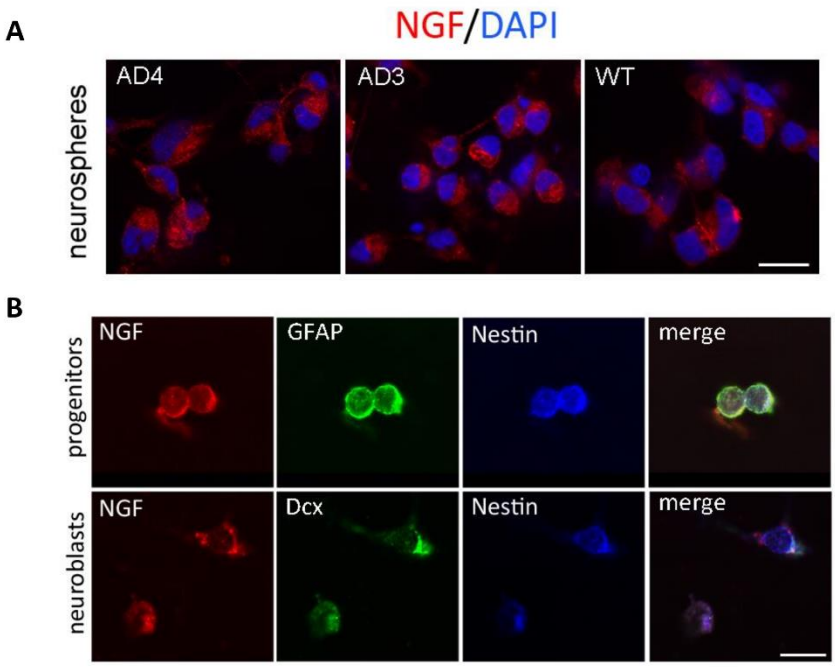


Figure 18. NGF expression in AD11 aNSC.
A) Immunofluorescence staining for NGF (red signal) in WT and AD11 neurospheres shows that NGF is equally expressed in both genotypes. DAPI staining on nuclei in blue. Scale bar 10 µm, 40 X magnification B) Immunofluorescence staining for NGF (red signal in left panels) and progenitors or neuroblasts/neuronal markers (green and blue signals in central panels) in DG-derived single cells. NGF is expressed in both DG-derived progenitors and neuroblasts. Scale bar 10µm, 40X magnification, zoom 2.

Consistently, total NGF measured by Alphalisa in the medium of WT neurospheres was 60 pg/ml/$5X10^6$ cells.

Finally, I tested the capacity of WT and AD11 neurospheres to differentiate into mature neurons by mitogens withdrawal for 5 days and labeling with anti-βIII-tubulin antibody (Tuj1). Although the percentage of Tuj1$^+$ cells was not significantly changed between WT and AD11 cultures, AD11 newborn neurons were poorly differentiated, displaying atrophic neurites (Fig. 19: % atrophic Tuj1$^+$ cells/tot Tuj1$^+$ cells: AD11 80±19; WT 30±11, p=0.02).

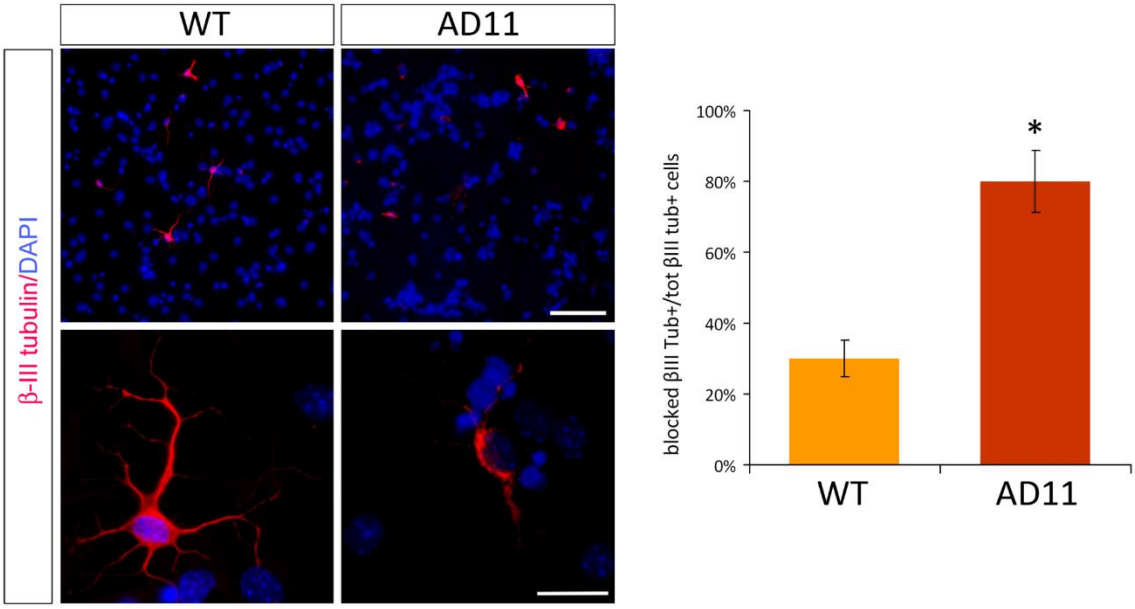


Figure 19. Impaired in vitro neurogenesis of AD11 aNSC.
*Immunofluorescence staining for βIII-tubulin on WT and AD11 differentiated neurospheres. AD11 βIII-tubulin$^+$ neurons display an atrophic morphology characterized by the absence of neurites outgrowth, compared to well-developed and branched WT βIII-tubulin$^+$ neurons. The histogram on the right represents the quantification of immature neuronal phenotype of WT and AD11 differentiated cultures, expressed as percentage of βIII-tubulin$^+$ cells without neurites elongation on the total number of βIII-tubulin$^+$ cells. * p <0.05, significantly different from WT, Student's t-test. Scale bar, 50 µm, 63X magnification. DAPI staining in blue.*

This defect of neuronal maturation, together with the increased proliferation rate, recapitulated *in vitro* the defective neurogenesis observed *in vivo* in AD11 adult hippocampus.

5.4 NGF and proNGF differentially affect proliferation of WT hippocampal progenitors

The concomitant expression of NGF and of αD11 antibody in AD11 cultures strongly suggested that NGF neutralization is occurring also *in vitro* and might be responsible for the increase in proliferation of AD3 and AD4 cultures. To verify this hypothesis, I tested whether anti NGF mAb αD11 antibody could increase the proliferation of WT aNSCs *in vitro.* Since αD11 antibody binds both pro and mature form of NGF, but with 2000 folds different affinities [331], I added αD11 to WT cultures at different concentrations (20 ng/ml, 100 ng/ml and 1000 ng/ml) in order to mimic two conditions: the neutralization of the mature form of NGF only or the overall neutralization of both mature NGF and proNGF precursor form. The dose range of the anti NGF mAb αD11 additions was calculated from a quantitative model for proNGF-NGF-interaction with the antibody (Fig. 20), based also on the actual concentration of NGF measured in our experimental conditions.

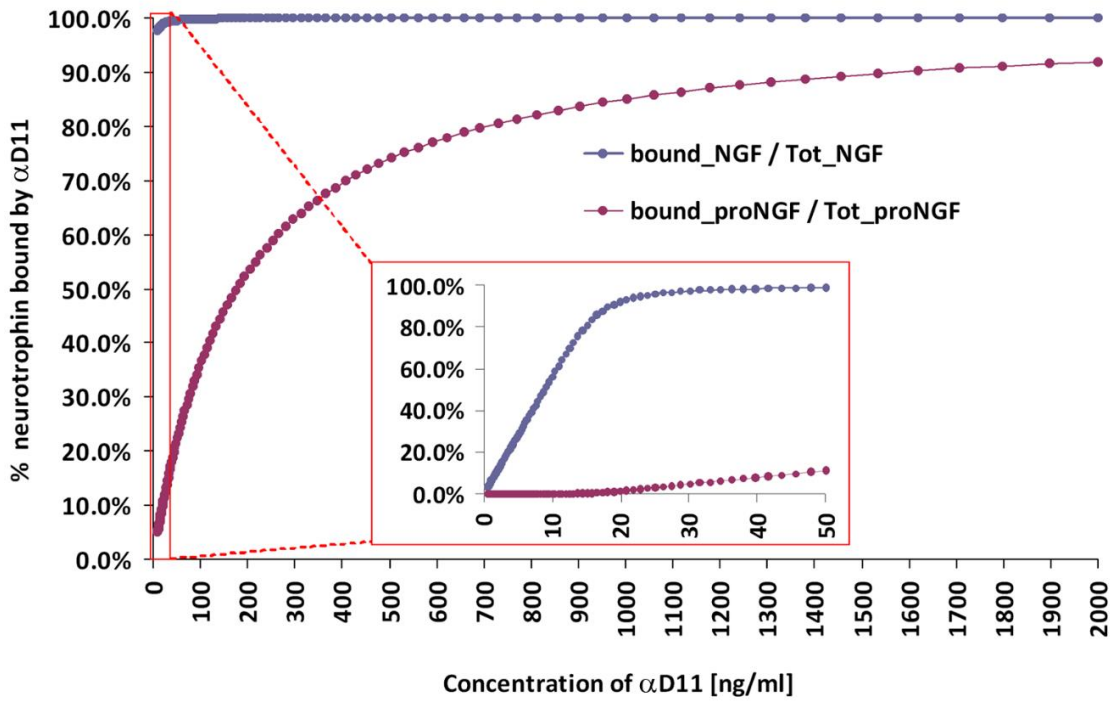


Figure 20. Neutralization of proNGF and NGF proteins by the αD11 anti-NGF antobody
Modeling of neutralization of proNGF and NGF proteins by the αD11 antibody, 72h after the treatment. The simulation is based on the NGF-proNGF-αD11 network model (see Methods) and NGF concentration measured in our experimental settings. In the lower range of concentrations, αD11 is unable to bind most of proNGF, while the NGF is almost completely neutralized. Only at saturating concentration αD11 is able to bind also most of proNGF.

After 2 weeks of treatment with 20 ng/ml anti NGF mAb αD11, a concentration that, according to the model, ensures the neutralization of the mature form only (Fig.20, see inset), proliferation of WT progenitors greatly increased, with respect to the untreated culture (Fig. 21, Fold Increase WT = 3.5±0.95; WT+αD11 = 47.8±3.1). At the intermediate concentration of 100 ng/ml, αD11 antibody continued to induce a 5 fold increase of the proliferative rate of WT aNSCs, but when I used the saturating concentration of 1000 ng/ml of the antibody, at which both NGF and proNGF are neutralized, the effect on the proliferative potential disappeared completely (Fig. 21), and the cultures proliferated at the same rate as untreated cultures.

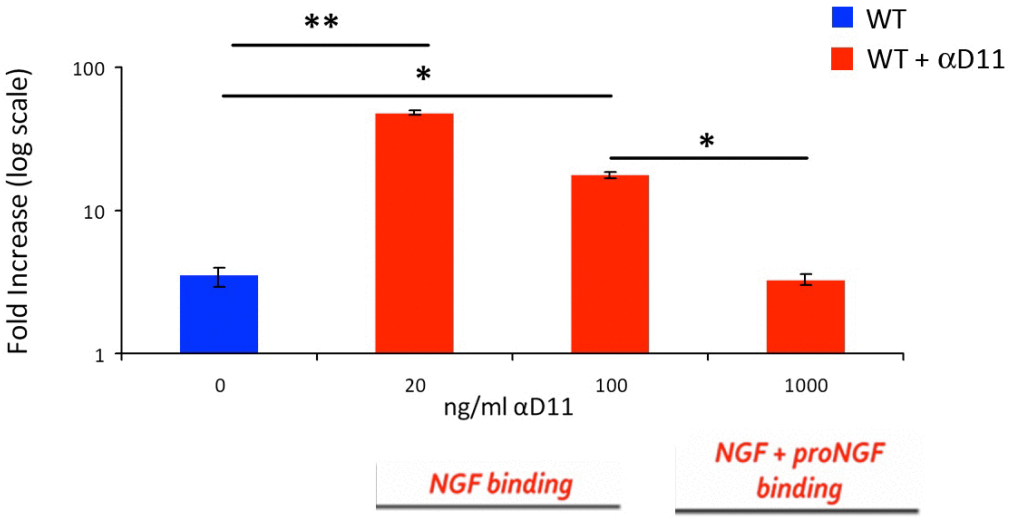


Figure 21. Effects of in vitro NGF neutralization on the proliferation of WT aNSCs.

*Fold Increase of proliferation of WT progenitors in the presence of mAb αD11. Progenitors treated with mAb αD11 at the lower concentration of 20 ng/ml (NGF binding) and 100 ng/ml (NGF binding and partially proNGF binding) proliferate more than those treated with the higher concentration of 1000 ng/ml (NGF+ ProNGF binding). 0 ng/ml corresponds to vehicle-treated cells. Data are mean ± SEM of 3 independent experiments. * p <0.05 and ** p <0.01 significantly different from WT, Student's t-test.*

This demonstrates that the selective neutralization of endogenously expressed mature NGF obtained *in vitro* accounts for the increased proliferative capacity of AD11 cultures respect the control WT, and also that the residual proNGF might play a role in controlling the proliferative rate of adult hippocampal stem/progenitor cells.

To directly evaluate the effects on aNSCs proliferation of mature NGF versus those of the precursor proNGF, I treated WT aNSCs with NGF or proNGF at equimolar concentrations (0.4 nM, 0.8 nM and 2 nM dose range). I used a recombinant furin-cleavage resistant form of proNGF (proNGF-KR) [345], to avoid uncontrolled cleavage of proNGF to NGF during the incubation time. The results (Figure 22) showed that pro and mature NGF had opposite effects on aNSCs proliferation.

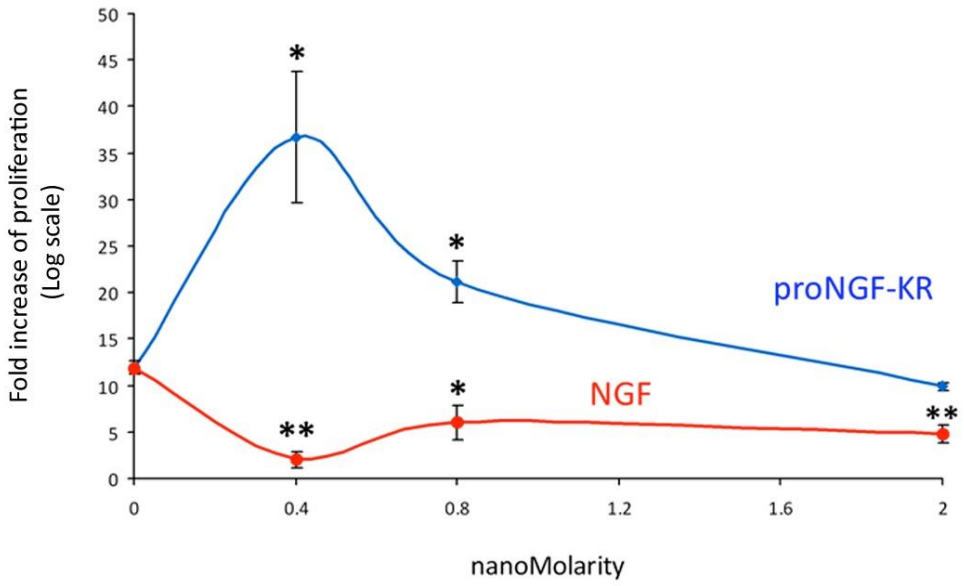


Figure 22. Effect of proNGF or NGF treatment on the proliferation of WT aNSCs.
*Fold Increase of WT progenitors in the presence of NGF (red line) or proNGF-KR (light blue line) at equimolar increasing concentrations. 0ng/ml corresponds to vehicle-treated cells. ProNGF-KR increases cell proliferation at low concentration but reduce it at higher concentration, while NGF reduces the proliferative rate regardless its concentration. Data are mean ± SEM of 3 independent experiments. * p <0.05 and ** p <0.01 significantly different from WT, Student's t-test.*

At the lowest neurotrophins concentration of 0.4 nM, while NGF treatment (red line in Fig. 22) significantly (p-value = 0.001) reduced WT DG cells proliferation, proNGF-KR (blue line in Fig. 22) had a positive effect on proliferation. At higher concentrations (> 0.4 nM), NGF trend to inhibit proliferation remained confirmed, while the proliferation inducing effect of proNGF-KR decreased in a dose-dependent manner. Indeed, while at the lowest concentration of 0.4 nM (10 ng/ml) proNGF-KR treated cells proliferate 3 times more than control (WT = 11.9±1.3, WT+proNGF-KR = 36.7±12.2, p-value = 0.02), at higher concentrations proNGF-KR mitigated its positive effect on proliferation, lowering the fold increase to 21.2±3.9 at 0.8 nM (20.8 ng/ml), while at 2 nM (50 ng/ml) the effect was totally abolished. Even though the presence of other cleavage enzymes, as serum metalloproteases, was limited by the fact that the neurospheres were cultured in serum free conditions, I could not exclude that proNGF-KR might be cleaved by other endogenous proteases. To this aim, the time course of proNGF cleavage to NGF was evaluated in conditioned medium from primary hippocampal neurons, cultured without serum for 12 days. Compared

to proNGF WT processing, which released ~ 44% of mature NGF, the degradation of proNGF-KR was negligible (13%, Fig. 23). In this scenario, the possibility that at higher concentrations proNGF-KR action is counterbalanced by mature NGF is unlikely to occur.

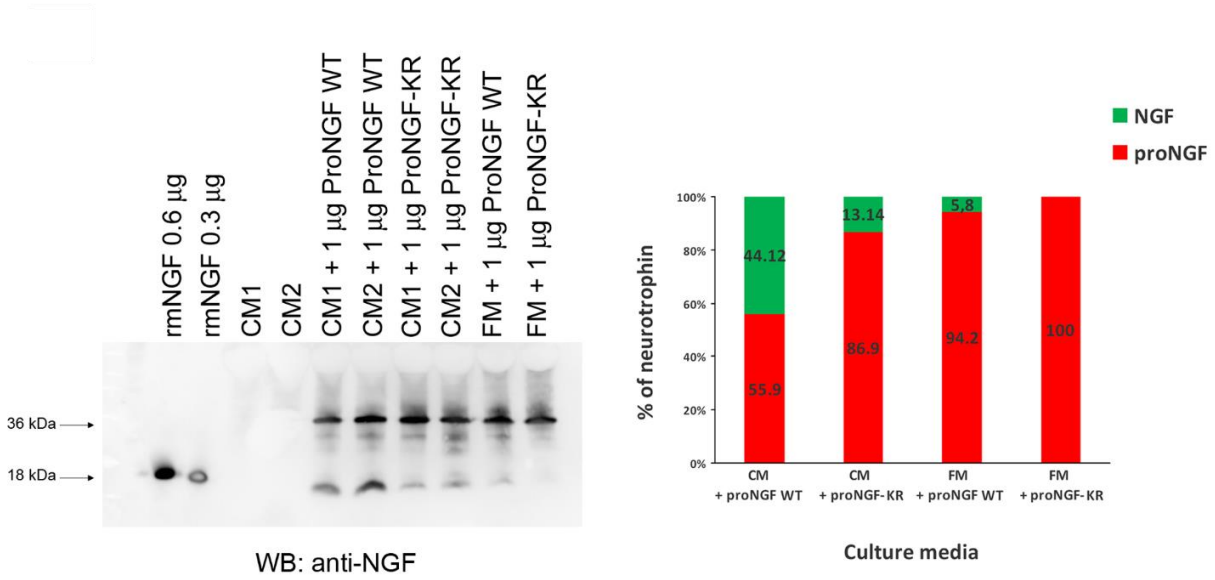
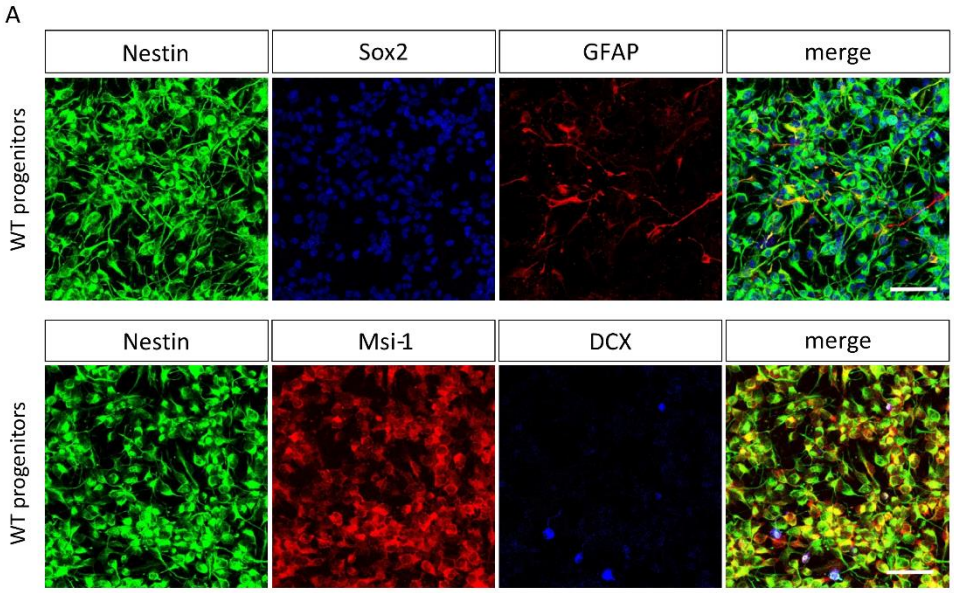


Figure 23. Analysis of proNGF processing.
Conditioned media (CM1 and CM2) from primary hippocampal neurons, incubated or not with 1 pg of proNGF-KR or proNGF WT for 72h, were run on SDS-PAGE for Western blotting analysis with an anti-NGF antibody. Mature NGF (18kDa) was released from proNGF WT only when incubated with CM but not with fresh media (FM1 and FM2), as quantified in the histogram. A relative small amount of mature NGF was also released from the unclivable, furin-resistant form of proNGF (proNGF-KR), suggesting the presence of other proteases in the CM. As reference, two different concentrations of recombinant NGF (rmNGF) were loaded in separate lanes.

5.5 Heterogeneity in cell-type composition and neurotrophin receptors of hippocampal aNSC cultures

Based on the previous results, I then hypothesized that increasing concentrations of proNGF might have differential effect on cells at different neurogenic stages (i.e. early versus late progenitors). Indeed, WT1 cultures are heterogeneous in terms of progenitor maturation, as demonstrated by immunostaining analysis performed with different cell type specific markers (Fig. 24A).



	Marker profile			
Cell Type	GFAP	Nestin	Msi-1	DCX
astrocytes	+	-	-	-
radial glial stems	+	+	-	-
early multipotent	-	+	-	-
middle multipotent	-	+	+	-
late multipotent	-	-	+	-
early neurals	-	+	+	+
Nest+ neurals	-	+	-	+
Msi-1+ neurals	-	-	+	+
late neurals	-	-	-	+
uncharacterized	-	-	-	-

Table I. Subpopulations of progenitors identified in proliferating WT1 aNSCs.

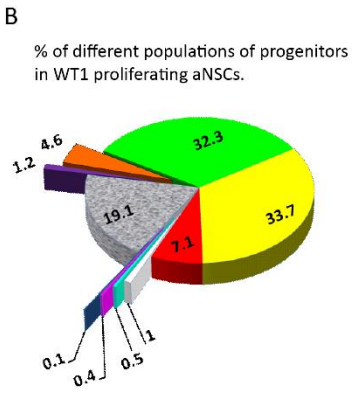


Figure 24. Heterogeneity of hippocampal aNSCs in vitro.
A) Immunofluorescence staining for early progenitors (Nestin⁺ cells green signal, GFAP⁺ cells red signal and Sox2⁺ cells blue signal) and for late multipotent progenitors (Nestin⁺ cells green signal, Msi-1⁺ cells red signal and DCX⁺ cells blue signal) show that WT1 cultures are heterogeneous in terms of cellular subtypes composition, as summarized in Table I. Scale bar, 50 µm, 40X magnification. B) Representation in percentage of the different population of progenitors. The majority (66%) of WT1 aNSCs are GFAP⁻/Nest⁺ multipotent progenitors, subdivided in early multipotent (32,3%, green) and middle multipotent (33,7%, yellow), while late multipotent progenitors are less represented (7%, red). A little portion (4.6%) of WT1 cultures is constituted by radial glia-like stem cells (orange). 2% of WT cells are neural committed progenitors (white, light blue, pink and dark blue).

Table I shows the different subpopulations identified in WT1 cultures based on the concomitant expression of Nest/Msi1/Dcx or GFAP/Nest/Sox2 markers: astrocytes (1.2% ± 0.6); radial glia-like stem cells (RGLs or Type-1, 4.6% ± 0.9); early multipotent (32.3% ± 2.6); middle multipotent (33.7% ± 5.1); late multipotent (7.1% ± 2.7); early

neural committed (1.0% ± 0.1); neural committed A (0.5% ± 0.1); neural committed B (0.4% ± 0.3); late neural committed (0.1% ± 0.2). As represented in the diagram of Fig. 24B, the major portion of WT1 cultures were $GFAP^{-}/Nest^{+}$ multipotent progenitors (66%), that I further subdivided in early multipotent (early Type-2a, in green, 32.3%) and middle multipotent (middle Type-2a, in yellow, 33.7%), by the fact that they were respectively negative or positive for the marker Musashi-1 (Msi1). Neural committed progenitors were less represented (about 2%). Of note, our cultures maintained a subpopulation of cells with the proper stem cells feature of quiescence, i.e. the RGLs (4.6%, in orange) that allow the long-term expansion of the culture.

In this view, the different progenitors could differentially respond to proNGF based on distinct differential expression of proNGF receptors $p75^{NTR}$ and TrkA. In order to investigate the neurotrophin receptor profile of my WT cultures, I performed immunostaining for $p75^{NTR}$ or TrkA in combination with GFAP, Nestin and Dcx markers. Co-immunostaining analysis on freshly isolated cells from WT hippocampus (*ex vivo* analysis) showed that RGLs (GFAP+/Nestin+) expressed more $p75^{NTR}$ than the late $Nestin^{+}/DCX^{+}$ progenitors, while TrkA was equally expressed at low level among the different populations (Fig 25).

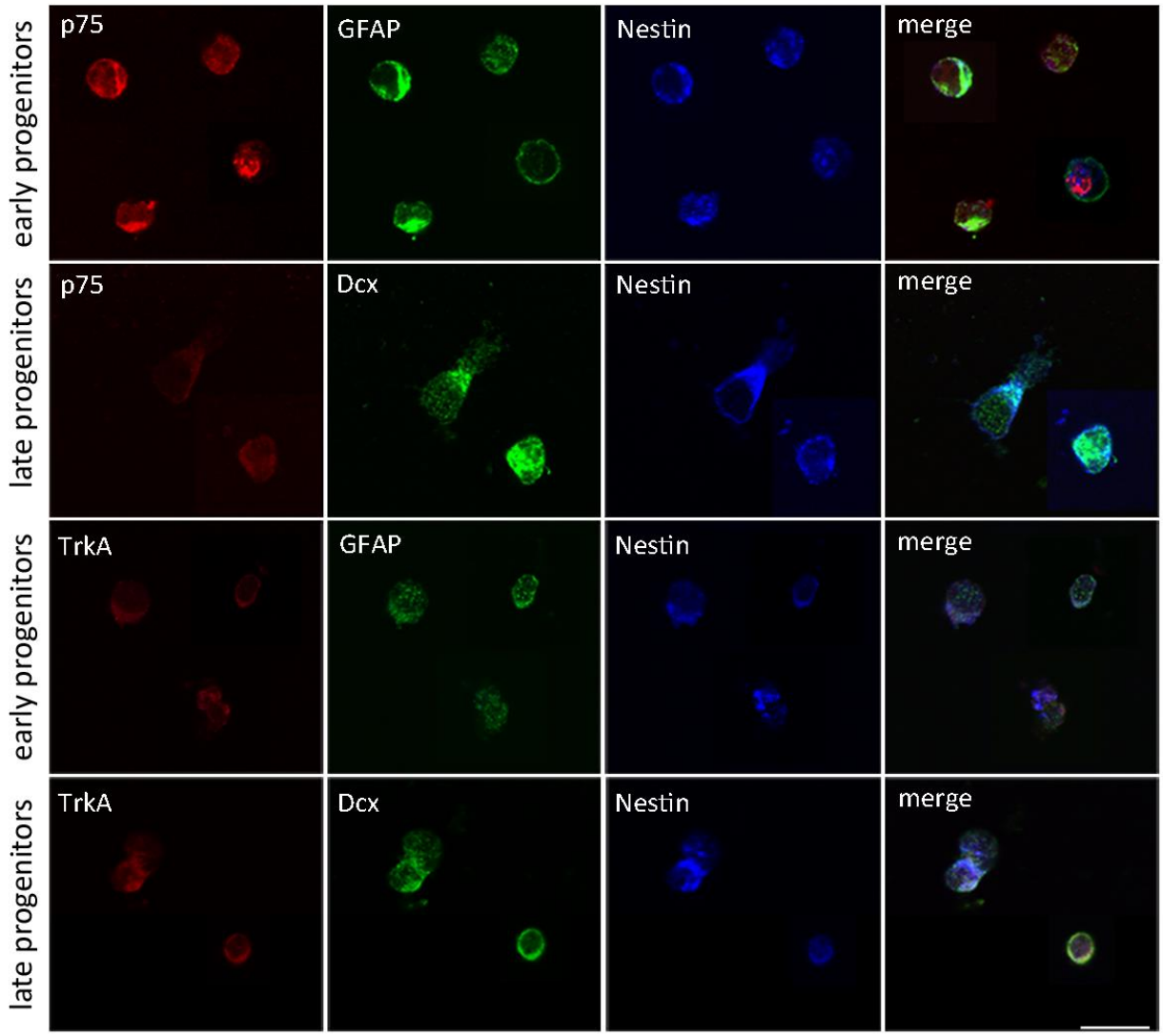


Figure 25. Ex vivo expression of p75^{NTR} and TrkA in stem cells and neuroblasts.
Immunofluorescence staining for p75^{NTR} or TrkA (red) in GFAP/Nestin double positive radial glial stem cells (green and blue signal respectively, early progenitor panels) and in Dcx/Nestin double positive neuroblasts (green and blue signal respectively, late progenitor panels) shows that p75^{NTR} is more expressed in radial glia-like stem cells than in neuroblasts, while TrkA is equally expressed at low level in all populations. Scale bar, 10 µm, 40X magnification.

Thus, difference in cell proliferation in response to proNGF concentration, previously observed (Fig. 22), are likely due to difference in p75^{NTR} expression among early and middle/late progenitors in my WT cultures. To further investigate this hypothesis, I analyzed the distribution of p75^{NTR} in WT progenitors (Fig. 26).

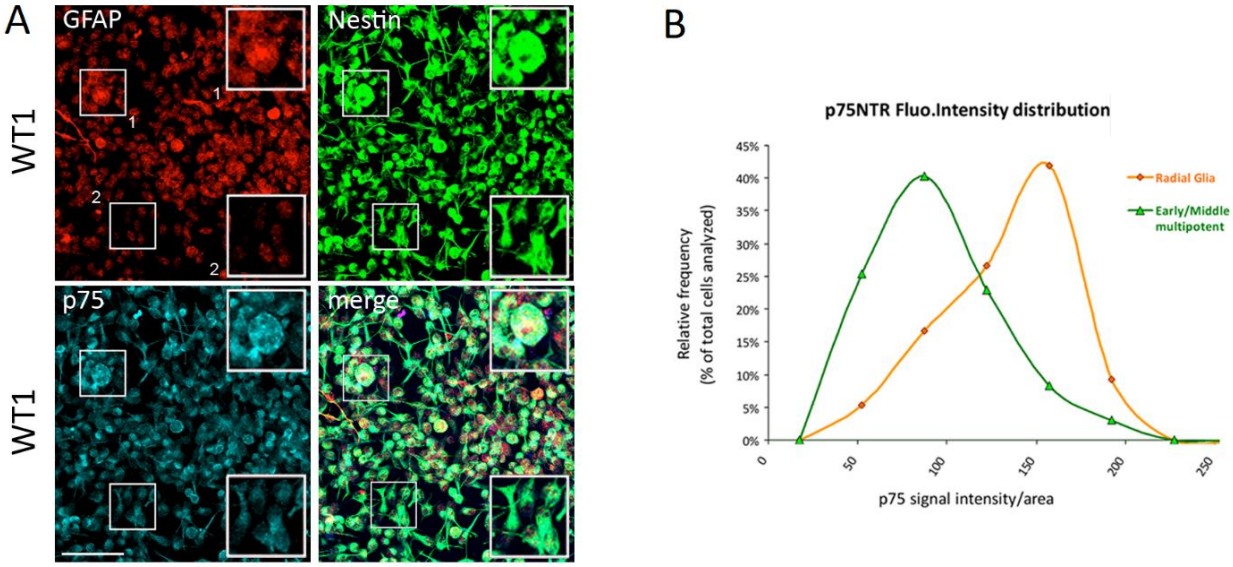


Figure 16. p75^{NTR} expression in vitro.

*A) Immunofluorescence staining for Nestin (green signal), GFAP (red signal) and p75^{NTR} (blue signal) in WT cells shows that p75^{NTR} is expressed in both radial glia-like stem cells and multipotent early and middle progenitors at different intensity levels, as quantified in the graphic on the right. White square boxes in each panel represent 2X magnification of the corresponding dot-lines insets. Scale bar, 100µm 40X magnification. B) Distribution of the fluorescent intensity of p75^{NTR}. Two independent statistical tests, Wilcoxon-Mann-Withney and Kolmogorov-Smirnov test, respectively, indicate that the two distribution, that of p75^{NTR} in the RGLs and that of p75^{NTR} in early/middle progenitors, are actually two distinct distributions. Data are mean of 3 independent experiments. p <1*10^{-5} and p <1*10^{-3} significant difference between the distributions according the p-value of each of two independent statistical tests.*

RGLs (GFAP$^+$/Nest$^+$/Dcx$^-$) expressed more p75^{NTR} than middle/late progenitors (GFAP$^-$/Nest$^+$/Dcx$^+$) (Fig. 26B), confirming also for DG what was previously reported in SVZ[346]. The two distributions, evaluated by measuring the p75^{NTR} fluorescence intensity among 300 events (cells), were significantly different, as calculated by two independent statistical tests (Wilcoxon-Mann-Withney test, p-value < 1*10^{-5}, and Kolmogorov-Smirnov test, p-value < 1*10^{-3}).

Based on this result, RGLs (that expressed high p75^{NTR}) might be more prone to respond to proNGF by re-entering cell cycle and starting to proliferate. Indeed, acute (48h) treatment of WT cells with 50 ng/ml proNGF-KR induced a significant increase in the expression levels of cyclin D1 in Nest$^+$/Dcx$^-$ cells, that correspond to RGLs, early and middle multipotent progenitors (Fig. 27A). As known, cyclin D1 is involved in G_1 phase progression and thus plays an important role to induce quiescent

cells to reentry the cell cycle. The distribution of cyclinD1 signal in the Nest^{+}/Dcx^{-} population (Fig. 27B) showed that, in the presence of proNGF-KR, a small fraction (6%) of RGLs/early-middle multipotent progenitors expressed cyclinD1 at high level (intensity value=260). This fraction could thus correspond to quiescent/early progenitors that respond to proNGF by increasing cyclinD1 expression and probably re-activating cell cycle.

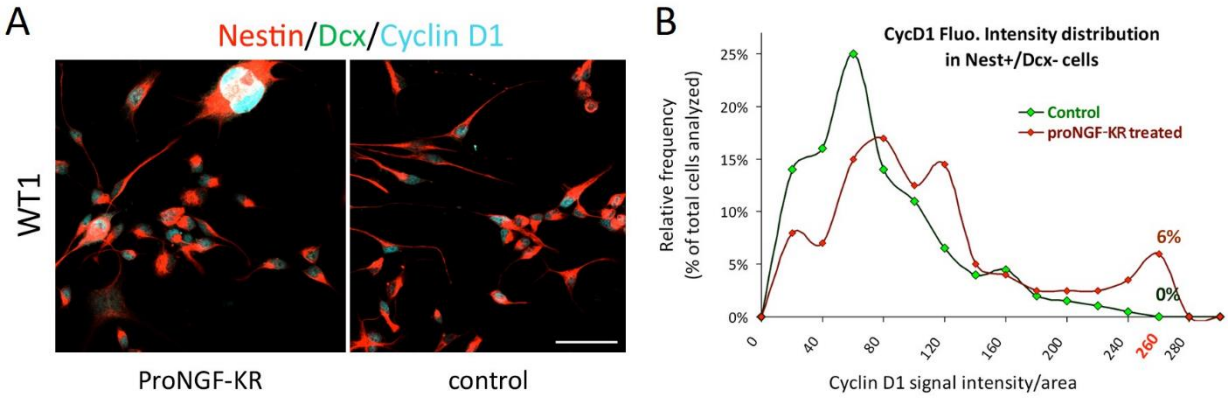


Figure 27. Cyc D1 up-regulation in a sub-population of pre-neural cells by proNGF-KR treatment.
A) proNGF-KR treatment of WT cells induced a significant increase in the expression of cyclin-D1 (light blue signal) in Nestin^{+}/Dcx^{-} cells (red and green signals, respectively), as quantified by the distribution of immunofluorescence intensity values of cyclin-D1 (B). Data are mean of 3 independent experiments. $p < 1*10^{-9}$ and $p < 1*10^{-6}$, significantly different from control according to Wilcoxon-Mann-Withney test and Kolmogorov-Smirnov test, respectively. Scale bar, 100 µm, 40X magnification.

It has been recently reported that the mitogenic induction of cyclin D1 expression in neural stem cells is driven by the phosphorylation of c-Jun protein [347], which is downstream of the p75^{NTR} signaling pathway [348]. This pathway is usually involved in promoting the apoptotic effect of p75^{NTR}, even though there are cumulative evidences showing a potential link between p75^{NTR} signaling and cell-cycle progression [349]. Interestingly, upon acute treatment of WT cells with proNGF-KR I found an increased amount of the phosphorylated c-Jun protein by Western blot analysis (Fig. 28), together with an increased level of cyclin D1 in treated cells, while both activated cleaved caspase-3 and phospho-AKT (TrkA signaling) levels remained unchanged between proNGF-KR-treated and untreated cells. This data confirms that

proNGF stimulates early progenitors proliferation through the activation of $p75^{NTR}$ signaling pathway and the induction of cyclin D1.

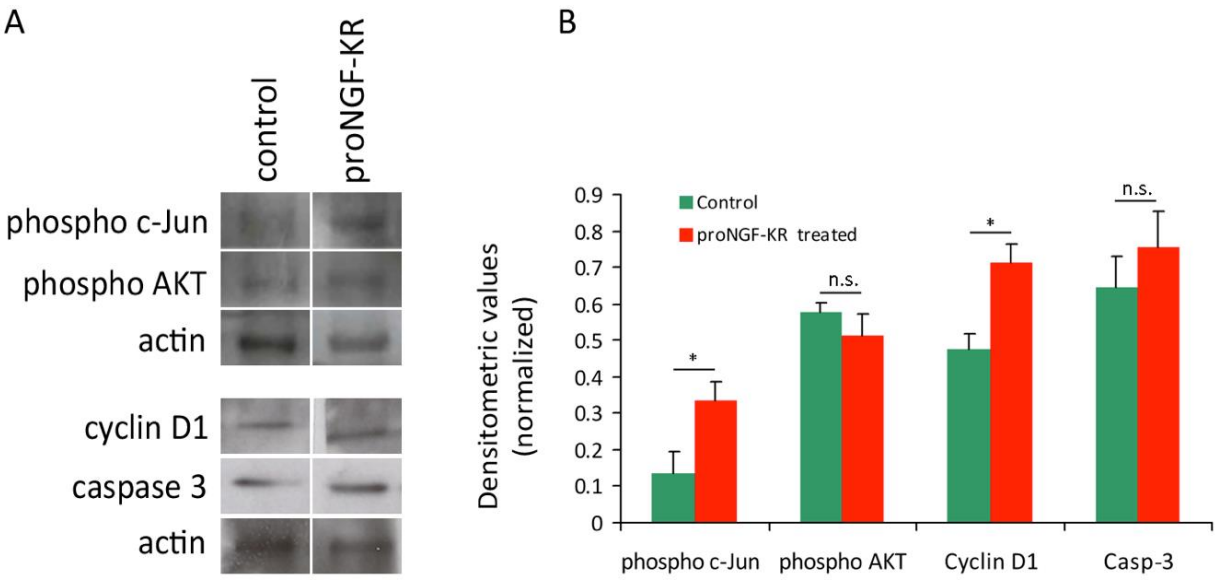


Figure 28. p75 signaling activation upon acute treatment with proNGF-KR.
*A) Western blot analysis of WT vehicle-treated (control) or proNGF-KR-treated cells shows up-regulation of phospho c-Jun and not of phospho AKT, indicating the activation of $p75^{NTR}$ signaling pathway upon proNGF-KR stimulation. This also leads to an increase in cyclin D1, while caspase-3 levels remain unchanged between untreated and treated cells, demonstrating that $p75^{NTR}$ signaling pathway, activated by proNGF-KR, triggers cell cycle progression. B) Densitometric quantification of Western blot signal. Data are mean ± SEM of 3 independent experiments. * p <0.05 significantly different from WT, Student's t-test.*

5.6 Mitogenic effect of proNGF on RGLs selected by LIF treatment

Due to the high heterogeneity of WT neurospheres cultures, I decided to better address this novel property of proNGF of inducing proliferation of quiescent stem cells, by testing its effects on proliferation specifically on a homogenous population of RGLs. To this aim, these putative stem cells were selected *in vitro* from the original WT culture by treating cells with Leukemia Inhibitory Factor (LIF) and by mitogen factors withdrawal as described (Fig. 29) (see Materials and Methods).

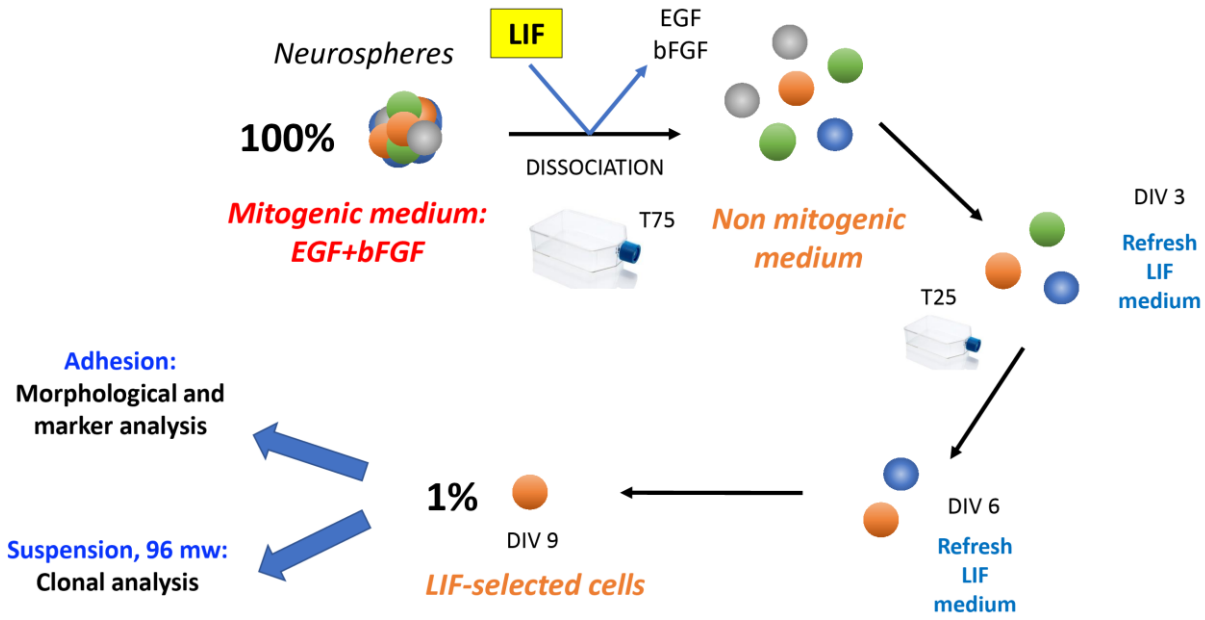


Figure 29. LIF-selection method.
Neurospheres growth in medium with mitogen are dissociated and transferred in medium mitogen free and in presence of LIF. At the end of 9 days in such condition (refreshing medium at day 3 and 6) only 1% of the starting cells survived. This selected population is then analysed for marker expression and for colony formation capacity (clonal analysis).

This method, based on the property of LIF to block differentiation pathway, increases GFAP expression and promotes symmetrical division during proliferation, allowing a reliable enrichment of quiescent/early progenitors, meanwhile the late progenitors die because of EGF and bFGF absence. LIF-treated progenitors were grown on glass slides double coated with laminin and poly-ornithin. In this way, I were able to obtain a homogenous population of cells with a radial elongation, resembling the typical radial glia morphology of RGLs observed *in vivo* (Fig. 30).

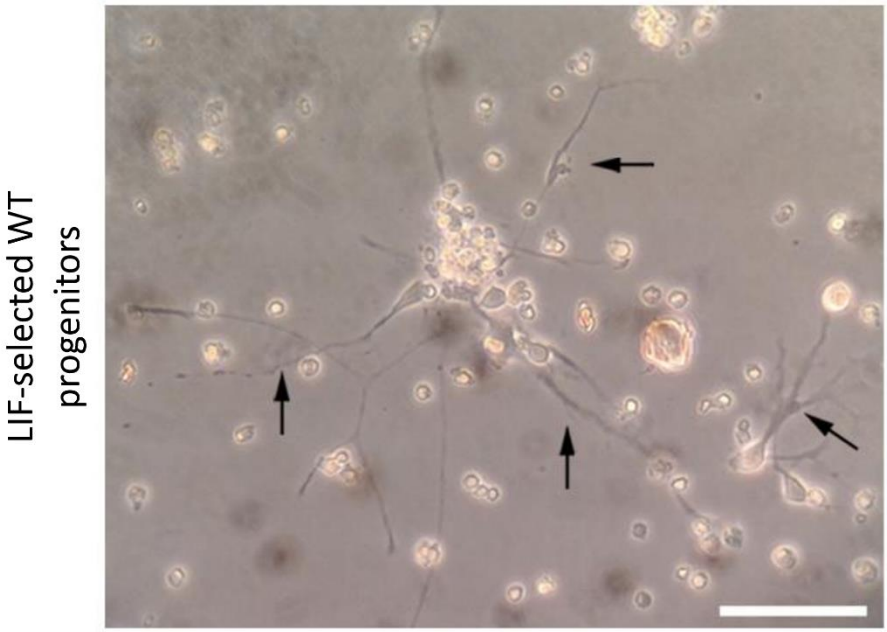


Figure 30. Phase-contrast micrograph of WT progenitors upon LIF selection.
Selected cells have a radial morphology typical of radial glia-like stem cells (RGLs), as indicated by the black arrows. Scale bar 50 µm, 40X magnification.

These selected cells are $GFAP^{+}$/$Nestin^{+}$ and $Mash1^{+}$/$Nestin^{+}$, thus representing early Type-1 and Type-2a progenitors, and expressed high level of $p75^{NTR}$ with respect to the un-treated heterogeneous population (Fig. 31A and 31B).

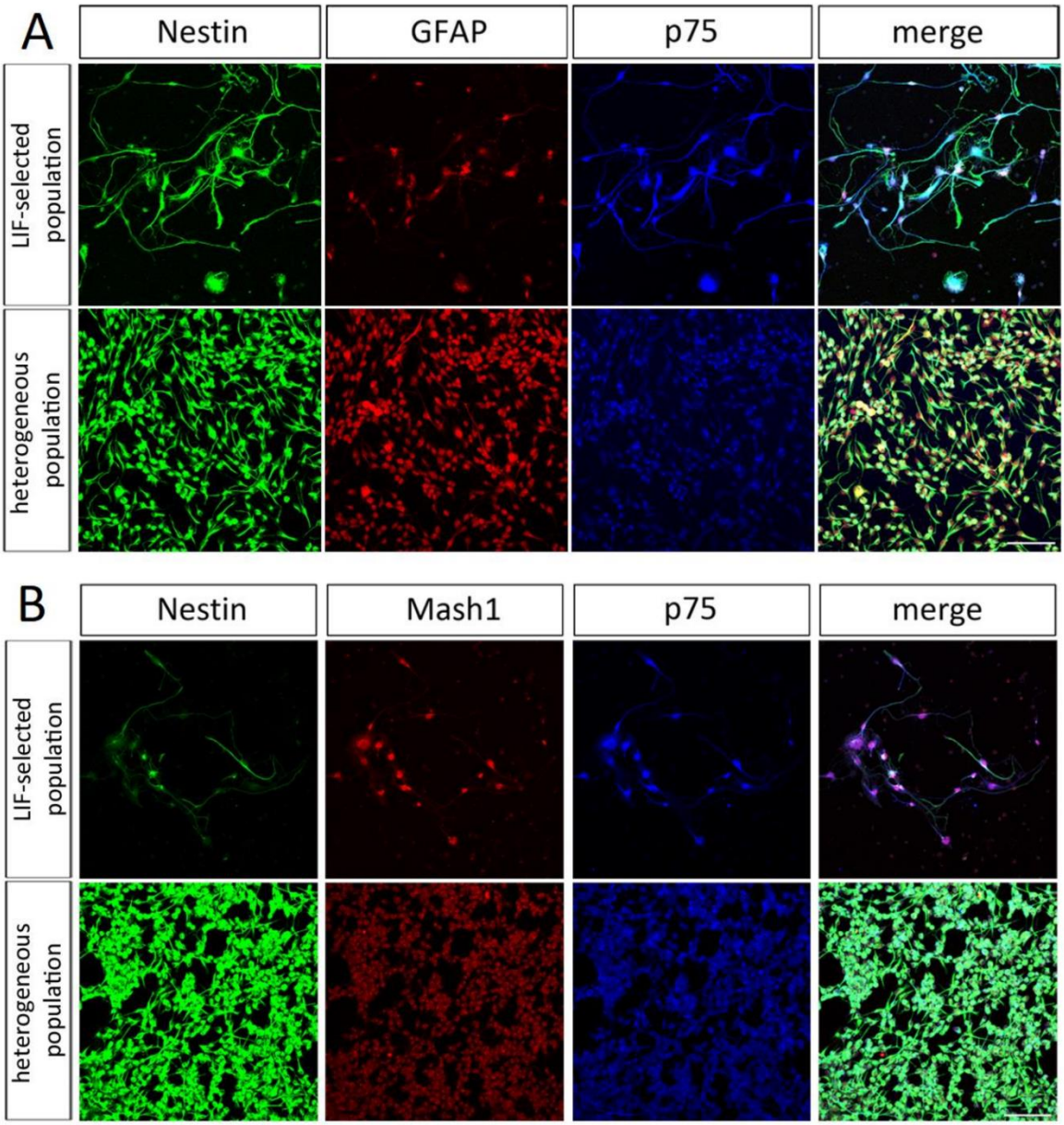


Figure 31. Expression of early markers and high p75^{NTR} signal in LIF-selected cells.
LIF-selected cells express high level of both RGLs markers (Nestin in green and GFAP in red in panel A), as well as of early Type-2a progenitors markers (Nestin in green and Mash1 in red in panel B) and p75^{NTR} (blue signal). Scale bar 100 µm, 40X magnification.

I then tested the effect of proNGF-KR on the proliferation of LIF-selected RGL cells, by a clonal assay (see Materials and Methods) in which I counted the number of neurospheres forming from 300 starting RGLs in the following conditions: mitogens (EGF + bFGF) or LIF in combination or not with proNGF-KR. The presence of LIF, in the absence of EGF+bFGF, was necessary for NSCs survival. The results shown in Fig. 32 demonstrated that, in the presence of mitogens, proNGF-KR increases the num-

ber of neurospheres in a concentration dependent manner (upper panels). Strikingly, proNGF-KR was also able to induce the formation of neurospheres in the absence of added mitogens, i.e. in non-mitogenic conditions (lower panels). This demonstrates that proNGF-KR acts as mitogenic factor in a concentration dependent manner on a homogenous population of putative stem cells.

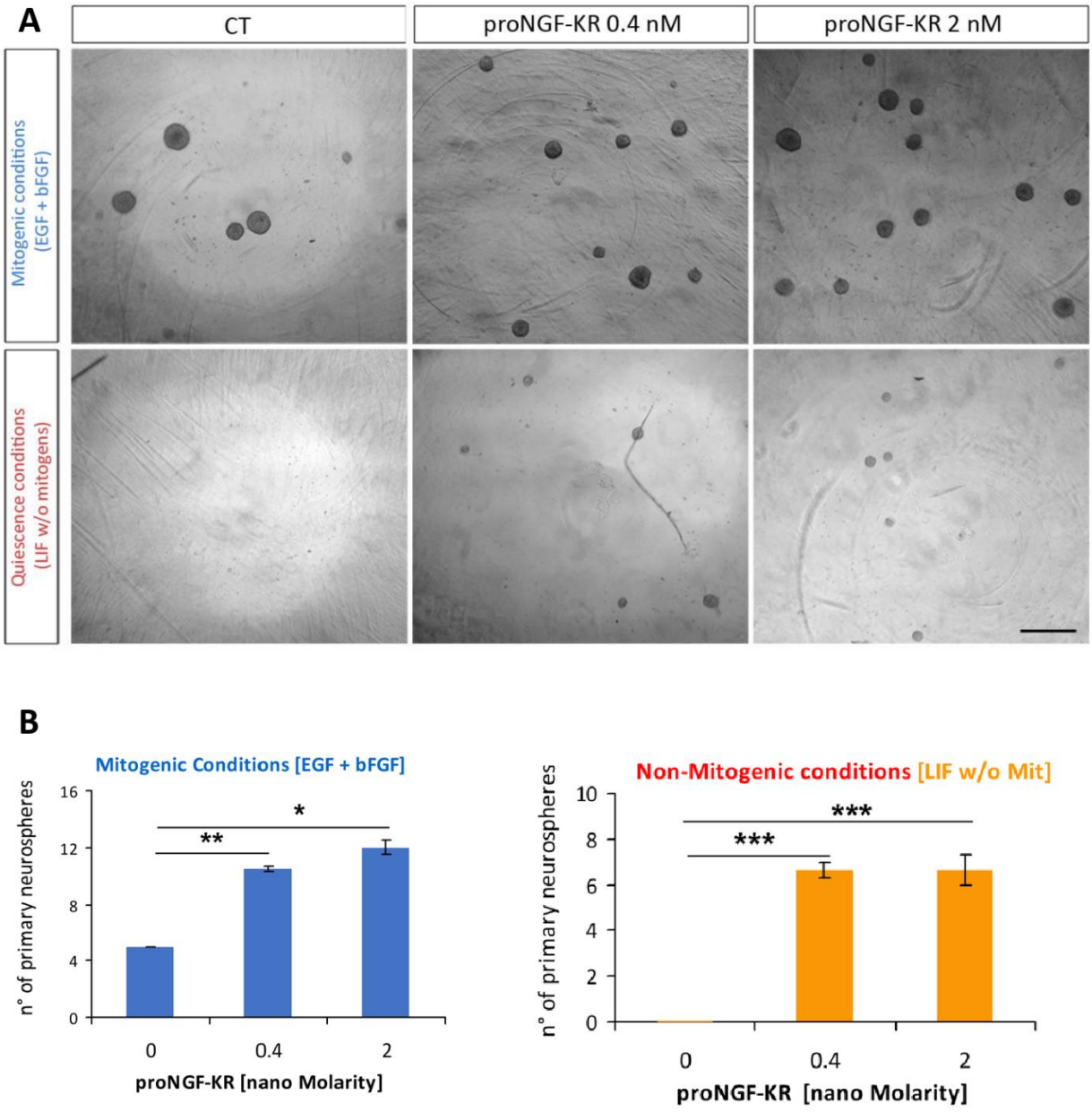


Figure 32. ***ProNGF-KR acts as mitogenic factor in a concentration-dependent manner on RGLs.***
*A) In the presence of mitogens, proNGF-KR increases the number of neurospheres in a concentration dependent manner (upper panels), as quantified in the B) histogram on the left. ProNGF-KR induces the formation of neurospheres in combination with LIF in non-mitogenic conditions (A, lower panels), as quantified in the B) histogram on the right. Scale bar 200µm, 5X magnification. Data are mean ± SEM of 3 independent experiments. * p <0.05, ** p <0.01, ***p <0.001 significantly different from control, Student's t-test.*

5.7 Mitogenic effect of proNGF on iNSCs and primary NSC cultures

I took advantage of this new biological property of proNGF-KR to improve the expansion capacity of induced neural stem cells (iNSCs) (see Materials and Methods). iNSCs are a promising reprogramming technology for future application in cell therapy [336]. We derived iNSCs cultures by infecting mouse embryonic fibroblast with the pRetro-Sox-2 [336] (Fig. 33).

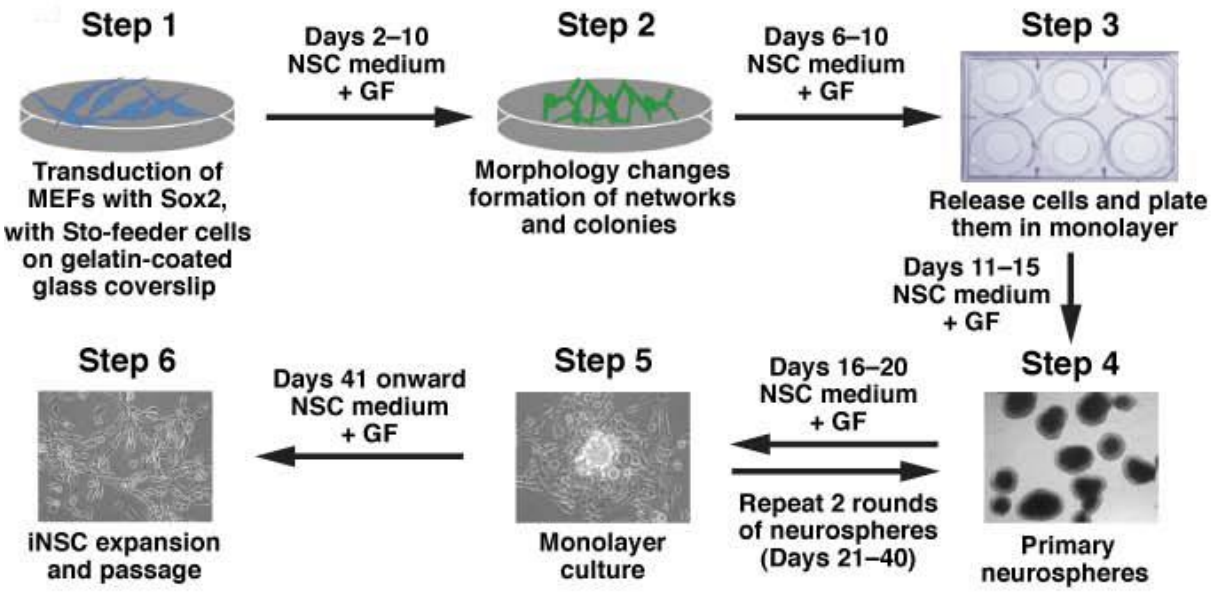


Figure 33. Protocol for direct reprogramming from Mouse Embryonic Fibroblasts (MEFs) to induced Neural Stem Cells.
MEFs are transduced with Sox2 Lentivirus and, the day after, MEFs cultured mediumis replaced neural stem cell medium(NSC) additioned with the mitogen factors. During the reprogramming process, cells go through morphological changes that allow to identify the reprogramming step. At the begin of the protocol, cells grow in adhesion, then they give rise to floating neurospheres (step 4). This growth in suspension allows to separate the reprogrammed cells (iNSCs) from the starting monolayer made of feeder, not-reprogrammed MEFs. Moreover, after the first purification of primary neurospheres, the reprogrammed monolayer of MEFs continue to give rise to other neurospheres. Once iNSCs cultures are purified, they can be expanded in adhesion or in suspension (neurospheres mode). (Figure from Ring, 2012).

One limitation of current iNSCs reprogramming protocols is that they produce mainly late Nestin^{+}/Dcx^{+} progenitors, with restrict ed propagation potential (≤ 8-9 passages) ([350]; this thesis). To explore the possibility of overcoming this limitation, I chronically exposed mouse iNSCs to 0.4 nM proNGF-KR from passage 0 to passage 6

(before the culture undergo spontaneous differentiation, despite the presence of mitogenic stimulus in the medium). At this passage, in control iNSCs there are more neural than early progenitors ($Dcx^+/Nestin^+$, 24.3%±4 vs $Nestin^+/Msi^-/Dcx^-$ cells, 11.5%±3.5; Fig 34, upper panels, and histogram). The remaining population is composed of the intermediate stages: the middle multipotent progenitors $Nest^+/Msi1^+/Dcx^-$ (38.4%±6.4), and the late multipotent $Nest^-/Msi1^+/Dcx^-$ (25.8%±2.4). This reflects the bias of this culture, characterized by a limited expansion potential, together with a tendency to neural differentiation.

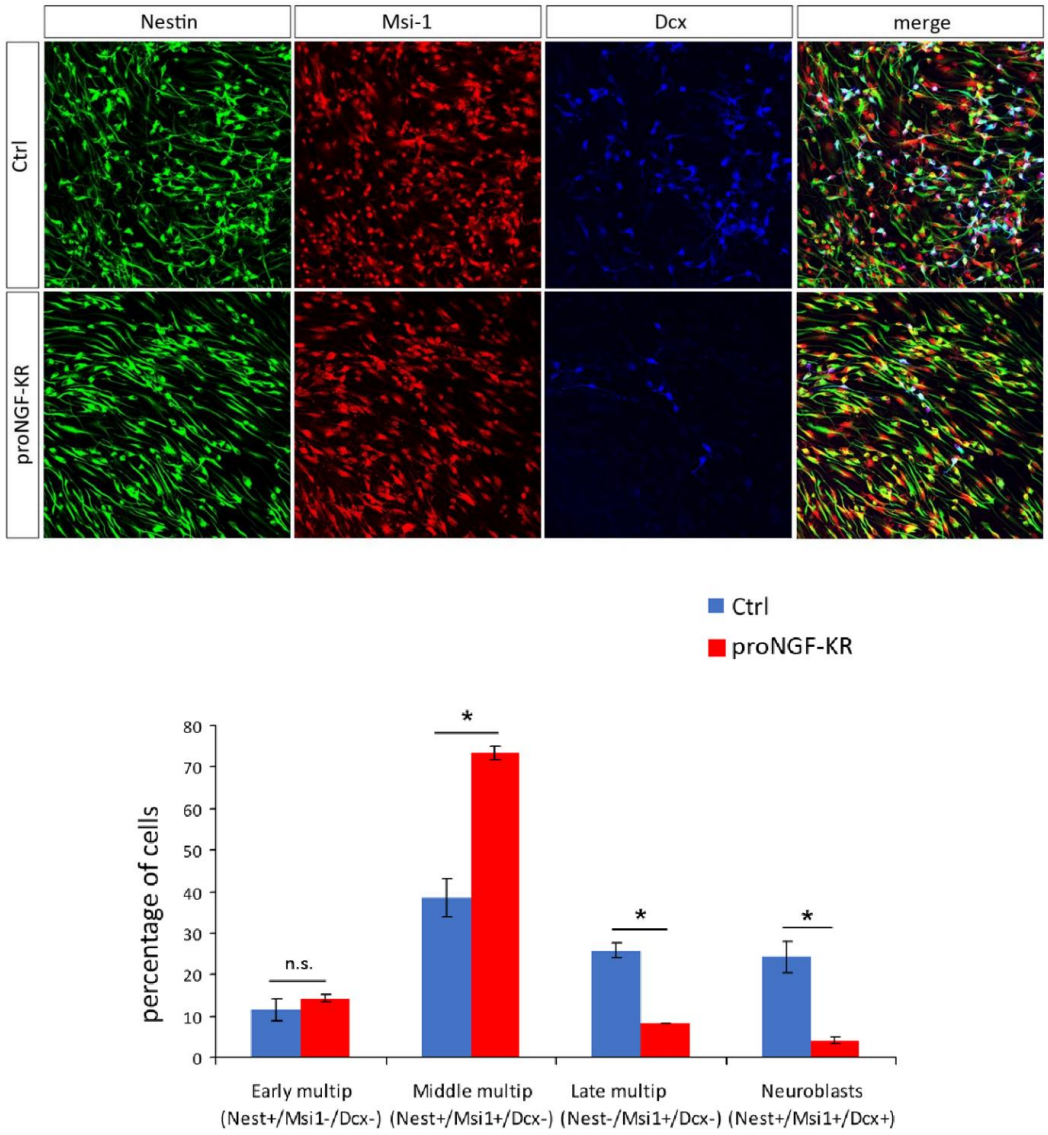


Figure 34. Immunofluorescence staining for different cellular subtype markers in iNSCs cultures treated with proNGF-KR.

*proNGF-KR induces an enrichment in middle multipotent progenitors ($Nest^+/Msi1^+/Dcx^-$, yellow cells) and a reduction of late neural progenitors ($Msi1^+/Nestin^+/Dcx^+$, cyan cells) and late multipotent progenitors ($Nest^-/Msi1^+/Dcx^-$, red cells), as quantified in the histogram on the right. Data are mean ± SEM of 3 independent experiments. * p <0.05 significantly different from control, Student's t-test. Scale bar 100 µm, 40X magnification.*

Notably, proNGF-KR chronic treatment of iNSCs significantly (p-value = 0.02) increased middle multipotent ($Nest^+/Msi1^+/Dcx^-$) progenitors from 38.4%±6.4 to 73.5%±2.3, while it drastically reduced the percentage of late ($Msi1^+/Nestin^+/Dcx^+$) neural and late multipotent ($Nest^-/Msi1^+/Dcx^-$) progenitors (from 28.4±6.4 to 4.0±1 and 25.8±2.4 to 8.2±0.04, respectively) (Fig. 34, lower panels). This change in subtype composition of the culture describes a population with a significantly higher expanding capacity. Moreover, neurospheres from proNGF-KR-treated iNSCs had greater dimensions than control neurospheres (Fig. 35) (diameter of neurospheres: control 62.3±21.3µm; treated 317±118.3µm, p = 0.0015).

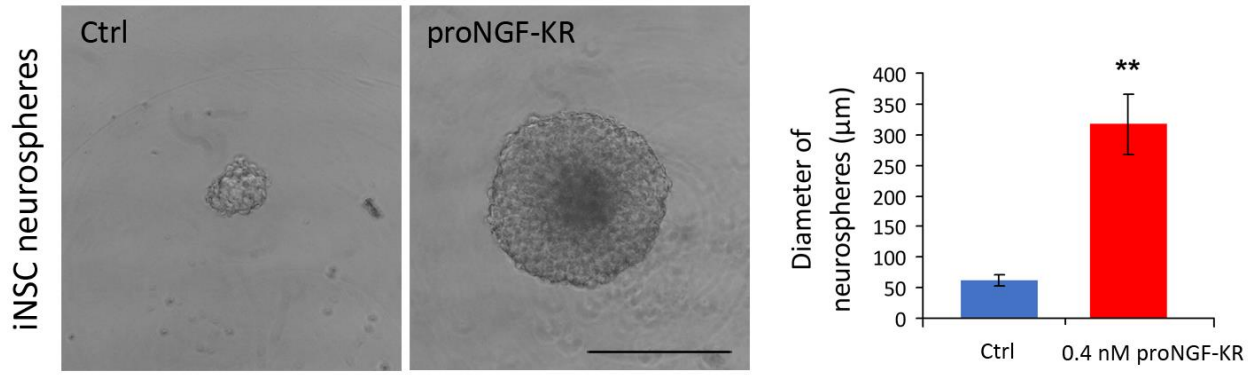


Figure 35. Phase-contrast micrographs of iNSCs neurospheres.

*ProNGF-KR-treated iNSCs are bigger in size compared to control, as quantified in the histogram on the right. Data are mean ± SEM of 3 independent experiments. ** p <0.01 significantly different from control, Student's t-test. Scale bar 200µm, 20X magnification.*

Interestingly, while the expansion capacity of control iNSCs was gradually lost over time, proNGF-KR treated iNSCs continued to grow exponentially up to 42 days in vitro (DIV, Fig. 36).

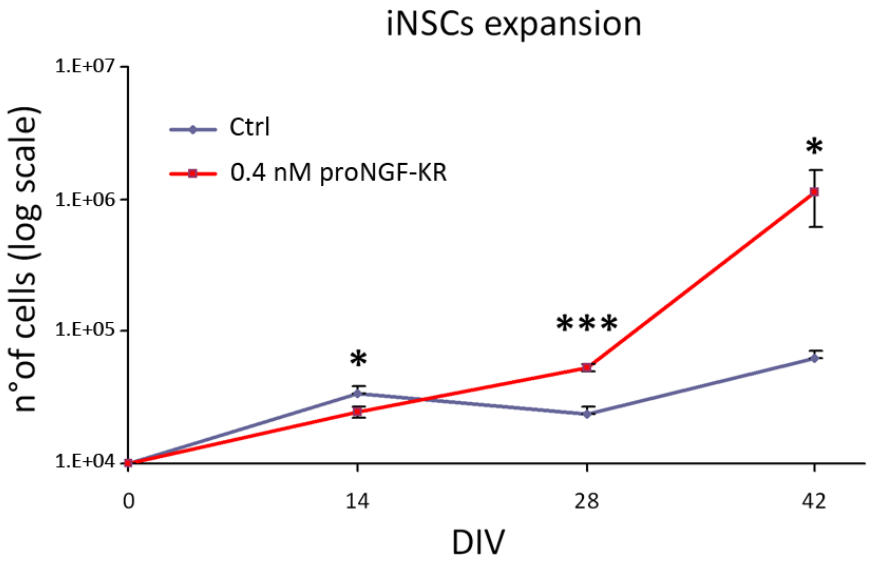


Figure 36. proNGF improve expansion capacity of iNSCs.
*The expansion capacity of proNGF-KR treated iNSCs (red line) continued to grow exponentially, while that of control iNSCs (blue line) is gradually lost over time. Data are mean ± SEM of 3 independent experiments. * p <0.05 and *** p <0.001 significantly different from control, Student's t-test.*

Therefore, proNGF-KR treatment induced an enrichment in early versus late progenitors. I further demonstrated the mitogenic effect of proNGF-KR on neural stem cells by its ability to increase the number of primary neurospheres derived per single hippocampus. I compared three culture conditions: NSC medium (with mitogens, EGF+bFGF), NSC medium + proNGF-KR and NSC medium (w/o mitogens) + proNGF-KR + LIF (see Materials and Methods). The presence of LIF, in the absence of EGF+bFGF, was necessary for NSCs survival. Fig. 37 shows that proNGF-KR, in synergy with EGF+bFGF, but not alone, increased the number of primary neurospheres already after 1 week of treatment (DIV7), and the overall number of primary neurospheres was 2 folds higher (p-value = 0.058), respect to control conditions, after 3 weeks in culture.

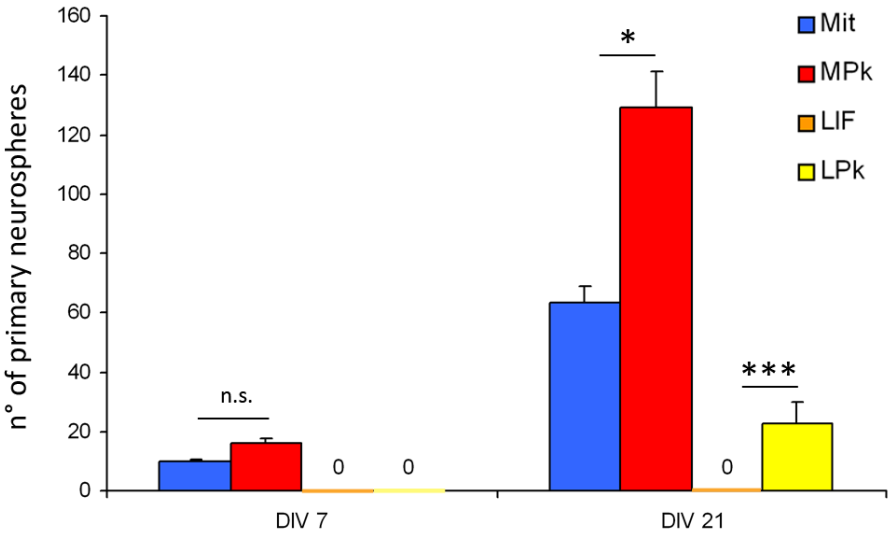


Figure 37. proNGF-KR improve primary hippocampal neurospheres derivation.
*After 3 weeks of treatment, proNGF-KR, in synergy with mitogens (EGF+bFGF, MPk, red), but not alone (LPk, yellow), increased the number of primary neurospheres and was also capable to induce the formation of some primary neurospheres in combination with LIF alone. Data are mean ± SEM of 3 independent experiments. * p <0.05 and *** p <0.001 significantly different from control, Student's t-test. Mit= mitogens; MPk= mitogens + proNGF-KR; LPk= LIF + proNGF-KR; DIV= days in vitro.*

Notably, proNGF-KR was also capable to induce formation of some neurospheres in combination with LIF alone, even though with a less extent than the other conditions.

Finally, to further confirm the mitogenic role of proNGF also *in vivo*, I took advantage of the proNGF#72 transgenic line, developed in our laboratory [335]. These mice constitutively express the furin-resistant mouse proNGF (proNGF-KR) under the control of the Thy1.1 promoter, in a background of normal endogenous proNGF\NGF production. Strikingly, neurospheres from proNGF mice proliferated significantly more than those derived from WT mice, demonstrating that neural stem cell exposed i*n vivo* to high levels of proNGF-KR display a greater proliferation capacity *in vitro* (Fig. 38).

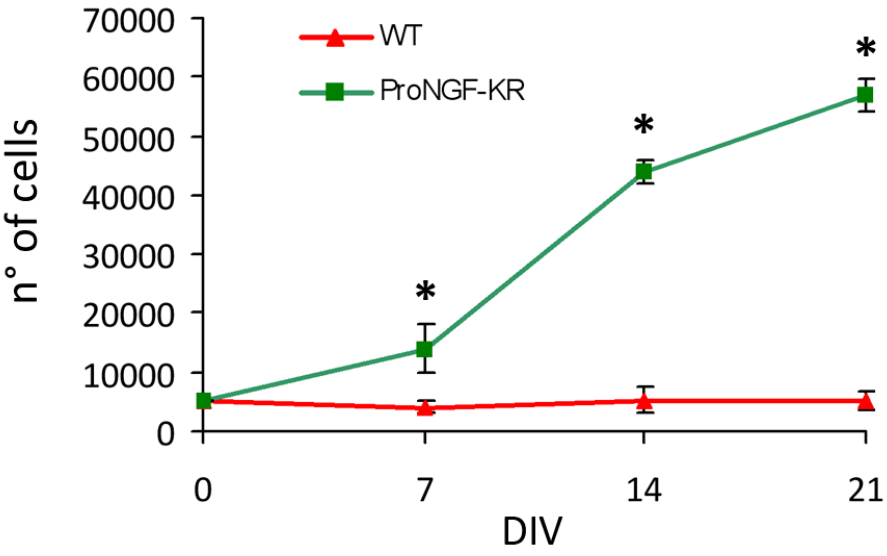


Figure 38. Proliferation curve of SVZ neurospheres derived from proNGF-KR transgenic and WT mice. *proNGF-KR cells (green line) proliferated significantly more than control (red line). Data are mean ± SEM of 3 independent experiments.* * p <0.05 *significantly different from WT, Student's t-test.*

6. DISCUSSION

6.1 proNGF and cell proliferation in the adult DG-hippocampus

6.1.a. proNGF increases cell proliferation

Here we report that proNGF increases the proliferation of neural stem cells (NSCs) of the adult mouse hippocampus. The mitogenic effect of proNGF is specifically addressed to a small subpopulation of highly $p75^{NTR}$ expressing cells, corresponding to the quiescent stem cells (RGLs) and slowly cycling, early progenitors that re-enter the cell cycle through cyclin D1 expression.

Our results are apparently in disagreement with those of a recent study [320], showing that uncleavable proNGF impairs proliferation of adult hippocampal NSCs through the reduction of cyclin E. In that work, the authors claimed a general anti-proliferative effect of proNGF in the adult mouse DG progenitors, without dissecting the specific action of proNGF at different concentration and throughout the different cell stages. Moreover, they examined cell proliferation by a vitality assay at 24 and 48 hours, while we analyzed our cultures after two weeks of treatment. In this way, we were able to reveal a novel mitogenic effect of proNGF specifically addressed to quiescent RGLs and at low proNGF concentration. Instead, high concentrations of proNGF-KR inhibits the overall proliferation of the NSCs culture, probably acting on the late progenitors that undertake neural commitment or became mature glia. We base this hypothesis on the fact that proNGF induces cell death of the mature cells of CNS [301, 303, 351]. It is also possible that proNGF-KR, at the beginning of treatment, slows down the growth of late progenitors, while RGLs are still in a quiescent state, as they need more days in cultures to be activated by proNGF. This could partially explain the difference between our data and those above mentioned.

In addition, it might be that RGLs are more sensitive to proNGF than their progeny, due to the higher expression of $p75^{NTR}$ in the stem cell compartment. So, at low concentration, proNGF would bind and activates only RGLs without affecting their progeny while, when present at high concentration, proNGF would arrest the proliferation of late progenitors. Interestingly, cyclin E has been recently proposed as a specific marker of quiescence, since its expression is required to maintain the quiescent state of hippocampal RGLs [352]. In this view, we can speculate that proNGF, through cyclin E downregulation [320] and cyclin D1 up-regulation (our paper), switches the RGLs from quiescence to the active state, while at later stages it affects neural maturation, probably by inducing apoptosis in neuroblasts [353].

6.1.b. Cyclin D1, proliferative increase and defective neurogenesis

We report that proNGF-KR treatment induces a significant activation of cyclin D1 in a subpopulation of hippocampal progenitors. Although proNGF is responsible for the increased proliferation of these cells, it remains to be investigated why this does not led to an increased neurogenesis. In particular, it remains to understand the final fate of the neuroblasts, derived from the highly-expressing $p75^{NTR}$ stem cells stimulated by proNGF. We know, from previously flow cytometry data obtained in our lab, that AD11 cultures contain more neuroblasts, among which there is a subpopulation of very late neuroblasts (highly expressing Dcx), compared to WT. *In vitro* differentiation shows that AD11 cell cultures give rise to βIII-tubulin positive neurons with severe atrophic morphology, suggesting that the newly formed neuroblasts do not complete their neurogenic path, probably due to the effect of proNGF. One hypothesis is that proNGF acts as an apoptotic factor for the late stage of maturation during neurogenesis. It is interesting to note that high level of cyclin D1 expression in the late progenitors might drive apoptosis in these cells, by a well-known mechanism of neuronal protection from the "cell cycle re-entry" [349]. This has been

previously demonstrated in sympathetic neurons, where programmed cell death induced by NGF withdrawal [354] is accompanied by an increase in cyclin D1 mRNA.

6.1.c. Variable expression of p75^{NTR} in aNSCs population

The differential expression of p75^{NTR} among the different stages of maturation is of considerable importance. A previous study on SVZ niche [346] showed that p75^{NTR} expression defines a population of stem or precursor cells that persist from development to adulthood and is able to respond to neurotrophin stimulation. In our study, we found that p75^{NTR} is highly expressed in the RGLs subpopulation of the hippocampus DG. These cells, selected *in vitro* by the "LIF method" show a specific mitogenic responsiveness to proNGF even in absence of other mitogens. In this view, p75^{NTR} is emerging as a marker of “stemness” in both neurogenic niches and other tissues. For instance, several tissues originating from migratory Neural Crest Stem Cells (NCSCs) have been shown to maintain a number of multipotent/bipotent undifferentiated cells that express p75^{NTR} [355-357]. In the enteric nervous system, differentiation of these cells is driven by a combination of NT-3 and other neurotrophic factors, trough up-regulation of TrkC and the concomitant down-regulation of p75^{NTR} [358], suggesting that p75^{NTR} is required to maintain the undifferentiated phenotype and survival of stem cells [359]. Thus, p75^{NTR} expression identifies cells that are Ki67-negative or slowly cycling *in vivo*, but retain high clonal potential *in vitro*, highlighting the importance of this receptor for the maintenance of a stem cells pool through the preservation of their quiescent state [360]. In this framework, our results show for the first time that the proNGF stimulation reactivates the cell cycle of a specific type of these p75^{NTR}-positive quiescent stem cells, *i.e.* the RGLs, in the hippocampal neurogenic niche.

6.1.d. LIF selection experiments and the RGLs of the DG

The interpretation of studies investigating signals involved in the proliferation of NSC is sometime hampered by the cellular heterogeneity of the cultures [361, 362]. The different cell types in the NSCs population have different responsiveness to stimuli depending on their receptor expression [362]. By selecting *in vitro* the neural stem cells with the "LIF method" we could demonstrate the existence of a population of truly quiescent stem cells derived from the DG and prove that proNGF signalling is very specific for those cells. According to some authors [363,364], in the adult hippocampal neurogenic niche there are not indefinitely self-renewable stem cells but only neurogenic precursors. Instead, our results demonstrate the existence of a long-term expandable and self-renewal quiescent subpopulation of stem cells, in line with the original classification of Palmer in 1997 [365]. The ability of these cells to survive in the absence of mitogens (likely entering in the in quiescent, G_0 phase) and to re-enter the cell cycle when mitogens are re-added to the culture is clearly a feature of quiescent stem cell. Moreover, the co-expression of GFAP and Nestin and the morphological analysis strengthen this concept by identifying them as Radial Glia.

Our experiments of *in vitro* selection show that the signalling we are analysing is something of very specific. An important consideration it must be done in comparing different study on proliferation of so much heterogeneous cultures. Based on what variable are under analysis (Vitality, Fold Increase, BrdU incorporation) and timing of analysis, different results can arise. In literature became increasingly clear the wide difference, at gene expression level, between the cellular type in the culture of NSCs [361, 362], so, at date the study of the signaling governing these cultures would require a specific focus on what is under analysis. The different cell types in the adult NSCs population have different responsiveness to stimuli because they have diversity in receptor expression. For example, it is to consider that classically,

in the NSCs medium there are EGF and bFGF as mitogen factors but, laking one of the specific receptors, not all the cell stages are responsive to all at any time during the maturation [362].

Of considerable importance is the fact that the "LIF selection method" allow us to confirm the existence of a population of truly quiescent stem cells derived from hippocampus. Indeed, at date some authors discuss that, in the adult hippocampal neurogenic niche, there are neurogenic precursors but not indefinitely self-renewable stem cells. Bull and Barlett (2005) showed that *in vitro*, these precursor of hippocampal derivation, are BDNF responsive cells, factor that is necessary for this amplifying cells to produce neurons during proliferation (for which EGF and bFGF is always request) *in vitro* [363]. They deny to assign to these cultures to contain a pool of truly stem cells on two bases: 1) The negative result of the Neural-Colony Forming Cell Assay (N-CFCA), that distinguish between colonies formed from extensively proliferating stem cells and more restricted progenitors, based on the extent of proliferation over a 3 week and subsequent colony size, with only the very largest colony (<1.5 mm diameter) being found to originate from a stem cell [366]; 2) the revelation of bFGF as critical mitogenic signal for expansion of this hippocampal derived cultures, rather than EGF. This is of relevance because bFGF is identified as a factor that stimulate the adult neural progenitors, with central role in signaling for hippocampal neurogenesis [364], instead EGF is emerging to control the early cell stages. For exemple in a study, EGF is found to increase the production of astrocyte (closely near to SVZ stem cells at lineage level) from rostral SVZ stem cells, but bFGF stimulated olfactory neuron production [367]. More precisely, the late neural precursors are responsive to both mitogens, but bFGF give a stronger stimulation. In our LIF experiments, a subpopulation of NSCs restarts to proliferate after a period of interrupted cell cycle in total absence of mitogen factors. The majority of cells dies during this period, but the 1% of cells that survive and are able to restart cell cycle, when mitogen factors are added, are properly showing features of truly stem cells (the capaci-

ty to enter in G_0 phase to survive during a period of negative environmental conditions, then re-enter in G_1 phase under optimal conditions). The co-expression of GFAP and Nestin and the morphological analysis strengthen this concept by classifying them in particular as Radial Glia. So, differently from Bull and Barlett (2005), we are addressed to assert the existence of a long-term expandable and self-renewal quiescent subpopulation of stem cells in the hippocampal neurogenic niche, in line with the original classification of Palmer (1997) [365]. Another aspect at favour of this position in our observation (data not shown) that neurospheres derived from LIF-selected cells, growing with EGF+bFGF over the time of our clonal analysis (5 days) continue to become larger and can reach, in 2 weeks, very big dimension (visible in the medium without microscope) probably until up the 1.5 mm of diameter needed to ascertain the stem cells feature in the N-CFCA. Indeed, as explained in *Materials and methods*, we used LIF also to restore the self-renewable capacity of our DG-aNSCs cultures when they are forthcoming to culturing collapse.

Someone could argue that the effect of LIF is not only to preserve the vitality of the stem cells pool during the non-mitogenic selective phase, but is, in absence of mitogen factors, to directly drive some late stage cell to regress to the quiescent and long term self-renewable state, even criticizing the original existence of quiescent stem cells pool in the primary DG-aNSCs cultures, rising the idea that our RGSCs obtained *in vitro* do not reflect an *in vivo* reality in the DG. Actually, it is demonstrated that LIF regulate many aspects of the aNSCs biology *in vitro*, that would let to think to a possible transition from late to early stage of aNSCs. First of all, Pitman *et all*. (2004) show that LIF strongly induce GFAP expression in aNSCs and that in a mitogenic context (EGF+bFGF) it increases the clonogenicity of the culture without exerting survival or proliferating effect [362]. They point out that this induction seems independent of the lineage commitment process, so not only at charge of cells addressed in the glial lineage. Finally, they argue that, the mechanism by which signaling pathways induced by EGF, bFGF, and LIF-related cytokines could interact to

maintain the stem cell state have emerged. LIF synergizes with EGF and bFGF in up-regulation of Notch1 [368], and Notch1 signaling, in the context of mitogen stimulation is well known to subserve this function by blocking the neural commitment of a part of population, in a lateral inhibition mechanism manner [134, 136, 369]. In aNSCs lineage, similar to the ultimate stage of nervous system development, when a cell is blocked to take the neural commitment, it remains in a transit amplifying state more similar to an activated glial cell. Indeed, in the absence of EGF and bFGF, Notch1 signaling impairs neuronal generation and enhances astrocytes formation [370]. As it is known that aNSC have glial features, and according to modern models, RGLs is considered to be a subpopulation of astrocyte with direct lineage derived from the end of nervous system development [371, 372], and, the concept of glia as neural precursor are expanding over the spatial limitation of the neurogenic niche [373], for example with the characterization of the Reactive Astrocytes [374], it is probably that the effect of LIF in absence of mitogen factors, is to convert some amplyfing cells, that do not die despite the mitogen absence, in RGLs by Notch1 up-regulation. However according to us, such eventuality does not change the relevance of our model of RGLs as representation of an *in vivo* situation. First of all because we report the presence of p75^{NTR}-high expressing GFAP^{+}/Nestin^{+} cells in *ex vivo* immunolabeling of freshly digested DG, so the same features that we found in our LIF-selected cells. The existence of an *in vivo* widespread LIF signaling in the adult nervous system is emerged [375], and its receptor is known to be well expressed in NSCs, despite LIF action in the adult neurogenic niche it has been confirmed much as a part of immune response systems rather than of physiological circuit [376]. A study shows that LIF alters the expression of a wide set of interesting gene patterns in NSCs, and all this alteration are consistent with modulation of immune responses, cell-cycle regulation and self-renewal improvement, always lying in a range of physiological cellular features (so, not leading transformation). Of note, for example, LIF exposure does not enhance telomerase expression, suggesting that LIF-dependent increase in growth rates and

clonogenicity improvement was not due to greater telomerase activity [377]. So, the natural mechanism by which adult stem cells senesce, that allow to avoid neoplastic transformation, is not altered. Therefore, it is much probably, that the use of LIF in the adult DG-aNSCs, is equivalent to expose cells to one of the factors the usually regulate their biology and that is a part of the complex system of factors involving also EGF, bFGF, NT-3, BDNF and, as reported by us, proNGF and NGF.

LIF receptor is known to be expressed in NSCs during development [378, 379], and previous data reported that LIF modulates NSCs self-renewal through the possible transition from late to early stage of progenitors [337]. Thus, it might be argued that the RGLs obtained *in vitro* after the LIF selection would not reflect a similar counterpart *in vivo* in the DG, but rather represent an *in vitro* artefact due to the ability of LIF to directly drive some cells at later stage to regress to the quiescent and long term self-renewable state. Indeed, we demonstrate the presence of $GFAP^{+}$/$Nestin^{+}$ cells expressing high levels of $p75^{NTR}$ (and with the same morphological features of our LIF-selected cells) in the DG-HP of WT animals by *ex vivo* immunolabeling of freshly dissociated tissue. Of note, the existence of an *in vivo* widespread LIF signaling in the adult nervous system has recently emerged [375], supporting the relevance of our finding.

6.3 Technical application and perspectives for the mitogenic activity of proNGF

6.3.a. The use of proNGF for producing adult hippocampal NSCs from single animal

Given the difficulty to produce NSCs from single adult hippocampus, the positive effect of proNGF on primary neurospheres formation is very important. As known for the SVZ, mitogens in the neurospheres protocol (EGF and bFGF) do not

support the proliferation of the quiescent stem cells, as these do not express EGFR [362] and because bFGF is more effective on late neural progenitors. This could explain the difficulty in isolating NSCs cultures from single adult hippocampus with current methods, since quiescent stem cells would not be stimulated by EGF and bFGF, and because Type 2 cells of hippocampus have a more limited expansion potential than their SVZ counterpart. So, the identification of a factor, like proNGF, that specifically stimulates the quiescent stem cells is very important. However, further investigations will be required, in terms of timing of treatment and proNGF concentration, for a better optimization of this protocol. Such eventual new protocol will need to be ameliorated for what concerns timing and concentration of treatment. Indeed, the treatment with proNGF-KR improves the number of neurospheres forming units, but seems that does not allow long-term propagation, probably because of its negative effect on the late amplifying cells that are necessary for culture propagation, as they exert a fundamental paracrine neutrophic signaling.

6.3.b. Mitogenic effect of proNGF-KR on iNSCs

We demonstrate the mitogenic role of proNGF also for the inducible NSCs (iNSCs). iNSCs technology is a powerful tool for studying neural development and neurological disorders, both *in vitro* and in animal model [336, 380]. Indeed, they are able to fully differentiate into mature neurons *in v*itro, and when transplanted into the mouse brain iNSCs successfully grafted and committed to neural lineage also *in vivo*. Importantly, on the issue of iNSCs safety, no sign of tumorigenesis was observed post transplantation *in vivo* [336, 380]. One main obstacle for an efficient use of iNSCs is their limited expansion potential, being composed mainly of late multipotent and late neural precursors that soon reach senescence [350], while earlier progenitors are poorly represented in the culture. In this view, the hereby demonstrated mitogenic effect of proNGF-KR on iNSCs cells is of valuable importance, as it can be

exploited as a new protocol aimed at expanding this population of cells prior to their differentiation into neurons. Moreover, the results obtained with iNSCs not only confirmed the mitogenic role of proNGF-KR on NSCs, but also allowed me to unravel the proNGF-KR specificity of action on the early progenitors even more precisely, respect to the DG-aNSCs experiments. Indeed, proliferation of mouse iNSCs cultures seems to be continually stimulated by proNGF-KR, unlike DG-aNSCs. It is important to point out that the two system are very different between them, with iNSCs cultures presenting a very large portion of neuroblasts and late multipotent stages compared to DG-aNSCs (50.1% Vs 9.1%). For this reason, due to their cell composition, iNSCs cultures have a very low expansion potential but, as reported above, are more prone to neural differentiation. My results show that proNGF-KR treatment leads to a great enrichment of the early stage progenitors at expense of the late ones. This is consistent with the fact that mouse iNSCs chronically treated with proNGF-KR overcame passage 9^{th} of culture (that we have identified as the limit of expansion potential for them) and seem to take the way of long-term self-renewal. As reported in the clonal analysis at low density, yet in the firsts passages, proNGF-KR allows expansion of few clones that reach dimension 6 folds major than untreated ones, while the majority of cells in culture do not give rise to new neurospheres at any passage, with no difference between proNGF-KR treated and untreated. This proliferation dynamic reflects the cell composition of the mouse iNSCs, which are mainly composed of late multipotent and neural precursors (that do not give neurospheres in the sub-culturing passages and do not respond to proNGF-KR), and few early progenitors, that we report are clearly stimulated to proliferate by proNGF-KR. I can thus conclude that mitogenic effect of the proNGF-KR is specifically addressed to the early progenitors.

The differentiation capacity of iNSCs after proNGF-KR treatment remains to be investigated in detail. For instance, we do not know whether the combination of LIF-selection and proNGF treatment can further improve the enrichment in the ear-

ly, expandable progenitors, as we did with DG-aNSCs. Also, we do not know whether we could obtain with this method an *in vitro* model of quiescent cells with NSC features (Astrocytic Like), in order to better control the maintenance and the expansion of iNSCs, with the perspective to be exploited in regenerative medicine studies.

6.4 Cyclins D1, E and proNGF/NGF equilibrium in the adult hippocampal neurogenesis

6.4.a. Cell cycle regulation during neurogenesis

The results of this thesis, and their comparison with previous published data, shed new light on the role of proNGF/NGF signaling equilibrium and their interplay with the related cyclins in regulating adult hippocampal neurogenesis. As mentioned before, Guo *et al.* [320] reports that proNGF treatment decreases cell proliferation of hippocampal progenitors through the reduction cyclin E protein level, which in turn results in a block of the cell cycle in the in G0/G1 phase. Thus, proNGF would affect cell proliferation by preventing cyclin E to exert its nominal function to promote the G1 to S phase transition. Actually, this could be what occurs in all NSCs that are in the amplification stage, but not in the RGLs. Indeed, it has been recently shown that the hippocampal RGLs stay in a quiescent state thanks to the expression of cyclin E, that is defined as a specific marker for quiescence of these cells [352]. So, the reduction of cyclin E protein level after proNGF treatment could result in the exit from the quiescent state, for what concerns the small subpopulation of RGLs, while in the amplifying NSCs a decrease of cyclin E would induce a slowdown or a block of the cell cycle. Guo et *al.* [320] also demonstrated that proNGF signaling, that involved cyclin E and cyclin D1, is $p75^{NTR}$ mediated. I have previously mentioned (see Discussion 6.1.c. section) that, in many tissues, $p75^{NTR}$ is a marker of quiescence, and the interplay between neurotrophins and the quiescent state of stem cells is still object of in-

tense study [359]. According to some authors [359, 360] $p75^{NTR}$ is important in maintaining stem cells in the undifferentiated state, as its expression levels correlate with the staminal potential of the cells (pluri-, multi-, bi- or uni-). Interstingly, one study demonstrates that NGF-mediated activation of $p75^{NTR}$ signalling pathway in HEK293 cells associates with the reduction of cyclin E levels by transcriptional repression [381].

For what concerns neuronal cells, cyclin E has been showed to have a role other than cell cycle regulator. For instance, cyclin E is highly expressed in mature hippocampal neurons, where, in complex with Cdk5, plays a critical role in synaptogenesis initiation [382]. In this view, cyclin E could play the same role of "cell-cycle stabilizer" by keeping in the G0 phase the two ends of the neurogenic path, *i.e.* the RGL and the late neuroblast/mature neuron. Indeed, both RGLs and neurons need to be kept out of the cell cycle, in a tight and controlled manner (such as the well-known mechanism of "apoptosis for cell cycle re-entry" [353]) (Fig.39).

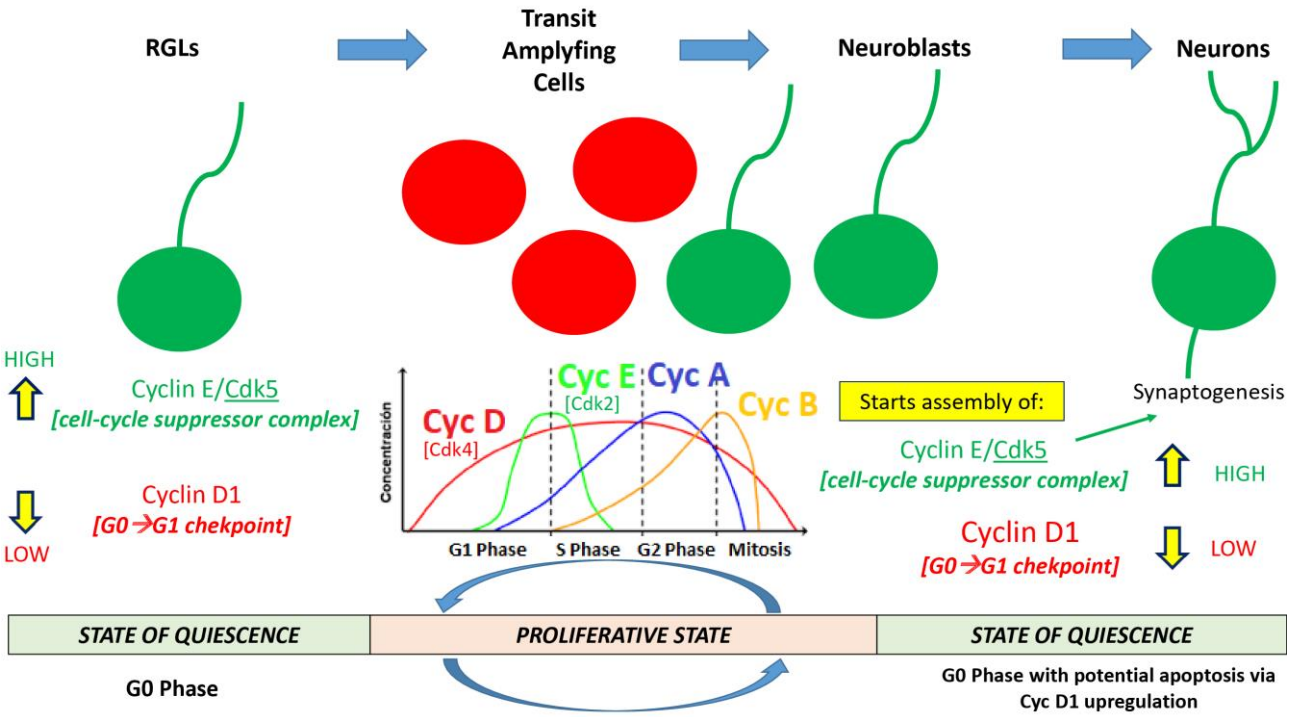


Figure 39. Hypothetical cyclin E and cyclin D1 dynamics during adult hippocampal neurogenesis.
In the begin of neurogenesis (i.e. in the RGL) cyclin E have a stably high expression level and acts as cell-cycle stabilizer, probably involved in cell-cycle suppressor complex with Cdk5. In this stage, cyclin D1 is instead not expressed. The RGL leaves the G0 phase and enter the G1 phase, starting the cell-cycle, when cyclin D1 expression is induced. From this point begin the amplifying stage of neurogenesis and all cyclins undertake their classical cell-cycle progression dynamic. Cyclin E, for example, take its classical role in G1/S transition, involved in active complex with Cdk2. It reaches a peak of expression in G1/S transition, then quickly disappears during S phase. Cyclin D family are globally important during all the cell-cycle. Meanwhile, cells mature and at one point, in the neuroblasts start assembling of the cell-cycle suppressor complex Cyclin E/Cdk5. When this stabilizer complex reaches an adequate level, cell is completely out of the cell-cycle and in this state of neuronal quiescence, the cyclin E/Cdk5 complex is determinant for synaptogenesis in newborn neurons. Finally, in the terminal maturation stages, cyclin D1 represent always the checkpoint to leave the G0 phase and, its induction activates the mechanism of apoptosis.

The radical difference between these type of cells would be in their response to the reduction of cyclin E protein level: cell cycle re-entry in the RGLs, which in turn would activates the neurogenic cascade by the intense proliferation of the amplifying progeny (which would show the classical cyclin E dynamic during cell-cycle, so whit peak in G1/S transition); block of maturation in the late neuroblasts, such as synaptogenesis; apoptosis in the mature neurons. At the same time, the increase of cyclin D1 protein levels in the RGLs, also caused by proNGF signalling, as demonstrated for the first time in this thesis, contributes to sustain RGLs activation. According to this general picture, at the beginning of neurogenesis proNGF would act as cell-cycle activator of the RGLs, by decreasing cyclin E and increasing cyclin D1;

later on, when the amplifying progenitors should undergo their terminal maturation and high level of cyclin E are required, the persistence of proNGF signalling, by keeping cyclin E low and thus preventing the neuronal differentiation, would rather affect the final phases of neurogenesis. So, in the late stages, the decrease of cyclin E level caused by proNGF could drive apoptosis in the neuroblasts, as a mechanism of cell-cycle control, to avoid that these cells, unable to differentiate, remained exposed to a persistent and thus uncontrolled mitogenic signal.

The same mechanism of action could be envisaged also *in vivo*, where proNGF, locally secreted in the hippocampal neurogenic niche, would activate, in a paracrine manner and through $p75^{NTR}$, the subpopulation of RGLs by reducing cyclin E level. proNGF signalling would thus activate the cell cycle by increasing cyclin D1 in the RGL, while later on it would drive cell death of the late amplifying and neuronal progenitors, as a protective mechanism aimed at preventing the production of neurons incapable to integrate and potentially cycling. Thus proNGF, to be functional effective for adult neurogenesis, may need of an adequate balancing support of mature NGF, which should increase cyclin E level in the late stage. Such a general picture could explain why *in vivo,* in the DG of AD11 mice (that can be viewed as a model of proNGF/NGF unbalance in favour of proNGF) I found increased proliferation but reduced neurogenesis. So, *in vivo*, the paracrine signal of proNGF needs to be counterbalanced rapidly by mature NGF, in order to "calibrate" proNGF effect on the late stages, thus allowing terminal differentiation of new neuroblasts and survival of new neurons (Fig. 40).

6.4.b. Dose-dependence of the proNGF-KR effect and the NGF hypothesis

The effect of proNGF-KR on proliferation, which is opposite to that of NGF and is cell-type specific, is also dose-dependent. This underscores the importance that *in*

vivo proNGF and mature NGF coexist and their ratios are subject to a complex homeostatic regulation [341, 383]. Moreover, proNGF and NGF mixtures can exert actions that neither exerts alone [384]. WT hippocampal NSCs proliferate less than AD11 but, unlike the latter, produce mature differentiated new βIII-Tub^{+} neurons. Accordingly, we propose a model for the modulation of adult hippocampal neurogenesis by the NGF system (Fig. 40), whereby proNGF locally produced in the neurogenic niche stimulates cell cycle activation in the quiescent stem cells and slowly dividing early Type-2a through p75^{NTR}, whereas the mature counterpart (NGF) is required to modulate cell proliferation of late progenitors and to drive their final neural maturation by binding to its high affinity receptor TrkA. The differential expression of p75^{NTR} and TrkA in our hippocampal progenitor culture (p75$^{NTR\ high}$/TrkA^{low} in early and p75$^{NTR\ low}$/TrkA^{high} in late progenitors) is in favour of our model. Our result highlight the overall importance of an adequate proNGF cleavage, in which the mature NGF acts as "calibrator" of the proNGF effect for a functional neurogenesis in the DG of hippocampus.

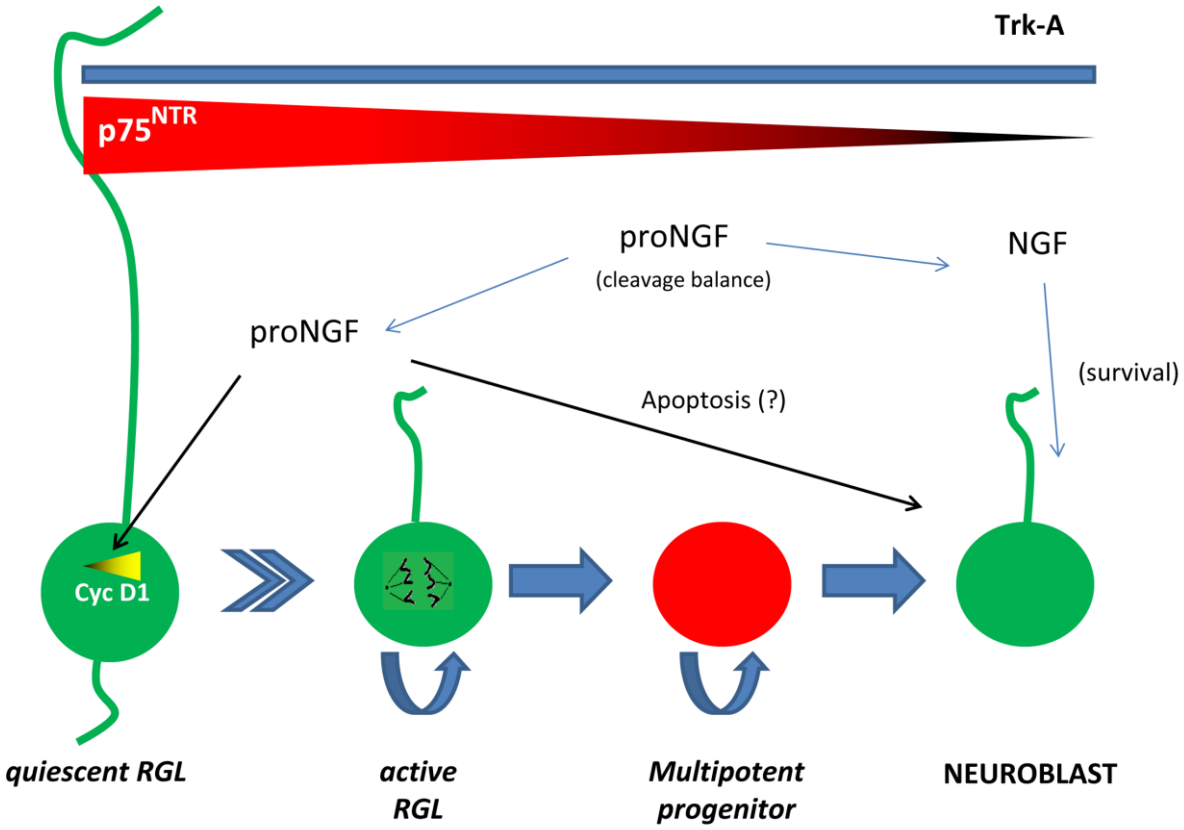


Figure 40. Possible mechanism of proNGF action in the hippocampal neurogenic niche.

The scheme represents the maturation from quiescent RGLs to neuroblasts. $p75^{NTR}$ protein level decreases during the maturation path. proNGF produced within the niche is partially cleaved to release mature NGF, while some proNGF remains unprocessed. This portion of proNGF switches the quiescent RGLs to the active state, by CycD1 induction. Mature NGF acts at the end of the maturation path by promoting neuroblast survival. Finally, in our hypothesis, proNGF drives programmed cell death in neuroblast, so the incessant regulation of proNGF cleavage is determinant for a balanced neurogenesis.

7. CONCLUSIONS

In summary, in this thesis I demonstrated that proNGF plays a critical role in hippocampal neurogenesis. It specifically acts as mitogen on RGLs (resident and tissue-derived), which express high levels of $p75^{NTR}$, and respond to proNGF re-entering cell cycle by increasing the cyclin D1 expression. The mitogenic effect of proNGF needs to be counteracted by mature NGF, which, conversely, is required for neuroblasts survival and differentiation. Thus, a fine balance between proNGF/NGF signaling is critical for a correct hippocampal neurogenesis. In fact, in an *in vivo* system of proNGF/NGF unbalance in favor of proNGF, *i.e.* the anti-NGF transgenic AD11 mice, hippocampal neurogenesis is severely compromised, with a drastic reduction of mature newborn neurons, despite the initial increase in cell proliferation specifically driven by driven by proNGF signaling.

In addition, I have further demonstrated the mitogenic property of proNGF in another cell system, the induced Neural Stem cells (iNSCs), which opens new perspectives for the implementation of cell-reprogramming protocols. The combination of proNGF, NGF and LIF for the establishment of highly expandable, genetically stable and always competent for neural differentiation of adult NSCs will offer the great opportunity to analyze their properties in terms of proliferation, differentiation and maturation on large scale, both in physiological and pathological context.
These results warrant further investigations into the role of proNGF/NGF signaling in neural stem cells biology, in the view of developing future therapeutic approaches based on the stimulation of endogenous adult neurogenesis or on cell-reprogramming protocols.

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Papers published:

1) **“ProNGF is a cell-type specific mitogen for adult hippocampal and for induced neural stem cells”**

Corvaglia V, Cilli D, Scopa C, Brandi R, Arisi I, Malerba F, La Regina F, Scardigli R and Cattaneo A (Stem Cells, 2019 May 27)

2) **“Impaired adult neurogenesis is an early event in Alzheimer’s disease neurodegeneration, mediated by intracellular Aβ oligomers”**

C. Scopa, F. Marrocco, V. Latina, F. Ruggeri, V. Corvaglia, F. La Regina, M. Ammassari-Teule**,** S. Middei, G. Amadoro, G. Meli, R. Scardigli and A. Cattaneo
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