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# Neural Progenitor Cell-astroglia cross-talk: involvement of the NF-κB p50 subunit.

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# Chapter 1.

## 1.1 Adult neurogenesis

Adult neurogenesis is a process of structural plasticity characterized by *de novo* generation and integration of new neurons into the existing circuitry in the adult central nervous system (CNS) (Ming and Song 2005; Christian et al. 2014). Basal level of neurogenesis occurs in two discrete regions: the subventricular zone (SVZ) in the lateral wall of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampal region (Lois and Alvarez-Buylla 1994; Kempermann and Gage 2000). These two areas, referred to as "canonical" sites of active adult neurogenesis, have been found in all mammalian species studied so far, including primates and humans (Eriksson et al. 1998; Gould et al. 1999; Knoth et al. 2010). The adult neurogenic process comprises a series of sequential developmental steps spanning from proliferation, survival, fate specification, neuronal migration, maturation and functional integration of neuronal progeny. The source of new neurons is the adult neural stem cell (NSC) and their progeny of neural progenitor cells (NPC), which were first isolated from the adult CNS of rodents by Reynolds and Weiss (1992) and Kilpatrick and Bartlett (1993). Their pioneering work on SVZ-derived NSC was pivotal, given that they reported methods for culturing and growing neural stem/progenitor cells (NSPC) in the neurosphere culture assay, where individual cells proliferate and generate clusters of cells in suspension. Additionally, these scientists obtained adhesive cultures of NSPC, grown as monolayers on coated substrates (such as laminin). In these proliferating conditions, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were defined as the primary mitogens used to propagate adult neural progenitors *in vitro* (Reynolds and Weiss 1992; Kilpatrick and Bartlett 1993). These studies established the basic protocols for keeping and maintaining NSPC self-renewal property among many passages, as well as their multipotentiality. Under these conditions the differentiation ability of NSPC is maintained over many passages and can be observed upon growth factor removal, when the molecular machinery drives the differentiation toward the

neural lineages, neurons, astrocytes and oligodendrocytes. Later on, similar protocols were also developed to expand and differentiate NSPC derived from the SGZ of the hippocampus (Gage et al. 1995).

The adult rodent SVZ contains four distinct cell types defined by their morphological features, molecular markers and electrophysiological properties: (1) migrating neuroblasts, type A cells; two distinct populations of NSPC, type B (2) and type C (3) cells; and (4) ependymal ciliated (type E) cells, that separate the SVZ from the ventricular cavity (Lledo et al. 2006). Type B cells are slow cycling cells lining the border between the striatum and the lateral ventricle and expressing the astrocytic marker glial fibrillary acidic protein (GFAP). Type B cells give rise to rapidly dividing intermediate progenitors, type C cells, that lose GFAP expression, and acquire distal-less homeobox-2 (Dlx-2). From type C cells type A cells arise, expressing PSA-NCAM (poly-sialylated neural cell adhesion molecule) and the neuroblast marker doublecortin (DCX) (Alvarez-Buylla and Garcia-Verdugo 2002). Neuroblasts leave the SVZ and move along the rostral migratory stream (RMS) in chains formed by glial cells before terminating into the olfactory bulb (OB), where they differentiate into granule and periglomerular interneurons (Doetsch et al. 1999) (Figure 1). Herein integrated newborn interneurons make local contacts and modulate sensory information. The current hypothesys is that they are involved in changes in the neural representation of an odor, thereby supporting long-term olfactory memory (Sultan et al. 2010).

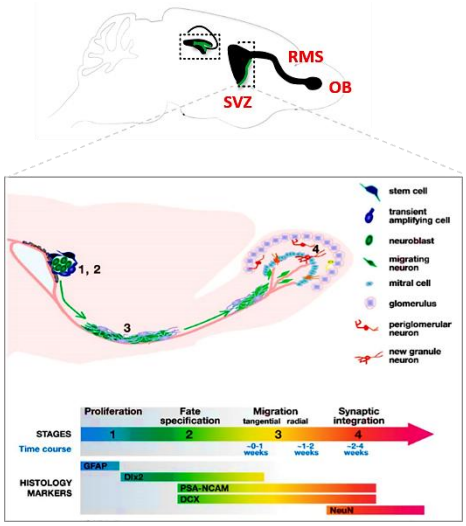


**Figure 1. Adult neurogenesis in rodent SVZ/olfactory systems undergoes four developmental stages.** A schematic illustration of the adult mouse brain with an enlarged view of the olfactory system. Four developing stages define SVZ neurogenesis. Stage 1. Proliferation: stem cells in the SVZ of the lateral ventricles give rise to transient amplifying cells (type C cells). Stage 2. Fate specification: transient amplifying cells differentiate into immature neurons or neuroblasts (type A cells). Stage 3. Migration: immature neurons migrate in chains through the RMS to the OB. Stage 4. Synaptic integration: immature neurons differentiate into either granule neurons (orange) or periglomerular neurons (red), (Modified from Ming and Song 2005).

Similarly, in the SGZ, stem cells termed type 1 cells, display a radial glial morphology and give rise to intermediate progenitors (type 2a and type 2b cells) which express neuronal specific markers before moving to the dentate granule cell layers where they integrate into the trisynaptic hippocampal circuit (Seri et al. 2001, 2004; Bonaguidi et al. 2012). The detailed developing process of adult hippocampal neurogenesis is described in the following section.

In addition to neurogenesis occurring in the two forebrain regions, emerging evidence arose for a novel neurogenic site along the walls of the third ventricle, in the hypothalamic area. The hypothalamus is a small brain area that surrounds the third ventricle and contains distinct nuclei. It serves as a central homeostatic regulator of numerous physiological and behavioural functions, such as feeding, metabolism, body temperature, thirst, fatigue, aggression, sleep, circadian rhythms, and sexual behaviour. Several studies using 5-bromo-2'-deoxyuridine (BrdU) as well as genetic lineage tracing techniques reported constitutive neurogenesis in the adult hypothalamus of mammals, including rats and mice (Kokoeva et al. 2005; Pierce et al. 2010; Lee et al. 2014). Under unstimulated

conditions, the hypothalamus displays a low rate of neurogenesis and it is suggested that the *de novo* formation of newborn neurons might serve as a compensatory mechanism contributing to homeostatic control of dietary and energy balance (Yon et al. 2013).

Within the adult hypothalamus, cells around the third ventricle form neurospheres, a hallmark of NSC. Cells like tanycytes, ependymocytes, subventricular astrocytes and parenchymal glial cells, all reside near the third ventricle and contribute to the neurogenic potential of the hypothalamus. Hypothalamic tanycytes constitute the main cell type lining the ventral third of the third ventricle, and resemble radial glia. Within the hypothalamus, tanycyte cell bodies are localised in the central and posterior hypothalamus, at the level of median eminence. Morphological studies have mapped and defined two subpopulations of tanycytes according to their position and process projection: i) β-tanycytes, lining the median eminence (ME); ii) α-tanycytes, extending into the arcuate nucleus (ARC) and ventromedial nucleus (VMN), lining the ventricular zone. Robins and colleagues (2013) have reported that α-tanycytes have NSC features and act as neural progenitors in the hypothalamus. It was demonstrated through a series of *in vitro* and *in vivo* experiments in adult mice (using a GLAST::CreER$^{T2}$ mice), that GLAST$^{+}$ (glutamate transporter) α-tanycytes are self-renewing cells, that constitutively give rise to new tanycytes, as well as astrocytes and neurons (Robins et al. 2013) (Figure 2).

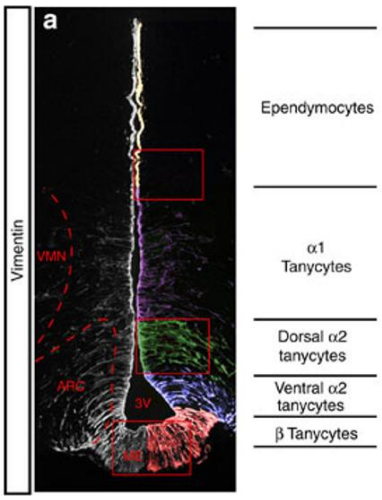


**Figure 2. Distinc tanycyte populations line the hypothalamic third ventricle**. Left hand panel: coronal section through third ventricle (3V), immunolabelled with Vimentin (intermediate filament marker, expressed by all tanycytes). The position and projection of the Vimentin$^{+}$ process defines different tanycyte subsets: β-tanycytes line the median eminence; α2-tanycytes reside adjacent to the arcuate nucleus; α1-tanycytes extend from the level of the ventromedial nucleus to the dorso-medial nucleus. VMN, ventromedial nucleus; ARC, arcuate nucleus; ME, median eminence. (Modified from Robins et al. 2013)

### 1.1.1 Adult hippocampal neurogenesis

Large efforts have been made in order to understand the function of adult-born neurons both in physio- and pathological conditions. The enormous interest in hippocampal neurogenesis was mainly due to the involvement of this brain region in cognition, and in particular in learning and memory (Aimone et al. 2010). In the hippocampus, neurogenesis is localized within the SGZ in the dentate gyrus, where excitatory granule cells are continuously produced throughout life. The adult SGZ is a narrow band of cells made only of one to three nuclei. The SGZ contains the cell bodies of radial glia-like cells, with processes extending to the densely packed granular cell layer and to the molecular layer (Nicola et al. 2015). The DG has a complex local circuitry, with both inhibitory interneurons and excitatory feedback neurons (mossy cells) constituting the network. The sensory information from specific cortical areas converge to the entorhinal cortex and flows through medial and lateral perforant pathways to the dentate DG, then to CA3 pyramidal cells via mossy fiber axons of granule cells, then to CA1 pyramidal cells via Schaffer collateral projections

of CA3 and back to the entorhinal cortex and subiculum regions, closing the "hippocampal loop" (Aimone et al. 2014).

According to a recent publication by Kempermann and colleagues, adult hippocampal neurogenesis can be divided into four phases, defined as precursor cell phase, early survival phase, postmitotic phase and late survival phase (Kempermann et al. 2015) (Figure 3). The proposed model goes through different developmental stages, identified based on cell morphology and protein marker expression (Kempermann et al. 2004). In general, from a radial glia-like precursor cell, adult neurogenesis progresses throughout three identifiable progenitor stages to a postmitotic maturation phase ending with the formation of a mature granule cell.

In the adult SGZ, four developmental stages define the precursor cell phase of NSPC pool expansion. *In vivo* clonal studies have revealed that radial glia-like precursors are multipotent stem cells capable of repeated self-renewal and generation of both neurons and astrocytes, but not oligodendrocytes, in the adult hippocampus (Bonaguidi et al. 2011). As it is, adult neurogenesis starts with a precursor cell population with morphological and antigenic properties of radial-glia-like cells, named as type 1 cells. The type 1 cells express GFAP, astrocytic marker and nestin. The cell body of type 1 cells is found in the SGZ whereas the processes extends into the molecular layer. Type 1 cells show also electrophysiological properties of astrocytes (Filippov et al. 2003). One transcription factor specifically expressed in SGZ radial glia stem cells is the SRY-related HMG box family member Sox2, which plays a key role in stem cells self-renewal and is critical for the balance between proliferation and differentiation (Julian et al. 2013). The developmental potential of type 1 cells has been demonstrated with tracing experiments (Seri et al. 2004, 2001). Radial glial-like type 1 cells divide asymmetrically, with one daughter cell giving rise to the stem cell and another one giving rise to the intermediate progenitor, or type 2 cells.

Type 2 cells or transient amplifying progenitors, are highly proliferative cells with short processes, that are oriented tangentially to the DG. Type 2 cells come in two subtypes, both nestin positive. One subtype called type-2a cells still express the GFAP, but lack the radial glia morphology, the other cellular phenotype called type-2b, is positive for the immature neuronal marker DCX. Another recently added marker for type 2 cells, is the T-box brain protein 2 (Tbr2), a transcription factor that identifies basal progenitor cells. Tbr2 is required for neurogenesis in the dentate gyrus of developing and adult mice. Tbr2 is critical for the transition from stem cells to intermediate progenitors, as it regulates Sox2 suppression (Hodge et al. 2008). Type 2 cells receive first synaptic GABAergic inputs, and respond to physiological stimuli, such as voluntary wheel running (Kronenberg et al. 2003) or pharmacological stimulation via serotoninergic-dependent mechanisms (Encinas et al. 2006) and GABA sets the pace for this regulation (Ge et al. 2007). Kempermann and colleagues propose that the fate choice decision occurs at the level of type 2a cells, given that already in type-2b cells there is expression of transcription factors NeuroD1 and Prox1, involved in neuronal fate choice (Kempermann et al. 2015).

Type 3 cells are DCX positive, but nestin negative. DCX expression extends from a proliferative stage, through cell cycle exit, for a period of two to three weeks (Couillard-Despres et al. 2005). DCX also overlaps with PSA-NCAM expression.

Next phase is the early survival phase. DCX expression still persists until next stage of newly formed neurons expressing the postmitotic marker NeuN and the transient $Ca^{2+}$ binding protein calretinin (Brandt et al. 2003). The majority of cells reaches this stage only three days after the initial division, then these immature neurons are subjected to a selection process, during which they can be eliminated or functionally recruited. Within few more days the number of $NeuN^{+}$ new neurons decreases via apoptosis (Kuhn et al. 2005). The majority of

cells are eliminated before that the functional integration is established in the CA3 region and before receiving dendritic input from the enthorinal cortex, in the molecular layer. Cells that survive retain a vertical morphology with a rounded nucleus and the apical dendrite projecting toward the molecular layer. Then cells send out their axon to target CA3 region, in what is called the mossy fiber path. The main synaptic input to these cells is still GABAergic, and GABA remains excitatory (depolarizing), until sufficient glutamatergic contact has been made (Wang et al. 2000). GABA plays different roles in NPC and their progeny. The response to GABA is depolarizing in NPC and young neurons. In NPC this depolarizing effect is necessary for calcium influx, that in turn promotes the expression of *NeuroD* gene and leads to neuronal differentiation. The absence of a strong GABAergic inhibition in immature neurons represents an important property of these newborn cells. In acute brain slices newborn neurons exhibit enhanced synaptic plasticity, due to lower induction threshold and larger amplitude of long-term potentiation (LTP) of perforant path synaptic inputs compared with mature granule cells. It is suggested that regulation occurs at the survival phase, where only stimuli that are more specific to the hippocampus can affect it as it is for exposure to an enriched environment or to learning and memory hippocampus-dependent tasks, that are known to increase survival at this stage (Gould et al. 1999; Döbrössy et al. 2003).

During the maturation phase, the depolarizing GABA has been shown to be critical for the maturation of adult-born neurons allowing the formation of glutamatergic synapses (Chancey et al. 2013). GABA remains depolarizing in young neurons until two week of age, which corresponds to the time in which glutamatergic spines develop on immature neurons. By 2.5 week of age young neurons have dendrites with spines and receive glutamatergic inputs. Once glutamatergic synaptic connections have been made, the new neurons go through a phase of late survival where increased synaptic plasticity occurs. Inhibitory inputs to adult-born neurons gradually increase with maturation. This

increased inhibition, together with the onset of glutamatergic synapses, sets up a critical period for newborn neurons in memory encoding.

The period of calretinin expression lasts for three to four weeks. Then newly formed cells switch the expression of calretinin to calbindin, that is found in all mature granule neurons (Brandt et al. 2003). At this time point the new neurons still require several additional weeks to become electrophysiologically indistinguishable from older granule neurons.

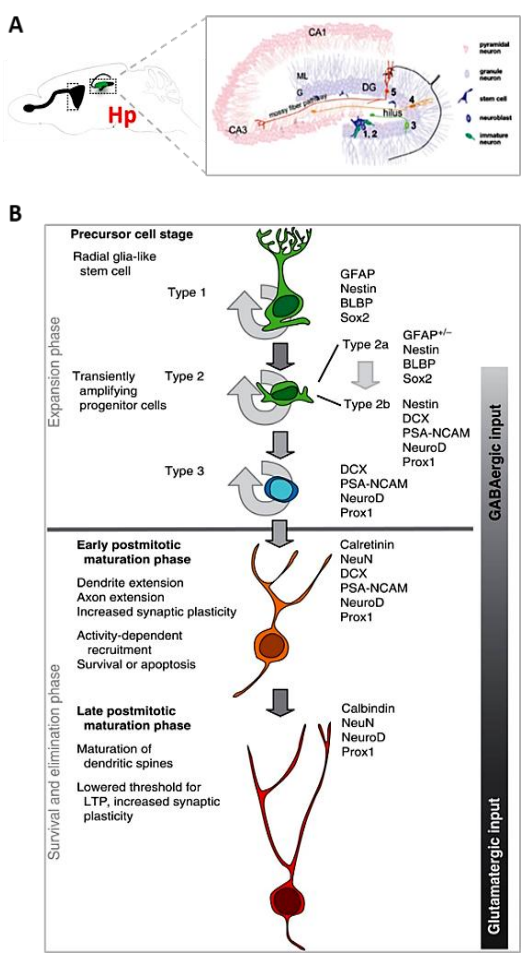


**Figure 3. Developmental stages in the course of adult hippocampal neurogenesis.** Panel **A**. Sagittal section view of an adult rodent brain and a detailed representation of the cytoarchitecture of the different layers of the hippocampus (hilus; SGZ; G, granular cell layer; ML molecular layer; area CA3 and CA1) and the hippocampal circuitry of the mossy fiber path connecting axonal projections of immature neurons to CA3 pyramidal cell layer; Schaffer collaterals (not shown) connect CA3 axon projections to CA1, (modified from Ming and Song 2005). Panel **B**. The precursor cell stage is characterized by cells with radial glia-like morphology (Type 1 cells) and three putative transient amplifying progenitor cells (Type 2a and type 2b cells and type 3 immature neuroblast). The early postmitotic phase is characterized by calretinin expression. The late postmitotic phase is characterized by terminally differentiated new granule cell, expressing calbindin. (Modified from Kempermann et al., 2015)

An intriguing question is about the functional importance of adult hippocampal neurogenesis. In other words, how newborn neurons contribute to adult brain functions. Many behavioural studies described the correlation between the

levels of adult hippocampal neurogenesis with performances in hippocampus-dependent tasks and thereby put a correlation link between neurogenesis and hippocampus-dependent processes of cognition and emotion. Adult hippocampal neurogenesis is a highly dynamic process, and many extrinsic positive modulators (i.e. enriched environment; physical activity; treatment with certain antidepressants; learning) have been reported to enhance performance in spatial navigation tasks (Morris water maze, MWM) and in tests of anxiety-related behaviour (Kim et al. 2012). On the other hand, physio- and pathological conditions like aging, stress and inflammation, have been reported as negative regulators, and result in decreased performance in tasks of spatial navigation and emotion-related behaviour. Methodological advances in conditional gene targeting improved temporal and tissue specific control of NSPC and neuronal progeny. The primary function that has been mainly associated with adult neurogenesis is pattern separation and hippocampal newborn granule neurons contribute to dentate gyrus pattern separation. Behaviourally, mice in spatial discrimination tasks (i.e. radial-arm maze; two choice discrimination task) must discriminate between two spatially proximate stimuli. Patter separation is the ability to distinguish similar stimuli, and it is linked to discrimination tasks. In has been demonstrated by several groups that by manipulating the rate of adult neurogenesis, new neurons are critical for making discrimination between close spatial location or similar environments in tests of working memory and long-term memory (Lacar et al. 2014; Vadodaria and Jessberger 2014). In this contest, Sahay et al. (2011) reported that by boosting adult hippocampal neurogenesis, utilizing transgenic mice where the proapoptotic gene BAX is conditionally deleted in nestin-expressing NSPC ($iBAX^{nestin}$ mice), enhances discrimination between similar contexts (Sahay et al. 2011). Thus, one of the current hypothesis is that adult hippocampal neurogenesis is critical for discriminating between highly dissimilar contexts, given that discrimination of proximal (in space) choices is selectively impaired without neurogenesis (two-choice discrimination task). Another possible role for adult hippocampal neurogenesis is in encoding time. Young neurons have

been shown to be suited to encode novel information better than the mature neurons (Aimone et al. 2006).

### 1.1.2 The neurogenic niche

The SGZ, as well the SVZ, form the proper microenvironment that is permissive for neurogenesis to occur. This microenvironment is called neurogenic "niche" (Morrens et al. 2012; Kempermann et al. 2015; Lin and Iacovitti 2015). Neurogenic niches can be defined as functional units within neurogenic areas, consisting of the stem cell pool, their progeny and the immature neurons, and other cellular components, like glial cells, microglia, and endothelial cells. The niche includes as well the extracellular matrix (ECM) and the vasculature and provides a milieu of non-cellular factors, such as secreted molecules and cell–to-cell contacts. All the components in the niche contributes to create a permissive microenvironment that tightly regulate neuronal development of the precursor cells. The local environment in the niche is pivotal in regulating the balance between self-renewal and multipotentiality maintenance for stem cells and for their progeny, and for controlling the different steps of lineage progression until the final commitment.

The architecture within the niche of the SGZ and SVZ differs because it reflects the location differences of the two neurogenic areas (Lin and Iacovitti 2015) (Figure 4). The SVZ is located in proximity to the cerebral ventricles and along the anterolateral ventricle multi-ciliated ependymal cells (E cells) are located. The cilium of B stem cells protrude the E cell wall and senses the cerebrospinal fluid (CSF) in order to gain access to its components, whereas the SGZ does not get in contact with the CSF.

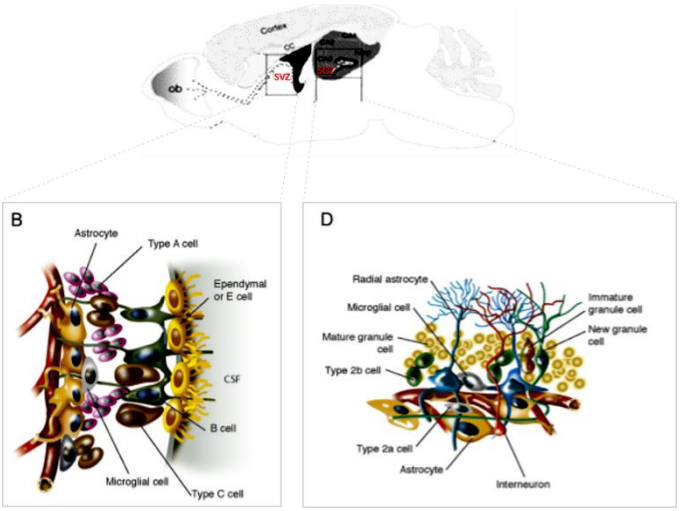


**Figure 4. Schematic representations of the cellular components that made up the canonical neurogenic niches of SVZ (panel B) and SGZ (panel D).** (modified from Lin and Iacovitti 2015)

A key factor of both neurogenic niches is the vasculature that surrounds the stem cells. For this reason, the niche is often referred also as "neurovascular niche" (Palmer et al. 2000). The vessels of the neurovascular niche lack the astrocytic end feet and the tight junctions that are found in endothelial cells of other tissues. These results in a highly permeable blood-brain barrier in the niche site (Shen et al. 2008). As a consequence, NSPC have access to both local endothelial derived factors and to systemic factors (i.e. vascular endothelial growth factor – VEGF; brain derived neurotrophic factor – BDNF; signaling components like cytokines and chemokines, etc.) (Lin and Iacovitti 2015).

Microglia are present in both SVZ and SGZ niches. They are considered important in regulating neurogenesis in both healthy and injured/diseased brain (Ekdahl 2012). The role of microglia in the niche includes phagocytosis. Sierra and colleagues (2010) showed that newborn cells undergoing death by apoptosis are rapidly cleared via phagocytosis by unchallenged microglia present in the adult SGZ niche (Sierra et al. 2010). In the healthy brain, microglia is also involved in synaptic elimination and pruning by phagocytic engulfment of synapses on mature neurons (Paolicelli et al. 2011) and by this way microglia keep the homeostasis in the presynaptic environment (Platel et

al. 2010). Altogether these functions contribute to the establishment of proper connections for newly formed neurons. Conversely, microglial activation and release of proinflammatory cytokines, upon brain inflammation, has been associated with decline in neurogenesis (Pluchino et al. 2008). Therefore, microglial activation state determines different, even opposite, effects on adult neurogenesis.

Astrocyte cells represent one of the major contributors of the neurogenic niches. Astrocytes have a broad diversity of subtypes, some behaving like stem cells (Seri et al. 2001) and some providing synaptogenic factors, that are critical for local dendritic spine maturation (Sultan et al. 2015). It has been shown that astrocytes also provide neurogenic signals, by instructing neural precursor cells to adopt a neuronal phenotype (Lim and Alvarez-Buylla 1999; Doetsch et al. 1999; Song et al. 2002). Astrocytes of the SGZ are coupled by gap junctions and create what is called the glial syncytium, by which they propagate signals and may regulate activation and differentiation of the stem cell pool. It has been shown that abolishment of gap junctions coupling, via inducible or constitutive depletion of connexin-30 and 43 in radial glia-like cells of the DG ($Cx43^{fl/fl}$/hGFAP-Cre/$Cx30^{-/-}$ mice), results in a dramatic decrease in the proliferation of precursor cells and in the percentage of newly born granule neurons (Kunze et al. 2009).

ECM molecules (i.e. laminin; heparan sulfate proteoglycans - HSPG; etc.) are also important because they modulate the signals coming from the surrounding parenchyma and blood vessels. Stem cells have laminin receptors (i.e. integrins) on their surface that interact with components of the basal lamina and this interaction helps stem cells to orient in the niche (Erickson and Couchman 2000). Moreover, HSPG of the matrix bind to mitogens like FGF, thereby regulating neurogenesis *via* ECM/growth factors interactions in structures called fractones (Kerever et al. 2007).

Astrocytes also contributes to the production of the ECM. Thrombospondins (TSP) are a family of astrocyte-secreted proteins that are components of the ECM. Previous work suggested that TSP may play a role in hippocampal synaptogenesis. In particular, TSP-1 has been included in the key neurogenic factors released by astrocytes, given that TSP-1 is able to enhance precursor cell proliferation and neuronal differentiation (Hughes et al. 2010; Lu and Kipnis 2010). In the neurogenic niche, astrocytes are as well a source of other important justacrine/paracrine signals (Notch; sonic hedgehog – SHH; bone morphogenetic proteins - BMP), neurotrophic factors (FGF2; BDNF; VEGF) and cytokines (Interleukine-1β - IL-1β; Interleukine-6 - IL-6). An interesting molecule, secreted by astrocytes is the high-mobility group box 1 (HMGB1) protein. HMGB1 is a common proinflammatory cytokine mediating inflammation, neuronal apoptosis and tissue damage. However emerging data suggest that HMGB1 appears to have beneficial effects in brain development, as it is associated with neurogenesis, in NPC survival, proliferation and neuronal differentiation via NF-κB signaling (Meneghini et al. 2013; Li et al. 2014). Altogether astrocytes may be considered as crucial contributors to neurogenic permissiveness and their contribution in CNS and neurogenesis is deeply discussed in the following section.

## 1.2 Astroglia in the central nervous system

Astrocytes are a heterogeneous cell population and represent the most abundant cells in the mammalian brain. Astrocytes exist in several morphologically different types all bearing multiple stellate processes. In the morphological classification the following phenotypes are included: i) the protoplasmic astrocytes (localized in brain grey matter), that provide para-arterial influx of nutrients, and para-venous clearance of toxic metabolites, with their perivascular endfeets; ii) the fibrous astrocytes (in brain white matter), involved mainly in the repair of damaged tissue; iii) the radial astrocytes that are the first developing cells from NSC during early embryogenesis and form a scaffold for neuronal migration and, after maturation, became stellate astrocytes. In the mammalian CNS astrocytes undergo differentiation from the NSC pool with the peak of astrogliogenesis occurring during late prenatal/early postnatal developmental (P1 to P10 in rodents), once the bulk of neurogenesis has occurred (Liddelow and Barres 2015). This switch from a largely neurogenic to a gliogenic potential of the NSC pool, reflects the temporal separation in the production of neurons *versus* astroglia. The increase in the number and complexity of glia during evolution suggests a possible function for astrocytes in human cognition (Molofsky et al. 2012). From an evolutionary point of view, both number and complexity of astrocytes increased. Invertebrates CNS contains only 20% of astrocytes, whereas human astrocytes constitute up to 50% of CNS cells, contacting up to 10-times more synapses than their rodent counterparts (Oberheim et al. 2009). Thus, human cortical astrocytes have a more complex branching profile than those of rodents. Given the astrocyte dimension in the human brain, Sloan and Barres (2014) raised the hypothesis that information processing and circuit organization modulated by astroglia may contribute to improved human cognitive abilities. Conversely, human neurodevelopmental disorders may represent an end-product of disrupted astrocyte development (Sloan and Barres 2014).

Originally astrocytes were considered mainly as passive bystanders, playing critical functions in energy metabolism, $K^+$ buffering and neurotransmitter recycling finalized to keep brain homeostasis. Nowadays, astrocytes cannot be considered anymore only as sustaining cells in the CNS, providing only metabolic and trophic support and passively maintaining neurons (Banker 1980). Great interest grew for the role of astrocytes in regulating neuron-glia interaction, in order to control several processes of brain development, like neurogenesis and differentiation (Barkho et al. 2006; Lim and Alvarez-Buylla 1999; Song et al. 2002), synapses formation (Pfrieger and Barres 1997) neuronal migration/axon guidance (Anton et al. 1997) and neuronal signaling (Fróes et al. 1999), all aspects that provide metabolic and electrophysiological interconnections between neurons and astrocytes. A vast array of data proves the importance of astrocyte-derived soluble factors in several steps of neuronal morphogenesis, ranging from precursor cell proliferation to neuronal differentiation and circuit formation (Gomes et al. 2001). Recently, the metabolic coupling between astrocytes and neurons has been considered to be essential for memory formation. The high metabolic demand for long-term memory formation is possible because astrocytes provide to neurons lactate as a source of energy, that is essential for long-term but not short-term memory formation, for synaptic plasticity and LTP (Suzuki et al. 2011).

Emerging evidence support the concept of regional differences in glia/neuron ratio across the different mammalian brain areas (Herculano-Houzel 2014). This issue highlights how important for brain physiology and function must be the interaction between glial cells and neurons.

### 1.2.1 Astroglial phenotypes in health and disease

Astrocytes become reactive and undergo reactive astrocytosis/astrogliosis in response to a vast array of CNS insults and the related mediators (such as cytokines and chemokines released from damaged surrounding neurons and active microglia) or in chronic neurological CNS diseases associated with aging, like Parkinson's, Huntington's and Alzheimer's diseases (Sofroniew 2015). In response to tissue damage or inflammation, the category of scar-forming astrocytes form scar borders that segregate the damaged tissue in the CNS. This scar-forming astrocytes demarcate the compartment where damage (trauma, stroke, or infection) has occurred, meanwhile preserving the adjacent tissue. Conversely, in response to mild or diffuse CNS insults, or in tissue that are adjacent to scar borders, astrocytes undergo hypertrophic reactive astrogliosis. The changes that are associated with hypertrophic astrogliosis vary with the nature of the triggering insult, the CNS region involved, the distance from the insult itself, and the presence of co-morbid peripheral infections (Khakh and Sofroniew 2015). Under these circumstances, reactive astrocytes display hypertrophic cell body and nucleus, and the expression of characteristic marker of astrocytes, like GFAP and S100β is upregulated. Reactive astrocytes are involved in neuroinflammatory processes through the release of proinflammatory molecules and reduced expression of genes involved in neuronal support.

I focused on two specific astrocytic players.

i) *Lipocalin-2* (LCN-2)

LCN-2 is a 24-kDa secreted protein, described first as neutrophil gelatinase-associated lipocalin (NGAL) and also known as 24-kDa superinducible protein (24p3). LCN-2 is a member of the lipocalin family of proteins, known to bind and transport hydrophobic molecules (Flower et al., 2000). LCN-2 acts as a carrier/transporter and binds to two distinct cell-surface receptors: brain type organic cation transporter (BOCT/24p3R), that is expressed constitutively and interestingly is present at high levels in astrocytes; megalin receptor, that is expressed in a wide variety of tissues and cell types, such as ependymal cells; neural progenitors in mice spinal cord; oligodendrocytes, neurons and cultured astrocytes (Lee et al., 2015).

LCN-2 has been implicated in a vast array of cellular processed ranging from cell death/survival (Devireddy et al., 2005), cell migration/invasion in cancer (Yang and Moses, 2009), cell differentiation (Bolignano et al., 2010). In physiological conditions LCN-2 is reported to have high affinity to bind and transport iron, therefore it modulates iron content into cells and this impacts on cell homeostasis (regulating proliferation *versus* apoptosis). Iron-containing (holo-) LCN-2 binds to 24p3R and is internalized, thereby increasing intracellular iron concentration. Instead, when the iron-free (apo-) LCN-2 is internalized upon 24p3R binding, it chelates iron and transfers it in the extracellular milieu, thereby impacting on cellular apoptosis (Devireddy et al. 2005) (Figure 5). As well, LCN-2 can transport iron when complexed with siderophores. This binding confers the ability to LCN-2 to work as part of the acute-phase response in innate immunity.

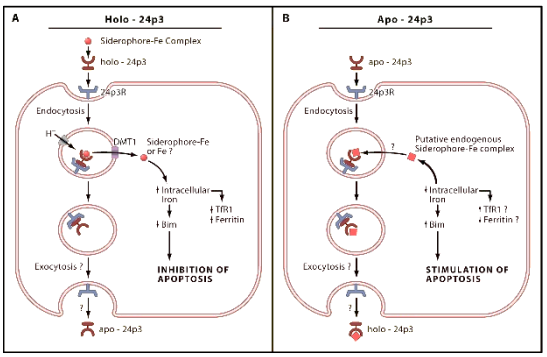


**Figure 5. Potential effects of LCN-2 iron binding activity on cellular homeostatsis.** ***A.*** Holo-24p3/LCN-2 containing the iron complex of the bacterial siderophore, donates iron to cells via the 24p3 receptor. Internalization of LCN-2 and its receptor leads to the uptake of iron from the siderophore-iron complex. Donation of iron to the cell leads to a decrease in TfR1 (Transferrin receptor protein 1) expression and an increase in ferritin levels. In addition, donation of iron to the cell prevents apoptosis by decreasing the expression of the proapoptotic protein Bim. ***B.*** Apo-24p3/LCN-2 binds to 24p3R and is internalized into the cell. Devireddy and colleagues (2005) suggest that a putative intracellular mammalian "siderophore iron complex", called catechol, becomes bound to apo-24p3 to form holo-24p3, which is subsequently released from the cell by exocytosis. Depletion of iron from the cell results in the upregulation of the proapoptotic molecule, Bim, which leads to apoptosis (Richardson 2005).

Initially, LCN-2 was proposed as an acute phase protein also in glial cells, based its role in the regulation of astrocyte death, morphology and migration. LCN-2 can be secreted by reactive astrocytes under inflammatory brain conditions. In astrocytes, released LCN-2 acts as an autocrine mediator of reactive astrocytosis. Following exposure to LCN-2, it was reported that, in parallel to increased GFAP immunoreactivity, there are also phenotypic changes in the number and lenght of cellular processes of astrocytes (Lee et al., 2015). LCN-2 upregulation also promotes astrocytes migration *via* release of chemokine ligand-10 (CXCL10), chemokine that promotes the migration of other CNS cell types. Altogether LCN-2 upregulation is consistent with the hallmarks of reactive astrocytes.

In the CNS, LCN-2 expression is described to occur in response to injury or in inflammatory conditions. On the other hand, Chia and colleagues (2011) reported the distribution and the expression of LCN-2 in normal rat brain in physiological conditions, with low levels of expression in most brain regions, comprising the hippocampus, and highest levels of expression in olfactory bulbs, brainstream and cerebellum (Chia et al. 2011). They reported that LCN-$2^+$ cells co-localize with $GFAP^+$ astrocytes of these regions.

In addition, in the CNS, LCN-2 has been suggested to play a modulatory action in cognitive functions (Ferreira et al. 2013), neuronal excitability and anxiety (Mucha et al. 2011) and depression in human beings (Naudé et al. 2013).

Interestingly, LCN-2 null mice (LCN-$2^{-/-}$) display anxious and depressive-like behaviors, cognitive impairment in spatial learning tasks and in parallel synaptic impairment in hippocampal long-term potentiation (Ferreira et al. 2013). Altogether behavioral studies, performed in order to assess animal anxiety-like behavior (i.e. elevated plus maze; light/dark box; open field test; Morris water maze), associated with hippocampal morphological analysis support the involvement of LCN-2 in emotion and cognition.

Recently, Mucha and collegues (2011) reported that both *Lcn2* gene and the encoded protein are upregulated in the hippocampus of male mice upon psychological stress (6h/daily restraint for 3days) and that LCN-2 contributes to stress-induced neuronal plasticity (Mucha et al., 2011). On one hand, they demonstrated through *in vitro* experiments that treating hippocampal neurons with lipocalin-2 (100 ng/ml), as an attempt to mimick stress induced upregulation, caused a decrease of mushroom spines (known as “memory spines”) and a concomitant increase of the thin spines (known as “learning spines”) in neuronal cultures. On the other hand, they observed that disruption of the *Lcn2* gene in mice, caused an increase in the percentage of mushroom spines and their density. These *ex vivo* data correlated also with higher anxious-

like behavior of LCN-2$^{-/-}$ mice upon stress. This scientific report suggested that lipocalin-2 might regulate brain allostasis, and act as a mechanism of cellular adaptation in response to harmful stimuli.

ii) *Thrombospondins* (TSPs)

TSPs are astrocyte-secreted glycoproteins, that participate in a vast array of biological functions as part of the ECM, and regulate cell-cell and cell-matrix interactions. The TSP family consist of two subgroups. Subgroup A includes TSP-1 and TSP-2, whereas subgroup B includes TSP-3/4/5 (Figure 6).

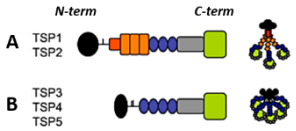


**Figure 6. Thrombospondin isoforms and oligomerization states.** TSP can be divided into two subgroups. TSP-1 and -2 (subgroup A) form trimers, whereas TSP-3, -4, and -5 (subgroup B) are assembled as pentameric proteins. All TSPs contain a variable number of epidermal growth factor (EGF)-like repeats (blue) that are contiguous with calcium binding repeats (gray) and a globular conserved C-terminal region. A distinctive characteristic of group A TSP is the presence of a pro-collagen (von Willebrand factor, red) homology domain and the three properdin-like repeats (orange). In contrast, the pentameric thrombospondins (TSP-3, -4, and -5) lack the procollagen homology domain and the properdin-like repeats and contain four (instead of three) copies of the EGF-like repeats (Modified from Eroglu et al. 2009)

Recently, it was demonstrated that TSPs promote the formation of new excitatory/gabaergic synapses in the developing CNS. This process is called synaptogenesis and is stimulated by astrocyte-secreted prosynaptogenic signals, such as TSP, and not only controlled by intrinsic neuronal mechanism (Risher and Eroglu 2012). Excitatory synaptogenesis occurs in the mammalian brain after birth, and in rodents this period occurs during the second/third postnatal weeks. TSP-1 and TSP-2 were found to play active roles by promoting the formation of excitatory synapses, and their deficiency resulted in reduced synaptic density during development (Liauw et al. 2008). The increase in synaptogenesis is mediated by the interaction between the TSP EGF-like

domains (common to all TSPs) and the α2δ-1 neuronal receptor (Eroglu et al. 2009).

Considering the critical role of TSPs in developmental synaptogenesis, it was also discovered that these ECM proteins are upregulated/downregulated in response to CNS injuries and in several neuropathological disorders. The role of TSPs was investigated in stroke recovery. It was reported that TSP-1/2 double KO mice exhibited significant reduction in synaptic density, as well as significant deficits in their ability to recover functions. Instead in WT mice TSP-1 and TSP-2 mRNA and protein expression levels increased following stroke and the proteins were found to be co-localized primarily in astrocytes (Liauw et al., 2008). These studies suggest that deficiency in TSP-1 and TSP-2 leads to impaired recovery after stroke and that TSPs are beneficial in term of synaptic formation. Altered TSPs expression has been reported in many neurological disorders. Down's syndrome (DS) is the most common genetic form of mental retardation and characterized by a reduced number of dendritic spines. DS astrocytes were found directly involved in the development of spine malformations and reduced synaptic density. This DS neurological feature has been linked to TSP-1 decreased protein expression in astrocytes (Garcia et al. 2010).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by impaired cognitive function and memory loss due to deposition of senile plaques (Amyloid-β), neurofibrillary tangles, loss of neurons, microgliosis and astrogliosis. Along with these features the clinical stage is characterized by massive synaptic loss that correlates with cognitive decline in AD patients. It has been demonstrated that TSP-1 expression levels are significantly decreased in brain of AD patients and in two AD mouse models (Tg2576 mouse model, expressing Aβ precursor protein - APP, Swedish mutation K670N; and Tg6799/5xFAD mouse model, overexpressing mutant human APP695 with the Swedish, Florida I716V , and London V717I familial AD mutations, as well as human Presenillin 1 harboring two familial mutations M146L and L286V), (Son et al. 2015). This authors demonstrated that TSP-1

plays a protective role against Aβ toxicity on dendritic spines. $A\beta_{1-42}$ was found able to regulate TSP-1 secretion levels in astrocyte and only TSP-1 was found reduced in astrocyte secretome. Furthermore, co-incubation of TSP-1 with $A\beta_{1-42}$ -treated hippocampal neurons, was shown to mitigate the dendritic spine loss *via* interaction with the TSP-1 receptor α2δ-1 *in vitro*.

Lu and Kipnis (2010) proposed TSP-1 as key regulator of NPC self-renewal and neuronal differentiation *in vivo* and *in vitro* (Lu and Kipnis 2010). The authors showed that deficiency of TSP-1 impairs cell proliferation and neuronal differentiation, both *in vivo* and *in vitro*. First, by quantifying BrdU and Ki67 proliferation markers in the neurogenic SGZ and SVZ areas in WT and TSP-$1^{-/-}$ mice (on a B6129SF1/J background), they reported a significant reduction in proliferating cells in mutant mice. In addition, in the same regions they observed a significant reduction in the number of $DCX^{+}/BrdU^{+}$ cells in TSP-$1^{-/-}$ mice compared with the WT counterparts. In the *in vitro* study, the authors confirmed that TSP-1 deficiency impairs cell proliferation and neuronal differentiation of SVZ-derived NPC. For the first time they demonstrated that incubation with exogenous TSP-1 (2 µg/ml) promotes WT NPC neuronal differentiation and that TSP-$1^{-/-}$ astrocytes are unable to promote neuronal differentiation of NPC. Altogether these observations suggest that TSP-1 is one of the astrocytes-derived molecules required for astrocyte induced neuronal differentiation.

Our group demonstrated that adult hippocampal NPC derived from wild type animals do express the α2-δ subunit of the voltage-gated calcium channels, and this receptor mediates the proneurogenic effect of antiepileptic drugs, pregabalin and gabapentin (Valente et al., 2012).

### 1.2.2 Astrocytes as modulators in adult neurogenesis

Astrocytes, as influential components of the neurogenic niches, can contribute to regulation of new neurons formation. An active role for astrocytes and astrocyte-secreted factors in neurogenesis, has been demonstrated in the last two decades. The group of Gage was among the first ones to provide evidence for the active and instructive role of astrocytes in the adult brain (Song et al. 2002). These scientists showed that hippocampal-derived astrocytes from postnatal mice actively regulate neurogenesis, instructing the adult stem cells to a neuronal fate commitment. They examined the fate choice of adult hippocampi-derived neural stem cells *in vitro*, establishing a co-culture system where NSPC were plated onto a feeder layer of primary hippocampal astrocytes from neonatal rodent brain. Furthermore, they showed that even adult hippocampal astrocytes retain the ability to promote neurogenesis from adult NSPC but are less effective compared to neonatal astrocytes. By examining the effects of astrocytes derived from neonatal and adult spinal cord, an area where multipotent stem cells can be isolated, they demonstrated the lower magnitude of neuronal differentiation of adult NSPC in presence of neonatal astrocytes. Conversely, astrocyte derived from adult spinal cord were ineffective in promoting neurogenesis, while were effective in promoting glial over neurogenic fate, data confirmed also by others (Lie et al., 2002). These results suggested for the first time the possibility that astrocytes from different CNS regions may provide regionally specific cues and that astrocytes at different developmental stages may exhibit different effects on NSPC fate choice.

Previously, in a similar work, Lim and Alvarez-Buylla reported that the interaction between NPC derived from adult SVZ and astrocytes is necessary to stimulate neurogenesis (Lim and Alvarez-Buylla, 1999). Given the ultrastructure of SVZ, with astrocytes in close contact with type B cells (slow cycling stem cells, $GFAP^+$) and with type C cells (rapidly dividing intermediate progenitors, $Dlx\text{-}2^+$), these researchers explored the possibility that astrocytes

provide a neurogenic microenvironment for SVZ cells. In their publication they showed that direct contact with astrocytes deriving from cortices supports the proliferation of SVZ precursor cells and the differentiation of these into type A neuroblasts ($DCX^+$). They suggested that it is necessary the direct contact between astrocytes and precursor cells for neurogenesis to occur. When they tested conditioned medium from astrocytes, they observed that the medium does not support neurogenesis, suggesting that the soluble factors produced by astrocytes may be necessary but not sufficient.

Later on the work by Song and colleagues was supported by the group of Zhao, who performed gene expression profiling studies on primary astrocytes isolated from different regions of the CNS. The gene expression analysis was performed on astrocytes isolated from regions with high plasticity, such as newborn and adult hippocampal astrocytes and newborn spinal cord astrocytes, shown to promote neuronal differentiation of adult NSPC, and on astrocytes isolated from the non-neurogenic area of adult spinal cord, known to inhibit neuronal differentiation (Barkho et al., 2006). Briefly, they demonstrated that astrocytes isolated from different brain areas have distinct gene expression profiles. They found out that neurogenesis-promoting astrocytes release interleukine-1β (IL-1β) and interleukine-6 (IL-6), which are proinflammatory citokines, that could promote NSPC neuronal differentiation in combination with other factors, such as vascular cell adhesion molecule-1 (VCAM-1) and interferon-induced protein 10 (IP-10). Instead, astrocytes isolated from the spinal cord express more insulin-like growth factor binding protein 6 (IGFBP6, a negative regulator of insulin-like growth factor II signaling) and decorin (proteoglycan that can inhibit transforming growth factor 2, TGFβ2) which negatively regulate neurogenesis. In fact, it was demonstrated that this factors inhibited neuronal differentiation *in vitro* when co-cultured NSPC and neurogenic astrocytes were treated with them. These authors proposed that the combo of factors secreted by astrocytes into the local microenvironment is affecting the neurogenic niche.

Recently, IL-6 was identified to promote neuronal, but not glial differentiation of adult hippocampal NPC when applied directly in culture, by increasing the fraction of class III β-tubulin ($TUJ1^+$) cells (Oh et al., 2010). Furthermore, was observed that also the average length of $TUJ1^+$ neurites was increased compared to vehicle-treated cells, and the effects observed were abolished when IL-6 was neutralized by anti-IL-6 blocking antibody. This recent study supported the work done by Barkho and colleagues (2006).

While proinflammatory cytokines generally inhibit neurogenesis, the fact that neurogenesis-promoting astrocytes express higher levels of inflammation related proteins than neurogenesis-inhibiting astrocytes, suggests that cytokines and chemokines may have context- and concentration-dependent effects.

Astrocytes have been shown to secrete many positive, as well as negative modulators of hippocampal neurogenesis. Neurogenesin-1 (Ng1) has been identified as an astrocyte-derived soluble factor that positively modulate neuronal differentiation both *in vitro* and *in vivo* (Ueki et al., 2003). Ng1 has been found prominently expressed in neurogenic brain areas and co-localize mainly within astrocytes of the SGZ of the adult brain. The implication of Ng1 in adult neurogenesis was confirmed by *in vitro* experiments on hippocampus-derived NSC cultures. In these experiments, Ng1 neurogenic effect was antagonized with a neutralizing antibody against-Ng1 applied to NSC cultures prior to treatment with the supernatant of hippocampal neurogenic region. Thus this work supported the concept that astrocytes from the hippocampus contribute to the favorable environment for adult neurogenesis.

Among other positive modulators, components related to glial metabolism also appear to be key regulators of adult hippocampal neurogenesis. ATP released from astrocytic metabolism, has been identified as a proliferative factor required for astrocyte-mediated NSC proliferation in the adult hippocampus and *in vitro* (Cao et al., 2013). Similarly, Sultan and colleagues reported that D-serine a secreted astrocyte gliotransmitter abundant in hippocampal astrocytes, is able to increase the number of NSPC *in vitro* and when administered exogenously, it increases both the number of RGL cells and the number of

transient amplifying progenitors in the dentate gyrus of adult mice (Sultan et al., 2013). Additionally the reduction of glutamine levels with fluorocitrate, that selectively inhibits glial metabolism, results in decreased levels of NPC proliferation (Fonnum et al., 1997).

An interesting protein is the neurotrophin brain-derived neurotrophic factor (BDNF), that has been reported to regulate adult hippocampal neurogenesis (Waterhouse et al., 2012). Exogenous BDNF injection into the hippocampus boost NPC proliferation *in vivo* and promotes the differentiation and maturation of adult NPC in culture. This is consistent with evidence that neurogenesis decreases in BDNF knockout mice. SGZ-derived NPC have been demonstrated to express BDNF-TrkB receptor, that is required for the neurogenic and behavioral response to antidepressive treatments, supporting the concept that SGZ-derived NPC are a required component in the amelioration of depression (Li et al., 2008). Studies have demonstrated that hippocampal astrocytes minimally produce BDNF (Rudge et al., 1992). During aging the levels of the neurotrophin does not change, whereas those of the receptor TrkB decreases with increasing age, as reported in the rat hippocampus. This phenomen might be one of those correlated with aging-related decline in adult hippocampal neurogenesis.

Instead, among the factors that are secreted by astrocytes and promote astroglial differentiation, there are bone morphogenetic proteins (BMP), that alter the fate of neural stem cells from neurogenesis to astrocytogenesis. BMP are members of the transforming growth factor β (TGFβ) superfamily, known to mediate the growth, differentiation and survival of different cell types (Dobolyi et al., 2012). First studies reported that BMPs (BMP2/4/5/6) induce astroglial differentiation of SVZ-generated neural progenitors and the concurrent decrease of oligodendroglial and neuronal lineages, and this instructive effect is merely differentiative with no influences on both proliferation and survival of astroglial lineage cells (Gross et al., 1996). Other reports, demonstrated that NSC treatment with low-dose of BMP4 (0.1 ng/ml) or BMP7 (1 ng/ml) stimulates NSC to differentiate exclusively into neurons (TUJ-$1^{+}$ cells), with other cells

remaining undifferentiated, suggesting a concentration-dependent effect of BMP (Chang et al., 2003). Astrocytes secrete ciliary neurotrophic factor (CNTF), that is also important for differentiation of NSC into astrocytic lineage. CNTF alone (20 ng/ml) has a dramatic effect on astrocytic differentiation, with $GFAP^+$ cells > 90% of total cells. Interestingly, the combined treatment of the astrogenic/neurogenic BMP with CNTF results in a decrease in astrocytes and an increase in neurons. This demonstrates the context-dependent regulation of BMP, regardless its concentration (Chang et al., 2003).

Furthermore, Ng1 involvement in neurogenic activity can be traced back to its structure (cysteine-rich domains), characteristic of BMP antagonists. Ueki and colleagues (2003) reported that Ng1 antagonize BMP-4 and alters that fate commitment of NSC from gliogenesis to neurogenesis, and suggests that Ng1 may influence hippocampal plasticity by downregulating the activity of BMP (Ueki et al., 2003).

Astrocytes supplement the local microenvironment also with mitotic factors and most notably FGF-2 and VEGF contribute to neurogenesis by enhancing the proliferation of NSC and their progeny both in the SVZ and SGZ. Bernal and colleagues (2011) asked whether a decreased availability of astrocyte-derived growth factors may contribute to the age-related decline in neurogenesis (Bernal and Peterson, 2011). GFAP-positive astrocytes express both VEGF and FGF-2. The authors found out that the gene expression levels of VEGF are significantly reduced in aged DG of rats compared to young one. In DG aging resulted also in the emergence of a phenotype of GFAP-positive astrocytes that did not co-express FGF-2, whereas in the young brain all GFAP-positive astrocytes co-localize with FGF-2. Anyway the overall density of astrocytes immunopositive for GFAP did not change. The authors suggested that the local environment during aging becomes less supportive for neurogenesis. VEGF and its receptor Flk-1 were detected also in Type-1 NSC and the co-expression of the two proteins is prior to neuronal commitment. Once $DCX^+$ cells are formed, VEGF expression is lost, but Flk-1-mediated sensitivity to VEGF signaling is still maintained indicating that immature neuroblasts may be influenced by VEGF

signals presented by neighboring astrocytes. Normal aging is accompanied by a decline in CNS function and reduced neurogenesis. Astrocytes might be considered as the major contributors in maintaining the neurogenic niche given that the altered profile of astrocytes in the aged hippocampus negatively affects neurogenesis.

In a recent publication a group of researchers investigated the neurogenic-inducing properties of astrocytes isolated from cortical, hippocampal and midbrain areas, by analyzing the secretome profile of astrocyte conditioned media (ACM) and the effect on neuronal differentiation of human NPC (Cordero-Llana et al., 2011). They cultured human NPC for one and four weeks in control media or in regional-specific ACM. They demonstrated that ACM derived from hippocampus and midbrain is able to promote neuronal differentiation of human NPC, whereas ACM from cortex does not. By using mass spectrometry (MS) they identified clusterin as a protein secreted only in hippocampal and midbrain and not in cortex ACM. Clusterin is an interesting secretory glycoprotein (75-80 kDa), with high expression in astrocytes. It has no effect on the proliferation of NPC, but it promotes both their neuronal differentiation and survival.

Generally, the control of NSPC proliferation and differentiation is influenced by several factors that are expressed by many cell types residing within the niche. The role of astrocytes on cell fate is different, with no clear knowledge of the instructive signals and the molecular pathways involved. Astrocytes are organizers of the neurogenic niche, and the signal they provide to the neurogenic process are a sum of complex microenvironmental cues, and influences of the surrounding cells. Astrocytes by releasing soluble factors, may play both instructive and permissive functions. On one hand astrocytes secreted molecules, may promote one fate at the expenses of another, on the other hand astrocytes cues may inhibit one fate, thereby allowing progenitors to differentiate in the other cell populations.

## 1.3 NF-κB signaling in adult neurogenesis

The term NF-κB (Nuclear Factor kappa-light chain enhancer of activated B cells) refers to a family of transcription factors, firstly discovered in immune B cells to bind to the intronic enhancer of the kappa light chain gene, the κB site. These proteins are ubiquitously expressed and responsible for regulating the expression of multiple genes whose products are involved in pleiotropic functions, including cell survival/death, cell proliferation and differentiation and immune and inflammatory responses (Grilli and Memo 1999; Kovacs et al., 2004; Vallabhapurapu and Karin 2009). Often NF-κB signaling appears to play opposite functions, since the final outcome of its activation depends on the cell type involved, the type of disease or injuries and the target genes activated or repressed. NF-κB signaling was initially studied mainly in the field of immunology, as major regulator of innate and adaptive immunity. In the CNS, NF-κB is expressed in both neurons and glia. In general, NF-κB in CNS glial cells, like microglia and astrocytes is mainly neurodestructive due to the overwhelming production of pro-inflammatory cytokines (Mattson and Meffert 2006). The role of NF-κB in the nervous system has gained interest because of its involvement in neuroprotection/neurodegeneration (Kaltschmidt and Kaltschmidt 2009), synaptic plasticity and neurite outgrowth (Gavalda et al. 2009) and formation of functional dendritic spines (Boersma et al. 2011). On one hand many *in vitro* studies have demonstrated that neurotoxic and pro-apoptotic stimuli including high concentrations of glutamate, amyloid-β or cytokines, cause NF-κB activation in neuronal cells, resulting in cell death (Uberti et al. 1999; Ryan et al. 2000; Zhang et al. 2005). On the other hand, it has been reported that neuron-restricted ablation of NF-κB-driven gene expression promotes neurodegeneration (Fridmacher et al. 2003), suggesting a neuroprotective role for NF-κB. Recent finding, suggest that neuronal NF-κB signaling is neuroprotective via maintaining neuronal survival, synaptogenesis, synapse to nucleus communication (Gutierrez and Davies 2011; Mattson and

Meffert 2006) and its role in regulating neural development and CNS functions such as learning and memory (Salles et al. 2014).
Herein, I will introduce NF-κB family members and their signaling pathways, focusing on the emerging evidence of a role for NF-κB in regulating adult hippocamapal neurogenesis.

### 1.3.1 The NF-κB family of transcription factors

The NF-κB family of transcription factors is composed of five subunits including p50/p105 (NF-κB1), p52/p100 (NF-κB2), p65 (RelA), RelB and c-Rel, which all share a conserved and multifunctional N-terminal Rel homology domain (RHD), that is responsible for homo- and hetero-dimerization of the different subunits. The RHD also contains nuclear localization signal (NLS), masked by binding of inhibitory IκB protein. Upon IκB inhibitory protein phosphorylation and degradation, NLS unmasking allows nuclear translocation of dimeric complexes which can then bind regulatory sequences in target genes. According to their structure, NF-κB family members can be divided into two subfamilies. One includes RelA, RelB and c-Rel, which are generally considered as transcriptionally activators, since they possess a transactivational domain (TAD), while the other subfamily includes p50 and p52 subunits, which are commonly considered as repressors, since lacking the TAD. The IκB family of inhibitors includes p100, p105, IκBα, IκBβ, IκBγ and Bcl-3. These inhibitors proteins are carachterized by the presence of ankyrin-repeat motives that mediate their binding with NLS/RHD of NF-κB subunits. The p105 and p100 C-terminal region, also containing ankyrin repeats, that acts like an auto-inhibitory domain, retaining the precursors of p50 and p52, respectively, into the cytoplasm. (Figure 7)

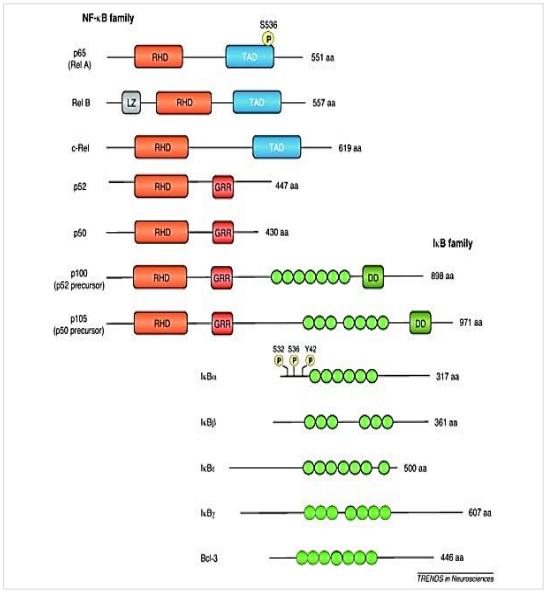


**Figure 7. Schematic diagram illustrating the mammalian members of the NF-κB and IκB families.** NF-κB family members have in common the RHD, with RelA, RelB and c-Rel additionally possessing a TAD [RelB has a leucine zipper (LZ), while p52 and p50 and their precursors have glycine-rich regions-(GRR); additionally, precursor possess regions with homology to death domains (DD)]. The ankyrin repeats of IκB members (denoted by green ovals), mediates the RHD binding. Within the IκBα phosphorylation sites relevant for regulation of NF-κB activation have been identified (Ser32, Ser36, Tyr42). (Gutierrez and Davies, 2011)

At the cellular level, under unstimulated conditions, NF-κB dimers are retained in the cytosol through the interaction with a member of the inhibitory IκB protein family. The IκB subunit masks NLS in the NF-κB complex, thereby maintaining it in the inactive state. There is a variety of potential combinations for the NF-κB complex. In the CNS, the most abundant forms are the p65/p50 heterodimer and the p50 homodimer, while IκBα is the most common inhibitory protein. A vast array of stimuli are able to trigger NF-κB activation by two main activating pathways, referred to as the canonical (or classic) and the non-canonical (or alternative) pathways. The canonical NF-κB signaling pathway is crucial for the activation of innate immunity and inflammation. In response to a several proinflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and agonists of the toll-like receptor, such as lipopolysaccharide (LPS), the IκB Kinase (IKK) complex is activated via phosphorylation of consensus motives in the IκB members (IκBα, IκBβ, IκBε and p105). More specifically, phosphorylation on Ser32 and Ser36 residues of

IκBα by the IκB Kinase-β (IKKβ) catalytic subunit of the IKK complex (composed of IKKβ and IKKα, the catalytic subunits and the IKKγ, the regulatory subunit) triggers ubiquitination and proteasome-mediated degradation of the inhibitory protein. Once activated, the NF-κB p65/p50 dimer translocates into the nucleus, where it binds to the DNA consensus sequence of target genes. The non-canonical NF-κB signaling pathway is critical for adaptative immunity and is activated by some particular members of the TNF family (i.e. CD40L; Lymphotoxin-β). The non-canonical pathway involves NF-κB inducing kinase (NIK)-induced phosphorylation of the IKKα subunit within the IKK complex, that in turn phosphorylates the p100 subunit of the heterodimeric complex p100/RelB. Following ubiquitination and proteasome-dependent processing of p100 into p52, the nuclear translocation of the dimer RelB/p52 occurs and regulates target genes expression. A third distinct NF-κB signaling pathway triggered by DNA damage and oxidative stress, is defined as atypical because it is independent of the IKK complex (Viatour et al. 2005). Although in this pathway proteasome-mediated degradation of IκB is involved, the upstream kinases and the phosphorylation sites are different and, as of today, not completely understood (Figure 8).

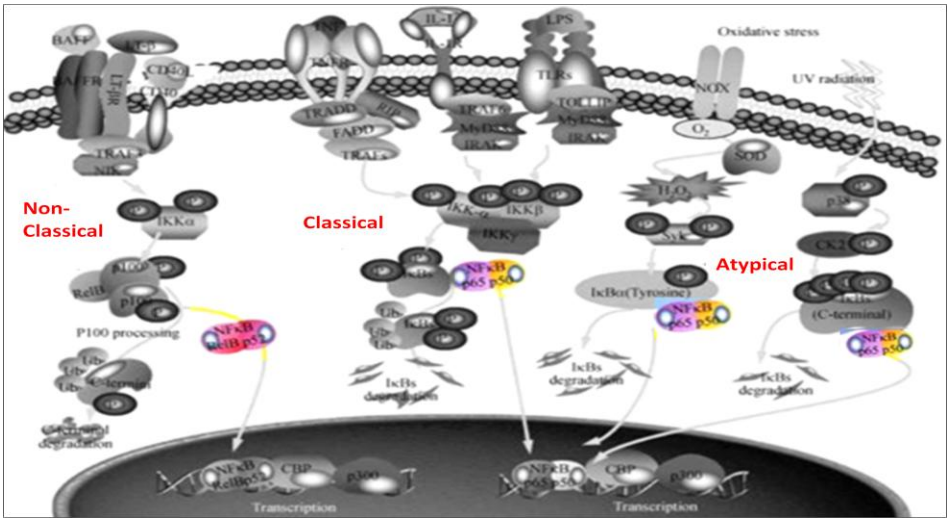


**Figure 8. Signaling pathways involved in NF-κB activation.** In the canonical pathway, IKK complex activation leads to IκBs phosphorylation, followed by their ubiquitination and degradation by the proteasome, allowing the release of the heterodimer of p65/p50, which then translocates into the nucleus. The non-canonical pathway is dependent on NIK and IKKα, but not on the trimeric IKK complex, and mediates the activation of RelB/p52 complex. In the non-canonical pathway NIK-induced phosphorylation of IKKα leads to the phosphorylation of p100 and proteasome-mediated processing of p100 into p52, allowing the nuclear translocation of RelB/p52 to regulate target gene expression. The atypical or IKK-independent pathway of NF-κB is stimulated by DNA damage (Modified from Zhang and Hu 2012)

### 1.3.2 NF-κB signaling in the central nervous system: a role in synaptic plasticity, learning and memory

In adult rodent brain all the DNA-binding subunits of NF-κB have been detected, with p50/p65 and p50/p50 dimers representing the most abundant forms (Kaltschmidt et al. 1993; Bakalkin et al. 1993; Schmidt-Ullrich et al. 1996). It has been reported that constitutive NF-κB activity (that is in the nucleus, free of IκB and capable of binding DNA) has been detected in hippocampal neurons *in vitro*, as well as it occurs *in vivo* in several rodent brain regions, within neurons of neocortex, olfactory bulbs, amygdala, hippocampus and hypothalamus (Bhakar et al. 2002; Schmidt-Ullrich et al. 1996). This piece information supports a physiological role for NF-κB in maintaining survival or functions of central neurons. In addition, NF-κB acts also as a signal transducer, not only as a transcriptional regulator, by delivering transient signals from distant active synaptic sites to the nucleus, for example upon glutamatergic stimulation or depolarization (with potassium chloride or kainate) (Wellmann et al. 2000). This supports the concept that the activated transcription factor has a central role in translating short-term synaptic events into long-term gene expression changes (Kaltschmidt and Kaltschmidt 2009).
Several studies based on genetic and pharmacological evidence have shown that NF-κB is implicated in the regulation of structural changes observed in synaptic connections and in controlling structural plasticity of neural processes (i.e. development, outgrowth, guidance and branching of axons and dendrites) (Gavalda et al. 2009; Salles et al. 2014), and that the signaling pathway is activated by the enhancement of synaptic activity (Meberg et al. 1996; Romano et al. 2006).

Genetic studies associated with a variety of behavioural paradigms are in support for the role of NF-κB signaling in learning and memory. The availability of transgenic and knock out (KO) mouse lines for specific NF-κB subunits has been instrumental to research efforts aimed at a better

understanding of their specific role in CNS-related physiological and pathophysiological functions. Mice with a null mutation of p50 ($p50^{-/-}$) show cognitive defects on novel task acquisitions (Kassed et al. 2002) and reduced short term spatial memory performance, without impairment of long term memory (Denis-Donini et al. 2008). However, the p50 subunit is a component of the transcriptional activator p65/p50 heterodimer, and a repressor in the p50/p50 homodimers, that lack the TAD. Therefore, some of the effects observed in $p50^{-/-}$ mice might be due to the loss of p50 function as repressor as well as activator in combination with p65. Recently, the group of Albensi, found that the lack of the NF-κB p50 subunit led to significant decreases in late long-term potentiation (LTP) and deficits in long-term memory (Oikawa et al., 2012), supporting the idea that p50 may also be required for spatial memory and long term plasticity. The deletion of p65 gene has been shown to be lethal during embryonic development, because of TNFα induced liver degeneration. To overcome this problems, the strategy of p65 deletion in association with concurrent deletion of type I TNF receptors, allowed to create a mouse line ($TNFR^{-/-}/p65^{-/-}$-double KO mice) which survive postnatally (Alcamo et al., 2001). These mice have normal brain cytoarchitecture but when analysed for memory performance using a spatial version of a radial arm maze, they showed selective learning deficits in spatial information storage (Meffert et al., 2003). These studies point out the role of NF-κB p50 and p65 in memory and hippocampal dependent tasks.

LTP and long-term depression (LTD) are models of synaptic plasticity, believed to be the molecular correlates of memory formation and forgetting, since able to increase or decrease the synaptic efficacy, respectively (de la Fuente et al., 2015; Snow et al., 2014). These paradigms are used to measure synaptic function and along with behavioral test of memory are used to assess hippocampal-dependent alterations in memory. *In vivo*, LTP induction increases the expression and the activation of NF-κB, with both p50 and p65 increase in the hippocampus in response to LTP (Meberg et al. 1996). Instead, the

inhibition of NF-κB reduces LTP. This suggests that NF-κB is required for LTP induced synaptic plasticity, and that NF-κB activation is at the basis of synaptic changes occurring during memory storage. The dendritic spines, site of excitatory and inhibitory synapses on neurons, are dynamic structures with a high turnover during development, whereas throughout life the spine turnover continues at a low rate. The finding that instructive experience leads to spine stabilization, changes in spine density and increase in spine volume during LTP (Alvarez & Sabatini, 2007; Saneyoshi et al., 2010), have linked structural spine plasticity occurring in learning and memory with NF-κB signaling.

As of today, many target genes being regulated by NF-κB in neurons have been discovered, and many of these genes are related to memory and plasticity. Those genes can be categorized in several groups. There are genes codifying for molecules involved in cell adhesion and growth, including neural cell adhesion molecule (NCAM) and β1-integrins, both controlling signals that induce neurite outgrowth during synaptic plasticity. Genes encoding for neurotrophins, such as BDNF, which results upregulated upon NF-κB activation in astrocytes and neurons and might be connected to cellular growth, differentiation and neurogenesis. Genes involved in cell signaling, such as protein kinase A (PKA). Kaltchmidt and colleagues (2006) reported that NF-κB regulates the PKA/CREB pathway that in turn controls spatial memory formation and synaptic plasticity (Kaltschmidt et al., 2006). Imielski and colleagues (2012) using a mouse model with neuronal specific ablation of NF-κB subunits (IκB/tTA mice - where neuronal NF-κB ablation can be established by CamKinaseII promoter-driven tetracycline controlled transactivator (tTA) regulating the expression of IκB in mice, where the transgene expression starts in type 2b $DCX^+$ cells) reported that NF-κB controls the transcription of the forkhead transcription factor (FOXO1) and PKA kinase cascade, involved in axonal outgrowth (Imielski et al., 2012).

In glial cells, there is no constitutive activity of NF-κB, suggesting that NF-κB activation might be connected to pathological events. It is suggested that NF-κB signaling in astrocytes might regulate chemokine-induced infiltration of immune cell into lesioned brain (Kaltschmidt and Kaltschmidt 2009). Astrocytes have beneficial functions during the early stages of injury, whereas sustained activation of astrocytes enhances neuroinflammation. Astroglial-derived mediators, like TNFα, recruit and enhance microglia⁄macrophage responses. This event is driven by astroglial NF-κB, which establishes an inflammatory intracellular environment. The group of Zheng and colleagues (2015) reported that in astrocytes, upon amyloid-β stimulation, there is a sustained activation of NF-κB, that leads to target upregulation and subsequent release of complement factor C3, which in turn, impairs neuronal function in Alzheimer's disease (Lian et al., 2015). Dvoriantchikova and colleagues (2009) showed that the transgenic inhibition of astroglial NF-κB (using GFAP-IκBα-dominant-negative mice, in which NF-κB is inactivated in astrocytes through overexpression of a dominant negative form of IκBα super-repressor under control of GFAP promoter) abrogates the establishment of the inflammatory cellular environment in a model of retinal ischemia-reperfusion injury (Dvoriantchikova et al., 2009). The same group reported that the transgenic inhibition of astroglial NF-κB has also neuroprotective effects in experimental autoimmune encephalomyelitis (Brambilla et al., 2009). Both publication demonstrate that selective inhibition of astroglial NF-κB significantly reduces the severity of the disease models. NF-κB role in astrocytes needs further investigation in order to explore the neuroprotective and and/or neurotoxic functions of the pathway in glial cells.

### 1.3.3 NF-κB signaling in adult neurogenesis: *in vitro* and *in vivo* supporting data

NF-κB family members are expressed in zones of active neurogenesis in the adult as well as in the postnatal mouse brain (Denis-Donini et al., 2005), suggesting that the signaling pathway may be actively involved in some of the steps accounting for neurogenesis, such as proliferation, survival, migration and differentiation (Widera et al. 2008; Zhang and Hu 2012). Direct evidence of the presence of NF-κB in neurogenic zones has been validated by immunofluorescent microscopy (Meneghini et al., 2010). The group of Zhang and colleagues (2012) has shown that NF-κB signaling is required for NSPC initial differentiation (Zhang et al., 2012). They demonstrated that selective inhibition of canonical NF-κB subunits and blocking of the signaling pathway (by pharmacological stimuli, such as SN50 - a cell-permeable direct inhibitor for NF-κB nuclear translocation; or with small hairpin RNA (shRNA) knockdown of NF-κB subunits; or with NSPC-specific transgene dominant-negative IκBα), it is possible to attenuate neural differentiation and enhance self-renewal capability of $Nestin^+$ and $Sox2^+$ NSPC (Zhang et al., 2012). Under physiological conditions, NF-κB is a crucial regulator of dentate gyrus tissue homeostasis (Imielski et al., 2012), however under pathological conditions, stress and/or aging, over-activation of NF-κB signaling could impair and reduce neurogenesis by depleting the hippocampal neural stem cell pool (Encinas and Sierra, 2012; Koo et al., 2010).

Many additional studies have implicated NF-κB signaling in regulating neurogenesis during development and in the adulthood. Several extracellular signals have been identified as being able to affect NSPC via NF-κB activation. Several cytokines have been shown to regulate neurogenesis by stimulating NF-κB signaling. IL-1β is a potent proinflammatory cytokine shown to mediate stress-induced depression via inhibiting adult neurogenesis. Koo and Duman (2008) reported that blockade of the IL-1β receptor (IL-1R1) by using either an antagonist or IL-1R1null mice (adult male IL-1R1 null mice bearing a C57BL/6

background) blocks the antineurogenic effect of stress. In the *in vitro* studies they demonstrated that hippocampal neural progenitor cells express IL-1β receptor-1 (IL-1R1) and that activation of this receptor decreases cell proliferation via the NF-κB signaling pathway (Koo and Duman, 2008). It has also been reported that IL-1β exerts also anti-proliferative, anti-neurogenic and pro-gliogenic effect on embryonic rat hippocampal NSPC, which is mediated by IL-1R1 (Green et al., 2012). On the contrary, the cytokine TNF-α was identified to trigger survival, proliferation, and neuronal differentiation from both adult rat and neonatal mice cultured SVZ NSPC, via NF-κB signaling *in vitro* (Widera et al. 2006; Zhang and Hu 2013). Other data suggest that TNF-α promotes gliogenesis but not neurogenesis (Ricci-Vitiani et al., 2006). A recent report suggests that the mechanism that may contribute to the neuroprotective activity of TNF-α is dependent on the regulation of Ephrin B2 gene by NF-κB pathway activation (Pozniak et al. 2013). Notwithstanding the negative effect of TNF-α on neuronal survival is dependent on the context, timing and dosage of its activity (Lenzlinger et al. 2001). Increasing interest has been devoted to another cytokine, interleukin-6 (IL-6), that has been shown to induce NSPC to differentiate specifically into glutamate-responsive neurons, as well as into phenotypically different glia types (Mukaino et al., 2008). Another example comes from studied from Wada and colleagues, who demonstrated that vascular endothelial growth factor (VEGF) directly promotes adult NSC survival (Wada et al., 2006). It was also demonstrated that VEGF can influence adult hippocampal neurogenesis and hippocampus-dependent memory (Licht et al., 2011), and the different VEGF phenotypes were blocked by inhibition of the downstream effector NF-κB. Taken together, these data support the hypothesis that in physiological conditions or in early stages of injuries, transient NF-κB activation by these cytokines/GF, may be beneficial by promoting neural progenitor cells differentiation for neural repair or maintaining daily neurogenesis. However, chronic activation of NF-κB would result in the reduction of the pool of NSPC (Koo et al., 2010). These results emphasise the

influence of local environment on the differentiation of NSPC during development,

Many receptors have been identified playing a pivotal role in NF-κB activation and NPC differentiation, upon ligand binding. Schwartz and colleagues, identified toll like receptors (TLR) as players in adult neurogenesis. TLR2 and TLR4 are found on adult NSPC and have distinct and opposing functions in NPC proliferation and differentiation both *in vitro* and *in vivo*. The activation of TLRs on the NPCs was mediated *via* activation of the NF-κB signaling pathway (Rolls et al., 2007).

Among the upstream receptors that activate NF-κB signaling pathway in NPC of adult mouse hippocampal and SVZ neurogenic region, there is the receptor for advanced glycation end-products (RAGE). RAGE ligands, like the alarmin HMGB1, S100β and AGE-BSA, are able to stimulate both proliferation and neuronal differentiation of SVZ-derived NPC *in vitro* (Meneghini et al., 2010). It was also demonstrated that HMGB1 and $A\beta_{1-42}$ oligomers, both involved in Alzheimer's disease pathophysiology (Takata et al., 2003) are able to promote neuronal differentiation of adult hippocampal NPC via activation of the RAGE/NF-κB axis (Meneghini et al., 2013).

Literature data supports the involvement of NF-κB signaling pathway in mediating the proneurogenic and antidepressant-like activity of some clinically relevant drugs. Increasing evidence demonstrate that neuroplasticity is disrupted in mood disorders and in animal models of depressive-like behaviour and it can be upregulated by antidepressant drugs (Pittenger and Duman, 2008; Hanson et al., 2011). An intriguing observation is that most antidepressant and environmental interventions (i.e. learning tasks; enriched environment; physical activity) that confer antidepressant like-behavioral effects also stimulate adult neurogenesis (Sahay and Hen, 2007).

Our group recently identified novel positive modulators of hippocampal neurogenesis, as potential new antidepressant drugs, that require NF-κB

activation for exerting their proneurogenic effects *in vitro* and *in vivo*. The first example is represented by pregabalin (PGB) and gabapentin (GBP) that are clinically relevant anticonvulsant, analgesic and anxiolytic drugs. Our group demonstrated that GBP and PGB produce a concentration dependent increase in the number of mature and immature neurons *in vitro*, acting through the α2-δ subunit of the voltage-gated calcium channels, which was found to be expressed on adult hippocampal NPC (Valente et al., 2012). The activation of NF-κB is involved in the proneurogenic effect elicited by α2-δ ligands, because the inhibition of both p50 and p65 nuclear translocation of IKKβ counteracted drug-mediated effects. Moreover, PGB antidepressant like activity was confirmed *in vivo* in adult mice. The appearance of depressive-like behaviour induced by chronic restraint stress was prevented by administration of the drug, and in parallel promoted adult hippocampal neurogenesis (Valente et al., 2012). Another drug reported to have proneurogenic and antidepressant-like activity *via* modulation of NF-κB signaling pathway is acetyl-L-carnitine (ALC) (Cuccurazzu et al., 2013). Our group demonstrated that the drug-mediated effect on neuronal differentiation of adult hippocampal NPC *in vitro*, is dependent on the acetylation of p65 subunit and subsequent NF-κB-mediated upregulation of metabotropic glutamate receptor 2 (mGlu2). In addition, when tested *in vivo*, chronic ALC treatment significantly increased the number of adult born neurons in mouse hippocampi and reverted depressive-like behaviour induced by unpredictable chronic mild stress.

Our group investigated the role of NF-κB p50 subunit in adult hippocampal neurogenesis by taking advantage of $p50^{-/-}$ mice generated in the laboratory of David Baltimore (Sha et al., 1995). In this genetically modified animal model, we demonstrated a marked deficiency in neurogenesis in the hippocampus , as a result of a defect in the late maturation of newly born neurons (Denis-Donini et al., 2008). Interestingly, no defects have been observed in the rate of NSPC proliferation in the SGZ of $p50^{-/-}$ animals compared to wt mice. However, the survival of neural progenitors after 21 days post BrdU injection was

significantly reduced in mutant compared to wt animals. A detailed phenotypic characterization of newly generated cells revealed no differences in the number of $DCX^+$ neuroblasts, but a marked reduction in calretinin post mitotic neurons in the DG of $p50^{-/-}$ animals. Based on this findings, our group proposed that the absence of the p50 subunit of NF-κB may trigger a selective defect in adult neurogenesis progression at the transition phase between neuroblasts and postmitotic neurons.

$p50^{-/-}$ mice were also evaluated at the behavioural level. When tested in the Morris water maze (MWM), wt and $p50^{-/-}$ performed equally in the acquisition test, with normal retrieval of hippocampal-dependent spatial memory in the probe test performed 24h after the last training trial. Conversely, when wt and $p50^{-/-}$ mice were subjected to the place recognition test for evaluating their ability to discriminate familiar versus a novel environment, the two genotypes performed differently. Specifically, $p50^{-/-}$ mice showed a selective impairment in short-term spatial memory. The correlation between neurogenesis defects and deficits in short-term spatial memory performances deserves further investigation.

Many other publications support the importance of NF-κB in synaptic plasticity and memory. The group of Albensi investigated the role of NF-κB in both synaptic plasticity and long term spatial memory in $p50^{-/-}$ mice (Oikawa et al., 2012). They used the same KO mice model to test if the absence of p50 negatively affected *in vivo* MWM performance and *in vitro* LTP response. LTP is an experimental paradigm used to assess hippocampal-dependent alterations and measure synaptic function, and is commonly regarded as the molecular correlate of memory formation. Albensi's group found that the lack of the NF-κB p50 subunit led to a significant decrease in late LTP and in selective alterations in MWM test, with defects in long term spatial memory. Given these contradictory findings, open questions remain concerning the complex contribution of NF-κB p50 subunit in long versus short memory.

The group of Imielski found out that NF-κB ablated mice (IκB/tTA mice) show a strongly increased number of activated astrocytes that is accompained with a

severe dentate gyrus atrophy. They proposed that this condition may rely on a dual function of NF-κB in the hippocampus. They suggest a cell autonomous role of NF-κB in axogenesis, with NF-κB being necessary for axogenesis and maturation in neuronal progenitors, whereas in mature granule cells NF-κB may play a distinct role by regulating neuroprotection as well as synaptic transmission. When NF-κB is inactivated, the dentate gyrus cannot be regenerated by the addition of new-born neurons (Imielski et al., 2012).

Several studies that demonstrated increased hippocampal neurogenesis following hippocampus-dependent tasks, have established a connection between neurogenesis in the adult hippocampus and learning and memory. The deficiency in hippocampal neurogenesis and the selective defect in short term spatial memory performance in adult $p50^{-/-}$ animals, provide evidence that NF-κB-regulated hippocampal neurogenesis, can influence aspects connected to hippocampal dependent learning.

## References

Aimone, J. B., Wiles, J., & Gage, F. H. (2006). Potential role for adult neurogenesis in the encoding of time in new memories. Nature Neuroscience, 9(6), 723–727.

Aimone, J. B., Deng, W., & Gage, F. H. (2010). Adult neurogenesis: integrating theories and separating functions. Trends in Cognitive Sciences, 14(7), 325–37.

Aimone, J. B., Li, Y., Lee, S. W., Clemenson, G. D., Deng, W., & Gage, F. H. (2014). Regulation and Function of Adult Neurogenesis: From Genes to Cognition. Physiological Reviews, 94(4), 991–1026

Alcamo, E., Mizgerd, J. P., Horwitz, B. H., Bronson, R., Beg, A. A., Scott, M., Doerschuk, C. M. Hynes, R O., Baltimore, D. (2001). Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-kappa B in leukocyte recruitment. Journal of Immunology (Baltimore, Md. : 1950), 167(3), 1592–600.

Alvarez, V. A., & Sabatini, B. L. (2007). Anatomical and physiological plasticity of dendritic spines. Annual Review of Neuroscience, 30, 79–97.

Alvarez-Buylla, A., & Garcia-Verdugo, J. M. (2002). Neurogenesis in adult subventricular zone. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 22(3), 629–34

Anton, E. S., Marchionni, M. A., Lee, K. F., & Rakic, P. (1997). Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. Development 124(18), 3501–10.

Bakalkin GYa, Yakovleva, T., & Terenius, L. (1993). NF-kappa B-like factors in the murine brain. Developmentally-regulated and tissue-specific expression. Brain Research. Molecular Brain Research, 20(1-2), 137–46.

Banker, G. A. (1980). Trophic interactions between astroglial cells and hippocampal neurons in culture. Science (New York, N.Y.), 209(4458), 809–10.

Barkho, B. Z., Song, H., Aimone, J. B., Smrt, R. D., Kuwabara, T., Nakashima, K., Gage, F. H., Zhao, X. (2006). Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. Stem Cells and Development, 15(3), 407–21.

Bernal, G. M., & Peterson, D. A. (2011). Phenotypic and gene expression modification with normal brain aging in GFAP-positive astrocytes and neural stem cells. Aging Cell, 10(3), 466–82.

Bhakar, A. L., Tannis, L.-L., Zeindler, C., Russo, M. P., Jobin, C., Park, D. S., MacPherson, S., Barker, P. A. (2002). Constitutive nuclear factor-kappa B activity is required for central neuron survival. Journal of Neuroscience, 22(19), 8466–75.

Boersma, M. C. H., Dresselhaus, E. C., De Biase, L. M., Mihalas, A. B., Bergles, D. E., & Meffert, M. K. (2011). A requirement for nuclear factor-kappaB in developmental and plasticity-associated synaptogenesis. Journal of Neuroscience, 31(14), 5414–25.

Bolignano, D., Coppolino, G., Donato, V., Lacquaniti, A., Bono, C., & Buemi, M. (2010). Neutrophil gelatinase-associated lipocalin (NGAL): a new piece of the anemia puzzle? Medical Science Monitor : International Medical Journal of Experimental and Clinical Research, 16(6), RA131–5.

Bonaguidi, M. A., Wheeler, M. A., Shapiro, J. S., Stadel, R. P., Sun, G. J., Ming, G., & Song, H. (2011). In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. Cell, 145(7), 1142–55.

Bonaguidi, M. A., Song, J., Ming, G., & Song, H. (2012). A unifying hypothesis on mammalian neural stem cell properties in the adult hippocampus. Current Opinion in Neurobiology, 22(5), 754–61.

Brambilla, R., Persaud, T., Hu, X., Karmally, S., Shestopalov, V. I., Dvoriantchikova, G., Ivanov, D., Nathanson, L., Barnum, S. R., Bethea, J. R. (2009). Transgenic inhibition of astroglial NF-kappa B improves functional outcome in experimental autoimmune encephalomyelitis by suppressing chronic central nervous system inflammation. Journal of Immunology, 182(5), 2628–40.

Brandt, M. D., Jessberger, S., Steiner, B., Kronenberg, G., Reuter, K., Bick-Sander, A., von der Behrens, W., Kempermann, G. (2003). Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. Molecular and Cellular Neurosciences, 24(3), 603–13.

Cao, X., Li, L.-P., Qin, X.-H., Li, S.-J., Zhang, M., Wang, Q., Hu, H-H., Fang, Y-Y., Gao, Y-B., Li, X-W, Sun, L-R., Xiong, W-C., Gao, T-M., Zhu, X.-H. (2013). Astrocytic adenosine 5’-triphosphate release regulates the proliferation of neural stem cells in the adult hippocampus. Stem Cells 31(8), 1633-43

Chancey, J. H., Adlaf, E. W., Sapp, M. C., Pugh, P. C., Wadiche, J. I., & Overstreet-Wadiche, L. S. (2013). GABA depolarization is required for experience-dependent synapse unsilencing in adult-born neurons. The Journal of Neuroscience, 33(15), 6614–22.

Chang, M.-Y., Son, H., Lee, Y.-S., & Lee, S.-H. (2003). Neurons and astrocytes secrete factors that cause stem cells to differentiate into neurons and astrocytes, respectively. Molecular and Cellular Neurosciences, 23(3), 414–26.

Chia, W.-J., Dawe, G. S., & Ong, W.-Y. (2011). Expression and localization of the iron-siderophore binding protein lipocalin 2 in the normal rat brain and after kainate-induced excitotoxicity. Neurochemistry International, 59(5), 591–9.

Christian, K. M., Song, H., & Ming, G. (2014). Functions and dysfunctions of adult hippocampal neurogenesis. Annual Review of Neuroscience, 37, 243–62.

Cordero-Llana, O., Scott, S. A., Maslen, S. L., Anderson, J. M., Boyle, J., Chowhdury, R.-R., Tyers, P., Barker, R. A., Kelly, C. M., Rosser, A. E., Stephens, E., Chandran, S., Caldwell, M. A. (2011). Clusterin secreted by astrocytes enhances neuronal differentiation from human neural precursor cells. Cell Death and Differentiation, 18(5), 907–13.

Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U., Winkler, J., Kuhn, H-G. Aigner, L. (2005). Doublecortin expression levels in adult brain reflect neurogenesis. The European Journal of Neuroscience, 21(1), 1–14.

Cuccurazzu, B., Bortolotto, V., Valente, M. M., Ubezio, F., Koverech, A., Canonico, P. L., & Grilli, M. (2013). Upregulation of mGlu2 receptors via NF-κB p65 acetylation is involved in the Proneurogenic and antidepressant effects of acetyl-L-carnitine. Neuropsychopharmacology, 38(11), 2220–30.

de la Fuente, V., Federman, N., Zalcman, G., Salles, A., Freudenthal, R., & Romano, A. (2015). NF-κB transcription factor role in consolidation and reconsolidation of persistent memories. Frontiers in Molecular Neuroscience, 8, 50.

Denis-Donini, S., Caprini, A., Frassoni, C., & Grilli, M. (2005). Members of the NF-kappaB family expressed in zones of active neurogenesis in the postnatal and adult mouse brain. Brain Research. Developmental Brain Research, 154(1), 81–9.

Denis-Donini, S., Dellarole, A., Crociara, P., Francese, M. T., Bortolotto, V., Quadrato, G., Orsetti, M., Ghi, P., Memo, M., Bonini, S. A., Ferrari-Toninelli, G., Grilli, M. (2008). Impaired adult neurogenesis associated with short-term memory defects in NF-kappaB p50-deficient mice. Journal of Neuroscience, 28(15), 3911–9.

Devireddy, L. R., Gazin, C., Zhu, X., & Green, M. R. (2005). A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. Cell, 123(7), 1293–305.

Dobolyi, A., Vincze, C., Pál, G., & Lovas, G. (2012). The neuroprotective functions of transforming growth factor beta proteins. International Journal of Molecular Sciences, 13(7), 8219–58.

Döbrössy, M. D., Drapeau, E., Aurousseau, C., Le Moal, M., Piazza, P. V, & Abrous, D. N. (2003). Differential effects of learning on neurogenesis: learning increases or decreases the number of newly born cells depending on their birth date. Molecular Psychiatry, 8(12), 974–82.

Doetsch, F., Caillé, I., Lim, D. A., García-Verdugo, J. M., & Alvarez-Buylla, A. (1999). Subventricular Zone Astrocytes Are Neural Stem Cells in the Adult Mammalian Brain. Cell, 97(6), 703–716.

Dvoriantchikova, G., Barakat, D., Brambilla, R., Agudelo, C., Hernandez, E., Bethea, J. R., Shestopalov, Valery I. Ivanov, D. (2009). Inactivation of astroglial NF-κB promotes survival of retinal neurons following ischemic injury. European Journal of Neuroscience, 30(2), 175–185.

Ekdahl, C. T. (2012). Microglial activation - tuning and pruning adult neurogenesis. Frontiers in Pharmacology, 3, 41.

Encinas, J. M., Vaahtokari, A., & Enikolopov, G. (2006). Fluoxetine targets early progenitor cells in the adult brain. Proceedings of the National Academy of Sciences of the United States of America, 103(21), 8233–8.

Encinas, J. M., & Sierra, A. (2012). Neural stem cell deforestation as the main force driving the age-related decline in adult hippocampal neurogenesis. Behavioural Brain Research, 227(2), 433–9.

Erickson, A. C., & Couchman, J. R. (2000). Still more complexity in mammalian basement membranes. The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society, 48(10), 1291–306.

Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A M., Nordborg, C., Peterson, D. a, & Gage, F. H. (1998). Neurogenesis in the adult human hippocampus. Nature Medicine, 4(11), 1313–7.

Eroglu, C., Allen, N. J., Susman, M. W., O'Rourke, N. A., Park, C. Y., Ozkan, E., Chakraborty, C., Mulinyawe, S. B., Annis, D. S., Huberman, A. D., Green, E. M., Lawler, J., Dolmetsch, R.Garcia, K. C., Smith, S. J., Luo, Z. D., Rosenthal, A., Mosher, D. F., Barres, B. A. (2009). Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell, 139(2), 380–92.

Ferreira, A. C., Pinto, V., Dá Mesquita, S., Novais, A., Sousa, J. C., Correia-Neves, M., Sousa, N., Palha, J. A., Marques, F. (2013). Lipocalin-2 is involved in emotional behaviors and cognitive function. Frontiers in Cellular Neuroscience, 7, 122.

Filippov, V., Kronenberg, G., Pivneva, T., Reuter, K., Steiner, B., Wang, L.-P., Yamaguchi, M., Kettenmann, H., Kempermann, G. (2003). Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. Molecular and Cellular Neuroscience, 23(3), 373–382.

Flower, D. R., North, A. C., & Sansom, C. E. (2000). The lipocalin protein family: structural and sequence overview. Biochimica et Biophysica Acta, 1482(1-2), 9–24.

Fonnum, F., Johnsen, A., & Hassel, B. (1997). Use of fluorocitrate and fluoroacetate in the study of brain metabolism. Glia, 21(1), 106–13.

Fridmacher, V., Kaltschmidt, B., Goudeau, B., Ndiaye, D., Rossi, F. M., Pfeiffer, J., Kaltschmidt, C., Israël, A., Mémet, S. (2003). Forebrain-specific neuronal inhibition of nuclear factor-kappaB activity leads to loss of neuroprotection. Journal of Neuroscience, 23(28), 9403–8.

Fróes, M. M., Correia, A. H., Garcia-Abreu, J., Spray, D. C., Campos de Carvalho, A. C., & Neto, M. V. (1999). Gap-junctional coupling between neurons and astrocytes in primary central nervous system cultures. Proceedings of the National Academy of Sciences of the United States of America, 96(13), 7541–6.

Gage, F. H., Coates, P. W., Palmer, T. D., Kuhn, H. G., Fisher, L. J., Suhonen, J. O., Peterson, D. A., Suhr, S. T. Ray, J. (1995). Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proceedings of the National Academy of Sciences of the United States of America, 92(25), 11879–83.

Garcia, O., Torres, M., Helguera, P., Coskun, P., & Busciglio, J. (2010). A role for thrombospondin-1 deficits in astrocyte-mediated spine and synaptic pathology in Down's syndrome. PloS One, 5(12), e14200.

Gavalda, N., Gutierrez, H., & Davies, A. M. (2009). Developmental switch in NF-κB signaling required for neurite growth. Development, 136(20), 3405–3412.

Ge, S., Pradhan, D. A., Ming, G.-L., & Song, H. (2007). GABA sets the tempo for activity-dependent adult neurogenesis. Trends in Neurosciences, 30(1), 1–8.

Gomes, F. C., Spohr, T. C., Martinez, R., & Moura Neto, V. (2001). Cross-talk between neurons and glia: highlights on soluble factors. Brazilian Journal of Medical and Biological Research 34(5), 611–20.

Gould, E., Reeves, A. J., Graziano, M. S., & Gross, C. G. (1999). Neurogenesis in the neocortex of adult primates. Science (New York, N.Y.), 286(5439), 548–52.

Gould, E., Tanapat, P., Hastings, N., & Shors, T. (1999). Neurogenesis in adulthood: a possible role in learning. Trends in Cognitive Sciences, 3(5), 186–192.

Green, H. F., Treacy, E., Keohane, A. K., Sullivan, A. M., O'Keeffe, G. W., & Nolan, Y. M. (2012). A role for interleukin-1β in determining the lineage fate of embryonic rat hippocampal neural precursor cells. Molecular and Cellular Neurosciences, 49(3), 311–21.

Grilli, M, and M Memo. 1999. "Nuclear Factor-kappaB/Rel Proteins: A Point of Convergence of Signaling Pathways Relevant in Neuronal Function and Dysfunction." Biochemical Pharmacology 57 (1): 1–7.

Gross, R. E., Mehler, M. F., Mabie, P. C., Zang, Z., Santschi, L., & Kessler, J. A. (1996). Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. Neuron, 17(4), 595–606.

Gutierrez, H., & Davies, A. M. (2011). Regulation of neural process growth, elaboration and structural plasticity by NF-κB. Trends in Neurosciences, 34(6), 316–325.

Hanson, N. D., Owens, M. J., & Nemeroff, C. B. (2011). Depression, antidepressants, and neurogenesis: a critical reappraisal. Neuropsychopharmacology, 36(13), 2589–602.

Herculano-Houzel, S. (2014). The glia/neuron ratio: how it varies uniformly across brain structures and species and what that means for brain physiology and evolution. Glia, 62(9), 1377–91.

Hodge, R. D., Kowalczyk, T. D., Wolf, S. A., Encinas, J. M., Rippey, C., Enikolopov, G., Kempermann, G., Hevner, R. F. (2008). Intermediate progenitors in adult hippocampal neurogenesis: Tbr2 expression and coordinate regulation of neuronal output. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 28(14), 3707–17.

Hughes, E. G., Elmariah, S. B., & Balice-Gordon, R. J. (2010). Astrocyte secreted proteins selectively increase hippocampal GABAergic axon length, branching, and synaptogenesis. Molecular and Cellular Neurosciences, 43(1), 136–45.

Imielski, Y., Schwamborn, J. C., Lüningschrör, P., Heimann, P., Holzberg, M., Werner, H., Leske, O., Püschel, A. W., Memet, S., Heumann, R., Israel, A., Kaltschmidt, C., Kaltschmidt, B. (2012). Regrowing the Adult Brain: NF-κB Controls Functional Circuit Formation and Tissue Homeostasis in the Dentate Gyrus. PLoS ONE, 7(2), e30838.

Julian, L. M., Vandenbosch, R., Pakenham, C. A., Andrusiak, M. G., Nguyen, A. P., McClellan, K. A., Svoboda, D. S., Lagace, D. C., Park, D. S., Leone, G., Blais, A., Slack, R. S. (2013). Opposing regulation of Sox2 by cell-cycle effectors E2f3a and E2f3b in neural stem cells. Cell Stem Cell, 12(4), 440–52.

Kaltschmidt, C., Kaltschmidt, B., & Baeuerle, P. A. (1993). Brain synapses contain inducible forms of the transcription factor NF-kappa B. Mechanisms of Development, 43(2-3), 135–47.

Kaltschmidt, B., Ndiaye, D., Korte, M., Pothion, S., Arbibe, L., Prüllage, M., Pfeiffer, J., Lindecke, A., Staiger, V., Israël, A., Kaltschmidt, C., Mémet, S. (2006). NF-kappaB regulates spatial memory formation and synaptic plasticity through protein kinase A/CREB signaling. Molecular and Cellular Biology, 26(8), 2936–46. http://doi.org/10.1128/MCB.26.8.2936-2946.2006

Kaltschmidt, B., & Kaltschmidt, C. (2009). NF-kappaB in the nervous system. Cold Spring Harbor Perspectives in Biology, 1(3), a001271

Kassed, C. A., Willing, A. E., Garbuzova-Davis, S., Sanberg, P. R., & Pennypacker, K. R. (2002). Lack of NF-kappaB p50 exacerbates degeneration of hippocampal neurons after chemical exposure and impairs learning. Experimental Neurology, 176(2), 277–88.

Kempermann, G., & Gage, F. H. (2000). Neurogenesis in the adult hippocampus. Novartis Found Symp, 231, 220–241,302–306.

Kempermann, G., Jessberger, S., Steiner, B., & Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. Trends in Neurosciences, 27(8), 447–52.

Kempermann, G., Song, H., & Gage, F. H. (2015). Neurogenesis in the Adult Hippocampus. Cold Spring Harbor Perspectives in Medicine, 5(7), a018812.

Kerever, A., Schnack, J., Vellinga, D., Ichikawa, N., Moon, C., Arikawa-Hirasawa, E., Efird, J. T. Mercier, F. (2007). Novel extracellular matrix structures in the neural stem cell niche capture the neurogenic factor fibroblast growth factor 2 from the extracellular milieu. Stem Cells (Dayton, Ohio), 25(9), 2146–57.

Khakh, B. S., & Sofroniew, M. V. (2015). Diversity of astrocyte functions and phenotypes in neural circuits. Nature Neuroscience, 18(7), 942–952.

Kilpatrick, T. J., & Bartlett, P. F. (1993). Cloning and growth of multipotential neural precursors: requirements for proliferation and differentiation. Neuron, 10(2), 255–65.

Kim, W. R., Christian, K., Ming, G.-L., & Song, H. (2012). Time-dependent involvement of adult-born dentate granule cells in behavior. Behavioural Brain Research, 227(2), 470–9.

Knoth, R., Singec, I., Ditter, M., Pantazis, G., Capetian, P., Meyer, R. P., Horvat, V.,Volk, B., Kempermann, G. (2010). Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PloS One, 5(1), e8809.

Kokoeva, M. V, Yin, H., & Flier, J. S. (2005). Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science (New York, N.Y.), 310(5748), 679–83.

Koo, J. W., & Duman, R. S. (2008). IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. Proceedings of the National Academy of Sciences of the United States of America, 105(2), 751–6.

Koo, J. W., Russo, S. J., Ferguson, D., Nestler, E. J., & Duman, R. S. (2010). Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. Proceedings of the National Academy of Sciences of the United States of America, 107(6), 2669–74.

Kovács, Attila D, Shikha Chakraborty-Sett, Servio H Ramirez, Lynn F Sniderhan, Andrea L Williamson, and Sanjay B Maggirwar. 2004. "Mechanism of NF-kappaB Inactivation Induced by Survival Signal Withdrawal in Cerebellar Granule Neurons." The European Journal of Neuroscience 20 (2): 345–52.

Kronenberg, G., Reuter, K., Steiner, B., Brandt, M. D., Jessberger, S., Yamaguchi, M., & Kempermann, G. (2003). Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. The Journal of Comparative Neurology, 467(4), 455–63.

Kuhn, H. G., Biebl, M., Wilhelm, D., Li, M., Friedlander, R. M., & Winkler, J. (2005). Increased generation of granule cells in adult Bcl-2-overexpressing mice: a role for cell death during continued hippocampal neurogenesis. The European Journal of Neuroscience, 22(8), 1907–15.

Kunze, A., Congreso, M. R., Hartmann, C., Wallraff-Beck, A., Hüttmann, K., Bedner, P., Requardt, R., Seifert, G., Redecker, C., Willecke, K., Hofmann, A., Pfeifer, A., Theis, M., Steinhäuser, C. (2009). Connexin expression by radial glia-like cells is required for neurogenesis in the adult dentate gyrus. Proceedings of the National Academy of Sciences of the United States of America, 106(27), 11336–41.

Lacar, B., Parylak, S. L., Vadodaria, K. C., Sarkar, A., & Gage, F. H. (2014). Increasing the resolution of the adult neurogenesis picture. F1000prime Reports, 6, 8.

Lee, D. A., Yoo, S., Pak, T., Salvatierra, J., Velarde, E., Aja, S., & Blackshaw, S. (2014). Dietary and sex-specific factors regulate hypothalamic neurogenesis in young adult mice. Frontiers in Neuroscience, 8, 157.

Lee, S., Jha, M. K., & Suk, K. (2015). Lipocalin-2 in the Inflammatory Activation of Brain Astrocytes. Critical Reviews in Immunology, 35(1), 77–84.

Lenzlinger, P. M., Morganti-Kossmann, M. C., Laurer, H. L., & McIntosh, T. K. The duality of the inflammatory response to traumatic brain injury. Molecular Neurobiology, 24(1-3), 169–81.

Li, M., Sun, L., Luo, Y., Xie, C., Pang, Y., & Li, Y. (2014). High-mobility group box 1 released from astrocytes promotes the proliferation of cultured neural stem/progenitor cells. International Journal of Molecular Medicine, 34(3), 705–14.

Li, Y., Luikart, B. W., Birnbaum, S., Chen, J., Kwon, C.-H., Kernie, S. G., Bassel-Duby, R., Parada, L. F. (2008). TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. Neuron, 59(3), 399–412.

Lian, H., Yang, L., Cole, A., Sun, L., Chiang, A. C.-A., Fowler, S. W., Fowler, S. W., Shim, D. J., Rodriguez-Rivera, J., Taglialatela, G., Jankowsky, J. L., Lu, H-C., Zheng, H. (2015). NFκB-Activated Astroglial Release of Complement C3 Compromises Neuronal Morphology and Function Associated with Alzheimer's Disease. Neuron, 85(1), 101–115.

Liauw, J., Hoang, S., Choi, M., Eroglu, C., Choi, M., Sun, G., Percy, M.,Wildman-Tobriner, B., Bliss, T., Guzman, R. G., Barres, B. A., Steinberg, G. K. (2008). Thrombospondins 1 and 2 are necessary for synaptic plasticity and functional recovery after stroke. Journal of Cerebral Blood Flow and Metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 28(10), 1722–32.

Licht, T., Goshen, I., Avital, A., Kreisel, T., Zubedat, S., Eavri, R., Segal, M., Yirmiya, R.,Keshet, E. (2011). Reversible modulations of neuronal plasticity by VEGF. Proceedings of the National Academy of Sciences of the United States of America, 108(12), 5081–6.

Liddelow, S., & Barres, B. (2015). SnapShot: Astrocytes in Health and Disease. Cell, 162(5), 1170–1170.e1.

Lie, D. C., Dziewczapolski, G., Willhoite, A. R., Kaspar, B. K., Shults, C. W., & Gage, F. H. (2002). The adult substantia nigra contains progenitor cells with neurogenic potential. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 22(15), 6639–49.

Lim, D. A., & Alvarez-Buylla, A. (1999). Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. Proceedings of the National Academy of Sciences of the United States of America, 96(13), 7526–31.

Lin, R., & Iacovitti, L. (2015). Classic and novel stem cell niches in brain homeostasis and repair. Brain Research, 1–16.

Lledo, P.-M., Alonso, M., & Grubb, M. S. (2006). Adult neurogenesis and functional plasticity in neuronal circuits. Nature Reviews. Neuroscience, 7(3), 179–93.

Lois, C., & Alvarez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. Science (New York, N.Y.), 264(5162), 1145–8.

Lu, Z., & Kipnis, J. (2010). Thrombospondin 1--a key astrocyte-derived neurogenic factor. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 24(6), 1925–34.

Mattson, M. P., & Meffert, M. K. (2006). Roles for NF-kappaB in nerve cell survival, plasticity, and disease. Cell Death and Differentiation, 13(5), 852–60.

Meberg, P. J., Kinney, W. R., Valcourt, E. G., & Routtenberg, A. (1996). Gene expression of the transcription factor NF-kappa B in hippocampus: regulation by synaptic activity. Brain Research. Molecular Brain Research, 38, 179–90. Meffert, M. K., Chang, J. M., Wiltgen, B. J., Fanselow, M. S., & Baltimore, D. (2003). NF-kappa B functions in synaptic signaling and behavior. Nature Neuroscience, 6(10), 1072–8.

Meffert, M. K., Chang, J. M., Wiltgen, B. J., Fanselow, M. S., & Baltimore, D. (2003). NF-kappa B functions in synaptic signaling and behavior. Nature Neuroscience, 6(10), 1072–8.

Meneghini, V., Francese, M. T., Carraro, L., & Grilli, M. (2010). A novel role for the Receptor for Advanced Glycation End-products in neural progenitor cells derived from adult SubVentricular Zone. Molecular and Cellular Neurosciences, 45(2), 139–50.

Meneghini, V., Bortolotto, V., Francese, M. T., Dellarole, A., Carraro, L., Terzieva, S., & Grilli, M. (2013). High-mobility group box-1 protein and β-amyloid oligomers promote neuronal differentiation of adult hippocampal neural progenitors via receptor for advanced glycation end products/nuclear factor-κB axis: relevance for Alzheimer's disease. The Journal of Neuroscience. 33(14), 6047–59.

Ming, G., & Song, H. (2005). Adult neurogenesis in the mammalian central nervous system. Annual Review of Neuroscience, 28, 223–50.

Molofsky, A. V, Krencik, R., Krenick, R., Ullian, E. M., Ullian, E., Tsai, H., Deneen, B., Richardson, W. D., Barres, B. A., Rowitch, D. H. (2012). Astrocytes and disease: a neurodevelopmental perspective. Genes & Development, 26(9), 891–907.

Morrens, J., Van Den Broeck, W., & Kempermann, G. (2012). Glial cells in adult neurogenesis. Glia, 60(2), 159–74.

Mucha, M., Skrzypiec, A. E., Schiavon, E., Attwood, B. K., Kucerova, E., & Pawlak, R. (2011). Lipocalin-2 controls neuronal excitability and anxiety by regulating dendritic spine formation and maturation. Proceedings of the National Academy of Sciences of the United States of America, 108(45), 18436–41.

Mukaino, M., Nakamura, M., Okada, S., Toyama, Y., Liu, M., & Okano, H. (2008). Role of IL-6 in regulation of inflammation and stem cell differentiation in CNS trauma. Japanese Journal of Clinical Immunology, 31(2), 93–8.

Naudé, P. J. W., Eisel, U. L. M., Comijs, H. C., Groenewold, N. A., De Deyn, P. P., Bosker, F. J., Luiten, P. G. M., den Boer, J. A., Oude Voshaar, R. C. (2013). Neutrophil gelatinase-associated lipocalin: a novel inflammatory marker associated with late-life depression. Journal of Psychosomatic Research, 75(5), 444–50.

Nicola, Z., Fabel, K., & Kempermann, G. (2015). Development of the adult neurogenic niche in the hippocampus of mice. Frontiers in Neuroanatomy, 9, 53.

Oberheim, N. A., Takano, T., Han, X., He, W., Lin, J. H. C., Wang, F., Xu, Q., Wyatt, J. D., Pilcher, W., Ojemann, J. G., Ransom, B. R., Goldman, S. A. Nedergaard, M. (2009). Uniquely hominid features of adult human astrocytes. The Journal of Neuroscience :: 29(10), 3276–87.

Oh, J., McCloskey, M. A., Blong, C. C., Bendickson, L., Nilsen-Hamilton, M., & Sakaguchi, D. S. (2010). Astrocyte-derived interleukin-6 promotes specific neuronal differentiation of neural progenitor cells from adult hippocampus. Journal of Neuroscience Research, 88(13), 2798–809.

Oikawa, K., Odero, G. L., Platt, E., Neuendorff, M., Hatherell, A., Bernstein, M. J., & Albensi, B. C. (2012). NF-κB p50 subunit knockout impairs late LTP and alters long term memory in the mouse hippocampus. BMC Neuroscience, Jul 11;13:45.

Palmer, T. D., Willhoite, A. R., & Gage, F. H. (2000). Vascular niche for adult hippocampal neurogenesis. The Journal of Comparative Neurology, 425(4), 479–94.

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T. A., Guiducci, E., Dumas, L., Gross, C. T. (2011). Synaptic pruning by microglia is necessary for normal brain development. Science (New York, N.Y.), 333(6048), 1456–8.

Pfrieger, F. W., & Barres, B. A. (1997). Synaptic efficacy enhanced by glial cells in vitro. Science, 277(5332), 1684–7.

Pierce, A. A., & Xu, A. W. (2010). De novo neurogenesis in adult hypothalamus as a compensatory mechanism to regulate energy balance. The Journal of Neuroscience, 30(2), 723–30.

Pittenger, C., & Duman, R. S. (2008). Stress, depression, and neuroplasticity: a convergence of mechanisms. Neuropsychopharmacology, 33(1), 88–109.

Platel, J.-C., Dave, K. A., Gordon, V., Lacar, B., Rubio, M. E., & Bordey, A. (2010). NMDA receptors activated by subventricular zone astrocytic glutamate are critical for neuroblast survival prior to entering a synaptic network. Neuron, 65(6), 859–72.

Pluchino, S., Muzio, L., Imitola, J., Deleidi, M., Alfaro-Cervello, C., Salani, G., Porcheri, C., Brambilla, E., Cavasinni, F., Bergamaschi, A., Garcia-Verdugo, J. M., Comi, G., Khoury, S. J., Martino, G. (2008). Persistent inflammation alters the function of the endogenous brain stem cell compartment. Brain : A Journal of Neurology, 131(Pt 10), 2564–78.

Pozniak, P. D., White, M. K., & Khalili, K. (2013). TNF-α/NF-κB Signaling in the CNS: Possible Connection to EPHB2. Journal of Neuroimmune Pharmacology, 9(2), 133–141.

Reynolds, B., & Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science, 255(5052), 1707–1710.

Ricci-Vitiani, L., Casalbore, P., Petrucci, G., Lauretti, L., Montano, N., Larocca, L. M., Falchetti, M. L., Lombardi, D. G., Gerevini, V., Di Giorgio Cenciarelli, C., D'Alessandris, Q. G., Fernandez, E., De Maria, R., Maira, G., Peschle, C., Parati, E., Pallini, R. (2006). Influence of local environment on the differentiation of neural stem cells engrafted onto the injured spinal cord. Neurological Research, 28(5), 488–92.

Richardson, D.R., 2005. 24p3 and its receptor: dawn of a new iron age? Cell, 123(7), pp.1175–7.

Risher, W. C., & Eroglu, C. (2012). Thrombospondins as key regulators of synaptogenesis in the central nervous system. Matrix Biology : Journal of the International Society for Matrix Biology, 31(3), 170–7.

Robins, S. C., Stewart, I., McNay, D. E., Taylor, V., Giachino, C., Goetz, M., Ninkovic, J., Briancon, N., Maratos-Flier, E., Flier, J. S., Kokoeva, M. V., Placzek, M. (2013). α-Tanycytes of the adult hypothalamic third ventricle include distinct populations of FGF-responsive neural progenitors. Nature Communications, 4, 2049.

Rolls, A., Shechter, R., London, A., Ziv, Y., Ronen, A., Levy, R., & Schwartz, M. (2007). Toll-like receptors modulate adult hippocampal neurogenesis. Nature Cell Biology, 9(9), 1081-8.

Romano, A., Freudenthal, R., Merlo, E., & Routtenberg, A. (2006). Evolutionarily-conserved role of the NF-kappaB transcription factor in neural plasticity and memory. The European Journal of Neuroscience, 24(6), 1507–16.

Rudge, J. S., Alderson, R. F., Pasnikowski, E., McClain, J., Ip, N. Y., & Lindsay, R. M. (1992). Expression of Ciliary Neurotrophic Factor and the Neurotrophins - Nerve Growth Factor, Brain-Derived Neurotrophic Factor and Neurotrophin 3-in Cultured Rat Hippocampal Astrocytes. European Journal of Neuroscience, 4(6), 459–471.

Sahay, A., & Hen, R. (2007). Adult hippocampal neurogenesis in depression. Nature Neuroscience, 10(9), 1110–5.

Sahay, A., Wilson, D. A., & Hen, R. (2011). Pattern separation: a common function for new neurons in hippocampus and olfactory bulb. Neuron, 70(4), 582–8.

Salles, A., Romano, A., & Freudenthal, R. (2014). Synaptic NF-kappa B pathway in neuronal plasticity and memory. Journal of Physiology-Paris, 108(4-6), 256–262.

Saneyoshi, T., Fortin, D. A., & Soderling, T. R. (2010). Regulation of spine and synapse formation by activity-dependent intracellular signaling pathways. Current Opinion in Neurobiology, 20(1), 108–15.

Scharfman, H., Goodman, J., Macleod, A., Phani, S., Antonelli, C., & Croll, S. (2005). Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. Experimental Neurology, 192(2), 348–56.

Schmidt-Ullrich, R., Mémet, S., Lilienbaum, A., Feuillard, J., Raphaël, M., & Israel, A. (1996). NF-kappaB activity in transgenic mice: developmental regulation and tissue specificity. Development (Cambridge, England), 122(7), 2117–28.

Seri, B., Garcia-Verdugo, J. M., McEwen, B. S., & Alvarez-Buylla, A. (2001). Astrocytes Give Rise to New Neurons in the Adult Mammalian Hippocampus. J. Neurosci., 21(18), 7153–7160.

Seri, B., García-Verdugo, J. M., Collado-Morente, L., McEwen, B. S., & Alvarez-Buylla, A. (2004). Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. The Journal of Comparative Neurology, 478(4), 359–78.

Sha, W. C., Liou, H.-C., Tuomanen, E. I., & Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF-κB leads to multifocal defects in immune responses. Cell, 80(2), 321–330.

Shen, Q., Wang, Y., Kokovay, E., Lin, G., Chuang, S.-M., Goderie, S. K., Roysam, B., Temple, S. (2008). Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. Cell Stem Cell, 3(3), 289–300.

Sierra, A., Encinas, J. M., Deudero, J. J. P., Chancey, J. H., Enikolopov, G., Overstreet-Wadiche, L. S., Tsirka, S. E., Maletic-Savatic, M. (2010). Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. Cell Stem Cell, 7(4), 483–95.

Sloan, S. A., & Barres, B. A. (2014). Mechanisms of astrocyte development and their contributions to neurodevelopmental disorders. Current Opinion in Neurobiology, 27, 75–81.

Snow, W. M., Stoesz, B. M., Kelly, D. M., & Albensi, B. C. (2014). Roles for NF-κB and gene targets of NF-κB in synaptic plasticity, memory, and navigation. Molecular Neurobiology, 49(2), 757–70.

Sofroniew, M. V. (2015). Astrogliosis. Cold Spring Harbor Perspectives in Biology, 7(2), a020420.

Son, S. M., Nam, D. W., Cha, M.-Y., Kim, K. H., Byun, J., Ryu, H., & Mook-Jung, I. (2015). Thrombospondin-1 prevents amyloid beta-mediated synaptic pathology in Alzheimer's disease. Neurobiology of Aging, 36(12), 3214–27.

Song, H., Stevens, C. F., & Gage, F. H. (2002). Astroglia induce neurogenesis from adult neural stem cells. Nature, 417(6884), 39–44.

Sultan, S., Mandairon, N., Kermen, F., Garcia, S., Sacquet, J., & Didier, A. (2010). Learning-dependent neurogenesis in the olfactory bulb determines long-term olfactory memory. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 24(7), 2355–63.

Sultan, S., Gebara, E. G., Moullec, K., & Toni, N. (2013). D-serine increases adult hippocampal neurogenesis. Frontiers in Neuroscience, 7, 155.

Sultan, S., Li, L., Moss, J., Petrelli, F., Cassé, F., Gebara, E., Lopatar, J., Pfrieger, F. W., Bezzi, P., Bischofberger, J., Toni, N. (2015). Synaptic Integration of Adult-Born Hippocampal Neurons Is Locally Controlled by Astrocytes. Neuron, 88(5), 957–72.

Suzuki, A., Stern, S. A., Bozdagi, O., Huntley, G. W., Walker, R. H., Magistretti, P. J., & Alberini, C. M. (2011). Astrocyte-neuron lactate transport is required for long-term memory formation. Cell, 144(5), 810–23.

Takata, K., Kitamura, Y., Kakimura, J., Shibagaki, K., Tsuchiya, D., Taniguchi, T., Smith, M. A., Perry, G., Shimohama, S. (2003). Role of high mobility group protein-1 (HMG1) in amyloid-beta homeostasis. Biochemical and Biophysical Research Communications, 301(3), 699–703.

Uberti, D, M Grilli, and M Memo. “Contribution of NF-kappaB and p53 in the Glutamate-Induced Apoptosis.” International Journal of Developmental Neuroscience : The Official Journal of the International Society for Developmental Neuroscience 18 (4-5): 447–54.

Ueki, T., Tanaka, M., Yamashita, K., Mikawa, S., Qiu, Z., Maragakis, N. J., Hevner, R. F., Miura, N., Sugimura, H., Sato, K. (2003). A novel secretory factor, Neurogenesin-1, provides neurogenic environmental cues for neural stem cells in the adult hippocampus. Journal of Neuroscience, 23(37), 11732–40.

Vadodaria, K. C., & Jessberger, S. (2014). Functional neurogenesis in the adult hippocampus: then and now. Frontiers in Neuroscience, 8, 55.

Valente, M. M., Bortolotto, V., Cuccurazzu, B., Ubezio, F., Meneghini, V., Francese, M. T., Canonico P.L. Grilli, M. (2012). α2δ Ligands Act as Positive Modulators of Adult Hippocampal Neurogenesis and Prevent Depressive-like Behavior Induced by Chronic Restraint Stress. Molecular Pharmacology. 82(2):271-80.

Vallabhapurapu, S., & Karin, M. (2009). Regulation and function of NF-kappaB transcription factors in the immune system. Annual Review of Immunology, 27, 693–733.

Viatour, P., Merville, M.-P., Bours, V., & Chariot, A. (2005). Phosphorylation of NF-κB and IκB proteins: implications in cancer and inflammation. Trends in Biochemical Sciences, 30(1), 43–52.

Wada, T., Haigh, J. J., Ema, M., Hitoshi, S., Chaddah, R., Rossant, J., Nagy, A., van der Kooy, D. (2006). Vascular endothelial growth factor directly inhibits primitive neural stem cell survival but promotes definitive neural stem cell survival. Journal of Neuroscience, 26(25), 6803–12.

Wang, S., Scott, B. W., & Wojtowicz, J. M. (2000). Heterogenous properties of dentate granule neurons in the adult rat. Journal of Neurobiology, 42(2), 248–57.

Waterhouse, E. G., An, J. J., Orefice, L. L., Baydyuk, M., Liao, G.-Y., Zheng, K., Lu, B., Xu, B. (2012). BDNF promotes differentiation and maturation of adult-born neurons through GABAergic transmission. Journal of Neuroscience, 32(41), 14318–30.

Wellmann, H., Kaltschmidt, B., & Kaltschmidt, C. (2000). Retrograde Transport of Transcription Factor NF-kappa B in Living Neurons. Journal of Biological Chemistry, 276(15), 11821–11829.

Widera, D., Mikenberg, I., Elvers, M., Kaltschmidt, C., & Kaltschmidt, B. (2006). Tumor necrosis factor alpha triggers proliferation of adult neural stem cells via IKK/NF-kappaB signaling. BMC Neuroscience, 7, 64.

Widera, D., Kaus, A., Kaltschmidt, C., & Kaltschmidt, B. (2008). Neural stem cells, inflammation and NF-kappaB: basic principle of maintenance and repair or origin of brain tumours? Journal of Cellular and Molecular Medicine, 12(2), 459–70.

Yang, J., & Moses, M. A. (2009). Lipocalin 2: a multifaceted modulator of human cancer. Cell Cycle (Georgetown, Tex.), 8(15), 2347–52.

Yon, M. A., Mauger, S. L., & Pickavance, L. C. (2013). Relationships between dietary macronutrients and adult neurogenesis in the regulation of energy metabolism. The British Journal of Nutrition, 109(9), 1573–89.

Zhang, Y., & Hu, W. (2012). NF-kapapB signaling regulates embryonic and adult neurogenesis. Frontiers in Biology, 7(4), 277–291.

Zhang, Y., Liu, J., Yao, S., Li, F., Xin, L., Lai, M., Bracchi-Ricard, V., Xu, H., Yen, W., Meng, W., Liu, S., Yang, L., Karmally, S., Liu, J., Zhu, H., Gordon, J., Khalili, K., Srinivasan, S., Bethea, J. R., Mo, X., Hu, W. (2012). Nuclear Factor Kappa B Signaling Initiates Early Differentiation of Neural Stem Cells. STEM CELLS, 30(3), 510–524.

Zhang Y., and Hu W. (2013). NFκB Signaling Directs Neuronal Fate Decision, Trends in Cell Signaling Pathways in Neuronal Fate Decision, Dr Sabine Wislet-Gendebien (Ed.), ISBN: 978-953-51-1059-0

# Chapter 2.

## Outline of the thesis

Neurogenesis occuring during postnatal life in restricted to two selected regions in the adult brain of mammalians. The canonical and well-studied neurogenic sites are the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, and the subventricular zone (SVZ) of the lateral ventricles (Ming and Song 2005; Bond et al. 2015; Kempermann et al. 2015). Emerging evidence recently suggested a novel neurogenic site along the walls of the third ventricle in the hypothalamic area, whose features and functions are under investigation (Kokoeva et al., 2005; Robins et al., 2013).

Large interest grew for hippocampal adult neurogenesis due to the involvement of this brain region in cognitive functions like learning and memory, processes relevant for human beings, as well as in emotional behavior and stress response (Aimone et al. 2010; Vadodaria and Jessberger 2014).

Adult hippocampal neurogenesis (ahNG) is a highly dynamic and plastic process strictly connected to environmental changes. Many extrinsic modulators (i.e. learning, enriched environment, physical activity; antidepressants drugs) have been reported to enhance hippocampal neurogenesis and, in parallel, long term potentiation and cognitive performances, reversal of depressive-like behaviour and resilience to stress in mouse models (Hanson et al., 2011). On the other hand, physio- and pathological conditions like aging, stress and inflammation, as well as addictive drugs, have been reported as negative regulators which result in decreased neurogenesis and performance in tasks of spatial navigation and alteration in emotion-related behaviour (Lazarov et al., 2010; Yirmiya and Goshen, 2011; Lucassen et al., 2015). Last, but not least, it is of great interest that hippocampal neurogenesis is deregulated in various neuropsychiatric disorders, such as major depressive disorder and anxiety, schizofrenia, and neurodegenerative diseases (Winner et al., 2011; Nath et al., 2012; Schoenfeld and Cameron, 2015; Miller and Hen, 2015).

Adult neurogenesis occurs in a permissive and instructive microenvironment referred to as the “neurogenic niche” where local environmental cues, both

membrane-associated and released by resident cells, interact with neural/stem progenitor cells (NSPC) and in turn affect their functionality (self-renewal and multipotential properties), their commitment and their survival. The niche is the result of the complex interplay among different cell types (NSPC, precursors, astroglia, microglia, neuroblasts), secreted molecules (i.e. cytokines, growth factors, secreted proteins), vasculature, and extracellular matrix (ECM) components (Lin and Iacovitti 2015). Cell-to-cell communication plays a key role in the homeostatic modulation of the neurogenic process under physiological conditions and also in response to damage in pathological conditions. Among the niche cellular components, astrocytes represent one important contributor which can instruct NSPC proliferation, affect the maintenance of their undifferentiated state, modulate their lineage differentiation through both contact-dependent interactions and secretion of soluble molecules. In addition, astrocytes can also influence maturation, survival and integration of newly generated neurons by providing signals which are critical for synaptogenesis and dendritic spine maturation (Song et al. 2002; Oh et al. 2010; Risher and Eroglu 2012).

In previous publications our lab proved that members of the NF-κB family of transcription factors are important contributors of signaling pathways in the SGZ neurogenic niche (Denis-Donini et al., 2005) and may be potentially involved in mediating the proneurogenic and antidepressive-like effect of some clinically relevant drugs (i.e. α2δ ligands, acetyl-L-carnitine) (Cuccurazzu et al., 2013; Valente et al., 2012) and in the proneurogenic effects of damage-associated signals (i.e. HMGB-1 and $A\beta_{1-2}$) (Meneghini et al., 2013). In the CNS NF-κB is involved in neuroprotection/neurodegeneration, differentiation, migration, synaptic plasticity, and neurite outgrowth, all functions making this family of transcription factors potential candidates for translating complex signals that regulate the neurogenic process (Grilli and Memo, 1999; Kaltschmidt and Kaltschmidt, 2009; Bortolotto et al., 2014). Within the family, the p50 subunit appears to play a crucial role since p50KO mice display

dramatically reduced adult hippocampal neurogenesis which is in association with short-term memory defects (Denis-Donini et al., 2008).

The main project for this PhD thesis developed from the observation that unlike *in vivo*, hippocampal NPC derived from WT and p50KO adult mice have similar rate of differentiation, proliferation and apoptosis *in vitro*.

Based on these observations, in our first investigation, we exploited the advantages of using primary cell culture systems from WT and p50KO mice to investigate the contribution made by astrocyte conditioned media (ACM) under basal conditions, on adult NPC fate specification. In particular, we wanted to elucidate the involvement of astroglial cells in the defective hippocampal neurogenesis observed *in vivo*. In our *in vitro* experimental context, we analyzed the role of the NF-κB pathway in the cross-talk between astrocytes and adult hippocampal NPC. For this purpose, we set up primary astroglial cultures, as well NPC cultures, from both WT and p50KO mice and analyzed the phenotypic commitment of WT/p50KO NPC when exposed to WT/p50KO astrocyte-conditioned media. On one hand, we attempted to identify the proneurogenic and/or antineurogenic signals that, under control of NF-κB p50, may be differentially expressed in astrocytes. On the other hand, we also focused our attention on the possibility that absence of p50 may produce cell-autonomous defects in NPC responsiveness to astrocytic-derived factors.

An additional line of investigation was devoted to explore region-specificity in astrocyte-derived signals that may affect cell fate commitment of hippocampal adult NPC. In particular, we analyzed differences in the pro-neurogenic and -gliogenic potential of hippocampal and hypothalamic astrocytes of both WT and p50KO origin. Additional work was performed with the purpose of understanding fate specification of hypothalamic WT NPC cultures in presence hippocampal ACM.

**References of the Outline of the thesis**

Aimone, J. B., Deng, W., & Gage, F. H. (2010). Adult neurogenesis: integrating theories and separating functions. Trends in Cognitive Sciences, 14(7), 325–37.

Bond, A. M., Ming, G.-L., & Song, H. (2015). Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. Cell Stem Cell, 17(4), 385–95.

Bortolotto V, Cuccurazzu B, Canonico PL, Grilli M (2014) NF-κB mediated regulation of adult hippocampal neurogenesis: relevance to mood disorders and antidepressant activity. BioMed Research International 2014, 612798.

Cuccurazzu, B., Bortolotto, V., Valente, M. M., Ubezio, F., Koverech, A., Canonico, P. L., & Grilli, M. (2013). Upregulation of mGlu2 receptors via NF-κB p65 acetylation is involved in the Proneurogenic and antidepressant effects of acetyl-L-carnitine. Neuropsychopharmacology : 38(11), 2220–30.

Denis-Donini, S., Caprini, A., Frassoni, C., & Grilli, M. (2005). Members of the NF-kappaB family expressed in zones of active neurogenesis in the postnatal and adult mouse brain. Brain Research. Developmental Brain Research, 154(1), 81–9.

Denis-Donini, S., Dellarole, A., Crociara, P., Francese, M. T., Bortolotto, V., Quadrato, G., Canonico, P LOrsetti, M.,Ghi, P.,Memo, M., Bonini, S. A., Ferrari-Toninelli, G. Grilli, M. (2008). Impaired adult neurogenesis associated with short-term memory defects in NF-kappaB p50-deficient mice. The Journal of Neuroscience, 28(15), 3911–9.

Grilli M, Memo M (1999). Nuclear factor-kappaB/Rel proteins: a point of convergence of signaling pathways relevant in neuronal function and dysfunction. Biochemical Pharmacology, 57(1), 1–7.

Hanson, N. D., Owens, M. J., & Nemeroff, C. B. (2011). Depression, antidepressants, and neurogenesis: a critical reappraisal. Neuropsychopharmacology, 36(13), 2589–602.

Kaltschmidt, B., & Kaltschmidt, C. (2009). NF-kappaB in the nervous system. Cold Spring Harbor Perspectives in Biology, 1(3), a001271.

Kempermann, G., Song, H., & Gage, F. H. (2015). Neurogenesis in the Adult Hippocampus. Cold Spring Harbor Perspectives in Medicine, 5(7), a018812.

Kokoeva, M. V, Yin, H., & Flier, J. S. (2005). Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science (New York, N.Y.), 310(5748), 679–83.

Lazarov, O., Mattson, M. P., Peterson, D. A., Pimplikar, S. W., & van Praag, H. (2010). When neurogenesis encounters aging and disease. Trends in Neurosciences, 33(12), 569–79.

Lin, R., & Iacovitti, L. (2015). Classic and novel stem cell niches in brain homeostasis and repair. Brain Research, 1–16.

Lucassen, P. J., Oomen, C. A., Naninck, E. F. G., Fitzsimons, C. P., van Dam, A.-M., Czeh, B., & Korosi, A. (2015). Regulation of Adult Neurogenesis and Plasticity by (Early) Stress, Glucocorticoids, and Inflammation. Cold Spring Harbor Perspectives in Biology, 7(9), a021303.

Meneghini, V., Bortolotto, V., Francese, M. T., Dellarole, A., Carraro, L., Terzieva, S., & Grilli, M. (2013). High-mobility group box-1 protein and β-amyloid oligomers promote neuronal differentiation of adult hippocampal neural progenitors via receptor for advanced glycation end products/nuclear factor-κB axis: relevance for Alzheimer's disease. The Journal of Neuroscience, 33(14), 6047–59.

Miller, B. R., & Hen, R. (2015). The current state of the neurogenic theory of depression and anxiety. Current Opinion in Neurobiology, 30, 51–8.

Ming, G., & Song, H. (2005). Adult neurogenesis in the mammalian central nervous system. Annual Review of Neuroscience, 28, 223–50.

Nath, S., Agholme, L., Kurudenkandy, F. R., Granseth, B., Marcusson, J., & Hallbeck, M. (2012). Spreading of Neurodegenerative Pathology via Neuron-to-Neuron Transmission of β-Amyloid. The Journal of Neuroscience, 32(26), 8767–77.

Oh, J., McCloskey, M. A., Blong, C. C., Bendickson, L., Nilsen-Hamilton, M., & Sakaguchi, D. S. (2010). Astrocyte-derived interleukin-6 promotes specific neuronal differentiation of neural progenitor cells from adult hippocampus. Journal of Neuroscience Research, 88(13), 2798–809.

Risher, W. C., & Eroglu, C. (2012). Thrombospondins as key regulators of synaptogenesis in the central nervous system. Matrix Biology, 31(3), 170–7.

Robins, S. C., Stewart, I., McNay, D. E., Taylor, V., Giachino, C., Goetz, M., Ninkovic, J, Briancon, N, Maratos-Flier, E, Flier, J S, Placzek, M. (2013). α-Tanycytes of the adult hypothalamic third ventricle include distinct populations of FGF-responsive neural progenitors. Nature Communications, 4, 2049.

Schoenfeld, T. J., & Cameron, H. A. (2015). Adult neurogenesis and mental illness. Neuropsychopharmacology : 40(1),113-28.

Song, H., Stevens, C. F., & Gage, F. H. (2002). Astroglia induce neurogenesis from adult neural stem cells. Nature, 417(6884), 39–44.

Vadodaria, K. C., & Jessberger, S. (2014). Functional neurogenesis in the adult hippocampus: then and now. Frontiers in Neuroscience, 8, 55.

Valente, M. M., Bortolotto, V., Cuccurazzu, B., Ubezio, F., Meneghini, V., Francese, M. T., Canonico, P. L., Grilli, M. (2012). α2δ Ligands Act as Positive Modulators of Adult Hippocampal Neurogenesis and Prevent Depressive-like Behavior Induced by Chronic Restraint Stress. Molecular Pharmacology. 82(2):271-80.

Winner, B., Kohl, Z., & Gage, F. H. (2011). Neurodegenerative disease and adult neurogenesis. The European Journal of Neuroscience, 33(6), 1139–51.

Yirmiya, R., & Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. Brain, Behavior, and Immunity, 25(2), 181–213.

# Chapter 3.

# Astrocyte-mediated neuronal fate specification of adult hippocampal neural progenitors is regulated by NF-κB p50.

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Running title: Role of NF-κB p50 in adult neural progenitor-astrocyte cross-talk

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**Abstract**

In previous work we demonstrated that NF-κB p50 acts as crucial regulator of adult hippocampal neural progenitor cells (ahNPC). Indeed, NF-κB p50KO mice are characterized by remarkably reduced hippocampal neurogenesis. As a follow up to that work, herein we show that when cultured *in vitro*, ahNPC from wild type (WT) and p50KO mice are not significantly different in their neurogenic potential. This observation prompted us to investigate cell autonomous and non-cell autonomous consequences of p50 absence on neuronal fate specification of ahNPC. In particular, we focused our attention on astrocytes, known to provide soluble proneurogenic signals, and investigated the influence of WT and p50KO astrocyte conditioned media (ACM) on WT and p50KO ahNPC differentiation. Interestingly, while WT ACM promoted both neuronal and astroglial differentiation, p50KO ACM only supported astroglial differentiation of WT ahNPC. By using a LC-MS/MS approach we identified several proteins which are significantly upregulated in p50KO compared to WT astrocytes. Among them, lipocalin-2 (LCN-2) was recognized as a novel astroglial-derived signal regulating neuronal fate specification of ahNPC. Interestingly, LCN-2 proneurogenic effect is greatly reduced in p50KO NPC. In addition, we demonstrated p50KO NPC unresponsiveness to both neuronal and astroglial fate specification signals from WT and p50KO ACM, and we identified reduced expression of α2δ1, a thrombospondin-1 receptor, as one phenotypic change occurring in ahNPC in absence of p50. Altogether our data suggest that reduced hippocampal neurogenesis in p50KO mice *in vivo* is to be ascribed to cell autonomous and, likely, to non-cell autonomous defects in the hippocampal neurogenic niche.

## Introduction

Adult neurogenesis occurs in restricted areas of postnatal and adult mammalian brain, namely the subventricular zone (SVZ) in the lateral wall of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (Ming and Song, 2005; Bond et al., 2015; Kempermann et al., 2015). Recent studies also suggested an additional neurogenic region in the adult hypothalamus (Kokoeva et al., 2005; Whalley, 2012).

Adult hippocampal neurogenesis (ahNG) has attracted great research interest due to its potential involvement in critical functions including cognition, mood and emotional behavior, stress response (Aimone et al., 2010, 2014; Lacar et al., 2014). The molecular mechanisms that modulate neurogenesis are very complex and deserve extensive investigation which may turn into a better understanding of homeostatic regulation of ahNG but also of neuropsychiatric/neurodegenerative disorders where this process is profoundly deregulated (Winner et al., 2011; Apple et al., 2016).

Within the CNS, NF-κB pathway activation has been involved in a wide range of functions both under physiological and pathological conditions (Grilli and Memo, 1999; Mattson and Meffert, 2006; Kaltschmidt and Kaltschmidt, 2009). A few years ago our group demonstrated that NF-κB proteins are expressed at considerable levels in areas of active neurogenesis in the postnatal and adult mouse brain (Denis-Donini et al., 2005). Since then, a vast array of information has been collected on the complex involvement of NF-κB proteins in different aspects of postnatal neurogenesis. In particular, several extracellular signals and membrane receptors have been identified as being able to affect neural stem cells/neural progenitor cells (NSC/NPC) and their progeny via NF-κB activation (Wada et al, 2006; Rolls et al., 2007; Widera et al., 2008; Meneghini et al., 2010, 2013).

Since the initial observations of NF-κB members being present in adult neurogenic areas, our group further explored the role of these regulatory proteins in adult neurogenesis with a specific focus on the NF-κB1 p50 subunit

(Grilli and Meneghini, 2012; Bortolotto et al., 2014). By taking advantage of p50KO mice (Sha et al., 1995) we demonstrated that absence of p50 can deeply affect the *in vitro* response of adult hippocampal NPC (ahNPC) to several endogenous signals (Meneghini et al., 2013), and to proneurogenic drugs (Valente et al., 2012). *In vivo* we also proved that p50KO mice display a dramatic reduction in adult hippocampal neurogenesis which correlates with a selective defect in hippocampal-dependent short-term memory (Denis-Donini et al., 2008). Interestingly, *in vivo* the proliferation rate of hippocampal NSC/NPC in p50KO mice appeared to be similar to that of WT mice. Similarly, the apoptotic rate in the hippocampal region was not increased in mutant mice compared to their WT counterpart. A detailed phenotypic characterization of newly generated hippocampal cells in p50KO mice suggested that absence of the NF-κB p50 subunit may trigger a rather selective defect in neuronal differentiation of ahNPC (Denis-Donini et al., 2008).

Homeostasis of adult neurogenesis requires permissive and instructive signals for aNSC/NPC. Several elegant studies have investigated the mechanisms through which local environment in the neurogenic niche may control fate specification of aNSC/NPC (Schänzer et al., 2004; Bonaguidi et al., 2005; Lu and Kipnis, 2010). Among others, a pivotal work by Song et al. (2002) demonstrated that astroglial cells are crucial regulators in the hippocampal niche, where they can instruct stem cells to adopt a neuronal fate (Song et al., 2002). Since the well documented role of NF-κB in neuronal but also in non-neuronal cells (Brambilla et al., 2009; Lian et al., 2015; de la Fuente et al., 2015), we recently decided to further dissect the role of NF-κB p50 in the cross-talk between adult neural progenitor cells and astrocytes. Exposure of WT and p50KO hippocampal NPC to conditioned media from WT and p50KO astrocytes was utilized as a strategy to study the potential influence of astroglia on adult NPC fate specification and the cell autonomous or non-cell autonomous role played by NF-κB p50 signaling in that context.

## Materials and Methods

*Animals.* Wild type (WT; C57BL/6; The Jackson Laboratories) and NF-κB p50$^{-/-}$ (p50KO; C57BL/6 Nfkb1$^{tm1Bal}$/J; The Jackson Laboratories) mice were housed under light- and temperature-controlled conditions in high-efficiency particulate air (HEPA)-filtered Thoren units (Thoren Caging Systems) at the University of Piemonte Orientale animal facility. Mice were kept 3-4/cage with *ad libitum* access to water and food. Animal care and handling were performed in accordance with European Community Directive and approved by the local IACUC (Institutional Animal Care and Use Committees).

*Isolation and culture of WT and p50KO adult mouse hippocampal neural progenitor cells (NPC).* For preparing NPC primary cultures from hippocampi, three adult (3-4 month-old) male WT and p50KO mice were sacrificed and cell suspension was prepared. Briefly, the brains were removed, and hippocampi were isolated and collected in ice-cold PIPES buffer pH 7.4 containing 20 mM PIPES, 25 mM glucose, 0.5 M KCl, 0.12 M NaCl (Sigma-Aldrich), and 100 U/100 µg/ml Penicillin/Streptomycin solution (Life Technologies). After 5 min at 110 x *g* centrifugation, the tissue was digested for 40 min at 37°C using the Papain Dissociation System (Worthington DBA). Cell suspension was plated onto 25 cm$^2$ cell-culture flask (Thermo-Fisher Scientific) and cultured as floating neurospheres through subsequent passages (Valente et al., 2012). Primary (Passage 1, P1) neurospheres were dissociated after 7-10 days *in vitro* (DIV), whereas P2-P30 neurospheres every 5 DIV. At each passage cells were plated in T25 flask at a density of 12,000 cells/cm$^2$ in growing medium: Neurobasal-A medium, supplemented with B27 supplement, 2 mM L-glutamine (Life Technologies), human Epidermal Growth Factor (hEGF, 20 ng/ml; Peprotech), basic Fibroblast Growth Factor (bFGF, 10 ng/ml; Peprotech) and Heparin sodium salt (0.0004 %, Sigma-Aldrich).

*Adult hippocampal NPC differentiation.* NPC (P5-P30) were used for differentiation experiments. Briefly, neurospheres were dissociated into single cells and plated onto laminin-coated Lab-Tek 8-well permanox chamber slides (Nunc) at a density of 43,750 cells/cm$^2$ in NPC differentiation medium (NDM, Neurobasal-A medium supplemented with B27, 2 mM L-glutamine and 100 U/100 µg/ml Penicillin/Streptomycin). NPC were treated in presence of hippocampal astrocyte conditioned media (ACM) derived from WT or NF-κB p50KO glial cultures, or in presence of NDM (referred to as standard, STD medium) for 24 h. WT NPC were treated in presence of 0.01–1 µg/ml recombinant mouse lipocalin-2 (LCN-2, Cell Signaling), 2 µg/ml purified human Thrombospondin-1 (hTSP-1, Amsbio), or corresponding vehicle for 24 h. For RAGE inhibition, 60 min before ACM or NDM treatment, 20 µg/ml neutralizing polyclonal anti-RAGE antibody (α-RAGE Ab; R&D System) was added to NPC.

*Primary Astrocyte Cultures.* Primary mixed glial cultures were prepared from cortex and hippocampus of neonatal (P1-2) C57BL/6 WT and p50KO mice and grown in DMEM high glucose, 10% FBS, 15 mM HEPES, 2 mM L-glutamine and 100 U/100 µg/ml Penicillin/Streptomycin at a density of 78,000 cells/cm$^2$. When cells reached confluence (around 10-12 D.I.V.), proliferation of non-astrocytic cells was blocked by 10 µM cytosine arabinoside (Sigma-Aldrich) for 96 h. Then cultures were switched to NDM. Phenotypic characterization of cultures was performed by immunocytochemistry with antibodies against GFAP (mouse monoclonal, 1:600, Millipore) and CD11b (rat monoclonal; 1:150, Millipore). Contaminating microglial cells were below 3% of total cells, with the remaining cells being GFAP$^+$. ACM was collected after 48h in presence of NDM. Briefly, ACM was centrifuged for 10 min at 16,000 x *g* to eliminate cells and debris and passed through 0.2 µm filter before use. ACM was used diluted 1:2 in fresh NDM.

*Immunolocalization studies in differentiated adult hippocampal NPC.* NPC were treated in presence of WT or p50KO ACM or STD medium for 24 h. Phenotypic characterization of NPC-derived cells was carried out by immunolocalization for MAP-2 (rabbit polyclonal, 1:600; Millipore), GFAP (mouse monoclonal, 1:600; Millipore), nestin (chicken monoclonal, 1:1,500; Neuromics), NG-2 (rabbit polyclonal, 1:500; Millipore). Secondary antibodies were as follows: Alexa Fluor 555-conjugated goat anti-rabbit (1:1,400; Molecular Probes); Alexa Fluor 488-conjugated goat anti-chicken antibody (1:1,400; Molecular Probes); Alexa Fluor 488-conjugated goat anti-rabbit (1:1,400; Molecular Probes), Alexa Fluor 488-conjugated goat anti-mouse (1:1,600; Molecular Probes). Nuclei were counterstained with 0.8 ng/ml Hoechst (Thermo Fisher scientific) diluted in PBS. In each experiment, 5 fields/well (corresponding to about 150-200 cells/well) were counted with a 60X objective by a Leica DMIRB inverted fluorescence microscope. Cells positive for each marker were counted and their percentage over total viable cells was calculated. All experiments were run in triplicates using different cell preparations and repeated at least three times.

*LC-MS/MS analysis and protein quantification.* WT and p50KO ACM were subjected to trichloroacetic acid (TCA) precipitation. Briefly, TCA was added to a final 12% (wt/vol) concentration and kept on ice for 2 h, followed by centrifugation (16,000 x g, 10 min). The supernatant was carefully removed, and 1 ml of tetrahydrofuran (pre-cooled in ice) was added to each pellet and vortexing was carried out until the pellet dissolved completely. Proteins were then reduced with dithiothreitol (DTT), alkylated by iodoacetamide, and digested with trypsin at 37°C O/N. Data acquisition was performed with a micro-LC Eksigent Technologies (Dublin, USA) system, with a stationary phase a Halo Fused C18 column (0.5 x 100 mm, 2.7 µm; Eksigent Technologies), interfaced with a Triple-TOF 5600+ system (AB SCIEX) equipped with a DuoSpray Ion source. The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B),

eluting at a flow-rate of 15.0 mL min-1 at an increasing concentration of solvent B from 2% to 40 % in 30 minutes. An initial data-dependent acquisition (DDA) analysis was performed on the p50KO and WT ACM samples to generate the SWATH-MS (Sequential Window Acquisition of Theoretical mass spectra) spectral library: the mass spectrometer analysis was performed using a mass range of 100–1500 Da (TOF scan with an accumulation time of 0.25 s), followed by a MS/MS product ion scan from 200 to 1250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps (35 candidate ions can be monitored during every cycle). The samples were then subjected to cyclic data independent analysis (DIA) of the mass spectra, using a 25-Da window: the mass spectrometer was operated such that a 50-ms survey scan (TOF-MS) was performed and subsequent MS/MS experiments were performed on all precursors. These MS/MS experiments were performed in a cyclic manner using an accumulation time of 40 ms per 25-Da swath (36 swaths total) for a total cycle time of 1.5408 s (Venable et al., 2004, Geromanos et al., 2009; Gillet et al., 2012). The ions were fragmented for each MS/MS experiment in the collision cell using the rolling collision energy. The MS data were acquired with Analyst TF 1.7 (AB SCIEX). Three replicates for each sample were subjected to the DIA analysis. The mass spectrometry files were searched using Protein Pilot (AB SCIEX) and Mascot (Matrix Science Inc.). For Protein Pilot the following parameters were used: cysteine alkylation, digestion by trypsin, no special factors and False Discovery Rate at 1%. For Mascot the following parameters were used: the digestion enzyme selected was trypsin, with 3 missed cleavages, a search tolerance of 0.4 Da was specified for the peptide mass tolerance, and 0.4 Da for the MS/MS tolerance. The charges of the peptides to search for, were set to 2+, 3+ and 4+, the search was set on monoisotopic mass and the following modifications were specified for the search: carbamidomethyl cysteins as fixed modification and oxidized methionine as variable modification. The search was conducted using the UniProt Swiss-Prot database of mouse proteins (version 25.03.2015, containing 41741 sequence entries). Dual filtering criteria for protein identification were employed by combining

FDR test from target-decoy database search with a cutoff *p*-value of 0.05, and protein/peptide confidence above 95% probability, with a minimum of two unique peptides per protein.

The label-free quantification was performed with Skyline (MacCoss Lab Software, University of Washington) by importing the SWATH-MS runs. The library of the identified proteins used for the processing of SWATH data was generated by combining the results of the database search performed with Protein Pilot and Mascot (Manfredi et al., 2016). The quantification was performed by integrating the extracted ion chromatogram of all the unique ions for a given peptide and by using MSstats, a Skyline external tool for statistical analysis as previously described (Choi et al., 2014).

*Western blot analysis*. Protein extracts from adult NPC cultures were obtained by lysis in 3X vol/vol of ice-cold hypotonic RIPA buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA pH 8, 1% (vol/vol) Triton X-100, 1% (wt/vol) SDS, 10 mM NaF, 1 mM $NaVO_4$, 1 mM DTT, protease inhibitor mix (Sigma-Aldrich)] for 30 min on ice. To complete the lysis, incubation at -80°C for 3 min followed by 2 min at 37°C was repeated three times. All lysates were centrifuged at 16,100 x *g* for 10 min at 4°C and supernatants were collected. Protein concentration was determined by Bradford assay (Sigma-Aldrich) and equivalent protein amounts (25 μg) were separated by SDS-PAGE gel (6%) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% BSA in TBS (Sigma-Aldrich) buffer for 60 min at RT. Immunoblots were carried out overnight in an antibody solution containing 3% (wt/vol) BSA in TBS-tween 0.1% with the primary antibody against the α2δ subunit of Dihydropyridine (DHP)/Thrombospondin-1 receptor (1:500; Sigma-Aldrich). After washing, blots were incubated with peroxidase-conjugated goat anti-mouse antibody (1:10,000; BIO-RAD) for 60 min at RT and immunocomplexes were visualized by the Supersignal West Pico Chemiluminescent substrate (Pierce). Densitometric analysis was performed using the Image Lab software

system (Bio-Rad Laboratories) and each band was normalized to β-actin signal (mouse monoclonal, 1:1,000; Sigma-Aldrich).

*Statistical analysis.* All experiments were run in triplicates using different cell preparations and repeated at least three times. Data were expressed as mean ± S.D. and analyzed using Student's *t*-test when only two independent groups were compared, or by one-way analysis of variance (ANOVA) followed by Tuckey's *post-hoc* test when three or more groups were compared. Statistical significance level was set for $p$ values $< 0.05$.

## Results

*In vitro WT and p50KO ahNPC do not show differences in neuronal differentiation and survival rates.*

Multipotent nestin$^+$, sox2$^+$ and GFAP$^-$ NPC isolated from adult mouse hippocampi can be maintained for several passages in an undifferentiated proliferative state (Cuccurazzu et al., 2013). When grown onto laminin-coated chamberslides, upon removal of growth factors and exposure to a serum-free defined medium (STD medium), NPC stop dividing and spontaneously differentiate toward the neuronal lineage. As previously published, by double immunolabeling for markers of neuronal cells (MAP-2) and undifferentiated progenitors (nestin), the appearance of newly generated MAP-2$^+$/nestin$^-$ neurons and MAP2$^+$/nestin$^+$ neuroblasts can be evaluated and quantified (Meneghini et al., 2013). Under these experimental conditions, both WT- and p50KO-derived NPC gave rise to the same percentage of neurons and neuroblasts (MAP-2$^+$/nestin$^-$ cells: 3.13 ± 0.63 and 3.34 ± 0.84 % for WT and p50KO NPC; MAP2$^+$/nestin$^+$ cells: 32.22 ± 3.16 and 33.15 ± 2.21 % for WT and p50KO NPC; Figure 1). No significant difference in the subpopulations of undifferentiated neural progenitors (MAP-2$^-$/nestin$^+$ cells: 12.86 ± 2.29 and 12.29 ± 3.20 % for WT and p50KO NPC; Figure 1) and non-neuronal cells (MAP-2$^-$/nestin$^-$ cells: 51.79 ± 3.83 and 51.23 ± 4.51 % for WT and p50KO NPC; Figure 1) was present. Interestingly, despite the role of the NF-κB signaling pathway in the regulation of cell fate, no significant difference in the basal apoptotic rate could also be observed between the two genotypes (11.84 ± 6.52 and 12.40 ± 5.87 % for WT and KO NPC; Figure 1), in agreement with the *in vivo* observation in hippocampi of adult WT and p50KO mice (Denis-Donini et al., 2008). Altogether we concluded that, unlike *in vivo* conditions, absence of NF-κB p50 did not correlate with altered neuronal differentiation of ahNPC *in vitro*.

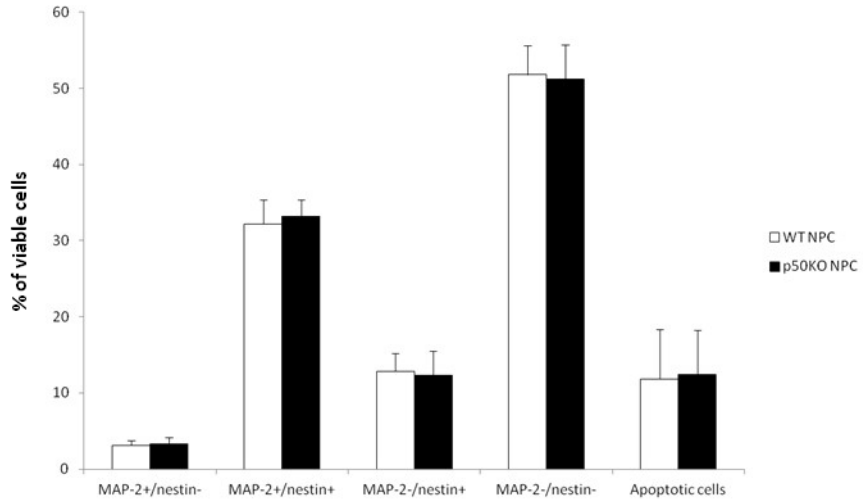


**Figure 1. Adult hippocampal NPC derived from WT and p50KO mice show similar *in vitro* differentiation and apoptotic rates**. Under differentiating conditions, WT and p50KO hippocampal NPC spontaneously give rise to four cell populations identified by double MAP-2 and nestin immunolabelling: MAP-$2^+$/$nestin^-$ neurons, MAP-$2^+$/$nestin^+$ neuroblasts, MAP-$2^-$/$nestin^+$ undifferentiated progenitors and MAP-$2^-$/$nestin^-$ cells (NG-$2^+$ and $GFAP^+$ cells). WT and p50KO hippocampal NPC show similar differentiation rate toward neuronal lineage. The apoptotic rate is not significantly different in WT and p50KO NPC. Data are expressed as mean values ± S.D. of $n = 4$ experiments, run in triplicates, and analyzed by Student's *t*-test.

*Effect of WT- and p50KO-derived ACM on neuronal differentiation of WT ahNPC cultures*

The dissonant consequences of NF-κB p50 absence between *in vitro* and *in vivo* data prompted us to investigate the possibility that non-cell autonomous effects were contributing to remarkably reduced hippocampal neurogenesis in p50KO mice (Denis-Donini et al, 2008). Soluble factors released by astrocytes have been shown to modulate differentiation of NPC (Song et al., 2002). Based on these observations we decided to evaluate the effect of astrocyte-generated

soluble factors on the differentiation rate of ahNPC toward neuronal and non-neuronal lineages. Primary mixed glial cultures were prepared from cortex and hippocampus of neonatal (P1-2) WT and p50KO mice. When cells reached confluence, proliferation of non-astrocytic cells was blocked by 10 μM cytosine arabinoside so to obtain an astrocytic-enriched cell population with less than 3% of contaminating microglia. Astrocyte-conditioned medium (ACM), diluted 1:2, was added to WT ahNPC cultures. Under these experimental conditions, we tested the effect of WT and p50KO ACM, in comparison with standard differentiation (STD) medium, on NPC differentiation. After 24 h, a significant increase in the percentage of *in vitro* generated neurons and neuroblasts was observed in WT cells exposed to WT-derived ACM, compared to STD medium condition (2.74 ± 0.70, 5.13 ± 0.88 %, for MAP-$2^+$/$nestin^-$ cells in STD and WT ACM, $p < 0.001$; 31.12 ± 3.75, 41.8 ± 3.03 %, for $MAP2^+$/$nestin^+$ cells in STD and WT ACM, $p < 0.001$; Figure 2 *A*, *B*). Under the same *in vitro* experimental conditions, no significant difference was observed between STD medium and p50KO ACM (2.74 ± 0.70, 2.91 ± 0.64 %, for MAP-$2^+$/$nestin^-$ cells in STD and p50KO ACM; 31.12 ± 3.75, 31.66 ± 4.63 % for $MAP2^+$/$nestin^+$ cells in STD and p50KO ACM; Figure 2 *A*, *B*), suggesting that, in absence of NF-κB p50, primary astrocytes could not produce proneurogenic molecule(s) or, alternatively, did produce antineurogenic molecule(s). To investigate whether ACM could affect cell survival in addition to neuronal differentiation, we also analyzed the apoptotic rate of ahNPC and their progeny. No difference in the percentage of apoptotic cells was observed in the different media conditions (16.37 ± 2.04, 14.92 ± 1.51, 14.56 ± 1.46 %, in presence of STD, WT and p50KO ACM, respectively; Figure 2 *C*). Altogether these data suggested that the absence of NF-κB p50 may affect the secretory profile of astrocytes, and in particular may reduce their proneurogenic potential on ahNPC, in absence of changes in their effect on the survival rate of NPC and/or their progeny.

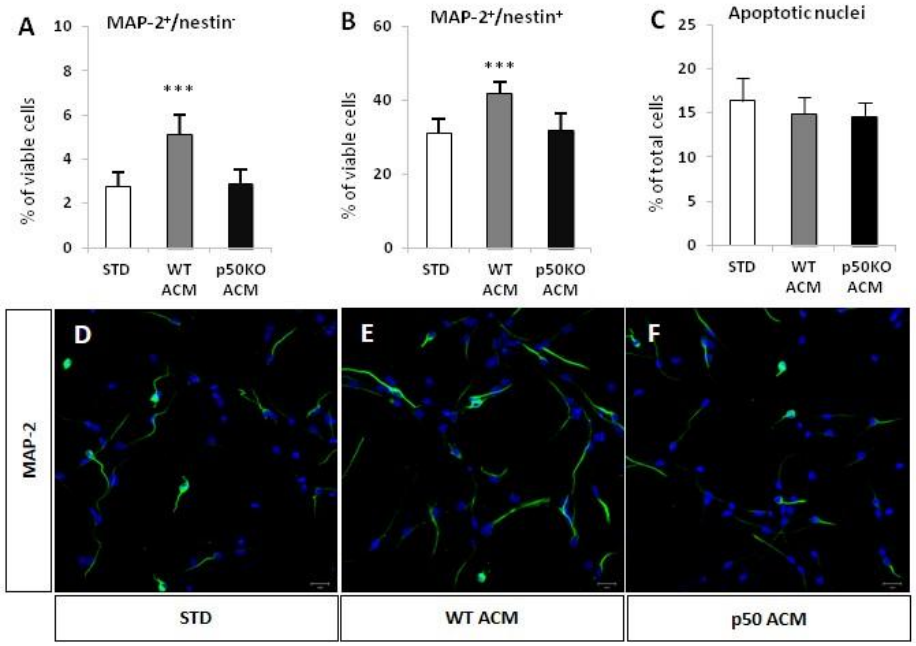


**Figure 2. WT, but not p50KO ACM, promote neuronal differentiation of WT ahNPC cultures**. ***A-B,*** Under differentiating conditions, 24h treatment of WT hippocampal NPC with WT ACM significantly increased the percentage of the subpopulations of MAP-2$^{+}$/nestin$^{-}$ neurons (***A***) and MAP-2$^{+}$/nestin$^{+}$ neuroblasts (***B***) compared with standard differentiation medium (STD). Conversely, p50KO ACM was devoid of proneurogenic effect. (***C***) No significant difference was observed in the apoptotic rate of cultures exposed to STD medium, WT and p50KO ACM. ***D-F,*** Representative confocal microscopy images of MAP2$^{+}$ cells (green) generated from ahNPC after 24h in presence of STD medium (***D***), WT ACM (***E***) and p50KO ACM (***F***). Nuclei were counterstained with Hoechst (blue). Magnification = 400X. Scale bar = 20 μm. Data are expressed as mean values ± S.D. of $n$ = 3 experiments, run in triplicates and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^{***}p < 0.001$ versus STD.

*Both WT- and p50KO-derived ACM significantly promote astroglial differentiation of WT ahNPC.*

When exposed to STD medium in absence of growth factors, ahNPC also spontaneously differentiate toward the astrocytic lineage and that commitment is already apparent within 24 h (Meneghini et al., 2013). Interestingly, we observed a significant increase in the percentage of $GFAP^+$ cells being generated *in vitro* when WT NPC were exposed to both WT and p50KO ACM compared to STD medium condition ($GFAP^+$ cells: 16.0 ± 1.71, 36.98 ± 6.36, 44.63 ± 6.70 % in presence of STD, WT and p50KO ACM, respectively; WT ACM vs vehicle, $p < 0.001$, p50KO ACM vs vehicle; $p < 0.001$; Figure 3 *A, C-E*). These data confirm that primary astrocytes produce soluble factors that promote astrogliogenesis *in vitro*, as previously demonstrated (Barkho et al., 2006). Moreover, they indicate that such soluble activities, unlike proneurogenic ones, are secreted even in absence of NF-κB p50. Actually, in our experimental setting p50KO ACM was more effective than WT ACM in promoting astrogliogenesis in ahNPC cultures (Student's *t*-test, $p < 0.05$; Figure 3 *A*). A subpopulation of NG-$2^+$ cells is also present both in proliferating and differentiating conditions in our cellular model. NG-$2^+$ cells, also known as polydendrocytes, are commonly regarded as oligodendrocyte precursor cells (Nishiyama et al., 2009) but also, although controversial, as multipotential progenitors (Kondo and Raff, 2000). Interestingly, under differentiating conditions, the percentage of NG-$2^+$ cells was not different in presence of the different media conditions (NG-$2^+$ cells: 23.44 ± 11.35, 23.50 ± 11.83, 23.17 ± 9.87 % in STD, WT and p50KO ACM, respectively; Figure 3 *B, F-H*). Altogether these *in vitro* data suggest that the absence of p50 selectively disrupts the proneurogenic potential of ACM, while astrogliogenic-derived signals are intact and even enhanced. Furthermore, these results support the hypothesis that defective hippocampal neurogenesis in p50KO mice could, at least in part, be ascribed to NF-κB p50-dependent changes in the secretome of astrocytes.

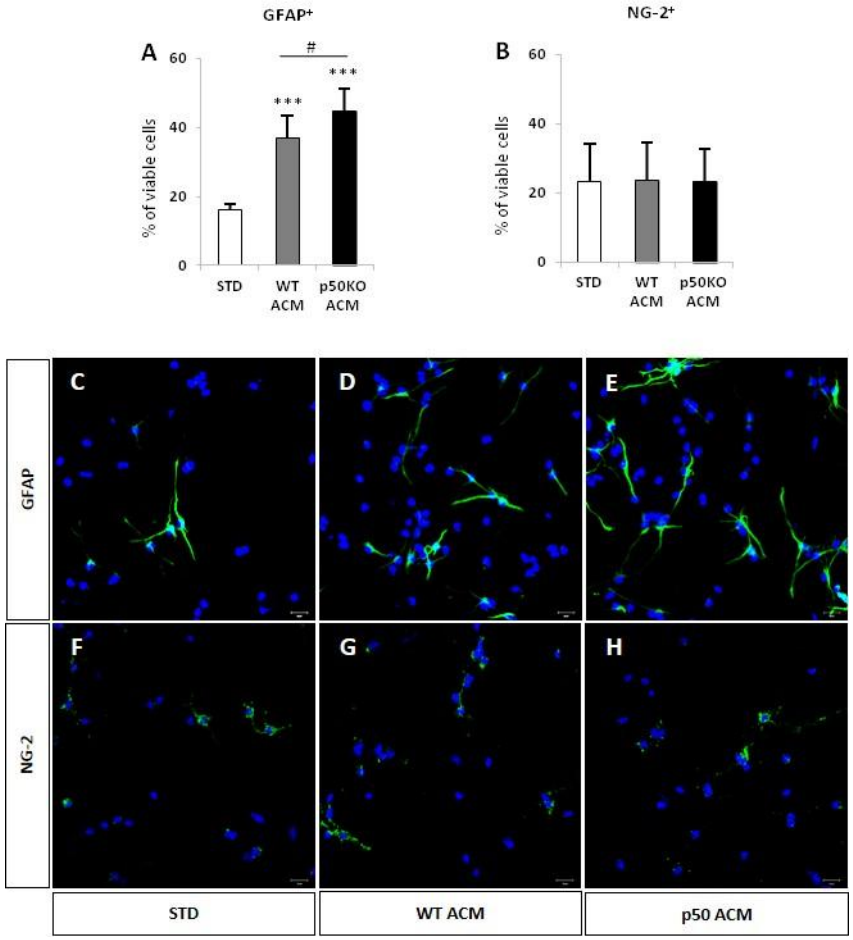


**Figure 3. Both WT and p50KO ACM promote astroglial differentiation of WT ahNPC.** ***A***, 24h treatment of WT ahNPC with WT and p50KO ACM significantly increased the percentage of $GFAP^+$ astroglial cells, compared with STD medium. p50KO ACM was more effective on astrogliogenesis than WT ACM. ***B***, No significant differences was observed in the percentage of $NG\text{-}2^+$ cells upon different media conditions. ***C-H,*** Representative confocal microscopy images of ahNPC cultures differentiated in presence of STD (***C, F***), WT ACM (***D, G***) and p50KO ACM (***E, H***), and immunolabelled for GFAP (***C-E***) and NG-2 (***F-H***). Nuclei were counterstained with Hoechst (blue). Magnification = 400X. Scale bar = 20 μm. Data are expressed as mean values ± S.D. of $n = 3$ experiments, run in triplicates and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^{***}p < 0.001$ versus STD; $^{\#}p < 0.05$ versus WT ACM.

*p50KO NPC are unresponsive to neuronal and astroglial differentiation signals from both WT- and p50KO astrocytes*

We then tested the effect of WT and p50KO ACM on p50KO NPC differentiation toward neuronal and non-neuronal lineages. Surprisingly, neither WT nor p50KO ACM promoted neuronal differentiation of p50KO NPC, and their effect was indistinguishable from that of STD medium (Figure 4 *A-B*). Specifically, the percentages of neurons (MAP-2$^+$/nestin$^-$ cells) were 3.04 ± 0.32, 3.05 ± 0.43, 3.14 ± 0.59 % in response to STD medium, WT and p50KO ACM treatment, respectively (Figure 4 *A*). Similarly, the percentage of neuroblasts (MAP2$^+$/nestin$^+$ cells) were 32.69 ± 2.19, 31.06 ± 3.54, 31.81 ± 2.34 % in presence of STD medium, WT and KO ACM, respectively (Fig. 4 *B*). In addition, unlike WT NPC, p50KO NPC did not even respond to the astrogliogenic effects of WT and p50KO ACM (GFAP$^+$ cells: 14.25 ± 2.57, 16.87 ± 3.64, 17.08 ± 2.14 %, for STD medium, WT and p50KO ACM treatment, respectively; Figure 4 *C*). Similarly, to what observed in WT NPC, we could not observe any difference in the percentage of NG-2$^+$ cells when p50KO NPC were exposed to different media conditions (NG-2$^+$ cells: 29.49 ± 5.13, 28.29 ± 5.48, 27.38 ± 6.82 %, in STD, WT and p50KO ACM, respectively, Figure 4 *D*). Finally, also in p50KO cultures apoptotic rates were not different in presence of the different media conditions (*data not shown*). Altogether these data suggested that, in absence of NF-κB p50, cell autonomous changes profoundly affect both neuronal and astroglial differentiation of ahNPC in response to astroglia-derived molecules.

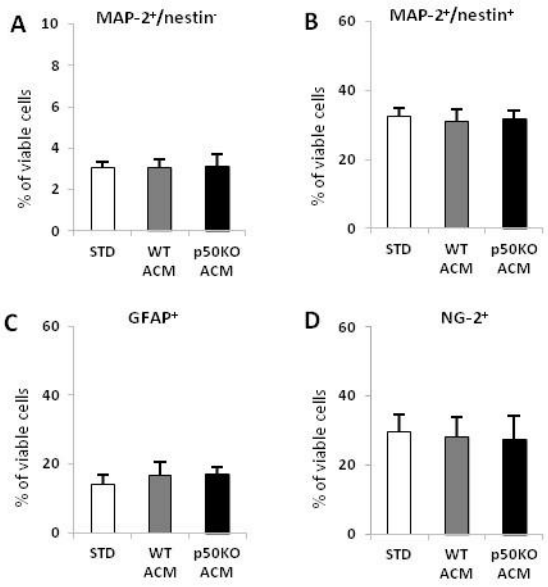


**Figure 4. p50KO NPC are unresponsive to neuronal and astroglial differentiation signals from both WT- and p50KO astrocytes.**

***A-B,*** Under differentiating conditions, 24h treatment of p50KO hippocampal NPC with WT and p50KO ACM did not affect the percentage of MAP-$2^+$/$nestin^-$ neurons (***A***) and MAP-$2^+$/$nestin^+$ neuroblasts (***B***), compared to STD condition. ***C-D***, No significant difference was observed in the percentage of $GFAP^+$ cells (***C***), and NG-$2^+$ cells (***D***) in presence of STD medium, WT and p50KO ACM. Data are expressed as mean values ± S.D. of $n = 3$ experiments, run in triplicates. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test.

*The Receptor for Advanced Glycated End-products (RAGE) is not involved in the proneurogenic activity of ACM on WT ahNPC differentiation*.

RAGE is functionally expressed by ahNPC (Meneghini et al., 2010) and its activation by astrocytes derived molecules, like HMGB-1 and S100B, results in the promotion of neuronal differentiation of ahNPC (Meneghini et al., 2013). In order to understand if the activation of RAGE could mediate the proneurogenic activity of WT ACM on WT ahNPC we pretreated cultures with a neutralizing

anti-RAGE antibody (α-RAGE Ab, 20 μg/ml). α-RAGE Ab was not able to counteract the increase of MAP-$2^+$ cells induced by WT ACM (MAP-$2^+$ cells: 3.56 ±0.31, 6.23 ± 0.71, 5.15 ± 0.62 %, for STD, WT ACM and WT ACM + α-RAGE Ab respectively, $p < 0.05$; Figure 5 *A*). These data suggested that proneurogenic soluble factors in WT ACM do not exert their action through RAGE engagement on ahNPC.

*p50KO NPC are defective in their response to thrombospondin-1 and display downregulated α2δ1 expression*

Thrombospondin-1 (TSP-1) is an astrocyte-derived proneurogenic factor (Lu and Kipnis, 2010). Recently our group demonstrated that α2δ1, a thrombospondin-1 (TSP-1) receptor (Eroglu et al., 2009), is functionally expressed by ahNPC (Valente et al., 2012). We evaluated α2δ1 expression levels in WT and p50KO ahNPC by western blot analysis. Interestingly, the α2δ1 subunit appeared strongly downregulated in p50KO compared with WT NPC (-59.5%; $p < 0.05$, Figure 5 *B, C*). As expected, recombinant human TSP-1 (2 μg/ml) promoted an increase in the percentage of newly formed neurons in WT NPC cultures, when compared to vehicle (MAP-$2^+$/nestin$^-$ cells: 5.90 ± 1.18 and 3.45 ± 0.64 % in TSP-1 and vehicle-treated cells; $p < 0.001$ vs vehicle; Figure 5 *D*). Conversely, TSP-1 treatment was ineffective in p50KO NPC (Figure 5 *D*). Overall these data confirmed that, in absence of p50, cell autonomous defects may reduce ahNPC responsiveness to astrocyte-derived proneurogenic signals.

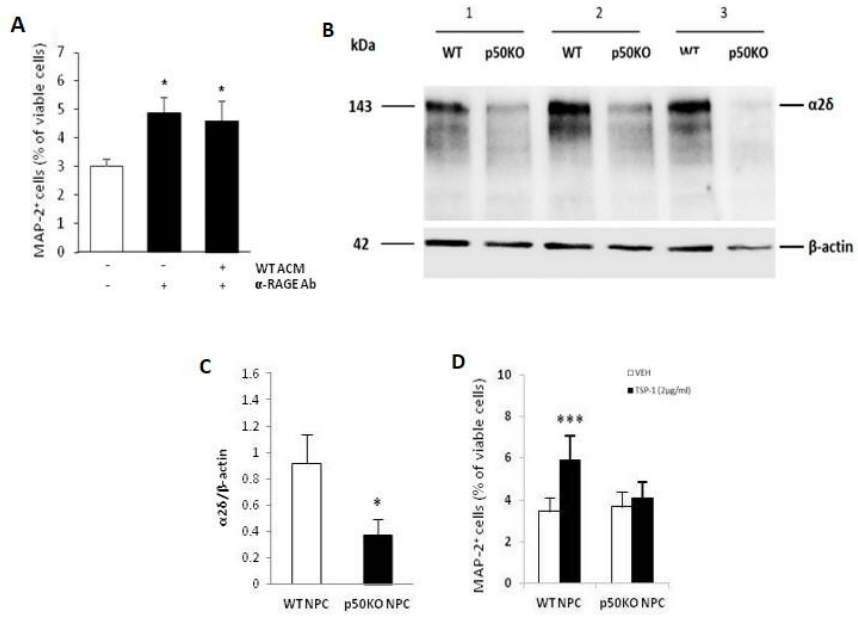


**Figure 5. p50KO NPC are defective in their proneurogenic response to thrombospondin-1 and display downregulated expression of α2δ1.** ***A,*** Pretreatment with the neutralizing anti-RAGE antibody (20 μg/ml) does not counteract the proneurogenic effects of WT ACM on WT ahNPC, as assessed by $MAP2^+$ cell counting. ***B***, Representative immunoblot analysis of α2δ1 subunit and β-actin expression levels in extracts of undifferentiated WT and p50KO ahNPC. Three (1-3) different WT and p50KO cell preparations were collected at different passages and analyzed. ***C,*** Quantification of α2δ1 expression levels in WT and p50KO NPC, normalized by β-actin, demonstrated downregulation in p50KO cultures. ***D,*** Under differentiating conditions, 24 h treatment of WT NPC with human recombinant TSP-1 (2 μg/ml) significantly increased the percentage of $MAP\text{-}2^+/nestin^-$ cells, compared to vehicle. No effect was observed in TSP-1-treated p50KO NPC. Data are expressed as mean values ± S.D. of $n = 4$ experiments, run in triplicates. $^{***}p < 0.001$ versus vehicle (Student's *t*-test).

*Proteomic analysis of soluble factors released by WT and p50KO ACM*

In order to identify proteins which are secreted by WT and p50KO astrocytes, we analyzed ACM samples using a proteomic approach based on a hybrid system that combines the triple quadrupole technology with a time of flight mass spectrometer platform (Andrews et al. 2011). The analysis was performed on peptides produced by tryptic digestion of ACM proteins. Each sample was injected separately into the HPLC columns in a data dependent acquisition mode in order to build the spectra library of proteins identified from the runs. The protein spectra were then subjected to SWATH (Sequential Window Acquisition of Theoretical mass spectra) analysis, that allows complete evaluation of fragmented ions from detectable peptides in biological samples. Through this approach we were able to identify and quantify, as listed in Table 1, four proteins differentially expressed in WT and p50KO ACM, with *p* values below 0.05. Specifically, the following proteins appeared upregulated in p50KO compared to WT ACM: neutrophil gelatinase-associated lipocalin-2 (NGAL/LCN-2), C-C motif chemochine-2 (CCL2), H-2 class I Histocompatibility antigen, K-K alpha chain (HA1K) and retinoic acid receptor responder 2 (RARRES2). The complete list of proteins identified in WT and p50KO ACM is enclosed as supplementary files.

**Table 1.** Differentially expressed proteins in WT and p50KO ACM as identified by LC-MS/MS. Peptide number, protein coverage percentage and fold change as KO/WT ratio are provided for each identified protein.

| Protein name | Accession number (UniProt Swiss-Prot) | Coverage (%) | Peptides | *p*-value | Fold change (KO/WT) |
|---|---|---|---|---|---|
| H-2 class I histocompatibility antigen, K-K alpha chain | sp\|P04223\|HA1K_MOUSE | 11.1 % | 1 | 0.0081 | 3.90 |
| C-C motif chemokine 2 (Monocyte chemoattractant protein 1) | sp\|P10148\|CCL2_MOUSE | 22.3 % | 4 | 0.0010 | 3.35 |
| Neutrophil gelatinase-associated lipocalin (NGAL) (Lipocalin-2) | sp\|P11672\|NGAL_MOUSE | 31.5 % | 4 | 0.0013 | 2.75 |
| Retinoic acid receptor responder protein 2 (Chemerin) | sp\|Q9DD06\|RARR2_MOUSE | 17.3 % | 1 | 0.0017 | 3.11 |

*Lipocalin-2 is an astrocyte-derived proneurogenic factor whose activity is strongly reduced in p50KO NPC.*

Lipocalin-2 (LCN-2) is 24 kDa iron-related protein whose modulatory role for diverse cell phenotypes in the CNS has recently attracted interest (Jha et al., 2015). Since no data are currently available on the role of LCN-2 on adult neurogenesis we focused our attention on this protein as a contributor to lack of proneurogenic effects elicited by p50KO ACM. We tested recombinant mouse LCN-2 (0.01-1 µg/ml) initially on differentiating WT ahNPC. LCN-2 promoted, in a concentration-dependent manner, neuronal differentiation of WT NPC, as assessed by counting the percentages of $MAP2^{+}$/$nestin^{-}$ cells in presence of the recombinant protein or vehicle (One-way ANOVA; $p < 0.001$, Fig. 6 *A*). Maximal proneurogenic effect was elicited in presence of 0.1 µg/ml LCN-2 (% of increase over vehicle-treated cells: + 84.11 ± 7.96, $p < 0.001$; Figure 6 *A*). Under the same concentration range, LCN-2 had no effect on cell survival (Figure 6 *B*). When tested on p50KO NPC LCN-2 was ineffective up to 0.3 µg/ml and only 1 µg/ml of the protein significantly increased the percentage of newly generated neurons (% of increase over vehicle-treated cells: + 67.83 ± 15.73, $p < 0.001$; Figure 6 *A*). Altogether, these data suggest, for the first time, that the protein LCN-2 is an astroglial-derived signal which promotes neuronal fate specification of ahNPC and whose activity is strongly reduced in p50KO NPC.

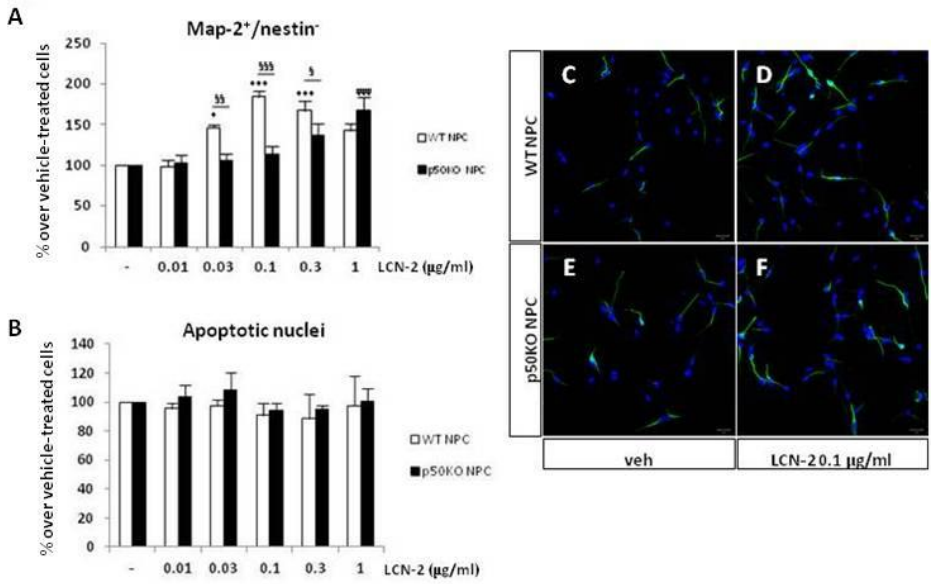


**Figure 6. Lipocalin-2 is a proneurogenic signal whose activity is reduced in p50KO NPC.** ***A,*** 24 h treatment with mouse LCN-2 (0.01-1 µg/ml) promoted neuronal differentiation of WT ahNPC in a concentration-dependent manner. Under the same experimental conditions, p50KO NPC responsiveness to LCN-2 is reduced by one order of magnitude. ***B,*** LCN-2 has no effect on the survival rate of WT and p50KO NPC and their progeny. ***C-F,*** Representative confocal microscopy images of WT (***C, D***) and p50KO (***E, F***) ahNPC differentiated in presence of vehicle (***C, E***) and 0.1 µg/ml of LCN-2 (***D***, ***F***) and immunolabeled for MAP-2 (green). Nuclei were counterstained with Hoechst (blue). Magnification = 400X. Scale bar = 20 µm. Data are expressed % as mean values ± S.D. of n = 3 experiments, run in triplicates, and analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. $^{*}$ $p$ <0.05, $^{***}p$ <0.001 versus WT vehicle; $^{\#\#\#}$ $p$ <0.001 versus p50KO vehicle; $^{\S}$ $p$ < 0.05, $^{\S\S}$ $p$ < 0.01, $^{\S\S\S}$ $p$ < 0.001 in WT versus p50KO NPC.

## Discussion

Since the initial observation of NF-κB members being present in adult neurogenic areas, data on the involvement of these regulatory proteins in the regulation of adult neurogenesis have exponentially grown (Grilli and Meneghini, 2012; Bortolotto et al., 2014). A few years ago we proved that p50KO mice display a dramatic reduction in adult hippocampal neurogenesis and, in parallel, a selective defect in hippocampal-dependent short-term memory. A detailed phenotypic characterization of newly generated hippocampal cells in p50KO mice suggested that absence of the NF-κB p50 subunit may trigger a rather selective defect in neuronal differentiation of ahNPC, in absence of alterations in survival and proliferation rates (Denis-Donini et al., 2008). As a follow up to that previous work, herein we show that when cultured *in vitro,* ahNPC from WT and p50KO mice are not significantly different in their neurogenic potential. Additionally, despite the well-known role of the NF-κB signaling pathway in the regulation of cell fate, no significant difference in the basal apoptotic rate could also be observed between the two genotypes. Altogether we concluded that, unlike *in vivo* conditions, absence of NF-κB p50 did not correlate with altered neuronal differentiation of ahNPC cultured alone *in vitro*.

Since the well documented role of NF-κB in non-neuronal cells (Brambilla et al., 2009; Lian et al., 2015; de la Fuente et al., 2015), we decided to further dissect the role of NF-κB p50 in the cross-talk between ahNPC and astrocytes. Exposure of WT and p50KO hippocampal NPC to conditioned media from WT and p50KO astrocytes was utilized as a strategy to study the potential influence of astroglia-generated soluble factors on adult NPC fate specification and the cell autonoumous or non-cell autonomous role played by NF-κB p50 signaling in that context. Initially we tested the effects of WT and p50KO astrocyte-conditioned media on WT ahNPC. As previously shown (Song et al., 2002), also in our hands WT ACM promoted neuronal differentiation of ahNPC. Additionally, WT primary astrocytes produced soluble factors that promoted

astrogliogenesis of ahNPC, again in line with published reports (Chang et al., 2003; Barkho et al., 2006). Interestingly, when ahNPC were exposed to p50KO ACM we could not observe proneurogenic effects. Conversely, p50KO ACM-derived astrogliogenic signals appeared intact and even enhanced, compared to WT ACM. Of note, no differences in the survival rate of NPC and/or their progeny could be observed after exposure to WT or p50KO ACM. Altogether these data suggested that the absence of NF-κB p50 could affect the secretory profile of astrocytes, and in particular reduce their proneurogenic potential on ahNPC. It could be hypothesized that, in absence of NF-κB p50, primary astrocytes could either be defective in the production of proneurogenic molecule(s) or, alternatively, they could produce antineurogenic molecule(s). Furthermore our data suggested that astrocyte-derived astrogliogenic potential was not affected by NF-κB p50 absence. Altogether these results supported the initial hypothesis that defective hippocampal neurogenesis in p50KO mice could, at least in part, be ascribed to NF-κB p50-dependent changes in the secretome of astrocytes. We then tested the effect of WT and p50KO ACM on p50KO NPC differentiation toward neuronal and non neuronal lineages. Surprisingly, neither WT nor p50KO ACM promoted neuronal and astroglial differentiation of p50KO NPC over basal conditions, suggesting that in absence of NF-κB p50 cell autonomous changes may also occur and affect responsiveness of ahNPC to astrocyte-derived proneurogenic and astrogliogenic signals.

In search for phenotypic changes in p50KO NPC which may explain their reduced responsiveness to astrocyte-proneurogenic signals we took into consideration the possibility that these cells may have lost membrane receptors for astrocyte-derived proneurogenic molecules. RAGE, the Receptor for Advanced Glycation End-products is expressed by adult NPC (Meneghini et al, 2010) and in hippocampal progenitors it mediates the proneurogenic effects of several molecules like HMGB-1 and S100B which can be secreted by astrocytes (Meneghini et al, 2013). Additionally, RAGE is the product of an NF-κB-regulated gene (Li and Schmidt, 1997). Based on these observations we

initially evaluated the contribution of RAGE engagement on the proneurogenic effects of WT ACM in WT ahNPC, by using a previously characterized neutralizing antibody (Meneghini et al., 2013). Receptor neutralization had no effect on WT ACM proneurogenic effects, suggesting that RAGE activation was not involved in ACM-mediated neuronal differentiation of ahNPC. Thrombospondin-1 (TSP-1) is an established astrocyte-derived proneurogenic factor (Lu and Kipnis 2010). A few years ago, our group demonstrated that α2δ1 a thrombospondin-1 (TSP-1) receptor (Eroglu et al., 2009), is functionally expressed by ahNPC (Valente et al., 2012). Interestingly, α2δ1 expression levels resulted dramatically reduced in p50KO ahNPC, compared to their WT counterpart. In line with this observation, recombinant human TSP-1 promoted an increase in the percentage of newly formed neurons in WT, but not in p50KO, NPC cultures. Overall these data confirmed that, in absence of p50, cell autonomous defects may reduce ahNPC responsiveness to astrocyte-derived proneurogenic signals, and in particular to TSP-1, via downregulation of its receptor.

We also actively searched for proteins which may be differentially secreted by astrocytes in absence of p50 and whose expression could correlate with the lack of proneurogenic effects of p50KO ACM. LC-MS/MS allowed us to identify several proteins in WT and p50KO ACM. More specifically, four molecules were differentially expressed in WT and p50KO ACM, namely C-C motif chemochine-2 (CCL-2), neutrophil gelatinase-associated lipocalin-2 (NGAL/LCN-2), H-2 class I Histocompatibility antigen, K-K alpha chain (HA1K), and retinoic acid receptor responder 2 (RARRES2, also known as chemerin). Interestingly, absence of NF-κB p50 is commonly linked to inflammation both in periphery and in brain (Bernal et al., 2014; Rolova et al., 2014) and CCL-2, NGAL/LCN-2 and RARRES2/chemerin all share an established role in inflammation. Indeed, CCL-2 and RARRES2/chemerin are proinflammatory chemokines, while LCN-2 is an acute phase-protein. Moreover, among them, CCL-2 and LCN-2 are both encoded by NF-κB target

genes and NF-κB signaling activation lies downstream their receptor activation (Ueda et al., 1994; Bu et al., 2006; Kohda et al., 2014).

We decided to focus our attention on NGAL/lipocalin-2 (LCN-2). It is a 24 kDa iron-related protein which belongs to a family of diverse secreted proteins (Flower, 2000) and whose modulatory role in the CNS has recently attracted interest (Jha et al., 2015), although its function is not completely understood (Ferreira et al., 2015). LCN-2 is secreted by brain astrocytes and regarded as an autocrine promoter of their classical proinflammatory activation (Bi et al., 2013; Jang et al., 2013). Since no information was available on the role of LCN-2 on adult neurogenesis, we decided to concentrate our attention on this protein, whose expression is increased in p50KO compared to WT ACM. To our surprise, LCN-2 promoted, in a concentration-dependent manner, neuronal differentiation of WT NPC, as assessed by counting the percentage of $MAP2^{+}/nestin^{-}$ and $MAP2^{+}/nestin^{+}$ cells in presence of the recombinant protein or of vehicle. In contrast with previous observations that LCN-2 can promote apoptosis of neurons and astrocytes (Lee et al., 2012; Tong et al., 2013), in our cellular model and within the tested concentration range the protein had no negative effect on the survival of ahNPC and their progeny. Interestingly, under the same experimental conditions, LCN-2 had no or little effect on neuronal differentiation of p50KO NPC, except at the highest concentration. Altogether, these data suggest, for the first time, that LCN-2 is an astroglial-derived signal which promotes neuronal fate specification of ahNPC and that p50KO NPC are less responsive to this protein. This observation points also to the possibility that, like for the TSP-1 receptor α2δ1, downregulation of LCN-2 receptor(s) may also occur in ahNPC and explain their reduced sensitivity (one order of magnitude) to this protein. In such respect increased expression of LCN-2 in conditioned media from p50KO astrocytes may represent a compensatory event to reduced LCN-2 receptor expression or sensitivity. Alternatively, the absence of p50 may disrupt intracellular signaling downstream several membrane receptors, including LCN-2 receptors, an aspect which deserves further investigation. LCN-2 is known to bind two cell-surface proteins. One is the

brain type organic cation transporter (BOCT or 24p3R or LCN2R) which is constitutively expressed at high levels in brain, including in the hippocampus, in neuronal cells and in the choroid plexus (Ip et al, 2011), but it also appears to be upregulated in astrocytes and microglia in EAE and peripheral nerve injury (Berard et al, 2012; Jha et al., 2013). Interestingly, NF-κB signaling activation has been shown to occur downstream of LCN-2/24p3R (Dizin et al., 2013). The second one is megalin, also known as low density lipoprotein receptor-related protein 2 (Lrp2), a multiligand endocytic receptor which is expressed by many cell types in the CNS. Lrp2 binds about 40 different ligands ranging from lipoprotein and protease inhibitor complex to growth factors and extracellular matrix proteins (Spuch et al., 2012). Increasing evidence has implicated Lrp2, in Aβ clearance in Alzheimer's Disease (Alvira-Botero et al., 2010). Recently, Lrp2 has also been involved in regulating the behaviour of neural stem and progenitor cells during development and in adulthood, although not in the hippocampus (Auderset et al., 2016). A detailed evaluation of BOCT and Lrp2 expression levels in WT and p50KO ahNPC is certainly needed to complete the picture.

Of course we cannot disregard other differentially expressed proteins as contributors in the lack of response of ahNPC to astrocyte-derived signals. Contradictory data have been gathered on the contribution of CCL-2 to neurogenesis. *In vitro* studies showed that incubation of NPC with CCL-2 increase neuronal differentiation as proven by an increase in the number of Tuj1-positive cells, in absence of effects on cell proliferation and survival (Liu et al, 2007). On the other hand an involvement of CCL-2 in the reduction of neurogenesis associated with cranial irradiation has also been suggested (Lee et al., 2013). Preliminary evidence suggest that exogenously added CCL-2 is also proneurogenic on WT ahNPC cultures (*data not shown*). As far as RARRES2/chemerin, limited information is available on the physiological role of this protein and its receptor (CMKLR1) in the CNS. Targeting CMKLR1:chemerin interaction has been proposed to be beneficial in preventing or treating multiple sclerosis (Graham et al, 2014). Although there

are no data pointing to its involvement in neurogenesis, recently a novel role for chemerin and its CMKLR1 receptor in regulating adipogenesis and osteoblastogenesis of bone marrow-derived precursor cells has been proposed (Muruganandan et al, 2010). Interestingly, chemerin is also an inducer of CCL-2 (Eisinger et al., 2011).

H-2 class I Histocompatibility antigen, K-K alpha chain (HA1K) was also identified by LC-MS/MS in astrocyte-conditioned media and appeared upregulated in p50KO ACM. Of course at this stage we cannot exclude that its presence in media may represent an artefact, but since exosomes bearing MHC Class I molecules have been previously demonstrated in other cell types (Zitvogel et al., 1998) in the future we will also explore the possibility that extracellular microvesicles of astrocytic origin could contribute, with their cargo, to ACM composition.

Last but not least, in our experimental setting p50KO NPC appeared also unresponsive to astrogliogenic astrocyte-derived signals and we could exclude astrogliogenic effects of LCN-2 treatment on ahNPC (*data not shown*). In the future we may also attempt identifying the nature of those astrocyte secreted signals that regulate astrogliogenesis.

In summary, although we cannot underestimate the contribution of other niche cell types which were not investigated (microglia in primis), herein we propose that reduced hippocampal neurogenesis in p50KO mice *in vivo* could be ascribed to complex cell autonomous and to non-cell autonomous defects in the cross-talk between astrocytes and adult hippocampal neural progenitor cells. Moreover, we show for the first time that LCN-2 is an astroglial-derived signal which promotes neuronal fate specification of ahNPC. Finally, these data add further complexity to the growing body of data suggesting the relevance of NF-κB signaling in the modulation of adult neurogenesis.

## References

Aimone JB, Deng W, Gage FH (2010) Adult neurogenesis: integrating theories and separating functions. Trends in Cognitive Sciences 14(7), 325–37.

Aimone JB, Li Y, Lee SW, Clemenson GD, Deng,W, Gage FH (2014) Regulation and Function of Adult Neurogenesis: From Genes to Cognition. Physiological Reviews 94(4), 991–1026.

Alvira-Botero X, Pérez-Gonzalez R, Spuch C, Vargas T, Antequera D, Garzón M, Bermejo-Pareja F, Carro E (2010) Megalin interacts with APP and the intracellular adapter protein FE65 in neurons. Molecular and Cellular Neurosciences, 45(3), 306–15.

Andrews GL, Simons BL, Young JB, Hawkridge AM, Muddiman DC (2011) Performance characteristics of a new hybrid quadrupole time-of-flight tandem mass spectrometer (TripleTOF 5600). Analytical Chemistry 83(13), 5442–6.

Apple DM, Fonseca RS, Kokovay E (2016) The Role of Adult Neurogenesis in Psychiatric and Cognitive Disorders. Brain Research S0006-8993(16)00043-3.

Auderset L, Landowski LM, Foa L,Young KM (2016). Low Density Lipoprotein Receptor Related Proteins as Regulators of Neural Stem and Progenitor Cell Function. Stem Cells International, 2016, 2108495.

Barkho BZ, Song H, Aimone JB, Smrt RD, Kuwabara T, Nakashima K, Gage FH, Zhao X (2006) Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. Stem Cells and Development 15(3), 407–21.

Berard JL, Zarruk JG, Arbour N, Prat A, Yong VW, Jacques FH, Akira S, David S (2012). Lipocalin 2 is a novel immune mediator of experimental autoimmune encephalomyelitis pathogenesis and is modulated in multiple sclerosis. Glia, 60(7), 1145–59.

Bernal GM, Wahlstrom JS, Crawley CD, Cahill KE, Pytel P, Liang H, Kang S, Weichselbaum RR, Yamini B (2014). Loss of NFκB1 leads to early onset aging. Aging, 6(11), 931–43.

Bi F, Huang C, Tong J, Qiu G, Huang B, Wu Q, Li F, Xu Z, Bowser R, Xia XG, Zhou H (2013) Reactive astrocytes secrete lcn2 to promote neuron death. Proceedings of the National Academy of Sciences of the United States of America, 110(10), 4069–74.

Bonaguidi MA, McGuire T, Hu M, Kan L, Samanta J, Kessler JA. (2005) LIF and BMP signaling generate separate and discrete types of GFAP-expressing cells. Development 132(24), 5503–14.

Bond AM, Ming G-L, Song H (2015) Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. Cell Stem Cell 17(4), 385–95.

Bortolotto V, Cuccurazzu B, Canonico PL, Grilli M (2014) NF-κB mediated regulation of adult hippocampal neurogenesis: relevance to mood disorders and antidepressant activity. BioMed Research International 2014, 612798.

Brambilla R, Persaud T, Hu X, Karmally S, Shestopalov VI, Dvoriantchikova G, Ivanov D, Nathanson L, Barnum SR, Bethea JR (2009) Transgenic inhibition of astroglial NF-kappa B improves functional outcome in experimental autoimmune encephalomyelitis by suppressing chronic central nervous system inflammation. Journal of Immunology 182(5), 2628–40.

Bu D, Hemdahl AL, Gabrielsen A, Fuxe J, Zhu C, Eriksson P, Yan Z (2006) Induction of neutrophil gelatinase-associated lipocalin in vascular injury via activation of nuclear factor-kappaB. The American Journal of Pathology, 169(6), 2245–53.

Chang MY, Son H, Lee YS, Lee SH (2003) Neurons and astrocytes secrete factors that cause stem cells to differentiate into neurons and astrocytes, respectively. Molecular and Cellular Neurosciences, 23(3), 414–26.

Choi M, Chang C-Y, Clough T, Broudy D, Killeen T, MacLean B, Vitek O (2014) MSstats: an R package for statistical analysis of quantitative mass spectrometry-based proteomic experiments. Bioinformatics 30(17), 2524–6.

Cuccurazzu B, Bortolotto V, Valente MM, Ubezio F, Koverech A, Canonico PL, Grilli M (2013) Upregulation of mGlu2 receptors via NF-κB p65 acetylation is involved in the Proneurogenic and antidepressant effects of acetyl-L-carnitine. Neuropsychopharmacology 38(11), 2220–30.

de la Fuente V, Federman N, Zalcman G, Salles A, Freudenthal R, Romano A (2015) NF-κB transcription factor role in consolidation and reconsolidation of persistent memories. Frontiers in Molecular Neuroscience 9;8:50.

Denis-Donini S, Caprini A, Frassoni C, Grilli M (2005) Members of the NF-kappaB family expressed in zones of active neurogenesis in the postnatal and adult mouse brain. Brain Research Developmental Brain Research, 154(1), 81–9.

Denis-Donini S, Dellarole A, Crociara P, Francese MT, Bortolotto V, Quadrato G, Canonico PL, Orsetti M, Ghi P, Memo M, Bonini SA, Ferrari-Toninelli G, Grilli M (2008) Impaired adult neurogenesis associated with short-term memory defects in NF-kappaB p50-deficient mice. The Journal of Neuroscience, 28(15), 3911–9.

Dizin E, Hasler U, Nlandu-Khodo S, Fila M, Roth I, Ernandez T, Doucet A, Martin PY, Feraille E, de Seigneux S (2013) Albuminuria induces a proinflammatory and profibrotic response in cortical collecting ducts via the 24p3 receptor. American Journal of Physiology. 305(7), F1053–63.

Eisinger K, Bauer S, Schäffler A, Walter R, Neumann E, Buechler C, Müller-Ladner U, Frommer KW (2012). Chemerin induces CCL2 and TLR4 in synovial fibroblasts of patients with rheumatoid arthritis and osteoarthritis. Experimental and Molecular Pathology, 92(1), 90–6.

Eroglu C, Allen NJ, Susman MW, O'Rourke NA, Park CY, Ozkan E, Chakraborty C, Mulinyawe SB, Annis DS, Huberman AD, Green EM, Lawler J, Dolmetsch R, Garcia KC, Smith SJ, Luo ZD, Rosenthal A, Mosher DF, Barres BA (2009). Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell, 139(2), 380–92.

Ferreira AC, Dá Mesquita S, Sousa JC, Correia-Neves M, Sousa N, Palha JA, Marques F (2015) From the periphery to the brain: Lipocalin-2, a friend or foe? Progress in Neurobiology, 131, 120–36.

Flower DR, North AC, Sansom CE (2000) The lipocalin protein family: structural and sequence overview. Biochimica et Biophysica Acta, 1482(1-2), 9–24.

Gillet LC, Navarro P, Tate S, Röst H, Selevsek N, Reiter L, Bonner R, Aebersold R (2012). Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. Molecular & Cellular Proteomics, 11(6), O111.016717.

Geromanos SJ, Vissers JPC, Silva JC, Dorschel CA, Li G-Z, Gorenstein MV, Bateman RH, Langridge, J. I. (2009) The detection, correlation, and comparison of peptide precursor and product ions from data independent LC-MS with data dependant LC-MS/MS. Proteomics 9(6), 1683–95.

Graham KL, Zhang JV, Lewén S, Burke TM., Dang T, Zoudilova M, Sobel, RA, Butcher EC, Zabel BA (2014) A novel CMKLR1 small molecule antagonist suppresses CNS autoimmune inflammatory disease. PloS One, 9(12), e112925.

Grilli M, Memo M (1999) Nuclear factor-kappaB/Rel proteins: a point of convergence of signaling pathways relevant in neuronal function and dysfunction. Biochemical Pharmacology, 57(1), 1–7.

Grilli M, Meneghini V (2012) in Transcription factors CREB and NF-κB: involvement in synaptic plasticity and memory formation, NF-κB proteins in adult neurogenesis: relevance for learning and memory in physiology and pathology, ed Albensi BC (Bentham Sciences Publishers, Oak Park, IL), pp 199–25

Ip JP, Noçon AL, Hofer M J, Lim SL, Müller M, Campbell IL (2011) Lipocalin 2 in the central nervous system host response to systemic lipopolysaccharide administration. Journal of Neuroinflammation, 8, 124.

Jang E, Kim J-H, Lee S, Kim J-H, Seo J-W, Jin M, Lee M-G, Jang IS, Lee W-H, Suk K (2013) Phenotypic polarization of activated astrocytes: the critical role of lipocalin-2 in the classical inflammatory activation of astrocytes. Journal of Immunology, 191(10), 5204–19.

Jha MK, Jeon S, Jin M, Lee WH, Suk K (2013) Acute Phase Protein Lipocalin-2 Is Associated with Formalin-induced Nociception and Pathological Pain. Immune Network, 13(6), 289–94.

Jha M K, Lee S, Park DH, Kook H, Park K-G, Lee I-K, Suk K (2015) Diverse functional roles of lipocalin-2 in the central nervous system. Neuroscience and Biobehavioral Reviews 49, 135–56.

Kaltschmidt B, Kaltschmidt C (2009) NF-kappaB in the nervous system. Cold Spring Harbor Perspectives in Biology 1(3), a001271.

Kempermann G, Song H, Gage FH (2015) Neurogenesis in the Adult Hippocampus. Cold Spring Harbor Perspectives in Medicine 5(7), a018812.

Kokoeva MV, Yin H, Flier JS (2005) Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science 310(5748), 679–83.

Kohda A, Yamazaki S, Sumimoto H (2014) DNA element downstream of the κB site in the Lcn2 promoter is required for transcriptional activation by IκBζ and NF-κB p50. *Genes to Cells : Devoted to Molecular & Cellular Mechanisms*, *19*(8), 620–8.

Kondo T, Raff M (2000) Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. Science 289(5485), 1754–7.

Lacar B, Parylak SL, Vadodaria KC, Sarkar A, Gage FH (2014) Increasing the resolution of the adult neurogenesis picture. F1000prime Reports, 6, 8.

Lian H, Yang L, Cole A, Sun L, Chiang A C-A, Fowler SW, Shim DJ, Rodriguez-Rivera J, Taglialatela G, Jankowsky JL, Lu H-C, Zheng H (2015) NFκB-Activated Astroglial Release of Complement C3 Compromises Neuronal Morphology and Function Associated with Alzheimer's Disease. Neuron 85(1), 101–115.

Lee S, Lee WH, Lee MS, Mori K, Suk K (2012) Regulation by lipocalin-2 of neuronal cell death, migration, and morphology. Journal of Neuroscience Research, 90(3), 540–50.

Lee SW, Haditsch U, Cord B J, Guzman R, Kim SJ, Boettcher C, Priller J,Ormerod B, Palmer TD (2013) Absence of CCL2 is sufficient to restore hippocampal neurogenesis following cranial irradiation. Brain, Behavior, and Immunity, 30, 33–44.

Li J, Schmidt AM (1997) Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. The Journal of Biological Chemistry, 272(26), 16498–506.

Liu XS, Zhang ZG, Zhang RL, Gregg SR, Wang L, Yier T, Chopp M (2007) Chemokine ligand 2 (CCL2) induces migration and differentiation of subventricular zone cells after stroke. Journal of Neuroscience Research, 85(10), 2120–5.

Lu Z, Kipnis J (2010) Thrombospondin 1- a key astrocyte-derived neurogenic factor. FASEB Journal 24(6), 1925–34.

Manfredi M, Martinotti S, Gosetti F, Ranzato E, Marengo E (2016) The secretome signature of malignant mesothelioma cell lines. Journal of Proteomics S1874-3919(16)30043-4.

Mattson MP, Meffert M K (2006) Roles for NF-kappaB in nerve cell survival, plasticity, and disease. Cell Death and Differentiation, 13(5), 852–60.

Meneghini V, Francese MT, Carraro L, Grilli M (2010) A novel role for the Receptor for Advanced Glycation End-products in neural progenitor cells derived from adult SubVentricular Zone. Molecular and Cellular Neurosciences 45(2), 139–50.

Meneghini V, Bortolotto V, Francese MT, Dellarole A, Carraro L, Terzieva S, Grilli M (2013) High-mobility group box-1 protein and β-amyloid oligomers promote neuronal differentiation of adult hippocampal neural progenitors via receptor for advanced glycation end products/nuclear factor-κB axis: relevance for Alzheimer's disease. Journal of Neuroscience 33(14), 6047–59.

Ming G, Song H (2005) Adult neurogenesis in the mammalian central nervous system. Annual Review of Neuroscience 28, 223–50.

Muruganandan S, Roman AA, Sinal CJ (2010) Role of chemerin/CMKLR1 signaling in adipogenesis and osteoblastogenesis of bone marrow stem cells. Journal of Bone and Mineral Research, 25(2), 222–34.

Nishiyama A, Komitova M, Suzuki R, Zhu X (2009) Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. Nature Reviews Neuroscience 10(1), 9–22.

Rolls A, Shechter R, London A, Ziv Y, Ronen A, Levy R, Schwartz M (2007) Toll-like receptors modulate adult hippocampal neurogenesis. Nature Cell Biology 9(9):1081-8.

Rolova T, Puli L, Magga J, Dhungana H, Kanninen K, Wojciehowski S, Salminen A, Tanila H, Koistinaho J, Malm T (2014) Complex regulation of acute and chronic neuroinflammatory responses in mouse models deficient for nuclear factor kappa B p50 subunit. Neurobiology of Disease, 64, 16–29.

Schänzer, A, Wachs, F-P, Wilhelm, D, Acker, T, Cooper-Kuhn, C, Beck, H., Winkler J, Aigner L, Plate KH, Kuhn HG (2004). Direct stimulation of adult neural stem cells in vitro and neurogenesis in vivo by vascular endothelial growth factor. Brain Pathology 14(3), 237–48.

Sha WC, Liou H-C, Tuomanen EI, Baltimore D (1995) Targeted disruption of the p50 subunit of NF-κB leads to multifocal defects in immune responses. Cell, 80(2), 321–330.

Song H, Stevens CF, Gage FH (2002) Astroglia induce neurogenesis from adult neural stem cells. Nature, 417(6884), 39–44.

Spuch C, Ortolano S, Navarro C (2012) LRP-1 and LRP-2 receptors function in the membrane neuron. Trafficking mechanisms and proteolytic processing in Alzheimer's disease. Frontiers in Physiology, 3, 269.

Tong J, Huang C, Bi F, Wu Q, Huang B, Liu X, Li F, Zhou H, Xia XG (2013) Expression of ALS-linked TDP-43 mutant in astrocytes causes non-cell-autonomous motor neuron death in rats. The EMBO Journal, 32(13), 1917–26.

Ueda A1, Okuda K, Ohno S, Shirai A, Igarashi T, Matsunaga K, Fukushima J, Kawamoto S, Ishigatsubo Y, Okubo T (1994) NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. J Immunology. 153(5):2052-63.

Valente MM, Bortolotto V, Cuccurazzu B, Ubezio F, Meneghini V, Francese, MT, Canonico, PL, Grilli M (2012) α2δ Ligands Act as Positive Modulators of Adult Hippocampal Neurogenesis and Prevent Depressive-like Behavior Induced by Chronic Restraint Stress. Molecular Pharmacology 82(2):271-80.

Venable JD, Dong M-Q, Wohlschlegel J, Dillin A, Yates JR. (2004) Automated approach for quantitative analysis of complex peptide mixtures from tandem mass spectra. Nature Methods 1(1), 39–45.

Wada T, Haigh JJ, Ema M, Hitoshi S, Chaddah R, Rossant J, Nagy A, van der Kooy D (2006) Vascular endothelial growth factor directly inhibits primitive neural stem cell survival but promotes definitive neural stem cell survival. Journal of Neuroscience 26(25), 6803–12.

Whalley K (2012) Neurogenesis: Hypothalamic neurogenesis regulates weight gain. Nature Reviews Neuroscience 13(5), 289.

Widera D, Kaus A, Kaltschmidt C, Kaltschmidt B (2008) Neural stem cells, inflammation and NF-kappaB: basic principle of maintenance and repair or origin of brain tumours? Journal of Cellular and Molecular Medicine 12(2), 459–70.

Winner B, Kohl Z, Gage FH (2011) Neurodegenerative disease and adult neurogenesis. The European Journal of Neuroscience 33(6), 1139–51.

Zitvogel, L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, Ricciardi-Castagnoli P, Raposo G, Amigorena S (1998) Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes. Nature Medicine, 4(5), 594–600.

## Supplementary material

**Table 2.** List of WT ACM proteins identified by Mascot and Protein Pilot database search, filtered for protein/peptide confidence above 95%, with a minimum of two unique peptides per protein.

| Cov. % | Accession | Protein names | Mass (Da) |
|---|---|---|---|
| 42.2 | CO3_MOUSE | Complement C3 (HSE-MSF) [Cleaved into: Complement C3 beta chain; C3-beta-c (C3bc); Complement C3 alpha chain; C3a anaphylatoxin; Acylation stimulating protein (ASP) (C3adesArg); Complement C3b alpha' chain; Complement C3c alpha' chain fragment 1; Complement C3dg fragment; Complement C3g fragment; Complement C3d fragment; Complement C3f fragment; Complement C3c alpha' chain fragment 2] | 186,484 |
| 29.7 | CLUS_MOUSE | Clusterin (Apolipoprotein J) (Apo-J) (Clustrin) (Sulfated glycoprotein 2) (SGP-2) [Cleaved into: Clusterin beta chain; Clusterin alpha chain] | 51,656 |
| 25 | NGAL_MOUSE | Neutrophil gelatinase-associated lipocalin (NGAL) (Lipocalin-2) (SV-40-induced 24P3 protein) (Siderocalin LCN2) (p25) | 22,875 |
| 22.2 | APOE_MOUSE | Apolipoprotein E (Apo-E) | 35,867 |
| 22 | CATA_MOUSE | Catalase (EC 1.11.1.6) | 59,795 |
| 17.3 | CH3L1_MOUSE | Chitinase-3-like protein 1 (BRP39 protein) (Breast regression protein 39) (Cartilage glycoprotein 39) (CGP-39) (GP-39) | 43,893 |
| 15.4 | K1C10_MOUSE | Keratin, type I cytoskeletal 10 (56 kDa cytokeratin) (Cytokeratin-10) (CK-10) (Keratin, type I cytoskeletal 59 kDa) (Keratin-10) (K10) | 57,770 |
| 15.1 | FIGL1_MOUSE | Fidgetin-like protein 1 (EC 3.6.4.-) | 74,850 |
| 12.4 | AL1A1_MOUSE | Retinal dehydrogenase 1 (RALDH 1) (RalDH1) (EC 1.2.1.36) (ALDH-E1) (ALHDII) (Aldehyde dehydrogenase family 1 member A1) (Aldehyde dehydrogenase, cytosolic) | 54,468 |
| 11.4 | CYTC_MOUSE | Cystatin-C (Cystatin-3) | 15,531 |
| 10.8 | CCL2_MOUSE | C-C motif chemokine 2 (Monocyte chemoattractant protein 1) (Monocyte chemotactic protein 1) (MCP-1) (Platelet-derived growth factor-inducible protein JE) (Small-inducible cytokine A2) | 16,326 |
| 10.4 | NT5D3_MOUSE | 5'-nucleotidase domain-containing protein 3 (EC 3.1.3.-) (GRP94-neighboring nucleotidase) | 63,170 |
| 8.7 | CERU_MOUSE | Ceruloplasmin (EC 1.16.3.1) (Ferroxidase) | 121,151 |
| 8.2 | CXL10_MOUSE | C-X-C motif chemokine 10 (10 kDa interferon gamma-induced protein) (Gamma-IP10) (IP-10) (C7) (Interferon-gamma induced protein CRG-2) (Small-inducible cytokine B10) | 10,789 |
| 8.1 | GRAG_MOUSE | Granzyme G (EC 3.4.21.-) (CTL serine protease 1) (MCSP-1) | 27,381 |
| 7.3 | PAFA_MOUSE | Platelet-activating factor acetylhydrolase (PAF acetylhydrolase) (EC 3.1.1.47) (1-alkyl-2-acetylglycerophosphocholine esterase) (2-acetyl-1-alkylglycerophosphocholine esterase) (LDL-associated phospholipase A2) (LDL-PLA (2)) (PAF 2-acylhydrolase) | 49,258 |
| 6.7 | K1C17_MOUSE | Keratin, type I cytoskeletal 17 (Cytokeratin-17) (CK-17) (Keratin-17) (K17) | 48,162 |
| 6.6 | SKIT1_MOUSE | Selection and upkeep of intraepithelial T-cells protein 1 (Skint-1) (Immunoglobulin-like and transmembrane domain-containing protein expressed in skin and thymus protein 1) | 41,792 |
| 6.5 | MCF2L_MOUSE | Guanine nucleotide exchange factor DBS (DBL's big sister) (MCF2-transforming sequence-like protein) | 129,113 |
| 6.3 | NAA50_MOUSE | N-alpha-acetyltransferase 50 (EC 2.3.1.-) (N-acetyltransferase NAT13) (NatE catalytic subunit) | 19,414 |
| 6.2 | RUND1_MOUSE | RUN domain-containing protein 1 | 67,865 |
| 6.2 | TRRAP_MOUSE | Transformation/transcription domain-associated protein (Tra1 homolog) | 291,557 |
| 6.1 | CBPE_MOUSE | Carboxypeptidase E (CPE) (EC 3.4.17.10) (Carboxypeptidase H) (CPH) (Enkephalin convertase) (Prohormone-processing carboxypeptidase) | 53,256 |

| | | | |
|---|---|---|---|
| 5.8 | LIFR_MOUSE | Leukemia inhibitory factor receptor (LIF receptor) (LIF-R) (D-factor/LIF receptor) (CD antigen CD118) | 122,574 |
| 5.5 | SCEL_MOUSE | Sciellin | 72,972 |
| 5.3 | ARAP3_MOUSE | Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 3 (Centaurin-delta-3) (Cnt-d3) (Dual specificity Rho- and Arf-GTPase-activating protein 1) | 169,741 |
| 5.2 | LG3BP_MOUSE | Galectin-3-binding protein (Cyp-C-associated protein) (CyCAP) (Lectin galactoside-binding soluble 3-binding protein) (Protein MAMA) | 64,491 |
| 5.2 | ESYT3_MOUSE | Extended synaptotagmin-3 (E-Syt3) | 100,068 |
| 5 | BRE1A_MOUSE | E3 ubiquitin-protein ligase BRE1A (BRE1-A) (EC 6.3.2.-) (RING finger protein 20) | 113,520 |
| 4.6 | SPRC_MOUSE | SPARC (Basement-membrane protein 40) (BM-40) (Osteonectin) (ON) (Secreted protein acidic and rich in cysteine) | 34,450 |
| 4.6 | K1C42_MOUSE | Keratin, type I cytoskeletal 42 (Cytokeratin-42) (CK-42) (Keratin-17n) (Keratin-42) (K42) (Type I keratin Ka22) | 50,133 |
| 4.6 | TDR12_MOUSE | Putative ATP-dependent RNA helicase TDRD12 (EC 3.6.4.13) (ES cell-associated transcript 8 protein) (Tudor domain-containing protein 12) | 137,627 |
| 4.5 | K22E_MOUSE | Keratin, type II cytoskeletal 2 epidermal (Cytokeratin-2e) (CK-2e) (Epithelial keratin-2e) (Keratin-2 epidermis) (Keratin-2e) (K2e) (Type-II keratin Kb2) | 70,923 |
| 3.9 | NAA20_MOUSE | N-alpha-acetyltransferase 20 (EC 2.3.1.88) (Methionine N-acetyltransferase) (N-acetyltransferase 5) (N-terminal acetyltransferase B complex catalytic subunit NAA20) (N-terminal acetyltransferase B complex catalytic subunit NAT5) (NatB complex subunit NAT5) (NatB catalytic subunit) | 20,368 |
| 3.8 | K2C5_MOUSE | Keratin, type II cytoskeletal 5 (Cytokeratin-5) (CK-5) (Keratin-5) (K5) (Type-II keratin Kb5) | 61,767 |
| 3.4 | WDR64_MOUSE | WD repeat-containing protein 64 | 123,893 |
| 3.3 | NCAN_MOUSE | Neurocan core protein (Chondroitin sulfate proteoglycan 3) | 137,200 |
| 3.2 | RAD54_HUMAN | DNA repair and recombination protein RAD54-like (EC 3.6.4.-) (RAD54 homolog) (hHR54) (hRAD54) | 84,352 |
| 3.1 | LBP_MOUSE | Lipopolysaccharide-binding protein (LBP) | 53,055 |
| 3 | BAI2_MOUSE | Adhesion G protein-coupled receptor B2 (Brain-specific angiogenesis inhibitor 2) | 169,863 |
| 2.9 | FNIP2_MOUSE | Folliculin-interacting protein 2 (FNIP1-like protein) (O6-methylguanine-induced apoptosis 1 protein) | 122,520 |
| 2.8 | CWC25_MOUSE | Pre-mRNA-splicing factor CWC25 homolog (Coiled-coil domain-containing protein 49) (Spliceosome-associated protein homolog CWC25) | 48,811 |
| 2.7 | FREM1_MOUSE | FRAS1-related extracellular matrix protein 1 (Protein QBRICK) | 244,544 |
| 2.3 | SYIM_MOUSE | Isoleucine--tRNA ligase, mitochondrial (EC 6.1.1.5) (Isoleucyl-tRNA synthetase) (IleRS) | 112,804 |
| 2.3 | ABHD8_MOUSE | Protein ABHD8 (EC 3.-.-.-) (Alpha/beta hydrolase domain-containing protein 8) (Abhydrolase domain-containing protein 8) | 48,229 |
| 2.3 | K2C79_MOUSE | Keratin, type II cytoskeletal 79 (Cytokeratin-79) (CK-79) (Keratin-79) (K79) (Type-II keratin Kb38) | 57,552 |
| 2.3 | CATZ_MOUSE | Cathepsin Z (EC 3.4.18.1) | 33,996 |
| 2 | FRAS1_MOUSE | Extracellular matrix protein FRAS1 | 442,369 |
| 1.9 | ACTBL_MOUSE | Beta-actin-like protein 2 (Kappa-actin) | 42,004 |
| 1.9 | PNCB_MOUSE | Nicotinate phosphoribosyltransferase (NAPRTase) (EC 6.3.4.21) (Nicotinate phosphoribosyltransferase domain-containing protein 1) | 58,265 |
| 1.7 | ZBT49_MOUSE | Zinc finger and BTB domain-containing protein 49 (Zinc finger protein 509) | 83,091 |
| 1.6 | PPARD_MOUSE | Peroxisome proliferator-activated receptor delta (PPAR-delta) (Nuclear hormone receptor 1) (NUC1) (Nuclear receptor subfamily 1 group C member 2) (Peroxisome proliferator-activated receptor beta) (PPAR-beta) | 49,715 |
| 1.5 | 5HT2B_MOUSE | 5-hydroxytryptamine receptor 2B (5-HT-2B) (5-HT2B) (5-HT-2F) (NP75 protein) (Serotonin receptor 2B) | 53,597 |
| 1.5 | TMPSD_MOUSE | Transmembrane protease serine 13 (EC 3.4.21.-) (Membrane-type mosaic serine protease) (Mosaic serine protease) | 59,806 |
| 1.5 | COBA2_MOUSE | Collagen alpha-2(XI) chain | 171,535 |
| 1.2 | NSD1_MOUSE | Histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific (EC 2.1.1.43) (H3-K36-HMTase) (H4-K20-HMTase) (Nuclear receptor-binding SET domain-containing protein 1) (NR-binding SET domain-containing protein) | 284,084 |

| | | | |
|---|---|---|---|
| 1.2 | MKKS_MOUSE | McKusick-Kaufman/Bardet-Biedl syndromes putative chaperonin (Protein Bbs6 homolog) | 61,924 |
| 1.1 | FAT2_MOUSE | Protocadherin Fat 2 (FAT tumor suppressor homolog 2) | 480,109 |
| 0.9 | SSH3_MOUSE | Protein phosphatase Slingshot homolog 3 (EC 3.1.3.16) (EC 3.1.3.48) (SSH-like protein 3) (SSH-3L) (mSSH-3L) | 72,227 |
| 0.8 | TLR4_MOUSE | Toll-like receptor 4 (CD antigen CD284) | 95,519 |
| 0.7 | ATP9B_MOUSE | Probable phospholipid-transporting ATPase IIB (EC 3.6.3.1) (ATPase class II type 9B) | 129,017 |
| 0.6 | RC3H2_MOUSE | Roquin-2 (EC 6.3.2.-) (Membrane-associated nucleic acid-binding protein) (RING finger and CCCH-type zinc finger domain-containing protein 2) | 131,295 |

**Table 3.** List of p50KO ACM proteins identified by Mascot and Protein Pilot database search, filtered for protein/peptide confidence above 95%, with a minimum of two unique peptides per protein.

| Cov (%) | Accession | Protein names | Mass (Da) |
|---|---|---|---|
| 48.2 | APOE_MOUSE | Apolipoprotein E (Apo-E) | 35,867 |
| 42.6 | SAA3_MOUSE | Serum amyloid A-3 protein | 13,774 |
| 36.4 | CO3_MOUSE | Complement C3 (HSE-MSF) [Cleaved into: Complement C3 beta chain; C3-beta-c (C3bc); Complement C3 alpha chain; C3a anaphylatoxin; Acylation stimulating protein (ASP) (C3adesArg); Complement C3b alpha' chain; Complement C3c alpha' chain fragment 1; Complement C3dg fragment; Complement C3g fragment; Complement C3d fragment; Complement C3f fragment; Complement C3c alpha' chain fragment 2] | 186,484 |
| 31.5 | NGAL_MOUSE | Neutrophil gelatinase-associated lipocalin (NGAL) (Lipocalin-2) (SV-40-induced 24P3 protein) (Siderocalin LCN2) (p25) | 22,875 |
| 28.1 | CLUS_MOUSE | Clusterin (Apolipoprotein J) (Apo-J) (Clustrin) (Sulfated glycoprotein 2) (SGP-2) [Cleaved into: Clusterin beta chain; Clusterin alpha chain] | 51,656 |
| 25.7 | CYTC_MOUSE | Cystatin-C (Cystatin-3) | 15,531 |
| 23.9 | SPA3N_MOUSE | Serine protease inhibitor A3N (Serpin A3N) | 46,718 |
| 22.3 | CCL2_MOUSE | C-C motif chemokine 2 (Monocyte chemoattractant protein 1) (Monocyte chemotactic protein 1) (MCP-1) (Platelet-derived growth factor-inducible protein JE) (Small-inducible cytokine A2) | 16,326 |
| 19.7 | CBPE_MOUSE | Carboxypeptidase E (CPE) (EC 3.4.17.10) (Carboxypeptidase H) (CPH) (Enkephalin convertase) (Prohormone-processing carboxypeptidase) | 53,256 |
| 18.1 | CERU_MOUSE | Ceruloplasmin (EC 1.16.3.1) (Ferroxidase) | 121,151 |
| 17.3 | RARR2_MOUSE | Retinoic acid receptor responder protein 2 (Chemerin) | 18,350 |
| 15.9 | SCG3_MOUSE | Secretogranin-3 (Secretogranin III) (SgIII) | 53,326 |
| 15 | CFAB_MOUSE | Complement factor B (EC 3.4.21.47) (C3/C5 convertase) [Cleaved into: Complement factor B Ba fragment; Complement factor B Bb fragment] | 85,004 |
| 12.4 | CCL7_MOUSE | C-C motif chemokine 7 (Intercrine/chemokine MARC) (Monocyte chemoattractant protein 3) (Monocyte chemotactic protein 3) (MCP-3) (Protein FIC) (Small-inducible cytokine A7) | 10,999 |
| 11.7 | HA1K_MOUSE | H-2 class I histocompatibility antigen, K-K alpha chain (H-2K(K)) | 41,646 |

| | | | |
|---|---|---|---|
| 9.3 | CATD_MOUSE | Cathepsin D (EC 3.4.23.5) | 44,954 |
| 9.3 | KIF5C_MOUSE | Kinesin heavy chain isoform 5C (Kinesin heavy chain neuron-specific 2) | 109,275 |
| 8.8 | TIMP1_MOUSE | Metalloproteinase inhibitor 1 (Collagenase inhibitor 16C8 fibroblast) (Erythroid-potentiating activity) (EPA) (TPA-S1) (TPA-induced protein) (Tissue inhibitor of metalloproteinases 1) (TIMP-1) | 22,628 |
| 8.2 | CXL10_MOUSE | C-X-C motif chemokine 10 (10 kDa interferon gamma-induced protein) (Gamma-IP10) (IP-10) (C7) (Interferon-gamma induced protein CRG-2) (Small-inducible cytokine B10) | 10,789 |
| 7.9 | NAA20_MOUSE | N-alpha-acetyltransferase 20 (EC 2.3.1.88) (Methionine N-acetyltransferase) (N-acetyltransferase 5) (N-terminal acetyltransferase B complex catalytic subunit NAA20) (N-terminal acetyltransferase B complex catalytic subunit NAT5) (NatB complex subunit NAT5) (NatB catalytic subunit) | 20,368 |
| 7.7 | K1C10_MOUSE | Keratin, type I cytoskeletal 10 (56 kDa cytokeratin) (Cytokeratin-10) (CK-10) (Keratin, type I cytoskeletal 59 kDa) (Keratin-10) (K10) | 57,770 |
| 7.7 | CATB_MOUSE | Cathepsin B (EC 3.4.22.1) (Cathepsin B1) [Cleaved into: Cathepsin B light chain; Cathepsin B heavy chain] | 37,280 |
| 7.6 | CAND1_MOUSE | Cullin-associated NEDD8-dissociated protein 1 (Cullin-associated and neddylation-dissociated protein 1) (p120 CAND1) | 136,332 |
| 7.1 | K1C42_MOUSE | Keratin, type I cytoskeletal 42 (Cytokeratin-42) (CK-42) (Keratin-17n) (Keratin-42) (K42) (Type I keratin Ka22) | 50,133 |
| 6.9 | FSTL1_MOUSE | Follistatin-related protein 1 (Follistatin-like protein 1) (TGF-beta-inducible protein TSC-36) | 34,554 |
| 6.8 | PAFA_MOUSE | Platelet-activating factor acetylhydrolase (PAF acetylhydrolase) (EC 3.1.1.47) (1-alkyl-2-acetylglycerophosphocholine esterase) (2-acetyl-1-alkylglycerophosphocholine esterase) (LDL-associated phospholipase A2) (LDL-PLA(2)) (PAF 2-acylhydrolase) | 49,258 |
| 6.7 | LBP_MOUSE | Lipopolysaccharide-binding protein (LBP) | 53,055 |
| 6.3 | SPRC_MOUSE | SPARC (Basement-membrane protein 40) (BM-40) (Osteonectin) (ON) (Secreted protein acidic and rich in cysteine) | 34,450 |
| 5.9 | CAB39_MOUSE | Calcium-binding protein 39 (MO25alpha) (Protein Mo25) | 39,843 |
| 5.9 | DDX27_MOUSE | Probable ATP-dependent RNA helicase DDX27 (EC 3.6.4.13) (DEAD box protein 27) | 85,939 |
| 5.6 | CTRO_MOUSE | Citron Rho-interacting kinase (CRIK) (EC 2.7.11.1) (Rho-interacting, serine/threonine-protein kinase 21) | 235,389 |
| 5.5 | CATA_MOUSE | Catalase (EC 1.11.1.6) | 59,795 |
| 5.5 | CH3L1_MOUSE | Chitinase-3-like protein 1 (BRP39 protein) (Breast regression protein 39) (Cartilage glycoprotein 39) (CGP-39) (GP-39) | 43,893 |
| 5.4 | MYH7_MOUSE | Myosin-7 (Myosin heavy chain 7) (Myosin heavy chain slow isoform) (MyHC-slow) (Myosin heavy chain, cardiac muscle beta isoform) (MyHC-beta) | 222,879 |
| 5.1 | PLTP_MOUSE | Phospholipid transfer protein (Lipid transfer protein II) | 54,453 |
| 4.4 | KRT35_MOUSE | Keratin, type I cuticular Ha5 (Hair keratin, type I Ha5) (Keratin-35) (K35) | 50,530 |
| 4.2 | ABCA1_MOUSE | ATP-binding cassette sub-family A member 1 (ATP-binding cassette transporter 1) (ABC-1) (ATP-binding cassette 1) | 253,912 |
| 4.1 | K2C79_MOUSE | Keratin, type II cytoskeletal 79 (Cytokeratin-79) (CK-79) (Keratin-79) (K79) (Type-II keratin Kb38) | 57,552 |
| 4 | ACTBL_MOUSE | Beta-actin-like protein 2 (Kappa-actin) | 42,004 |
| 3.6 | NCAN_MOUSE | Neurocan core protein (Chondroitin sulfate proteoglycan 3) | 137,200 |
| 3.5 | FAM3C_MOUSE | Protein FAM3C (Interleukin-like EMT inducer) | 24,753 |

| | | | |
|---|---|---|---|
| 3.3 | K22E_MOUSE | Keratin, type II cytoskeletal 2 epidermal (Cytokeratin-2e) (CK-2e) (Epithelial keratin-2e) (Keratin-2 epidermis) (Keratin-2e) (K2e) (Type-II keratin Kb2) | 70,923 |
| 3.2 | PPARD_MOUSE | Peroxisome proliferator-activated receptor delta (PPAR-delta) (Nuclear hormone receptor 1) (NUC1) (Nuclear receptor subfamily 1 group C member 2) (Peroxisome proliferator-activated receptor beta) (PPAR-beta) | 49,715 |
| 3.1 | TMCC1_MOUSE | Transmembrane and coiled-coil domains protein 1 | 71,642 |
| 2.8 | CWC25_MOUSE | Pre-mRNA-splicing factor CWC25 homolog (Coiled-coil domain-containing protein 49) (Spliceosome-associated protein homolog CWC25) | 48,811 |
| 2.7 | MOT10_MOUSE | Monocarboxylate transporter 10 (MCT 10) (Aromatic amino acid transporter 1) (Solute carrier family 16 member 10) (T-type amino acid transporter 1) | 55,320 |
| 2.3 | RC3H2_MOUSE | Roquin-2 (EC 6.3.2.-) (Membrane-associated nucleic acid-binding protein) (RING finger and CCCH-type zinc finger domain-containing protein 2) | 131,295 |
| 2.3 | ABHD8_MOUSE | Protein ABHD8 (EC 3.-.-.-) (Alpha/beta hydrolase domain-containing protein 8) (Abhydrolase domain-containing protein 8) | 48,229 |
| 2 | K1C15_MOUSE | Keratin, type I cytoskeletal 15 (Cytokeratin-15) (CK-15) (Keratin-15) (K15) | 49,138 |
| 1.9 | PNCB_MOUSE | Nicotinate phosphoribosyltransferase (NAPRTase) (EC 6.3.4.21) (Nicotinate phosphoribosyltransferase domain-containing protein 1) | 58,265 |
| 1.8 | LIFR_MOUSE | Leukemia inhibitory factor receptor (LIF receptor) (LIF-R) (D-factor/LIF receptor) (CD antigen CD118) | 122,574 |
| 1.7 | DDX46_MOUSE | Probable ATP-dependent RNA helicase DDX46 (EC 3.6.4.13) (DEAD box protein 46) | 117,448 |
| 1.5 | TMPSD_MOUSE | Transmembrane protease serine 13 (EC 3.4.21.-) (Membrane-type mosaic serine protease) (Mosaic serine protease) | 59,806 |
| 1.4 | CO4A2_MOUSE | Collagen alpha-2(IV) chain [Cleaved into: Canstatin] | 167,325 |
| 1.2 | LG3BP_MOUSE | Galectin-3-binding protein (Cyp-C-associated protein) (CyCAP) (Lectin galactoside-binding soluble 3-binding protein) (Protein MAMA) | 64,491 |
| 1 | AKAP4_MOUSE | A-kinase anchor protein 4 (AKAP-4) (A-kinase anchor protein 82 kDa) (AKAP 82) (mAKAP82) (FSC1) (Major sperm fibrous sheath protein) (Protein kinase A-anchoring protein 4) (PRKA4) (p82) | 93,796 |
| 0.5 | BAI2_MOUSE | Adhesion G protein-coupled receptor B2 (Brain-specific angiogenesis inhibitor 2) | 169,863 |

**Table 4.** Raw data file of peptides used in SWATH-MS analysis for the identification and quantitation of the 4 differentially expressed proteins listed in Table 1.

| Protein | Peptide | Replicate | Precursor Result | Total Area MS1 | Total Area Fragment |
|---|---|---|---|---|---|
| sp\|P04223\|HA1K_MOUSE | GNEQIFR | SWATH_WTrep3 | 2471 | #N/D | 2471 |
| sp\|P04223\|HA1K_MOUSE | GNEQIFR | SWATH_WTrep2 | 2702 | #N/D | 2702 |
| sp\|P04223\|HA1K_MOUSE | GNEQIFR | SWATH_WTrep1 | 2340 | #N/D | 2340 |
| sp\|P04223\|HA1K_MOUSE | GNEQIFR | SWATH_KOrep_4 | 10868 | #N/D | 10868 |
| sp\|P04223\|HA1K_MOUSE | GNEQIFR | SWATH_KOrep_2 | 10213 | #N/D | 10213 |
| sp\|P04223\|HA1K_MOUSE | GNEQIFR | SWATH_KOrep_1 | 11466 | #N/D | 11466 |
| sp\|P04223\|HA1K_MOUSE | GNEQIFR | SWATH_KOrep_3 | 9993 | #N/D | 9993 |
| sp\|P10148\|CCL2_MOUSE | EAVVFVTK | SWATH_WTrep3 | 153687 | 128204 | 25483 |
| sp\|P10148\|CCL2_MOUSE | EAVVFVTK | SWATH_WTrep2 | 164498 | 140179 | 24319 |
| sp\|P10148\|CCL2_MOUSE | EAVVFVTK | SWATH_WTrep1 | 146597 | 122183 | 24414 |
| sp\|P10148\|CCL2_MOUSE | EAVVFVTK | SWATH_KOrep_4 | 661337 | 554219 | 107119 |
| sp\|P10148\|CCL2_MOUSE | EAVVFVTK | SWATH_KOrep_2 | 662528 | 562099 | 100429 |
| sp\|P10148\|CCL2_MOUSE | EAVVFVTK | SWATH_KOrep_1 | 605459 | 508588 | 96871 |
| sp\|P10148\|CCL2_MOUSE | EAVVFVTK | SWATH_KOrep_3 | 583708 | 492348 | 91360 |
| sp\|P10148\|CCL2_MOUSE | KEWVQTYIK | SWATH_WTrep3 | 32001 | 31099 | 902 |
| sp\|P10148\|CCL2_MOUSE | KEWVQTYIK | SWATH_WTrep2 | 20523 | 19090 | 1433 |
| sp\|P10148\|CCL2_MOUSE | KEWVQTYIK | SWATH_WTrep1 | 38266 | 36820 | 1446 |
| sp\|P10148\|CCL2_MOUSE | KEWVQTYIK | SWATH_KOrep_4 | 71301 | 68320 | 2981 |
| sp\|P10148\|CCL2_MOUSE | KEWVQTYIK | SWATH_KOrep_2 | 85960 | 83370 | 2591 |
| sp\|P10148\|CCL2_MOUSE | KEWVQTYIK | SWATH_KOrep_1 | 63109 | 59543 | 3566 |
| sp\|P10148\|CCL2_MOUSE | KEWVQTYIK | SWATH_KOrep_3 | 94789 | 91994 | 2794 |
| sp\|P10148\|CCL2_MOUSE | EWVQTYIK | SWATH_WTrep3 | 155424 | 128374 | 27050 |
| sp\|P10148\|CCL2_MOUSE | EWVQTYIK | SWATH_WTrep2 | 160812 | 128365 | 32447 |
| sp\|P10148\|CCL2_MOUSE | EWVQTYIK | SWATH_WTrep1 | 178413 | 146207 | 32206 |
| sp\|P10148\|CCL2_MOUSE | EWVQTYIK | SWATH_KOrep_4 | 329334 | 282540 | 46794 |
| sp\|P10148\|CCL2_MOUSE | EWVQTYIK | SWATH_KOrep_2 | 329952 | 279782 | 50170 |
| sp\|P10148\|CCL2_MOUSE | EWVQTYIK | SWATH_KOrep_1 | 329134 | 281025 | 48109 |
| | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| sp\|P10148\|CCL2_MOUSE | EWVQTYIK | SWATH_KOrep_3 | 332781 | 289702 | 43079 |
| sp\|P10148\|CCL2_MOUSE | SSAPLNVK | SWATH_WTrep3 | 153153 | 144243 | 8909 |
| sp\|P10148\|CCL2_MOUSE | SSAPLNVK | SWATH_WTrep2 | 160286 | 150394 | 9892 |
| sp\|P10148\|CCL2_MOUSE | SSAPLNVK | SWATH_WTrep1 | 191953 | 182631 | 9322 |
| sp\|P10148\|CCL2_MOUSE | SSAPLNVK | SWATH_KOrep_4 | 516756 | 476254 | 40502 |
| sp\|P10148\|CCL2_MOUSE | SSAPLNVK | SWATH_KOrep_2 | 487764 | 448050 | 39714 |
| sp\|P10148\|CCL2_MOUSE | SSAPLNVK | SWATH_KOrep_1 | 499476 | 461775 | 37701 |
| sp\|P10148\|CCL2_MOUSE | SSAPLNVK | SWATH_KOrep_3 | 459034 | 422266 | 36768 |
| sp\|P11672\|NGAL_MOUSE | WYVVGLAGNAVQK | SWATH_WTrep3 | 135741 | 120467 | 15273 |
| sp\|P11672\|NGAL_MOUSE | WYVVGLAGNAVQK | SWATH_WTrep2 | 199234 | 183843 | 15391 |
| sp\|P11672\|NGAL_MOUSE | WYVVGLAGNAVQK | SWATH_WTrep1 | 171925 | 155501 | 16423 |
| sp\|P11672\|NGAL_MOUSE | WYVVGLAGNAVQK | SWATH_KOrep_4 | 398726 | 350009 | 48717 |
| sp\|P11672\|NGAL_MOUSE | WYVVGLAGNAVQK | SWATH_KOrep_2 | 439424 | 392870 | 46555 |
| sp\|P11672\|NGAL_MOUSE | WYVVGLAGNAVQK | SWATH_KOrep_1 | 421031 | 371489 | 49542 |
| sp\|P11672\|NGAL_MOUSE | WYVVGLAGNAVQK | SWATH_KOrep_3 | 435008 | 391324 | 43684 |
| sp\|P11672\|NGAL_MOUSE | TFVPSSR | SWATH_WTrep3 | 160713 | 124169 | 36544 |
| sp\|P11672\|NGAL_MOUSE | TFVPSSR | SWATH_WTrep2 | 150379 | 117681 | 32698 |
| sp\|P11672\|NGAL_MOUSE | TFVPSSR | SWATH_WTrep1 | 164657 | 128344 | 36312 |
| sp\|P11672\|NGAL_MOUSE | TFVPSSR | SWATH_KOrep_4 | 414944 | 320077 | 94868 |
| sp\|P11672\|NGAL_MOUSE | TFVPSSR | SWATH_KOrep_2 | 423173 | 326578 | 96596 |
| sp\|P11672\|NGAL_MOUSE | TFVPSSR | SWATH_KOrep_1 | 414689 | 319453 | 95236 |
| sp\|P11672\|NGAL_MOUSE | TFVPSSR | SWATH_KOrep_3 | 462786 | 369185 | 93601 |
| sp\|P11672\|NGAL_MOUSE | YPQVQSYNVQVATTDYNQFAMVFFR | SWATH_WTrep3 | 88085 | 84257 | 3828 |
| sp\|P11672\|NGAL_MOUSE | YPQVQSYNVQVATTDYNQFAMVFFR | SWATH_WTrep2 | 86929 | 84550 | 2379 |
| sp\|P11672\|NGAL_MOUSE | YPQVQSYNVQVATTDYNQFAMVFFR | SWATH_WTrep1 | 72685 | 69959 | 2727 |
| sp\|P11672\|NGAL_MOUSE | YPQVQSYNVQVATTDYNQFAMVFFR | SWATH_KOrep_4 | 257914 | 248498 | 9416 |
| sp\|P11672\|NGAL_MOUSE | YPQVQSYNVQVATTDYNQFAMVFFR | SWATH_KOrep_2 | 288924 | 277626 | 11298 |
| sp\|P11672\|NGAL_MOUSE | YPQVQSYNVQVATTDYNQFAMVFFR | SWATH_KOrep_1 | 284240 | 271784 | 12456 |
| sp\|P11672\|NGAL_MOUSE | YPQVQSYNVQVATTDYNQFAMVFFR | SWATH_KOrep_3 | 223409 | 213629 | 9780 |
| sp\|P11672\|NGAL_MOUSE | ELSPELK | SWATH_WTrep3 | 146315 | 128375 | 17940 |
| sp\|P11672\|NGAL_MOUSE | ELSPELK | SWATH_WTrep2 | 181324 | 154967 | 26357 |
| sp\|P11672\|NGAL_MOUSE | ELSPELK | SWATH_WTrep1 | 123902 | 109046 | 14856 |
| sp\|P11672\|NGAL_MOUSE | ELSPELK | SWATH_KOrep_4 | 399498 | 352069 | 47429 |

| | | | | | |
|---|---|---|---|---|---|
| sp\|P11672\|NGAL_MOUSE | ELSPELK | SWATH_KOrep_2 | 399837 | 352828 | 47008 |
| sp\|P11672\|NGAL_MOUSE | ELSPELK | SWATH_KOrep_1 | 380892 | 328979 | 51913 |
| sp\|P11672\|NGAL_MOUSE | ELSPELK | SWATH_KOrep_3 | 356387 | 316640 | 39746 |
| sp\|Q9DD06\|RARR2_MOUSE | HPPVQLAFQEIGVDR | SWATH_WTrep3 | 1874 | #N/D | 1874 |
| sp\|Q9DD06\|RARR2_MOUSE | HPPVQLAFQEIGVDR | SWATH_WTrep2 | 1877 | #N/D | 1877 |
| sp\|Q9DD06\|RARR2_MOUSE | HPPVQLAFQEIGVDR | SWATH_WTrep1 | 1707 | #N/D | 1707 |
| sp\|Q9DD06\|RARR2_MOUSE | HPPVQLAFQEIGVDR | SWATH_KOrep_4 | 5360 | #N/D | 5360 |
| sp\|Q9DD06\|RARR2_MOUSE | HPPVQLAFQEIGVDR | SWATH_KOrep_2 | 6580 | #N/D | 6580 |
| sp\|Q9DD06\|RARR2_MOUSE | HPPVQLAFQEIGVDR | SWATH_KOrep_1 | 5245 | #N/D | 5245 |
| sp\|Q9DD06\|RARR2_MOUSE | HPPVQLAFQEIGVDR | SWATH_KOrep_3 | 5373 | #N/D | 5373 |

# Chapter 4.

**Modulatory events within the adult hippocampal neurogenic niche: novel insights into region-specific astrocyte-derived signaling cues and the role played by NF-κB p50.**

[UNPUBLISHED RESULTS]

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## Introduction

Active adult neurogenesis is well documented to occur in the adult mammalian brain in two discrete areas, the subventricular zone (SVZ) of the later ventricles, and the subgranular zone (SGZ) of the dentate gyrus (Ming and Song, 2005; Bond et al., 2015; Nicola et al., 2015). Growing evidence suggest the presence of another neurogenic area in the hypothalamus, along the walls of the third ventricle (Kokoeva et al., 2005). The neurogenic areas called niches are composed of different cell types and non-cellular components, such as soluble cues (i.e. growth factors, cytokines, hormones) originated both locally and distantly. In the neurogenic niches neural stem/progenitor cells (NSPC) are housed and supported by the different cellular and molecular components, that balance their proliferation and differentiation rates (Lin and Iacovitti, 2015). Astrocytes represent one major contributor of the hippocampal neurogenic niche. Hippocampal-derived astrocytes were demonstrated to promote neurogenesis both via directly interacting with adult hippocampal NSPC and through secreted factors (Song et al., 2002). On the other hand, astrocytes isolated from non-neurogenic areas, such as those isolated from the adult spinal cord were proven ineffective in promoting neurogenesis, while were effective in promoting gliogenesis (Barkho et al., 2006). Overall these data suggested that the astrocytes in adult neurogenic regions contribute to generate an instructive environment for neuronal differentiation of adult NSPC.

Our group reported that the NF-κB family of transcription factors are expressed at considerable levels in areas of active neurogenesis in the postnatal and adult mouse brain (Denis-Donini et al., 2005). Previous publications from our lab also reported that mice lacking NF-κB p50 subunit display a dramatic reduction in adult hippocampal neurogenesis associated with short-term memory defects (Denis-Donini et al., 2008). Interestingly, NPC derived from WT and p50KO hippocampi do not display any differences in neuronal differentiation and apoptotic rates *in vitro*. These data suggested that impaired hippocampal neurogenesis observed *in vivo* could also be due to inadequate signaling

between niche cells. In previous work in our laboratory (Cvijetic et al., under submission) we investigated NPC-astroglia communication and, in particular, the effect of soluble factors released by astrocytes derived from mixed culture of cortex/hippocampi on cell fate specification of WT and p50KO adult hippocampal NPC (ahNPC). We demonstrated that WT ACM promote both neuronal and astroglial differentiation of WT NPC, whereas p50KO ACM can only promote astrogliogenesis. Additionally, when p50KO NPC were exposed to WT and p50KO ACM, they showed unresponsiveness to both treatment conditions.

As a follow up of these experiments, in the first part of this study, we addressed the importance of region-specificity in the effect of ACM on ahNPC. More specifically, we compared the proneurogenic and progliogenic properties of hippocampal and hypothalamic ACM deriving from WT and p50KO astrocytes on adult hippocampal WT NPC fate specification.

A second line of investigation was aimed at assessing the differentiation of WT hypothalamic NPC in response to hippocampal ACM deriving from WT and p50KO astrocytes cultures.

## Materials and Methods

*Adult mouse hippocampal (Hp) and hypothalamic (Hyt) neural progenitor cell (NPC) cultures.* Hippocampi and hypothalami from three adult (3-4 month-old) C57BL/6 male mice were dissected and cell suspension was prepared as previously described (Valente et al, 2012). Cells were maintained as floating neurospheres through subsequent passages (P1-P30).

*NPC differentiation experiments.* Differentiation experiments were carried out as previously described (Valente et al., 2012). Briefly, neurospheres (passages 5-30) were dissociated into single NPC and plated onto laminin-coated Lab-Tek 8-well permanox chamber slides (Nunc) at a density of 43,750 cells/$cm^2$ in NPC differentiation medium (NDM, Neurobasal-A medium supplemented with B27, 2 mM L-glutamine and 100 U/100 µg/ml Penicillin/Streptomycin). NPC were treated in presence of astrocyte conditioned media (ACM) derived from WT or NF-κB p50KO glial cultures (referred to as hippocampal, Hp – or hypothalamic, Hyt - ACM), or in presence of NDM (referred to as standard, STD medium) for 24 h.

*Primary Astrocyte Cultures.* Primary mixed glial cultures were prepared from hippocampus and hypothalamus of neonatal (P1-2) C57BL/6 mice and grown in DMEM high glucose, 10% FBS, 15 mM HEPES, 2 mM L-glutamine and 100 U/100 µg/ml Penicillin/Streptomycin at a density of 78,000 cells/$cm^2$. When cells reached confluence (around 10-12 D.I.V.), proliferation of non-astrocytic cells was blocked by 10 µM cytosine arabinoside (Sigma-Aldrich) for 96h. Then cultures were switched to NDM. Phenotypic characterization of cultures was performed by immunocytochemistry with antibodies against GFAP (mouse monoclonal, 1:600, Millipore) and CD11b (rat monoclonal; 1:150, Millipore). Contaminating microglial cells were below 3% of total cells, with the remaining cells being $GFAP^+$.

*Preparation of astrocyte-conditioned media (ACM).* To prepare astrocyte-conditioned medium we used cells at confluence. ACM was collected after 48h of conditioning of enriched glial cultures in presence of Neurobasal-A medium with B27 supplement. Medium collected was centrifuged for 10 min x 16,100 x *g* at 4°C, to detach cells and large debris, and filtered using 0.20 μm filter before use in NPC differentiation studies. Collected conditioned medium was stored at -80°C. The ACM was used with 1:2 dilutions in all experiments.

*Immunolocalization studies in adult hippocampal and hypothalamic NPC.* Phenotypic characterization of NPC-derived cells was carried out by immunolocalization for MAP-2 (rabbit polyclonal, 1:600; Millipore), GFAP (mouse monoclonal, 1:600, Millipore), nestin (chicken monoclonal, 1:1500, Neuromics), NG-2 (rabbit polyclonal, 1:500; Millipore). Nuclei were counterstained with 0.8 ng/ml Hoechst (Thermo Fisher scientific) diluted in PBS. In each experiment, 5 fields/well (corresponding to about 150-200 cells/well) were counted with a 60X objective by a Leica DMIRB inverted fluorescence microscope. Cells positive for MAP-2, GFAP and NG-2 were counted and their percentage over total viable cells was calculated.

*Statistical analysis.* All experiments were run in triplicate using different cell preparations and repeated at least three times. Data were analyzed using Student's *t*-test when only two independent groups were compared, or by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc when comparing more than three groups. Results were expressed as mean ± S.D. Statistical significance level was set for $p$ values $< 0.05$.

## Results

*Hippocampal but not hypothalamic ACM can promote neuronal differentiation of hippocampal NPC.*

Soluble factors released by astrocytes have been shown to modulate differentiation of adult neural progenitor cells (NPC) (Song et al., 2002). Based on these observations we evaluated the effect of astrocyte-generated soluble factors on the differentiation rate of adult hippocampal NPC toward neuronal and non neuronal lineages. Multipotent $Sox2^+$/$nestin^+$/$GFAP^-$ NPC isolated from adult mouse hippocampi can be maintained for several passages in an undifferentiated proliferative state (Cuccurazzu et al., 2013). Upon removal of growth factors from medium, NPC stop dividing and differentiate onto laminin-coated dishes. By double immunolabeling for markers of neurons (MAP-2) and undifferentiated progenitors (nestin), the appearance of newly generated MAP-$2^+$/$nestin^-$ neurons and MAP-$2^+$/$nestin^+$ neuroblasts, can be evaluated and quantified. Under these experimental conditions we tested the effect of hippocampal (Hp) and hypothalamic (Hyt) astrocyte-conditioned medium (ACM) in comparison with standard differentiation medium (STD). After 24 h, a significant increase in the percentage of mature neurons and neuroblasts was observed in cells exposed to Hp ACM, but not to Hyt ACM, compared to standard medium (mean % ± S.D. over viable cells of MAP-$2^+$/$nestin^-$ cells: 3.49 ± 0.40, 5.69 ± 0.67, 3.46 ± 0.33 % in presence of STD medium, Hp and Hyt ACM, respectively; ANOVA, $p < 0.001$ vs STD; Fig. 1 *A*; MAP-$2^+$/$nestin^+$ cells: 32.10 ± 4.08, 40.46 ± 2.66, 33.49 ± 2.69 %, in presence of STD medium, Hp and Hyt ACM, respectively; ANOVA, $p < 0.001$ vs STD; Fig. 1 *B*). Under the same experimental *in vitro* conditions adult hippocampal NPC can also differentiate toward the astrocytic lineage. Interestingly, both Hp and Hyt ACM resulted in an increased percentage of $GFAP^+$ cells when compared to standard medium ($GFAP^+$ cells: 14.53 ± 1.48, 29.18 ± 6.74, 36.42 ± 5.10 in presence of STD medium, Hp and Hyt ACM, respectively; ANOVA, $p < 0.001$ vs STD;

Fig. 1 *C*). Conversely, the NG-2$^+$ population of oligodendroglial precursor cells was not affected by the treatment with Hp and Hyt ACM compared to STD medium (NG-2$^+$ cells: 30.35 ± 6.00; 29.15 ± 6.04, 34.91 ± 4.59 %, in presence of STD medium, Hp and Hyt ACM; Fig. 1 *D*). To investigate whether ACM could promote cell survival in addition to neuronal differentiation, we also analyzed NPC apoptotic rate. No difference in the percentage of apoptotic cells was reported in presence of the different media (mean % ± S.D. over total cells: 9.08 ± 1.23, 7.13 ± 1.43, 6.86 ± 1.73 % in presence of STD medium, Hp and Hyt ACM, respectively; Fig. 1 *E*). Altogether these data suggest that a certain degree of region-specificity in the proneurogenic but not in the astrogliogenic signals provided by astrocytes on adult hippocampal NPC. Apparently, only hippocampal astrocytes but not hypothalamic astrocytes can secrete proneurogenic signals for ahNPC. Conversely, both hippocampal and hypothalamic astrocytes can trigger astrogliogenesis from ahNPC.

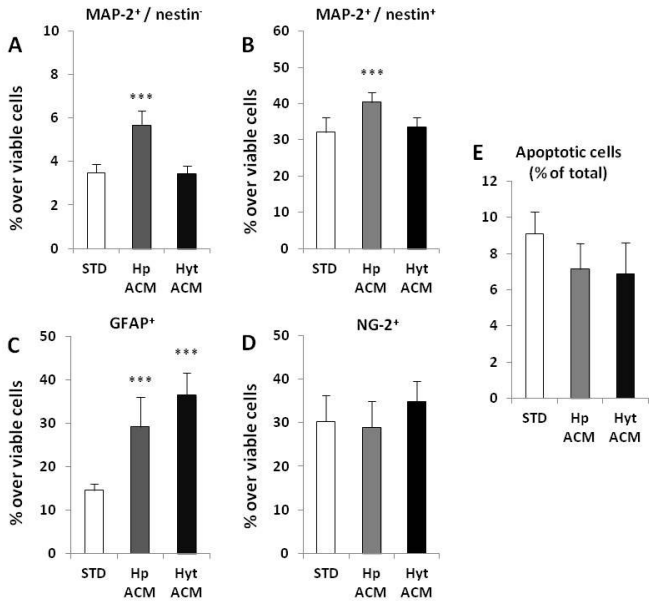


**Figure 1. Hypothalamic ACM, unlike Hippocampal ACM, does not promotes hippocampal NPC neuronal differentiation. Both ACM promote astrogliogenesis.** ***A-B*** Under differentiating conditions, 24h treatment of WT

hippocampal NPC with Hp ACM significantly increased the percentage of the subpopulations of MAP-$2^+$/$nestin^-$ neurons (***A***) and MAP-$2^+$/$nestin^+$ neuroblasts (***B***) compared with STD. Conversely, Hyt ACM was devoid of proneurogenic effect. ***C***, Astroglial population of $GFAP^+$ cells increased upon both Hp and Hyt ACM treatment. ***D***, No significant differences was observed in the percentage of NG-$2^+$ cells upon different media conditions. ***E***, No significant difference was observed in the apoptotic rate of cultures exposed to STD medium, Hp and Hyt ACM. Data are expressed as mean values ± S.D. of $n = 3$ experiments, run in triplicates and analyzed by one-way ANOVA followed by Tukey's post-hoc test. *** $p < 0.001$ versus STD medium.

*Hypothalamic NPC do not respond to proneurogenic soluble signals coming from the neurogenic astrocytes of the hippocampal niche.*

Recently hypothalamus was identified as a novel adult proneurogenic niche where newly formed neurons might contribute to the homeostatic control of many physiological behaviors related to hypothalamic functions, including energy balance regulation (Pierce and Xu 2010; Whalley 2012). In our laboratory we have set up and characterized an *in vitro* model of adult hypothalamic (Hyt) NPC (Salem et al, unpublished observations).

We then asked how adult Hyt NPC respond to astrocyte-secreted signals. In order to do so, we tested hypothalamic WT NPC differentiation rate toward neuronal and glial lineages upon treatment with astrocyte conditioned media derived from the hippocampal niche. When we tested the effect of Hp ACM on hypothalamic NPC, we did not observe any effect on neuronal differentiation (mean % ± SD over viable cells of MAP-$2^+$/$nestin^-$ cells: 4.61 ± 0.54, 4.48 ± 0.46 %, in presence of STD medium and Hp ACM, respectively; Fig. 2 *A*; MAP-$2^+$/$nestin^+$ cells: 35.27 ± 4.15, 35.88 ± 3.73 %, in presence of STD medium and Hp ACM, respectively; Fig. 2 *B*). Under the same experimental conditions we observed a significant increase in newly generated astroglial cells upon ACM treatment ($GFAP^+$ cells: 29.11 ± 6.03, 44.09 ± 2.35 %, in presence of STD medium and Hp ACM, respectively; Fig. 2 *C*). Interestingly, a small, but significant increase in the percentage of NG-$2^+$ cells in Hyt NPC cultures

was observed upon Hp ACM treatment (NG-2$^+$ cells: 33.12 ± 3.49, 37.19 ± 2.47 %, in presence of STD medium and Hp ACM, respectively; Student's *t*-test vs STD, Fig. 2 *D*). No difference in the percentage of apoptotic cells was reported between STD medium and Hp ACM treatment (mean % ± S.D. over total cells: 6.91 ± 1.01, 6.03 ± 1.32 %, STD medium and WT ACM, respectively; Fig. 2 *E*). Altogether, these preliminary data suggest that soluble molecules from hippocampal astrocytes are not able to promote neuronal differentiation of Hyt NPC. On the contrary, similarly to what we observed on Hp NPC, hippocampal ACM can promote astrogliogenesis from Hyt NPC. Additionally, Hp ACM increase the NG-2$^+$ cell population only from Hyt NPC and not from Hp NPC. NG-2$^+$ cells are commonly considered oligodendrocyte precursors (Nishiyama et al., 2009). Interestingly, it has been proposed that, although controversial, hypothalamic NG-2$^+$ cells, distributed throughout the mediobasal hypothalamus, may represent a population able to give rise to new neurons (Robins et al., 2013). Future studies will be needed for understanding the nature of this population in our cell culture model, for example by assessing, under long-term culture conditions, the fate of these cells toward oligodendrocytic and neuronal lineages.

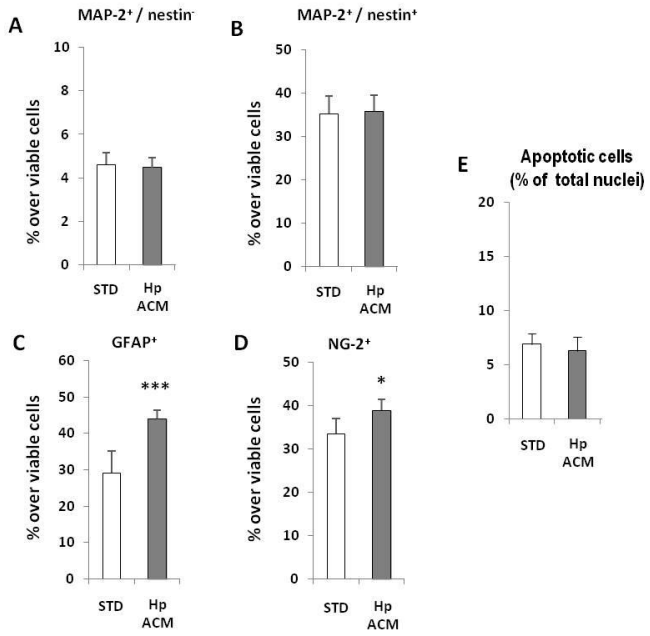


**Figure 2. Hippocampal ACM promotes an increase in GFAP$^+$ and NG-2$^+$ cells from Hypothalamic NPC.** ***A-B*** Under differentiating conditions, 24 h treatment of hypothalamic NPC with hippocampal ACM did not affect the percentage of the subpopulations of mature MAP-2$^+$/nestin$^-$ neurons (***A***) and MAP-2$^+$/nestin$^+$ neuroblasts (***B***) compared with standard differentiation medium (STD). ***C***, Conversely, hippocampal ACM was able to promote astroglial differentiation. ***D***, Hyt NPC showed a significant increase in the percentage of NG-2$^+$ cells upon treatment with hippocampal ACM. ***E***, No significant difference was observed in the apoptotic rate of cultures exposed to STD medium or hippocampal ACM. Data are expressed as mean values ± S.D. of $n = 3$ experiments, run in triplicates and analyzed by Student's *t*-test, * $p < 0.05$ and *** $p < 0.001$ versus STD medium.

*Similar effects of p50KO Hp and Hyt ACM on WT hippocampal NPC.*

We then evaluated if NF-κB p50 absence has different consequences on the soluble signals released by hippocampal and hypothalamic astrocytes. In order to do so, primary astrocyte cultures were set up also from both regions of p50KO animals, ACM were collected and then tested in parallel on Hippocampal NPC. As previously shown (see Cvijetic et al. under submission) Hp p50KO ACM promotes astrogliogenesis (Fig. 3 *B*) but not neurogenesis (Fig. 3 *A*). Very similar effects were observed with p50KO Hyt ACM. In detail: MAP-$2^+$/$nestin^-$ cells: 3.31 ± 0.43, 3.51 ± 0.82, 3.34 ± 0.67 % in presence of STD medium, Hp and Hyt p50KO ACM, respectively (Fig. 3 *A*); $GFAP^+$ cells: 13.16 ± 1.40, 36.00 ± 3.24, 30.38 ± 5.15 % in presence of STD medium, Hp and Hyt p50KO ACM, respectively (ANOVA, $p < 0.001$ vs STD; Fig. 3 *B*). When we checked for NG-$2^+$ oligodendroglial precursor cells, we did not observe any significant effect on NG-$2^+$ cells differentiation upon any treatment (NG-$2^+$ cells: 30.36 ± 0.98, 27.21 ± 5.21, 35.58 ± 4.28 % in presence of STD medium, Hp and Hyt p50KO ACM, respectively; Fig. 3 *C*). No difference in the percentage of apoptotic cells was reported in presence of the different media (mean % ± SD over total cells: 8.33 ± 0.54, 6.92 ± 1.18, 7.18 ± 1.52 % in presence of STD medium, Hp and Hyt p50KO ACM, respectively; Fig. 3 *D*). This subset of data suggests that absence of p50 subunit may not affect the astrogliogenic potential of both hippocampal and hypothalamic astrocytes on Hippocampal NPC.

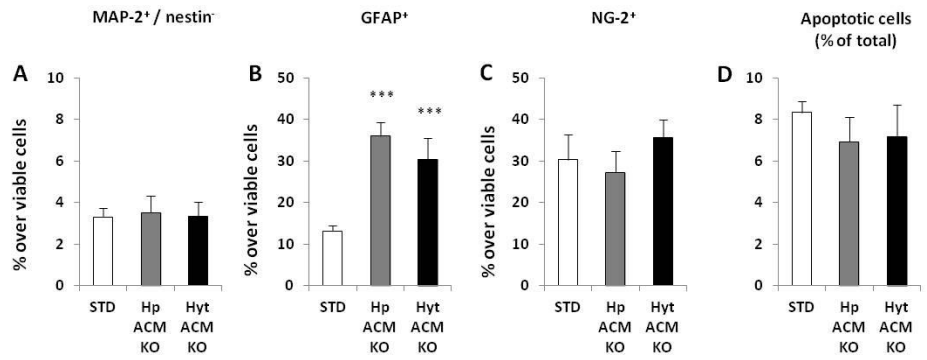


**Figure 3. No significant differences in the effects of p50KO Hippocampal and Hypothalamic ACM on WT hippocampal NPC.** ***A,*** Under differentiating conditions, 24h treatment of WT hippocampal NPC with Hp or Hyt ACM deriving from p50KO astrocytes does not induce neuronal differentiation compared with standard differentiation medium (STD). ***B,*** Conversely, both media are able to increase astroglial population of $GFAP^+$ cells. ***C***, No significant difference was observed in the percentage of NG-$2^+$ cells upon different media conditions. ***D***, No significant difference was observed in the apoptotic rate of cultures exposed to STD medium, Hp and Hyt ACM p50KO. Data are expressed as mean values ± S.D. of $n = 3$ experiments, run in triplicates and analyzed by one-way ANOVA followed by Tukey's post-hoc test. *** $p < 0.001$ versus STD medium.

We extended our analysis by testing the effect of Hp p50KO ACM on WT hypothalamic NPC. Also in this experiment, we confirmed that hippocampal p50KO ACM (like WT hippocampal ACM on hypothalamic NPC) could not produce proneurogenic effects compared to STD condition. In detail the percentages of newly generated populations were as follows: MAP-$2^+$/$nestin^-$ cells, 4.61 ± 0.54, 3.99 ± 0.57 % in presence of STD medium and Hp p50KO ACM, respectively (Fig. 4 *A*)*;* MAP-$2^+$/$nestin^+$ cells: 35.27 ± 4.15, 33.57 ± 3.83 % in presence of STD medium and Hp p50KO ACM, respectively (Fig. 4 *B*); $GFAP^+$ cells: 29.12 ± 6.03, 47.35 ± 1.95 % in presence of STD medium and Hp p50KO ACM, respectively (Student's *t*-test, *** $p < 0.001$ vs STD; Fig. 4 *C*). Interestingly, Hp p50KO, unlike Hp WT ACM, was devoid of effect on the NG-$2^+$ population (NG-$2^+$ cells: 33.12 ± 3.50, 37.11 ± 5.13 % in presence of STD

medium and Hp p50KO ACM, respectively; Fig. 4 *D*). No difference was also present in the apoptotic rate in presence of the different media conditions (mean % ± SD over total cells: 7.54 ± 1.20, 6.32 ± 1.53 % in presence of STD medium and Hp p50KO ACM, respectively; Fig. 4 *E*). These very preliminary data suggest that the absence of p50 may affect astrocyte secretion of soluble molecules which may promote an increase in the NG-2$^{+}$ population originating from Hyt NPC.

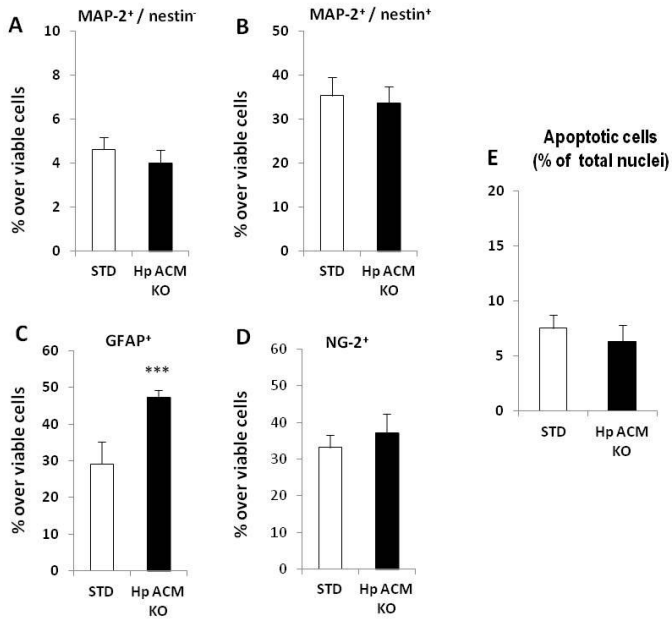


**Figure 4. Hypothalamic WT NPC, in presence of Hippocampal ACM originating from p50KO astrocytes, differentiate only toward the astroglial lineage.** ***A-B*** Under differentiating conditions, 24 h treatment of WT hypothalamic NPC with Hp ACM deriving from p50KO astrocytes does not induce neuronal differentiation compared with standard differentiation medium (STD). ***C,*** Conversely, Hp p50KO ACM is still able to increase astroglial population of GFAP$^{+}$ cells. ***D***, No significant differences was observed in the percentage of NG-2$^{+}$ cells upon Hp p50KO ACM treatment. ***E***, No significant difference was observed in the apoptotic rate of Hyt NPC cultures exposed to STD medium versus Hp p50KO ACM. Data are expressed as mean values ± S.D. of $n = 3$ experiments, run in triplicates and analyzed by Student's *t*-test *** $p < 0.001$ versus STD medium.

## References

Barkho BZ, Song H, Aimone JB, Smrt RD, Kuwabara T, Nakashima K, Gage FH, Zhao X (2006) Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. Stem Cells and Development 15(3), 407–21.

Bond, A. M., Ming, G.-L., & Song, H. (2015). Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. Cell Stem Cell, 17(4), 385–95.

Cuccurazzu, B., Bortolotto, V., Valente, M. M., Ubezio, F., Koverech, A., Canonico, P. L., & Grilli, M. (2013). Upregulation of mGlu2 receptors via NF-κB p65 acetylation is involved in the Proneurogenic and antidepressant effects of acetyl-L-carnitine. Neuropsychopharmacology, 38(11), 2220–30.

Denis-Donini, S., Caprini, A., Frassoni, C., & Grilli, M. (2005). Members of the NF-kappaB family expressed in zones of active neurogenesis in the postnatal and adult mouse brain. Brain Research. Developmental Brain Research, 154(1), 81–9.

Denis-Donini S, Dellarole A, Crociara P, Francese MT, Bortolotto V, Quadrato G, Canonico PL, Orsetti M, Ghi P, Memo M, Bonini SA, Ferrari-Toninelli G, Grilli M (2008) Impaired adult neurogenesis associated with short-term memory defects in NF-kappaB p50-deficient mice. The Journal of Neuroscience, 28(15), 3911–9.

Kokoeva, M. V, Yin, H., & Flier, J. S. (2005). Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science 310(5748), 679–83.

Lin, R., & Iacovitti, L. (2015). Classic and novel stem cell niches in brain homeostasis and repair. Brain Research, 1–16.

Meneghini, V., Francese, M. T., Carraro, L., & Grilli, M. (2010). A novel role for the Receptor for Advanced Glycation End-products in neural progenitor cells derived from adult SubVentricular Zone. Molecular and Cellular Neurosciences, 45(2), 139–50.

Ming, G., & Song, H. (2005). Adult neurogenesis in the mammalian central nervous system. Annual Review of Neuroscience, 28, 223–50.

Nicola, Z., Fabel, K., & Kempermann, G. (2015). Development of the adult neurogenic niche in the hippocampus of mice. Frontiers in Neuroanatomy, 9, 53.

Nishiyama, A., Komitova, M., Suzuki, R., & Zhu, X. (2009). Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. Nature Reviews. Neuroscience, 10(1), 9–22.

Pierce, A. A., & Xu, A. W. (2010). De novo neurogenesis in adult hypothalamus as a compensatory mechanism to regulate energy balance. The Journal of Neuroscience 30(2), 723–30.

Robins, S. C., Trudel, E., Rotondi, O., Liu, X., Djogo, T., Kryzskaya, D., Bourque CW. Kokoeva, M. V. (2013). Evidence for NG2-glia derived, adult-born functional neurons in the hypothalamus. PloS One, 8(10), e78236.

Song, H., Stevens, C. F., & Gage, F. H. (2002). Astroglia induce neurogenesis from adult neural stem cells. Nature, 417(6884), 39–44.

Valente MM, Bortolotto V, Cuccurazzu B, Ubezio F, Meneghini V, Francese, MT, Canonico, PL, Grilli M (2012) α2δ Ligands Act as Positive Modulators of Adult Hippocampal Neurogenesis and Prevent Depressive-like Behavior Induced by Chronic Restraint Stress. Molecular Pharmacology 82(2):271-80.

Whalley, K. (2012). Neurogenesis: Hypothalamic neurogenesis regulates weight gain. Nature Reviews Neuroscience, 13(5), 289.

# Chapter 5.

## Discussion

Scientific background

Adult neurogenesis is a plastic and dynamic process that persists throghout post-natal life in mammalian species, including humans. Active sites of adult neurogenesis are the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Ming and Song, 2005; Bond and Song, 2015). Growing evidence suggests the presence of another neurogenic area in the hypothalamus, along the walls of the third ventricle (Kokoeva et al., 2005). The process of adult neurogenesis is supported by a pool of neural stem/progenitor cells (NSPC), which reside in a permissive microenvironment called "neurogenic niche", where the different cellular types and molecular components provide a supportive milieu that balance NSPC quiescence along with proliferation and differentiation and survival (Lin & Iacovitti, 2015).

Astrocytes represent one major contributor within the hippocampal neurogenic niche. Astrocytes isolated from neurogenic and non-neurogenic brain areas have been reported to exhibit different effects on the neuronal differentiation of NSPC suggesting that functional heterogeneity of astrocytes may reflect the different brain region environment (Song et al. 2002; Barkho et al. 2006).

In previous publications our lab proved that members of the NF-κB family of transcription factors are important contributors of signaling pathways in the SGZ neurogenic niche (Denis-Donini et al., 2005). Within the NF-κB family, the p50 subunit appears to act as a crucial regulator of adult hippocampal neural progenitor cells (ahNPC). Specifically focusing on NF-κB p50 subunit, our group has demonstrated that NF-κB is involved in mediating the proneurogenic and antidepressive-like effect of some clinically relevant drugs (i.e. α2δ ligands; acetyl-L-carnitine) (Cuccurazzu et al., 2013; Valente et al., 2012), in the proneurogenic effects of damage-associated signals (i.e. HMGB-1 and $A\beta_{1-2}$) (Meneghini et al., 2013), and that the absence of p50 affects the *in vitro*

response to those drugs/signals. *In vivo* our group also proved that p50KO mice display a dramatically reduced adult hippocampal neurogenesis which is in association with hippocampal-dependent short-term memory defects (Denis-Donini et al., 2008).

Working hypothesis

The major project for the PhD thesis developed from the observation that unlike *in vivo*, hippocampal NPC derived from WT and p50KO adult mice have similar rate of differentiation, proliferation and apoptosis *in vitro*. We hypothesized that deficient signaling within the niche and/or intrinsic defects of NPC, might contribute to the reduced neurogenesis observed *in vivo* in p50KO mice. In particular, we focused on astrocytes as one of the relevant components in niche signaling and well-studied modulators in neurogenesis. We decided to explore ahNPC-astrocyte cross-talk *in vitro* in order to further dissect the contribution of non-cell autonomous signaling and cell autonomous defects, played by NF-κB p50, in fate specification.

Principal findings of the thesis work

Based on these observations, we exploited the advantages of using primary cell culture systems to investigate the contribution made by astrocyte conditioned media (ACM), under basal conditions, on adult NPC fate specification. The most relevant finding concerning WT NPC cultures, was that neuronal fate determination is mediated only by WT ACM treatment, whereas p50KO ACM has no proneurogenic activity. On the contrary, both WT and p50KO ACM display astrogliogenic activity. The effect we observe in our experimental conditions are merely differentiative since ACM had no influence on the survival of NPC and their progeny. Interestingly, when we exposed p50KO NPC to WT and p50KO ACM, we did not observe any difference in lineage specification compared to STD condition, suggesting that also cell autonomous defects may affect the responsivity to proneurogenic and astrogliogenic signals secreted from the astrocytes. Previously our group demonstrated that α2δ1, a

thrombospondin-1 (TSP-1) receptor (Eroglu et al., 2009), is functionally expressed by ahNPC (Valente et al., 2012). During this PhD thesis we proved that α2δ1/TSP-1 receptor expression is downregulated in p50KO NPC. When we tested thrombospondin-1, a well-known proneurogenic glycoprotein of the extracellular matrix released by astrocytes (Lu & Kipnis, 2010) we observed a defective response to the proneurogenic molecule only in p50KO NPC and not in WT NPC. Overall these data suggest that absence of p50 may also reduce ahNPC responsiveness to TSP-1, an astrocyte-derived signal.

A step forward in this study was done by analyzing the secretory profile of WT and p50KO astroglial cultures. By analyzing WT and p50KO ACM, we wanted (i) to explore if there were proteins differentially modulated under the control of NF-κB p50, (ii) to identify, among the detected proteins, potential proneurogenic or antineurogenic molecules, to be further validated in our *in vitro* system. Through LC-MS/MS proteomic analysis of ACM, we identified four proteins that were upregulated in p50KO compared to WT ACM: neutrophil gelatinase-associated lipocalin-2 (NGAL/LCN-2), C-C motif chemochine-2 (CCL-2), H-2 class I Histocompatibility antigen, K-K alpha chain (HA1K) and retinoic acid receptor responder 2 (RARRES2, chemerin).

As a starting point, we focused our attention on Lipocalin-2 (LCN-2). No information is currently available on its role on adult neurogenesis. LCN-2 is mainly known as an acute phase protein secreted from activated microglia, reactive astrocytes and neurons. In the CNS, LCN-2 has been suggested to play a modulatory action in cognitive functions (Ferreira et al., 2013), neuronal excitability and anxiety (Mucha et al., 2011) and depression in human beings (Naudé et al., 2013). However, its precise role in the pathophysiology of the CNS remains to be outlined. We discovered for the first time that LCN-2 is able to elicit neuronal differentiation of WT ahNPC in a concentration-dependent fashion. Conversely, LCN-2 protein was found less active on p50KO ahNPC. These results, suggest for the first time that LCN-2 is an astroglial-derived signal that may promote neuronal differentiation of ahNPC and the lack of p50 results in a reduced response to its proneurogenic activity.

As describerd in the unpublished section of this thesis, we also collected some preliminary data on the cross-talk between ahNPC and ACM originating from the hypothalamic area. The hypothalamic region has been discovered recently as a new neurogenic site, where neurogenesis might be regulated by growth factors, diet and hormones, given the involvement of this region in homeostatic and physiological processes (Kokoeva et al., 2005; Lee et al., 2014). As a follow up of the experiments performed before, we compared the proneurogenic and progliogenic properties of hippocampal and hypothalamic ACM deriving from WT astrocytes on adult hippocampal WT NPC fate specification. Additionally we assessed the contribution in NPC cell fate determination of hippocampal and hypothalamic astrocytes cultures deriving from NF-κB p50KO mice.

Our finding reports that hippocampal but not hypothalamic ACM can promote neuronal differentiation of hippocampal NPC, confirming that astrocytes may provide region-specific signals for the neurogenic process. This subset of data suggested us also that the astrogliogenic signals are still retained in absence of p50 subunit and independently of the origin of the astrocytes. We further explored the lineage fate determination of adult hypothalamic WT NPC in basal conditions and in response to hippocampal WT and p50KO ACM. We found out that hypothalamic WT NPC do not respond to proneurogenic soluble signals coming from WT hippocampal astrocytes. Conversely, hypothalamic NPC in presence of both WT and p50KO hippocampal ACM only differentiate toward astroglial lineage. Furthermore, we observed for the first time a pro-differentiative effect of WT hippocampal ACM on oligodendroglial NG-2 cell population of WT hypothalamic NPC, and this effect appeared p50-dependent.

## Conclusions and future perspectives

This work supports the concept that non-cell autonomous defects in astrocyte signaling might be relevant for the neurogenic process *in vitro* and, presumably, *in vivo*. We suggest that the absence of NF-κB p50 subunit in astrocytes is a critical aspect that negatively influence the proneurogenic activity of cortico-hippocampal and hippocampal ACM.

On the other hand we comfirm that NPC intrinsic defects might be at the basis of the deregulated neurogenesis observed in p50KO mice, since hippocampal NPC have downregulated α2δ1 expression levels. Additionally we can confirm the importance of NF-κB p50 in the regulation of proteins secreted by astrocytes, including new proneurogenic molecules such as LCN-2.

We also confirm the concept of region-specific regulation of ahNPC by molecular cues arising from the same neurogenic region. The field of region-specific proneurogenic potential deserves further investigation. Moreover, it would be interesting to assess the responsiveness of hypothalamic NPC to proneurogenic moleclules relevant in hippocampal NPC neuronal differentiation (i.e. TSP-1, LCN-2).

Since LCN-2 was identified as an astrocyte-derived factor that exerts proneurogenic activity in WT NPC and to a lesser extent in p50KO NPC, we would like to further explore lipocalin-2 receptor expression, BOCT1/24p3R and megalin, in hippocampal WT and p50KO NPC.

Potential future work could also be aimed at understanding the involvement in neuronal fate determination of ahNPC for other proteins identified in astrocyte secretome, for example CCL-2. There are many reports claiming both negative and positive modulatory effects of the chemokine CCL-2 in neuronal differentiation of ahNPC. On one hand increased systemic levels of CCL-2 observed during aging have been associated with decreased neurogenesis and age-related cognitive impairment (Villeda et al., 2011), on the other hand other results indicate that CCL-2 can influence both the migration, survival and fate choice of SVZ-derived neural precursor cells (Gordon et al., 2012). Therefore,

it could be interesting to evaluate WT and p50KO NPC responsiveness to this chemokine.

Another potential field of future investigation is that of intercellular communication via extracellular vesicles (EV), such as exosomes and shedding microvesicles, which are involved in neuronal protection, neuronal development, as well as neurodegeneration (Kalani et al., 2014; Rajendran et al. 2014). It could be of interest exploring the involvement of EV as a possible novel mechanism of glia-to-NPC transfer of molecules which may affect NPC fate determination. Interestingly, LCN-2 and TSP-1 have both been reported to be secreted in exosomes (Burke et al., 2014).

## References

Bond, A. M., Ming, G.-L., & Song, H. (2015). Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. Cell Stem Cell, 17(4), 385–95.

Bortolotto, V., Cuccurazzu, B., Canonico, P. L., & Grilli, M. (2014). NF-κB mediated regulation of adult hippocampal neurogenesis: relevance to mood disorders and antidepressant activity. BioMed Research International, 2014, 612798.

Burke, M., Choksawangkarn, W., Edwards, N., Ostrand-Rosenberg, S., & Fenselau, C. (2014). Exosomes from myeloid-derived suppressor cells carry biologically active proteins. Journal of Proteome Research, 13(2), 836–43.

Cuccurazzu, B., Bortolotto, V., Valente, M. M., Ubezio, F., Koverech, A., Canonico, P. L., & Grilli, M. (2013). Upregulation of mGlu2 receptors via NF-κB p65 acetylation is involved in the Proneurogenic and antidepressant effects of acetyl-L-carnitine. Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 38(11), 2220–30.

Denis-Donini, S., Caprini, A., Frassoni, C., & Grilli, M. (2005). Members of the NF-kappaB family expressed in zones of active neurogenesis in the postnatal and adult mouse brain. Brain Research. Developmental Brain Research, 154(1), 81–9.

Denis-Donini, S., Dellarole, A., Crociara, P., Francese, M. T., Bortolotto, V., Quadrato, G., Orsetti, M., Ghi, P., Memo, M., Bonini, S. A., Ferrari-Toninelli, G., Grilli, M. (2008). Impaired adult neurogenesis associated with short-term memory defects in NF-kappaB p50-deficient mice. Journal of Neuroscience, 28(15), 3911–9.

Eroglu, C., Allen, N. J., Susman, M. W., O'Rourke, N. A., Park, C. Y., Ozkan, E., Chakraborty, C., Mulinyawe, S. B., Annis, D. S., Huberman, A. D., Green, E. M., Lawler, J., Dolmetsch, R.Garcia, K. C., Smith, S. J., Luo, Z. D., Rosenthal, A., Mosher, D. F., Barres, B. A. (2009). Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell, 139(2), 380–92.

Ferreira, A. C., Pinto, V., Dá Mesquita, S., Novais, A., Sousa, J. C., Correia-Neves, M., Sousa, N., Palha, J. A., Marques, F. (2013). Lipocalin-2 is involved in emotional behaviors and cognitive function. Frontiers in Cellular Neuroscience, 7, 122.

Gordon, R. J., Mehrabi, N. F., Maucksch, C., & Connor, B. (2012). Chemokines influence the migration and fate of neural precursor cells from the young adult and middle-aged rat subventricular zone. Experimental Neurology, 233(1), 587–94.

Kalani, A., Tyagi, A., & Tyagi, N. (2014). Exosomes: mediators of neurodegeneration, neuroprotection and therapeutics. Molecular Neurobiology, 49(1), 590–600.

Kokoeva, M. V, Yin, H., & Flier, J. S. (2005). Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science (New York, N.Y.), 310(5748), 679–83.

Lee, D. A., Yoo, S., Pak, T., Salvatierra, J., Velarde, E., Aja, S., & Blackshaw, S. (2014). Dietary and sex-specific factors regulate hypothalamic neurogenesis in young adult mice. Frontiers in Neuroscience, 8, 157.

Lin, R., & Iacovitti, L. (2015). Classic and novel stem cell niches in brain homeostasis and repair. Brain Research, 1–16.

Meneghini, V., Bortolotto, V., Francese, M. T., Dellarole, A., Carraro, L., Terzieva, S., & Grilli, M. (2013). High-mobility group box-1 protein and β-amyloid oligomers promote neuronal differentiation of adult hippocampal neural progenitors via receptor for advanced glycation end products/nuclear factor-κB axis: relevance for Alzheimer's disease. Journal of Neuroscience, 33(14), 6047–59.

Ming, G., & Song, H. (2005). Adult neurogenesis in the mammalian central nervous system. Annual Review of Neuroscience, 28, 223–50.

Mucha, M., Skrzypiec, A. E., Schiavon, E., Attwood, B. K., Kucerova, E., & Pawlak, R. (2011). Lipocalin-2 controls neuronal excitability and anxiety by regulating dendritic spine formation and maturation. Proceedings of the National Academy of Sciences of the United States of America, 108(45), 18436–41.

Naudé, P. J. W., Eisel, U. L. M., Comijs, H. C., Groenewold, N. A., De Deyn, P. P., Bosker, F. J., Luiten, P. G. M., den Boer, J. A., Oude Voshaar, R. C. (2013). Neutrophil gelatinase-associated lipocalin: a novel inflammatory marker associated with late-life depression. Journal of Psychosomatic Research, 75(5), 444–50.

Valente, M. M., Bortolotto, V., Cuccurazzu, B., Ubezio, F., Meneghini, V., Francese, M. T., Canonico P.L. Grilli, M. (2012). α2δ Ligands Act as Positive Modulators of Adult Hippocampal Neurogenesis and Prevent Depressive-like Behavior Induced by Chronic Restraint Stress. Molecular Pharmacology. 82(2):271-80.

Villeda, S. A., Luo, J., Mosher, K. I., Zou, B., Britschgi, M., Bieri, G., Stan, T. M., Fainberg, N., Ding, Z., Eggel, A., Lucin, K. M., Czirr, E., Park, J-S., Couillard-Després, S., Aigner, L., Li, G., Peskind, E. R., Kaye, J. A., Quinn, J. F., Galasko, D. R., Xie, X. S., Rando, T. A., Wyss-Coray, T. (2011). The ageing systemic milieu negatively regulates neurogenesis and cognitive function. Nature, 477(7362), 90–4.

# Chapter 6.

**Manuscript under submission to Journal of Neuroscience**

**Astrocyte-mediated neuronal fate specification of adult hippocampal neural progenitors is regulated by NF-κB p50.**

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**Poster sessions**

*NF-κB–mediated regulation of astrocyte-secreted signals affects the neurogenic potential of adult hippocampal neural progenitors.* V. Bortolotto, **S. Cvijetic**, M. Manfredi, E. Ranzato, E. Marengo, P.L. Canonico, M. Grilli. (45th annual meeting of the Society for Neuroscience, Chicago, USA, October 17-21, 2015)

*NF-κB-mediated regulation of astrocyte-secreted signals modulating the differentiation potential of adult hippocampal neural progenitor cells.* **S. Cvijetic**, V. Bortolotto, E. Marengo, M. Manfredi, E. Ranzato, P.L. Canonico, M. Grilli. (37° Congresso Nazionale della Società Italiana di Farmacologia "I Nuovi Orizzonti Della Ricerca Farmacologica: Tra Etica E Scienza" Napoli, October 27-30, 2015)

*Involvement of the NF-κB p50 subunit in adult Neural Progenitor Cell-astroglia cross-talk.* **S. Cvijetic**, V. Bortolotto, S. Lovecchio, R. Salem, P.L. Canonico, M. Grilli. (Abcam event: Adult Neurogenesis: Evolution, Regulation and Function, Dresden, Germany, May 6-8, 2015)

*Potential role of NF-κB p50 in astrocyte-neural progenitor cell communication within the neurogenic niche.* V. Bortolotto, **S. Cvijetic**, S. Lovecchio, P.L. Canonico, M. Grilli. (44th Annual meeting of the Society for Neuroscience, Washington DC, USA, November 2014)

*Neural progenitor cell-astroglia cross-talk: involvement of NF-κB p50 subunit and extracellular vesicles.* **S. Cvijetic**, V. Bortolotto, S. Lovecchio, P.L. Canonico, M. Grilli. (17° Seminario SIF Dottorandi, Assegnisti di Ricerca, Postdottorandi e Specializzandi, Rimini, September 16-18, 2014)

*Role of NF-κB p50 in microglia-neural progenitor cell cross-talk.***S. Cvijetic**, V. Bortolotto, S. Lovecchio, P.L. Canonico, M. Grilli. (9th FENS Forum of Neuroscience, Milan, July 5-9, 2014)

*NF-κB p50 subunit contribution to microglia-neural progenitor cells cross-talk: potential relevance in adult neurogenesis deregulation.* **S. Cvijetic**, V. Bortolotto, S. Lovecchio, P.L. Canonico, M. Grilli *(*Monotematico SIF: Mood disorders: from neurobiology to novel therapeutic strategies, Modena, March 20-21, 2014)

## Awards

Premio miglior comunicazione orale al 17° Seminario SIF - Dottorandi, Assegnisti di Ricerca, Postdottorandi e Specializzandi (Rimini, September 16-18, 2014). Titolo del lavoro premiato: Neural progenitor cell-astroglia cross-talk: involvement of NF-κB p50 subunit and extracellular vesicles. **S. Cvijetic**, V. Bortolotto, S. Lovecchio, P.L. Canonico, M. Grilli

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