Università degli Studi del Piemonte Orientale

"Amedeo Avogadro"

Department of Pharmaceutical Sciences

Ph.D in Chemistry&Biology

XXIX cycle a.y. 2013-2016

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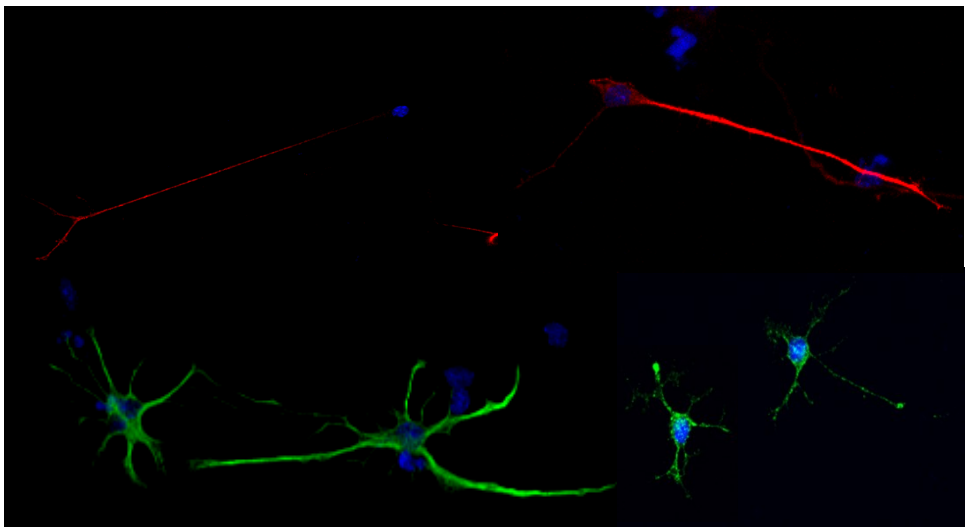
Drug Discovery and Development



Rita Salem

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Supervised by Prof. Mariagrazia Grilli

PhD program coordinator Prof. Domenico Osella

« Il faut savoir s'instruire dans la gaieté.

Le savoir triste est un savoir mort. L'intelligence est joie. »

"Learning should be a cheerful process.

Sad knowledge is dead knowledge. Intelligence is joy."

De Voltaire

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List of Abbreviations

5-HT 5-HydroxyTriptamine

ACh Acetylcholine

ahNPC adult hippocampal Neural Progenitor Cells

ahNSC adult hippocampal Neural Stem Cells

AR Adrenergic receptors

BDNF Brain Derived Neurotrophic Factor

BrDU BromoDioxyUridine

CA3 Cornus Ammonis - 3

CNS Central Nervous System

DA Dopamine

DAT Dopamine Transporter

DCX DoubleCourtin

DDC aromatic amino acid decarboxylase

DG Dentate Gyrus

EE Enriched Environnement

FST Forced Swim Test

GABA Gamma-AminoButyric Acid

GCL Granular Cell Layer

GF Growth Factors

GFAP Glial-Fibrillary Acidic Protein

IGF-1 Insulin-like Growth Factor

IR Immediate Release

LTP Long Term Potentiation

m-CPP meta-ChloroPhenylPiperazine

MDD Major Depressive Disorder

MWM Morris Water Maze

NE NorEpinephrine

NET Noradrenaline Transporter

NeuN Neuronal Nuclear antigen

OB Olfactory Bulb

PCPA DL-P-Chlorophenylalanine

PFC PreFrontal Cortex

PSA-NCAM Polysialylated-Neural Cell Adhesion Molecule

RN Raphe Nucleus

SGZ Subgranular Zone

SOX-2 Sry-related HMG box transcription factor

SVZ Subventricular Zone

TPH Tryptophan Hydroxylase

TST Tail Suspension Test

CMS Chronic Mild Stress

VEGF Vascular Endothelial Growth Factor

VTA VentroTegmental Area

SSRI Selective Serotonin Reuptake Inhibitors

SNRI Serotonin and Noradrenaline Reuptake Inhibitors

SARI Serotonin Antagonist and Reuptake Inhibitor

TCA TriCyclic Antidepressants

MAOI MonoAmine Oxidase inhibitors

Chapter I

INTRODUCTION

1. Adult neurogenesis

For long time scientists assumed that neurogenesis was restricted to embryonic and early postnatal development period in our central nervous system (CNS). Since two decades several key findings - such as the remodeling of vocal centers in singing birds each year through new born neurons and the isolation and culture of neural cells with undifferentiated cells properties - consolidated the discovery of newly generated and integrated neurons during adult life.

1. Adult neurogenesis in human and rodent brain

Adult neurogenesis is a particular form of neural plasticity described in many brain regions of multiple species such as birds, reptiles, fish and mammalian brain, including human. In adult mammalian brain the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle are the two major niches of neural stem cells where a basal level of neurogenesis is described (Kempermann and Gage 1999, Kempermann et al., 2003, Zhao et al., 2008). In rodent brain thousands of neural progenitors are produced on a daily basis in the SVZ and migrate along the rostral migratory stream (RMS) towards the olfactory bulb (OB) where they differentiate into local interneurons (Malvaut et al., 2016). In the SGZ newly born neurons are integrated in the DG and play a physiological role in hippocampal function (Zhao et al., 2008). Retrospective birth dating by ^{14}C incorporation in CNS neurons showed that one third of human hippocampal neurons are subjected for turn over during life time. The extent of hippocampal neurogenesis between middle aged human and middle aged mice was comparable (Spalding et al., 2013). The importance of adult neurogenesis is different between rodents and human. While it is pronounced in both DG and OB of rodent brain, only DG neurogenesis appears to be substantial in human brain (Bergmann et al., 2012, Spalding et al., 2013). A considerable level of newly generated neurons was also described in other brain regions such as

neocortex (Gould et al., 2007) hypothalamus (Kokoeva et al., 2005) and human striatum (Ernst et al., 2014). However, the role of adult neurogenesis outside the SGZ and SVZ remains to be fully described.

2. Hippocampal neurogenic niche and neurogenesis process

Adult CNS "stem cells" exhibit three prime features: (i) they are self-renewing, with the ability to regenerate progeny identical to themselves, (ii) proliferative, undergoing mitosis with long cell cycle and (iii) multipotent, able to generate neuronal and glial lineages cells. Adult hippocampal Neural Stem Cells (ahNSC) reside in a unique microenvironment called neurogenic niche confined within the SGZ hilus region. The neurogenic niche is a complex zone composed of NSC surrounded by ependymal cells, astroglial cells, vessels and mature neurons (Fig. 1). Diffusible molecule and cell-cell contacts within the niche and from outside can influence NSC proliferation, differentiation and survival (Alvarez-Buylla et al., 2004, Quinones-Hinojosa at al., 2006). The generation of new neurons is a complex multistep process (Fig. 2) that involves a series of developmental phases before the complete maturation and functional integration into neuronal networks (Fig. 2, A). Cellular maturation phases could be identified by morphological characteristics as well as the expression of specific molecular markers (Fig. 2, B). In the SGZ, dormant radial glial-like cells have processes spanning the granule layer and the inner molecular layer of the hippocampus. They express glial fibrillary acidic protein (GFAP), Sry-related HMG box transcription factor (Sox2), nestin (nerve cells intermediate filament) ($GFAP^+$, $Sox2^+$, $nestin^+$, $S100\beta^-$) and are negative for the astroglial marker S100β (Filipov et al., 2003). They are usually referred as type 1 cells or NSC. By asymmetric division, a NSC gives rise to an identical progeny that remains in quiescent state and to a transiently amplifying neural progenitor cell (NPC).

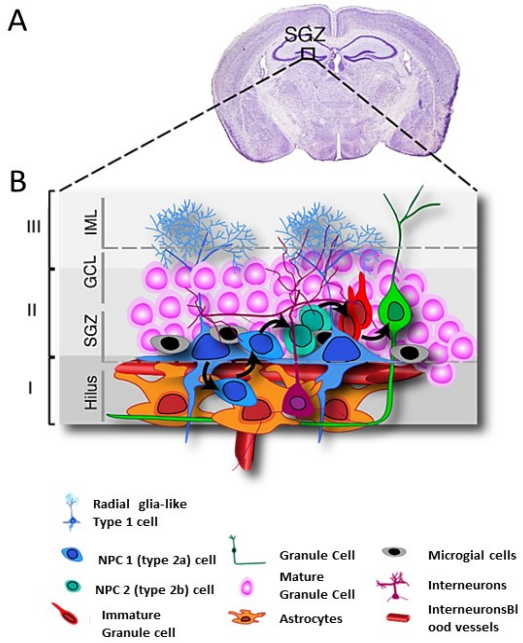


Figure 1. Schematic representation of the cytoarchitecture in the hippocampal neurogenic niche. A. Coronal section of adult mouse brain showing the localization of the subgarnular zone (SGZ) in the hippocampus. **B**. Cell population in the dentate gyrus (DG) of the mammalian brain. GCL: Granular cell Layer; IML: Inner Molecular Layer. *(Modified from Donegà et al. 2013)*

NPCs are non-radial cells with short processes positioned tangentially to the granular layer. They are GFAP^{-}, S100β^{-}, Sox2^{+}, Nestin^{+} highly proliferative and referred to as type 2 cells (Kronenberg et al., 2003). NPCs generate neuronal progenitors expressing doublecortin (DCX) and polysialylated-neural cell adhesion molecule (PSA-NCAM) named neuroblasts type 3 cells, and a population of glial progenitors GFAP^{+}, S100β^{+} (Ma et al., 2009, Bonaguidi et al., 2012). Immature newborn neurons (DCX^{+}) migrate a small distance in the granule cell layer (GCL) of the DG and start to receive synaptic inputs from existing neuronal circuits. The surviving cells continue their maturation and have more complex apical dendrites connecting the enthorinal cortex and hippocampal cornu ammonis (CA3) subfield. Mature neurons express neuronal nuclear antigen (NeuN) and Calbindin (Fig. 2 B). It takes roughly 1-2 months for newly generated neurons to reach morphological maturity and functionally integrate in neuronal networks (Fig. 2) (Lucassen et al., 2010).

In the early 1990s several laboratories concomitantly showed that NSC could be isolated from adult brain and expanded *in vitro* in presence of growth factors. Isolated stem cells in presence of epidermal growth factors (EGF) formed spheres after days in proliferation and expressed nestin (Colucci-D'Amato et al., 2006; Reynolds and Weiss, 1992). The *in vitro* model of stem cells deriving from brain tissue and specifically from the hippocampus was validated and well characterized over the years in our and other research groups. It is used as a tool to identify molecular events underlying adult hippocampal neurogenesis and its possible modulators.

3. Adult hippocampal neurogenesis modulators

Proliferation of ahNPC, complete maturation, integration into neuronal circuits and spines development of newly generated neurons are plastic processes strongly regulated by neuronal network activity, cell-cell interactions, endogenous and exogenous modulators (Fig. 3).

1. Network activity and neurotransmitters

Enriched environment (EE) and voluntary exercise increase the DG network activity and result in Gamma-Aminobutyric acid (GABA) spill over from local GABAergic synapses into the neurogenic niche. $Nestin^+$ quiescent NSCs in the SGZ respond to spilled GABA through $GABA_A$ receptors containing the γ_2 subunit. GABA halts the proliferation and favors the differentiation and maturation of new neurons (Dranovsky et al., 2011). One peculiarity of newborn DCX^+ young neurons is having high chloride levels due to high expression of chloride importer (NKCC1) that are lost with maturation and replaced by chloride transporter (KCC2).

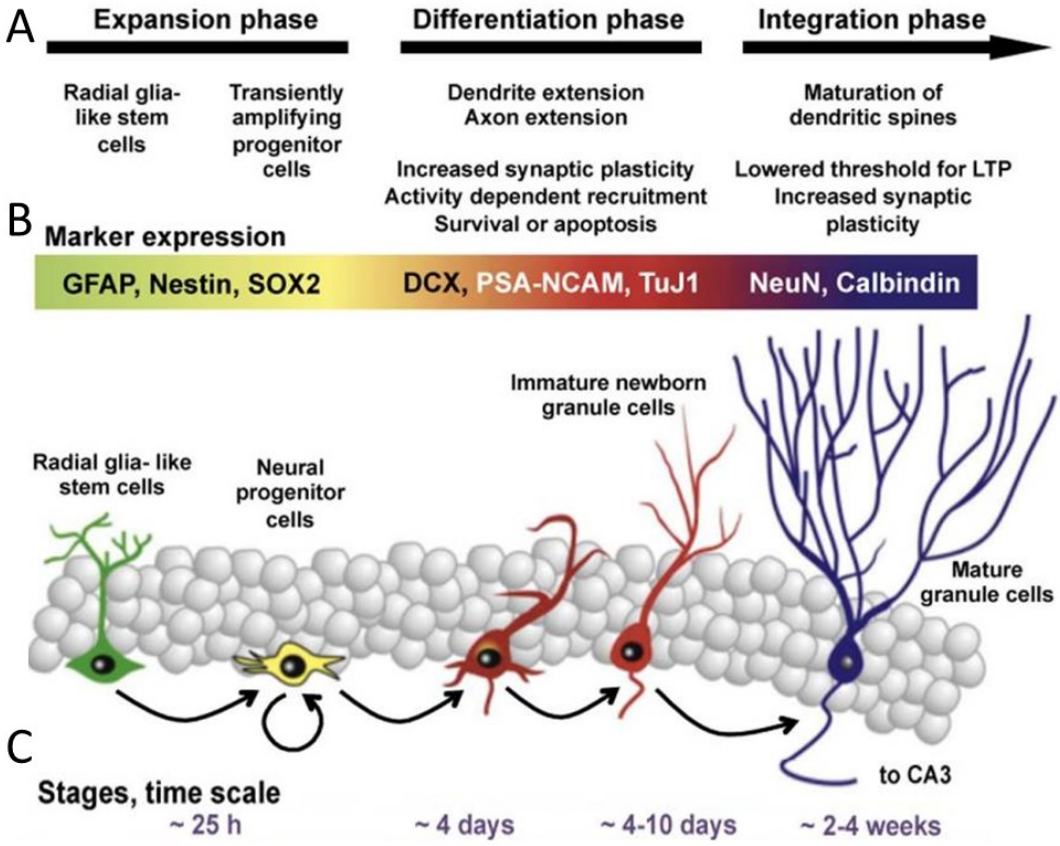


Figure 2. **Representative scheme for the phases and hallmarks of adult hippocampal neurogenesis. A.** Summary for the different phases of hippocampal neurogenesis. **1**. Expansion by activation of radial glia-like cells and proliferation of intermediate adult hippocampal neural progenitor cells (ahNPC). **2.** Differentiation toward immature neurons (neuroblasts). **3.** Integration into the dentate gyrus networks (Cornu ammonis, CA3) and maturation of young granule neurons. **B.** Different markers are expressed by ahNPC at different phases of maturation. Immature cells markers (GFAP, Nestin, and Sox-2); neuroblasts markers (DCX, PSA-NCAM and Tuj-1); mature neurons markers (NeuN and Calbindin). **C.** Time scale of the different steps of maturation. (*Modified from Schouten et al., 2012).*

EE increases the level of stimulatory neurotransmitter glutamate in the synapses. Newly generated neurons express NMDA glutamate receptors with NR2B subunit. This particular feature facilitates long term potentiation (LTP) induced by glutamate that enhances the maturation, integration and survival of new neurons (Ge et al, 2007). In summary, intrinsic cell properties and network activity dictate the quality and/or the quantity of the proliferation of ahNPC and the maturation of young neurons.

Major CNS neurotransmitters such as acetylcholine (ACh), dopamine (DA), norepinephrine (NE) and serotonin (5-HT) modulate neurogenesis. The cholinergic system has a substantial role in the hippocampal functions and is one of the major candidates suggested to influence adult neurogenesis. Indeed the ablation of basal forebrain in adult rats (cholinergic center) decreased the number of double labeled cells for 5-bromo-2-deoxyuridine BrdU (DNA intercalant during proliferation) and NeuN (mature neurons) in the SGZ (Cooper-Kuhn et al., 2004). Dopaminergic axons originate from ventral tegmental area (VTA) and the adjacent substantia nigra to innervate the DG (Mu et al., 2011). The hallmark of Parkinson's disease is cerebral dopamine depletion. Indirect studies on the brain of patients or rodent models of Parkinson's disease were used to study the effect of dopamine on adult neurogenesis. The proliferation of ahNPC is reduced in the SGZ in dopamine depleted brains (Höglinger et al., 2004). Noradrenergic innervation of the DG originates from the locus coeruleus. Lesions of NE projecting fibers decreased neurogenesis considerably (Kulkarni et al., 2002). Intra-hippocampal injection of NE and β3-adrenergic receptor (β3-AR) agonist increased the proliferation and the number of nestin^{+}/GFAP^{+} neural precursors (Jhaveri et al., 2010). A more recent study suggested that NE increase ahNPC proliferation through β2-AR (Masuda et al., 2012). Serotonin is synthesized in the brain stem raphe nuclei and serotonergic fibers project into the DG of the hippocampus where they connect with granule

cells and interneurons (Alenina and Klempin, 2015). The role of serotonin in adult hippocampal neurogenesis will be detailed in a separate paragraph (II.3).

2. Astrocytes

With regard to the importance of astrocytes in the CNS, Nedergaard and Goldman stated that "astrocytes might serve neurons not so much as servants but as parents … astrocytes tell neurons what to do beside just cleaning up their mess" (Nedergaard et al., 2003). Astrocytes are the most abundant cell population in the CNS and the major components of the neurogenic niche (Song et al., 2002). Traditionally astrocytes were assigned only a supportive and trophic role in all brain functions (Pixley, 1992). After years of research now astrocytes are acknowledged aside from the trophic role also for their major contribution to embryonic and adult neurogenesis (Gomes et al., 2001). Specifically, astrocytes through the release of diffusible signals and also direct contact to neurons play a role in neuronal differentiation, synapse formation, axonal guidance and electrophysiological properties regulation (Pfrieger and Barres 1997; Lim and Alvarez-Buylla 1999; Song et al. 2002). In particular, hippocampal astrocytes are the energy providers during memory formation. The lactate hydrogenase provided by astrocytes sustains the long term potentiation (LTP) and memory retention in the hippocampus (Suzuki et al. 2011). Astrocytes have intercellular crosstalk between themselves, neurons, microglia cells and endothelial cells. Importantly, astrocytes communicate bidirectionally with neurons by the tripartite synapse, a structure where astrocytes are associated with presynaptic and postsynaptic neurons membrane. The interaction is physical and functional since astrocytes function is dependent on neuronal activity (reviewed in Pekny et al., 2016). Astrocytes employ several molecular mechanisms such as exocytosis, diffusion through cellular membrane and membrane transporters to release numerous neurotransmitters and trophic factors. These elements are signals to neurons and microglial cells that regulate the synaptic and neuronal activity (Newman, 2003). For example,

thrombospondin is an extracellular glycoprotein secreted by astrocytes and specifically upregulated after an ischemic injury. Astrocytes through thrombospondin control the post-ischemic angiogenesis and synaptic reformation (Liauw et al., 2008).

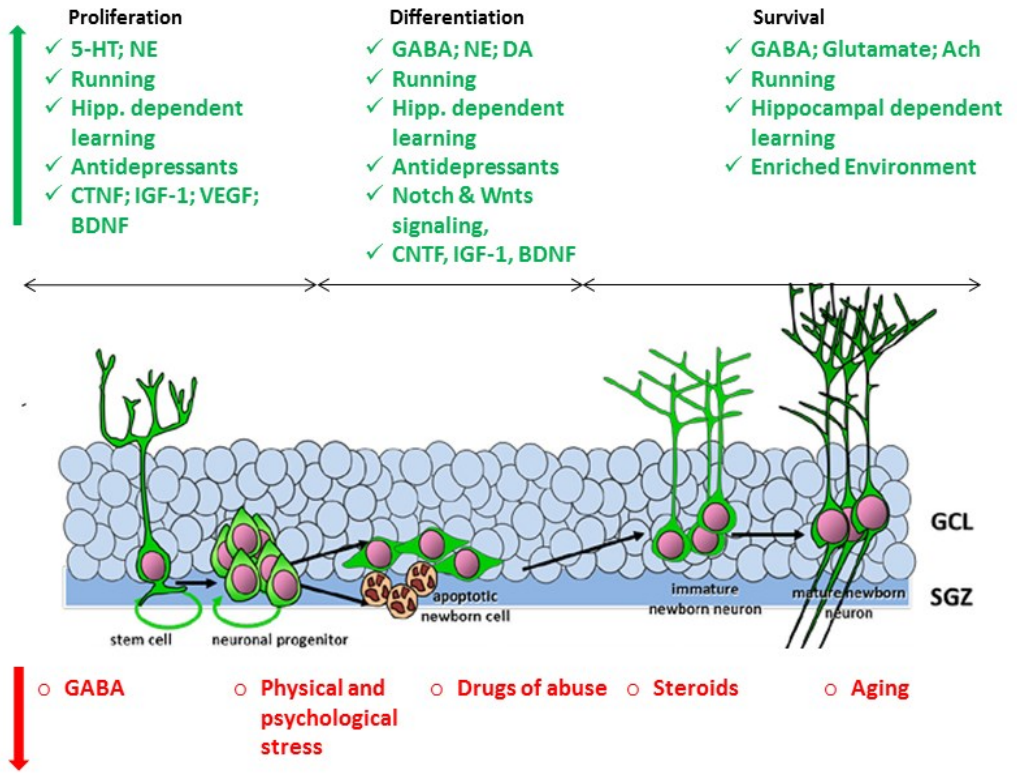


Figure 3. Adult hippocampal neurogenesis is modulated by several endogenous and exogenous factors. The different phases of neurogenesis could be increased by several modulators such as neurotransmitters, growth factors, environment and learning (Green factors) or decreased by stress, drugs and aging (Red factors) (*Modified from Koehl, 2015*).

In addition to their role in physiological and pathological conditions in the CNS astrocytes are also important contributors to adult neurogenesis specifically in the hippocampus. Song and colleagues showed that co-culture of hippocampal NPCs

and astrocytes promoted the proliferation of NPCs and favored their differentiation toward the neuronal lineage. An intriguing outcome of this study was that astrocytes originating from the spinal cord did not have neurogenic effect, therefore the neurogenic potential could be niche specific (Song et al., 2002). Another study showed that astrocytes coming from proneurogenic regions secreted cytokines and chemokines such as IL-1β and IL-6 that promoted neuronal differentiation at low concentrations (Barkho et al., 2006). Additionally, astrocytes through Wnt signaling regulate the expression of transcription factors such as: (i) Prox1 that has a role in the proliferation of NPCs and maturation of newly generated neurons and (ii) NeuroD1 that is crucial for survival and maturation of new neurons (Karalay and Jessberger, 2011).

3. Extrinsic and environmental regulators

Neurogenesis is also regulated by behavioral and environmental positive modulators such as voluntary exercise/running, EE, learning and negative modulators such as stress, aging and drug of abuse (Kempermann and Gage, 2000). Running is one of the most potent inducers of neural progenitor cell proliferation. It dramatically increases the number of BrdU positive ($BrdU^+$) cells in the DG of adult mice. Additionally, running increases the survival of newborn neurons after 4 weeks from exercise and synaptic plasticity since it enhances LTP in the DG (Van Praag et al. 1999 a,b). EE has a complementary effect increasing the survival of neurons at a critical stage of their maturation (Kobilo et al., 2011). For example, running itself increases the number of $BrdU^+$ cells. Subsequent learning increases the number of $BrdU^+/NeuN^+$ and Tuj-1^+ after 4 weeks from running. Hence learning rescues immature neurons from death and favors integration in the DG neuronal circuits (Leuner et al., 2004; Shors, 2008). Increased number and survival of 1- to 3-week-old newborn neurons are influenced by spatial learning and exposure to EE (Kee et al., 2007; Tashiro et al., 2007; Vivar et al., 2013). In

contrast, stress and aging are severe negative regulators of proliferation and differentiation (Kempermann, 2000). Proliferation in the DG is drastically reduced in aged (21 month-old) rats compared to middle-aged (6 month-old) rats (Kuhn et al., 1996). Reduced proliferation resulted in a decreased number of new neurons (eight to nine folds) from middle-aged to aged rats, indicating a dramatic decrease in neurogenesis associated with aging (Heine et al., 2004).

4. Role in physiology and changes under stressful conditions

The DG network activity regulates *de novo* neurogenesis and the post-mitotic neurons contribute to hippocampal dependent learning, memory and behavior (Kempermann et al., 1997, Gould et al., 1999). The dorsal hippocampus has a preferential role in learning and memory, whereas the ventral hippocampus is involved in affective behavior (Bannerman et al., 2004). For example, Morris Water Maze (MWM) learning tasks (conventional paradigm to assess spatial learning) increased the number of $BrdU^+/DCX^+$ cells along the SGZ of the DG. These young neurons had more complex and a higher number of DCX^+ arborizations (Tronel et al., 2010). Furthermore, learning elicits different influences on neural precursors at different developmental stages. For instance, in contrast to the 7-day old cells, the survival of 3-day old newborn cells is inhibited by MWM training (Dupret et al., 2007). This implicated that there is a selective integration of newborn neurons in spatial learning and memory. Stress reduces neurogenesis and MWM learning capacity. This effect was reversed by housing the mice in EE cages that normalized the number of $BrdU^+$ in the hippocampal DG and restored spatial learning in MWM (Nilsson et al., 1999; Veena et al., 2009). Among the elements of EE, the use of running wheels was the most effective in increasing $BrdU^+$ ahNPC after 24 h and $BrdU^+/Calbindin^+$ new neurons after 4 weeks. Increased neurogenesis was correlated with improved performance in MWM tasks (Kempermann et al., 1997). Moreover, running increases the level of brain derived

neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) and induces LTP in the hippocampus (Vivar et al., 2013; Kronenberg et al., 2003). Supporting the role of active adult neurogenesis in hippocampal-dependent tasks, the age-related reduction of hippocampal neurogenesis was also paralleled with MWM learning and memory deficits (reviewed by Lieberwirth et al., 2016).
Despite its extensive characterization in rodents, the functions of adult hippocampal neurogenesis in human CNS remain a big question. Several researchers suggested that adult hippocampal neurogenesis might have an important role in hippocampal-dependent contextual pattern discrimination. Computational modeling suggests that new stimuli cause the inhibition of old consolidated networks and the activation of new ones. This network remodeling is potentially mediated by newly integrated interneurons that play a role in higher pattern separation capacity between very similar memories and contexts (Bakker et al., 2008; Sahay et al., 2012).

Adult hippocampal neurogenesis (ahNG) is affected diversely in several CNS disorders. It is downregulated in mood disorders, specifically major depression disorder (MDD), epilepsy, stroke, and Parkinson disease (Kempermann et al., 2008). In contrast, cell proliferation was increased in the SVZ and SGZ of Alzheimer's patients and the SVZ of Huntington's patients postmortem brains (Zhao et al., 2008). Adult neurogenesis alterations could not explain the entire pathophysiology of neuropsychiatric disorders such as depression, schizophrenia and dementia. However, it was strongly correlated to hippocampal related impairments such as memory loss, difficulties in learning and increased incidence of depression (Kempermann et al., 2008). In 1992, Gould and colleagues were the first to correlate the decrease of ahNPC proliferation in the SGZ and elevated levels of stress hormones in the plasma of adult rats (Gould et al., 1992). Restraint and immobilization used as stressor in rodents inhibited the proliferation of NPC and reduced the survival of newly generated PSA-NCAM cells, therefore resulting in a

decrease of the DG volume and GCL thickness (Lee et al, 2009; Pham et al., 2003). The odor of predators, a natural stressor, is also shown to rapidly decrease the number of BrdU-labeled cells in the SGZ. Moreover, there was also a decrease in the percentage of $NeuN^+$ neurons at 3 weeks after the odor exposure (Zhao et al, 2007). Similarly, chronic unpredictable mild stress (UCMS) (a paradigm used to induce depressive-like behavior in rodent) decreased also $Ki67^+$ cells (endogenous marker for proliferation) and young neurons survival in the DG (Heine et al., 2004). In summary, psychological stress that causes depressive state decreased neurogenesis at different levels: proliferation rate of NPC, survival of young neurons (neuroblasts) and the number of mature neurons (Kempermann, 2002).

2. Serotonin

1. Serotoninergic circuit and receptor expression in the CNS

Serotonin (5-hydroxytryptamine, 5-HT) was first discovered in mid-thirties by the pharmacologist V. Erspamer in the gastrointestinal tract and the blood. Serotonin induced smooth muscle fibers contraction, thus it was first described as vasoconstrictor, hence the name serotonin (serum that gives tone) (Rapport et al., 1948). Particularly during early embryonic development 5-HT is secreted by the placenta and enters the fetus blood stream. It is one of the first and essential signaling cues in the embryonic brain developmental stages (Bonnin et al., 2011). In mammals, including human, serotonin is synthesized from the amino acid L-tryptophan by two enzymes: tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase (DDC). TPH enzymes exist in two forms: TPH1, found in several tissues and TPH2 that is a neuron-specific isoform (Walther et al., 2003). The peripheral and central brain serotonin systems are separated but equally important for several physiological functions. Peripherally 5-HT is important for energy balance, food intake, gastrointestinal tract, cardiovascular system. Centrally

5-HT is involved in behavioral and neuropsychological processes including mood perception, reward, anger, aggression, appetite, memory, sexuality and attention (Berger et al., 2009).

While it was thought that 5-HT is exclusively produced in raphe nuclei (RN) neurons located in the midline of the brainstem, recent *in situ* hybridization experiments showed that TPH2 is also expressed in the hippocampus and the striatum (Gutknecht et al., 2009). Serotoninergic neurons are the most abundant and complex system in the brain since they innervate almost all brain regions. They are among the earliest differentiated neurons found in a variety of organisms from C. Elegans to vertebrates (Lesch and Waider, 2012). Serotoninergic neurons originating from the rostral-dorsal and medial raphe terminate in the cortical, limbic (hippocampus), midbrain (ventral tegmental area VTA) and hindbrain (cerebellum) regions. Neurons originating from the caudal raphe are descending and innervate the spinal cord (Fig. 4). Indeed Gershone and Tack (2007) reported that "virtually all brain cells are close to at least one serotoninergic fiber". Dysregulation of the serotoninergic system was implicated mainly in the pathogenesis of neuropsychiatric disorders (Roth et al., 2004).

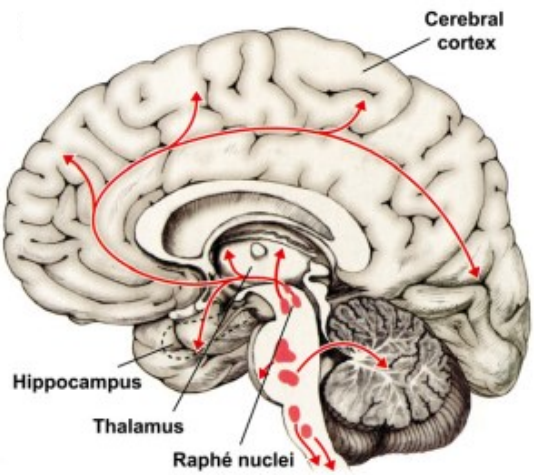


Figure 4. Central serotoninergic efferent fibers originate from the raphe nucleus to innervate several brain structures. Serotonin is a neurotransmitter produced in neurons originating in the raphe nuclei located in the midline of the brainstem. The most caudal raphe innervates the spinal cord, while the more rostral raphe, the dorsal raphe nucleus and the medial raphe nucleus, innervate the rest of the central nervous system by diffused projections (*Dalley and Roiser, 2012*).

After synthesis in the RN, 5-HT is packed into synaptic vesicles till its release into the synaptic cleft upon stimulatory signals. Extracellular levels of serotonin are regulated by presynaptic 5-HT reuptake transporter (5-HTT or SERT) and degraded by the monoamine oxidase enzymes (MAO) (Fig. 5). Serotonin transporter SERT belongs to the family of monoamine transporters together with noradrenaline transporter (NET) and dopamine transporter (DAT) (Scholze et al., 2008). More insights in the function of 5-HT and its mechanism of action emerged with the cloning of at least 15 receptor subtypes. 5-HT receptors are grouped in 6 classes of G-protein coupled receptors (GPCRs) named 5-$HT_{1,2,4,5,6,7}$ and one class of ligand-gated ion channel 5-HT_3 (Fig. 5) (Kroeze et al., 2002). The expression of 5-HT receptors has been described in the mammalian brain including human. Receptors autoradiography revealed a region specific distribution of different receptors as shown in Fig. 6. Serotonin receptors 5-$HT_{1A,\ 2A,\ 2C,\ 3,\ 6\ and\ 7}$ and auto-receptors 5-$HT_{1B/2B}$ were found in the hippocampus (Brichta et al., 2013). Since 5-HT receptors and SERT modulate virtually all brain functions, they became the major focus of CNS drug development, and many current medications modulate serotonin neurotransmission.

2. Serotonin and mood regulation

To study the impact of early and adult life serotonin dysregulations in the brain, scientists relied on genetically modified animal models. Mice lacking SERT ($SERT^{-/-}$) and MAO ($MAO^{-/-}$) have constant overexposure to serotonin during early development and adult life. Anatomical studies on these mice showed that they have failed segregation and barrel structure formation of the thalamo-cortical axons. Altered anatomical structure and inhibited cortical neurons migration are under the CTRL of 5-HT_{1B} and 5-HT_6 receptors (Cases et al., 1996; Salichon et al., 2001; Meffre et al., 2012). In contrast, mice models lacking TPH2 ($TPH2^{-/-}$) were depleted for 5-HT in the brain during embryonic and adult life. These mice did not

show any anatomical abnormalities in the serotoninergic neurons formation (Gutknecht et al., 2012). In fact the lack of 5-HT during early life is thought to be compensated by 5-HT secreted by the placenta (Gutknecht et al., 2008, Dayer, 2014). In contrast several studies showed that $TPH2^{-/-}$ mice have juvenile and adult fear/aggressive behavior in all experimental paradigms, lack of social interaction and severe maternal neglect to pups (Lerch-Haner et al., 2008, Lesch et al., 2012). The indoleamine hypothesis stated that the vulnerability to either depression or mania was related to the decrease of serotonergic activity. It is attributed to either less serotonin release or to fewer serotonin receptors or impaired serotonin receptor-mediated signal transduction (Mann, 1999). Later studies reported more complication and proved that indeed serotonin dysregulation is associated with impulsive aggression, anxiety, cognitive dysfunction and eating disorders (reviewed in Dayer, 2014). For example, researchers subjected female mice to chronic unpredictable stress and scored their male offspring in forced swim test and glucose consumption to assess their depressive-like behavior and in open field and exploratory paradigms to assess their aversive reactions to environment. The offspring showed depressive- and anxiety-like behavior, aggressiveness and anhedonia (Franklin et al., 2010). Studies on human confirmed that maternal depression could cause cognitive disabilities such as delayed maturation of language discrimination in infants. Within the same study scientists showed that infants from mothers treated with SERT inhibitors antidepressants exhibited more mature language discrimination abilities compared to infants from untreated mothers (Weikum et al., 2012).

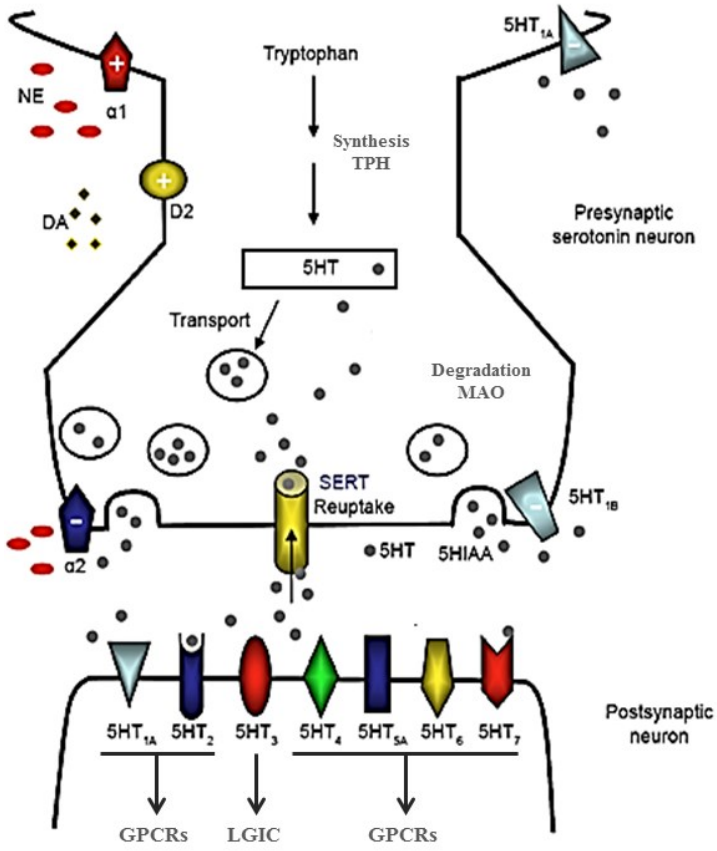


Figure 5. Serotonin Neuron Synapse. Serotonin (5-HT) is produced from tryptophan by the tryptophan hydroxylase (TPH) and packaged into storage vesicles until its release into the synapse. Multiple postsynaptic 5-HT receptors mediate 5-HT signaling. Serotonin receptors are 7 subfamilies grouped in 2 types as: Ligand-gated ion channel (LGIC) and G-protein coupled receptors (GPCR). 5-HT levels in the synaptic cleft are regulated by SERT or degraded to inactive metabolites by monoamine oxidases (MAO).

Presynaptic 5-HT_{1A} and 5-HT_{1B} auto-receptors detect the presence of 5-HT in the synapse and shut down (-) further 5-HT release. Dopamine receptors D_2 and α_1-α_2- adrenergic receptors positively (+) influence 5-HT transmission. *(Modified from Saltiel and Silvershein, 2015).*

Functional magnetic resonance imaging (FMRI) studies showed that depression induced by chronic mild stress exposure, reduced the connectivity between the RN and the hippocampus, hence serotonin dependent-functions in the hippocampus was reduced (Gordon and Goelman, 2016). DL-P-Chlorophenylalanine (PCPA), an irreversible inhibitor of TPH2, induces acute 5-HT depletion. Two studies used PCPA and showed that 5-HT depletion in human brain induced depression and cognitive function deficits and more specifically memory impairments. Depressed patients that received PCPA treatment had a transient return of depressed

symptoms even after treatment with SERT inhibitors antidepressants (Delgado et al. 1990, Shopsin et al. 1976). Therefore antidepressants therapeutic effect required the enhancement of serotoninergic activity.

Taking into account that 5-HT receptors and SERT are distributed diversely in the brain, several studies dissected their role in deleterious effects of stress, onset of depression and response to antidepressants. For instance, partial reduction of SERT expression using silencing RNA (SERT-siRNA), increased the level of 5-HT in the synapse followed by a reduced expression of 5-HT_{1A} autoreceptor. Silencing SERT in mice brains caused decreased immobility in the tail suspension test that is used to evaluate antidepressant-like activity (Ferrés-Coy et al., 2013). Presynaptic and

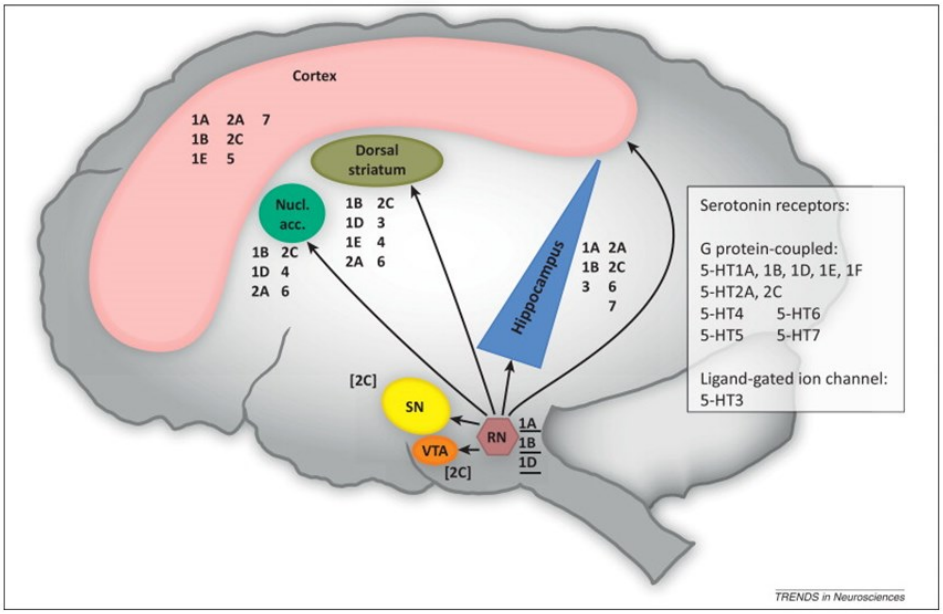


Figure 6. Serotonin receptors distribution in mammal's brain. *(Brichta et al., 2013)*

postsynaptic 5-HT_{1A} receptors are described as the major mediators of antidepressant activity. For example, 5-HT_{1A} autoreceptor agonists inhibited the feedback loop that usually leads to 5-HT reuptake from the synapse. Thus the increased availability of 5-HT in the synaptic cleft induced an anxiolytic and

antidepressant effect (Baldwin and Rudge, 1995). Moreover 5-HT by binding the postsynaptic 5-HT_{1A} reduced the firing in the post-synaptic neurons inducing and anxiolytic effect (Chilmonczyk et al., 2015). Receptors binding studies as well as autoradiography studies showed that depressed patients have an increased 5-HT_{2A} binding and activity (Ferrier et al. 1986; McKeith et al. 1987).

3. Serotonin and plasticity: emphasis on adult neurogenesis

As highlighted previously, 5-HT is an essential neurotransmitter that promotes neuronal development during embryonic as well as adult life. Particularly the medial raphe provides substantial inputs to various interneurons throughout the entire hippocampus and in the hilus (Fig. 7). While the dorsal raphe projections terminate preferentially in the SGZ region of the DG, with small axonal terminations that appear to be positioned for diffused release of 5-HT (Djavadian, 2004). The dorsal projections are positioned the best to modulate directly adult hippocampal neurogenesis, however also medial projections could modulate the neurogenic process through indirect effect on interneurons and network activity (Fig. 7) (Gordon and Goelman, 2016). The ablation of the RN (by 5,7-dihydroxytryptamine injection in the medial/dorsal raphe) or PCPA treatment depleted 5-HT levels in the brain, including the hippocampus. This depletion caused a drastic reduction of $BrdU^+$ and PSA-$NCAM^+$ cells in the DG (Brezun et al., 2000). A RN graft restored the levels of 5-HT, the number of proliferative ahNPCs and newly generated neurons (neuroblasts) (Brezun and Daszuta, 2000). Interestingly, $TPH2^{-/-}$ mice do not show a drastic reduction in the number of proliferative cells, but a severe deficient transition from type 1 stem cells to type 2 progenitor cells and increased cell death rate. Moreover, DG neurogenesis in $TPH2^{-/-}$ mice did not increase after voluntary wheel running, a well-known positive modulator of neurogenesis (Klempin et al., 2013).

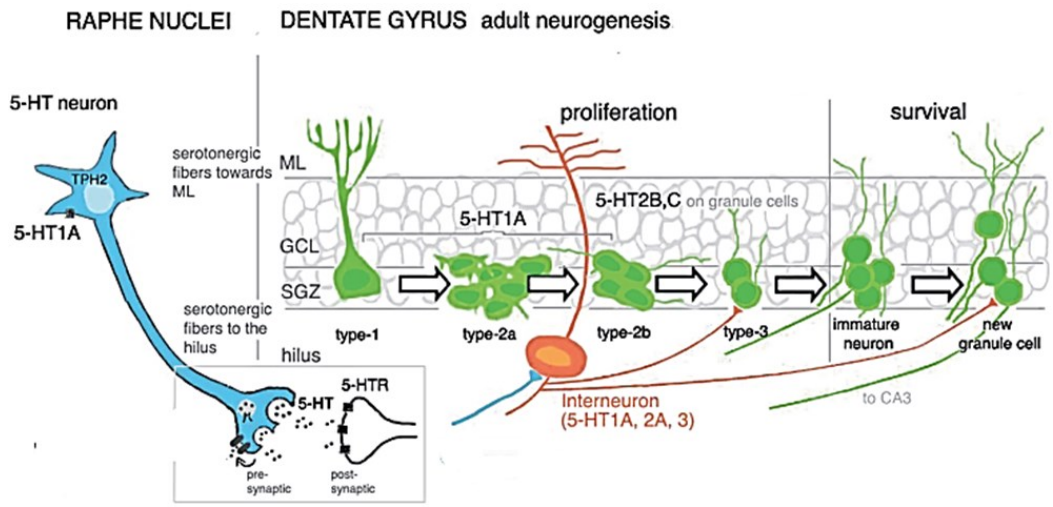


Figure 7. Illustration of serotonin mode of action during adult hippocampal neurogenesis. Serotonergic neurons located in the brainstem raphe nuclei synthesize TPH2 to be released in the synaptic cleft. Tracts of serotonergic fibers terminate in the hippocampus projecting into the molecular layer (ML) and the hilus, where they make synapses with principle neurons (Blue) and interneurons (in orange). Interneurons in the hilus in turn contact immature and newly generated granule cells in the SGZ and GCL. Several 5-HT receptors are expressed by the cell populations of the DG. 5-HT_{1A}R in type-1 to type-2b ahNPC in the SGZ; 5-HT_{2B} and $_{2C}$ receptors in adult granule cells of the GCL and 5-HT_{2A} in the hilus *(modified from Brichta et al., 2013).*

Several studies investigated the different contribution of 5-HT receptors in the synaptic plasticity and neurogenesis specifically in the hippocampus. For example, 5-HT_{1A} is involved in dendrites and spine formation in the hippocampus. 5-HT_{1A} antagonist decreased the number of dendritic spines of granule neurons and the total dendritic length of DG. This deleterious effect could be reversed by administrating 5-HT_{1A} agonist (Yan et al., 1997). Interestingly 5-HT_{1A} is expressed in type 1 (NSC) and type 2 (NPC) in the SGZ (Fig. 7). Blocking postsynaptic 5-HT_{1A} *in vivo* by a selective antagonist caused a 30% reduction of $BrdU^+$ in the SGZ of treated vs. not treated rats (Radley and Jacobs 2002). Depleting 5-HT secretion by PCPA followed by treatment with postsynaptic 5-HT_{1A} agonist (8-OH-DPAT)

rescued the number of proliferative ahNPC $BrdU^+$ in the SGZ (Banasr et al, 2003). Hippocampal NPC grown *in vitro* in proliferation expressed TPH2 and produced 5-HT in the medium. 5-HT_{1A} antagonist and PCPA treatment counteracted the proliferation of ahNPC *in vitro* as well as *in vivo*. Proliferation was rescued by administrating exogenous 5-HT or 5-HT_{1A} agonist (Benninghoff et al., 2010). Moreover, 5-HT_{1A} KO mice, even though they do not have lower baseline neurogenesis, were not responsive to 5-HT_{1A} agonist (Santarelli et al., 2003). Therefore, genetic animal model further confirmed that 5-HT_{1A} is usually required for positive modulation of neurogenesis.

Serotonin receptors 5-HT_2 are also expressed in the hippocampus. In details 5-HT_{2A} expression is very dense in the hilus neurons and interneurons, while 5-HT_{2C} mostly marked the granular layer cells. Type 2 NPC in the SGZ expressed 5-$HT_{2B/2C}$ (Fig. 7) (Alenina and Klempin, 2015). Stimulation of 5-HT_{2C} *in vivo* decreased mRNA and protein levels of BDNF, known to increase adult neurogenesis; *vice versa* blocking 5-HT_{2C} restored the levels of BDNF and increased the number of proliferative cells ($BrdU^+$) in the SGZ (Vaidya et al., 1997; Klempin et al. 2010). Another group also investigated the implication of 5-HT_2 in the regulation of ahNPC proliferation and neuronal differentiation in the SGZ. Adult rats were treated with DOI (5-$HT_{2A/2C}$ agonist) or ketanserine (5-$HT_{2A/2C}$ antagonist). DOI did not change the percentage of proliferative cells, but ketanserine produced a decrease in the number of BrdU-labeled cells (Banasr et al, 2003). In addition to the highlighted role of 5-HT_{1A} and 5-HT_{2C}, also 5-HT_4 could play a role in adult hippocampal neurogenesis. For example chronic treatment with RS6733, a 5-HT_4 agonist, increased the number of newborn DCX^+ cells, and more specifically the ones with a more mature profile determined by more developed dendrites (Mendez-David et al., 2014). Altogether these studies showed that despite the opposite role of 5-HT receptors in neurogenesis, serotonin remains necessary for the response to positive modulators of neurogenesis such as exercise and antidepressants (Klempin et al., 2013).

3. Mood disorders: focus on Major Depressive Disorder

Mood disorders are complex pathologies of the brain where the mood spectrum involves both depressed and manic episodes. Unipolar, bipolar and major depression disorders (MDD) are the most complex and prevalent mood disorders. Major depressive disorder is the second leading cause of disability in patients aged between 15-44 years. In addition to being ranked as the second in global disease burden, MDD is also projected to be the leading cause worldwide by 2030 (World Health Organization, 2012; Manji et al., 2001). Despite the discovery of first and second generation medications for MDD, at least 50% of patients who begin therapy with newer-generation of medications either fail to respond or discontinue therapy due to intolerable side effects. Thus, there remains an ongoing need for antidepressants that are more effective and/or better tolerated (Berton and Nestler, 2006; Hunot et al., 2007).

1. Definition and pathophysiology of MDD

MDD is a pathology characterized by multiple functional and structural impairments in the CNS and behavior. The most commonly listed characteristics are:

1. Emotional/motivational symptoms such as sadness, hopelessness, misery, anhedonia (loss of interest and joy), guilt and suicidal thoughts.
2. Cognitive symptoms such as negative cognition of self and the world (worthlessness).
3. Decreased mental productivity such as memory loss and learning disabilities.
4. Somatic symptoms such as loss of appetite, lack of energy, sleep difficulties and weight loss. Under stress the hippocampus and the hypothalamic-

pituitary-adrenal axis (HPA) are activated and stress hormones such as corticosteroids are released by adrenal glands. Stress hormones are the main inducers to the somatic symptoms of depression (Eisch and Petrick 2012).

To make an MDD diagnosis, clinicians rely on 3 obligatory symptoms: deep sadness, loss of interest and loss of energy that last at least two weeks in the first episode (Mill and Petronis 2007). Among others, one animal model of depression stood out as the most reliable and closes to human MDD. This animal model helped to study the physiological basis of depression. Chronic mild stress (CMS) procedure was developed in 1980s and used to induce depressive-like behavior in rodents. It is considered reliable since the conditions are realistic, reversible (specifically by antidepressant treatment) and the time course is suitable for chronic drug treatments (Willner, 2005). CMS could be induced by several factors such as: early maternal isolation, adult social isolation, and restraint/immobilization episodes. Depressive-like behavior could be assessed by behavioral tests such as: the forced swim test (FST), tail suspension test (TST) and hedonic reactivity. In fact animals with depressed-like behavior have longer immobility times in the FST, TST and have decreased palatable (sucrose) food intake (anhedonia) compared to naïve animals. These parameters are indications for helplessness and loss of interest. Additionally, MWM test is commonly used to assess the hippocampal-dependent spatial memory that is impaired by stress (Ehninger and Kempermann, 2005). Moreover, the advantage of this model is to predict *in vivo* the mechanisms of action of known or putative antidepressants and their effect on depressive symptoms and adult neurogenesis (Willner, 2005; Mirescu and Gould, 2006).

The complex pathophysiology of MDD involves many combined factors such as genetic susceptibility, anatomical and morphological alterations and neurochemical unbalance.

Genetic susceptibility. Studies on twins of families with mood disorder history showed a heritability of 50% and a significantly higher risk to develop mood

disorder during their life. Additionally genetic studies showed that a number of chromosomal abnormalities were associated with the development of MDD. Most of the genes were linked to neurotransmitters or neurotrophic systems such as: 5-HT_{2A} receptor, SERT, TPH2 and BDNF (Marchand et al., 2005; Fakhoury, 2015). Genetic factors increased the risk of developing MDD when combined with environmental factors such as stress and traumatic experience. For example, a study showed that patients holding two short (S) alleles instead of 1 or 2 long (L) alleles of SERT locus were more sensitive to stressful life events, exhibited more severe depressive symptoms and higher number of depressive episodes (Kendler et al., 2005).

Anatomical changes. MDD is accompanied or could cause impaired connectivity among neuroanatomical structures involved in the regulation of mood and stress response. For example, physical connections between the hippocampus, amygdala and prefrontal cortex (PFC) are disrupted physically and chemically in patients with MDD. Moreover, the PFC and hippocampus have smaller volumes in patients with MDD compared to healthy individuals (Bremner et al., 2000, Cole et al., 2011, McKinnon et al., 2009; Boldrini et al., 2012). Emotional and cognitive aspects of depression such as worthlessness, hopelessness, guilt and memory loss could be caused by the loss of these connections.

Morphological changes. Exposure to stress causes atrophy of neurons, reduced number of synapses (specifically glutamatergic synapses), loss of neurons and glia in the PFC and the hippocampus in human and rodent brain (Fig. 8) (Duman et al., 2012). Moreover, postmortem studies on brain tissue showed that the number of neurons in the GCL of the DG is reduced in patients with untreated MDD, which could explain the reduced volume of the hippocampus in these patients (Boldrini et al., 2012).

Neurotrophic factor downregulation. Neurotrophins are considered key regulators of neuroplasticity hence they underlie the compromised plasticity aspects of MDD (Fig. 9). In rodents, acute and chronic immobilization stress as well as chronic pain stimuli resulted in decreased BDNF mRNA expression in rat hippocampus. This study suggested also that probably depression and pain have similar pathways (Maletic et al., 2007).

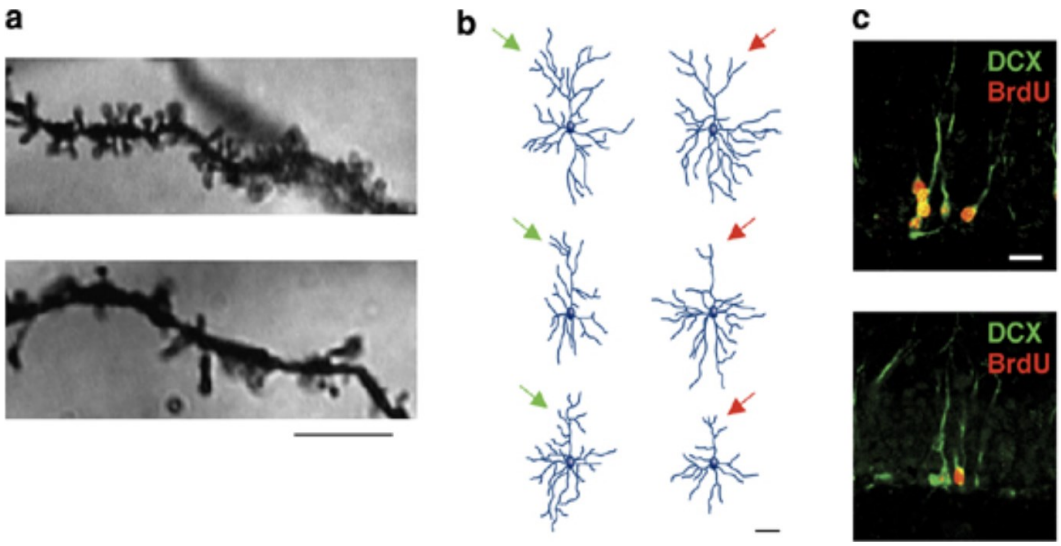


Figure 8. Stress alters several forms of neuroplasticity including adult neurogenesis. a. Post-weaning social isolation stress for 8 weeks significantly reduced dendritic spines in the prefrontal cortex (PFC) (visualized by Golgi-Cox staining) (Upper panel CTRL vs. Lower panel stressed). **b.** Chronic restraint stress reduced the length and complexity of cortical dendrites (Reconstitution of CTRL left panel vs. stressed right panel). **c.** Chronic unpredictable stress reduced the number of proliferative cells in the hippocampal SGZ. Visualized by bromodeoxyudridine BrdU and doublecortin (DCX) labeling markers of neuroblasts (CTRL upper panel vs. stressed lower panel). *(Pittenger and Duman, 2008).*

Decreased BDNF is followed by a low CREB activity, reduced arborization and spine formation (Fig. 9) (Duman and Monteggia, 2006). Brains of depressed patients have a severe reduction in the number of synapses specifically in the PFC and hippocampus (Maletic et al., 2007). A postmortem study on human serum

showed that BDNF levels are reduced in samples from depressed patients, and antidepressant therapy restored brain BDNF levels to the normal range simultaneously with improvement in depressive state (Shimizu et al., 2003). Mice lacking for BDNF did not show depressive-like symptoms in the FST. The infusion of BDNF into the hippocampus and the RN region of depressed wild type mice mimicked the effects of antidepressants (Eisch et al., 2003). Similarly, fibroblast growth factor (FGF) is downregulated in the hippocampus of patients with MDD. Additionally insulin-like GF- I (IGF-I) is increased in the hippocampus by antidepressants treatment. IGF-I infusion into the brain has an anti-depressant like effect similarly to BDNF (reviewed in Duman et al., 20-16).

Neurochemical unbalance: emphasis on monoamine deficiency. Back in 1960, European and American researchers simultaneously suggested that depression was caused by deficiency in the catecholamine norepinephrine (NE) and the indoleamine 5-HT in the brain (Fig. 9). In fact NE from the locus coeruleus (LC) and 5-HT from the RN are the most dominant innervations in the CNS regions involved in MDD (Berton and Nestler, 2006). The involvement of the monoaminergic system was initially postulated after the finding that monoamine depleting agents such as “reserpine” (anti-hypertension drug) could produce depressive symptoms. Animal research studies showed that reserpine depleted 5-HT and NE from neurons. Simultaneously, patients showed severe depressive state and sedative behavior. In contrast monoamine oxidase inhibitors (MAOI) increased the levels of monoamines in the synapse and antagonized the effect of reserpine (Manji et al., 2001; Lopez-Munoz and Alamo, 2009). Subsequently, pharmacological studies showed that 5-HT receptors might add more complication to antidepressants effect. Serotonin receptors 5-HT_{1A}, 5-$HT_{2A/2C}$ and 5-HT_4 emerge as candidates to mediate antidepressant response. For instance, they regulate normal development of dendritic spine density, synapse formation of pyramidal and granule cells in the DG, long-term plastic changes that decrease anxiety-like behavior and mediate ahNPC maturation at the late phases (Djavadian, 2004). For

example, 5-HT_{1A} is overexpressed in the hippocampus of MDD patients. This compensatory mechanism is a response to decreased levels of 5-HT (Santarelli et al., 2003). In contrast, increased binding on 5-$HT_{2A/2C}$ receptors is responsible for adverse side effects related mainly to weight changes and sleep deprivation (De Vry and Schreiber, 2000). Activated 5-HT_4 increases the levels of BDNF and ahNPC proliferation in the DG through cAMP signaling cascade (Djavadian, 2004; Mattson et al., 2004; Ferrés-Coy et al., 2013).

Similarly to 5-HT, low levels of NE were specifically associated with cognitive dysfunction, fatigue and apathy. These associations solicited scientist to study more deeply the role of NE in cognitive and psychiatric disorders that will not be detailed in the context of this work. Many other neurotransmitters systems were found to be involved in MDD but to a less extent compared to 5-HT and NE. For example, the synthesis and release of GABA are reduced in response to acute and chronic stress whereas the glutamate levels are high after stress. Likewise increased levels of histamine in the limbic system have been associated with depression and sleeping problems in patients with MDD (reviewed in Marchand et al., 2005).

2. Antidepressants: classes and mechanisms of action

During the 1950s, while carrying out trials on a new medication for tuberculosis, researchers noticed that the medication had also a mood improving effect on tuberculosis patients. Clinicians showed that anti-tuberculosis drug improved the depressive state of patients (raised mood, weight gain, better interpersonal capacity, increased interest in themselves and surroundings). Scientists showed that “iproniazid” had indeed MAO inhibitory activity (thus the name MAOI, Fig. 10 A). It increased the levels of 5-HT and NE in the synaptic cleft by inhibiting their degradation (Sandler, 1990). MAOI caused severe side effects thus their use became limited.

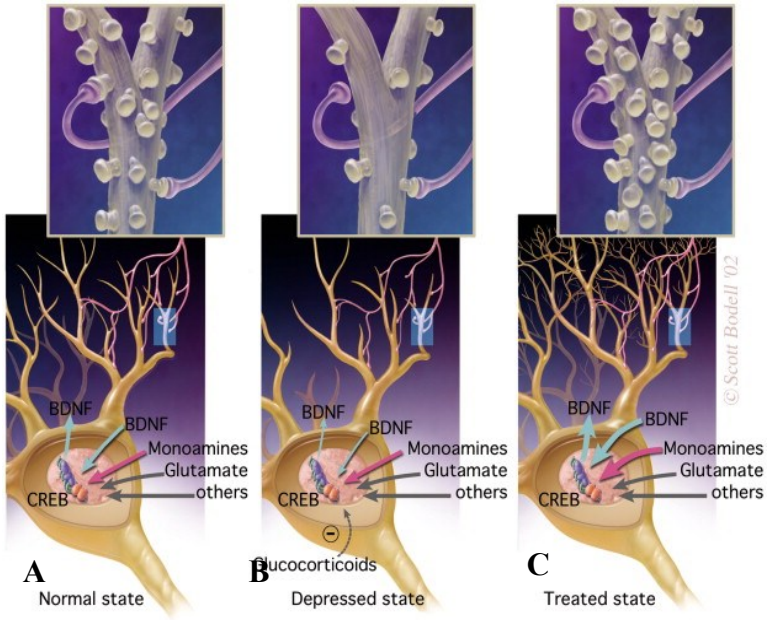


Figure 9. Synaptic connectivity and chemical balance is lost in stressed brain. A. Normal state: healthy hippocampal pyramidal neurons receive physiological levels of monoamines (5-HT, NE), glutamate and BDNF (Thick arrows). **B**. Severe stress increases the levels of glucocorticoids that, in turn inhibit CREB signaling causing reduction of BDNF, monoamines and stimulatory glutamate thus inducing radical loss of dendritic arborizations and spines (Thin arrows). **C.** Antidepressants, by activating CREB signaling or by increasing monoamines restore BDNF levels, dendritic arborizations and spine number. (*Modified from E. Nestler et al., 2002*)

At the same year of MAOI discovery in 1957 tricyclic (TC) molecules were characterized chemically similar to antihistaminic drugs. Therefore, they were tested for their potential antihistaminic and sedative effects. R. Kuhn, a famous psychiatrist at that time, showed that they improved the symptoms in patients with depressive psychosis and raised the first suggestion of potential antidepressant effect. TC molecules started to be used as antidepressants (hence the name TCA, Fig. 10 A). TCA such as imipramine showed anti-histaminic activity as well as NE and 5-HT reuptake inhibition (NRI and SRI). TCA had low MAOI activity therefore they had safer profile in patients (Lopez-Munoz and Alamo, 2009). In

summary, the first-generation of antidepressants was effective because it enhanced serotonergic or noradrenergic mechanisms or both. Unfortunately, even TCA were still non-selective drugs with considerable side effects due to: anti-histaminic activity (sedation, sleep), cholinergic activity (tremors), anti-muscarinic M1 (constipation, dry mouth, urinary retention) and α1-adrenergic receptor sites (orthostatic hypotension, sexual dysfunction) (Duman et al., 2016). Moreover, TCA and MAOI had a fast chemical effect on the amine levels in the synaptic cleft, but the therapeutic effects did not take place before 4 weeks of treatment (Pittenger and Duman, 2008). It is important to point out that these initial drugs were approved and prescribed merely upon clinical observation without real knowledge of the mechanism of action. Since then, rational drug design (relying on the knowledge of the biological target and its role) led to the introduction of selective, faster, and better tolerated antidepressants.

The second generation of antidepressants included: selective serotonin reuptake inhibitors (SSRI), serotonin and norepinephrine reuptake inhibitors (SNRIs) and noradrenaline reuptake inhibitors (NARI) (Fig. 10 B). Selective SSRI had similar efficacy as TCA but lacked the anti-muscarinic activity, thus they had less cardiovascular side effects. Their major side effects were headaches, sexual dysfunction, and weight gain due to 5-HT_{2C} agonistic activity. SNRI were more balanced on both receptors system, had the same efficacy as TCA and SSRI yet they lacked the muscarinic activity and α1 AR blockade, thus their major side effect was hypertension. NARI had mainly analgesic activity but they dangerously increase the arterial blood pressure, hence they were subjected to prescription restrictions (Berton and Nestler, 2006).

As part of the effort to address the unmet need of better tolerated antidepressants, attention has been given to multimodal acting drugs and new mechanisms of action for depression treatment. Multifunctional or multimodal drugs could be used in multiple disorders and have less severe side effects controlled by dosage regulations. As an example (i) serotonin antagonist and reuptake inhibitors (SARI) (Fig. 10, B) such as trazodone and nefazodone that have the same efficacy as TCA and SSRI with additional anxiolytic and hypnotic activity (Lopez-Munoz and Alamo, 2009); (ii) agomelatine that is melatonergic receptor agonist and 5-HT_{2C} receptor antagonist (Soumier et al., 2009); (iii) vortioxetine that is a 5-$HT_{3,7,1D}$ antagonist, 5-HT_{1B} partial agonist, 5-HT_{1A} agonist and SERT inhibitor (Guilloux et al., 2013). Several studies are focusing on understanding how multimodal drugs induce their antidepressant effect, their off-label therapeutic use and their side effects.

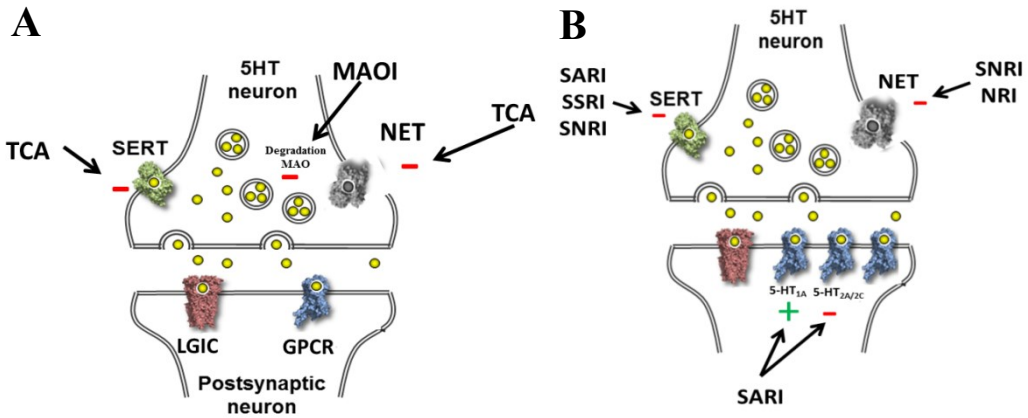


Figure 10. Antidepressant Mechanism of action. A. Monoamine oxidase inhibitors (MAOI) inhibit mitochondrial oxidation of monoamines in the presynaptic neuron. Tricyclic antidepressants (TCA) inhibit 5-HT transporter (SERT) and/or noradrenaline transporter (NET) **B**. Serotonin Antagonist and Reuptake Inhibitors (SARI) antagonize serotonin receptor 2 (5-HT_2) and block SERT; Selective serotonin reuptake inhibitors (SSRI) block serotonin reuptake transporter (SERT) on the presynaptic neuron; Serotonin and Noradrenaline Reuptake Inhibitors (SNRI) block SERT and NET. All mechanisms increase the level of 5-HT and NE in the synaptic cleft. *(Modified from Brigitte Schiott, Biomodeling, Semper Ardens Research project, 2016)*

3. Adult hippocampal neurogenesis, MDD and antidepressants

Environmental interventions such as learning tasks, enriched environment and physical activity stimulate adult neurogenesis and have an antidepressant-like effect on behavior (Sahay and Hen, 2007). By the same token scientists suggested that antidepressants might also increase adult hippocampal neurogenesis. "The neurogenic hypothesis of depression" postulated that decreased production of new granule cells in the dentate gyrus of the hippocampus is linked to the pathophysiology of depression and that the increase in hippocampal neurogenesis and plasticity are required for the behavioral effect of antidepressant treatment (Eisch and Petrick, 2012).

The ablation of the SGZ by X-ray irradiation inhibited the onset of behavioral improvement mediated by fluoxetine (SSRI) on non-human primate animal model of MDD (Perera et al., 2011). Boldrini and colleagues showed that the number of ahNPC in the hippocampal SGZ of patients with MDD is increased in SSRI antidepressant-treated versus non-treated patients (Boldrini et al., 2009). Fluoxetine accelerated synaptogenesis (increase spines formation) and enhanced LTP in the hippocampal neurogenic niche. These improvements were accompanied with improved depressive behavior (Wang et al., 2008). Moreover, several studies showed that chronic (14 days or more) treatment with fluoxetine upregulates NPC proliferation rate (increase the number of $BrdU^+$ cells in the SGZ) and immature neuron survival rate (Malberg et al, 2000; Kodama et al, 2004; Huang and Herbert, 2006; Marcussen et al, 2008). Similar results have been seen in experiments using citalopram and escitalopram (SSRI). In details, scientists induced a depressive state by olfactory ablation in rats. Rats were treated with citalopram in short and long term paradigms to assess the proliferation and survival of newly generated neuron. They showed that citalopram increases the number of proliferative $BrdU^+$ cells and restore the number of PSA-$NCAM^+$ surviving new neurons to levels compared to control rats (Jaako-Movits et al, 2006). Another study used CMS paradigm and

assessed the effect of escitalopram on ahNPC proliferation and differentiation. They showed that, similarly to fluoxetine and citalopram, also long term treatment with escitalopram increased the neurogenesis in the SGZ with simultaneous increase in glucose consumption (hedonic behavior) as a sign of alleviated depressive state (Jayatissa et al, 2006). Citalopram and reboxetine (NARI) may potentially upregulate neurogenesis through BDNF signaling since serum and hippocampal regions BDNF levels are increased following treatment (Russo-Neustadt et al, 2004). Several studies used chronic treatments with antidepressants such as SSRI, TCA, NRI and MAOI to enhance several steps of the neurogenic process in the DG of rodents and non-human primates. Notably, the increase of neurogenesis was simultaneous to the onset of therapeutic effect of antidepressants (Malberg et al., 2000, Santarelli et al., 2003, Airan et al., 2007). Neurotrophic factors such as BDNF and IGF-1 activate ERK in neurons to induce neuroprotection and in ahNPC to increase proliferation and differentiation (Yamada et al. 2001; Shelton et al. 2004). Similarly, mood stabilizers such as lithium and valproate activate ERK and enhance both proliferation and survival of ahNPC in the SGZ (Hao et al, 2004; Hanson et al, 2011a). Low serum levels of BDNF were associated with depression in patients. Treatment with antidepressant and, specifically, with SSRI was associated to normalized levels of BDNF (Molendijk et al., 2011). Agomelatine that is a multimodal antidepressant also increased the survival of ahNPC *via* melatonergic receptor agonistic and 5-HT_{2C} antagonistic activities accompanied with increased BNDF signaling (Soumier et al., 2009). Similarly, acute and repeated dosing of vortioxetine (multimodal antidepressant) significantly increased the number of $BrdU^+$ cells and DCX^+ with tertiary dendrites (more mature cells). Vortioxetine increased proliferation, cell survival and stimulated maturation of immature granule cells in the hippocampal SGZ (Guilloux et al., 2013).

Our group also participated in consolidating the neurogenic hypothesis of depression and the role of antidepressants. Pregabalin (PGB) and gabapentin (GBP)

are clinically relevant anticonvulsant, analgesic and anxiolytic drug. Interestingly, PGB and GBP are also prescribed to MDD patients with post-traumatic stress and anxiety (Stein et al., 2008). Our group showed that PGB and GBP increase, in a concentration-dependent manner, the number of mature and immature neurons generated from the *in vitro* model of ahNPC. They act through α2-δ1 subunit of the voltage-gated calcium channels expressed by hippocampal NPC (Valente et al., 2012). More interestingly, this effect was blunted by inhibiting the nuclear translocation of NF-κB p50/p65 subunits to the nucleus (Valente et al., 2012). Within the same study PGB interestingly decreased depressive-like behavior in mice that received unpredictable CMS, since it reduced immobility time in both TST and FST. Thus the antidepressant activity of PGB and GBP could be potentially mediated by neurogenesis (Valente et al., 2012). In another study, our group focused on Acetyl-L-carnitine (ALC) that can (i) cross the blood brain barrier, (ii) improve mood disorders in human particularly in elderly and (iii) have an analgesic effect in rodent studies (Zanardi et al., 2006; Cuccurazzu et al., 2013; Bortolotto et al., 2014). ALC is an acetyl group donor to proteins including NF-*κ*B p65 subunit (Chiechio et al., 2006), but it was not known how ALC can have an antidepressant effect. Our group hypothesized that the antidepressant activity of ALC could be due to a proneurogenic effect mediated by NF-*κ*B signaling pathway. ALC showed a potent pro-neurogenic activity on ahNPC *in vitro*. This effect was mediated by the activation of NF-*κ*B pathway and subsequent upregulation of metabotropic glutamate receptors 2 (mGlu2) expression *in vitro* and *in vivo*. These cellular mechanisms were inhibited by blocking p65 translocation to the nucleus. *In vivo* ALC reversed depressive-like behavior in mouse model of MDD (Cuccurazzu et al., 2013). The antidepressant activity described by our group was also confirmed by another study on genetic rat model of depression. Moreover they compared ALC to TCA and showed that rats had reduced immobility in FST and increased palatable food consumption. More interestingly in their model the onset of ALC effect required three days of

treatment while TCA required 14 days of treatment (Nasca et al., 2013). Altogether our group and literature studies suggested that increasing neurogenesis might be a new mechanism of action for antidepressants. It is still not known if multimodal antidepressants such as trazodone (SARI) could have an effect on neurogenesis and by which mechanisms.

4. Trazodone

Trazodone hydrochloride (TZD) is a dose dependent multifunctional drug that has advantages over other antidepressants in terms of therapeutic use. It is prescribed as antidepressant at high doses and has multiple off-label uses mainly for sleep and anxiety disorders at low doses. TZD detailed mechanism of action and diverse therapeutic benefits are still not fully characterized.

1. Trazodone: peculiar mechanism of action

Trazodone hydrochloride is a phenylpiperazine-triazolopyridine compound first discovered by Angelini research laboratories in Italy in the 1960s. Trazodone, similarly to SSRI and their derivatives, was introduced as the second generation of antidepressants after TCA and MAOI. Trazodone had comparable efficacy to TCA and SSRI antidepressants and yet was more tolerated. Early studies in rat brain showed that given acutely or chronically with doses similar to other SSRI antidepressants, trazodone appeared inactive as an inhibitor of serotonin reuptake by SERT but showed 5-$HT_{2A/C}$ antagonistic activity (Fuller et al., 1984). Affinity binding studies showed that Trazodone has 50 to 100 fold higher affinity to 5-$HT_{2A/C}$ than SERT (Fig. 12 A). To elicit a therapeutic effect trazodone is administrated at a dose that recruits both the weaker binding to SERT and the strong binding 5-$HT_{2A/C}$ receptor (Fig. 11, B) (Knight et al., 2004; Stahl et al., 2009). Together these properties annotated trazodone as a dose-dependent 5-$HT_{2A/C}$ antagonist and serotonin reuptake inhibitor (SARI). Based on the knowledge of

both mechanisms of action outcome, trazodone mechanisms are suggested to act synergistically (Stahl et al., 2009). On one hand antagonizing 5-$HT_{2A/C}$, trazodone similarly to TCA antidepressants, increase the release of dopamine in the cortex inducing antidepressant activity (Chagraoui et al., 2015).

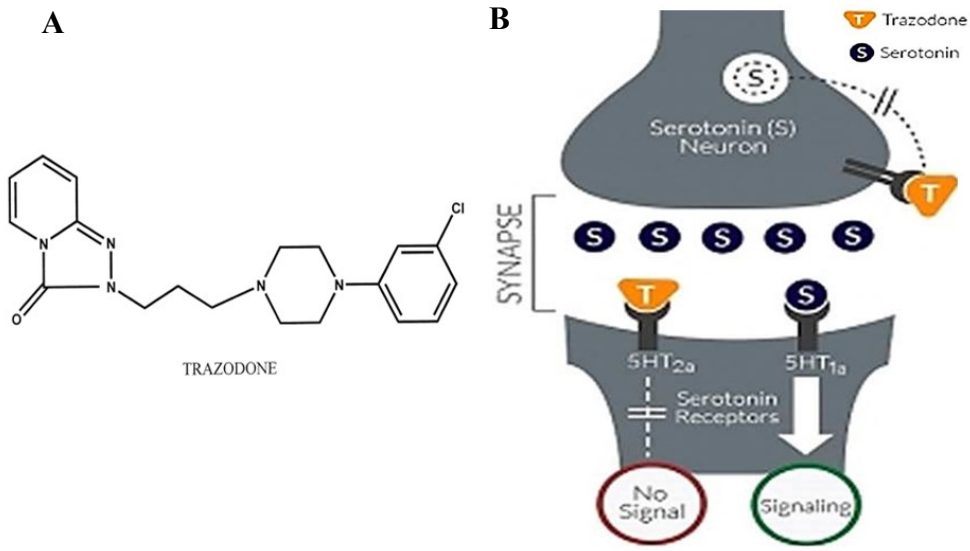


Figure 11. Chemical structure of trazodone and its antidepressant mechanism. A. Chemical structure of phenylpiperazine-triazolopyridine, trazodone **B.** Schematic representation of trazodone mechanism of action. Trazodone is a serotonin antagonist and reuptake inhibitor (SARI). It inhibits serotonin reuptake by blocking SERT on the presynaptic membrane allowing serotonin to activate it 5-HT_{1A} receptor and antagonizes 5-HT_{2A} receptor. *(Stahl, Trends in Psychopharmacology, 2009)*

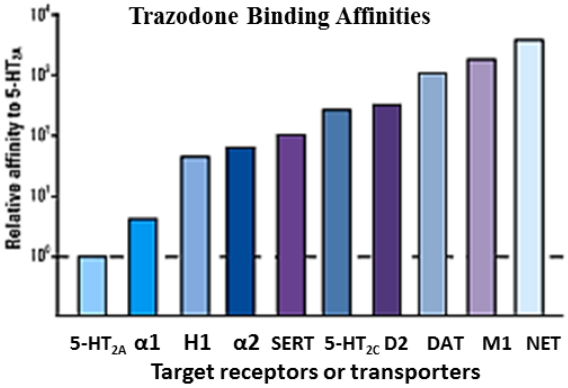
On the other hand, by blocking SERT, trazodone increases the availability of serotonin in the synapse and allows the activation of 5-HT_{1A} resulting in hyperpolarization and reduced neuronal excitability and firing (Chilmonczyk et al., 2015). The hyperpolarization makes the neurons less excitable and leads to antidepressant and anxiolytic activity. Additionally, trazodone has different binding affinities to various receptors such as α_1 and α_2 adrenergic receptors, histamine

receptor 1 (H1), dopamine receptor 2 (D2) and muscarinic receptor (M1) (Fig. 12 A). Trazodone acts as agonist or antagonist with different binding affinities (Fig. 12 B). Electrophysiological studies in the dorsal raphe and hippocampus of rats showed that administration of trazodone sustain serotonin availability not only by blocking SERT but also by desensitizing the 5-$HT_{1A/1B}$ presynaptic auto-receptors. The desensitization of 5-HT auto-receptors induced a tonic release of serotonin in the synapse resulting in a sustained firing rate of 5-HT neurons (Ghanbari et al., 2010).

Multifunctional activity of trazodone has a high pharmacological relevance with regards to side effects and off-label uses. 5-$HT_{2A/C}$ receptors activation is known to induce antidepressants side effects such as insomnia, sexual dysfunction and anxiety. Trazodone antagonistic activity on 5-$HT_{2A/C}$ alleviates anxiety and sexual dysfunction and induce hypnosis (Stahl et al., 2009). Arousal mechanisms are known to involve the activation of several neurotransmitter systems such as serotonin, norepinephrine, dopamine, acetylcholine, and histamine. By antagonizing all these systems simultaneously trazodone impairs arousal and induces sleep. Thus low doses act as hypnotic drug to improve sleep disorders related and not related to MDD.

Trazodone is absorbed rapidly with peak in plasma attained after 1-2 hours following an immediate release (IR) tablet. It is metabolized by cytochrome P450 (CYP450) isoforms that generate different metabolites including active ones such as *meta*-chlorophenylpiprazine (*m*-CPP). Trazodone metabolite *m*-CPP inhibits SERT, antagonizes 5-HT_{2A} and act as partial agonist of 5-HT_{1A} and 5-HT_{2C}. The levels of *m*-CPP (0.056 μg/ml) are only 10% of TZD levels (0.62 μg/ml) in the plasma of treated patients (Mittur, 2011). Some researchers claim that such low levels of m-CPP are negligible. Thus, antagonist activity of trazodone overwhelms *m*-CPP activity that does not influence trazodone's clinical outcomes (Stahl et al., 2009).

A



B

Receptor	TZD activity	pKi
5-HT1A	Partial Agonist	7
5-HT1B	Antagonist	
5-HT2A	Antagonist	7.4
5-HT2B	Antagonist	7.1
5-HT2C	Antagonist /agonist ?	6.7
5-HT7	Antagonist	
5-HTT	Antagonist	6.2-6.8
a1	Antagonistic	6.9-7.9
a2	Antagonistic	6.5-7
H1	Antagonistic	6-6.7
D2	?	5.5
M1	?	4.5
NET	Antagonistic	4.4
DAT	?	5.1

Figure 12. Trazodone binds different receptors and transporters populations A. Graph representing trazodone binding affinity to different receptor population relatively to the strongest binding on 5-HT_{2A}. **B.** Affinity of trazodone for various human pharmacological targets. *(Modified from stahl et al., 2009).*

2. Therapeutic doses, efficacy and side effects

Trazodone is marketed as an antidepressant approved by U.S. Food and Drug Administration (FDA) in 1982. Today, it is sold under different brands names such as Desyrel®, Oleptro™, Deprax and others. Generic formulation is also available and is manufactured by several pharmaceutical firms. Trazodone hydrochloride is available in different formulations such as IR tablets, prolonged release tablets (TzCOAD), oral drops and injectable solution. Therapy with IR tablets must be gradual starting with low doses 75-150 mg/day, and reaching 300 mg/day. In the elderly, it is given at lower doses of maximum 100 mg/day (Fagiolini et al., 2012).

The novel TzCOAD prolonged release (once a day tablets) was developed in 2010 and approved recently by FDA and many European countries.

Trazodone efficacy was compared to TCA (amitriptyline and imipramine), SSRIs (fluoxetine, paroxetine, sertraline, citalopram and escitalopram), SNRIs (venlafaxine and mirtazapine) and noradrenaline/dopamine reuptake inhibitors (bupropion). The Hamilton rating Scale for Depression (HAM-D) and Geriatric Depression Scale (GDS) scores showed that trazodone has the same efficacy as other antidepressants (reviewed by Fagiolini et al., 2012). Additionally, to its efficacy, it had advantages for patients with major depression who have sleeping difficulties or show prevalent sleep disturbance. The great improvement of sleep was measured by a HAM-D-related sleep disturbance score. Trazodone was efficacious in treating insomnia and anxiety associated to MDD. Patients diagnosed with MDD under trazodone treatment showed improved insomnia, depression and anxiety symptoms (Saletu-Zyhlarz et al., 2003). It is frequently prescribed as a concomitant agent along with an SSRI/SNRI or as monotherapy.

Trazodone is a well-tolerated drug with side effects similar to SSRI including drowsiness (sedation and somnolence), hypotension, headache, dizziness and dry mouth. Drowsiness is the major side effect that accompanies the peak concentration of trazodone in the plasma. Elderly patients are more likely to experience the sedative or hypotensive effects of trazodone than young adults. By avoiding this peak mainly related to IR tablets may reduce the severity of the side effects (Mittur, 2011). Therefore by developing TzCOAD prolonged release tablets, patients were able to describe small differences in side effects severity compared to IR tablets. Namely, patients that were treated with TzCOAD reported less severe dizziness, drowsiness, dry mouth, headaches and nausea (Moon et al., 2008). Dry mouth has been reported in approximately 15–30% of patients during trazodone therapy and probably results from α-adrenergic blockade. Some case studies reported spontaneous penile erection and rare priapism. Priapism is also a known effect of

m-CPP mediated by its activity at 5-HT_{1C} receptors. Trazodone was not associated with malformations in infants when administrated during pregnancy. In summary, and importantly, trazodone showed an overall significant lower rate of discontinuous treatment related to side effects compared to SSRI antidepressants (Anderson et al., 2000).

3. Trazodone as a neuroprotective antidepressant

In addition to their therapeutic activities, several antidepressants have also displayed important neuroprotective activity. Trazodone has also a potential neuroprotective activity specifically important taking into consideration its multifunctional properties. First, recent studies on animal models of Huntington Disease (HD) revealed that trazodone reduces free radicals and improves mitochondrial respiratory complex activity after 2 weeks of treatment in HD rat models. Additionally, it improved memory performance and motor activity in HD rats (Lauterbach, 2013). These studies need to be extended beyond rat models and be assessed in human patients under conventional doses. The mechanism by which trazodone improved memory and motor activity in HD rats is still unknown (Fagiolini et al., 2012).

Several studies reported that antidepressant treatments of glial cells had anti-inflammatory effects. For example in a co-culture model of astroglia-microglia, an inflammatory status was induced by increasing the proportion of microglia. Treatment with venlafaxine (SNRI) reversed the pro-inflammatory status within the model (Vollmar et al., 2008). Likewise, fluoxetine (SSRI) was shown to counteract astrocytic cell loss in animal models of depression, and increase astrocyte glucose release, that provides essential energy substrates for neurons to sustain normal function and cellular integrity (Czéh et al., 2006). Up till a year ago, it was still unknown if astrocytes are a target for trazodone or if it has a neuroprotective effect in physiological conditions and after an inflammatory insult. Daniele and

colleagues were the first to study the potential role of trazodone in neuroprotection and neuroinflammation. They treated cultured human astrocytes with trazodone before and after an inflammatory challenge, namely by exposure to lipopolysaccharide (LPS). TZD treatment of astrocytic culture enhanced mRNA expression of growth factors and lactate release from astrocytes. It activated AKT signaling pathway while simultaneously decreased pro-inflammatory signaling by inhibiting interferon γ (IFN-γ) release, constitutive phosphorylation of ERK1/2 and JNK constitutive phosphorylation. These effects were mediated by 5-HT_{1A} stimulation, 5-$HT_{2A/C}$ blockade and α_1 and α_2 AR antagonism. Moreover, after LPS/TNF-α insult trazodone reversed the inflammatory status by: (i) normalizing astrocyte proliferation specifically through the activation of 5-HT_{1A} and antagonism of 5-$HT_{2A/C}$, (ii) restoring the release of lactate, (iii) inhibiting the secretion of inflammatory mediators such as IL-6 and IFN-γ production, (iv) counteracting the decrease of neurotrophic factors and (v) counteracting the activation of ERK1/2 and JNK (Daniele et al., 2015 a). In another study of the same year, the same group was able to show that TZD is neuroprotective not only on astrocytes but also on human neuron-like cell. Indeed trazodone also reversed the inflammatory status and restored cell viability through the inhibition of JNK/p38 pathway (Daniele et al., 2015 b).

In essence, TZD is a well-tolerated drug with multifunctional properties. It has an important relevance in treating depression-related symptoms and insomnia. The newly discovered astrocytic and neuronal protective effect of trazodone raises the question about additional novel therapeutic actions and their cellular and subcellular pathways. For example, it is unknown if trazodone like other antidepressants may modulate adult hippocampal neurogenesis.

5. NF-κB

1. NF-κB signaling pathway and subunits in the CNS

NF-κB proteins are transcription factors ubiquitously expressed in all tissues including the brain. They regulate genes involved in immunity, apoptosis, cell survival, cellular growth, cellular repair and oncogenesis. The NF-κB family is composed by p50, p52, p65 (RelA), c-Rel, and RelB subunits that form transcription factors after dimerization. They all share the Rel homology domain responsible for DNA binding, dimerization, nuclear translocation and interaction with the inhibitor protein κB (IκB). Non-active proteins are retained in the cytoplasm by IκB. There are two activation pathways of NF-κB: the canonical pathway relying on the IκB inactivation and an alternative activation pathway mediated by NF-κB inducing kinase (NIK). Upon activation, NF-κB members are transferred to the nucleus to repress or activate target gene expression (Kaltschmidt et al. 1993). NF-κB subunits dimers such as p50/p65 and p50/p50 are expressed in the CNS by neuronal and non-neuronal cells (Schmidt-Ullrich et al. 1996). Interestingly, IκB-NF-κB dimers complexes were also found in synapses distant from the nucleus. Upon glutamatergic stimulation, NF-κB also acted as a signal transducer by delivering signals from active synapses to the nucleus (Mikenberg et al., 2007). Hence increased synaptic activity induces NF-κB pathway activation (Romano et al. 2006). Moreover, constitutive NF-κB activity was described in hippocampal neurons *in vivo* as well as *in vitro* (Bhakar et al. 2002; Schmidt-Ullrich et al. 1996).

2. NF-κB: relevance to plasticity and adult neurogenesis

Initially NF-κB family was described to have a role in proliferation, survival, differentiation, migration and apoptosis of neurons during development and upon

neuronal activity (Bortolotto et al., 2014). Recently, multiple studies showed that many synaptic plasticity events regulate the NF-κB subcellular distribution, DNA binding activity, and transcription. Indeed NF-κB activation and/or inhibition regulate neurite outgrowth, arborization and guidance (Gavalda et al. 2009; Salles et al. 2014). Therefore, NF-κB transcription factors have a central role in translating synaptic events into gene expression changes that might have a longer duration (Kaltschmidt and Kaltschmidt, 2009).

Our group was among the first to demonstrate that NF-κB family members are expressed in the adult active neurogenic zone SVZ and SGZ (Denis-Donini et al., 2005; Denis-Donini et al., 2008; Meneghini et al., 2010). NF-κB signaling pathway is involved in the different steps of the neurogenic process including proliferation, survival, differentiation and migration of embryonic and adult newly generated neurons (Widera et al. 2008; Denis-Donini et al., 2008; Zhang and Hu 2012). Upstream ligands and receptors that activate NF-κB could affect adult neurogenesis. One of the first studies showed that VEGF directly promotes adult NSC survival and hippocampal-dependent memory via NF-κB downstream effectors (Licht et al., 2011). Therefore, growth factors in physiological conditions and in injuries are able to increase adult neurogenesis through NF-κB transient activation. Our group demonstrated that receptors for advanced glycation end-products (RAGE) are expressed by hippocampal NSC and NPCs. Upon RAGE engagement, the NF-κB canonical pathway is activated and NF-κB dimers are translocated to the nucleus. Several RAGE ligands such as alarmin HMGB-1, S100β, and AGE-BSA, stimulated both proliferation and neuronal differentiation of SVZ-derived NPC *in vitro*. This effect was blocked by SN-50 that inhibits the nuclear translocation of NF-κB subunits (Meneghini et al., 2010). Moreover, mice lacking for NF-κB p50 ($p50^{-/-}$) subunits were developed by David Baltimore in 1995 to study the role of p50 in the immune system (Sha et al., 1995). Interestingly active NF-κB was associated with increased proliferation of $nestin^{+}/Sox\text{-}2^{+}$ NPC

and early neuronal differentiation in the SVZ (Zhang et al., 2012). Since our group already showed that NF- κB p50 subunit is expressed in the SGZ, this model was a huge advantage to study the role of p50 in adult hippocampal neurogenesis. Our group was able to show that p50KO mice have a net reduction in the newly generated neurons in the hippocampal SGZ (Fig. 13). Reduced ahNG was correlated to a selective impairment in hippocampal-dependent short-term spatial memory (Denis-Donini et al., 2008).

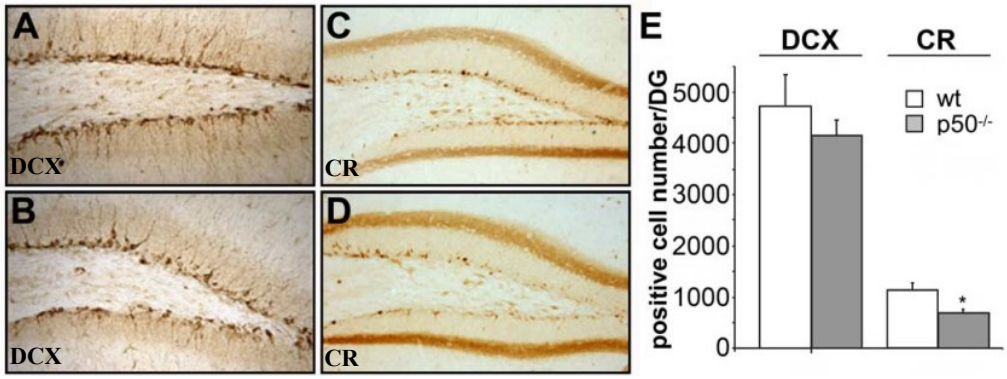


Figure 13: Reduction of newborn neurons in NF-κB p50KO mice. A-B Representative images of doublecortin (DCX, marker of immature neurons) in the DG of WT (**A**) and p50KO mice (**B**). **C-D** Representative images of Calretinin (CR, marker of mature neurons) in WT (**C**) and p50KO mice (**D**). **E.** Quantification of total number of DCX^+ and CR^+ cells in the DG of both genotypes. *(Denis-Donini et al., 2008)*

Other groups also showed that treatment with SN50 resulted in impaired memory consolidation in mice (Lubin et al., 2007). NF-κB downstream target genes such as monocyte chemoattractant protein-1 (MCP-1) stimulates the migration of newly generated neurons in adult rat SGZ (Widera et al., 2004). Using the same approach our group showed that Aβ 1−42 oligomers and HMGB-1, both involved in Alzheimer's disease, could promote neuronal differentiation by the activation of RAGE/NF-κB axis thus consolidating the role of NF-κB in neurogenesis (Meneghini et al., 2013). Other groups also showed the role of NF-κB in synaptic

plasticity and memory. p50KO mice showed decreased LTP (the main electrophysiological parameter used to study the formation and consolidation of memory formation) and long term memory alterations in MWM test (Overstreet and Wegener, 2013).

Many studies showed that NF-κB signaling pathway contribute to neural plasticity (Mattson, 2005) and cognitive tasks (Meffert and Baltimore, 2005). Synaptic plasticity and adult neurogenesis are reduced in depression therefore we could hypothesize that NF-κB could also be involved in reduced neurogenesis observed in MDD. Indeed NF-κB could be the way for certain antidepressants to exert their antidepressant activity via increased neurogenesis (Pittenger and Duman, 2008; Hanson et al., 2011). Our lab also participated in showing that members of NF-κB are important contributors in the SGZ neurogenic response to multiple clinically relevant drugs with antidepressant-like activity (Bortolotto et al., 2014; Meneghini et al. 2014; Cuccurazzu et al., 2013).

Chapter II

OUTLINE OF THE THESIS

Outline of the thesis

Adult hippocampal neurogenesis (ahNG) is a peculiar form of neural plasticity involved in cognition, mood regulation and stress response (Aimone et al., 2010, 2014; Eisch and Petrik, 2012). The neurogenic niche of the hippocampus, located in the subgranular zone (SGZ), is a microenvironment where neural stem cells (NSCs) are logged, proliferate and start differentiation (Alvarez-Buylla et al., 2004, Quinones-Hinojosa at al., 2006). Within the niche NSCs differentiation could be regulated by astrocytes released factors such as neurotransmitters, trophic factors and several neuro-modulatory proteins (Newman, 2003; Liauw et al., 2008; Song et al., 2002). AhNG is profoundly deregulated in several neurodegenerative and neuropsychiatric disorders where also astrocytes had altered phenotype and function (Pekny et al., 2016). The mechanisms by which astrocytes control adult hippocampal neural progenitor cells (ahNPC) are still poorly understood. The discovery and characterization of potential pro-neurogenic or anti-neurogenic molecules and their downstream signaling is still an open field of research. In previous work our laboratory showed that NF-κB p50 is a crucial signaling for ahNPC differentiation (Bortolotto et al., 2014). Indeed NF-κB p50 knockout (KO) mice had reduced late neuronal maturation correlated with selective defects in hippocampal-dependent short-term memory (Denis-Donini et al., 2008). *In vitro,* ahNPC from wild type (WT) and p50KO mice are not significantly different in their neurogenic potential. These observations triggered us to study possible cell-autonomous and non cell-autonomous defects caused by NF-κB p50 depletion on both astrocytes released factors and ahNPC differentiation. In chapter III we present a published work where we studied the effect of astrocytes released factors in astrocytes conditioned medium on ahNPC differentiation. In this *in vitro* work, we focused on studying the role of NF-*κ*B transcription factors, specifically the absence of NF-*κ*B p50 subunit in: (i) WT and p50KO ahNPC differentiation and response to medium deriving from WT and p50KO astrocytes, (ii) potential

differences between WT and p50KO astrocytes released factors, (iii) the neurogenic effect of differentially released factors from WT and p50KO astrocytes on ahNPC and (iv) proneurogenic molecules receptors expression in ahNPC.

Even though NSCs reside in a microenvironment their behavior could be also modulated by exogenous factors such as stress (Kempermann, 2002) and pharmacological treatment (Aimone et al., 2014). While ahNPC proliferation and differentiation is downregulated after acute and chronic stress in animal models of major depressive disorder (MDD) (Kempermann and Kronenberg, 2003; Pittenger and Duman, 2007), the pharmacological treatment with antidepressants such as selective serotonin reuptake inhibitors (SSRI), serotonin and noradrenaline reuptake inhibitors (SNRI), restore stress depleted ahNG in these rodent models (Eisch and Petrick, 2012). Our interest in antidepressants effect on ahNG was even more substantiated when their modulation of ahNG was confirmed also in postmortem human brain. Interestingly, the number of proliferative cells is increased in the SGZ of patients diagnosed for MDD and treated with SSRI (Boldrini et al., 2009). The neurogenic hypothesis of depression stated that "increased adult hippocampal neurogenesis is required for the onset of antidepressants therapeutic action" (Eisch and Petrick, 2012). Notably, more complexity was added to this hypothesis with the discovery that different serotonin receptors targeted by antidepressants have distinct and even opposite role in ahNG (Klempin et al., 2010). Trazodone is a serotonin antagonist and reuptake inhibitor (SARI) antidepressant (Stahl, 2009). Our interest in trazodone lies in its peculiar binding affinities to different serotonin and adrenergic receptors and its activity as an agonist or antagonist. Trazodone is a well-tolerated drug with also several off-label uses including hypnosis. It is still not known if trazodone is a proneurogenic multimodal antidepressant and how its multimodal activity on serotonin receptors could mediate its potential effect. In chapter IV we present a work under preparation for publication where we focused on: (i) investigating the effect of

trazodone on ahNPC neuronal differentiation, (ii) evaluated the mechanism by which trazodone exert its effect and (ii) studied potential downstream signaling required for trazodone activity.

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Chapter III

CHAPTER III

Cell autonomous and non cell-autonomous role of NF-κB p50 in astrocyte-mediated fate specification of adult neural progenitor cells.

Suzana Cvijetic[1,2]*, Valeria Bortolotto[1,2]*, Marcello Manfredi[4], Elia Ranzato[3], Emilio Marengo[3,4], Rita Salem[1,2], Pier Luigi Canonico[2], Mariagrazia Grilli[1,2#]

[1]Laboratory of Neuroplasticity, [2]Dipartimento di Scienze del Farmaco, Università del Piemonte Orientale, 28100 Novara, Italy; [3]Dipartimento di Scienze ed Innovazione Tecnologica, Università del Piemonte Orientale, 15121 Alessandria, Italy; [4]ISALIT srl - Dipartimento di Scienze ed Innovazione Tecnologica, Università del Piemonte Orientale, 15121 Alessandria, Italy.

*Equal contributors

[#]Corresponding author: Mariagrazia Grilli, MD, Laboratory of Neuroplasticity, Dipartimento di Scienze del Farmaco, University of Piemonte Orientale, Via Bovio, 6 28100 Novara Italy. Phone number: +39 0321 375828 Email: mariagrazia.grilli@uniupo.it

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Main points. NF-κB p50 absence in astrocytes reduces the pro-neurogenic effects of their soluble signals on ahNPC. p50 absence in ahNPC impairs their responsiveness to astrocyte-derived signals. Lipocalin-2 is a novel astrocyte-derived proneurogenic molecule.

Key words: Adult neurogenesis, hippocampus, neuronal differentiation, lipocalin-2, α2δ1.

Abstract

In previous work, we demonstrated that NF-κB p50 acts as crucial regulator of adult hippocampal neural progenitor cells (ahNPC). Indeed, NF-κB p50 knockout (KO) 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 pro-neurogenic 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 some 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 pro-neurogenic effect was greatly reduced in p50KO NPC, where LCN-2 receptor gene expression appeared downregulated. In addition to that, 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 another phenotypic change occurring in ahNPC in absence of p50. Altogether our data suggest that dysregulated NPC-astrocyte communication may contribute to reduced hippocampal neurogenesis in p50KO mice *in vivo.*

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 (Bond et al., 2015; Kempermann et al., 2015; Ming and Song, 2005). Recent studies also suggested an additional neurogenic region in the adult hypothalamus (Kokoeva et al., 2005).

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; Eisch and Petrik, 2012; Lacar et al., 2014). Although complex, the molecular mechanisms that modulate neurogenesis deserve investigation since this may turn into a better understanding of ahNG in brain homeostasis and in neuropsychiatric/neurodegenerative disorders where this process is profoundly deregulated (Apple et al., 2016; Winner et al., 2011).

Within the CNS, NF-κB pathway activation has been involved in a wide range of functions both under physiological and pathological conditions (Kaltschmidt and Kaltschmidt, 2009; Mattson and Meffert, 2006; Oikawa et al., 2012). 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 (Meneghini et al., 2010, 2013; Rolls et al., 2007; Wada et al., 2006; Widera et al., 2008; Zhang et al., 2012).

Since the initial observation of NF-κB presence 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 (Bortolotto et al., 2014; Grilli and Meneghini, 2012). 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 pro-neurogenic drugs (Valente et al., 2012). *In vivo* we also proved that p50KO mice display a dramatic reduction in adult hippocampal neurogenesis which correlated with a selective defect in hippocampal-dependent short-term memory (Denis-Donini et al., 2008). Interestingly, *in vivo* and *in vitro* the proliferation rate of hippocampal NSC/NPC in p50KO mice appeared to be similar to that of WT mice. Moreover, 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 late maturation of newly generated neurons (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. Among others, a pivotal work demonstrated that astroglial cells which 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), 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 aNPC 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 centrifugation 5 min at 110 x *g*, 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 47,000 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 (LCN2, Cell Signaling), 2 µg/ml purified human Thrombospondin-1 (hTSP1, 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 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 DIV), 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$^+$. Astrocyte-conditioned medium (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 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 as stationary phase a Halo Fused C18 column (0.5 x 100 mm, 2.7 µm; Eksigent Technologies Dublin, USA), interfaced with a Triple-TOF 5600+ system (AB Sciex, Concord, Canada) 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 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 (Geromanos et al., 2009; Gillet et al., 2012; Venable et al., 2004). 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, Concord, Canada). Three replicates for each sample were subjected to the DIA analysis. The mass spectrometry files were searched using Protein Pilot (AB SCIEX, Concord, Canada) and Mascot (Matrix Science Inc., Boston, USA) with the following parameters: cysteine alkylation, digestion by trypsin, no special factors and False Discovery Rate at 1% for Protein Pilot. 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 cysteines as fixed modification and oxidized methionine as variable modification. The search was conducted using the UniProt Swiss-Prot database containing 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 (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 [50mM Tris-HCl pH 7.5, 150mM NaCl, 0.5mM EDTA pH 8, 1% (vol/vol) Triton X-100, 1% (wt/vol) SDS, 10mM NaF, 1mM $NaVO_4$, 1mM 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 (25µg) 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).

Quantitative reverse transcriptase PCR (qRT-PCR). RNA was extracted and cDNA generated using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Penzberg, Germany). qRT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad), and the CFX384 Real-Time PCR Detection System (Bio-Rad). The following qRT-PCR primer pairs were used (KiCqStart® SYBR® Green Primers Predesigned): for LCN-2 receptor (LCN-2R/24p3R), 5′-CATTATGGCTCTTCGGTTTC-3′ (forward), 5′-TAGAAATCGCCAGTCCTTAG-3′ (reverse); for β-actin, 5'-GATGTATGAAGGCTTTGGTC-3' (forward), 5'-TGTGCACTTTTATTGGTCTC -3' (reverse). LCN-2R/24p3R expression levels were normalized against β-actin as housekeeping gene and their relative ratio was calculated.

Statistical analysis. All experiments were run in triplicates using different cell preparations and repeated at least three times. Data were calculated 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 Tukey'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 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 chamber slides, upon removal of growth factors and exposure to a serum-free defined medium (STD medium), NPC stop dividing and spontaneously differentiate toward both neuronal and glial lineages. 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 MAP-2$^+$/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 (mean % ± S.D. of MAP-2$^+$/nestin$^-$ cells: 3.1 ± 0.6 and 3.3 ± 0.8 for WT and p50KO NPC; MAP-2$^+$/nestin$^+$ cells: 32.2 ± 3.2 and 33.1 ± 2.2 for WT and p50KO NPC; Fig. 1). When exposed to STD medium in absence of growth factors, ahNPC spontaneously differentiate also toward the astrocytic lineage (Meneghini et al., 2013). The percentage of astrocytes (GFAP$^+$ cells) generated *in vitro* by WT and p50KO NPC was not different (mean % ± S.D. of GFAP$^+$ cells: 16 ± 1.7 and 14.2 ± 2.6 for WT and p50KO NPC; Fig. 1). Finally, no significant difference in the basal apoptotic rate could be observed between the two genotypes (11.8 ± 6.5 and 12.4 ± 5.9 % for WT and p50KO NPC, respectively).

Effect of WT- and p50KO-derived astrocyte-conditioned media (ACM) on neuronal differentiation of WT ahNPC cultures.

We investigated the possibility that non cell-autonomous effects were contributing to remarkably reduced hippocampal neurogenesis in p50KO mice (Denis-Donini et al., 2008).

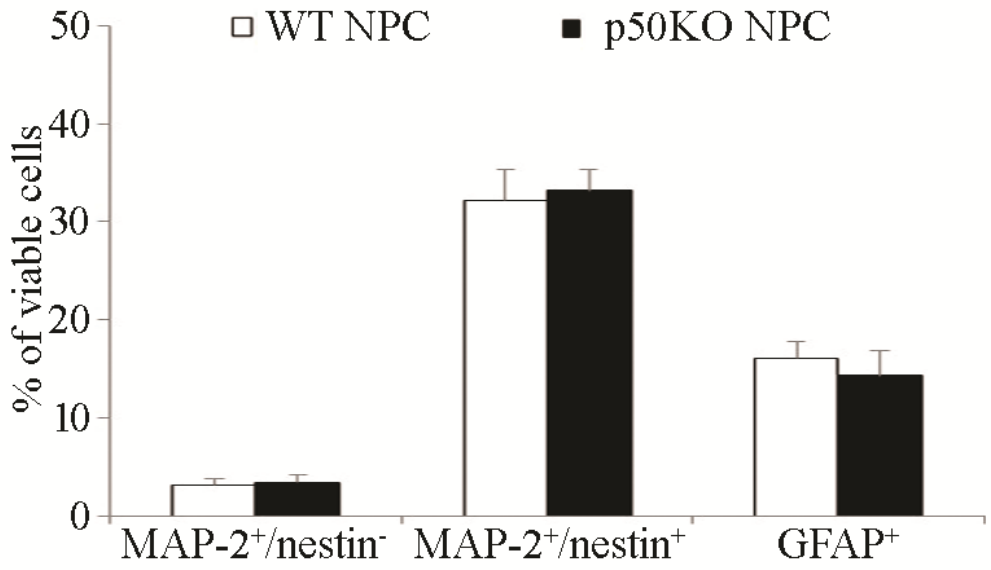

FIGURE 1. Adult hippocampal NPC derived from WT and p50KO mice show similar in vitro neuronal and glial differentiation rates. Under differentiating conditions, WT and p50KO ahNPC spontaneously give rise to similar percentages of MAP-2$^+$/nestin$^-$ neurons, MAP-2$^+$/nestin$^+$ neuroblasts and GFAP$^+$ astrocytes. Data are expressed as mean % ± S.D. of n = 4 experiments, run in triplicates, and analyzed by Student's *t*-test.

Since soluble factors released by astrocytes have been shown to modulate differentiation of NPC (Song et al., 2002), we evaluated their effect on the differentiation rate of ahNPC toward neuronal and non-neuronal lineages. Primary mixed glial cultures were prepared from hippocampi 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) 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 neuronal 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 (% increase over STD medium-treated cells: +93.8 ± 43.3 for MAP-2^+/$nestin^-$ cells, $P < 0.001$; +36.6 ± 23.3 for MAP-2^+/$nestin^+$ cells, $P < 0.001$; Fig. 2A, B). Under the same *in vitro* experimental conditions, no significant difference was observed between WT NPC treated with STD medium or p50KO ACM on both neuron and neuroblast subpopulations (Fig. 2A, B). To investigate whether ACM could affect cell survival in addition to neuronal differentiation, we analyzed the apoptotic rate of ahNPC and their progeny. No difference in the percentage of apoptotic cells was observed in the different media conditions (Fig. 2C).

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

We then tested the effect of ACM on astroglial differentiation of ahNPC. 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 (% increase over STD medium: +130.2 ± 28.1 and +180 ± 44.8 for WT and p50KO ACM, respectively; $P < 0.001$; Fig. 3A). Actually, in our experimental setting p50KO ACM was more effective than WT ACM in promoting astrogliogenesis (Tukey post hoc, $P < 0.01$; Fig. 3A). 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 STD, WT ACM and p50KO ACM (Fig. 3B).

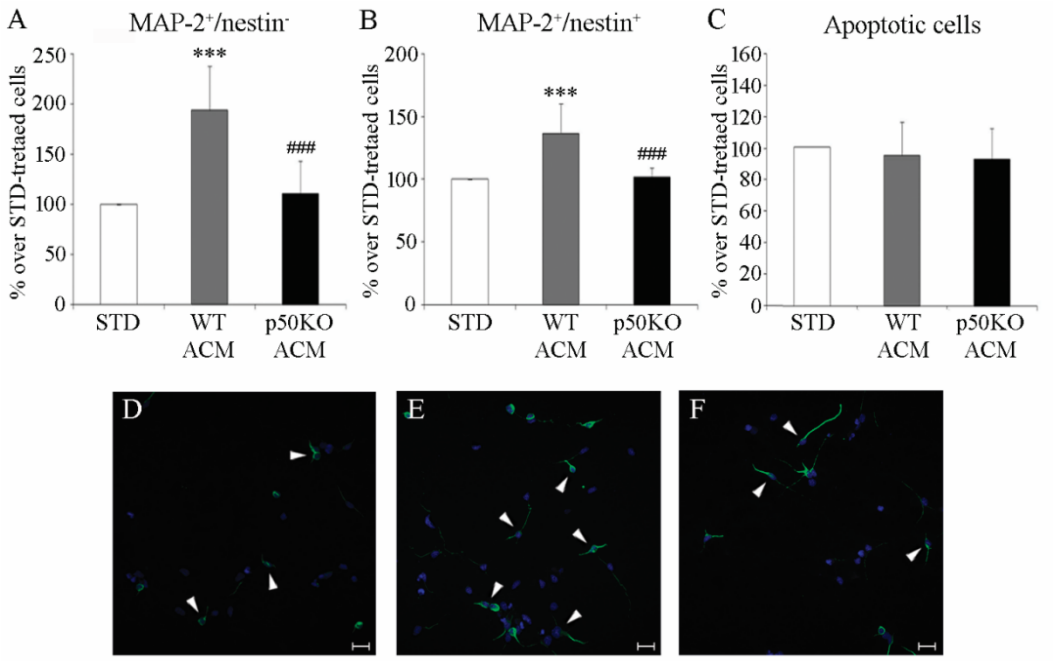

FIGURE 2. WT, but not p50KO ACM, promote neuronal differentiation of WT ahNPC cultures. (**A-B**) Under differentiating conditions, 24h treatment of WT hippocampal NPC with WT ACM significantly increased the percentage of MAP-2^+/$nestin^-$ neurons (**A**) and MAP-2^+/$nestin^+$ neuroblasts (**B**) compared with standard differentiation medium (STD). Conversely, p50KO ACM was devoid of any pro-neurogenic 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 MAP-2^+ cells (green, white arrowheads) 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 were calculated 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; $^{###}P < 0.001$ versus WT ACM. Data are expressed as % of increase over STD treated cells.

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 KO NPC, and their effect was indistinguishable from that of STD medium (Fig. 4A, B). In addition, unlike WT NPC, KO NPC did not even respond to the astrogliogenic effects of WT and KO ACM (Fig. 4C). 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 (Fig. 4D). Finally, also in p50KO cultures apoptotic rates were not different in presence of the different media conditions (*data not shown*).

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

RAGE is functionally expressed by ahNPC (Meneghini et al., 2010) and its activation by astrocytes released molecules, like HMGB-1 and S100B, promotes neuronal differentiation of ahNPC (Meneghini et al., 2013). In order to understand if the activation of RAGE could mediate the pro-neurogenic activity of WT ACM on WT ahNPC we pre-treated cultures with a neutralizing anti-RAGE antibody (α-RAGE Ab, 20 μg/ml). α-RAGE Ab was not able to block the increase of MAP-2^+ cells induced by WT ACM (% increase over STD medium: +70.07 ± 24 for WT ACM, $P < 0.001$; +50.6 ± 27.8 for WT ACM + α-RAGE Ab, $P < 0.01$; Fig. 5A).

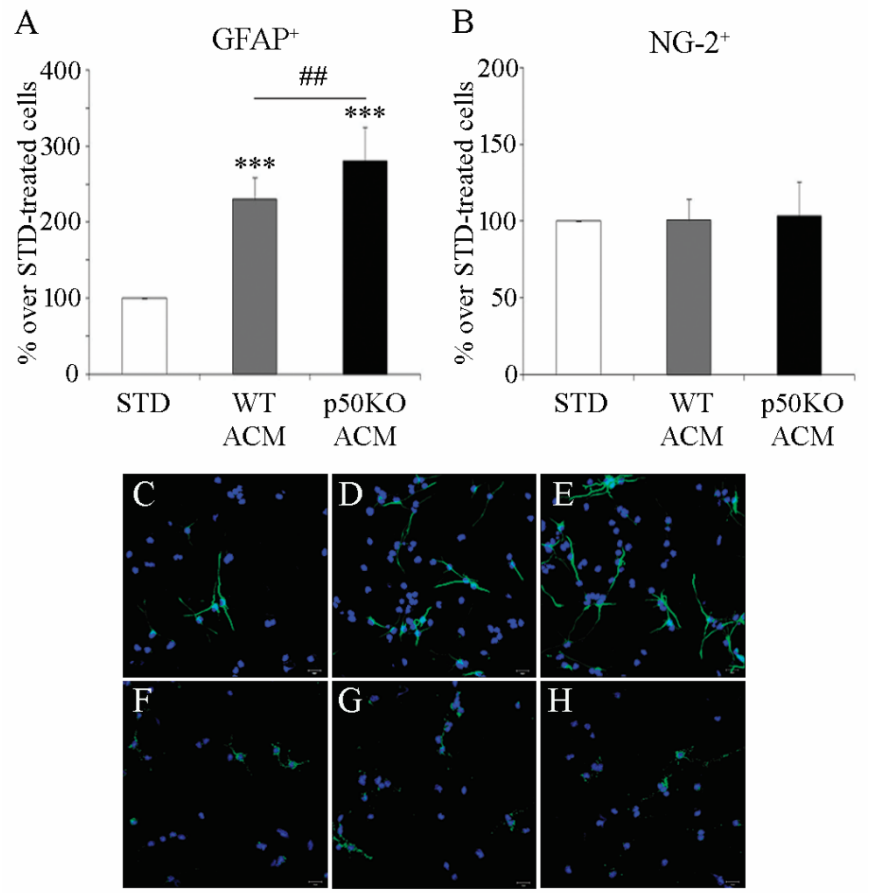

FIGURE 3. Both WT and p50KO ACM promote astroglial differentiation of WT ahNPC. (**A**) 24h treatment of WT ahNPC with WT and p50KO ACM significantly increased the percentage of GFAP$^+$ astroglial cells, compared with STD medium. p50KO ACM was more effective on astrogliogenesis than WT ACM. (**B**) No significant differences were observed in the percentage of NG-2$^+$ cells upon exposure to 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 were calculated 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; $^{\#\#}P < 0.01$ versus WT ACM. Data are expressed as % of increase over STD treated cells.

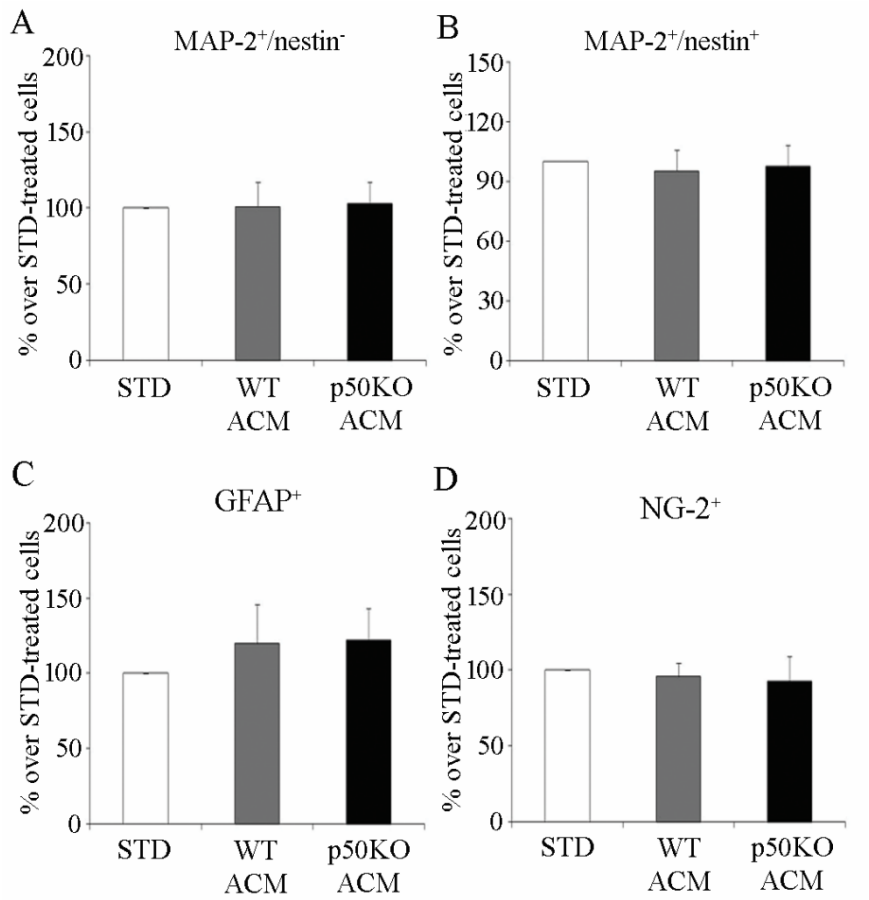

FIGURE 4. p50KO ahNPC are unresponsive to neuronal and astroglial differentiation signals from both WT and p50KO astrocytes. (**A-B**) Under differentiating conditions, 24h treatment of p50KO hippocampal NPC with WT and p50KO ACM did not affect the percentage of MAP-2$^+$/nestin$^-$ neurons (**A**) and 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.

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

Thrombospondin-1 (TSP-1) is an astrocyte-derived pro-neurogenic 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). 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 (% increase over vehicle: +71.7 ± 29.5 in TSP-1-treated cells; $P < 0.001$; Fig. 5B). Conversely, TSP-1 treatment was ineffective in p50KO NPC (Fig. 5B). Then 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%; $P < 0.05$, Fig. 5C).

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 HPCL 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, three proteins which were 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). Fold-change increase of each identified protein in p50KO ACM *vs* WT ACM is reported in Table 1.

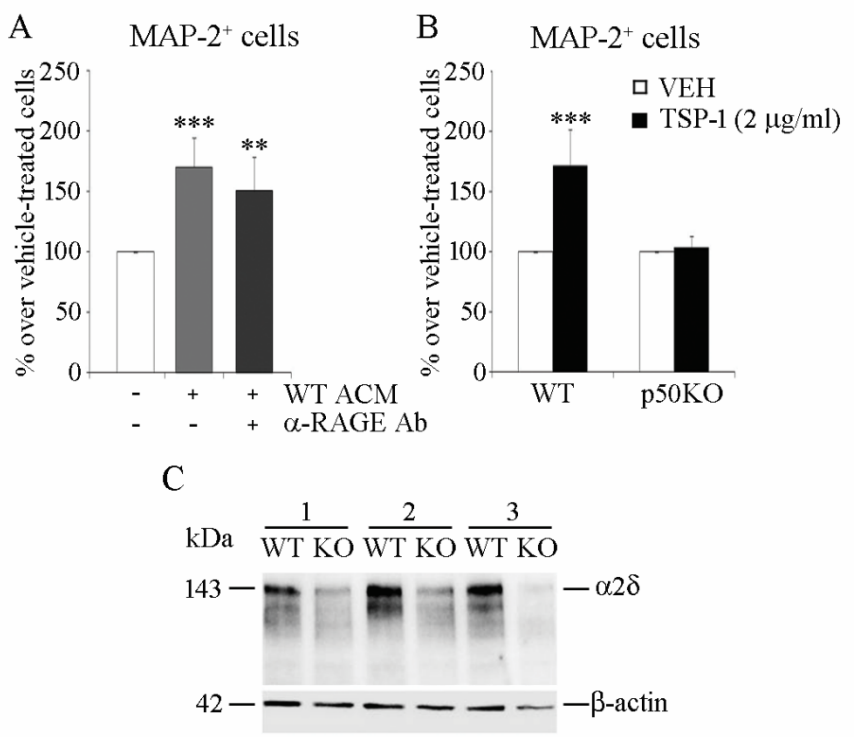

FIGURE 5. p50KO NPC are defective in their pro-neurogenic response to thrombospondin-1 and display downregulated expression of α2δ1. (**A**) RAGE activation does not mediate the proneurogenic effects of WT ACM. Pretreatment with a neutralizing anti-RAGE antibody (20 μg/ml) does not counteract the pro-neurogenic effects of WT ACM on WT ahNPC, as assessed by MAP-2$^+$ counting. (**B**) Under differentiating conditions, 24 h treatment of WT NPC with human recombinant TSP-1 (2 μg/ml) significantly increased the percentage of MAP-2$^+$/nestin$^-$ cells, compared to vehicle. No effect was observed in TSP-1-treated p50KO NPC. Data have been calculated as mean values ± S.D. of n = 4 experiments, run in triplicates. $^{***}P < 0.001$ versus vehicle (Tukey's *post hoc* test). Data are expressed as % of increase over vehicle-treated cells. (**C**) 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.

Lipocalin-2 is an astrocyte-derived pro-neurogenic 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 upregulated protein as a potential contributor to the absence of pro-neurogenic effects by p50KO ACM. We tested recombinant mouse LCN-2 (0.01-1 μg/ml) initially on differentiating WT ahNPC. To our surprise LCN-2 promoted, in a concentration-dependent manner, neuronal differentiation of WT NPC, as assessed by counting the percentages of MAP-2^{+}/$nestin^{-}$ cells generated in presence of recombinant protein or vehicle (One-way ANOVA; $P < 0.001$, Fig. 6A). Maximal pro-neurogenic effect was elicited in presence of 0.1 μg/ml LCN-2 (% increase over vehicle-treated cells: +86.78 ± 29.18, $P < 0.001$; Fig. 6A). When tested on p50KO NPC LCN-2 was ineffective up to 0.3 μg/ml and only 1 μg/ml of protein significantly increased the percentage of newly generated neurons (% of increase over vehicle-treated cells: +70.27 ± 31.1, $P < 0.001$; Fig. 6A). Under the same concentration range, LCN-2 had no effect on cell survival in both genotypes (Fig. 6B). We then evaluated mRNA levels for LCN-2 receptor (24p3R) and β-actin in WT and p50KO NPC by qRT-PCR. qRT-PCR analysis showed that LCN-2R/24p3R gene expression is markedly reduced in p50KO compared to WT cells (% reduction: -64.4 ± 2.4, Student's *t*-test: ** $P < 0.01$; Fig. 6C).

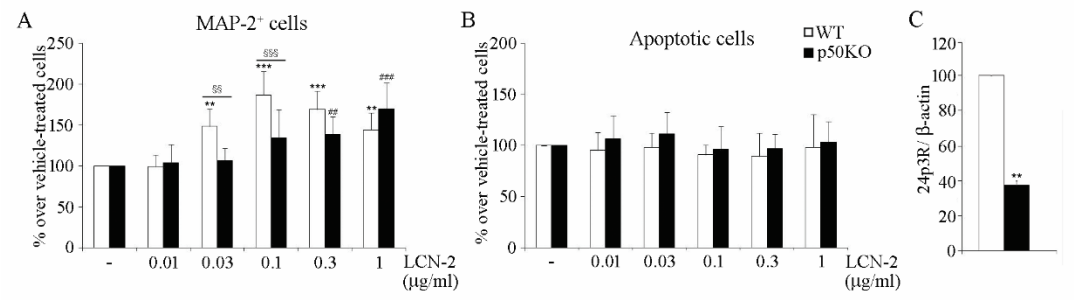

FIGURE 6. Lipocalin-2 is a novel astrocyte-derived pro-neurogenic signal whose activity is reduced in p50KO NPC. (**A**) 24 h treatment with mouse LCN-2 (0.01-1 µg/ml) promoted neuronal differentiation of WT ahNPC in a concentration-dependent manner. Under the same experimental conditions, p50KO NPC responsiveness to LCN-2 is significantly reduced. (**B**) LCN-2 has no effect on the survival rate of WT and p50KO NPC and their progeny. Data were calculated 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. $^{**}P < 0.01$, *** $P < 0.001$ versus WT vehicle; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus p50KO vehicle; $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ in WT vs P50KO NPC. Data are expressed as % of increase over vehicle-treated cells. (**C**) QRT-PCR evaluation of LCN-2R/24p3R expression levels in WT and p50KO NPC. LCN-2R expression levels were normalized against β-actin as housekeeping gene and their relative ratio was calculated. LCN-2R expression was downregulated in p50KO NPC. ** $P < 0.01$ vs WT NPC, Student's *t*-test.

Discussion

In recent years, experimental data on the involvement of NF-κB proteins in the regulation of adult neurogenesis have been generated (as reviewed in Grilli and Meneghini, 2012; Bortolotto et al., 2014). A few years ago, our group 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 strongly

suggested that lack of p50 is associated with defects in the late maturation of newly generated neurons, in absence of alterations in survival and proliferation rates of ahNPC (Denis-Donini et al., 2008). As a follow up to that work, herein we show that when cultured *in vitro,* ahNPC from WT and p50KO mice are not significantly different in their neurogenic potential. By using double immunocytochemistry, we could distinguish two distinct $MAP2^+$ cell populations which are generated *in vitro* by ahNPC: $MAP2^+/nestin^+$ cells, which may be indicative of NPC commitment toward the neuronal lineage and $MAP2^+/nestin^-$ cells, which may better reflect neuronal maturation. Under basal conditions we could not observe significant differences in the two populations between WT and p50KO NPC cultures. Altogether we concluded that absence of NF-κB p50 did not correlate *in vitro* with alteration in NPC commitment to neuronal lineage or with maturation of newly generated neurons, as observed *in vivo* (Denis-Donini et al., 2008).

Since the well documented role of NF-κB in non-neuronal cells (Brambilla et al., 2009; Lian et al., 2015), we dissected the role of p50 in 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 influence of astroglia-generated soluble factors on NPC fate specification and the cell-autonomous or non cell-autonomous role played by p50 signaling in that context. Initially we tested the effects of WT and p50KO ACM 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 (Barkho et al., 2006; Chang et al., 2003). Interestingly, when ahNPC were exposed to p50KO ACM we could not observe pro-neurogenic 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.

One interpretation for the lack of proneurogenic effects elicited by p50KO ACM is that absence of the NF-κB subunit could directly affect the secretory profile of astrocytes. It could be hypothesized that, in absence of NF-κB p50, primary astrocytes either become defective in the production of pro-neurogenic molecule(s) or, alternatively, produce antineurogenic molecule(s). In such context, direct and indirect effects of p50 absence could be envisioned. p50 absence may indeed affect transcription of genes encoding for astrocyte-secreted molecules. This could occur as a consequence of the fact that target gene(s) transcription is repressed by p50 homodimers (in that case the gene would become overexpressed) or because, in absence of p50, a profound rearrangement occurs in the available pool of NF-κB transcriptional dimeric complexes, which may in turn result in activation and/or repression of target gene(s). Of course, we cannot even exclude that p50 absence may affect astrocyte secretory profile also by altering the expression of genes encoding proteins which may influence the activity of secreted proteins via posttranscriptional modifications, i.e kinases, phosphatases, proteases. Although unlikely, p50 absence may even affect astrocyte secretory pathways or transcription of proteins directly participating in secretory mechanisms. Of course, we do not disregard the possibility that NF-κB p50 absence may change, in addition to their secretory profile, other phenotypic features of astrocytes, including receptor expression and intracellular signaling pathways, but we did not experimentally address this.

We also tested the effect of WT and p50KO ACM on p50KO NPC differentiation. Surprisingly, neither WT nor p50KO ACM promoted neuronal and astroglial differentiation of p50KO NPC over basal conditions, suggesting that in absence of p50 cell autonomous changes may also occur and affect responsiveness of ahNPC to astrocyte-derived pro-neurogenic and astrogliogenic signals.

In search for phenotypic changes in p50KO NPC which may explain their reduced responsiveness to astrocyte-derived pro-neurogenic signals we took into

consideration the possibility that these cells may be deficient in membrane receptors for astrocyte-derived pro-neurogenic molecules. RAGE, the Receptor for Advanced Glycation End-products, is expressed by adult NPC where it mediates the pro-neurogenic effects of several molecules like HMGB-1 and S100B which are secreted by astrocytes (Meneghini et al., 2010, 2013). Receptor neutralization had no effect on WT ACM pro-neurogenic effects, suggesting that RAGE activation was not involved in ACM-mediated neuronal differentiation of ahNPC. Thrombospondin-1 (TSP-1) is also an established astrocyte-derived pro-neurogenic factor (Lu and Kipnis, 2010). Our group demonstrated that α2δ1, a TSP-1 receptor (Eroglu et al., 2009), is functionally expressed by ahNPC (Valente et al., 2012). Interestingly, α2δ1 expression levels were significantly reduced in p50KO ahNPC, compared to their WT counterpart. In line with this observation, 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 pro-neurogenic 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 pro-neurogenic effects of KO ACM. LC-MS/MS allowed us to identify proteins differentially expressed in WT and p50KO ACM. More specifically, three molecules were upregulated in p50KO compared to WT ACM, namely C-C motif chemochine-2 (CCL2), neutrophil gelatinase-associated/lipocalin-2 (NGAL/LCN-2), H-2 class I Histocompatibility antigen, K-K alpha chain (HA1K). Interestingly, absence of NF-κB p50 is commonly linked to inflammation in periphery and in brain (Bernal et al., 2014; Rolova et al., 2014) and both CCL-2 and LCN-2 share an established role in inflammation. 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 (Bu et al., 2006; Kohda et al., 2014; Ueda et

al., 1994). We hypothesized that one of these proteins may indeed exert anti-neurogenic activities on NPC and we focused our attention on LCN-2.

LCN-2 is a 24 kDa iron-related protein 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 concentrated our attention on this protein, whose expression is increased by about threefold in p50KO compared to WT ACM. To our surprise, LCN-2 promoted, in a concentration-dependent manner, neuronal differentiation of WT NPC. In contrast with previous observations that LCN-2 can promote apoptosis of neurons and astrocytes (Lee et al., 2012), in our cellular model and within the tested concentration range the protein had no negative effect on the survival of ahNPC and their progeny. 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. Such reduced sensitivity correlates with downregulation of LCN-2R/24p3R mRNA levels in p50KO ahNPC. Although it has been demonstrated that LCN-2R is constitutively expressed at high levels in brain, including the hippocampus (Chia et al., 2015), to our knowledge this is the first demonstration that this receptor is expressed in aNPC. Interestingly, NF-κB signaling activation has been shown to occur downstream of LCN-2/24p3R activation (Dizin et al., 2013).

At this stage of knowledge, we have not yet identified a culprit for reduced pro-neurogenic effects of p50KO ACM on WT NPC. As far as CCL-2, literature data suggested both pro-neurogenic (Liu et al., 2007) and antineurogenic effects (Lee et al., 2013) of this chemokine. In our cellular model also CCL-2 exerted pro-

neurogenic activity (*data not shown*), suggesting that upregulated expression of this chemokine cannot justify reduced pro-neurogenic effects of p50KO ACM on WT NPC. 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 demonstrated in other cell types (Zitvogel et al., 1998), in the future we would like to explore the possibility that extracellular exosomes/microvesicles of astrocytic origin could contribute, with their cargo, to ACM composition and, potentially, to astrocyte-NPC communication. The proteins that we identified in ACM by LC-MS/MS is likely to represent a small fraction of all astrocyte secreted proteins, so we may have missed other contributors to the reduced proneurogenic potential of p50KO ACM. Among NF-κB target genes that are expressed in astrocytes and whose product is secreted there are indeed many additional inflammatory and anti-inflammatory cytokines and chemokines (for example, IL-1, IL-6, IL-8, IL-10, IL-12, IL-23, TNF) (Choi et al., 2014), as well as growth factors like BDNF (Marini et al, 2014). The role of BDNF in neurogenesis is well established, but preliminary experiments in our laboratory did not reveal significant differences in its expression levels or its maturation process between WT and p50KO astrocytes (*data not shown*).

An additional explanation for reduced pro-neurogenic effects of p50KO ACM on WT NPC could be that p50KO ACM components may negatively affect expression or functionality of receptor(s) for astrocyte-derived pro-neurogenic molecules on WT NPC. Since this event occurs in 24 h, internalization/downregulation or posttranslational modifications of receptors may potentially take place in WT NPC when exposed to p50KO ACM.

In our experimental setting p50KO NPC were not only unresponsive to pro-neurogenic but also to astrogliogenic astrocyte-derived signals. In the future, we

may also attempt identifying the nature of astrocyte-secreted signals that promote astrogliogenesis and of the complex cell-autonomous changes that take place in NPC in absence of p50. We cannot exclude that the inability of p50KO NPC to respond to both pro-neurogenic and astrogliogenic signals may also depend on p50-requirement downstream of ACM treatment.

Altogether we propose that reduced hippocampal neurogenesis in p50KO mice *in vivo* could be ascribed to complex defects in the cross-talk between astrocytes and ahNPC. Of course, we cannot exclude the contribution of other cell phenotypes to reduced hippocampal neurogenesis in p50KO mice and in particular of microglial cells which are well known to affect NPC fate specification (Sierra et al., 2014). Our experimental work demonstrates that complex cell autonomous and non cell-autonomous changes can affect NPC fate specification in absence of NF-κB p50. We also show, for the first time, that LCN-2 is an astroglial-derived signal which promotes neuronal fate specification of ahNPC and whose activity, in parallel with a downregulation of LCN-2R, is strongly reduced in p50KO NPC. Altogether, these data add further complexity to a growing body of data suggesting the relevance of astrocytes and NF-κB signaling in the modulation of adult neurogenesis.

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Notes. Conflict of Interest: none declared

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Chapter IV

CHAPTER IV

TRAZODONE AS A NOVEL PRONEUROGENIC ANTIDEPRESSANT

[ACS Chemical Neuroscience]

Valeria Bortolotto[1,2], Rita Salem[1,2], Michele Bianchi[2], Erika del Grosso[2], Pier Luigi Canonico[2], Mariagrazia Grilli[1,2#]

[1]Laboratory of Neuroplasticity and Pain,[2]Department of Pharmaceutical Sciences, University of Piemonte Orientale, Novara, Italy.

[#]**Corresponding author:**

Mariagrazia Grilli, MD, Laboratory of Neuroplasticity, Dept. Pharmaceutical Sciences, University of Piemonte Orientale "A. Avogadro", Via Bovio, 6 28100 Novara, Italy. Phone: +39 0321375828; e-mail: mariagrazia.grilli@uniupo.it

Abstract

Decreased adult hippocampal neurogenesis (ahNG) is believed to contribute to reduced hippocampal volume in major depressive disorder (MDD). Several antidepressants (AD) also increase ahNG simultaneously to the onset of the therapeutic effect in a rodent model of depression. Trazodone (TZD) is a multifunctional antidepressant with off-label uses including hypnosis. It is a dose-dependent serotonin receptor 5-HT_2 antagonist, reuptake inhibitor (SARI), 5-HT_{1A} partial agonist and α_1 and α_2 adrenergic receptor (AR) antagonist. We asked whether trazodone is a proneurogenic antidepressant and which are the molecular mechanisms underlying its activity. To answer this question, we first assessed the expression of receptors involved in TZD pharmacological activity in murine adult hippocampal neural progenitor cells (ahNPC). Then we tested TZD activity to promote neuronal differentiation of ahNPC *in vitro*. We found that TZD increases neuronal differentiation in a concentration and time-dependent manner. The proneurogenic effect was mediated by 5-HT_{2A} and 5-HT_{2C} antagonism. Serotonin depletion in ahNPC blunted TZD proneurogenic effect. Moreover NF-κB p50 subunit presence was required for TZD proneurogenic effect. Altogether we concluded that trazodone proneurogenic effect is through 5-$HT_{2A/2C}$ antagonism and NF-κB p50 dependent. Our results suggest that ahNG is a pharmacological target for trazodone and might be involved in its therapeutic activities.

Introduction

Adult hippocampal neurogenesis (ahNG) is a compelling form of neural plasticity susceptible to pharmacological modulation (Duman et al., 2001; Dranovsky and Hen, 2006; Xu et al., 2016). Lifelong generation of new neurons in the hippocampus takes place in the subgranular zone (SGZ) of the dentate gyrus (DG) from a pool of adult hippocampal neural progenitor cells (ahNPC) (Kempermann et al., 2003; Zhao et al., 2008; Bond et al., 2015). Newly generated neurons can integrate into neuronal networks and contribute to hippocampal-dependent learning, memory and to mood regulation (Aimone et al., 2014). The failure of ahNG is correlated to cognitive impairment and mood alterations in several neuropsychiatric disorders mainly in major depressive disorder (MDD) (Kempermann and Kronenberg, 2003; Mirescu and Gould, 2006; Kempermann et al., 2008). Notably, acute and chronic stress reduce neuroplasticity at multiple levels including ahNPC proliferation and neuronal differentiation (Pittenger and Duman, 2007; Eisch and Petrick, 2012). Despite the extensive research studies that tried to explain this effect, there is still a lot to discover about the involvement of specific receptors in this depletion.

After being ranked as the second in global disease burden, MDD is also projected to be the leading cause worldwide by 2030 (World Health Organization, 2012; Manji et al., 2001). Interestingly, pharmacological treatment of MDD with several antidepressants (AD), including tricyclic AD (TCA), selective serotonin reuptake inhibitors (SSRI) and serotonin and noradrenaline reuptake inhibitors (SNRI), restored ahNG to physiological levels in rodent models of MDD-like disorder (Pittenger and Duman, 2007; Eisch and Petrick, 2012). Increased number of proliferative cells was also reported in a postmortem study on human brain from patients diagnosed with MDD and treated with SSRI (Boldrini et al., 2009). These findings suggest that positive modulation of ahNG is required for AD effect. However, there is more complexity in this hypothesis since distinct serotonin

receptor subtypes might contribute diversely and even mediate opposite effects on ahNG (Klempin et al., 2013). AhNPC express serotonin receptors 5-HT_{1A}, 5-$HT_{2C/B}$ and neuron specific serotonin synthesis enzyme tryptophan hydroxylase (TPH2) (Benninghoff et al., 2010). Research studies showed that 5-HT_{1A} agonists increase ahNPC proliferation (Banasr et al., 2003) whereas 5-HT_2 agonists decreased ahNPC neuronal differentiation in mice (Klempin et al. 2010). Despite extensive research on positive modulation of ahNG by antidepressants, little is explored on the role of 5-HT receptors targeted by AD on ahNPC differentiation and apoptosis. The complexity of these systems *in vivo* contributed to the scarce precision in this context.

More than 50% of patients diagnosed for MDD quit antidepressant therapy due to side effects or unresponsiveness (Hunot et al., 2007; Duman et al., 2016). Trazodone is a triazolopyridine derivative multimodal AD. Trazodone is a dose-dependent 5-$HT_{2A/2C}$ antagonist and serotonin reuptake transporter (SERT) inhibitor (SARI) (Stahl et al., 2009). Moreover, trazodone is a 5-HT_{1A} partial agonist and has binding affinities for α_1 and α_2 adrenergic receptors (AR), histamine receptor 1 (H1) with minimal anticholinergic activity (Knight et al., 2004; Stahl, 2009). Several studies described the therapeutic effect of trazodone as a well-tolerated antidepressant, commonly also prescribed as add-on hypnotic drug to improve sleep disorders (Saletu-Zyhlarz et al., 2003; Stahl et al., 2009). However, it is not known if trazodone characterized as multimodal antidepressant is a positive modulator of ahNG and which mechanisms are employed in this effect.

In our study, we used a well characterized simplified *in vitro* model of ahNPC to determine whether trazodone modulate their proliferation, neuronal and non-neuronal differentiation and survival. We also investigated the receptors and downstream signaling pathways potentially involved in the *in vitro* effect of trazodone.

Materials and methods

Animals. Male C57BL/6 Wild type (WT; C57BL/6, The Jackson Laboratories, US) and NF-κB $p50^{-/-}$ (p50KO; C57BL/6 Nfkb1tm1Bal/J; The Jackson Laboratories, US) mice were acclimated under light- and temperature-controlled conditions in high efficiency particulate air (HEPA)- filtered Thoren units (Thoren Caging System, Hazelton, PA). Mice were housed in number of 3-4/cage with *ad libitum* access to food and water at the animal facility of University of Piemonte Orientale. Animal care and handling were carried out according to protocols approved by the European Community Directive and the local Institutional Animal Care and Use Committees (IACUC).

Drugs. All drugs concentrations were chosen based on binding affinity to target receptors. The sources of compounds were as follows: 5-Hydroxytryptamine hydrochloride (5-HT); 2-[3-[4-(3-Chlorophenyl)-1-piperazinyl]propyl]-1,2,4-triazolo[4,3-a]pyridin-3(2H)-one HCl, a full 5-HT_{2C} receptor agonist (Trazodone, TZD); Venlafaxine HCl, a 5-HT and noradrenaline (NE) RI (SNRI, VEN); DL-P-Chlorophenylalanine, an irreversible tryptophan hydroxylase inhibitor (PCPA); Phenylephrine HCl, an α_1 AR agonist (PHE) were purchased from Sigma-Aldrich, Milan. IT8,9-Dichloro-2,3,4,4a-tetrahydro-1*H*-pyrazino[1,2-*a*]quinoxalin-5(6*H*)-one HCl, a selective and potent 5-HT_{2C} antagonist (WAY161503); (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide, a high affinity 5-HT_{2A} agonist (TCB-2); (2*R*)-1-[(3-Hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine HCl, a 5-HT_7 receptor antagonist (SB 269970); (3R)-3-(Dicyclobutylamino)-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide HCl, a high affinity and selective 5-HT_{1A} antagonist (NAD 299); idazoxan, an α_2 AR antagonist (IDZ) were purchased from Tocris (Bioscience, Bristol, UK) distributed by Space import export, Milan, IT.

Isolation and culture of adult hippocampal Neural Progenitor Cells (NPC). Adult murine hippocampal NPC were isolated WT and p50KO mice as previously shown (Meneghini et al., 2014). Briefly, for each culture preparation three adult male mice (3-4-month-old) were euthanized by cervical dislocation. Brains were removed and hippocampi were dissected out 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 of penicillin/streptomycin (Pen/Strep, Gibco, Life Technologies, Monza, IT). After centrifugation for 10 min at 110 x g, tissue was digested for 40 minutes (min) at 37^0C with Papain Dissociation System (Worthington DBA, Lakewood, NJ). Cell suspension was cultured in NUNC EasYFlask 25 cm^2 (Thermo Scientific, Waltham, MA) with serum-free complete proliferation medium containing Neurobasal-A supplemented with B27, 2 mM glutamine (Gibco), 20 ng/ml recombinant human Epidermal Growth Factor (rhEGF), 10 ng/ml of recombinant human basic Fibroblast Growth Factor (rhbFGF/FGF-2, PeproTech, Rocky Hill, NJ), 4 µg/ml heparin sodium salt (Sigma-Aldrich), and 100 U/100 µg/ml of Pen/Strep (Gibco). Passage 1 (P1) neurospheres were dissociated for the first time after ten days in vitro (DIV). From P2, dissociation was performed every five days and NPC were replated at a density of 12,000 cells/cm^2. NPC were used for experiments from P5 to P30. Proliferating NPC were routinely tested for their undifferentiated state by expression of sox-2 (Sry-related HMG box transcription factor) and nestin (Type VI intermediate filament).

For irreversible inhibition of tryptophan hydroxylase (TPH2), cells were treated with 50 µM of PCPA or vehicle after 48 hours in proliferation. At day 5 (72 hours after treatment) cells were dissociated and plated for differentiation as detailed in section of NPC differentiation.

RNA Reverse transcription and Polymerase Chain Reaction analysis. Total mRNA was extracted from murine NPC and human NSC using TRIzol reagent (Sigma-

Aldrich) followed by chloroform precipitation (Sigma-Aldrich). RNA was treated with RQ1 RNase-Free DNase (Promega, Milan, IT). cDNA was generated from 1 μg of RNA by reverse transcription using ImProm-II Reverse Transcription System (Promega) according to manufacturer's protocol. PCR reactions were performed using a GoTaq Flexi DNA polymerase kit (Promega) in a 25 μl mix containing (1 μl of cDNA, Buffer 1x, 1.2 mM $MgCl_2$, 0.6 mM dNTPs, and 0.5 μM of each primer). Primers for serotonin receptors: 5-$HT_{2A,}$ sense 5′-ATAGCCGCTTCAACTCCAGA-3′, antisense 5′-ACGGCCATGATATTGGTGAT-3'; 5-$HT_{2C,}$ sense 5′-TGATTGGACTGAGGGACGAAAGCA-3′, antisense 5′-TTCCCACAAAGCACCGACAGGATA-3′; 5-$HT_{7,}$ sense 5'-CTGGTGGTGGTTCTCAGTAGTATC-3', antisense 5'-CAGCATAAGCAAAGTGGGGAGGTA-3'. Murine cDNA from adult hippocampal tissue was used as positive control.

Mouse Hippocampal NPC differentiation. For NPC differentiation, single NPC cells were plated onto laminin-coated Lab-Tek 8-well permanox chamber slides (Nunc) in differentiation medium containing Neurobasal-A, B27 and 2 mM glutamine, at a density of 43,750 cells/cm^2. NPC were treated with indicated drug concentrations or vehicle for 24h. To investigate the involvement of different serotoninergic and adrenergic receptors in antidepressants or serotonin effects, before addition of drugs, cells were first treated for 30 min with selective agonists/antagonists such as TCB-2 (5-HT_{2A} agonist), WAY151606 (5-HT_{2c} agonist), phenylephrine HCl (α_1 AR agonist), NAD299 (5-HT_{1A} antagonist), SB269970 (5-HT_7 antagonist).

Immunolocalization studies in differentiated murine NPC. Differentiated cells in chamber slides were fixed with 4% paraformaldehyde/4% saccharose solution (Sigma) for 20 min and processed for immunocytochemistry. After fixation cells were washed 3 times with saline phosphate buffer (PBS) and permealised in PBS containing 0.48% Triton X-100 (Sigma-Aldrich), 5 min at room temperature (RT).

Primary antibodies directed against: microtubule-associated protein-2 (MAP-2; rabbit polyclonal, 1:600; Millipore, Milan, IT), nestin (Chicken monoclonal, 1:1500; Neuromics, DBA, IT), glial fibrillary acidic protein (GFAP, mouse polyclonal, 1:600, Millipore), SRY-related HMG-box gene (Sox-2, rabbit polyclona, 1:500 Millipore) were incubated for 150 min in humid chamber at RT. Secondary antibodies Alexa Fluor 555–conjugated goat anti-rabbit (1:1,400), Alexa Fluor 488-conjugated goat anti-chicken (1:1,400) (all from Molecular Probes, Life Technologies) were incubated for 50 min in humid chamber at RT . Nuclei were counterstained with 0.8ng/ml Hoechst (Thermo Fisher Scientific). In each experiment, five fields/well (corresponding to about 150-200 cells/well) were counted with a 60X objective by a Leica DMIRB inverted fluorescence microscope. Positive cells for each marker were counted and their percentage was calculated over total viable cells. Data are presented as percentage of increase over vehicle unless indicated otherwise.

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 two independent groups were compared. One-way analysis of variance (ANOVA) followed by Tuckey's post-Hoc test were performed to compare three or more groups. Statistical significance level was set for p values < 0.05.

Results

Trazodone increases neuronal differentiation from ahNPC in a concentration and time dependent manner.

Multipotent and proliferating ahNPC isolated from adult mouse hippocampi are cultured *in vitro* in presence of EGF and bFGF-2 as floating neurospheres. They are phenotypically characterized by the expression of SRY-related HMG-box gene 2 (Sox-2) and nestin, markers of undifferentiated cells (Fig. 1 A) and by the absence of mature (MAP-2) and immature (class III beta-tubulin, Tuj-1) neuronal markers (Rietze and Reynolds, 2006; Meneghini et al., 2010). Upon removal of growth factors, ahNPC stop dividing and differentiate toward neuronal and astroglial lineages (Fig. 1 A). Cells cultured onto laminin-coated chamber slides for 24 h in absence of EGF and FGF, were treated with a wide concentration range of trazodone (0.0003 - 100 μM). These cells were fixed and double labeled for MAP-2 and nestin. We quantified neuronal differentiation by counting MAP-2^+/$nestin^-$ cells representing a more mature neuronal population and double MAP-2^+/$nestin^+$ cells representing an immature cell population of neuroblasts. Double negative MAP-2^-/$nestin^-$ cells represented a heterogenous population including both glial-committed and $sox2^+$/$nestin^-$ undifferentiated progenitors. MAP2^-/$nestin^+$ cells represented the starting NPC population. As shown in Fig. 1 B, C *in vitro* trazodone promoted neurogenesis in a concentration dependent manner. We observed an increased percentage of MAP-2^+/$nestin^-$ (ANOVA, $p < 0.001$) and MAP-2^+/$nestin^+$ cell population (ANOVA, $p < 0.001$) with maximal effect elicited by100 nM trazodone. Increased neuronal differentiation was paralleled with a decrease in non-neuronal MAP-2^-/$nestin^-$ cells (ANOVA, $p< 0.001$, Fig. 1 D). We did not observe any significant difference in the apoptotic rate of trazodone-treated cells at any concentration compared to vehicle-treated cells (Fig. 1 E).

At each time point cells were fixed and double stained for MAP-2 and nestin and counted. Results in Fig. 2 A, B showed that at 12 h trazodone increased significantly the percentage of immature MAP-2^+/$nestin^+$ cells (Tukey post hoc, $p < 0.01$), while 5-HT increased significantly the percentage of both MAP-2^+/$nestin^-$ (Tukey, $p < 0.01$) and MAP-2^+/$nestin^+$ cells (Tukey, $p < 0.05$). In contrast VEN did not have a significant effect on neuronal differentiation at 12 h. At 24 h trazodone, 5-HT and VEN increased significantly the percentage of MAP-2^+/$nestin^-$ (ANOVA, $p< 0.001$) and immature MAP-2^+/$nestin^+$ cells (ANOVA, $p< 0.001$). Interestingly at 48 h only trazodone maintained a proneurogenic effect on MAP-2^+/$nestin^-$ cells (Tukey post-hoc, $p<0.001$). We observed a parallel significant decrease in non-neuronal MAP-2^-/$nestin^-$ population in all treatments at 12 h (ANOVA, $p < 0.01$) and 24 h (ANOVA, $p < 0.01$) whereas this decrease was observed only in trazodone at 48 h (Tukey, $p < 0.001$) (Fig. 2 C). Neither trazodone nor 5-HT and VEN had a significant effect on the apoptotic rate within the same time point (Fig. 2 D). In summary, trazodone is proneurogenic in the *in vitro* model of ahNPC in a concentration and time-dependent manner. When compared to 5-HT and VEN, only trazodone increased the number of mature neurons after 48 h.

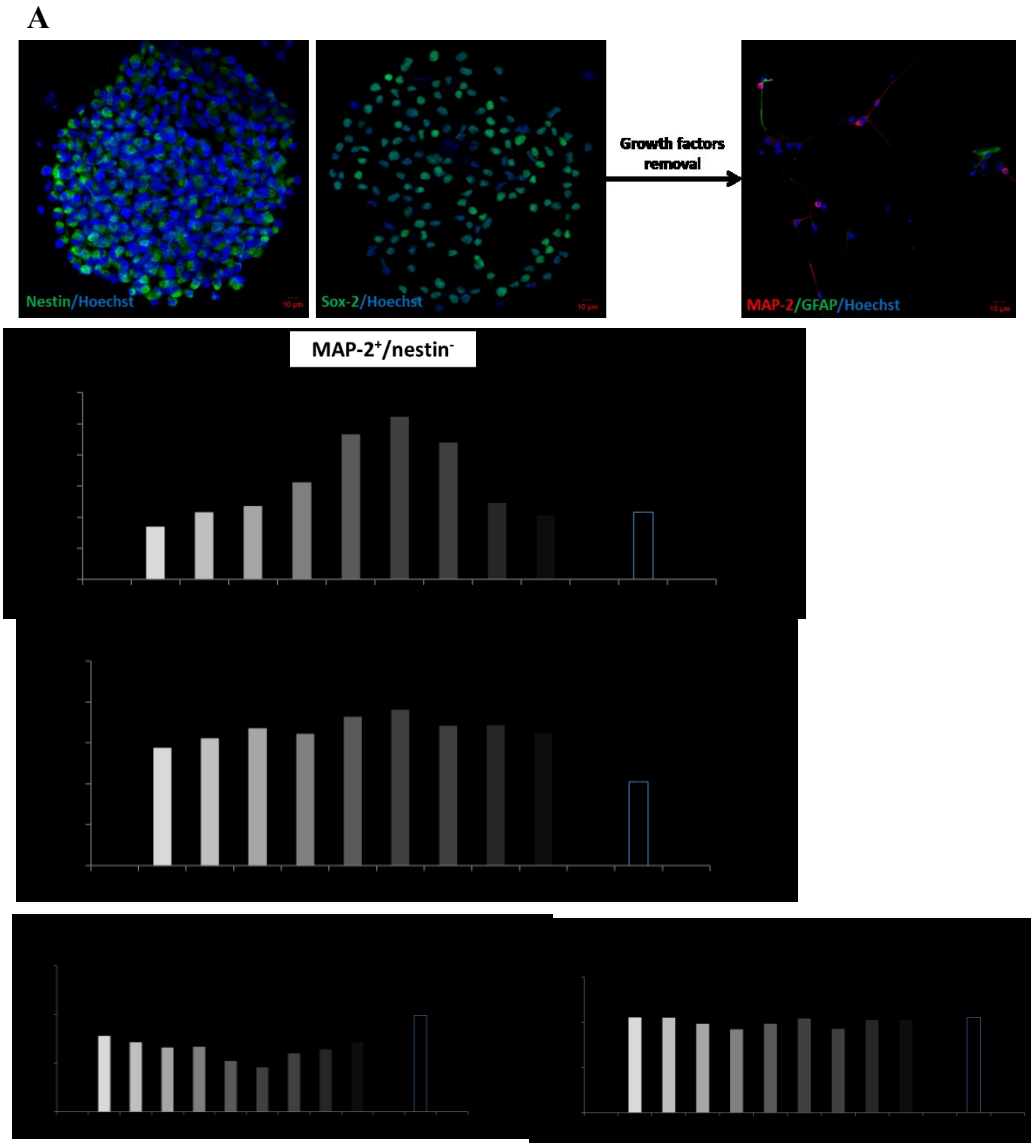

Figure 1: Trazodone effect on neuronal differentiation of adult hippocampal neural progenitor cells (ahNPC) *in vitro*. (A) Fluorescent confocal microscope images of adult hippocampal NPC neurospheres immunolabeled for Sox-2 and nestin, markers of undifferentiated cells. In absence of growth factors ahNPC differentiate toward neuronal (MAP-2, red) and astroglial (GFAP, green) cells. Nuclear staining: Hoechst. Magnifications 400X. Scale bar = 10 μm. **(B-C-D-E)** ahNPC were differentiated for 24 h in presence of trazodone (TZD, 0.0001 – 100 μM) and corresponding vehicle. Then cells were fixed and double stained for MAP-2 (neurons marker) and nestin. Cells were counted/quantified under fluorescent microscope and categorized as: MAP-2^+/$nestin^-$, MAP-2^+/$nestin^+$, MAP-2^-/$nestin^-$ and apoptotic cells. Data are expressed as percentage of increase over vehicle-treated cells ± SD of experiments performed in triplicate. Statistical analysis was performed by one-way repeated measure ANOVA followed by Tukey's post hoc multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001.

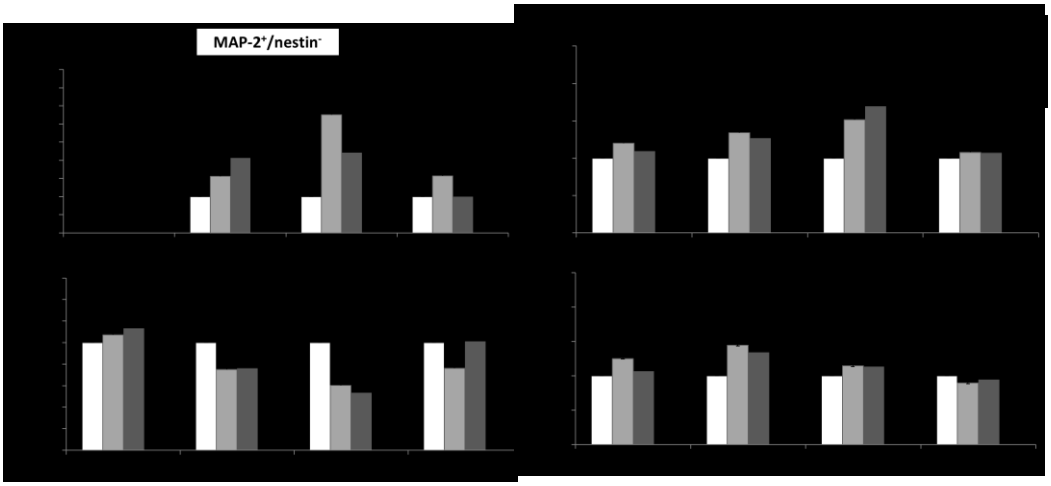

Figure 2: Time course assessment of trazodone proneurogenic effect compared to serotonin and venlafaxine. Cells were differentiated for 2, 12, 24 and 48 h in presence of vehicle, trazodone (TZD, 100 nM), serotonin (5-HT, 1 μM) and venlafaxine (VEN, 10 μM) then fixed and stained for MAP-2 and nestin. We show MAP-2^+/$nestin^-$ and MAP-2^+/$nestin^+$, MAP-2^-/$nestin^-$ and apoptotic cells quantification. Data are expressed as percentage of increase over vehicle-treated cells ± SD of experiments performed in triplicate. Statistical analysis was performed by one-way repeated measure ANOVA followed by Tukey's post hoc multiple comparisons test vs. vehicle of the same time point. *P < 0.05, **P < 0.01, ***P < 0.001.

5-$HT_{2A/2C}$ receptors are expressed by hippocampal neural progenitor cells and their antagonism mediates trazodone proneurogenic effect.

In order to determine the contribution of distinct receptors in the proneurogenic effect of trazodone we treated ahNPC with trazodone and/or specific receptor agonist/antagonists. Since transcripts for 5-HT_{1A} are also expressed by the *in vitro* model of ahNPC (Benninghoff et al., 2010), we investigated whether the effect of trazodone on neuronal differentiation is dependent on its agonistic activity on 5-HT_{1A}. For that target we exposed ahNPC to trazodone (100 nM) in presence of NAD299 (30 nM), a selective 5-HT_{1A} antagonist [$K_{i\ (5\text{-}HT1A)}$ = 0.6 nM] or its corresponding vehicle for 24h. As shown in Fig. 3 A, B NAD299 did not block trazodone proneurogenic effect neither on MAP-2^+/$nestin^-$ (Tukey, $p < 0.001$) nor on immature MAP-2^+/$nestin^+$ cells (Tukey, $p < 0.001$). The increase in neuronal

differentiation by trazodone was paralleled by a decrease in MAP-2^-/$nestin^-$ cells (Tukey, $p < 0.001$) (data not shown). Exposing cells to NAD 299 by itself or in presence of trazodone did not affect the apoptotic rate of ahNPC and their progeny (Fig. 3 C).

We evaluated the expression of 5-HT_{2A} and 5-HT_{2C} serotonin receptors in proliferative ahNPC by (RT)-PCR analysis. Results showed that ahNPC express mRNA for 5-HT_{2A} and 5-HT_{2C} serotonin receptors (Fig. 3 D). We studied the involvement of 5-HT_2 in trazodone proneurogenic effect on WT ahNPC. For that purpose, we treated ahNPC with proneurogenic trazodone concentrations (30, 100 and 300 nM) in presence of TCB-2 (10 nM), a 5-HT_{2A} high affinity agonist [$K_{i\ (5\text{-}HT2A)}$ = 0.73 nM], or WAY151603 (WAY, 10 nM), a potent and selective 5-HT_{2C} agonist [$K_{i\ (5\text{-}HT2C)}$ = 4 nM]. (Fig. 3 E, F). The agonists by themselves, compared to vehicle, did not have a significant effect on neuronal differentiation (Fig. 3 E, F) or on the apoptotic rate (data not shown). As expected, trazodone 30, 100 and 300 nM concentrations were proneurogenic as proven by increased percentage of MAP-2^+/$nestin^-$ (ANOVA, $p < 0.001$) and MAP-2^+/$nestin^+$ (ANOVA, $p < 0.001$) cells. Interestingly, both WAY and TCB-2 shifted trazodone response curve to the right (Tukey, $p< 0.001$) (Fig. 3, E, F). Overall these results suggest that trazodone binding to serotonin receptors 5-$HT_{2A/2C}$ but not 5-HT_{1A} mediated its proneurogenic effects.

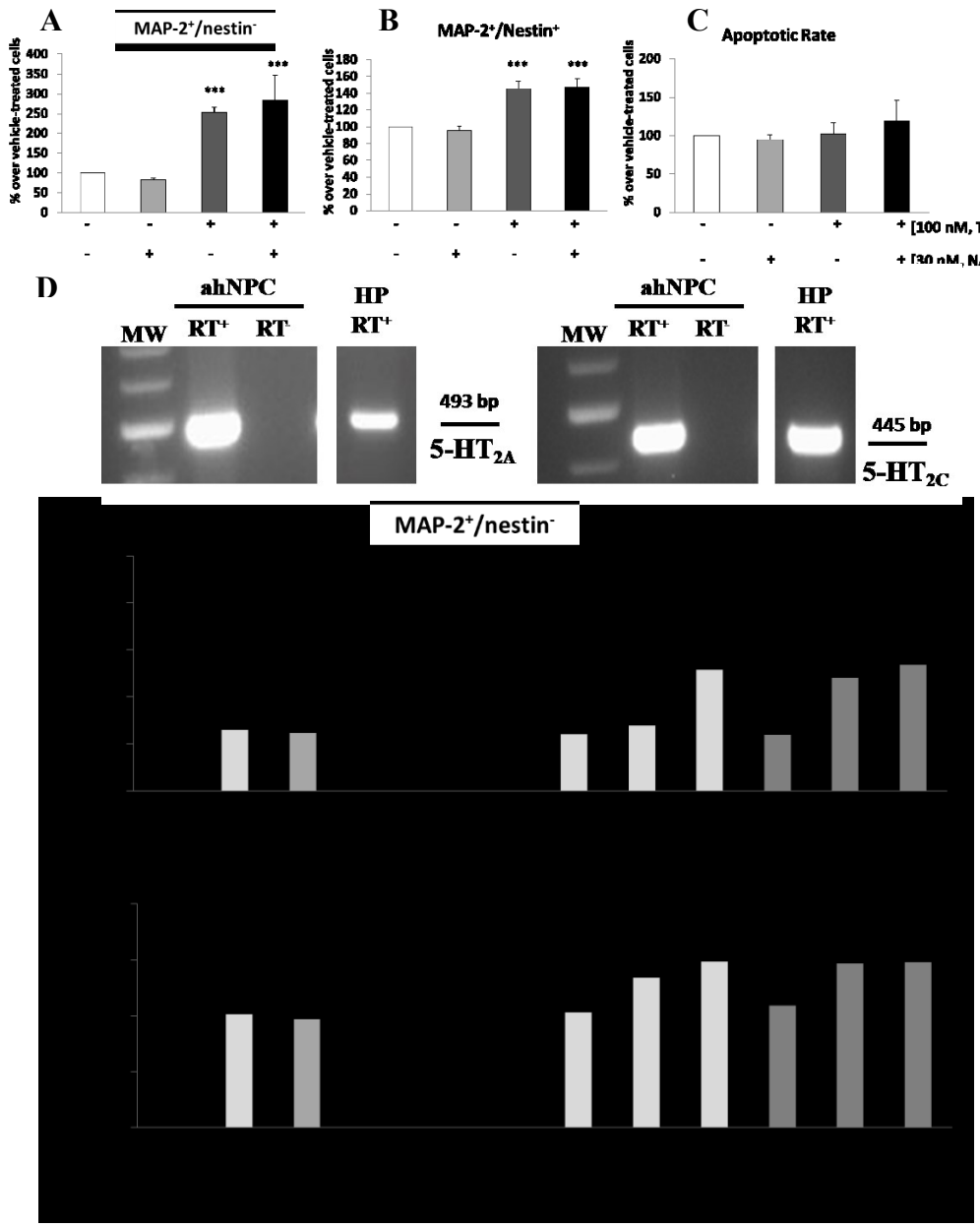

Figure 3: Involvement of serotonin receptors 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ in trazodone proneurogenic effect on ahNPC. (A-C) ahNPC in differentiation were treated for 24 h with trazodone (TZD, 100 nM) in presence of 5-HT$_{1A}$ antagonist NAD299 (30 nM). We show MAP-2$^+$/nestin$^-$ and MAP-2$^+$/nestin$^+$ and apoptotic cells quantification. **(D)** Representative images of (RT)-PCR analysis for the expression of 5-HT$_{2A}$ (493bp), 5-HT$_{2C}$ (445bp) receptors (RT$^+$). Positive control for mRNA expression was cDNA from adult hippocampus tissue (HP RT$^+$). Negative reverse transcription reactions (RT) for ahNPC were used as negative control (RT$^-$). The 100-base pair (bp) DNA ladder was used as molecular weight (mw) marker. **(E-F)** ahNPC in differentiation were treated for 24 h with trazodone (30, 100 and 300 nM) in presence or absence of WAY151603 (5-HT$_{2C}$ agonist, 10 nM) or TCB-2 (5-HT$_{2A}$ agonist, 10 nM). We show MAP-2$^+$/nestin$^-$ and MAP-2$^+$/nestin$^+$ quantification. Data are expressed as percentage of increase over vehicle-treated cells ± SD of experiments performed in triplicate. Statistical analysis was performed by one-way repeated measure ANOVA followed by Tukey's post hoc multiple comparison tests. ***P < 0.001. NAD299 "NAD"; WAY151603 "WAY".

Trazodone effect on neuronal differentiation does not require its binding to α_1 and α_2 adrenergic receptors.

Since trazodone is also an antagonist of α_1 and α_2 AR we investigated if this activity could mediate its proneurogenic effect. We treated ahNPC with trazodone in presence of L-phenylephrine, an α_1 AR agonist [$K_{i\ (\alpha_{1a})}$ = 4.7 nM] (PHE). As shown in Fig. 4 A, B L-phenylephrine did not counteract the proneurogenic effect of trazodone on MAP-2^+/$nestin^-$ cells (ANOVA, $p < 0.0001$) and MAP-2^+/$nestin^+$ neuroblasts (ANOVA, $p < 0.0001$) at any tested concentration. We also treated cells with idazoxan, an α_2 AR antagonist [$K_{i\ (\alpha_{2a})}$ = 8.01 nM]. We did not observe any effect on neuronal differentiation compared to vehicle (Fig. 4 C, D). In summary, these results showed that adrenergic receptors do not mediate the proneurogenic effect of trazodone.

Serotonin depletion in ahNPC inhibits the proneurogenic effect of trazodone but not 5-HT and venlafaxine.

Serotonin could be synthetized by ahNPC through the enzyme TPH2 (Benninghoff et al., 2010). Since we observed that trazodone antagonistic activity on $5\text{-}HT_{2A/2C}$ mediate its proneurogenic effects, we hypothesized the presence of an endogenous serotoninergic tone. We decided to deplete serotonin in ahNPC and evaluate its effect on ahNPC under basal and drug-treated conditions. To deplete 5-HT we treated ahNPC for 72 h with TPH2 irreversible inhibitor PCPA (50 μM) or vehicle. PCPA-treated and vehicle-treated neurospheres were dissociated and plated at the same density for differentiation in absence of growth factors (Fig. 5 A, experimental timeline). Cells were differentiated with proneurogenic concentrations of trazodone (30, 100 and 300 nM), 5-HT (1 μM), VEN (10 μM) or vehicle for 24 h. As expected, trazodone, 5-HT and VEN increased the percentage of both neuronal populations from vehicle-treated cells (ANOVA, $p< 0.001$, Tukey post hoc test, $p < 0.001$, Fig. 5 B, C). We observed that PCPA-treated ahNPC gave

rise to a lower percentage of MAP-2$^+$/nestin$^-$ compared to vehicle-treated (Tukey post hoc test, PCPA vs. vehicle treated cells, $p < 0.05$) while the percentage of i

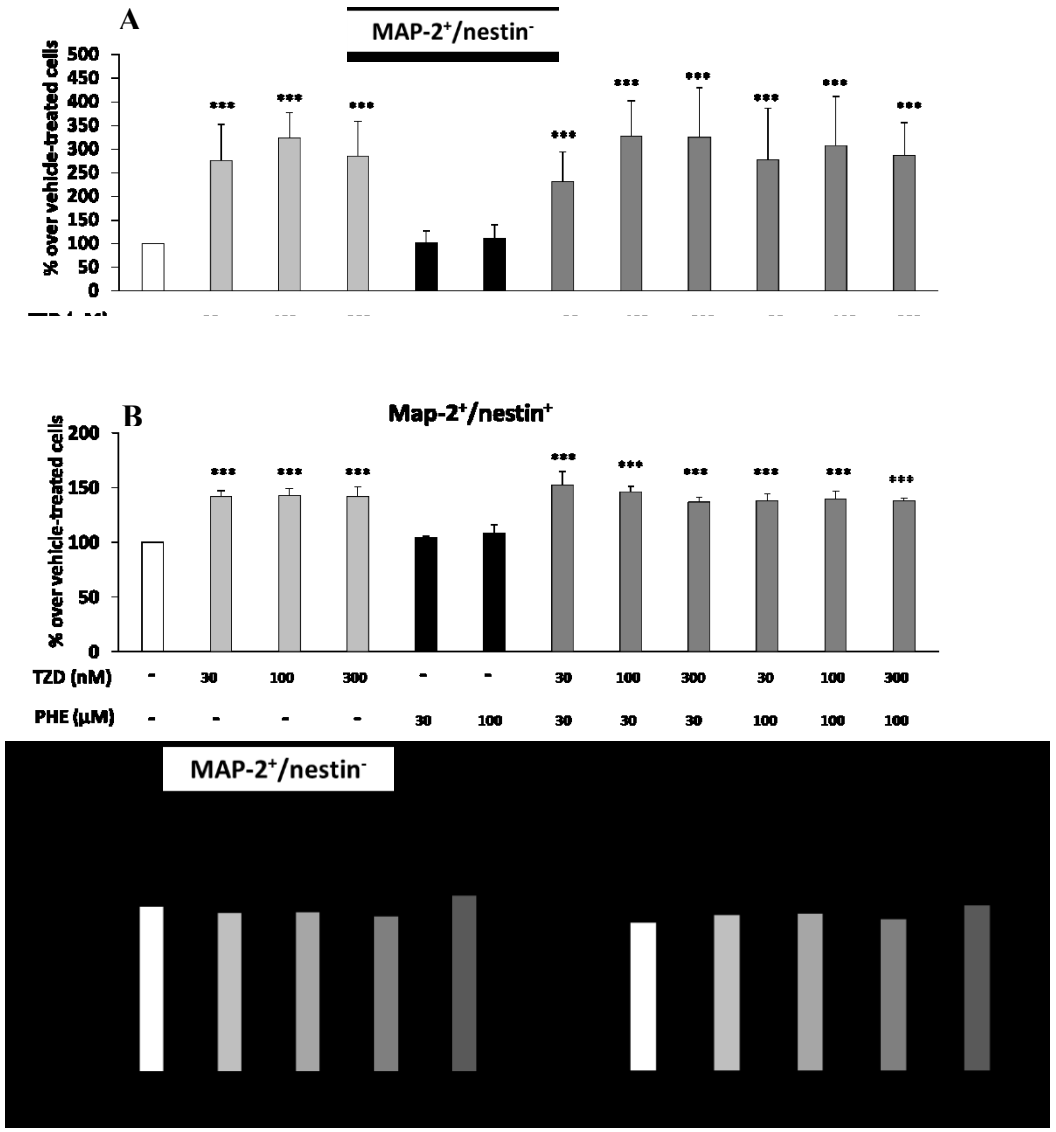

Figure 4: Involvement of adrenergic receptors α_1 and α_2 in trazodone proneurogenic effect on ahNPC. **A-B**- Cells were differentiated for 24 h with trazodone (TZD, 30-100 and 300 nM) in presence or absence of L-phenylephrine (α_1 agonist, 30 or 100 µM) or **(C-D)** idazoxan (α_2 antagonist, 0.001 - 1 µM). We show MAP-2$^+$/nestin$^-$ and MAP-2$^+$/nestin$^+$ quantification. Data are expressed as percentage over vehicle-treated cells ± SD of experiments performed in triplicate. Statistical analysis was performed by one-way repeated measure ANOVA followed by Tukey's post hoc multiple comparison tests. ***P < 0.001. L-phenylephrine "PHE"; Idazoxan "IDZ"

Trazodone proneurogenic effect was inhibited totally in PCPA-treated cells, while 5-HT and VEN produced a significant increase of MAP-2^+/nestin$^-$ (ANOVA, $p < 0.001$, Tukey post hoc test, $p < 0.01$) and immature MAP-2^+/Nestin$^+$ (ANOVA, $p < 0.001$, Tukey post hoc test, $p < 0.001$) cells (Fig.5 B, C). The lack of response to trazodone by PCPA-treated cells could not be due to increased apoptotic rate since we did not observe significant differences between vehicle-treated and PCPA-treated ahNPC (Fig. 5 D). Altogether these results further confirmed that trazodone proneurogenic effects were due to its antagonistic activity on 5-$HT_{2A/2C}$ receptors.

The NF-κB p50 signaling pathway is potential player in trazodone effect.

Since NF-κB transcription factors and in particular p50 mediate the proneurogenic response to several drugs (Valente et al., 2012; Meneghini et al., 2013), we asked whether trazodone proneurogenic activity is also regulated by NF-κB signaling. We prepared ahNPC from wild type (WT) and p50KO mice. In differentiating conditions WT and p50KO ahNPC were treated with trazodone (100 nM), 5-HT (1 μM) and VEN (10 μM). As expected, trazodone, 5-HT and VEN increased significantly MAP-2^+/nestin$^-$ (ANOVA, $p < 0.001$) and MAP-2^+/Nestin$^+$ (ANOVA, $p < 0.001$) cells generated by WT ahNPC. Remarkably p50KO ahNPC did not respond to proneurogenic effect of trazodone while 5-HT (Tukey, $p < 0.001$) and VEN (Tukey, $p < 0.001$) increased significantly both MAP-2^+/nestin$^-$ and MAP-2^+/Nestin$^+$ cells (Fig. 6, A, B). The significant increase in neuronal population was paralleled by a significant decrease in non-neuronal MAP-2^-/Nestin$^-$ (ANOVA, $p < 0.001$) cells (Fig. 6, C). We did not observe significant differences in the apoptotic rate of WT and p50KO ahNPC under basal and drug treatment conditions (Fig. 6, D). Together these results show that trazodone proneurogenic effect depend on the presence of NF-κB p50 subunit in ahNPC.

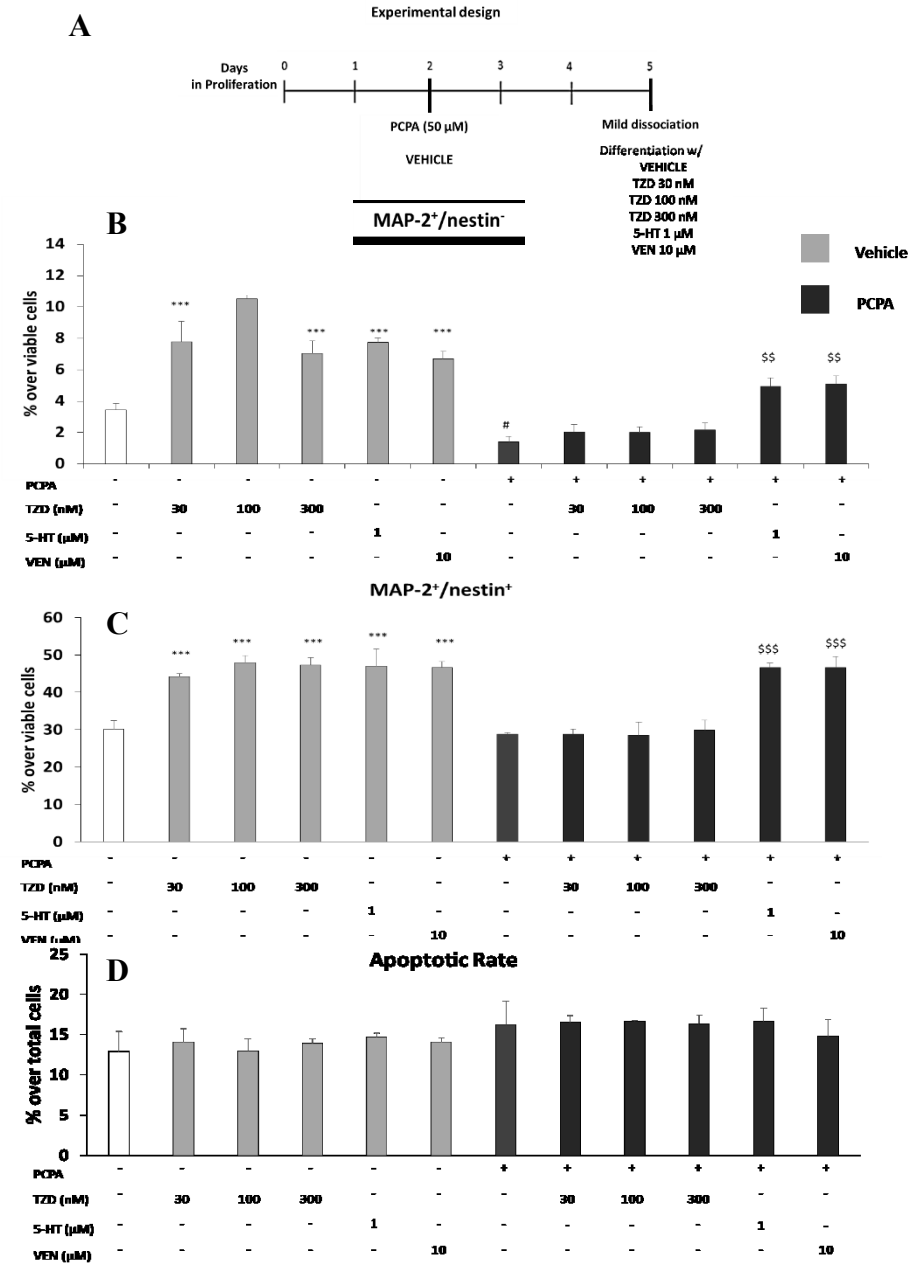

Figure 5: The effect of serotonin depletion on ahNPC differentiation and trazodone proneurogenic effect. (A) Experimental time line. **(B-C-D)** ahNPC in proliferation were treated with PCPA (50) for 72 h then differentiated in presence of trazodone (TZD, 30-100 and 300 nM), serotonin (5-HT, 1 µM) and venlafaxine (VEN, 10 µM). Vehicle treated cells (light grey bars) and PCPA-Treated cells (dark grey bars). We show MAP-2^+/nestin$^-$ and MAP-2^+/nestin$^+$ and apoptotic cells quantification. Data are expressed as percentage over viable cells ± SD and over total cells for the apoptotic rate. Experiments were run in triplicate. Statistical analysis was performed by one-way repeated measure ANOVA followed by Tukey's post hoc multiple comparison tests. ***P < 0.001 *vs.* vehicle-treated cells. $^{\$\$}$P < 0.01, $^{\$\$\$}$P < 0.001 *vs.* PCPA-treated cells. #p < 0.05, vehicle- *vs.* PCPA-treated cells. DL-P-chlorophenylalanine, PCPA.

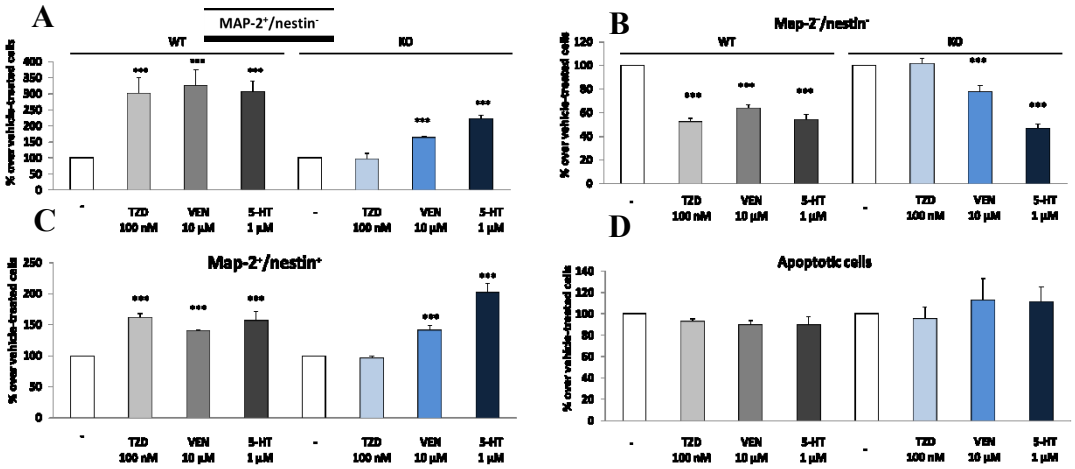

Figure 6: The role of NF-κB p50 signaling pathway in mediating the increase of neuronal differentiation by trazodone. ahNPC from WT and NF-κB p50 KO mice were isolated for culture *in vitro* in proliferation. Both genotypes were differentiated in presence of **(A-B)** Trazodone (TZD, 100 nM), 5-HT (1 µM) and VEN (10 µM). We show MAP-2^+/$nestin^-$ and MAP-2^+/$nestin^+$, MAP-2^-/$nestin^-$ and apoptotic cells quantification. Data are expressed as percentage of increase over vehicle-treated cells ± SD of experiments performed in triplicate. Statistical analysis was performed by one-way repeated measure ANOVA followed by Tukey's post hoc multiple comparison tests. ***P < 0.001. NF-κB p50 Knock out ahNPC "KO".

5-HT proneurogenic effect is mediated by 5-HT_7 receptors.

Altogether our data suggested that trazodone and 5-HT induce neurogenesis *in vitro* via distinct mechanisms. For this reason, we characterized which serotonin receptors mediate *in vitro* the proneurogenic effect of the endogenous neurotransmitter. First, we studied a possible role of 5-HT_{1A}. We tested the effect of a wide range of 5-HT concentrations (0.0001 – 100 µM) in presence or absence of NAD299 (10 nM). Results in Fig. 7 A, B showed that 5-HT concentrations (0.001 – 10 µM) increase both MAP-2^+/$nestin^-$ (ANOVA, $p < 0.001$) and MAP-2^+/$nestin^+$ cells (data not shown). NAD299 by itself did not affect neurogenesis and did not inhibit the proneurogenic effect of 5-HT.

Last but not least we could demonstrate by (RT)-PCR analysis that ahNPC express mRNA specific for 5-HT_7 receptor (Fig. 7, B). To assess the potential contribution of 5-HT_7 in serotonin effect, we treated ahNPC with 5-HT (1 μM) in presence or absence of SB269970 (SB), a 5-HT_7 antagonist [$K_{i\ (5\text{-}HT7)}$ = 8.9 nM]. As shown in Fig. 7 C, 5-HT (Tukey, $p < 0.001$) increased significantly the percentage of MAP-2^+/$nestin^-$ cells. SB per se did not affect the neuronal differentiation of ahNPC. SB 1, 10 and 100 nM counteracted the proneurogenic effect of 5-HT. Altogether these data showed that 5-HT_7 but not 5-HT_{1A} might be involved in 5-HT proneurogenic effect on ahNPC *in vitro* model.

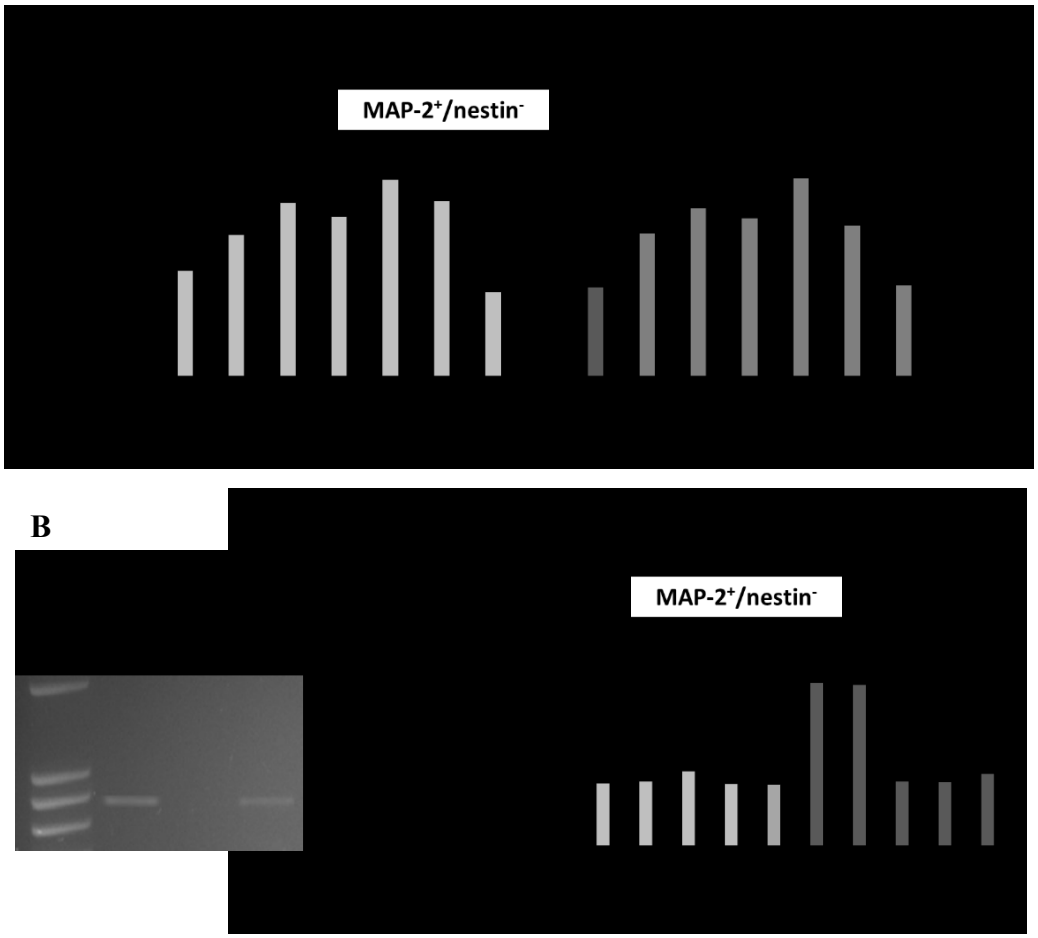

Figure 7: Serotonin receptors 5-HT$_{1A}$ and 5-HT$_7$ involvement in 5-HT proneurogenic effect. (A) ahNPC were treated with a wide range of 5-HT (0.0001 – 100 μM) in presence or absence of NAD299 (5-HT$_{1A}$ antagonist, 10 nM). **(B)** Representative images of (RT)-PCR analysis showing the expression of 5-HT$_7$ (852bp) in WT proliferative ahNPC (RT$^+$). Positive control for mRNA expression was cDNA from adult hippocampus tissue (HP RT$^+$). Negative reverse transcription reactions (RT) for ahNPC were used as negative control (RT$^-$). The 100 bp DNA ladder was used as molecular weight (mw) marker. **(C)** ahNPC were differentiated for 24 h in presence of 5-HT (1 μM) in presence of SB269970 (5-HT$_7$ antagonist) concentrations (1- 10 - 100 nM). We show MAP-2$^+$/nestin$^-$ cells quantification. Data are expressed as percentage of increase over vehicle-treated cells ± SD of experiments performed in triplicate. Statistical analysis was performed by one-way repeated measure ANOVA followed by Tukey's post hoc multiple comparison tests. **P < 0.01, ***P < 0.001.

Discussion

The generation of new neurons during post-natal and adult life is a highly plastic process regulated by exercise, learning, stress and markedly downregulated in neuropsychiatric disorder specifically in MDD (Kempermann et al., 2008; Aimone et al., 2014). Interestingly adult hippocampal neurogenesis is necessary for the therapeutic effect of several antidepressants in rodents, non-human primates and human mammalian brain (Santarelli et al, 2003; Malberg et al., 2000; Boldrini et al, 2009). Evidence correlating antidepressants to increased adult hippocampal neurogenesis support the possibility that such effect participates to the therapeutic effect of these drugs. Trazodone is a 5-HT_2 antagonist, SERT inhibitor and 5-HT_{1A} partial agonist antidepressant, with unknown effect on adult neurogenesis. In our study, we used an *in vitro* model of adult hippocampus derived NPC culture to test the effect of trazodone on neuronal differentiation. In such model, we showed, for the first time, that trazodone increased the percentage of both more mature and immature neuroblasts in a concentration and time dependent manner. In the time course assay, we also treated cells with 5-HT and venlafaxine (VEN) as reference compounds for trazodone effect.

The endogenous neurotransmitter 5-HT is the physiological agonist of all serotonin receptors and is known to increase the proliferation and maturation of ahNPC mainly through 5-HT_{1A} receptor (Alenina and Klempin, 2015). VEN is an SNRI antidepressant that increases the number and long term viability of proliferative NPC and mature neuron ($NeuN^+$) in the SGZ of rats (Khawaja et al., 2004; Xu et al., 2006; Asokan et al., 2014). We confirmed *in vitro* that trazodone, 5-HT and VEN increase neuronal differentiation from ahNPC. Moreover, we observed that only trazodone, and not 5-HT nor VEN, maintained a significant increase on mature neurons after 48h. We could not report any effect by trazodone and reference compounds on the survival of ahNPC and their progeny. Therefore, we

suggest that the effect of trazodone in our settings is exclusive on ahNPC differentiation and not survival.

We then determined the receptors involved in trazodone proneurogenic effect. We confirmed by qualitative PCR analysis that ahNPCs express 5-HT_{2A}, 5-HT_{2C} and 5-HT_{1A} receptors (data not shown). Previous reports showed that hippocampal NPC *in vivo* and *in vitro* express 5-HT_{2B}, 5-HT_{2C}, 5-HT_{1A} as well as the 5-HT producing enzyme TPH2 (Benninghoff et al., 2010; Brichta et al., 2013). We investigated whether 5-HT_{1A} receptors mediate the proneurogenic effect of trazodone on ahNPC. Results showed that a 5-HT_{1A} antagonist (NAD299) *per se* did not affect neuronal differentiation and did not inhibit the proneurogenic effect of trazodone at any concentration, without affecting the apoptotic rate. Literature data suggest that 5-HT_{1A} is involved in NPC proliferation but not in neuronal differentiation. Earlier studies reported that 5-HT_{1A} antagonist caused a 30% reduction of proliferative ahNPC *in vivo* (Radley and Jacobs 2002). Moreover, serotonin depletion by *DL-P-chlorophenylalanine* (PCPA), an irreversible inhibitor of TPH2, followed by treatment with postsynaptic 5-HT_{1A} agonist (8-OH-DPAT) rescued the number of proliferative ahNPC $BrdU^+$ in the SGZ (Banasr et al, 2003). *In vitro* PCPA treatment and 5-HT_{1A} antagonist hampered the proliferation but not the differentiation of ahNPC. Finally, proliferation was rescued by 5-HT or 5-HT_{1A} agonist treatment (Benninghoff et al., 2010).

We studied the possible involvement of 5-HT_2 receptors in trazodone effect. 5-HT_{2A} agonist (WAY151603) or 5-HT_{2C} agonist (TCB-2) *per se* did not produce any effect on neuronal differentiation of ahNPC (Fig. 3 E,F). We then tested their effect in presence of trazodone. Pretreatment of cells with 5-HT_{2A} agonist (WAY151603) or 5-HT_{2C} agonist (TCB-2) occupy 5-HT_2 receptors and prevent the binding of trazodone. Results showed that 5-HT_{2C} and 5-HT_{2A} agonists inhibited the effect of trazodone in a concentration-dependent fashion. These observations further support the idea that both receptors mediate trazodone proneurogenic effect.

In vivo studies showed that 5-HT_{2C} antagonist restored the levels of BDNF, a positive modulator of neurogenesis, followed by an increased number of proliferative cells ($BrdU^+$) in mice SGZ. *Vice versa* the activation of 5-HT_{2C} *in vivo* decreased mRNA and protein levels of BDNF (Vaidya et al., 1997, Klempin et al. 2010). Agomelatine, a melatonergic receptor (M1 and M2) agonist and 5-HT_{2C} antagonist antidepressant, *in vivo* increases ahNPC proliferation in the SGZ (BrdU labeling) and stimulates BDNF signaling pathway in adult rat hippocampus. This effect appears to be due to both melatonergic agonistic activity and 5-HT_2 antagonism (Soumier et al., 2009). Controversial results reported that ketanserin (5-$HT_{2A/2C}$ antagonist) produced a decrease in the number of BrdU-labeled cells whereas DOI (5-$HT_{2A/2C}$ agonist) treatment did not change cells proliferation (Banasr et al, 2003). Based on our as well as literature data, we can postulate that 5-$HT_{2A/2C}$ antagonism could have a significant role in neuronal differentiation of ahNPC. Trazodone can also bind α_1/α_2 AR and H1 receptors. We showed in this study that trazodone proneurogenic effect does not involve α_1 and α_2 AR. Moreover, we could also exclude a contribution of H1 receptors in its effect (*data not shown*).

Irreversible inhibition of TPH2 by PCPA decreases the proliferation of murine ahNPC without affecting their basal neuronal differentiation; moreover, exogenously added 5-HT can restore proliferation rate under the same experimental conditions (Benninghoff et al., 2010). Since we observed that trazodone proneurogenic effect was mediated by 5-$HT_{2A/2C}$ antagonism, we then evaluated the effect of the drug in PCPA-treated ahNPC. In these conditions the proneurogenic effect of trazodone was abolished, further supporting the idea that the antagonistic activity of trazodone on 5-$HT_{2A/2C}$ was mediating its activity. In the same experimental setting, 5-HT and venlafaxine were still proneurogenic. The proneurogenic effect of the endogenous agonist suggests that serotonin receptors expression is likely not affected by TPH2 irreversible inhibition. Venlafaxine

proneurogenic effect in PCPA-treated cells also confirms that ahNPC are still responsive to antidepressants with a different (noradrenergic) mechanism of action. Altogether we may conclude that trazodone proneurogenic effect is indeed 5-$HT_{2A/2C}$ antagonism dependent. Interestingly, we also observed a small but significant reduction in differentiation toward more mature neuronal cells (stained MAP-2^{+}/$nestin^{-}$ cells) but not toward immature neuroblasts from PCPA-treated compared to vehicle-treated cells. We doubt that these observations could be due apoptosis of that specific cell population, since we did not observe significant differences in the apoptotic rate of ahNPC and their progeny in that experimental condition. Our results are in line with Benninghoff et al. (2010) who did not observe a difference in Tuj-1 (another marker of neuroblasts) expressing cells after PCPA-treatment. One possible explanation is that serotonin depletion may specifically affect later neuronal maturation of newly generated cells. Additionally, several *in vivo* studies showed that 5-HT depletion in the brain does not cause a dramatic reduction of neurogenesis but inhibits the response to positive modulators such as running, enriched environment, learning and antidepressants (Klempin et al., 2013, Alenina and klempin, 2015).

Our group previously reported that the inhibition of NF-κB activation counteract the proneurogenic effect of clinically relevant drugs such as α2δ ligands and acetyl-L-carnitine, all characterized by antidepressant activity in murine models of unpredictable mild stress (Cuccurazzu et al., 2013; Meneghini et al. 2014). We therefore tested trazodone on ahNPC deriving from NF-κB p50 knock out mice. In contrast to 5-HT and venlafaxine, trazodone proneurogenic effect appeared to require the presence of NF-κB p50.

In this study, we also further explored the receptor subtype(s) mediating 5-HT proneurogenic effect. Based on literature reports, we initially took into consideration an involvement of 5-HT_{1A} receptors (Banerjee et al., 2007; Kaufman et al., 2016). To our surprise a 5-HT_{1A} antagonist did not counteract the *in vitro*

proneurogenic effect of 5-HT. The 5-HT_7 receptors are the latest addition to 5-HT receptors family and are characterized for their high affinity to 5-HT (Sleight et al., 1995). 5-HT_7 receptor binds with high affinity the antidepressant amitriptyline (Mullins et al., 1999). Moreover, chronic antidepressant treatment downregulates 5-HT_7 receptors expression in rat hypothalamus (Guscott et al., 2005). We tested the potential involvement of 5-HT_7 in mediating 5-HT proneurogenic effect *in vitro*. We evaluated the effect of the 5-HT_7 antagonist SB269970 alone and in presence of 5-HT on ahNPC. SB269970 *per se* did not affect neuronal differentiation but it inhibited the proneurogenic effect of 5-HT. Thus, at least in part, serotonin might increase neuronal differentiation of ahNPC through 5-HT_7 activation. The involvement of 5-HT_7 in mediating antidepressant activity is still controversial and will certainly require further investigation *in vitro* and *in vivo* (Nandam et al., 2007).

In summary, our results indicated that trazodone is a proneurogenic antidepressant and that at least *in vitro*, this activity is mediated by 5-$HT_{2A/2C}$ antagonism. Moreover NF-κB p50 subunit is required for trazodone, but not serotonin, proneurogenic activity. Finally, for the first time, we propose the involvement of 5-HT_7 receptors in the *in vitro* regulation of adult hippocampal neurogenesis.

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CHAPTER V

Discussion and Conclusions

Discussion and conclusions

The initial work of the scientist "Santiago Ramón y Cajal" showed that neurons do not proliferate and are not replaced in adult life. For long times scientists were convinced that we are born with the neurons that we will ever have. Indeed, the adult generation of new neurons in the brain was a taboo until the 60's when scientists showed that: (i) radioactive thymidine was detected in the adult brain (ii) proliferative cells could differentiate toward neurons and (iii) proliferative cells can be isolated from adult brain and culture *in vitro* (Reynolds and Weiss, 1992). Since the beginning of the 80's decades of studies investigated the nature, the zones and the role of adult neurogenesis. A great interest was given to adult neurogenesis in the hippocampus since it is the main center of cognitive functions like learning and memory (Bannerman et al., 2004). Several neurodegenerative and neuropsychiatric disorders that are characterized by loss of cognitive functions were also described for a reduced ahNG and altered astrocytes function (Kempermann and Kronenberg, 2003; Pekny et al., 2016).

My PhD work, embedded in the general topic of ahNG studies, focused on studying the effect of potential endogenous neuro-modulatory proteins secreted by local astrocytes and exogenous pharmacological compounds on ahNPC neuronal differentiation by *in vitro* approaches. We studied the role of NF-κB transcription factors, specially the NF-κB p50 subunit, as downstream signaling required for effective proneurogenic activity.

We gave a particular attention to the astrocytes-ahNPC cross-talk based on literature observations and earlier studies performed in our lab. Our group observed that *in vivo* p50KO ahNPC have defective late neuronal differentiation and p50 KO mice have impaired hippocampal-dependent spatial memory (Denis-Donini et al., 2008). In contrast, *in vitro,* spontaneous differentiation of p50KO ahNPC, was not different from their WT counterpart. An elegant earlier work done by Song and

colleagues (2002), showed that astrocytes and NPC co-culture increased the neuronal differentiation from the NPC. Moreover, this increase was region-specific since hippocampal but not spinal cord-derived astrocytes increased the neuronal differentiation from hippocampal NPC (Song et al., 2002). The mechanisms employed by astrocytes are still poorly understood and little is known about the identity of astrocytes-secreted proteins and their effect on ahNPC neuronal differentiation. The hypothesis of our work stated that possibly astrocytes signaling and protein secretion is altered in absence of NF-κB p50 explaining altered ahNG observed *in vivo*. Our aim during PhD years was to understand (i) how astrocytes communicate with ahNPC, (ii) determine proteins and receptors involved in this regulation and (iii) study possible defects caused by NF-κB p50 depletion in both astrocytes and ahNPC. Results by LC-MS/MS analysis identified proteins differentially expressed in WT and p50KO astrocytes conditioned media. We found three proteins upregulated in p50KO compared to WT astrocytes conditioned media. Two of these proteins are encoded by NF-κB target genes and NF-κB signaling activation. Thus we could show that astrocytes express and release soluble factors in the medium. Moreover we showed that p50KO ahNPC have reduced response to astrocyte-derived pro-neurogenic signals such as thrombospondin- 1 (molecule already known as proneurogenic) and Lipocalin- 2 (characterized for the first time in our study as proneurogenic molecule). In attempt to explain this lack of response we found that in fact the absence of p50 caused a downregulation in α2δ1 subunit (thrombospondin receptor) and LCN-2R/24p3R (lipocalin receptor) expression in p50KO ahNPC compared to WT. Therefore, ahNPC express receptors for astrocytes released factors an respond in changing their differentiation. Ultimately, the outcome of this study showed that the absence of NF-κB p50 subunit cause complex cell autonomous and non-cell autonomous defects in the astrocytes-ahNPC cross-talk. These findings could explain reduced ahNG and memory defects observed *in vivo.* Altogether, these data add further

complexity to the relevance of astrocytes and NF-κB signaling in the modulation of adult neurogenesis. Moreover, it suggests new proteins and mechanisms employed by astrocytes to regulate ahNPC neuronal differentiation.

Our laboratory also participated for many years in studying ahNG *in vivo* and *in vitro* for its response to drugs with antidepressants-like effect and the role of NF-κB signaling in mediating their proneurogenic effect. This work is based on several studies showing that both acute and chronic stress reduce ahNPC proliferation and differentiation in the SGZ (Pittenger and Duman, 2007). Studies on rodents, non-human primates and human mammalian brain showed that ahNG is decreased in depression disorder and increased after antidepressant treatment (Santarelli et al, 2003; Malberg et al., 2000; Boldrini et al, 2009). Recently the neurogenic hypothesis of depression states that "the production of new granule cells in the dentate gyrus of the hippocampus is linked to the pathophysiology of depression and that the increase in hippocampal neurogenesis and plasticity are required for the behavioral effect of antidepressant treatment" (Eisch and Petrick, 2012).
Antidepressants such as TCA, SSRI and SNRI increase the levels of monoamines neurotransmitters in the synaptic cleft, thus alleviating the depressive symptoms. These antidepressants were not selective only on the serotoninergic or noradrenergic receptors but had also dopaminergic and cholinergic activity underlying their several side effects (Lopez-Munoz and Alamo, 2009). Even though the neurochemical effect of these antidepressants was fast the therapeutic activity onset took 3 to 4 weeks (Pittenger and Duman, 2008). Altogether these reasons caused that more than 50% of patients quit therapy (Berton and Nestler, 2006). Molecular studies allowed us to understand that 5-HT have several receptors types with different and even opposite roles. Despite these findings, the effect of antidepressants is still poorly studied with regard to its specificity to distinct serotonin receptors. Based on this gap of knowledge we chose Trazodone. It is a serotonin antagonist and reuptake inhibitor antidepressant (SARI) (Stahl, 2009).

The choice of trazodone was based on its peculiar distinct activity on serotonin receptors. It is an antagonist of 5-$HT_{2A/2C}$ serotonin receptors. 5-HT_2 activation mediates the side effects of antidepressants such as anxiety and sleeplessness. Therefore, beside antidepressant, trazodone is commonly prescribed as a hypnotic and anxiolytic drug. Moreover, trazodone is 5-HT_{1A} partial agonist, the mechanism by which 5-HT induce the antidepressant (Chilmonczyk et al., 2015). Last but not least, trazodone is also a dose-dependent antagonist for α_1 and α_2 adrenergic receptors and histamine receptor H1. Trazodone interestingly has a negligible anticholinergic activity usually causing tremors as side effects in antidepressant treatment. Taking into consideration the knowledge about trazodone therapeutic effect and its mechanism of action, we suggested that its multimodal activity that is the asset of its antidepressant activity, could also modulate ahNG. Our study is the first to show that trazodone is proneurogenic in a concentration and time-dependent manner. Trazodone did not influence apoptosis even at high concentrations thus trazodone it is has also a safe profile with regard to cytotoxicity. We also observed that trazodone, compared to 5-HT and venlafaxine (SNRI), was the only drug that increased the percentage of mature neurons after 48 h of treatment. Therefore, trazodone might have an additive effect on late neuronal maturation. Interestingly, late maturation phase is one of the most critical phases of neuronal differentiation since it determines the integration of young neurons in the network activity (Ge et al, 2007). When we studied the involvement of different receptors in trazodone effect we showed that 5-HT_{2A} and 5-HT_{2C} antagonistic activity mediated the increase in neuronal differentiation. We also found that neither 5-HT_{1A} nor α_1 and α_2 adrenergic receptors were involved in trazodone effect. Literature studies reported that 5-HT_{1A} activation increase ahNPC proliferation (Vaidya et al., 1997, Klempin et al. 2010) whereas the effects of 5-HT_2 activation or blockade on ahNG are still controversial. Our results also showed that 5-$HT_{2A/2C}$ agonists did not influence differentiation but they blocked trazodone effect. Altogether we suggest that 5-HT_{1A} activation could be involved in ahNPC proliferation whereas 5-$HT_{2A/2C}$

inhibition could be involved in neuronal differentiation. These observations add more insight into the diversity of serotonin receptors activity on ahNG and the importance of multimodal antidepressants. Moreover, for the first time we suggested that 5-$HT_{2A/2C}$ antagonism has a positive effect on neuronal differentiation.

Recent studies showed that ahNPC in fact may secrete serotonin since they express the TPH2 (5-HT synthesis enzyme in neurons). Trazodone proneurogenic effect was completely inhibited in TPH2 depleted cells while 5-HT and venlafaxine maintained their effect. By that we confirmed that in absence of the endogenous serotoninergic tone, trazodone antagonistic activity on 5-$HT_{2A/2C}$ does not produce a proneurogenic effect. Serotonin depletion downregulates ahNPC proliferation but does not affect the generation of Tuj-1 (immature neurons marker) marked cells (Benninghoff et al., 2010). When we depleted serotonin in our cells we also confirmed that the number of immature neurons was not different compared to non-depleted cells. But interestingly the mature neurons number was significantly less in serotonin-depleted cells compared to vehicle. This important observation *in vitro* is consistent with *in vivo* studies showing that the absence of 5-HT counteracts the effect of positive modulators of ahNG on differentiation and specifically the maturation of ahNPC (Klempin et al., 2013).

To study downstream signaling potentially involved in trazodone signaling we suggested the involvement of NF-κB transcription factors specifically the p50 subunit. Our idea was based on previous studies in our lab and other groups showing that NF-κB transcription factors are expressed in the neurogenic niches and might be involved in ahNG (Denis-Donini et al., 2005; Valente et al., 2012; Cuccurazzu et al., 2013). We showed that p50KO ahNPC do not respond to trazodone proneurogenic activity. An interesting observation was also that 5-HT and venlafaxine effect was not counteracted by the absence of NF-κB p50. Thus, we suggested that trazodone effect require the presence of NF-κB p50 subunit

while 5-HT do not. It would be of interest to further investigate downstream gene expression modifications mediated by trazodone. Taking into consideration the differences we observed between 5-HT and trazodone in absence of NF-κB p50 we hypothesized that different receptor subtypes mediate 5-HT proneurogenic effect. We showed that 5-HT_7 and not 5-HT_{1A} mediate at least in part the proneurogenic effect of 5-HT *in vitro*. 5-HT_7 has high affinity to 5-HT and it is possibly involved in antidepressant activity. We could suggest for the first time possible involvement of 5-HT_7 in ahNG. Our conclusions and suggestions on trazodone are guided by the observations on *in vitro* cultured ahNPC. Extended *in vivo* studies should confirm that increased ahNG is required for trazodone antidepressant effect.

In conclusion, the main take home message is that ahNG is modulable by several factors. The principle findings are that astrocytes secrete molecules such as Lipocalin-2 to increase neuronal differentiation from ahNPC. We showed that intrinsic defects in astrocytes and ahNPC are caused by NF-κB p50 absence since astrocytes change their secretory profile and ahNPC receptors expression. We showed for the first time that ahNG is a pharmacological target for trazodone and that 5-$HT_{2A/2C}$ antagonism and 5-HT_7 activation could be novel mechanisms that positively modulate ahNG. Finally, NF-κB transcription factors specifically the p50 subunit play an important role in mediating astrocytes and antidepressants proneurogenic effects.

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CHAPTER VI

List of Publications and Posters

Acknowledgments

Publications

"Cell autonomous and noncell-autonomous role of NF-κB p50 in astrocyte-mediated fate specification of adult neural progenitor cells."

Cvijetic S, Bortolotto V, Manfredi M, Ranzato E, Marengo E, **Salem R**, Canonico PL, and Grilli M. *Glia*, 2017, **65**: 169–181.

Manuscript under preparation for submission

"Trazodone as a proneurogenic antidepressant"

Valeria Bortolotto[1,2], Rita Salem[1,2], Michele Bianchi[2], Erica del Grosso[2], Pier Luigi Canonico[2], Mariagrazia Grilli[1,2#]

[1]Laboratory of Neuroplasticity and Pain, [2]Department of Pharmaceutical Sciences, University of Piemonte Orientale, Novara, Italy.

List of poster presentations

- Poster and Short oral presentation "**The hypothalamus as a novel adult neurogenic niche**". **R. Salem**, V. Bortolotto, P.L. Canonico, M. Grilli. 17th National Congress for PhD students and Postdocs of the Italian Society of Pharmacology, 16-18 September 2014, Rimini, Italy.
- Poster "**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, May 6-8, 2015: "Adult Neurogenesis: Evolution, Regulation and Function" Dresden, Germany.
- Poster "**The hypothalamus as a pharmacologically relevant neurogenic zone**". **R. Salem**, V. Bortolotto, P.L. Canonico, M. Grilli. 27-30 October 2015, 37th National Congress of the Italian Society of Farmacology, "I nuovi orizzonti della ricerca farmacologica: tra etica e scienza", Napoli, Italy.

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