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TITOLO TESI DI DOTTORATO DI RICERCA

Hydroxytyrosol, a phenolic compound present in extra virgin olive oil and in olive mill waste-water, stimulates the adult and aged hippocampal neurogenesis

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Abbreviations

AHN: Adult Hippocampal Neurogenesis
ANOVA: Analysis of Variance
BrdU: 5-Bromo-2'-Deoxyuridine
Btg1: B-Cell Translocation Gene 1
CA: *Cornu Ammonis*
DCX: Doublecortin
DG: Dentate Gyrus
EVOO: Extra Virgin Olive Oil
GFAP: Glial Fibrillary Acidic Protein
HTyr: Hydroxytyrosol
Iba1: Ionized Calcium-Binding Adapter Molecule
KO: Knockout
MD: Mediterranean Diet
NeuN: Neuronal Nuclei
NSCs: Neural Stem Cells
NPCs: Neural Progenitor Cells
PLSD: Protected Least Significant Difference
SEM: Standard Error of the Mean
SGZ: Sub-Granular Zone
SOX2: Sex Determining Region Y-Box2
SVZ: Sub-Ventricular Zone
TRITC: Tetramethylrhodamine
Tyr: Tyrosol
WT: Wild-Type

Abstract

The management and disposal of olive mill waste-water (OMWW) are among the main environmental problems of the Mediterranean area because the phenolic nature of OMWW makes it highly resistant to biodegradation and highly phytotoxic. However, in the OMWW many high added-value compounds can be recovered to use in other ways. Among these, Hydroxytyrosol (HTyr) is a phenolic compound with various beneficial effects on human health and it can cross the blood-brain-barrier. Considering the previous data on neuroprotective effects of HTyr, we asked whether HTyr could stimulate hippocampal neurogenesis *in vivo* in adult and aged wild-type mice as well as in the Btg1 knockout, a mouse model of accelerated neural aging. We found that treatment with HTyr activates neurogenesis in the dentate gyrus of adult, aged and Btg1-null mice, by increasing the survival of new neurons and decreasing apoptosis. Notably, however, in the aged and Btg1-null dentate gyrus, HTyr treatment stimulates also the proliferation of stem and progenitor cells, whereas in the adult dentate gyrus HTyr lacks any proliferative effect. Moreover, the new neurons generated in aged mice after HTyr treatment are recruited to existing circuits, as shown by the analysis of specific neural markers. Finally, HTyr treatment also reduces the markers of aging lipofuscin and Iba1.

Overall, our findings suggest that the recovery of HTyr from OMWW could be an advantage for the environment, and its use as a diet supplement an important ingredient to counteract neurogenesis decline during aging.

1 Introduction

1.1 Mediterranean Diet and Extra Virgin Olive Oil (EVOO)

The Mediterranean diet (MD) is a set of food, social behavior, and a way of life. In fact, in addition to the foods consumed, many other variants define MD: method of conservation, culinary processes, eating behaviors, etc.; all these variables are related to cultural, economic and ethnic differences in the Mediterranean countries.

The first definition of MD is based on dietary traditions from the late 1950s and early 1960s in Greece and Italy, and it is based on the dietary analysis conducted by Ancel Keys and colleagues in seven countries of MD regions, where they found a strong inverse correlation between the type of dietary fat and mortality, specifically in mortality due to coronary heart disease and cancer (Keys, 1995; Keys et al., 2017). Subsequently, the model of MD pattern was defined in 1993 as a dietary pattern found in the olive-growing areas of the Mediterranean region (Willett et al., 1995).

The dietary pattern of MD is characterized by the consumption of abundant plant foods in the form of fruit, vegetable, bread, other forms of cereal, potatoes, beans, nuts, and seeds; dietary products are principally cheese and yogurt; a low to moderate consumption of fish, depending on the proximity of sea; a low to moderate consumption of poultry; fewer than four eggs consumed per week; low amount of red meat; wine consumed in a low to moderate amounts, normally during the meal; and olive oil consumption (Willett et al., 1995). Therefore, MD is a very variable diet which includes the consumption of several classes of food and nutrients with well documented beneficial effects on cardiovascular diseases and cancer (Lichtenstein et al., 2006; Renaud et al., 1995). Furthermore, several studies showed that the high adherence to MD is also associated with a slower cognitive decline during aging and with a reduction in the risk to develop neurodegenerative diseases, such as Alzheimer's disease (Samieri et al., 2013).

1.1.1 Extra virgin Olive Oil (EVOO) production and polyphenols compounds

The Extra Virgin Olive oil (EVOO) is a key component of MD and its daily consumption is between 25 to 50 g/day. Historically, the culture of the olive tree has begun 6000 years ago. The Phoenicians, Syrians, and Palestinians spread the olive tree in the regions bordering the Mediterranean Sea. Subsequently, Greeks and Romans taught its cultivation and exploitation to the colonized people and the production of olive oil has begun a key element of the diet in the country bordering the Mediterranean Sea and, despite their lack of knowledge about the many effects of EVOO on health, the inhabitants of Mediterranean countries have always cited

olive oil as being responsible for population longevity. Currently, the beneficial effects of olive oil on health are well characterized and they can be attributed to oleic acid and some monounsaturated fatty acids (MUFA), several micro-constituents, and some polyphenol compounds (Casamenti and Stefani, 2017). In total, approximately 230 chemical compounds including aliphatic and triterpene alcohols, sterols, hydrocarbons, volatile carotenes and polyphenol compounds, such as lipophilic and hydrophilic phenols. Due to the beneficial properties of polyphenol on human health, the European Regulation No 1348/2013 establishes that the quality of EVOO is referred to the content in free fatty acids and polyphenols, both quantitatively and qualitatively. At optimal conditions, the polyphenol content in the EVOO can reach levels exceeding 60mg/100g (Ranalli et al., 2009; Servili and Montedoro, 2002).

The phenolic compounds in EVOO can be classified as phenolic alcohols, phenolic acids, flavonoids, lignans, and glycosides. The glycosides are a subclass of iridoids that are found in the plant, and they are geraniol-derived monoterpenes composed by a six-atoms heterocycle including oxygen heteroatoms fused to cyclopentane ring. When this ring is broken the molecules are known as secoiridoids (Casamenti and Stefani, 2017).

The phenolic concentration in EVOO is influenced by several variables, from cultivation techniques of the olive trees to olive processing to the EVOO product. Indeed, the cultivar conditions don't influence the phenolic profile, however, several conditions such as altitude, irrigation, olive ripening can modify the concentration of several phenolic compounds, and this makes the quality of EVOO very variable from breeding traditions. For instance, the secoiridoid oleuropein is always present but its concentration is greater in the EVOO obtained from ripe olive, whereas the concentration of other hydrophilic polyphenols decrease, indicating that the fruit ripening can influence the polyphenolic composition of olive oil (Ranalli et al., 2009; Servili and Montedoro, 2002).

The phenolic profile of EVOO is mainly influenced during its production, which can be subdivided into several phases: washing, destoning, crushing, malaxation, separation (Figure 1.1); except for the initial washing step, all the other steps influence the phenolic composition of EVOO.

Initially, the harvested olives are washed and pitted. The seeds of olives are rich in peroxidase, which catalyze the phenolic oxidation during the subsequent steps of EVOO production. In fact, it was demonstrated that the destoning of olive influence the phenolic compounds of EVOO (Frega et al., 2005).

Subsequently, the olives are crushed. The crushing step aims to break down the cellular membranes of olive fruits and thus release small drops of oil from the vacuoles (Rodis et al., 2002).

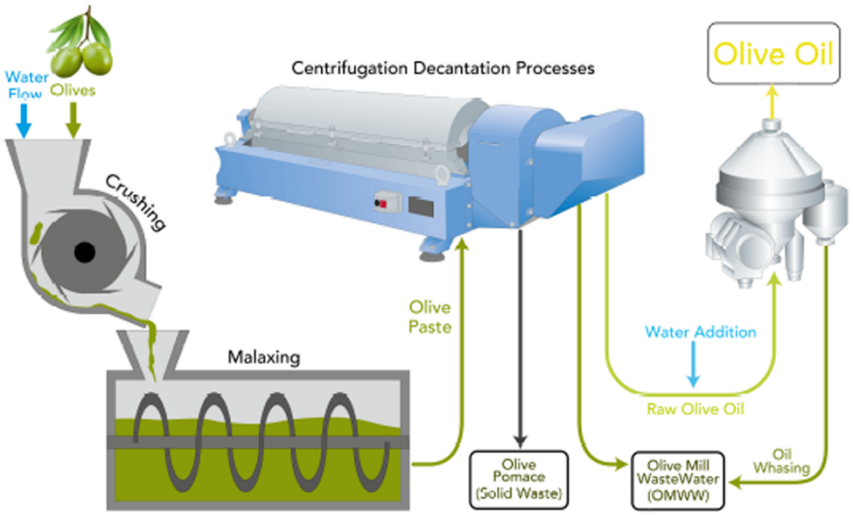


Figure 1.1 Flowchart of the three-phase procedure to obtain olive oil through three fractions: olive oil, olive pomace (solid residue), and OMWW (Ahmed et al., 2019).

This operation produces a mixture of two distinct liquid phases (raw oil and water) and a heterogeneous solid phase (pit, skin, and pulp fragments). Crushing is a critical phase in the EVOO production because it influences the quality of final products, especially its content in polyphenol content (Servili et al., 2015). This step allows a direct contact of the peroxidases and polyphenol oxidase enzymes with phenolic compounds. For instance, during this phase, the endogenous β-glucosidases catalyze the hydrolysis of oleuropein, demethyloleuropein, and ligstroside generating the main hydrophilic phenols, including the secoiridoid aglycons, which include the Hydroxytyrosol (HTyr) and tyrosol (Tyr; Figure 1.2). There are other secoiridoids derived from oleuropein and these include the dialdehydic form of decarboxymethyl elenolic acid bound to either HT, also known as oleacein, or to tyrosol, also known as oleocanthal (Figure 1.2; Casamenti and Stefani, 2017).

The paste obtained by the crushing phase is mixed in the malaxation phase. This phase essentially consists of heating olive paste up to 25-27°C under slow and continuous stirring. This step permits to break the oil-water emulsion, formed during the crushing, and to join the oil droplets into an ever-larger droplet that facilitates the next step of extraction. During the malaxation, the distribution of secoiridoid aglycons and the phenolic alcohols between oil and water phases is related to their solubility in water and oil, and it is inversely related to the time and temperature of the malaxation phase (Frega et al., 2005).

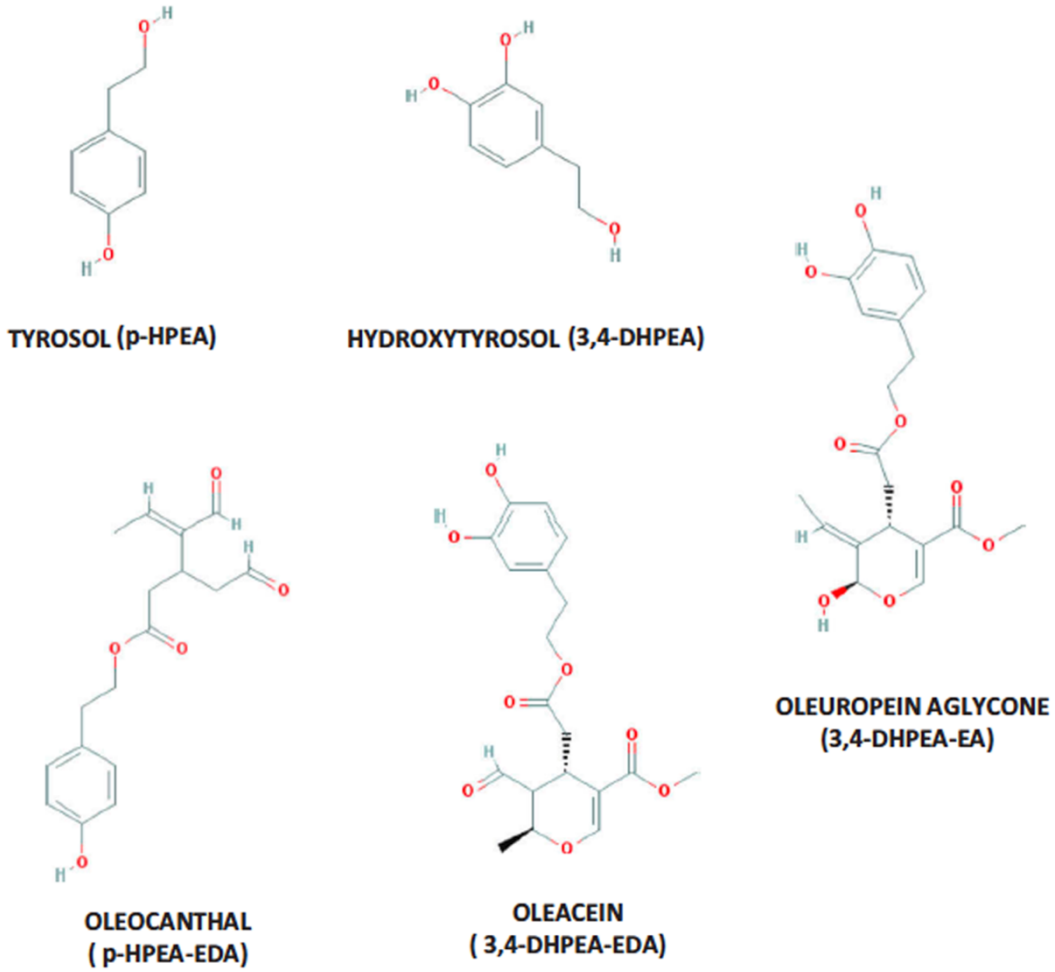


Figure 1.2 Chemical formulas of the main olive leaf and olive oil polyphenols in the aglycone form (Casamenti and Stefani, 2017).

Subsequently, the oil must be separated from pomace, and it is performed using several techniques. In Italy, Greece, and Portugal, the major olive oil-producing countries, the main separation system is a traditional system that uses a battery of presses to separate the two products of the malaxation phase. In addition to the traditional system, other separation systems were developed, such as 2-phases separating decanter or 3-phases separating system (A.R.A) or Sinolea method. The choice of the separation system significantly influences the phenolic profile of EVOO. For instance, the use of 2-phases separating decanter increases the concentration of polyphenol compounds, but EVOO is more bitter, compared to EVOO obtained with other systems.

The oil-must obtained after separation is composed of 80% of EVOO and 20% of vegetation water, the final step is the centrifugation of this oil-must that permits the purification of the final product: EVOO.

Overall, these data demonstrated that all phases in the production of EVOO influence its phenolic profile and, considering the beneficial effects of these compounds, the choice of the extraction system or development of new techniques allowing the production of high-quality EVOO with an enhanced health effect.

1.1.2 Olive mill waste-water (OMWW)

Production of wastes is an important issue in the production of olive oil. In fact, at the end of olive oil production, two types of waste are generated: a solid residue and an effluent known as olive oil mill waste-water (OMWW). The management and the disposal of OMWW are one of the main environmental problems of the Mediterranean area where, from November to February, are produced great quantities of olive oil with a large volume of waste-water. The annual worldwide production of OMWW is estimated to be from 10 to 30 million m^3, dependent on the used oil extraction system, the processed fruit and the operating conditions (McNamara et al., 2008). For instance, the manufacture of olives by the traditional milling and pressing system, a common system used in Italy, produced 1-2 tons of OMWW during the processing of 1 ton of olives (Paraskeva and Diamadopoulos, 2006).

Typical OMWW composition by weight is 83-94% water, 4-16% organic compounds, and 0,4-2,5% phenolic compounds, divided into high-molecular weight (tannins, anthocyanins, etc.), and low-molecular weight compounds (Tyr, HTyr, etc.). The high phenolic nature of OMWW makes it highly resistant to biodegradation and highly phytotoxic (Zirehpour et al., 2014). For these reasons, several pre-treatments techniques have been developed for the polluting power of OMWW. However, at present, in Italy, the disposal of OMWW is regulated by the law n°574/1996. This law permits to spread OMWW on agricultural and industrial soils with a series of restrictions, because of the expense of new technologies for pre-treatments and the difficulty or conventional treatment methods. This practice causes extensive pollution of the soil and even transfers harmful compounds into other media, such as groundwater and surface water.

Therefore, the management of OMWW is a very important issue, and have been proposed several strategies:

- Waste reduction via olive production system (i.e. using two-phases instead of the three-phases continuous system).
- Detoxification methods aiming at the reduction of the impact of the pollution load to the recipient.
- Recovery of recycling of components from OMWW.

The third point can be considered the one with more advantages. In fact, more integrated methodologies should be developed combining treatment, recycling, enhancement, and energy-producing process, allowing the recovery of high added-value compounds.

Enhancement and recycling constitute new concepts that are increasingly necessary worldwide. Typically, by-products, wastes, and effluents from fruit and vegetable processing consist in high amounts of protein, sugars, and lipids along with peculiar aromatic and aliphatic compounds; thus, they could be considered as cheap and abundant raw material for the synthesis of value-added chemicals and biomaterials (Federici et al., 2009).

In relation to EVOO production, in the OMWW there are many high added-value compounds. Indeed, when olives are milled to produce EVOO, the important phenolic compounds are quite completely conveyed in OMWW. More than 99% of active polyphenols in olive fruits are present from 100 to 300-fold more concentrated in OMWW. Several techniques have been used to recover phenolic compounds from OMWW, including enzymatic preparation, solvent extraction, membrane separation, centrifugation and chromatographic procedures (Dermeche et al., 2013).

Among high added-value compounds, there is Hydroxytyrosol (HTyr) a phenolic compound present in the EVOO but in higher concentration in the OMWW, where it is one of the compounds responsible for the great toxicity of this waste (Fiorentino et al., 2003).

1.2 Hydroxytyrosol (HTyr)

The Hydroxytyrosol [4-(2-Hydroxyethyl)-1,2-benzenediol; 3,4-DHPEA; HTyr] is a phenylethanoid, structurally composed by a benzene ring with a 2-hydroxyethyl and two hydroxyl groups, which are responsible for its high ORAC index, or the ability to absorb radical oxygen, of 40,000 µmolTE/g, about 10 times more than green tea and coenzyme Q10, two known antioxidant compounds (Martínez et al., 2018).

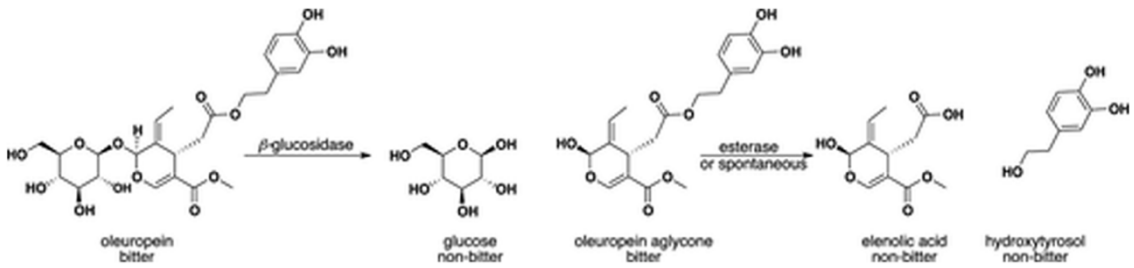


Figure 1.3 Hydrolysis of Oleuropein in elenoic acid and Hydroxytyrosol.

HTyr is a phenolic compound present in the olive oil, and its concentration increase during the production of olive oil in the phases of crushing and malaxation when the endogenous β-glucosidases hydrolyze oleuropein in oleic acid and HTyr (Figure 1.3), which ends up in the EVOO and OMWW (Gambacorta et al., 2007). The final concentration of HTyr in EVOO can be influenced by several factors. For instance, its concentration depends on the type of olives or techniques used during the production of olive oil can influence the concentration of HTyr in the final product, indeed, its concentration in virgin oil is 1.74 ± 0.84 mg kg^{-1}, and it is lower compared to the concentration in EVOO, which is 14.32 ± 3.01 mg kg^{-1} (Blekas et al., 2002; Rodis et al., 2002). It has been estimated that the consumption of HTyr per day is 5.6 mg (Martínez et al., 2018). However, most of the HTyr, ends up in the OMWW, during the olive processing, from which it can be recovered and used as diet supplementation.

After its oral ingestion, HTyr reaches easily the intestine because it is resistant to gastric juices of the stomach. In the intestine, it is absorbed through bi-directional passive diffusion mechanism (Imran et al., 2018; Manna et al., 2000), with an efficiency that oscillates from 75% up to 100% (Martínez et al., 2018). The food matrix influences the absorption of HTyr, indeed, its stability is higher after its administration as a natural component of EVOO (Pastor et al., 2016), compared to its administration to a water vehicle (González-Santiago et al., 2010).

After its absorption, HTyr is rapidly metabolized in two steps. In the first step are involved the alcohol and aldehyde dehydrogenases (ALDH) localized in the cytoplasm of enterocytes. Subsequently, the 98% of HTyr absorbed is sulfonated or glucuronidated, by sulphotransferases (SULT), uridine-5'-diphosphoglucuronosyl transferases (UGT) and catechol-O-methyltransferases (COMT), respectively; forming the main HTyr metabolites detected in the biological sample (Figure1.4*A*; Marković et al., 2019). Besides their accumulation in kidney and liver, HTyr and its metabolites reach several tissues such as muscle, testis, liver, and brain; this widespread diffusion is responsible for its various beneficial effects (Robles-Almazan et al., 2018). For these reasons, several ways of synthesis of HTyr to study its beneficial effects were developed in last decades (Bernini et al., 2013). Furthermore, various derivates of HTyr were synthesized to improve its adsorption. One of these chemical modifications is the acetylation of HTyr, which is also present at low concentration in EVOO and it crosses the intestinal barrier more easily than HTyr, increasing thus the pharmaceutical efficacy (Bernini et al., 2013; Mateos et al., 2011).

HTyr mainly acts as a free radical-scavenger and metal chelator. This efficient activity is attributed to the presence of the o-dihydroxyphenyl moiety, which acts as a chain breaker by donating a hydrogen atom to peroxyl-radicals (ROO*). In this way, fairly reactive ROO* is

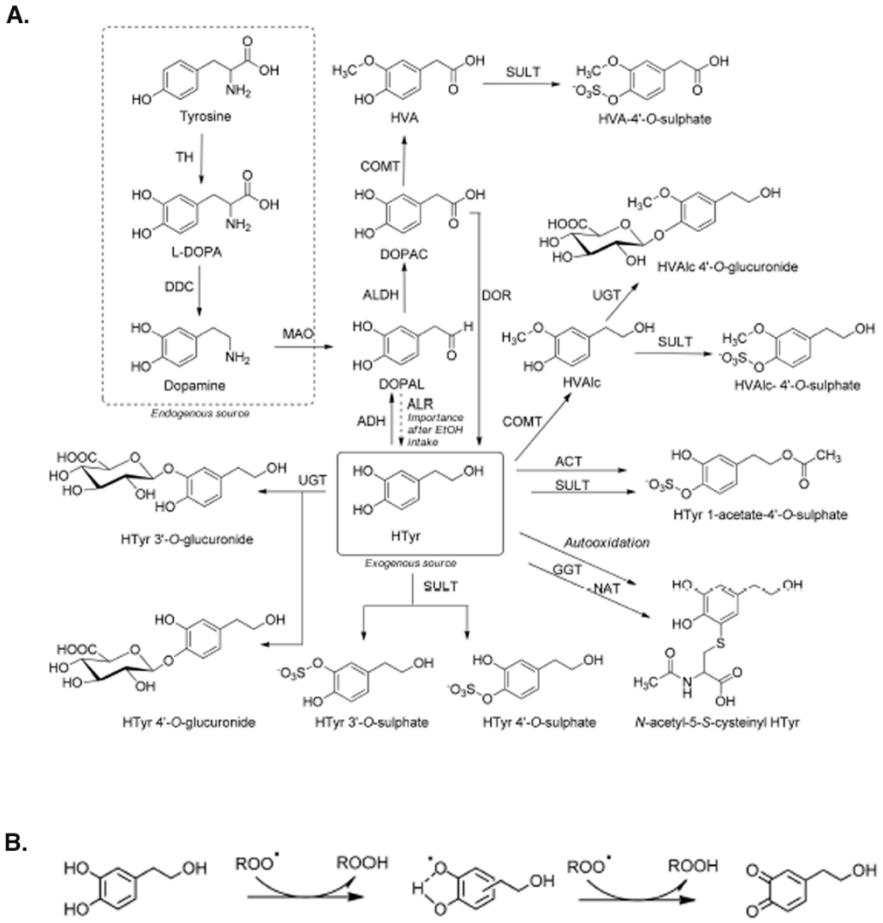


Figure 1.4 Metabolism of HTyr and its mechanism of free radical scavenging.
(A) Metabolic pathways of endogenous and exogenous HTyr. **HVAlc**: homovanillic alcohol; **HVA**: homovanillic acid; **EtOH**: ethanol; **TH**: tyrosine hydroxylase; **DDC**: dopa decarboxylase; **MAO**: monoaminoxidase; **ALDH**: aldehyde dehydrogenase; **ALR**: aldehyde/aldosa reductase; **ADH**: alcohol dehydrogenase; **DOR**: DOPAC reductase; **COMT**: catechol-O-methyltransferase; **UGT**: uridine 50-diphosphoglucuronosyl transferases; **SULT**: sulphotransferase; **ACT**: O-acetyltransferase; **GGT**: ɣ-glutamyl transpeptidase; NAT: N-acetyl transferase. **(B)** Mechanism of free radical scavenging by HTyr (Marković et al., 2019).

replaced with HTyr* radical, unreactive due to the presence of an intramolecular hydrogen bond in the phenoxy radical (Figure 1.4*B*; Marković et al., 2019).

Furthermore, it was demonstrated the involvement of HTyr in the modulation of several cell signaling. For instance, it was demonstrated that HTyr inhibits the growth of human hepatocellular carcinoma cells via inactivating the Akt and nuclear factor–kappa B (Nf-kB) pathway (Zhao et al., 2014). Otherwise, it was observed that HTyr reduces oxidative stress in the heart by induction of Nfr2-dependent gene expression (Bayram et al., 2012).

All the biological features involving the HTyr produce a wide range of biological effects, such as cardio-protective, anti-cancer, anti-inflammatory, anti-microbial, neuroprotective, and other effects (Marković et al., 2019).

1.2.1 Hydroxytyrosol and cardiovascular system

HTyr is involved in the inverse correlation between MD and cardiovascular disease (Keys, 1995; Keys et al., 2017; Marković et al., 2019). Several works suggested a possible therapeutic use of HTyr in the treatment of atherosclerosis. The atherosclerosis is a vascular disease in which the inside of the artery narrows due to the build of the plaque and can result in coronary artery disease, stroke, peripheral artery disease, or kidney problems, depending on which arteries are affected. The atherosclerotic vessel wall contains a high level of ROS, which affects several redox-sensitive pathways in vascular cells, with an expression of adhesion molecule and chemotactic factors that induce the migration and proliferation of vascular smooth muscle cells. Moreover, increased arterial adhesiveness creates an excellent environment for local infiltration of circulating immune cells, resulting in chronic inflammation (De Nigris et al., 2001; Spagnoli et al., 2007). Several works demonstrated that the action of HTyr in atherosclerosis disease involves the anti-oxidant properties of HTyr on low-density lipoprotein (LDL) and high-density lipoprotein (HDL) of cholesterol.

The LDL oxidation is one of the key steps in the initiation of atherosclerosis, and HTyr is very efficient in preventing lipid peroxidation and protecting LDL from oxidation. At this regard, the European Food Safety Authority (EFSA) has published a report in which it is indicated that on LDL oxidation "*a daily intake of 20 g of olive oil, which contains at least 5 mg of HTyr and its derivate (e.g. oleuropein and Tyr) provides the expected beneficial effect*" (2010). However, in this activity of HTyr is not only involved the free-radical scavenging function, but also the modulation of gene expression, such as the CD40 and its downstream products, which are associated with a decrease in oxidized LDL in plasma (Castañer et al., 2012). Moreover, it has been also observed beneficial effects of HTyr on high-density lipoprotein (HDL), through the prevention of oxidative modification of Apolipoprotein A-1, the main HDL protein involved in cholesterol efflux capacity, and other HDL proteins (Calabriso et al., 2018).

Besides these functions, it was observed that a regular intake of HTyr modulates the expression of inflammation and oxidative stress-related genes (Colica et al., 2017) and downregulates the expression of vascular cell adhesion molecules involved in atherosclerosis (Scoditti et al., 2014). Furthermore, HTyr prevents platelet aggregation, with effects similar to acetylsalicylic acid (González-Correa et al., 2008). Finally, it was demonstrated that a diet with HTyr induces a change in proteomic profile in the heart and aorta (Catalán et al., 2016).

1.2.2 HTyr and cancer

A vast number of *in vitro* and *in vivo* studies have shown significant anticancer effects of HTyr against various types of malignant cells, with different mechanisms of action (Bernini et al., 2013; Marković et al., 2019).

Considering that HTyr reaches easily the intestine after oral administration, many works were focused on its effect on colon cancer. It was observed that the treatment with HTyr has anti-proliferative and pro-apoptotic effects on LDL1 human colon cancer cells, mediated by activation of PI3K/Akt/FOXO3a pathway and by an increase in the concentration of ROS due to a decline in the antioxidant defensive capacity of these cells (Sun et al., 2014). The same effects have been observed in the colon cancer cells Caco-2 and HT-29 (López De Las Hazas et al., 2017). Finally, in xenograft of HT-29, treatment with HTyr reduces cancer cell proliferation which results in a decrease in cancer mass and expression of EGFR on colon cancer cells (Bernini et al., 2017).

In addition to colon cancer, the beneficial anticancer effects of HTyr have been investigated in the breast, hepatic, skin, blood, brain and other cancer suggesting its possible wide use in cancer prevention and treatment (Bernini et al., 2013; Marković et al., 2019). For instance, in hepatocellular carcinoma cells (HCC), the treatment with HTyr *in vitro* blocks the cell cycle in G2/M transition and induce their apoptosis, whereas *in vivo* it has been demonstrated that HTyr as the same effects on tumor by a suppression of the activation of Akt and NF-kB pathway (Zhao et al., 2014).

These works demonstrated that HTyr induces an arrest of cell cycle and their apoptosis in various types of cancer cells. These investigation has revealed that the anti-proliferative activity of HTyr is inversely correlated to the ability of the different cell lines to remove H_2O_2 from the culture medium (Rosignoli et al., 2016). Therefore, overall, these data demonstrate a possible role of HTyr as an anticancer molecule.

1.2.3 HTyr and Brain

The HTyr administrated by oral ingestion can reach the brain because it can cross the blood-brain barrier (Hornedo-Ortega et al., 2018). Moreover, HTyr is naturally present in the brain as a by-product of dopamine and tyramine metabolism (Figure 1.4*A*; De La Torre et al., 2006; Rodríguez-Morató et al., 2016).

Several beneficial effects of HTyr were demonstrated in the brain. In fact, HTyr reduces oxidative stress in brain slices undergoing oxidative stress, i.e., lipid peroxidation, peroxynitrite formation and production of inflammatory mediators (prostaglandin E2 and interleukin-1β; De

La Cruz et al., 2015); similarly, HTyr protects *in vitro* from oxidative stress, by rescuing either PC12 cells or SH-SY5Y neuroblastoma cells from 6-hydroxydopamine-induced damage, from L-DOPA toxicity or preventing hydrogen peroxide-induced death (Funakohi-Tago et al., 2018; Omar et al., 2018; Peng et al., 2015).

Recent studies showed the role of HTyr in several neurodegenerative diseases. In fact, treatment with HTyr exhibited beneficial effects in Alzheimer's disease (AD), the most common cause of dementia. This amyloid disease is characterized by the deposition of typical protein/peptides aggregates in tissue that is associated with brain degeneration and progressive cognitive impairment. Several works demonstrated that aromatic molecules interfere with amyloid aggregation, possibly by remodeling the amyloid intermediates through different mechanisms of interaction (Bastianetto and Quirion, 2004; Pawar et al., 2005), or by redirecting or speeding up the aggregation cascade towards non-toxic species (Necula et al., 2007; Wang et al., 2008a). It was demonstrated that aromatic ring present in phenol interacts with aromatic residues in amyloidogenic proteins and these interactions prevent the formation of amyloid fibrils (Cheng et al., 2013). Recently, Leri and colleagues demonstrated that HTyr and other phenols in EVOO interfere with the fibrillar formation, aggregate seeding and cytotoxicity of $A\beta_{1-42}$, in a dose-dependent manner. These data suggest a possible therapeutic use of HTyr and other phenols in olive oil or OMWW in the treatment of AD and its prevention (Leri et al., 2019; Nardiello et al., 2018).

Moreover, HTyr is a promising compound to treat Parkinson's disease. This neurodegenerative disease is characterized by the progressive loss of dopaminergic neurons in the midbrain region known as *substantia nigra pars compacta* and by the presence of cytoplasmic protein aggregates, called Lewy body, and Lewy neurites in remaining neurons (Bellucci et al., 2016). The monoamine oxidase B (MAO-B) inhibitors are the main therapeutic approach for Parkinson's disease and increase synaptic dopamine by blocking its degradation (Connolly and Lang, 2014). On the other hand, the rise of cytoplasmic dopamine induces its enzymatic and spontaneous oxidation to Dopamine-species, including 5-S-cysteinyl-dopamine (Bellucci et al., 2016). It was demonstrated that HTyr inhibits both enzymatic and spontaneous oxidation of endogenous dopamine and mitigates the increase in spontaneous oxidation during MAO inhibition, suggesting that the use of HTyr could be used to enhance the efficiency of the clinical treatment of Parkinson's disease (Goldstein et al., 2016).

Finally, studies *in vivo* showed that treatment with HTyr attenuates spatial-cognitive deficits, restores learning capacity and memory performance, and improves cognitive functions (Davinelli et al., 2016; Zheng et al., 2015a). Furthermore, Zheng and colleagues showed that

HTyr treatment rescued the mRNA levels of BDNF, GAP43 and other neural markers reduced by stress in the whole hippocampus but no indication on the production of new neurons in the neurogenic niches was provided.

However, the effects of HTyr on the production of new neurons in post-natal life are not yet known.

1.3 Adult Neurogenesis

Adult neurogenesis can be defined as the capacity to generate new functional neurons from neural stem cells during adulthood. This argument has always been a topic of debate. In 1928 Ramon y Cajal, a father of modern neuroscience, wrote "*Once development was ended, the founts of growth and regeneration…dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended and immutable. Everything must die, nothing may be regenerated"* and, for many years, the brain was considered a static structure, incapable of generating new neurons in adult age. This dogma has fallen in 1965 when Altman and colleagues, using thymidine analogs, found the evidence of the existence of newborn neurons in adult rat brains (Altman, 1969; Altman and Das, 1965). Subsequently, several types of research confirmed the presence of adult neurogenesis in rodents and in other species, such as songbird, where newly generated neurons were found in the adult age and a connection between adult neurogenesis and the ability of songbird to learn some new syllables (Nottebohm, 2004).

While several works demonstrated a presence of adult neurogenesis in several species, its presence in human is still a topic of debate among the neuroscience researchers. The first evidence of adult neurogenesis in humans was obtained in cancer patients treated with the thymidine analog 5-Bromo-2'-deoxyuridine (BrdU), for diagnostic purposes. The *post-mortem* analysis of these patients has shown the presence of proliferating cells in the brains; moreover, these cells were positive for neuronal or astrocytic markers (Eriksson et al., 1998). Subsequently, measuring the concentration of nuclear bomb-test-derived 14C in genomic DNA, Spaldini and colleagues demonstrated that a large subpopulation of hippocampal neurons is subjected to exchange, with an addition of 700 new neurons in hippocampus per day, corresponding to an annual turnover of 1.75% (Spalding et al., 2013).

Although these data support the presence of neurogenesis in the adult human brain, a recent work published by Sorrells and colleagues argue otherwise (Sorrells et al., 2018). In this work, the authors examined 18 adult and 19 perinatal or postnatal samples of *post-mortem* brain tissues obtained from individuals of wide age range (14 gestational weeks to 77 years) with various causes of death, and upon immunohistochemistry analysis, the authors did not detect young

neurons in the brain of adult patients (Sorrells et al., 2018). Simultaneously, a second work demonstrated the persistence of adult hippocampal neurogenesis in the adult human brain (Boldrini et al., 2018). In this work, using unbiased stereological methods on *post-mortem* brain on 28 women and men from 14 to 79 years of age, Boldrini and colleagues, characterized and quantified the angiogenesis, volume, and cells at different maturational stages in the dentate gyrus of the hippocampus, one of the adult neurogenic niches, described later, demonstrating the persistence of adult hippocampal neurogenesis up to 80 years old. Besides, they suggest that the age-related cognitive decline may be caused by a variety of factors, such as a smaller quiescent neural progenitor pool, diminished angiogenesis, or decreasing neuroplasticity in the dentate gyrus (Boldrini et al., 2018).

It may appear surprising that two studies used similar methods and reached the opposite conclusion as to whether adult human neurogenesis exists. However, the explanation is found in the methodologies used in collecting the brain sample, as demonstrated by Moreno Jimenez and colleagues (Moreno-Jiménez et al., 2019). Indeed, they found that the detection of adult neurogenesis markers in the human brain is critically dependent on fixation conditions and histological pretreatment of the tissue (Moreno-Jiménez et al., 2019). In this works, the authors analyzed *post-mortem* brain samples obtained from 13 neuralgically healthy subjects between 43 and 87 years of age. The samples have been obtained under tightly controlled conditions and state state-of-the-art tissue processing methodologies and upon immunohistochemistry assays, they found the persistence of AHN in the adult human dentate gyrus (DG) until the ninth decade of life (Moreno-Jiménez et al., 2019).

Overall, these studies showed that the production of new neurons continues during the adult age in different species, including rodents and humans. However, the production of new neurons in primates in adulthood is lower than that observed in rodent species.

1.3.1 Neural stem niches

Adult Neural Stem cells (NSCs) can self-renew and differentiate in any of the neural cell lineages such as astrocytes, oligodendrocytes, and neurons (Gage, 2000).

After embryonic development, the NSCs are localized in specific microenvironments, called neurogenic niches, that support the self-renewal and differentiation of NSCs (Gage, 2000).

In the adult mammalian brain, there are two main neurogenic niches able to generate new neurons: the sub-ventricular zone (SVZ) of lateral ventricle and the sub-granular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (Figure 1.5; Zhao et al., 2008). Despite various

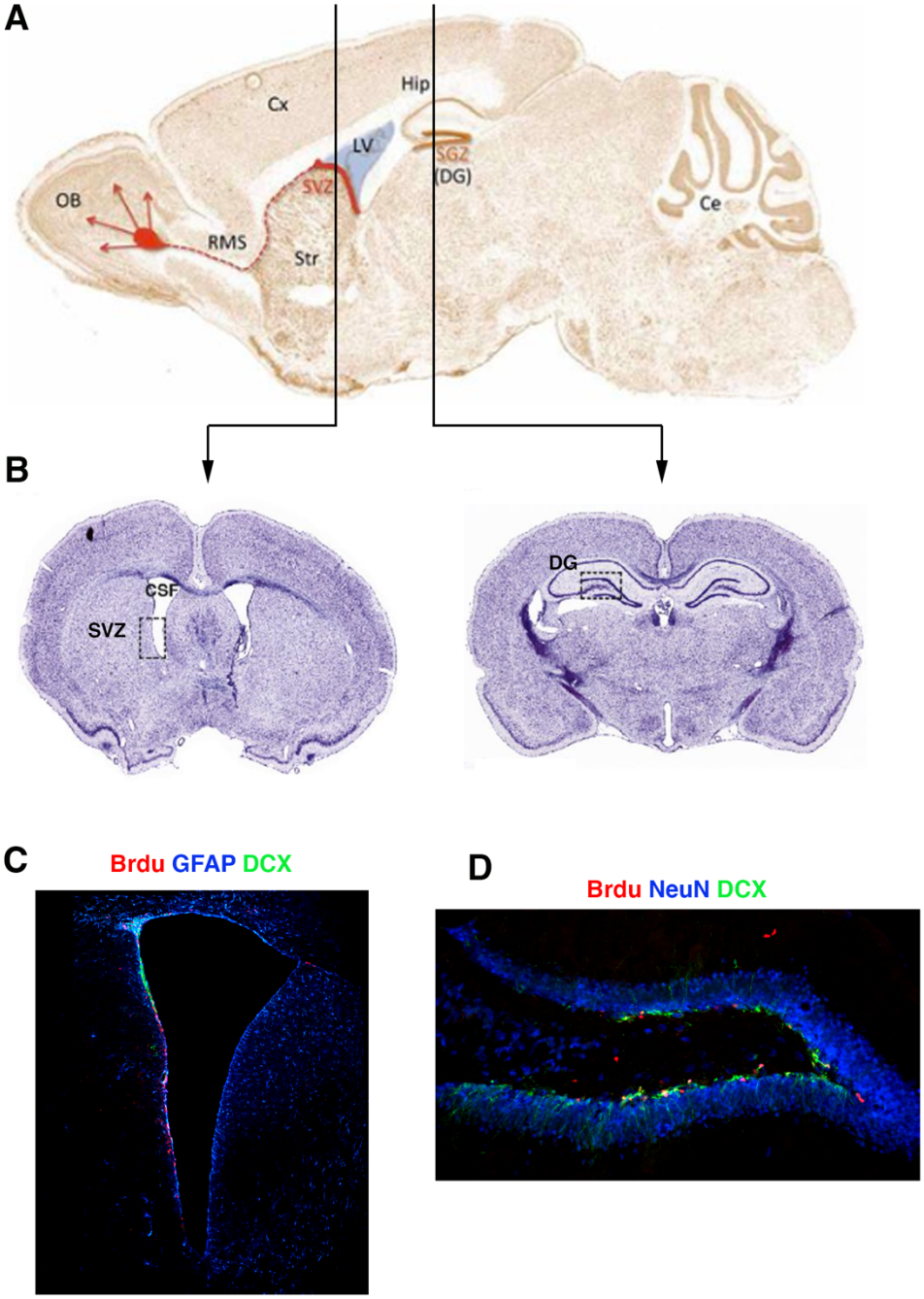


Figure 1.5 Schematic representation of two main neurogenic niches in the mammalian brain.
(A) Representations of a sagittal section of the mouse brain and the two main neurogenic niches: the sub-granular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Hip) and the sub-ventricular zone (SVZ) of the lateral ventricle (LV). Neurons generated in the SVZ migrate through the rostral migratory stream (RMS) and are incorporated into the olfactory bulb (OB). **(B)** Coronal views of the two neurogenic niches. **(C-D)** Immunofluorescence images of SVZ **(C)** and DG **(D).**

common elements in the two niches, they generate neurons that are morphologically and functionally different (Ming and Song, 2011). Besides these regions, the NSCs have been isolated from other regions of the adult mammalian brain: the neocortex (Gould et al., 1999a; Homman-

Ludiye et al., 2012), the striatum (Jensen et al., 2004), the amygdala (Bernier et al., 2002), the *substantia nigra* (Zhao et al., 2003), the spinal cord (Weiss et al., 1996). However, the cells isolated from these brain regions showed an active proliferation and differentiation only *in vitro* experiments, while *in vivo* these cells remain in a quiescent state (Kim and Sun, 2012).

1.3.1.1 Neurogenesis in Sub-Ventricular Zone (SVZ)

The subventricular zone (SVZ) of the lateral ventricles is one of the two neurogenic niches, together with the DG of the hippocampus, where new neurons are continuously generated throughout adulthood (Alvarez-Buylla and García-Verdugo, 2002; Zhao et al., 2008).

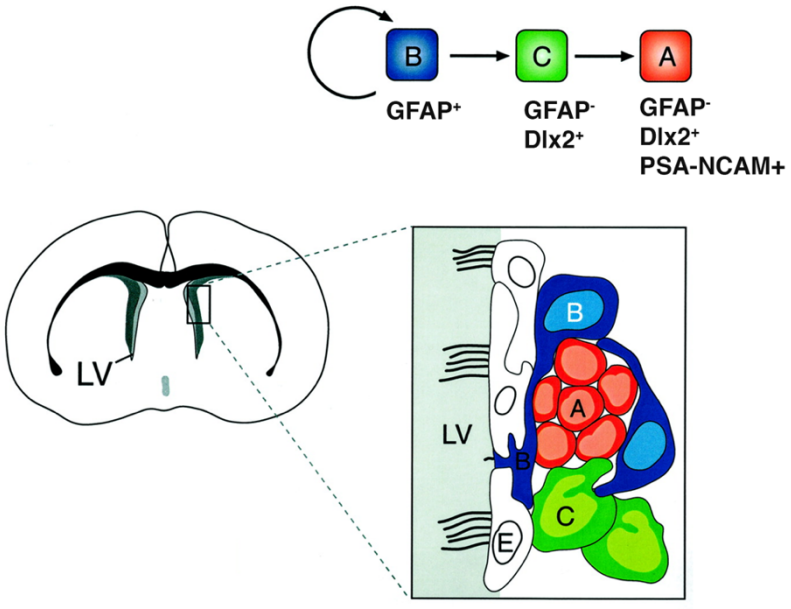


Figure 1.6 Neurogenesis in adult SVZ.
Coronal representation of Sub-ventricular zone (SVZ) of the lateral ventricle (LV), and schematic representation of neurogenesis in SVZ. **B:** Type-B cells, slowly dividing radial cells; **A:** Type-A Cells Neuoblasts; **C:** Type-C cells, immature precursors; **E:** Type E- cells, ependymal cells (Muñoz and Guha, 2011).

The SVZ is a layer of dividing cells extending along the lateral wall of the lateral ventricle (Figure 1,5, 1.6). The new neurons born from SVZ migrate along the rostral migratory stream until reaching the olfactory bulb, where they differentiate into granular and perigranular neurons and are integrated into pre-existing neural circuitry, contributing to form the olfactory memory (Figure 1.5; Alvarez-Buylla and García-Verdugo, 2002; Doetsch and Scharff, 2001). The neural cells into the SVZ can be classified by morphology and markers expression. This characterization divides cells into four many cell types: neuroblasts (Type A cells), SVZ astrocytes (Type B cells), immature precursors (Type C cells) and ependymal cells (Type E cells; Figure 1.6; Zhao et al., 2008). Ependymal cells form a barrier between cerebrospinal fluid

(CSF) and brain parenchyma but they are also involved in the control of adult neurogenesis through the production of protein, such as Noggin or PEDF (Zhao et al., 2008).

Type-B cells are slowly dividing radial cells that can be subdivided into two sub-classes: Type-B1 and Type-B2. Type B1 cells are in contact both with lateral ventricle through their apical cilia and with blood vessels via their basal ends. While Type-B2 cells are located in the brain parenchyma and have no contacts. Type-B cells have the morphology of astroglial cells and express GFAP (glial fibrillary acid protein), Nestin, Sox2, BLBP, and CD133. Type-C cells (transit-amplifying progenitors) originate from Type-B cells. They loss ramified morphology and are actively dividing NSCs, and are characterized by the expression of Nestin, Mash1, Dlx, and EGFR. Type-C cells mature in Type-A cells, which are proliferating neuroblasts that differentiate into immature neurons when they start to migrate from the SVZ to the olfactory bulb. Type-A cells migrate towards Rostral Migratory Stream to reach the olfactory bulb where they mature and differentiate in GABA inhibitory neurons, which are integrated into pre-existing neural circuitry and participate in forming the olfactory memories (Alvarez-Buylla and García-Verdugo, 2002; Zhao et al., 2008).

1.3.1.2 Neurogenesis in Sub-granular Zone (SGZ) of Dentate Gyrus

The Sub-granular zone (SGZ) is localized in the adult hippocampus. The hippocampus is a bilateral structure positioned under the cortex of the brain, and subdivided into three regions: the proper Hippocampus or *Cornu Ammonis* (CA), the Subiculum, and the Dentate gyrus (DG) (Figure 1.7).

The *Cornu Ammonis* (CA) can be subdivided into four regions: CA1, CA2, CA3, and CA4. The region CA1 sends two major outputs towards the subiculum and the entorhinal cortex, while the region CA2 receives input from the entorhinal cortex. CA3 receives input both from the entorhinal cortex and, more importantly, from the mossy fibers of the granule cells of the dentate gyrus. Most of the pyramidal cells send their projected axons towards CA1 and CA2, whereas some pyramidal cells send fibers back to the DG. Finally, there is the CA4 region, also known as Hilus, considered part of the dentate gyrus, which mainly receives inputs from granule cells of the dentate gyrus and the pyramidal cells of CA3. These neural networks are summarized in Figure 1.7*B*.

The Subiculum is the most inferior component of the hippocampal formation and it is localized in the telencephalon, between the entorhinal cortex and region CA1. The subiculum consists of

four regions: *parasubiculum*, *presubiculum*, *postsubiculum* e *prosubiculum*. This structure receives input from the CA1 region and entorhinal cortex and it is the main source of output signals of the hippocampus.

Finally, the DG is considered the neurogenic niche of the adult hippocampal and can be subdivided into 3 distinct layers. There is a molecular layer (ML), a layer relatively cells-free, which

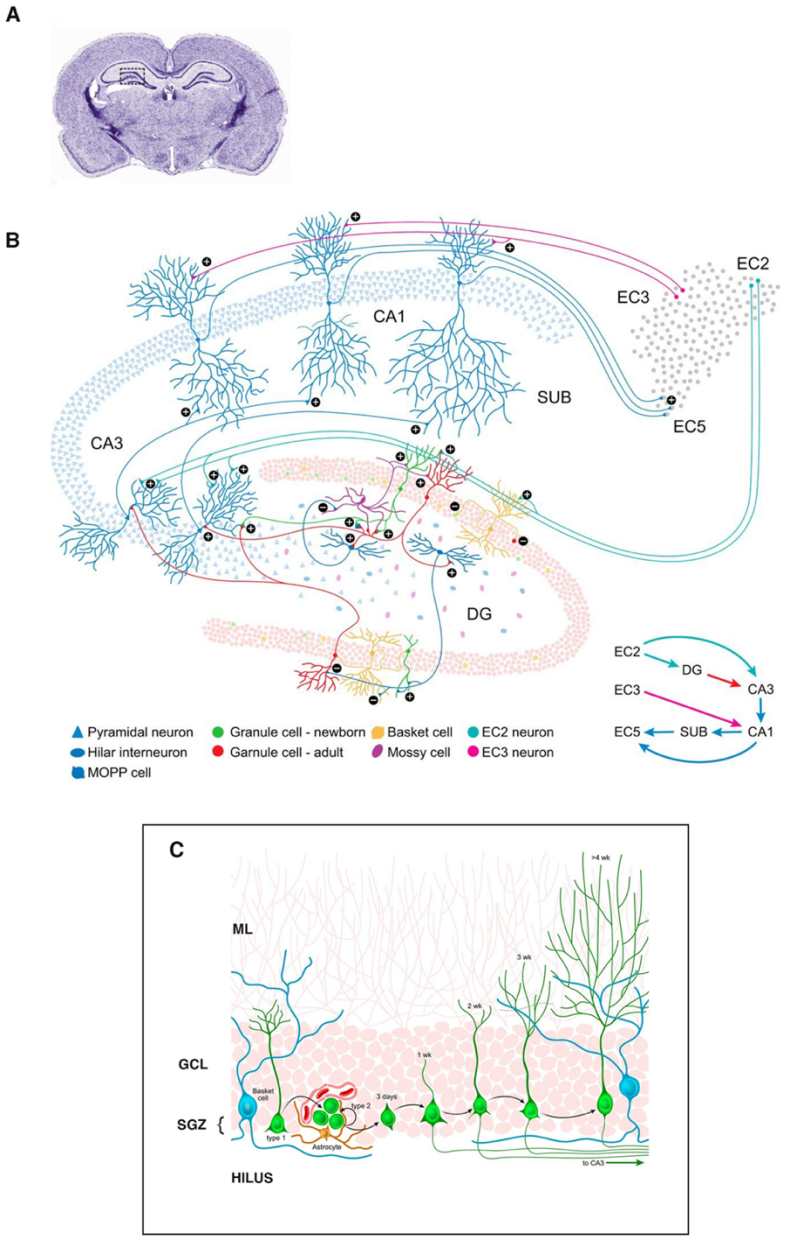


Figure 1.7 Schematic representation of adult hippocampus and neurogenesis.
(A) Sagittal section of mouse brain; **(B)** Schematic representation of neural circuitry of the adult hippocampus; **(C)** Schematic representation of Dentate Gyrus (DG) and its subdivision in layers. **CA:** corpus ammonis; **SUB:** Subiculum; **EC:** entorhinal cortex; **DG:** dentate gyrus; **ML:** molecular layer; **GCL:** Granule cell layer; **SGZ:** subgranular zone (Modified by Aimone et al.,2011)

is mainly occupied by the dendrites of granule cells and the fibers of the perforant path that originate from the entorhinal cortex. The main cell layer is the granule cell layer (GCL), which

contains the granule cells, the principal cellular component of DG and the only type of new neurons products during adult hippocampal neurogenesis (AHN). These cells receive input from the entorhinal cortex and with their axonal projections send excitatory output to pyramidal cells in the CA3 region. Finally, there is the polymorphic layer itself lies within the granule cell layer. The most abundant cells found here are the mossy cells (Figure 1.7*C*).

The NSCs and neural progenitor cells (NPCs) of the dentate gyrus are localized in the sub-granular zone (SGZ), a layer of cells located between the GCL and the Hilus. These cells can proliferate and differentiate until they form new granule neurons that will be integrated into the pre-existing neural circuitry. It has been proposed a six-stage model for the differentiation from putative NSCs to functionally integrated neurons, and every stage of this model is characterized by a different morphology and different pattern of protein expression (Figure 1.8; Kempermann et al., 2004a).

Based on this characterization, the NSCs are called Type-1, they are localized in SGZ, and express the proteins Glial fibrillary acid protein (GFAP), Sox2, Nestin and Hes5, but not the astrocytic marker S100β (Filippov et al., 2003; Komitova and Eriksson, 2004; Kronenberg et al., 2003; Seri et al., 2001). Morphologically, Type-1 cells showed two different shapes: 54% of Type-1 cells present a morphology radial-glia like, with a triangular cell body located into SGZ and long dendrites that cross GCL to reach the more external layer of DG, the molecular layer, where they connect with endothelial cells of capillaries; whereas the last 46% of Type-1 cells have not a radial-like morphology but are characterized by rounded cell body that is localized in SGZ, and sometimes from cell body starts a short process extended in parallel to the edge of the dentate gyrus (Lugert et al., 2010; Suh et al., 2007). All Type-1 cells are semi-quiescent, only a few of them expressing proliferation markers PCNA (Lugert et al., 2010) and MCM2 (Bonaguidi et al., 2011). However, it has been demonstrated that external stimuli can induce the proliferation of Type-1 cells. For instance, the addition of potassium chloride at depolarizing concentration to the culture medium of primary cells of the hippocampus induces non-proliferating Type-1 cells to enter in the cell cycle (Walker et al., 2008).

Type-1 cells mature into progenitor cells, classified as Type-2 cells. These cells haven't long dendrites but short tangentially-oriented processes that originated from the cell body. This cell population can be subdivided into two subclasses which differ for the expression of protein doublecortin (DCX): Type-2a (GFAP-negative, Nestin-positive, DCX-negative) and Type-2b (GFAP-negative, Nestin-positive, DCX-positive) (Fukuda et al., 2003; Kempermann et al., 2004a).

Type-3 cells are the proliferating cells characterized by the rounded cell body, and the expression of polysialated neural cell adhesion molecule (PSA-NCAM) and DCX, whereas are negative to the expression of Nestin (Kempermann et al., 2004a). Moreover, these cells have a high

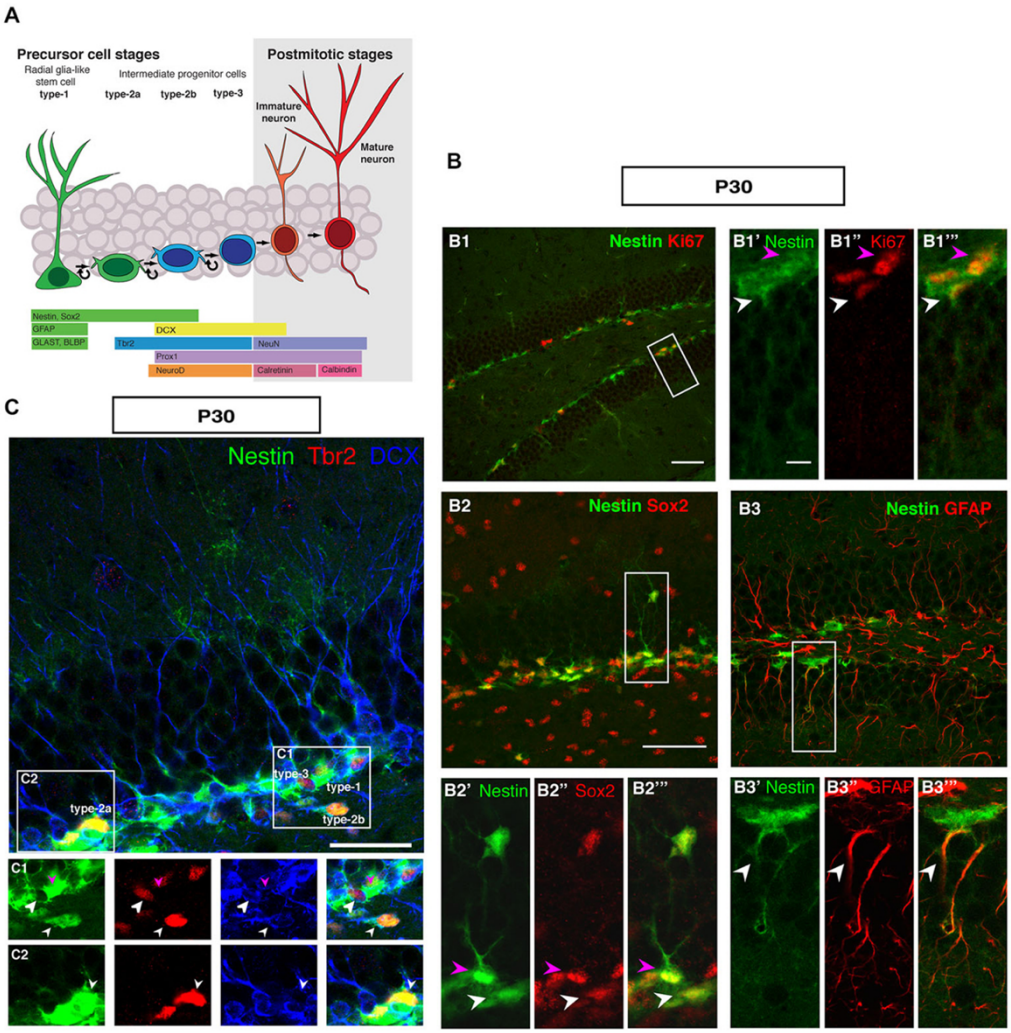


Figure 1.8 Neurogenesis in the adult dentate gyrus (DG).
(A) Schematic diagram of neural progenitor cell development in AHN. **(B)** Characterization of Type-1 and Type-2 cells using proliferation and precursor markers in the adult dentate gyrus at p30. **(B1–B1‴)** Nestin-GFP (green), Ki67 (red), both type-1 cells with their radial morphology and type-2 cells, which lack the radial process are positive for Ki67 (white and magenta arrowheads respectively). **(B2–B2‴)** Sox2 expression (red) is found in type-1 cells (magenta arrowhead) and type-2 cells in the subgranular zone (SGZ) (white arrowhead). **(B3–B3‴)** GFAP-positive cells (red) co-label with Nestin-GFP in type-1 cells in the SGZ (arrowheads). Scale bar, 100 µm for the overview and 20 µm for the insets. **(C)** The co-localization of Nestin-GFP, Tbr2 (red) and DCX (blue) identifies different types of cells in the SGZ of the adult dentate gyrus. **(C1)** Radial glia-like type-1 cells are Nestin-GFP positive but negative for Tbr2 and DCX (arrowhead; magenta), type-2b cells are Nestin-GFP, Tbr2 and DCX positive (arrowhead; small white). The cells, which express only Tbr2 and DCX are type-3 cells (arrowhead; big white). **(C2)** Type-2a cells express Nestin-GFP and Tbr2 but do not express DCX (arrowhead; white). Scale bars are 100 µm for overview and insets (Nicola et al., 2015).

presence of polysialic acid on the membrane surface, which is involved in the migration of these cells from the proliferative niche (SGZ) to GCL (Gascon et al., 2007). When these cells

reach their final position in GCL, they downregulate PSA-NCAM and differentiate in post-mitotic neurons (Seki, 2002).

Type-3 cells differentiate in early post-mitotic cells, also known as Stage 5 immature neurons. These cells exit from the cell cycle, and are positive to the expression of two proteins: NeuN and Calretinin. NeuN (Neuronal Nuclei) is a protein expressed in all types of post-mitotic neurons in the brain (Mullen et al., 1992; Seki, 2002), while Calretinin is a Ca-binding protein that is transiently expressed in immature granule cells, and which is replaced by another Ca-binding protein, Calbindin, when cells in stage 5 differentiate in mature granule cell, or Stage 6 (Brandt et al., 2003). After 4-7 weeks, the new cells become functionally indistinguishable from older granule cells, and they find their place in the hippocampal circuitry, including their connection with the local network of interneurons (Kempermann et al., 2004a, 2004b).

A common method utilized to measure and analyze the AHN *in vivo* is the use of thymidine analogs, as 5-Bromo-2'-deoxyuridine (BrdU). In mice, this molecule can be administered through drinking water or intraperitoneal injections, and it will be incorporated in all proliferating cells, including NSCs and NPCs. Therefore, the analysis of BrdU positive cells at different

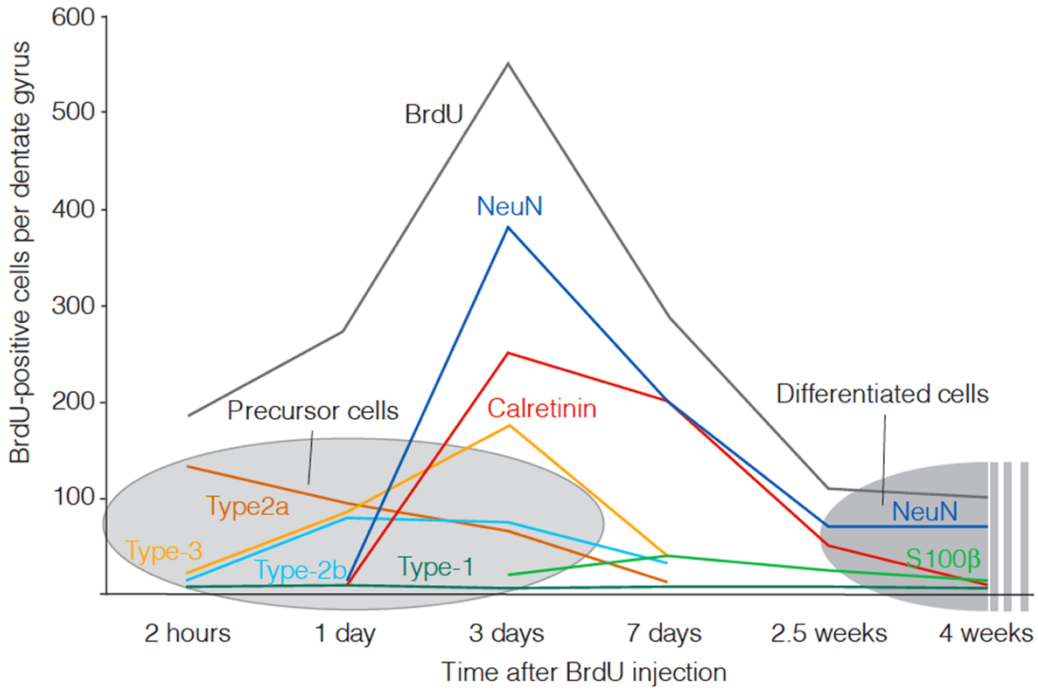


Figure 1.9 Numbers of new cells during different stages of neuronal development.
The curves show numbers of cells of different phenotypes labeled with the thymidine analog bromodeoxyuridine (BrdU), overtime after the injection of BrdU. The gray line on top gives the total number of BrdU-labeled cells; the colored curves below give the number of BrdU-marked cells expressing calretinin, NeuN and S100b (a marker for mature astrocytes), and the number of BrdU-marked cells of the different precursor cell types. The largest expansion of new cells occurs within the first three days after the injection of BrdU. Selection, or otherwise elimination, of new cells occurs on the level of calretinin-positive, early post-mitotic neurons (Kempermann et al., 2004b).

time points after its administration allows measuring the cell proliferation and their birth date, allowing to follow their fate. As illustrated in Figure 1.9, after one intraperitoneal injection of BrdU there is an increase of BrdU-positive cells with a peak after three days from the administration, with a four- or five-fold expansion of the progenitor pools. This increase is followed by a dramatic drop in BrdU positive cells. After 2,5 weeks from BrdU injection, the number of new neurons ceased to decrease and become stable (Kempermann et al., 2004b). Proliferation, maturation, survival, and differentiation of NSCs, NPCs, and their progenies are highly regulated by intrinsic and extrinsic signals.

1.3.2 Regulation of Adult Hippocampal Neurogenesis

In addition to NSCs and NPCs and their progenies, in the neurogenic niche of DG, there are other types of cells: astrocytes, GABAergic interneurons, microglia, and endothelial cells.

Table 1.1 Extrinsic factors involved in regulation of AHN

Extrinsic Factors	Effects on AHN
Wnt	*Induces the proliferation and differentiation of NSCs and NPCs*
Sonic Hedgehog (Shh)	*Induces the proliferation of NSCs (Faigle and Song, 2013)*
Notch	*In Type-1 cells, it induces the self-renewal (Liu et al., 2010). In immature neurons it modulates dendritic arborization (Breunig et al., 2007)*
Bone morphogenic genes (BMP)	*Inhibits the proliferation of NPCs, inducing their glial differentiation (Faigle and Song, 2013)*
Vascular endothelial growth factor (VEGF)	*Induces cell proliferation and angiogenesis (Palmer et al., 2000)*
Brain-derived neurotrophic factor (BDNF)	*Induces the proliferation, differentiation and synaptogenesis (Benarroch, 2013)*
Insulin-like growth factor 1 (IGF1)	*Induces the neurogenesis, and the differentiation of NPCs in oligodendrocytes (Faigle and Song, 2013)*
Epidermal growth factor (EGF)	*Induces the differentiation of NSCs (Doetsch et al., 2002)*
Glutamate	*Induces the survival of newborn cells and dendritic maturation (Benarroch, 2013)*
Serotonin	*Stimulates the proliferation of NSCs (Benarroch, 2013)*
Dopamin	*Induces the proliferation of NPCs (Suh et al., 2009)*

All these cells in neurogenic niches with blood vessels and extracellular matrix create a set of extrinsic factors (Table 1.1) which regulate the AHN. Among these extrinsic signals there are: morphogens, as Wnt or Sonic Hedgehog (Shh); growth factors, as BDNF and VEGF; neurotransmitters, such as glutamate (Benarroch, 2013). These extrinsic signals are translated into

intrinsic signals in NSCs, NPCs and their progenies. Among the intrinsic factors there are: cell cycle regulators, transcription factors, epigenetic regulations (methylation of DNA, histone modifications and microRNA signaling), summarized in Table 1.2.

Table 1.2 Table 1.2 Intrinsic factors involved in regulation of AHN (Benarroch, 2013).

Intrinsic Factors	Effects on AHN
Sox2	*Transcriptional factor essential in the maintenance of NSCs and to self-renewal (Suh et al., 2009)*
Trb2	*Transcriptional factors expressed in intermediate progenitor cells, where induces their differentiation (Faigle and Song, 2013)*
TLX	*Nuclear receptor. It induces the proliferation inhibiting the expression of $p21^{Cip1/WAF1}$. Moreover, it actives the Wnt pathway (Faigle and Song, 2013)*
NeuroD	*It is involved in the survival and maturation of new neurons (Benarroch, 2013)*
CREB	*Induces the phosphorylation of cAMP, inducing the proliferation and survival of newborn neurons (Benarroch, 2013)*
Methyl-CpG-binding domain protein-1 (MBD1)	*Neuronal differentiation*
HDAC2 (Histone deacetylase 2)	*Regulates the maturation and proliferation of NSCs*

Therefore, the AHN is regulated by an orchestra of extrinsic and intrinsic factors that influence the stemness/pluripotency, the proliferation, the survival, the migration, the fate specification and the integration of the new neurons into the pre-existing neural circuitry.

The maintenance of the stemness of NSCs is the first step in the regulation of AHN, and it is important to preserve the neurogenic capacity throughout life. Indeed, NSCs have a limited number of proliferation rounds before they terminally differentiate and lose the stem cell capacity (Encinas et al., 2011); therefore, several cell signaling pathways regulate the balance between the proliferation and self-renewal of NSCs, such as Notch signaling (Imayoshi et al., 2010). The activation of Notch signaling induces proliferation of NSCs, and through an asymmetric distribution of Numb, a repressor protein of Notch, generates two different cell types from one NSC. The daughter cell where there is Numb remains an NSC, whereas the daughter cell where there is not Numb proliferates with the symmetric division until exhaustion of its stem capacity (Shen et al., 2002).

In the regulation of quiescence, proliferation and differentiation of NSCs and NPCs play a key role in the molecular network of the cell cycle (Beukelaers et al., 2012; Farioli-Vecchioli and

Tirone, 2015). Indeed, using various murine models it was demonstrated that a dysregulation of the cell cycle significantly influences the homeostasis of AHN. For example, the overexpression of cyclin D1-Cdk4 induces an expansion of stem and progenitor pool altering the differentiation (Artegiani et al., 2011), whereas the genetic ablation of cyclin D2 induces a total arrest of AHN (Kowalczyk et al., 2004). Moreover, it was demonstrated the role of inhibitors of cell cycle progression in the regulation of AHN. For instance, the deletion of $p21^{CIP1/WAF1}$ increases the proliferation of NPCs in DG of 2-month-old mice (Pechnick et al., 2008), whereas $P16^{Ink4a}$ prevents the activation of the quiescent NSCs in aged mice by running (Micheli et al., 2019). Another cell cycle inhibitor involved in the maintenance of the NSCs pool is Btg1. It was demonstrated that Btg1 plays a role in the control of the balance between proliferation and quiescence of NSCs. Indeed, its genetic ablation induces the depletion of NSCs pool in the adult mice before they reach adulthood (Farioli-Vecchioli et al., 2012). The role of Btg1 in adult neurogenesis will be discussed later.

Successively, NSCs and NPCs choose whether to continue to proliferate or to exit the cell cycle to differentiate, and many factors are involved in this choice. For example, the protein Sox2 (sex-determining region Y-box 2), which is expressed in Type-1 and Type-2 cells, controls the mitotic activity and the pluripotency of stem and progenitor cells. Moreover, Sox2 regulates positively the expression of nuclear receptor TLX (Beckervordersandforth et al., 2015). In turn, also TLX is essential to maintain the self-renewal and proliferative capacities of NPCs, whereas its inhibition causes an arrest in the proliferation of the progenitor cells and induces their differentiation. Its function is mediated by activation of the WNT/β-catenin pathway and by inhibition of cell cycle inhibitor $p21^{CIP1/WAF1}$ (Lie et al., 2005; Qu et al., 2010).

Another regulation of adult hippocampal neurogenesis is the cell fate specification. Under physiological conditions, most cells derived from NSCs will differentiate into mature granule cells, while only a small subset will give rise to astrocyte. This choice is regulated by expression or inhibition of specific neural genes, regulated by several epigenetic mechanisms (Benarroch, 2013; Hsieh and Gage, 2004). Furthermore, as demonstrated in transplantation experiment, the neurogenic niche plays a key role in the fate specification of new cells, in fact when the NSCs are transplanted in ectopic brain regions differentiate in astrocytes, suggesting that the neurogenic niche induces their differentiation in new neurons rather than in astrocytes (Shihabuddin et al., 2000).

The survival of new cells is an important step in the regulation of AHN because in this way the organism regulates the final number of new neurons, checking that the organism does not produce an excess of new cells and also that the quality of the newly generated cells is preserved,

selecting out unfit cells. During AHN, there are two critical points for the survival of new cells: during the transition from amplifying progenitors to post-mitotic cells, and during the integration stage of immature neurons. The majority of hippocampal newborn cells undergo apoptosis in the first few days of cells' life through adulthood and are immediately recognized and degraded by "unchallenged" microglia (Sierra et al., 2010). Microglia are the brain professional phagocytes compared with other cell types (Sierra et al., 2013) and prevent the release of toxic intracellular contents (Nagata et al., 2010), and thus, this process is essential to avoid alterations of the surrounding tissue. A recent study proposes that microglial phagocytosis does not conclude with the physical elimination of apoptotic cells, but is followed by a coordinated transcriptional program that triggers the production of neurogenic modulatory factors, which directly contribute to the maintenance and correct regulation of the adult hippocampal neurogenic cascade (Diaz-Aparicio et al., 2020). Besides, using a combined in vitro and in vivo based experimental strategy, they found that the secretome of phagocytic microglia limits the production of new neurons to maintain the homeostasis of the adult hippocampal neurogenic niche (Diaz-Aparicio et al., 2020). Indeed, microglial cells play a key role also in the regulation of proliferation, and differentiation of NSCs and NPCs (Gemma and Bachstetter, 2013). In vitro studies demonstrate that cultured microglia promote differentiation of precursor cells (Aarum et al., 2003), whereas microglia-conditioned media enhances neuroblast production and neuronal survival (Morgan et al., 2004; Walton et al., 2006). Furthermore, microglia were suggested to inhibit the proliferation of hippocampal NSCs, as their number inversely correlates with adult hippocampal neurogenesis (Gebara et al., 2013). The specific molecular mechanisms though which microglia regulate different steps of neurogenesis are only beginning to be explored. Under normal conditions, microglia have highly ramified morphology with thin processes, and they dynamically move in the brain parenchyma in what has been called a surveillance state; in contrast, reactive microglia adopt several altered morphologies (Helmut et al., 2011). However, neurons are not only passive targets of microglia but rather they control the activity of microglial cells through a system of ON and OFF signals (Biber et al., 2007). CD200, CX3CL1 (also known as fractalkine), CD47, CD55, HMGB1 are all OFF neuro-immune regulatory proteins constitutively expressed on healthy neurons and keep microglia in their resting state and antagonized proinflammatory activity. ON signals are inducible and include chemokine, purine and glutamate and they are found in damaged neurons. In aged mice, it was observed that the OFF signals produced by CD200 and fractalkine are destroyed, inducing the activation of microglia with a reduction in hippocampal neurogenesis (Gemma et al., 2010). Besides, it was observed that a blockade of microglia activity with minocycline, an anti-inflammatory drug, improves

spatial learning in aged mice, even if it is not connected to an increase in neurogenic activity (Kohman et al., 2013). However, in pathological conditions, it was observed the opposite effect of the activation of microglia: its activation induces an increase in hippocampal neurogenesis while its inhibition causes a decrease in hippocampal neurogenesis (De Lucia et al., 2016). Furthermore, it was observed that voluntary exercise, a known neurogenic stimulus, reduce the density of microglial cells in DG (Gebara et al., 2013), and reduce the expression of pro-inflammatory cytokines such as TNF-α, and increasing the expression of the anti-inflammatory cytokines such as IL-1ra (Pervaiz and Hoffman-Goetz, 2011) or the chemokine CX3CL1, that induces a neuroprotective microglia phenotype and promotes neurogenesis (Vukovic et al., 2012). Further studies are necessary to understand the complex role of microglia in control of adult hippocampal neurogenesis.

Another critical point in the regulation of AHN is the control of the migration of the newborn cells. During the maturation and differentiation, the newborn cells do not remain in the same position but migrate along the SGZ and in GCL until to reach their final position (Figure 1.10). In the first seven days, the Type-3 cells exit from the cell cycle and extend their long processes tangential to the SGZ where contacts blood vessels directly, and use them as the substrate to migrate tangentially away from their initial position. After seven days, the newborn Type-3 cells extend radial dendritic processes and differentiate into stage 5/6 cells and exhibit limited radial migration through the GCL. This migration is controlled by several chemoattractants,

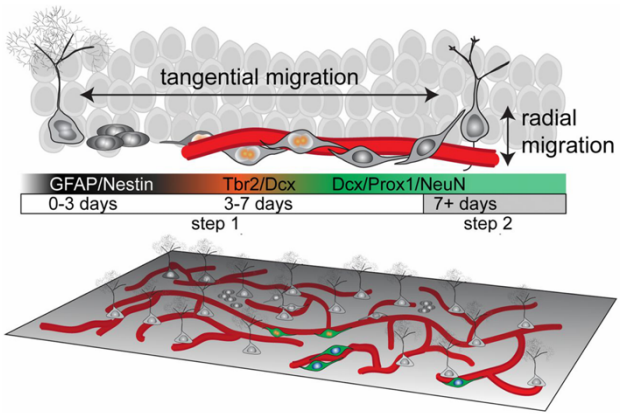


Figure 1.10 Two-step model for neuronal migration during adult hippocampal neurogenesis.
During adult hippocampal neurogenesis, radial glia-like cells give rise to $Tbr2^+$ intermediate progenitor cells within 3 days. In the next 4 days, the cells become DCX^+ proliferating neuroblasts that extend long processes tangential to the SGZ and contact blood vessels directly. During this phase, neuroblasts use blood vessels as a substrate to migrate tangentially away from their parental RGL. After 7 days, newborn neural progeny extends radial dendritic processes and develops into $NeuN^+Prox1^+$ dentate granule neurons and exhibits limited radial migration through the granule cell layer. (*Lower*) Global-view illustration of RGLs, neural progeny, and vasculature in the adult SGZ. (Sun et al., 2015).

such as Reelin, PSA-CAM, and DCX (Sun et al., 2015). Moreover, it was demonstrated that aberrant migration reduces dendritic complexity (Ibrahim et al., 2016).

Finally, when the new mature granule cells reach their final position in GCL, they start to form axonal and dendritic processes and develop a dendritic spine to allow for neuron-to-neuron communication (Kempermann et al. 2004). The establishment of this connection is important to the survival of new cells, indeed, the absence of synaptic input generally induce selective cell apoptosis (Kempermann et al., 2004b; Kim and Sun, 2011; Tashiro et al., 2006).

The newborn neurons will be integrated into the pre-existing neural circuitry, where will be involved in the learning processes. Learning is one of the most important stimuli to AHN. In rodent species, trace eyeblink conditioning and Morris water maze, two behavioral tests used to measure the hippocampus-dependent learning, don't affect the proliferation of neural stem e progenitor cells, however, enhance the survival in one-week-old neurons but triggers the apoptosis in new neurons three days and two weeks old (Ambrogini et al., 2004; Dupret et al., 2007; Gould et al., 1999b). According to this model, learning saves neurons of one-two weeks of age from death, in a period when they express enhanced plasticity, while suppresses older and younger neurons, by a feedback mechanism that ensures a critical number of cells in DG (Leuner et al., 2004).

Therefore, learning influences the AHN but it was also demonstrated the opposite: the AHN influences learning processes; this theory was demonstrated by several works. In a work published by Saxe and colleagues (2006), were employed transgenic mice expressing herpes simplex virus thymidine kinase under the control of the GFAP promoter to kill the stem and progenitor cells with ganciclovir administration. Using this strategy, it was demonstrated that neurogenesis is necessary for learning in contextual fear conditioning, a behavioral task (Saxe et al., 2006). In another work, it was observed that a premature differentiation of newborn neurons, through a nestin-driver expression of the PC3 gene, altered the learning processes (Farioli-Vecchioli et al., 2008). In fact, the overexpression of PC3 in neural stem and progenitor cells doesn't influence the total number of proliferating cells but the timing of differentiation, accelerating the transition from Type-1 cells to Type-3 progenitor cells. This premature differentiation severely reduces the incorporation of newborn neurons into spatial memory networks, and this reflects in a consistent deficit in spatial memory tests (Farioli-Vecchioli et al., 2008).

Therefore, the new neurons in DG play a key role in memory coding, as they are more excitable until the age of 4 weeks, thus improving the resolution and correlation between new memories and old memories of events encoded by mature neurons (Aimone et al., 2011; Farioli-Vecchioli et al., 2008, 2013). Therefore, AHN is related to cognition (Zhao et al., 2008).

Various environmental factors can influence both positively and negatively the AHN, and the different types of cells in AHN respond differently to these stimuli. Generally, it has been observed that Type-1 cells do not change in response to neurogenic stimuli, such as running or environmental enrichment, and this is in agreement with the stem phenotype. Indeed, they don't participate in acute fluctuations of adult neurogenesis, preventing the exhaustion of the stem pool. Nevertheless, the Type-1 cells are in contact with their processes with the molecular layer of the DG and with blood vessels in SGZ, therefore these cells might mediate information without this resulting in their increased proliferation. However, the number of Type-1 cells can be influenced by several pathological conditions, such as chronic stress, aging, neurodegenerative disease (Kempermann et al., 2004b). In contrast to Type-1 cells, the other cells in AHN respond to various neurogenic stimuli, which influence their proliferation, survival and differentiation.

1.5 Aging

The aging can be considered a physio-pathological condition characterized by a cognitive decline. Several works suggest that hippocampal neurogenesis persists throughout the lifespan, however, it undergoes a decline with age. The first evidences were obtained in rodent species, where, using the thymidine analogs and immunohistochemistry assay, a decline in mitotic activity of neural progenitors in DG of 12- and 21-months old animals and a decrease in newborn neurons was demonstrated (Kempermann et al., 1998; Kuhn et al., 1996). Subsequently, the persistence and decline of hippocampal neurogenesis were also detected in primates. Initially, it was observed the persistence of hippocampal neurogenesis in Macaque monkeys up to 23 years old, which is equivalent to the old human age. However, the rate of neurogenesis in non-human primates occurs during the adult age at significantly lower levels, than in rodent species (Gould et al., 1999c). Subsequently, several works confirmed the persistence of hippocampal neurogenesis in aged humans. An analysis conducted on post mortem brains demonstrated the persistence of hippocampal neurogenesis into the eight decades of human life. Moreover, this work suggests that the age-related decline is not correlated with a decline in NPCs proliferation but with other factors, such as a smaller quiescent neural progenitor pool, diminished angiogenesis, or decreasing neuroplasticity in the DG, indicating an involvement of neurogenic niche in age-related decline (Figure 1.11; Boldrini et al., 2018). Another study analyzed the brain of 18 patients with an average age of 90.6 years, and identifies both NPCs and neuroblasts in the human hippocampus, albeit with high variation between subjects, with an inverse correlation between the number of these immature neurons and cognitive functions of patients. Moreover, the number of immature neurons dropped sharply in patients with Alzheimer's disease (AD),

suggesting that the impairment of adult neurogenesis may be a mechanism underlying the memory deficit and decline in cognitive functions in aging and neurodegenerative diseases, such as AD (Boldrini et al., 2018; Moreno-Jiménez et al., 2019).

The age-related decline in hippocampal neurogenesis has been associated with changes in the concentration of several modulators involved in the regulation of AHN. For instance, it was demonstrated that in neurogenic niches of aged rats there is a dramatic decrease in BDNF and CREB, causing a decrease in the number of proliferating cells (Hattiangady and Shetty, 2008; Hattiangady et al., 2005). Furthermore, many cell signaling pathways are involved in the age-related decline of hippocampal neurogenesis. For instance, it was demonstrated an age-related increase in expression of Wnt antagonist Dickkopf-1 (Seib et al., 2013), which inhibits the Wnt signaling with a decrease in the expression of the mitotic factor Survivin (Miranda et al., 2012).

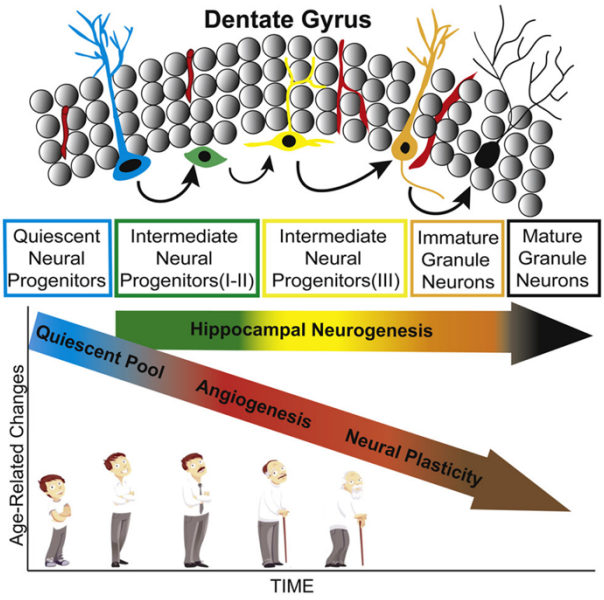


Figure 1.11 Schematic representation of age-related changes in AHN.
Persistent adult neurogenesis in humans into the eighth decade of life, despite declines in quiescent stem cell pools, angiogenesis, and neuroplasticity (Boldrini et al., 2018).

Furthermore, it was observed that mice deficient in Dickkopf-1 protein exhibited enhanced hippocampal neurogenesis during aging and an increase in performance at neurogenesis-dependent tasks, compared to aged mice control when the expression of Dickkopf-1 was not modulated (Seib et al., 2013).

Other important modulators of AHN are the growth factors. Indeed, it has been demonstrated their involvement in age-related decline in neurogenesis. In fact, in aged rats, there is a decrease in levels of fibroblast growth factor (FGF-2) as well as Insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF) (Kempermann, 2015), all of these factors being

directly correlated with age-related decline in adult neurogenesis. For instance, the infusion of IGF-1 ameliorated the decrease in hippocampal neurogenesis in aged rats (Lichtenwalner et al., 2001), when in a model of long-lived mice, enhanced hippocampal neurogenesis coupled with increased levels of IGF-1 was observed during aging (Sun et al., 2005).

Other factors correlated with age-related decline in hippocampal neurogenesis are Glucocorticoid, whose secretion increases with age (Sapolsky, 2000). Glucocorticoid is a class of steroid hormones released in the bloodstream from the adrenal gland; this secretion is induced by the Adrenocorticotropic hormone (ACTH), whose secretion is in turn controlled by Corticotropin-releasing hormone (CRH) released from the hypothalamus. The main glucocorticoid hormone is cortisol. Its secretion is induced by physical and emotional stress and is linked with hippocampal atrophy and negative regulation of neurogenesis (Odaka et al., 2017; Sapolsky, 2000). Interestingly, a study where aged mice are infused with vascular and neurogenic factors of young mice showed a rejuvenated neurogenic niche and a restoration of hippocampal neurogenesis, thus demonstrating the importance of neurogenic niche (Katsimpardi et al., 2014).

In the neurogenic niches, there are other cell types besides NSCs and NPCs; these cells are involved in the regulation of adult neurogenesis and they also undergo age-related changes. A cell population involved in the regulation of adult hippocampal neurogenesis and aging is Microglia. Microglia are the resident brain immune cells and have many important roles in the healthy and diseased CNS, and it was observed that in aged mice there is a significant increase in the density of activated-microglial cells, measured by expression of Iba1 (Gebara et al., 2013). Furthermore, in aged mice was observed an increase in expression of mRNA for the inflammatory cytokines Tumor Necrosis Factor α (TNFα), Interleukin 1β (IL1β), Interleukin 6 (IL6), and Interferon γ (IFNγ), a decrease in the anti-inflammatory cytokines Interleukin 10 (IL10) and Transforming Growth Factor β (TGFβ; Frank et al., 2006; Sierra et al., 2007), and increased inflammatory receptors MHCII (Henry et al., 2009) and CD86 (Frank et al., 2006), suggesting a shift to a more inflammatory phenotype of these cells. The same results were observed after ischemia (Ito et al., 2001) and brain disease (Cornejo and von Bernhardi, 2016). Therefore, activated microglial cells in aged mice create an adverse milieu for neurons, that may favor neurodegenerative diseases (Cornejo and von Bernhardi, 2016).

Overall, these data highlight the complex interplay of different factors within neurogenic niches that may be affected by the aging process and which thereby ultimately affect the number of newborn neurons produced in the aged brain.

Understanding the causes involved in age-related decline in hippocampal neurogenesis could be a key to develop therapeutic strategies and thus counteract the loss of cognitive function in

aging. Several works demonstrated that corrects habits, such as running or diet, can prevent the age-related decline in hippocampal neurogenesis. Due to the longer average life span of the human population, cognitive impairment associated with aging is an increasingly important aspect of health.

1.4 Neurogenic Stimuli

There are many types of stimuli that can modulate hippocampal neurogenesis in adulthood or aging. In this thesis, some of them will be discussed, in particular the voluntary running, the antidepressants, and the diet and natural compounds (Figure 1.12)

1.4.1 Voluntary Exercise or Running

Voluntary exercise or running is a powerful neurogenic stimulus. The first indirect link between running and the production of new neurons in DG was published by Kempermann and colleagues (1997). They housed adult mice in a cage with toys, tunnels, tubes and a free access running wheel, and after 40 days, they observed an increase in newborn cells in adult DG of mice housed in an enrichment environment (Kempermann et al., 1997). Subsequently, it was demonstrated that, among the various elements in an enrichment environment, the voluntary exercise in a free-entry running wheel is a potent neurogenic stimulus in the dentate gyrus, with a 2-3 times increase of newborn-cells (Van Praag et al., 1999; Vivar et al., 2012). Specifically, running induces the proliferation of type-2 and type-3 cell precursors (Kronenberg et al., 2003), with an increase in stage-5 and stage-6 cells in DG. Furthermore, it has been observed that under physiological condition, running is a specific neurogenic stimulus for the neurogenic niche of DG, indeed it has not any effect on adult neurogenesis in SVZ (Brown et al., 2003). Anyway, recent works demonstrated that running can restore adult neurogenesis in the SVZ in the neurogenesis-defective mouse model Btg1 knockout (Mastrorilli et al., 2017), and

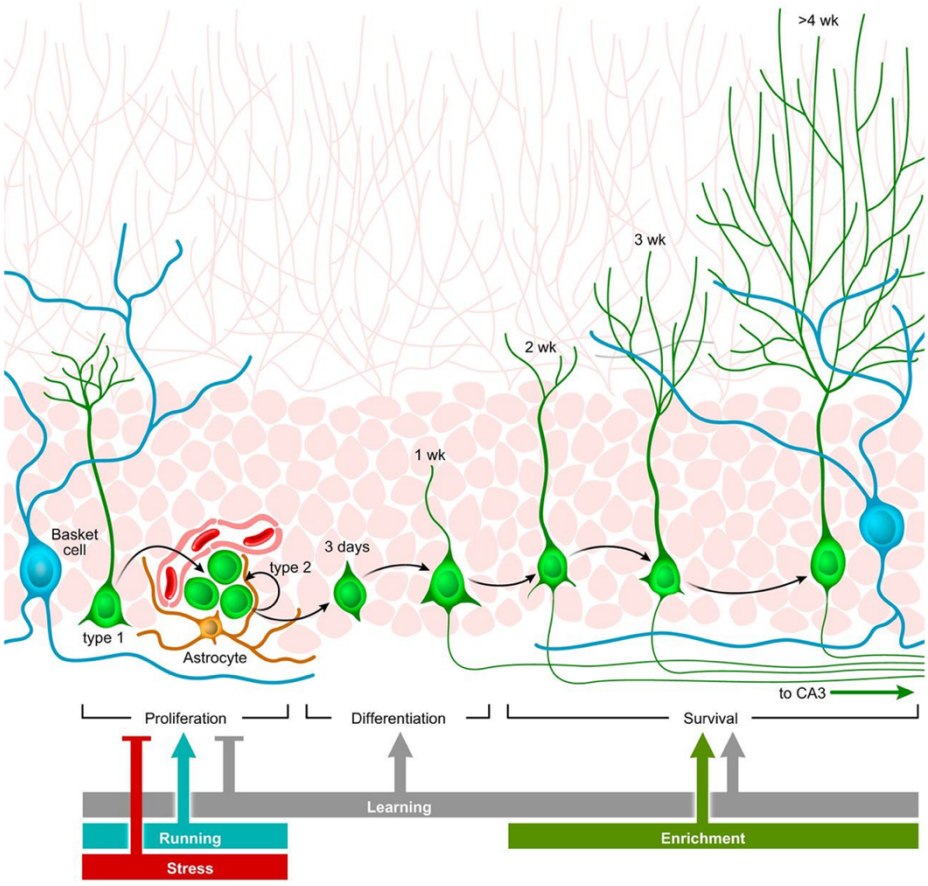


Figure 1.12 Neurogenic stimuli.
Regulation of neurogenesis by behaviors. Neurogenesis is regulated by many behavioral factors as well. Running is one of the most potent inducers of neurogenesis, targeting the proliferation of neural progenitor cells. Enrichment has a complementary effect, increasing the survival of neurons at a critical stage of their maturation. In contrast, stress is a severe negative regulator of new neuron birth, suppressing proliferation. The effects of learning are more complex, suppressing the neurogenesis process at some stages while increasing it at other stages (Aimone et al., 2011).

$p21^{Cip1/WAF1}$ knockout (Nicolis di Robilant et al., 2019). Furthermore, in this latter model, it was observed that voluntary running improves the olfactory memories (Nicolis di Robilant et al., 2019).

Running hasn't only a pro-proliferative effect on hippocampal neurogenesis. In fact, it was observed anti-apoptotic effects of running (Baek and Kim, 2016; Chen and Russo-Neustadt, 2009); further, it regulates the neural plasticity of new neurons by increasing dendritic complexity and spine density (Eadie et al., 2005; Stranahan et al., 2007), as well as the short-term synaptic plasticity from lateral entorhinal cortex, thus favoring the integration of contextual and spatial information (Vivar et al., 2012, 2016).

The main neurogenic mediator of running on the AHN is BDNF, a neurotrophin that positively modulates neural survival, growth and synaptic plasticity by the induction of the long-term

potentiation (LTP) and synaptic transmission (Binder, 2004). Other trophic factors involved in the pro-neural effect of running are endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1). Similarly to BDNF, IGF-1 expression is enhanced both in the hippocampus and the blood of mice within 1 hour of running (Gomez-Pinilla et al., 2008), and it was demonstrated that an increase of the level of IGF-1 plays an important role in enhancing AHN and in hippocampus-dependent learning. Furthermore, the depletion of circulating IGF-1 eliminated the exercise-induced hippocampal cell proliferation (Glasper et al., 2010). In another work, it was demonstrated an involvement of negative neurogenesis regulators bone morphogenetic protein (BMP) in the increase of neurogenesis and learning-induced by running. Indeed, an increase in expression of BMP4 reduces the pro-neurogenic effects of running (Gobeske et al., 2009), and this is in agreement with the role of BMP/BMPR1A signaling in the quiescence maintenance of NSCs (Mira et al., 2010). Wnt signaling is also involved in the neurogenic effects of running. It was demonstrated that running induces a decrease in expression of Secreted frizzled-related protein (SFRP3), which is involved in maintenance quiescence of NSCs and NPCs (Jang et al., 2013). Finally, it was observed that running improve memory and activates c-fos, an immediate early gene used as a molecular marker of neural activity (Clark et al., 2010).

Overall, these works showed the involvement of several pathways in neurogenic effects of running on adult neurogenesis and learning.

1.4.2 Antidepressant drugs and Fluoxetine (FLX)

Various types of drugs can influence the adult neurogenesis, comprising the antidepressant. The first evidence has been provided by Malberg and colleagues (2000), which demonstrated that a chronic administration of antidepressants increases proliferating cells in the dentate gyrus and the hilus (Malberg et al., 2000). Subsequently, many works have been published on the roles of antidepressant drugs on AHN, which led to the formulation of the "neurogenic hypothesis of depression".

FLX is an antidepressant with well-characterized effects on AHN. It belongs to the class of the Selective Serotonin Inhibitors (SSRIs) and acts by blocking serotonin re-uptake back to presynaptic neurons, increasing its concentration in the presynaptic cleft. Its effect on AHN is mediated by receptor 5HT1, which is involved in the self-renewal of precursor cells (Klempin et al., 2010). Indeed, its genetic ablation blocks the neurogenic effects of FLX on AHN (Santarelli et al., 2003). Conversely, other serotonin receptors play an inverse role. For instance, 5-HT2c receptor, which is the long term stimulate an increase of late-stage progenitor cells and

early post-mitotic neurons, may antagonize the acute effect of 5-HT1 receptors, thus causing the known delay in FLX effect (Klempin et al., 2010).

To understand the target cells of FLX in AHN, a mouse line where the cyan fluorescent protein with nuclear localization (CFPnuc) was under the control of the promoter of Nestin was generated. This mouse line has been used to show that FLX induces an increase in the proliferation of dividing dentate gyrus type-2 and type-3 progenitor cells, but it is ineffective on stem cells (Encinas et al., 2006; Hanson et al., 2011). However, recent work showed that in Btg1 knockout mice, FLX induces the proliferation of NSCs, suggesting that FLX can acts on these cells in compromised conditions (Micheli et al., 2018). Moreover, it was observed that the chronic treatment with FLX is correlated with inhibition of expression of cell cycle inhibitor $p21^{Cip1/WAF1}$, which is expressed in transit-amplifying progenitors and neuroblasts, suggesting the involvement of cell cycle genes in the mechanism of action of FLX. However, unlike voluntary exercise, the treatment with FLX doesn't influence the length of the cell cycle (Micheli et al., 2017).

Like running, the FLX does not only affect cell proliferation of NSCs and NPCs, but it is also involved in the regulation of differentiation and maturation of newborn cells. Indeed, it was demonstrated that chronic treatment with FLX accelerates the rate of maturation in DCX positive cells (Wang et al., 2008b) with a mild but significant increase by chronic FLX treatment in the rate of terminal differentiation of stage 6 adult neurons (Micheli et al., 2017).

The neurotrophins, such as BDNF, are implicated in the action of antidepressants on AHN. It was observed that the chronic treatment with antidepressants increases the levels of mRNA of BDNF and its receptor TrkB in the hippocampus (Kim et al., 2013), and infusion of exogenous BDNF induce similar effects to chronic treatment with antidepressants (Jun et al., 2012; Shirayama et al., 2002). However, the chronic treatment with antidepressants increases the proliferation of NPCs in DG of transgenic mice with the reduction in BDNF signaling, indicating that the neurotrophins don't regulate the proliferation phase of AHN (Sairanen et al., 2005).

Very interestingly, FLX suppresses BMP signaling in the adult mouse hippocampus by both decreasing the level of BMP4 and increasing the levels of BMP inhibitors noggin; furthermore, the overexpression of BMP4 blocks the effects of FLX on the proliferation of DG while noggin infused into lateral ventricles exerts antidepressant activity (Brooker et al., 2017).

Overall, FLX and antidepressants stimulate neurogenesis in a double way: on one hand, they enhance NPCs proliferation (Encinas et al., 2006), while on the other hand, they induce NPCs differentiation (Wang et al., 2008b) and survival (Santarelli et al., 2003). It has been demonstrated that an increase in the turnover of newborn neurons improves the possibility that some

of these neurons established a profitable connection with pre-existing circuits, and thus promoted their survival, with a BDNF dependent system (Sairanen et al., 2005).

1.4.3 Diet and natural compounds

Consumption of healthy food is one of the key allies in protecting brain function and preserving cognitive abilities in adulthood and aging. Similarly to environmental stimulation, exposure to specific dietary factors can influence neuronal function and synaptic plasticity, influencing cognitive abilities, in particular, those which are hippocampus-dependent (Stangl and Thuret, 2009).

Calorie intake, meal frequency, meal texture, and meal content are all variables of diet able to influence the hippocampal neurogenesis and are the results of social habits, economic availability and environmental resources.

The calorie restriction enhances the spatial memory and behavior outcome in murine models of neurodegenerative diseases, probably increasing the AHN through the regulation of BDNF levels (Lee et al., 2002a). Meal texture is another variable in the diet able to influence AHN. Indeed, in rats, it was demonstrated that chewing in solid/hard diet influences the corticosterone levels, inducing a major proliferation of NPCs compared with rats fed with a soft diet (Aoki et al., 2005). However, during the last decades, there was a steady increase in publications on the nutrients able to influence the brain health, indicating that the meal content is the main variable of the diet involved in its effects on cognitive function and AHN (Table 1.3; Stangl and Thuret, 2009).

Vitamins are organic molecules that are essential micronutrients that an organism needs in small quantities for the proper functioning of its metabolism. Essential nutrients cannot be synthesized in the organism, either at all or not in sufficient quantities, and therefore must be obtained through the diet. Vitamins can be subdivided in water-soluble (B and C vitamins) and fat-soluble (A, D, K, and E vitamins), and both classes are involved in several aspects of health, including the brain health.

Among the water-soluble vitamins, the B-type of vitamins, which we find in several types of foods, show the effects on cognitive functions and hippocampal neurogenesis. For instance, it was demonstrated that a low concentration of Vitamin B-9, also known as acid folic or folate, in the diet causes a decrease in cognitive functions (Kado et al., 2005) whereas a diet supplementation with Vitamin B-9 slows down the age-related cognitive decline (Durga et al., 2007; De Jager et al., 2012). Furthermore, Vitamin-B-9 influences the DNA methylation and other

epigenetics phenomena in NSCs, NPCs and their progenies, modulating the AHN, and the same effects have been observed for vitamin B-6 and B-12 (Kronenberg et al., 2008).

Table 1.3 Modulation of AHN by diet.

Diet	Animal Model	Effect on AHN	References
Calorie Restriction/ Dietary Restriction	*Rat/Mouse*	*Increased survival*	*(Bondolfi et al., 2004; Kitamura et al., 2006; Lee et al., 2000, 2002a, 2002b)*
Omega 3 fatty acids (DHA, docosahexaenoic acid)	*Rat*	*Increased*	*(Kawakita et al., 2006)*
Flovonoids	*Rat, chronically stressed*	*Increased proliferation*	*(An et al., 2008)*
Blueberry	*Rat*	*Increased proliferation*	*(Casadesus et al., 2004)*
Curcumin	*Mouse*	*Increased proliferation*	*(So et al., 2008)*
Retinoic Acid excess	*Mouse*	*Decreased proliferation*	*(Crandall et al., 2004)*
Vitamin B-9 deficiency	*Mouse*	*Inhibited proliferation*	*(Kronenberg et al., 2008)*
Vitamin E	*Mouse*	*Increased proliferation; decrease survival*	*(Ciaroni et al., 1999, 2002)*
Vitamin A deficiency	*Rat*	*Decrease proliferation (rescue with retinoic acid)*	*(Bonnet et al., 2008)*
Ethanol	*Rat/Mouse*	*Decreased proliferation*	*(He et al., 2005; Nixon and Crews, 2002; Stevenson et al., 2009)*

Among the fat-soluble vitamins with roles in brain health, there is vitamin E, which is found in nuts, seed oil, and leafy green vegetables, and it is known for its antioxidant and anti-inflammatory proprieties with many effects on body health. Its effect on AHN is controversial because it has been observed that a deficiency of vitamin E in diet induces an increase in proliferation in DG of the hippocampus (Ciaroni et al., 1999), but this effect is related to an increase in cell death (Ciaroni et al., 2002). Therefore, further studies are necessary to understand the role of this Vitamin in the regulation of AHN and cognitive functions.

Another class of nutrients extensively studied for their positive roles in brain health are ω-3 FAs, a form of polyunsaturated fatty acid (PUFA) commonly found in fatty fish, walnuts, flax-seed, and their respective oils. In adult rats, it was observed that a diet with Docosahexaenoic acid (DAHA), one of the principal ω-3 FAs, induces the NPCs in adult DG to exit from the cell cycle to differentiate in mature granule cells and increase the survival of newborn cells (Kawakita et al., 2006). Moreover, this increase in AHN after DAHA intake seems to be related to an improvement in specific memory domains with an improvement in cognitive functions (Yurko-Mauro et al., 2015).

Polyphenols are a wide class of phytochemical compounds present in several plant foods, and they are known for their antioxidant and anti-inflammatory proprieties, which are involved in their positive effects on health, comprising brain health. Indeed, a diet rich in polyphenol or their use as diet supplementation shows positive effects on cognitive functions in several models of brain injury, and AHN (Stangl and Thuret, 2009). For instance, it has been demonstrated that a diet with consumption of strawberry and blueberry induces respectively an increase in proliferation and survival of NPCs in DG of the hippocampus (Shukitt-Hale et al., 2015; Yurko-Mauro et al., 2015), with the improvement in spatial working memory (Casadesus et al., 2004). A polyphenolic compound widely consumed in the Mediterranean diet (MD) and known for its biological action is the Resveratrol. This molecule belongs to the stilbenoids group, characterized by two phenyl rings linked to each other by an ethylene bridge, and is considered responsible for the health benefits attributed to regular and moderate wine consumption, also known as “French Paradox” (Renaud and de Lorgeril, 1992). This natural polyphenol has been detected in more than 70 plant species, where it acts as a phytoalexin, and in various human foods, such as peanuts, tree nuts, grapes, cocoa, wine, and berry fruit, and as natural food ingredient resveratrol shows anti-inflammatory, anti-carcinogenesis, cardioprotective, vasorelaxant, phytoestrogenic and neuroprotective effects (Salehi et al., 2018). The neuroprotective effects are mediated by an increase in the activation of AMP-activated kinase (AMPK), leading to neurite outgrowth and stimulation of mitochondrial biogenesis (Dasgupta and Milbrandt, 2007). Its role in the regulation of AHN is still intensely debated because different works show opposing and contradictory. Recently it has been demonstrated that resveratrol consumption activates Sirtuin 1, a histone deacetylase, and increases the AHN, suggesting the use of resveratrol as a possible pro-plasticity inducer (Baur et al., 2006; Moriya et al., 2011). However further studies are necessary to understand the role and the range of actives concentration of resveratrol in the modulation of AHN.

Another polyphenol with beneficial effects on health is curcumin. This molecule is the main curcuminoid of turmeric (*Curcuma longa*), a plant member of the ginger family. In Asian cuisine, it is used as an ingredient in the preparation of sauces, but it is also used in traditional medicine due to its beneficial effects on health, whereas in Europe it is mainly used as dye food. Recently, it has been demonstrated a possible role of this molecule in the modulation of AHN. Indeed, *in vitro*, curcumin exerted biphasic effects on NPCs: a low concentration of curcumin activates p38 and ERK kinases and stimulates cell proliferation, whereas at high concentrations curcumin is cytotoxic. *In vivo*, administration of curcumin to adult mice resulted in a significant increase in the number of newly generated neurons in the dentate gyrus of the hippocampus, indicating that curcumin enhances the AHN and that has a biological activity that may enhance neural plasticity and repair (So et al., 2008).

Overall, these data indicate that dietary compounds by modulating the hippocampal neurogenesis can improve cognitive functions. Considering the differential concentration and assimilation of these nutrients in the various diet, to understand the beneficial effects of each nutrient on brain health is useful to design a diet with a combination of specific nutrients, to improve their therapeutic effects on the age-related decline of cognitive functions or in neurodegenerative disease. For instance, it was demonstrated that LMN diets, a diet rich in polyphenols and polyunsaturated fatty acid, has a significant effect on AHN with an increase of Type-2b/3 cells and mature granule cells in GCL in adult DG of mice fed with this diet (Valente et al., 2009).

1.6 Murine Model Btg1 Knockout

The murine models play a key role in the study of the regulation of AHN, the mechanism of aging, and neurogenic conditions. Their utility is due to their accelerated growth rate and the easy possibility to modify their genome to create knockout, knock-in and transgenic mice.

In our laboratory, several murine models have been studied and characterized demonstrating the role of several genes in the control of adult neurogenesis. Noteworthy is the Btg1 knockout model.

1.6.1 B-cell translocation gene 1 (Btg1)

B-cells translocation gene-1 (Btg1) is a member of the Btg family, which include more genes that encode for anti-proliferative activity, but with a different expression pattern (Figure 1.13; Tirone, 2001; Winkler, 2010). Initially, Btg1 has been identified as a gene involved in chromosomal translocation t(8;12)(q24;q22) presents in B cells of patients with chronic lymphatic leukemia (Rimokh et al., 1991). The encoding sequence of this gene is composed of two exons

separated by an intron, which are transcribed into an mRNA of 1,8 kb, which is translated into a protein of 171 amino acids with a molecular weight of 19297 Da. Like other members of the Btg family, Btg1 is characterized by the Btg domain, a sequence in its N-terminal, which spans for 104-106 amino acids with two highly conserved regions the BoxA and BoxB, structured in 5 α-helix and 4 β-sheets (Winkler, 2010).

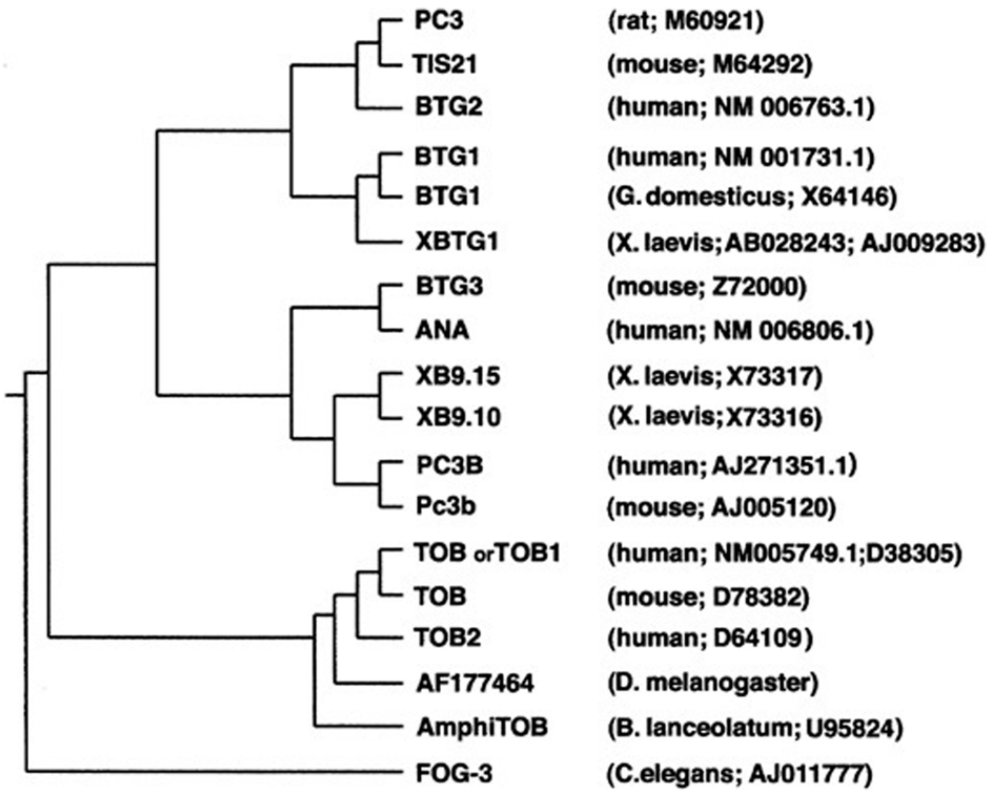


Figure 1.13 Phylogenetic relationship of Btg/Tob protein family (Tirone, 2001).

Btg1 is expressed in several cellular contexts (Rimokh et al., 1991), such as muscle (Rouault et al., 1992), CNS (Farioli-Vecchioli et al., 2012) and various types of tumors (Zheng et al., 2015b), and, through the interactions with other proteins, it is involved in several cellular mechanisms (Winkler, 2010).

In CNS, it has been found a function of this gene in granule cells precursor (GCL) of the cerebellum during the development (Ceccarelli et al., 2015), and in NSCs and NPCs of adult neurogenic niches (Farioli-Vecchioli et al., 2012).

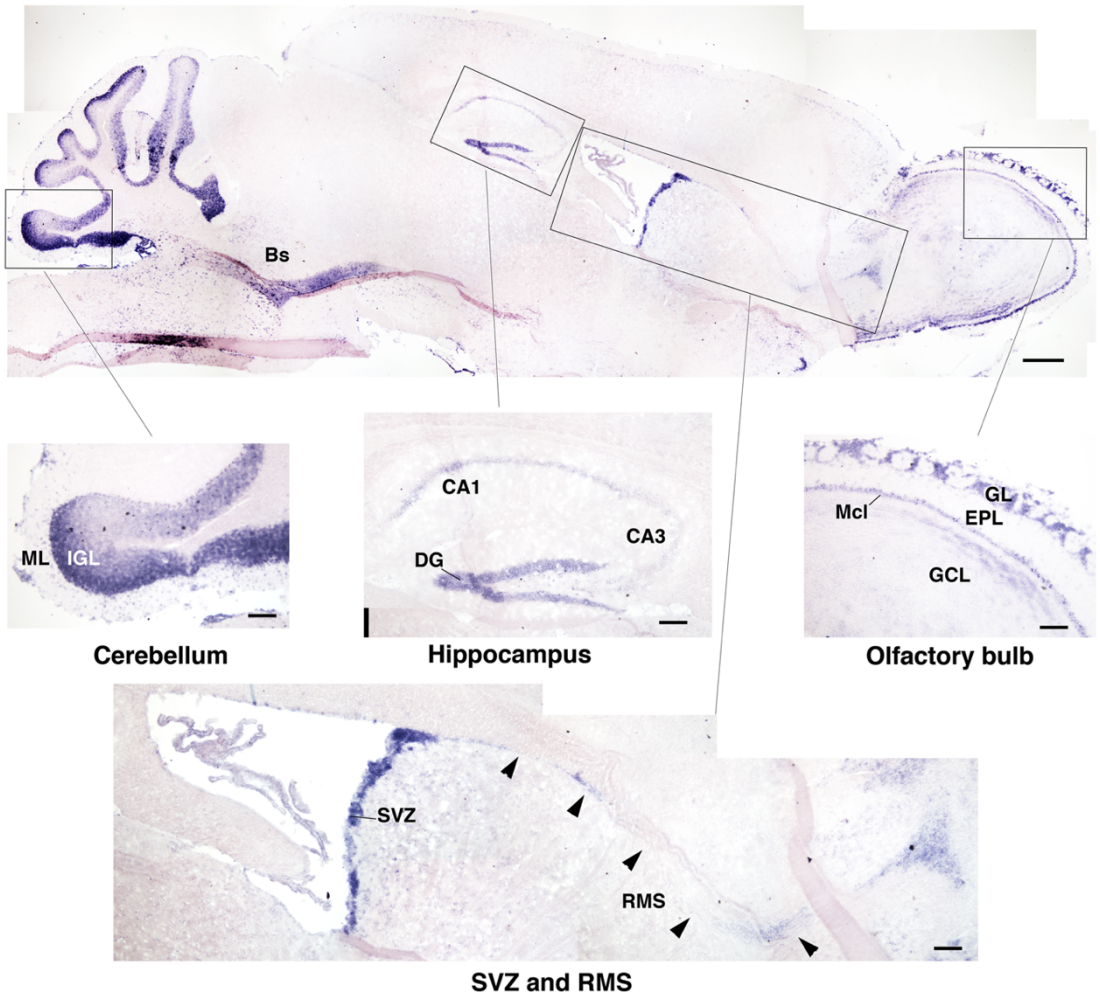


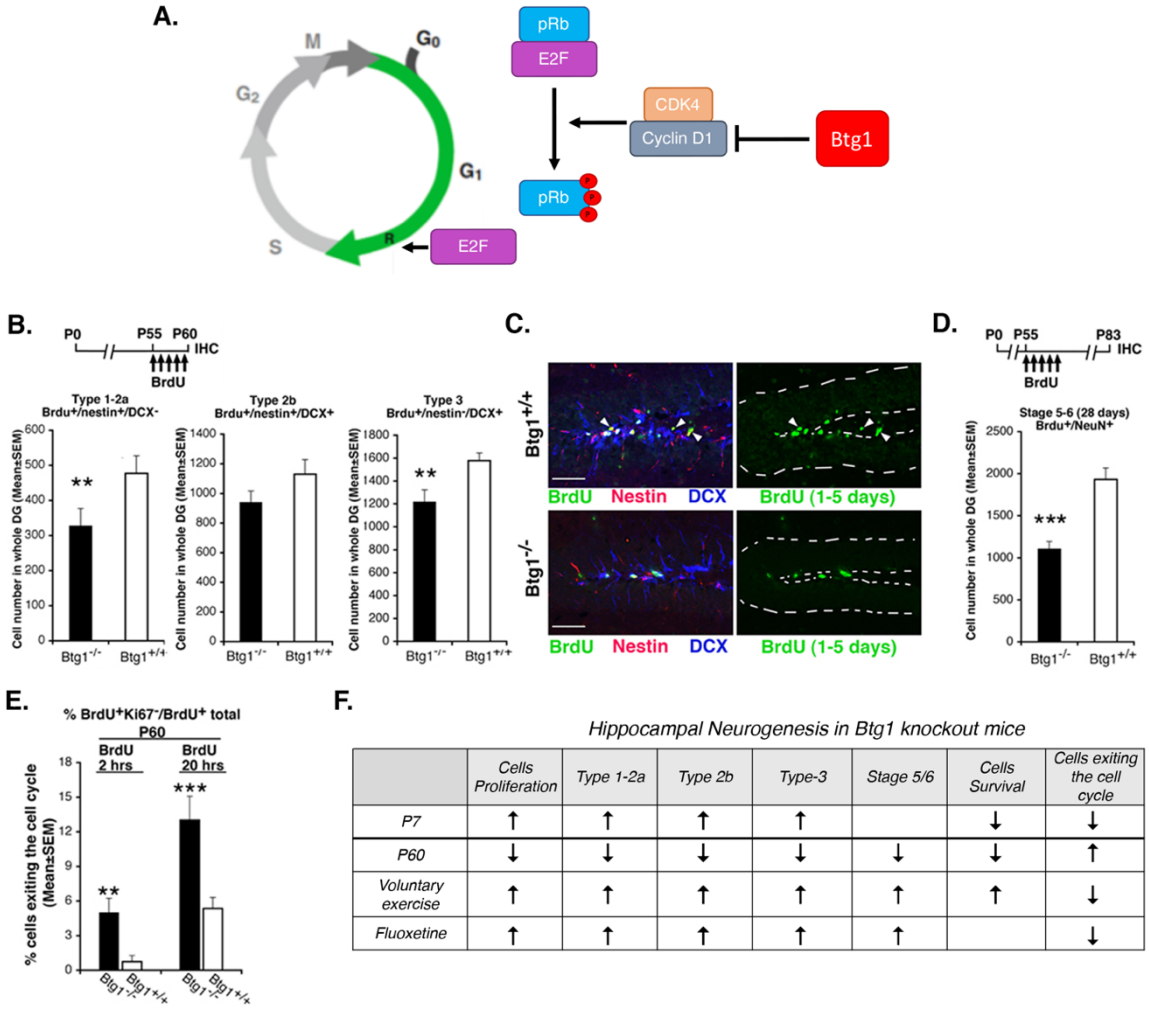
Figure 1.14 BTG1 expression in adult murine brain.
A representative sagittal section of the brain from a 2-month-old mouse, showing the expression of Btg1 mRNA labeled by in situ hybridization. Btg1 mRNA is clearly detectable (see enlargements of boxed areas): **(i)** in all neurons within the cell layers in the dentate gyrus blades of the hippocampus (DG) and to a lower extent in CA3 and CA1; **(ii)** in the subventricular zone (SVZ) and in neurons migrating from it along the rostral migratory stream (RMS); **(iii)** in the olfactory bulb in the glomerular layer (GL) and in the mitral cell layer (Mcl), while it is present to a lower level in the granule cell layer (GCL) and is absent in the external plexiform layer (EPL); **(iv)** in the cerebellum, in the molecular layer (ML) and the internal granular layer (IGL); **(v)** in the brainstem (Bs; upper panel). Scale bars: 500µm (panel above) or 100µm (enlargements) (Farioli-Vecchioli et al., 2012).

1.6.2 Roles of Btg1 in Brain and adult hippocampal neurogenesis

Using RNA *in situ* hybridization, it has been demonstrated expression of the Btg1 in several regions of CNS of 2-month-old mice. As shown in Figure 1.14, Btg1 is expressed at low levels in CA3 and CA1 areas of the hippocampus and the rostral migratory stream (RMS), while it is expressed at medium and high levels in DG of the hippocampus and SVZ of the lateral ventricle, and in the internal granule layer (IGL) of the cerebellum; and in these CNS regions it has been

characterized a function of Btg1 utilizing the murine model Btg1 knockout, where it was generated the genetic ablation of Btg1gene (Ceccarelli et al., 2015; Farioli-Vecchioli et al., 2012). During cerebellar development, a complex pattern of neurogenesis and cell movements generate the cerebellar cortex, with two principal class of neurons: the granule cells and the Purkinje cells. During embryonic development, granule cells precursor (GCPs) proliferate and migrate from the rhombic lip along the outer surface of the cerebellar anlage to form the external granule layer (EGL). GCPs in the EGL proliferate until the second week of post-natal day, after that they exit from the cell cycle and migrate inward to form the internal granule layer (IGL), below the Purkinje cell soma. In mice, the cerebellar neurogenesis ends at P20 (P, post-natal day; Roussel and Hatten, 2011). Using the murine model BTG1 knockout, it has been demonstrated that Btg1 regulates the proliferation of GCPs by inhibition of cyclin D1. These data have been confirmed in vitro on C17.2 cells, a cell line derived from the cerebellar GCPs, where overexpression of Btg1 induces an arrest of the cell cycle through the inhibition of cyclin D1 expression, and only the overexpression of this latter can rescue the Btg1-dependent G1 arrest (Figure 1.15*A*; Ceccarelli et al., 2015).

The role of Btg1 in adult neurogenesis has been well characterized in a work published by Farioli Vecchioli and colleagues (Farioli-Vecchioli et al., 2012). Initially, this study analyzed the proliferation of NSCs and NPCs and their survival/differentiation in both neurogenic niches of wild-type and Btg1 knockout mice, at P7 and P60. To this aim, the mice were treated with a daily intraperitoneal injection of BrdU for five days and then sacrificed, and the samples have been analyzed using immunofluorescence assay. The published results show that the genetic ablation of Btg1 in adult mice (P60) causes a decrease of BrdU uptake in all populations of NSCs and NPCs indicating a decrease in cell proliferation and survival/differentiation in both neurogenic niches of Btg1 knockout, compared to wild-type mice (Figure 1.15 *B, C, D, F*). Considering the anti-proliferative activity of Btg1, these data were unexpected; however, in mice at P7, they observed the opposite effects with an increase of proliferation, measured with the expression of Ki67, which is expressed in mitotic cells during all phases of cell cycle excepted for G0/G1 (Scholzen and Gerdes, 2000). Notably, the reduction of proliferation at P60 in neurogenic niches is correlated with a significant increase of the ratio $BrdU^{+}Ki67^{-}/BrdU^{+}$ (Figure 1.15*E*) and in the expression of marker like p53, $p21^{Cip1/WAF1}$, and cleaved (activated) caspase 3; suggesting that after genetic ablation of Btg1 a higher number of cells exit the cell cycle ($BrdU^{+}Ki67^{-}/BrdU^{+}$) to entry into quiescence, as measured by expression of $p21^{Cip1/WAF1}$, or die by apoptosis, as measured by expression of cleaved (activated) caspase 3.



	Cells Proliferation	Type 1-2a	Type 2b	Type-3	Stage 5/6	Cells Survival	Cells exiting the cell cycle
P7	↑	↑	↑	↑		↓	↓
P60	↓	↓	↓	↓	↓	↓	↑
Voluntary exercise	↑	↑	↑	↑	↑	↑	↓
Fluoxetine	↑	↑	↑	↑	↑		↓

Figure 1.15Roles of Btg1 in the control of cell cycle progression and adult hippocampal neurogenesis. **(A)** A schematic representation of the molecular mechanism of Btg1 in the control of cell cycle progression. **(B)** Scheme of BrdU treatment and quantification of the number of new 1- to 5-day-old type-1–2a (BrdU+/Nestin+/DCX−), type-2b (BrdU+/Nestin+/DCX+), and type-3 (BrdU+ /Nestin−/DCX+) stem and progenitor cells in 2-month-old mice (P60). **(C)** Confocal microscopy representative images showing new stem and progenitor cells in 2-month-old mice (type-1–2a; BrdU+/Nestin+/DCX−, marked by green and red and negative to blue, respectively, indicated by white arrowheads; scale bar, 50µm). **(D)** 28-day-old terminally differentiated neurons (BrdU+ /NeuN+; above the graph is the scheme of treatment of mice with five BrdU injections 28 days before perfusion at P83). **(E)** Percentage of cells exiting the cell cycle (ratio between BrdU+/Ki67− and total BrdU+ progenitor cells; n = 3 mice) after a BrdU pulse of 2 hours or of 20 h. *p < 0.05, **p < 0.01, or ***p < 0.001 vs. Btg1+/+ dentate gyrus; Student's t test. **(G)** Summary table of the effects of Btg1 gene ablation on hippocampal neurogenesis in young (P7) and adult (p60) Btg1 knockout mice and after voluntary exercise or treatment with fluoxetine. (Modified by Farioli Vecchioli et al., 2012).

These data obtained *in vivo* are supported by neurospheres assay. Using NSCs isolated from adult mice (P60), it has been shown that, after 7 days in culture, the number of neurospheres obtained from Btg1 knockout mice is lesser than those obtained from Btg1 wild-type mice. Moreover, it has been shown that Btg1 ablation causes a reduction in cell expansion of primary neurospheres (measured as the ratio between the number of cells at the end of the culture and

the initial number of cells) and smaller secondary neurospheres, suggesting that genetic ablation of Btg1 causes a decrease in asymmetrical divisions of NSCs, compared to wild-type conditions. The same analyzes showed inverse results in neurospheres obtained from mice at P7.
Overall, these data indicate that the genetic ablation of Btg1 removes control of cell cycle progression, thereby enhancing the division rate in NSCs, and this explains the enhanced proliferative potential in P7 mice. However, the miss-regulation of the cell cycle triggers the inhibitors p53 and $p21^{Cip1/WAF1}$, which leads cells to apoptosis and gradual depletion of the stem cell pool. Such depletion is responsible, in the long period (P60), of the reduction in proliferation and asymmetric divisions. Consistently with impairment of neurogenesis, BTG1 knockout mice of 2 to 4 months of age have defects in hippocampus-dependent memory tasks (Farioli-Vecchioli et al., 2012).
It was demonstrated that running and fluoxetine, can reverse the phenotype of Btg1 knockout mouse (Farioli-Vecchioli et al., 2014; Micheli et al., 2018). Moreover, it was demonstrated that overexpression of Sox2 in the dentate gyrus of BTG1 knockout mice can overcome, at least in part, the defect of proliferation. Given that Sox2 has been demonstrated to preserve the self-renewal of stem cells (Remboutsika et al., 2011), and rescue the defective proliferation of RBPJ-null dentate gyrus cells (Ehm et al., 2010), these results suggest that Sox2 and Btg1 may share common pathways (Micheli et al., 2018).
Overall these data indicate that in BTG1 knockout mice there is a depletion of NSCs pool, such as aging. However, suggest the existence of hidden, residual proliferative potential in NSCs that are brought into evidence by a neurogenic stimulus (fluoxetine or running) after deletion of the quiescence-maintaining gene Btg1. All these findings make the Btg1 knockout mouse an excellent model to study other neurogenic stimuli and their correlation with aging.

2 Aim

HTyr is a phenolic compound present in the olive oil, but it is also found in olive oil waste-water, a waste product potentially dangerous for the environment. HTyr can be conveniently recovered by innovative technologies to obtain HTyr-enriched extracts exhibiting health beneficial effects (Bernini et al., 2017; Marković et al., 2019; Romani et al., 2016). The multiple biological properties of HTyr are well known, especially the antioxidant, anticancer, anti-inflammatory and neuroprotective effects (Bernini et al., 2013; Imran et al., 2018; Romani et al., 2019; Schaffer et al., 2010). Furthermore, HTyr can cross the blood-brain-barrier (Hornedo-Ortega et al., 2018), and several beneficial effects were demonstrated in the brain. Indeed, HTyr reduces oxidative stress in brain slices undergoing oxidative stress, i.e., lipid peroxidation, peroxynitrite formation and production of inflammatory mediators (prostaglandin E2 and interleukin-1 β; De La Cruz et al., 2015); similarly, HTyr protects *in vitro* from oxidative stress, by rescuing either PC12 cells or SH-SY5Y neuroblastoma cells from 6-hydroxydopamine-induced damage, from L-DOPA toxicity or preventing hydrogen peroxide-induced death (Funakohi-Tago et al., 2018; Omar et al., 2018; Peng et al., 2015). Moreover, treatment with HTyr in a mouse model of Alzheimer's disease (TgCRND8 mice) caused a significant reduction of the amyloid-β plaque number in hippocampal areas (Leri et al., 2019; Nardiello et al., 2018).

However, despite the extensive literature about the modulation of adult neurogenesis by several phenolic compounds (Dias et al., 2012), little is known about the potential role of HTyr in this process *in vivo*. Only a report by Zheng and colleagues showed that HTyr treatment rescues the mRNA levels of BDNF, GAP43 and other neural markers reduced by stress in the whole hippocampus but no indications on the production of new neurons in the neurogenic niches were provided (Zheng et al., 2015a).

The neurogenesis in post-natal life occurs mainly in two neurogenic niches of the mammalian brain: the sub-ventricular zone (SVZ) of the lateral ventricle and the dentate gyrus (DG) of the hippocampus (Gage, 2000). In this latter neurogenic niche, the new neurons are generated from radial glia-like stem cells (named Type-1), which express the glial fibrillary acid protein (GFAP), Nestin and Sox2 (Filippov et al., 2003; Komitova and Eriksson, 2004; Kronenberg et al., 2003; Seri et al., 2001). DG stem cells mature initially into proliferating progenitor cells, classified as type-2a (negative for GFAP but positive for Nestin and Sox2), type-2b (positive for Nestin and Doublecortin [DCX]), or type-3, positive for DCX and negative for Nestin (Fukuda et al., 2003; Kempermann et al., 2004a). The progenitor cells mature then into early post-mitotic cells (stage 5), which express the Ca^{2+}-binding protein, and into terminally

differentiated neurons (stage 6), positive for calbindin and the late differentiation marker NeuN (Brandt et al., 2003; Kempermann et al., 2004a). The new DG neurons play a key role in memory coding, as they are more excitable up to the age of 4 weeks, thus improving the resolution and correlation between new memories and old memories of events encoded by mature neurons (Aimone et al., 2011; Farioli-Vecchioli et al., 2008, 2013). Therefore, adult hippocampal neurogenesis contributes to cognition (Zhao et al., 2008). Remarkably, during aging, the generation of progenitor cells and neurons in the DG undergoes a progressive decrease (Kuhn et al., 1996). This alteration contributes to cognitive decline, loss of working and episodic memory, impaired learning capacity and motor coordination, not only in the context of human neurodegenerative disorders (Vivar et al., 2016) but also during normal aging (Bizon and Gallagher, 2003).

Considering the high concentration of HTyr in OMWW, its beneficial effects on health and its ability to cross the blood-brain barrier, the purpose of this work aims to study the possible use of HTyr as a neurogenic stimulus.

First of all, we purposed to evaluated if HTyr could be able to control the survival of newly-formed neurons in the DG of adult and aged mice, thanks to its known neuroprotective activity. Therefore, we analyzed the potential beneficial effect of HTyr on the proliferation of different cell populations in the hippocampal niche, in aged and adult mice. Furthermore, we analysed the effects of HTyr treatment in the adult BTG1 knockout mouse model. In this model, stem and progenitor cells of the dentate gyrus undergo an early postnatal increase of proliferation, but rapidly and age-dependently lose their capability to proliferate and to self-renew, starting as early as at two months of age, i.e., during adulthood (Farioli-Vecchioli et al., 2012). Furthermore, we have previously observed that neurogenic stimuli such as running or an antidepressant (fluoxetine) rescue the defective self-renewal of Btg1-null stem and progenitor cells (Farioli-Vecchioli et al., 2014; Micheli et al., 2018).

This study is part of an ongoing analysis aimed at evaluating the neurogenic potential of stem and progenitor cells of the dentate gyrus challenged with different neurogenic stimuli in physiological and defective models (Farioli-Vecchioli et al., 2014; Micheli et al., 2018, 2019).

3 Materials and Methods

3.1 Synthesis of Hydroxytyrosol

All the HTyr synthesis procedures were performed by the laboratory of Dr. Bernini of the University of Tuscia (Viterbo, Italy) and previously published (Bernini et al., 2008). A patent was filed by the University of Tuscia on the procedure of synthesis of HTyr (WO2008110908).

3.1.1 Reagents

Tyrosol [2-(4'-hydroxyphenyl)ethanol], was purchased from Sigma Aldrich as all other solvents and reagents. All chemicals used were of analytical grade. IBX and DMP were prepared in the laboratory as described in the literature (Frigerio et al., 1999; Ireland and Liu, 1993). Silica gel 60 F254 plates and silica gel 60 were furnished by Merck.

3.2.1 Synthesis reactions

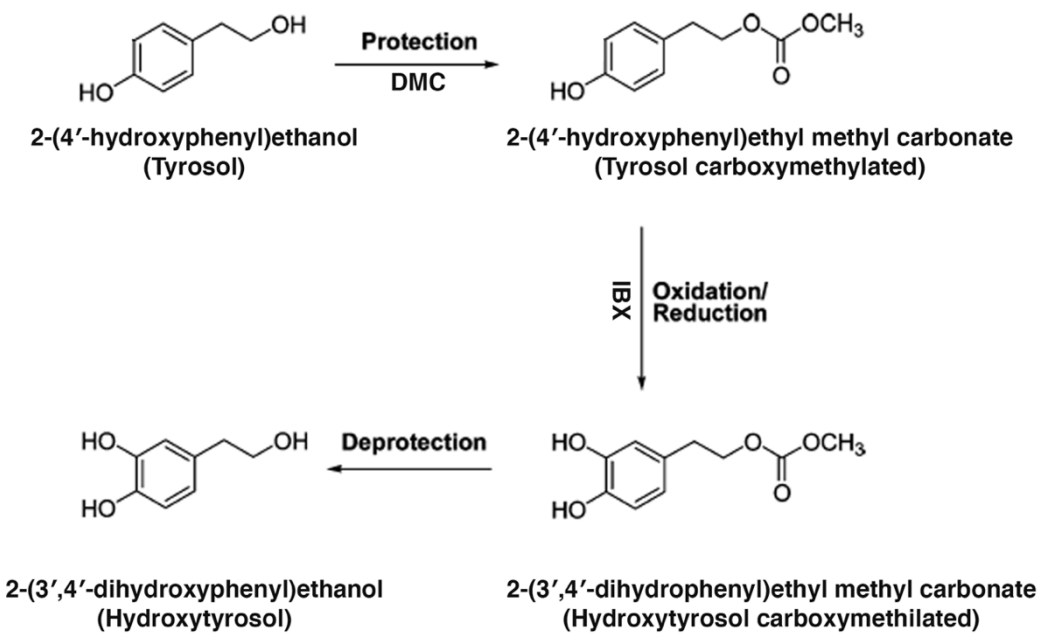


Figure 3.1 Synthetic Procedure to Obtain Hydroxytyrosol from Tyrosol.
DMC: dimethyl carbonate; **IBX:** 2-iodoxybenzoic acid

HTyr was synthesized on a scale of the order of grams using a patented procedure optimized in the laboratory of Dr. Bernini at the University of Tuscia, Viterbo, Italy and published (Bernini et al., 2008). Briefly, this procedure of synthesis is based on three steps: (1) selective derivatization of the alcoholic group of tyrosol (2-(4'-hydroxyphenyl)ethanol) with dimethyl carbonate in acidic medium to afford tyrosol carboxymethylated; (2) oxidation of tyrosol

carboxymethylated with 2-iodobenzoic acid and in situ reduction with sodium dithionite to obtain hydroxytyrosol carboxymethylated; (3) hydrolysis of hydroxytyrosol carboxymethylated under basic conditions to get hydroxytyrosol. The reactions as summarized in figure 3.1. At the end of each step, the obtained product was purified by chromatographic column and characterized by Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) to verify both the chemical structure and purity. Hydroxytyrosol was isolated as a 98% pure yellow oil (Bernini et al., 2008).

3.2 Mouse line, genotyping and husbandry

Btg1 knockout and Btg1 wild-type were generated as previously described (Farioli-Vecchioli et al., 2012). Initially, a mouse genomic clone was isolated from the 129/Sv mouse library of phage lambda by standard techniques. A fragment of 6 Kb encompassing the mouse Btg1 gene was cloned in pBluescript II. A phosphoglycerate kinase-neomycin resistance cassette was inserted in the SacII restriction site located in mouse Btg1 exon 1 (49 bp after ATG). A Herpes simplex virus thymidine kinase gene cassette (negative selection) was cloned adjacent to the 3' end of the genomic region.

Subsequently, the linearized targeting vector was inserted in embryonic stem cells (ES) and cells were selected them with G418 (250 μg/ml) and ganciclovir (0.5 μg/ml). A resistant clone was injected into 3.5-day C57BL/6 blastocysts to obtain male chimeras. The genotype of resistant ES cells and of agouti pups was determined, following digestion of DNA with AccI, by Southern blotting using as probes a genomic fragment of about 0.5 kb comprising the EcoRI-BglII region at 50 of the gene or a fragment of the neomycin sequence (wild-type or knockout alleles generated 5 or 6.1 kb fragments, respectively).

The Btg1 heterozygotes mice obtained from chimera animals were crossed several times, until as isogenic progeny was obtained, also referred throughout the paper to as Btg1 KO and Btg1 WT for 2-month-old mice, or referred to as WT for 15-month-old wild-type mice. Genotyping of mice was routinely performed by PCR, using genomic DNA from tail tips. Three primers were used to identify mice carrying the different genotype Btg1 knockout, Btg1 heterozygote and Btg1wild-type, one complementary to the neo cassette (mBtg1-Neo-R 5'-CGGAGAACCTGCGTGCAATC-3') and the other two complementary to the targeted exon I (mBtg1-F 5'-CCATGCATCCCTTCTACACCC-3'; mBtg1-R 5'- TGCAGGCTCTGGCTGAAAGT-3') and were amplified together in the PCR reaction (Figure 3.2). By PCR reaction were obtained patterns of amplification specific for each of the three combinations of alleles (knockout, 388 bp amplification by mBtg1-F and mBtg1-Neo-R primers; wild-type, 136 bp amplification of exon I by mBtg1-F and mBtg1-R primers).

Temperature	Time	
94°C	10 minutes	
94°C	1 minute	
55°C	1 minute	35 cycles
72°C	1 minute	
72°C	10 minutes	
4°C		

Figure 3.2 PCR program to Btg1 knockout and wild type genotyping.

Mice were maintained under standard specific-pathogen-free conditions and were housed in standard cages until 2 or 15 months of age. Then, mice were randomly assigned to untreated/control group (H2O, maintained with drinking water) or treated group (HTyr, administered with Hydroxytyrosol).

All animal procedures were performed on male mice and completed in accordance with the current European (directive 2010/63/EU) Ethical Committee guidelines and the protocol of the Italian Ministry of Health (authorization 442-2016-PR).

3.3 Experimental design

15-month-old wild-type mice and 2-month-old Btg1 wild-type and Btg1 knockout mice were randomly assigned to the untreated/control group (H2O, maintained with drinking water) or treated group (HTyr, administered with Hydroxytyrosol in drinking water).

100 mg/Kg/day of HTyr (human equivalent dose 8.1 mg/Kg/day; Nair and Jacob, 2016) was administered to mice for 30 days. The daily dosage was selected within a range of antioxidant effects free of cytotoxicity (Auñon-Calles et al., 2013). Moreover, the duration of the treatment ensures an adequate accumulation of HTyr and its metabolites in the brain (López de las Hazas et al., 2018). The intake of HTyr was calculated considering the weight of mice and the average water consumption per mouse (7 ml/day) (Bachmanov et al., 2002). The weight was measured before and during the administration of HTyr.

For the examination of stem and progenitor cells, mice were euthanized at the end of HTyr or H2O treatment (Figure 3.3*A*)

To study survival and maturation of newly-formed neurons, mice were subjected to one daily injection of BrdU (95 mg/kg i.p.) during the first five days of treatment with HTyr or water and sacrificed at the end of the treatment (Figure 3.3*B*).

To analyze the integration of new neurons in 15-month-old wild-type mice, the mice treated with HTyr and H20 were randomly placed in the cage with a running wheel during the last seven days of treatment. Run distances were recorded daily with an automatic counter. The average running wheel distance over the whole experiment (7 days) did not significantly change in treated compared to untreated mice (data not shown). Mice were sacrificed 1.5 hours after the end of voluntary running (Figure 3.3*C*).

At the end of different treatments, the brains were collected after transcardiac perfusion with 4% paraformaldehyde (PFA) in PBS 1X and kept overnight in PFA. Brains were then equilibrated in 30% sucrose and cryopreserved at -80°C.

3.4 Immunohistochemistry and lipofuscin detection

Immunohistochemistry was performed on serial free-floating coronal sections cut at 40 µm thickness at -25°C in a cryostat from brains embedded in Tissue-Tek OCT (Sakura, Torrence, CA, USA). Sections were then processed for multiple labelling immunohistochemistry using fluorescent methods.

To detect Ki67, the sections were previously pretreated with Glycine 0.1M for 5 minutes and then permeabilized with 0,3% TRITON X-100 in PBS [NaCl 137mM, KCl 2.7 mM, KH_2PO_4 1.4 mM, Na_2PO_4 x H_2O_2 6.45 mM]. Subsequently, the sections were incubated with the blocking solution [0,3% TRITON X-100 and 3% Normal Donkey Serum (NDS) in PBS] for 1 hour at 4°C, and then incubated with primary antibody diluted in blocking solution for 16-18 hours at 4°C.

To detected BrdU incorporation, the sections are incubated in 2N HCl for 45 minutes at 37°C, followed by 2 washes with 0.1 M sodium borate buffer pH 8.5 for 10 minutes each. This treatment denatures the DNA molecules allowing the antibody to bind the molecule of BrdU incorporated in the DNA. After that, the sections were permeabilized with TBS plus [0,1% TRITON X-100, 0,03% TWEEN-20 in TBS (50mM Tris-HCL and 150 mM NaCl)] for 10 minutes and then were incubated with the blocking solution [TBS PLUS with 3%NDS] for 1 hour at 4°C and then incubated with primary antibody diluted in blocking solution for 16-18 hours at 4°C.

Using these protocols, the antibodies against Ki67 or BrdU were incubated together with other primary antibodies, so as to label the specific subpopulations of dentate gyrus stem, progenitor cells, and neurons.

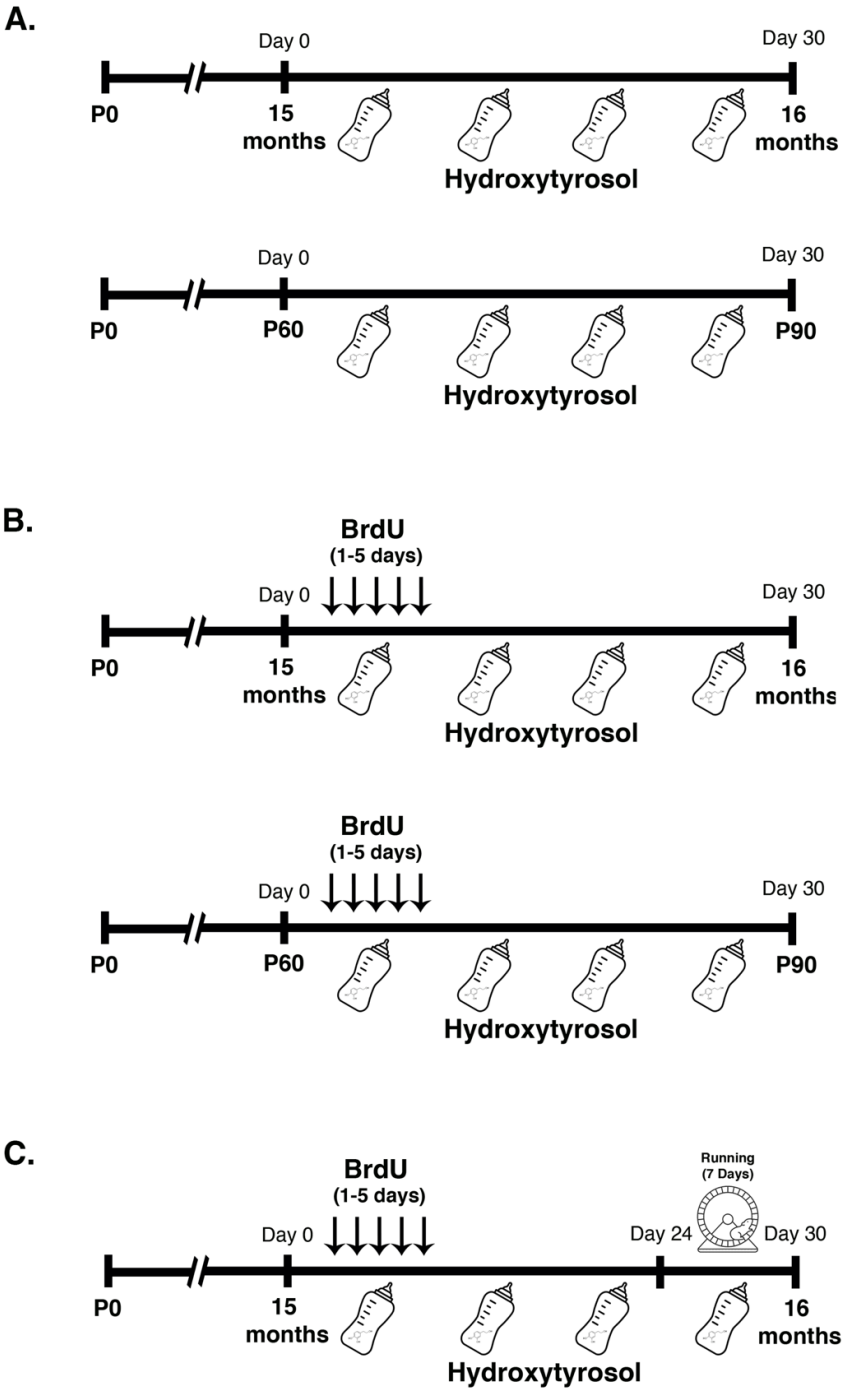


Figure 3.3 Hydroxytyrosol treatments.
Schematic representation of treatment with HTyr (100 mg/kg/die) to measure proliferation of NSCs and NPCs **(A)**, the survival of new-born cells **(B)** and the activation of c-fos **(C)**.
P: Post Natal Day

Another protocol was used to detect c-fos and BrdU together. The sections were incubated with 1N HCl for 30 minutes at 45°C followed by two washes with 0.1 M sodium borate buffer pH 8.5 for 10 minutes each. This treatment denatures the molecule of DNA but in a milder way, allowing the detection of both BrdU and c-fos. After that, the sections were permeabilized with

0.3% TRITON X-100 in PBS for 10 minutes and then were incubated with the blocking solution [0.3% TRITON X-100 and 3% Normal Donkey Serum (NDS) in PBS] at room temperature. Finally, the sections were incubated with primary antibody diluted in blocking solution for 16-18 hours at room temperature.

The primary antibodies used are listed below:

- 5-Bromo-2'-deoxyuridine (BrdU): rat monoclonal, Abcam, Cambridge, UK; AB6326; 1:400;
- Ki67: rabbit monoclonal, Biocare Medical, Pacheco, CA, USA; clone SP6, CRM325; 1:100;
- Glial fibrillary acid protein (GFAP): mouse monoclonal, Sigma-Aldrich, St. Louis, MO, USA; G6171; 1:200;
- NeuN: mouse monoclonal, Millipore Burlington, MA, USA; MAB377; 1:400;
- Sox2: goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA; SC-17320; 1:300;
- Doublecortin (DCX): goat polyclonal, Santa Cruz Biotechnology; SC-8066; 1:300;
- Iba1: goat polyclonal, Abcam, Cambridge, UK; AB5076; 1:600;
- Cleaved (activated) Caspase-3: rabbit polyclonal, Cell Signaling Technology, Danvers, MA, USA; 9661; 1:100;
- c-fos: rabbit polyclonal, Millipore; Ab-5 PC38; 1:500.

The bind between antigen and primary antibody was detected using secondary antibodies conjugated to fluorophores. Secondary antibodies to visualize the antigen were all obtained from Jackson ImmunoResearch (West Grove, PA, USA) and were used as follows: a donkey anti-rabbit antiserum Cy3-conjugated (Ki67), a donkey anti-rat antiserum TRITC-conjugated (BrdU), a donkey anti-goat conjugated to Alexa-488 (DCX, Iba1) or Alexa-647 (Sox2), a donkey anti-mouse conjugated to Alexa-647 (NeuN, GFAP), or a donkey anti-rabbit conjugated to Cy3 (Caspase-3) or Cy2 (c-fos).

Nuclei were counterstained by Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA, 1 mg/ml in PBS).

At the end of the procedure, to reduce auto-fluorescence due to lipofuscin deposits, slices were treated with 0.3% Sudan Black (Sigma-Aldrich) in 70% Ethanol for 30 seconds and rinsed thoroughly with PBS 1x.

Alternatively, to quantify lipofuscin deposits, slices were directly stained with Hoechst 33258 (Sigma Aldrich; 1 mg/ml in PBS) and auto-fluorescence was analyzed in three different channels (Kempermann et al., 2002).

Confocal Z-stacks and single-plane images of the immune-stained sections were obtained using a TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Analyses were performed in sequential scanning mode to rule out cross-bleeding between channels.

3.5 Quantification of cell Numbers

A stereological study of the number of cells was performed by analyzing with confocal microscopy one-in-six or one-in-eight series of 40-µm free-floating coronal sections (240 µm apart), to count cells expressing the indicated marker throughout the whole rostro-caudal extent of the dentate gyrus. The total estimated number of cells within the dentate gyrus, positive for each of the indicated markers, was obtained by multiplying the average number of positive cells per section by the total number of 40 µm sections including the entire dentate gyrus (about 50–60 sections), as described (Farioli-Vecchioli et al., 2008, 2012; Gould et al., 1999b; Jessberger et al., 2005; Kee et al., 2007). Therefore, about 10 sections (20 dentate gyri) per mouse and at least three animals per group were analyzed. Cell number analyses were performed manually by trained experimenters using the I.A.S. software to register positive cells (Delta Sistemi, Rome, Italy).

3.6 Statistical Analysis

The effect of HTyr treatment on the cell number of each cell population was statistically analyzed in wild-type 15-month-old mice treated with HTyr or with water using Student's *t*-test. In experiments where the number of cells was low (Caspase-3^+ cells), we used – after verifying with the Levene's test that the equality of variances was not satisfied – a non-parametric test, namely, the Mann-Whitney *U* test in place of Student's *t*-test.

The effect of HTyr treatment in Btg1 WT and Btg1 KO 2-month-old mice was statistically analyzed in all experiments using two-way ANOVA, in order to test the main effects of the two factors, i.e., genotype or HTyr treatment, on the cell number of each cell population. Individual between-group comparisons to test simple effects were performed by Fisher's PLSD ANOVA *post hoc* test. These analyses were carried out using the StatView 5.0 software (SAS Institute, Cary, NC, USA) and XLSTAT (Addinsoft, Paris, France). Differences were considered statistically significant at $P < 0.05$. All data were expressed as mean values ± SEM (standard error of the mean).

4 Results

HTyr is a phenolic compound found in the EVOO and OMWW, the main waste products during the processing of olive. It is known for its antioxidant activity, where two hydroxyl groups on the benzene ring are responsible for its activity as free radical scavenging, increasing its efficacy under stress conditions. HTyr can be orally administered, and it is absorbed by the intestine wall where its metabolism starts. After that, HTyr and its metabolites reach several tissues. This widespread diffusion, after oral administration of HTyr, is responsible for various beneficial effects of this molecule, such as well-documented beneficial effects in cardio-vasculature diseases and several types of cancer. Furthermore, HTyr has shown neuroprotective effects and a role in the prevention of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases (Bernini et al., 2013; Marković et al., 2019; Romani et al., 2019).

Considering these neuroprotective effects of HTyr, and beneficial properties of other phenolic compounds on AHN, our purpose was to analyze the effects of HTyr oral administration on hippocampal neurogenesis.

To this aim, the mice were treated with HTyr (100 mg/Kg/day) in drinking water for 30 days, and they were compared to control mice treated with vehicle (standard drinking water). This daily dosage was selected within a range of antioxidant effects free of cytotoxicity (Auñon-Calles et al., 2013). The oral administration of HTyr was used to mimic diet consumption. Moreover, the duration of the treatment ensures an adequate accumulation of HTyr and its metabolites in the brain, since the HTyr can cross the blood-brain barrier (Hornedo-Ortega et al., 2018).

4.1 Hydroxytyrosol stimulates adult neurogenesis in aged mice and enhances the survival of newborn cells

Hippocampal neurogenesis persists throughout the lifespan. Using thymidine analogues, in mice was observed a decline in mitotic activity of neural progenitors in DG of 12- and 21-month-old mice, and a decrease in newborn neurons (Kempermann et al., 1998; Kuhn et al., 1996).

Therefore, we sought to examine whether HTyr administration was able to revert the deficit on the production of new neurons in the DG of 15-month-old wild-type mice. To this aim, we treated the mice with HTyr (100 mg/kg/day) in drinking water, where cells were birth dated by subjecting mice to daily injection of BrdU, at a concentration of 95 mg/kg, during the first 5 days of treatment with HTyr. After 30 days, the mice were sacrificed by trans-cardiac perfusion

for DG cells examination; the results were compared to those obtained in control mice treated with vehicle (drinking water).

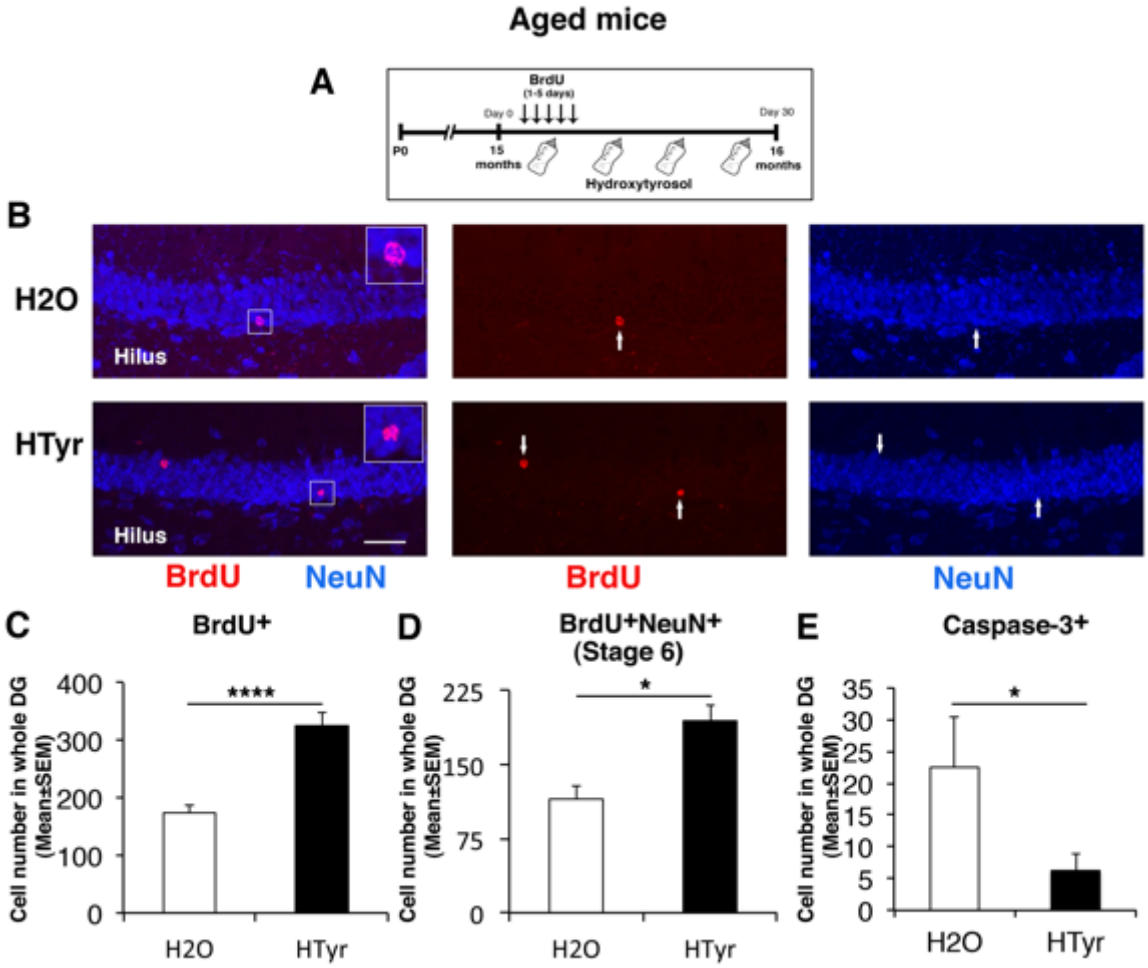


Figure 4.1 HTyr administration enhances the production of new neurons in aged mice by promoting their survival.
A) Experimental timeline: 15-month-old wild-type mice were treated with HTyr in the drinking water for 30 days; mice received 5 daily injections of BrdU (95 mg/kg) during the first days of treatment. At the end of the treatment, mice were subjected to immunohistochemistry analysis. **B)** Representative images by confocal microscopy showing that HTyr treatment induces the production of 26 to 30-day-old neurons (in red BrdU+ and in red/blue BrdU+/NeuN+ cells). Arrows indicate double-labeled cells. The white box area is shown with 2.5x digital magnification. Scale bar, 50 µm. **C)** Total number of newly-formed neurons – as detected by BrdU incorporation – and (**D**) number of stage-6 neurons (BrdU+/NeuN+) was augmented by treatment with HTyr in aged wild-type mice (* $P < 0.05$, **** $P < 0.0001$; Student's t-test). **E)** Quantification of the total number of neurons positive for activated Caspase-3 in the same 15-month-old mice analyzed for the quantification shown in graph (**C**) and (**D**). Caspase-3+ cells decreased in dentate gyri treated with HTyr (* $P < 0.05$, Mann-Whitney U test). **C-E)** The numbers of dentate gyrus cells are means ± SEM; 5 mice per group were used.

The newly-formed neurons were analyzed in the DG by recording the total number of 26- to 30-day-old BrdU positive cells and the number of BrdU-positive cells co-labeled with NeuN, the protein expressed in all types of post-mitotic neurons in the brain and used to mark the mature granule cells or stage 6 cells in the hippocampal neurogenic niche (Brandt et al., 2003; Kempermann et al., 2004a).

We observed a significant increase of total BrdU positive cells in mice treated with HTyr compared with untreated mice (hereafter indicated as WT-HTyr and WT-H20, respectively; WT-HTyr vs. WT-H20, 88% increase, $p < 0.0001$, Student's *t*-test; Figure 4.1*B*, *C*). Moreover, the number of $BrdU^+/NeuN^+$ neurons was expanded by the treatment (WT-HTyr vs. WT-H20, 68% increase, $p = 0.0216$, Student's *t*-test; Figure 4.1*B*, *D*).

These results show that HTyr can revert the physiological decline of neurogenesis observed in the hippocampus of aged mice.

The survival of the newborn cells is an important issue because it regulates the functional balance during hippocampal neurogenesis. Indeed, only a few newborn neurons survive after birth while a majority of newborn neurons undergo programmed cell death, by apoptosis, within 1 to 4 days after birth, during the maturation from amplifying progenitors to neuroblasts (Sierra et al., 2010). The second wave of death occurs when the neurons are in the process of attaining terminal differentiation, 1-3 weeks after birth (Kempermann et al., 2004b; Tashiro et al., 2006). It is possible to detect apoptosis in DG measuring the number of cells positive to activated Caspase-3, which is a crucial mediator of apoptosis cascade.

To verify whether the increased number of newly-formed neurons was related to the neuroprotective/pro-survival activity of HTyr treatment, we measured the apoptotic marker activated Caspase-3 in the dentate gyrus. The total number of activated Caspase-3 positive cells was very low, as expected. However, a marked decrease of activated $Caspase\text{-}3^+$ cells was recorded in treated mice compared to untreated mice (WT-HTyr vs. WT-H20, 72% decrease, $p < 0.0443$, Mann-Whitney *U* test; Figure 4.1*E*), suggesting that apoptotic cell death was counteracted by HTyr administration.

4.2 Hydroxytyrosol increases the proliferation of stem and progenitor cells

We then investigated if the observed pro-neurogenic effects were correlated exclusively to the neuroprotective activity of HTyr or also to a stimulation of the proliferation of neural stem/progenitor cells.

Initially, we analysed the total number of proliferating cells in DG of aged mice treated with HTyr (100 mg/kg/die) in drinking water for 30 days, compared to control mice treated with drinking water (Figure4.2*B*). To this aim, we measured the number of cells expressing the protein Ki67, which marks cycling cells (Scholzen and Gerdes, 2000), and we observed a significant increase (81%) of proliferating cells at the end of the treatment with HTyr compared to treatment with water (WT-HTyr vs. WT-H2O, $p = 0.002$, Student's *t*-test, Figure 4.2*A*, *C*).

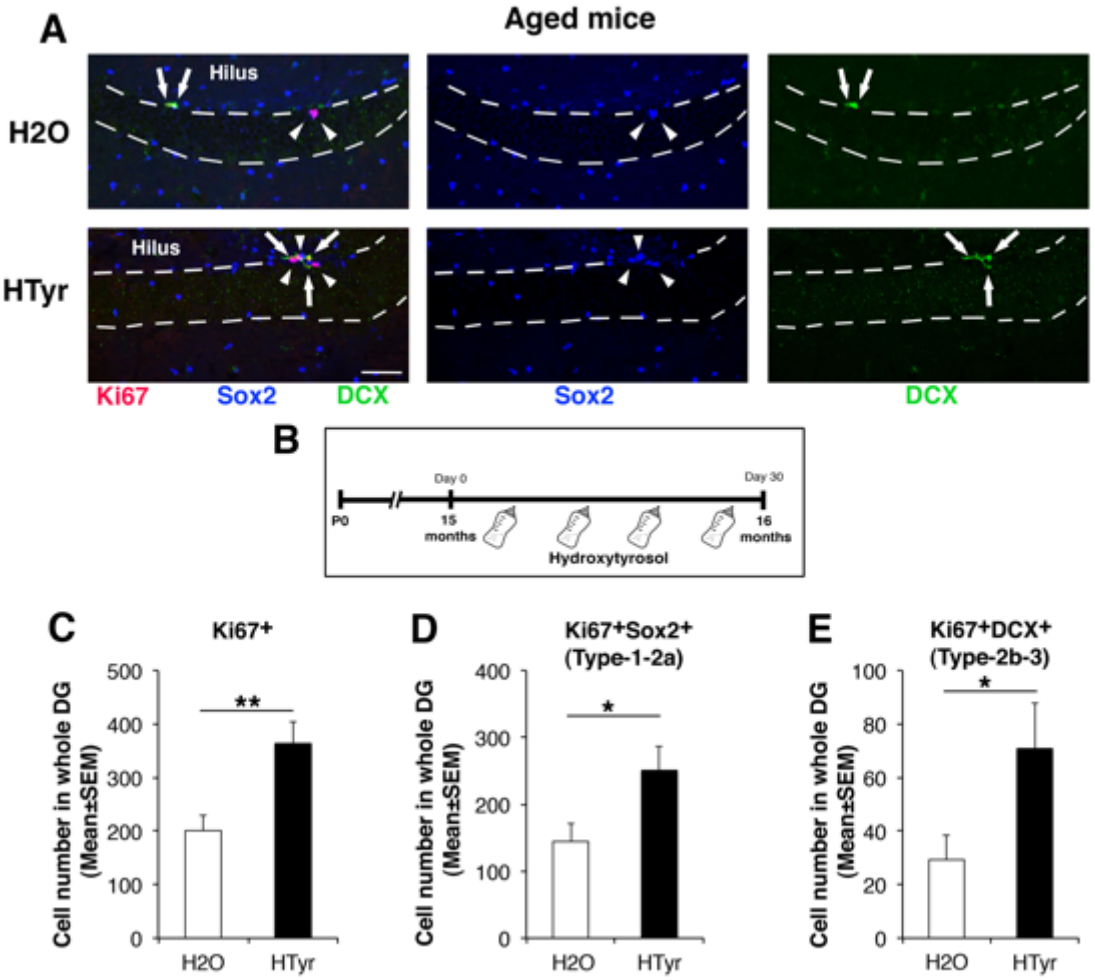


Figure 4.2 HTyr treatment promotes the proliferation of aged dentate gyrus stem/progenitor cells.
***A*)** Representative images by confocal microscopy showing that treatment with HTyr, whose timeline is shown in **(*B*),** significantly enhances the total number of proliferating progenitor cells (Ki67 positive) and, in particular, the number of type-1/2a stem/progenitor cells (Ki67+/Sox2+; in red/blue, indicated by arrows) and type-2b/3 progenitors (Ki67+/DCX+; in red/green, indicated by arrowheads). The white dashed line labels the outer and inner boundaries of the dentate gyrus. Scale bar, 50 μm. ***B*)** Experimental diagram of mice treatment: 15-month-old mice were administered with HTyr for 30 days and analyzed. ***C*)** The total number of Ki67 positive cells is increased by HTyr compared with H2O treatment**.** ***D*)** Proliferating type-1/2a progenitor cells (Ki67+/Sox2+) and **(*E*)** type-2b/3 cells (Ki67+/DCX+) are significantly amplified by HTyr administration. ***C*–*E*)** The numbers of dentate gyrus cells are means ± SEM; 4 mice per group were analyzed. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test.

Subsequently, we asked if treatment with HTyr influences the proliferation of stem and progenitor cells. To this aim, we used the expression of several proteins to distinguish the proliferation of various cell types in hippocampus neurogenesis (Kempermann et al., 2004a). Measuring the cells co-labelled with Ki67 and Sox2, we observed that treatment with HTyr induces an increase of proliferation of Type-1 and -2a cells, which are respectively stem and progenitor cells in SGZ of DG (WT-HTyr vs. WT-H2O, 73% increase, $p = 0.019$; Student's *t*-test, Figure 4.2*A*, *D*).

We also measured the proliferating Type-2b and -3, also known as neuroblasts, marked with the expression of Ki67 and DCX, and we observed that HTyr treatment induce a significant increase in proliferation of these cells (WT-HTyr vs. WT-H2O, 141% increase, $p = 0.04$, Student's *t*-test, Figure 4.2*A*, *E*).

These observations showed that in aged wild-type mice HTyr was able to stimulate also the proliferation of stem and progenitor cells.

4.3 Hydroxytyrosol influences the neurogenic niche of the dentate gyrus in aged mice

Neurogenic niche plays many roles in the regulation of hippocampal neurogenesis, including the age-related cognitive decline.

Therefore, we asked if the treatment with HTyr had beneficial effects on the neurogenic niche of aged mice. Considering the antioxidant activity of HTyr, we have chosen to analyse the presence of non-specific deposits of lipofuscin because they are residues of lipid peroxidation and are considered signs of aging correlated to chronic oxidative stress (Flood et al., 1995).

After dietary supplementation with HTyr (Figure 4.3*A*), we examined the levels of autofluorescent lipofuscin pigment deposits in the dentate gyrus and we observed an 18% decrease of their number in the HTyr-treated group in comparison to control group (WT-HTyr vs WT-H2O, $p = 0.042$, Student's *t*-test, Figure 4.3*B*, C), demonstrating that HTyr supplementation increased the neuronal health, by protecting neurons against degeneration.

Microglia are recognized as the resident brain immune cells and they play a role in apoptosis, proliferation, and differentiation of neural stem/progenitor cells (Gemma and Bachstetter, 2013). Moreover, some neurogenic stimuli, such as voluntary exercise, induce an increase in proliferation of progenitor cells reducing also the number of microglial cells (Gebara et al., 2013). Microglial cells play an important role in aging. In fact, with aging, microglia acquire a phenotype that is increasingly inflammatory and cytotoxic, generating a hostile environment for neurons (Cornejo and von Bernhardi, 2016).

Therefore, we thought to verify whether the pro-neurogenic effect of HTyr was related to its influence on microglia. To this aim, we analyzed the number of microglial cells, by mean of the marker Iba1. Iba1 positive cells in WT-HTyr mice turned out to be less numerous than in control WT-H2O mice (WT-HTyr vs WT-H2O 24% decrease, $p < 0.0002$, Student's *t*-test, Figure 4.3*D*, *E*). This reduction of microglial cells could be in part responsible for the pro-proliferative effect of HTyr.

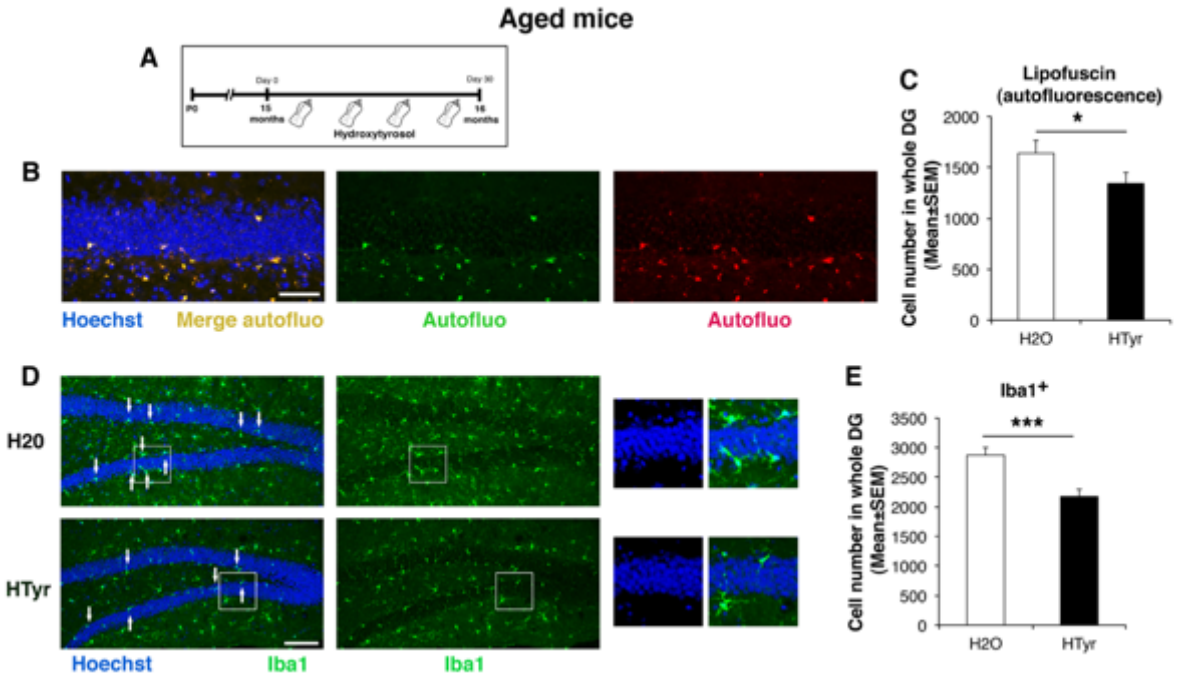


Figure 4.3 HTyr treatment reduces lipofuscin deposits and activated microglia.
A) Experimental timeline: 15-month-old WT mice were treated with HTyr for 30 days, followed by immunohistochemistry analysis. ***B)*** Fluorescence confocal images representing lipofuscin autofluorescent cells in the dentate gyrus. Nuclei, stained with Hoechst 33258, are in blue. Scale bar, 50 µm. ***C)*** The number of lipofuscin-loaded cells significantly decreased in treated dentate gyri. ***D)*** Representative images by confocal microscopy of activated microglia cells (Iba1 positive, in green). Nuclei were counterstained with Hoechst 33258 (blue). Arrows indicate representative Hoechst+/Iba1+ cells. The white box area is shown with 2.5x digital magnification. Scale bar, 100 µm. (***E***) The total number of Iba1 positive cells was significantly decreased by treatment with HTyr. In (***C***) and (***E***) the numbers of dentate gyrus cells are means ± SEM; 4 mice per group were analyzed; * $p < 0.05$, *** $p < 0.001$, Student's *t*-test.

Overall, our results revealed that in aged wild-type mice HTyr exerts a marked pro-survival activity and increases the number of newly-born neurons also by stimulating the proliferation of stem and progenitor cells.

4.4 HTyr-induced enhancement of neurogenesis stimulates the activation of new neurons and their incorporation into memory circuits of the aged dentate gyrus

It is known that newly generated neurons of the dentate gyrus are progressively integrated into spatial memory-related circuits after 4–6 weeks. This integration process is evaluated by measuring c-fos expression, whose activation specifically occurs in dentate gyrus neurons of mice that have undergone spatial memory training and are thus correlated with the recruitment of new neurons into spatial memory circuits (Kee et al., 2007). Voluntary exercise also is a

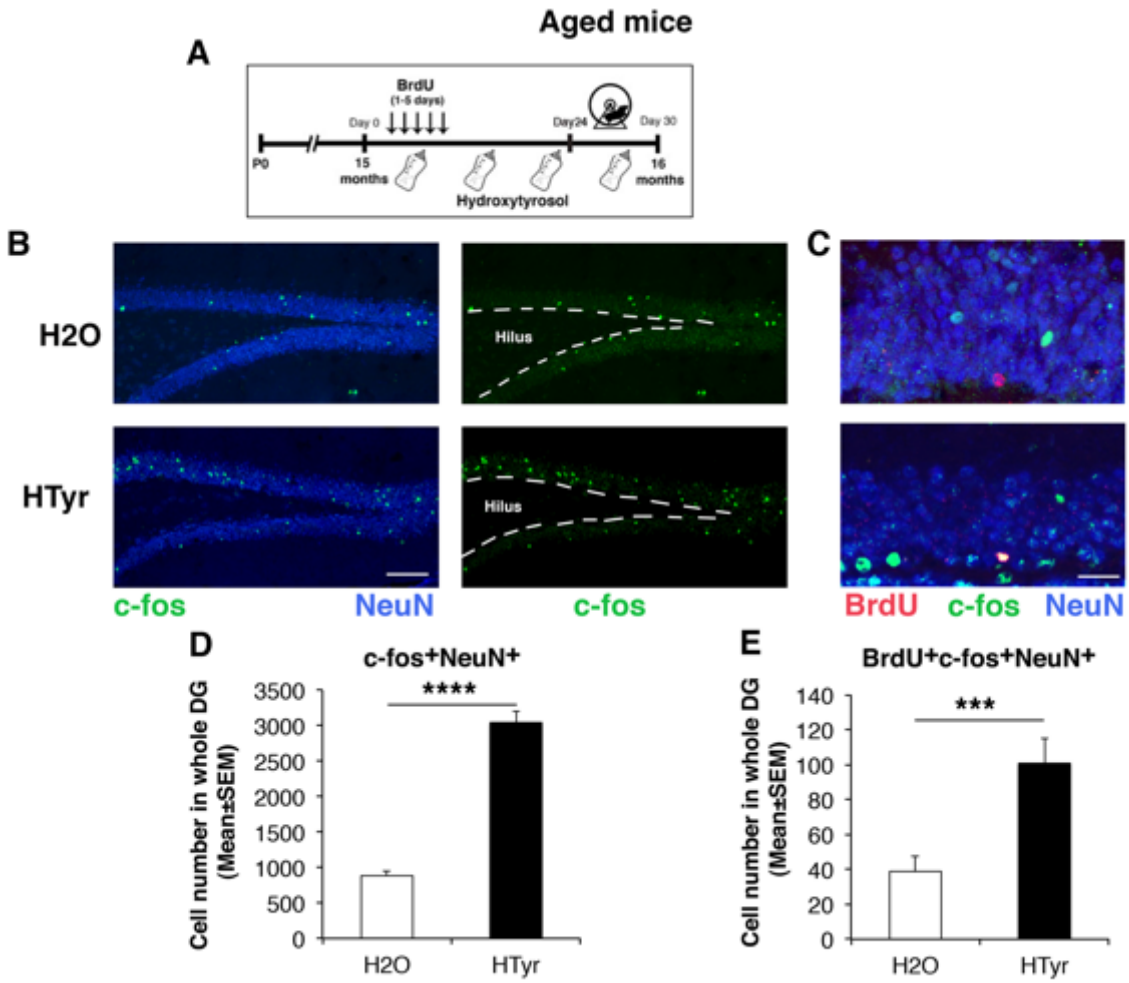


Figure 4.4 HTyr stimulates the activation of new neurons and their incorporation into memory circuits of the aged dentate gyrus.
A) Experimental timeline: 15-month-old mice received HTyr or water for 30 days. During the first 5 days, mice were injected with BrdU, and the last 7 days of treatment were exposed to running wheel until their sacrifice. ***B)*** Representative confocal images of c-fos+ (green) and NeuN+ cells (blue; merged with c-fos) and (***C***) of BrdU+/c-fos+/NeuN+ cells (red/green/blue merged) in the dentate gyrus following treatment with HTyr or water, as described in (***A***). The white dashed line in (***B***) labels the inner boundaries of the dentate gyrus. Scale bar are 100 µm in (***B***) and 25 µm in (***C***). Activation of new neurons, identified as c-fos+/NeuN+ cells ***(D)*** and as c-fos+/BrdU+/NeuN+ cells ***(E),*** is highly improved by treatment with HTyr. The numbers of dentate gyrus cells are means ± SEM; 3 mice per group were analyzed. *** $p < 0.001$, ***** $p < 0.0001$, Student's *t*-test.

neurogenic stimulus able to improve memory and to activate c-fos. Thus, we wished to define whether the increased production of new neurons, after HTyr treatment, affected the extent of their activation by running. To this aim, we measured the number of new neurons that became activated by running, using the following experimental paradigm (Clark et al., 2010). 15-month-old mice, treated with the HTyr or with the vehicle for 30 days, were subjected to daily injection of BrdU during the first 5 days of treatment and were exposed to a running wheel during the last 7 days (Figure 4.4*A*). Mice were sacrificed 1.5 hours after the end of running,

i.e., at the moment of the highest activation of c-fos (Clark et al., 2010). c-fos/NeuN-positive neurons and $BrdU^+/c\text{-}fos^+/NeuN^+$ neurons were measured in the whole dentate gyrus. A striking increase was observed of both cellular populations in the dentate gyrus of mice treated with HTyr (WT-HTyr *vs*. WT-H2O, $c\text{-}fos^+/NeuN^+$, 3.4-fold increase, $P < 0.0001$, Figure 4.4*B*, *D*; WT-HTyr *vs*. WT-H2O, $BrdU^+/c\text{-}fos^+/NeuN^+$, 2.5-fold increase, $P = 0.0004$, Figure 4.4*C*, *E*; Student's *t*-test). This demonstrated the efficient integration of newly-formed neurons in preexisting memory circuitries, suggesting a positive effect of HTyr on the formation of new memories.

As a whole, HTyr dietary supplementation exerts a beneficial influence on the neurogenic process and appears to be a candidate for the prevention and improvement of age-related brain dysfunctions.

4.5 In adult dentate gyrus, HTyr has a proliferation effect only in a neurogenesis-defective mouse model

The results discussed above were related to the condition of neurogenic decline that takes place during aging. To investigate whether HTyr positively affected also physiological neurogenesis in adult mice or whether acted only in defective conditions, we tested the compound in 2-month-old wild-type (Btg1 WT) and Btg1 knockout (Btg1 KO) mice, the latter presenting reduced neurogenesis that resembles a condition of aging. In fact, the ablation of Btg1 reduces, after a burst of hyperproliferation at an early postnatal stage, the pool of dividing adult stem and progenitor cells in the adult neurogenic niches by decreasing their proliferative capacity (Farioli-Vecchioli et al., 2012).

Initially, we examined the effect of HTyr administration on the proliferative state of stem/progenitor cells and neuroblasts. To this aim, the 2-month-old Btg1 WT and Btg1 KO mice were treated with HTyr (100 mg/kg/day) in drinking water for 30 days, while the control mice were treated with vehicle (drinking water; Figure 4.5*B*). We observed that the number of total proliferating cells, marked with Ki67, in Btg1 WT mice treated with HTyr (WT-HTyr) didn't change, compared to control Btg1 WT mice treated with vehicle (WT-H2O) (Ki67+ cells: WT-HTyr vs. WT-H2O, P = 0.80, Fisher's PLSD ANOVA post hoc test; Figure 4.5*A*, *C*).

The same results were obtained from the analysis of the proliferation of different subpopulations of AHN. Indeed, no significant changes were observed in proliferation of Type-1 cells, stem cells in SGZ of DG recognized by the co-expression of Ki67, GFAP and SOX2 (WT-HTyr vs. WT-H2O, $P = 0.67$ Fisher's PLSD ANOVA *post hoc* test; Figure 4.5*E*), or

subpopulation Type1-2a, marked by the expression of Ki67 and SOX2 (WT-HTyr vs. WT-H2O, $P = 0.98$; Figure 4.5*D*), or neuroblasts, type2b-3 cells expressing Ki67 and DCX (WT-HTyr vs. WT-H2O, $P = 0.29$ Fisher's PLSD ANOVA *post hoc* test; Figure 4.5*F*). These results suggest that the treatment with HTyr doesn't influence the proliferation of stem and progenitor cells in the dentate gyrus of the adult hippocampus.

Different results were obtained in 2-month-old Btg1 KO mice. Indeed, as expected, in Btg1 KO untreated mice (KO-H2O) compared to WT untreated mice (WT-H2O), we observed a significant decrease of total Ki67$^+$ cells (KO-H2O vs. WT-H2O, 25% decrease $P = 0.0139$, Fisher's PLSD ANOVA *post hoc* test) as well as of proliferating type-1-2a and type-2b-3 cells (respectively, 22% and 42% decrease, with $P = 0.042$ and $P = 0.0026$, Fisher's PLSD ANOVA *post hoc* test; Figure 4.5*C*-*F*). These observations confirmed our previously published data (Farioli-Vecchioli et al., 2012; Micheli et al., 2018).

Using the same experimental paradigm, previously described, we tested the effect of treatment with HTyr on the proliferation of stem and progenitor cells in the DG of adult Btg1 KO mice. In contrast to what observed in wild-type, 2-month-old Btg1 knockout mice treated with HTyr presented a two-fold expansion of Ki67-positive, proliferating cells compared to the control mice (total Ki67$^+$, KO-HTyr vs. KO-H2O, $P < 0.0001$; Figure 4.5*A*, *C*). Subpopulations analysis showed a marked increase by HTyr treatment of proliferating type-1 stem cells number, i.e., Ki67$^+$/Sox2$^+$/GFAP$^+$, and of type-1-2a progenitor cells, Ki67$^+$/Sox2$^+$ (respectively, 79% increase and 119% increase in KO-HTyr vs. KO-H2O comparison, both with $P < 0.0001$, Fisher's PLSD ANOVA *post hoc* test; Figure 4.5*A*, *D* and *E*). The proliferating type-2b-3 cells also were amplified by the treatment in the knockout genotype (KO-HTyr vs. KO-H2O, 91% increase and $P < 0.0001$, Fisher's PLSD ANOVA *post hoc* test; Figure 4.5*A*, *F*). Thus, HTyr treatment rescued the deficit of proliferating cells in Btg1 knockout mice, also in excess of the number observed in WT untreated dentate gyri (total Ki67$^+$, KO-HTyr vs. WT-H2O, $P < 0.0001$; Figure 4.5*C*).

Our observations revealed that HTyr administration had the property to induce a remarkable increase of proliferation of stem/progenitor cells in the dentate gyrus in conditions of aging-like defective neurogenesis – occurring in the Btg1 knockout model – but not in adult physiological conditions.

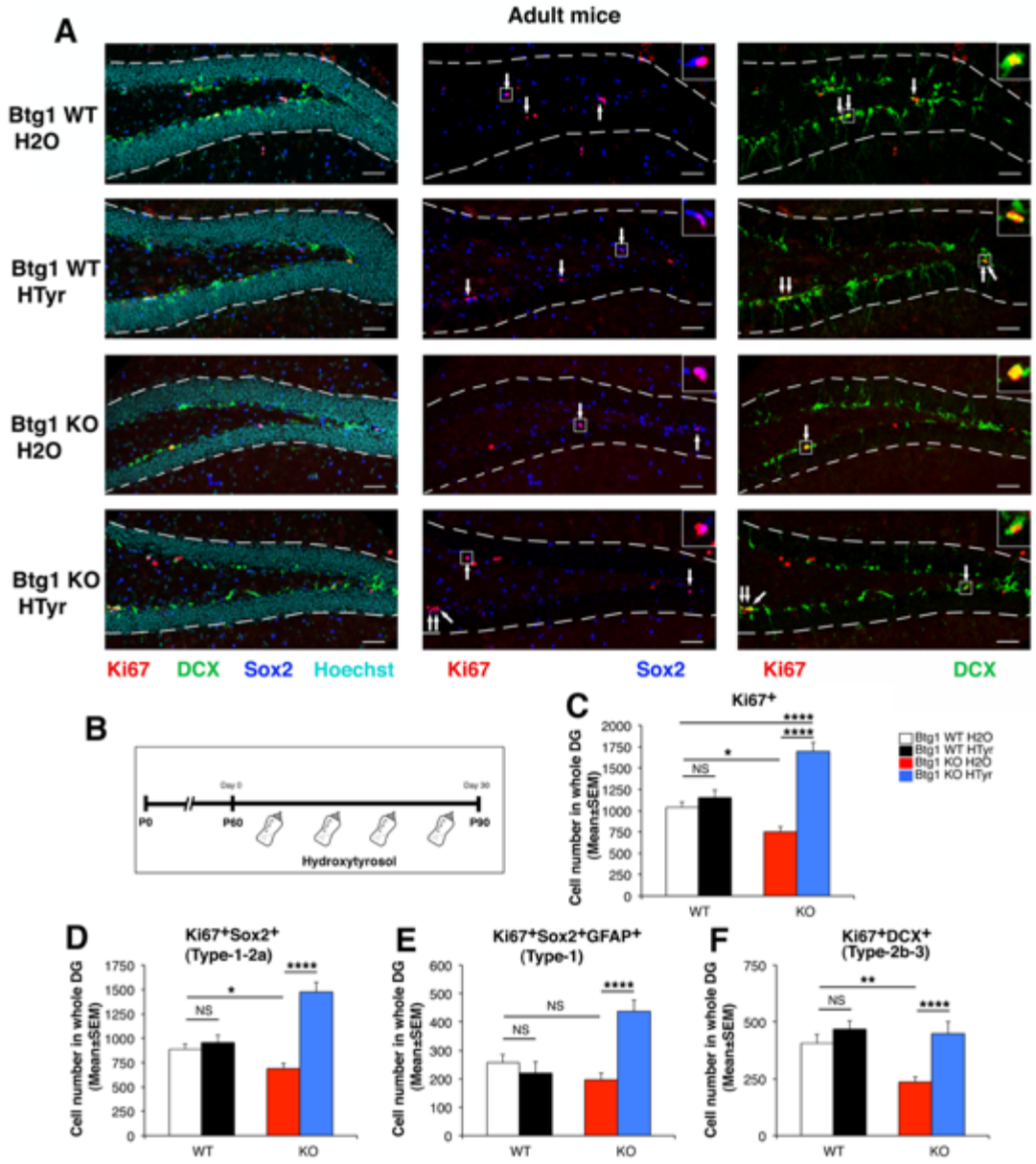


Figure 4.5 HTyr promotes the proliferation of stem/progenitor cells in dentate gyrus of adult Btg1 knockout mice, a model of defective neurogenesis.

A*)** Representative images by confocal microscopy showing that treatment with HTyr, as outlined in **(*B*)**, significantly enhances the total number of proliferating cells (Ki67+) and, in particular, the number of type1/2a stem/progenitor cells (Ki67+/Sox2+, in red/blue) and type 2b/3 progenitors (Ki67+/DCX+, in red/green) in Btg1 knockout (Btg1 KO) but not in Btg1 wild-type (Btg1 WT) mice. Arrows indicate double labelled cells. Nuclei were counterstained by Hoechst 33258 (in cyan). The white dashed line labels the outer boundaries of the dentate gyrus. The white box area is shown with 3x digital magnification. Scale bar 50, µm. ***B*)** Experimental paradigm: 2-month-old Btg1 WT and Btg1 KO mice were treated with HTyr or with water for 30 days, followed by immunohistochemistry analysis. ***C*)** The number of Ki67 positive cells is not affected by treatment in WT dentate gyri, while the significant decrease of Btg1 KO-proliferating cells, relative to Btg1 WT, is reversed above control levels in KO-HTyr (two-way ANOVA HTyr effect: $F_{(1,179)} = 37.003$, $p < 0.0001$; Genotype effect: $F_{(1,179)} = 4.773$, $p < 0.032$; Genotype x Treatment effect: $F_{(1.179)} = 33.052$, $p < 0.0001$. Followed by analysis of simple effects: $*p < 0.05$, $****p < 0.0001$, or NS $p > 0.05$, Fisher's PLSD ANOVA *post hoc* test). ***D*)** The number of stem/ progenitor cells (type-1/2a, Ki67+/Sox2+), (E***) of stem cells (type-1, ki67+/Sox2+/GFAP+) and (***F***) of type-2b/3 progenitors (ki67+/DCX+) is significantly increased in Btg1 KO dentate gyri after treatment with HTyr compared to untreated mice (two-way ANOVA HTyr effect, type-1-2a: $F_{(1.179)} = 31.713$, $p < 0{,}0001$; type-1: $F_{(1.179)} = 20.303$, $p < 0.0001$; type-2b-3: $F_{(1.175)} = 12.087$, $p = 0.0006$. Analysis of simple effect: $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$ or NS > 0.05, Fisher's PLSD ANOVA *post hoc* test). The numbers of dentate gyrus cells are means ± SEM; 4 mice per group were analyzed.

4.6 HTyr has a pro-survival effect in the adult dentate gyrus, both in physiological and defective conditions

The data described above did not show a pro-proliferative effect of HTyr in the AHN of adult Btg1 wild-type mice. Nevertheless, we wondered if the treatment with HTyr can stimulate neurogenesis by influencing the survival rate of newly-formed neurons.

To this aim, we treated the 2-month-old mice with HTyr (100 mg/kg/day) in drinking water, and cells were birth-dated by subjecting mice to daily injection of BrdU, at a concentration of 95 mg/kg, during the first 5 days of treatment. After 30 days, the mice were sacrificed by transcardiac perfusion for DG cells examination; the results were compared to those obtained in control mice treated with vehicle (drinking water; Figure 4.6*B*). The newly-formed neurons were analyzed in the DG by recording the total number of 26- to 30-day-old BrdU positive cells and the number of BrdU-positive cells co-labeled with NeuN.

We observed that the survival rate of newly-formed neurons was significantly increased by treatment both in Btg1 wild-type and knockout genotypes, as demonstrated by the expanded number of total BrdU positive 26- to 30-day-old cells (WT-HTyr vs. WT-H2O, 29.5% increase, $P = 0.0116$; KO-HTyr vs. KO-H2O, 62% increase, $P < 0.0001$, Fisher's PLSD ANOVA *post hoc* test; Figure 4.6*A*, *C*) and of $BrdU^+/NeuN^+$ 26- to 30-day-old neurons (WT-HTyr vs. WT-H2O, 31% increase, $P = 0.0277$; KO-HTyr vs. KO-H2O, 69% increase, $P < 0.0001$, Fisher's PLSD ANOVA *post hoc* test; Figure 4.6*A*, *D*).

As a whole, this demonstrated that HTyr is able to activate the proliferation of stem and progenitor cells only in presence of a genetic background displaying a neurogenic deficit or during aging, whereas HTyr stimulates neuronal survival and maturation not only in aged mice but also in adult mice.

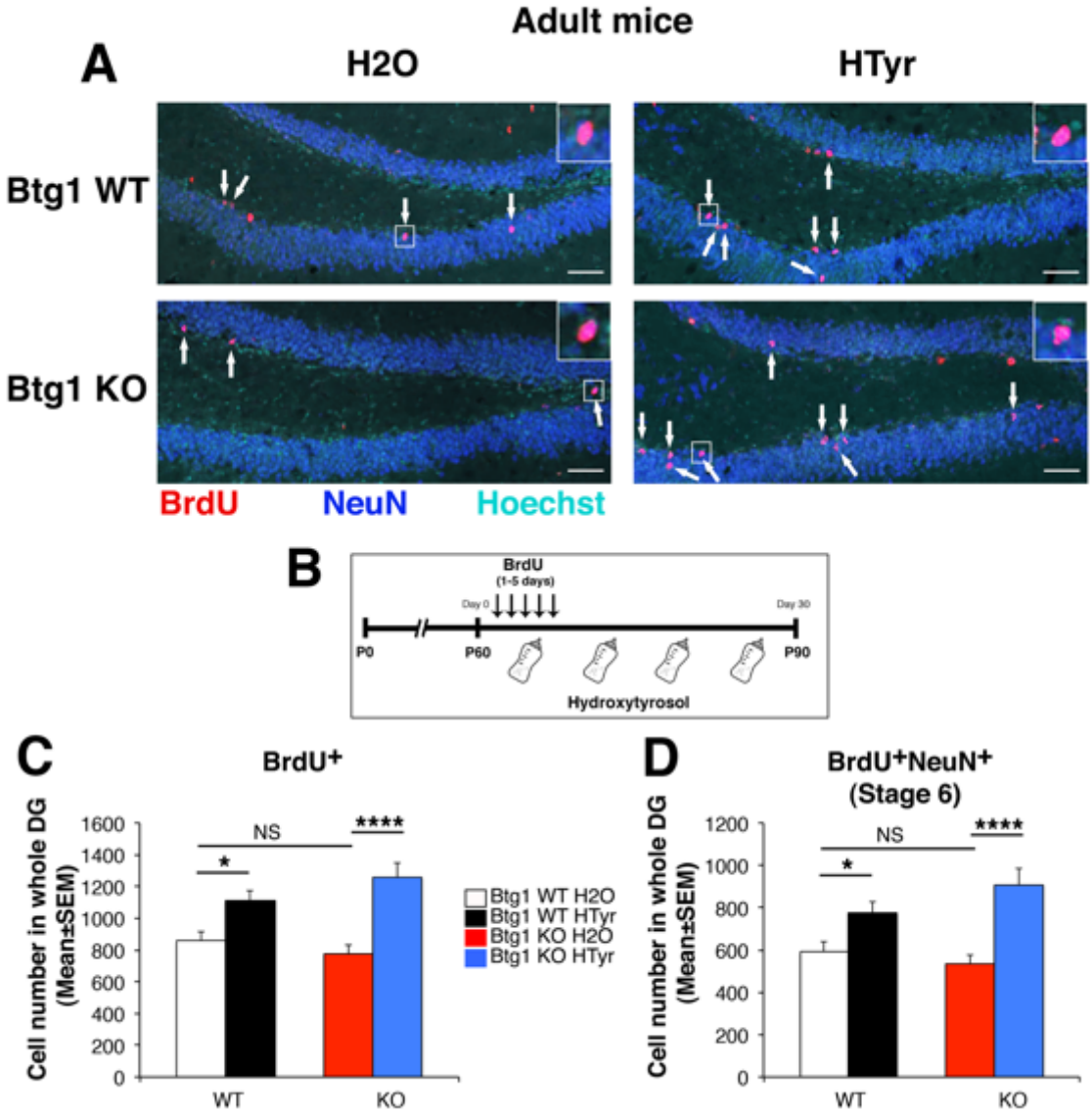


Figure 4.6 HTyr promotes survival of newly-formed neurons in 2-month-old dentate girus.
A) Representative images by confocal microscopy showing that HTyr treatment induces the production of 26- to 30-day-old neurons (identified as BrdU+ and BrdU+/NeuN+, stage 6) both in Btg1 WT and in Btg1 KO mice. Nuclei were counterstained by Hoechst 33258. BrdU, NeuN, and Hoechst are in red, blue and cyan, respectively. Arrows indicate triple labeled granule cells (BrdU+/NeuN+/Hoechst). The white box area is shown with 3x digital magnification. Scale bar, 50 µm. ***B)*** Experimental timeline: 2-month-old wild-type (Btg1 WT) and Btg1 KO mice were treated with HTyr in the drinking water for 30 days; mice received five daily injections of BrdU (95 mg/kg) during the first days of treatment, then they were sacrificed. ***(C)*** Total number of BrdU positive cells and (***D***) number of stage 6 neurons (BrdU+/NeuN+) was significantly augmented by treatment with HTyr at 2 months of age both in Btg1 WT and in Btg1 KO mice compared to untreated mice (two-way ANOVA HTyr effect, BrdU positive cells: $F_{(1.197)} = 27.005$, $p < 0.0001$; Brdu+/NeuN+: $F_{(1.197)} = 29.974$, $p < 0.0001$. Analysis of simple effect: *$p < 0.05$, ****$p < 0.0001$ or NS > 0.05, Fisher's PLSD ANOVA *post hoc* test). The numbers of dentate gyrus cells are means ± SEM; 5 mice per group were analyzed.

5 Discussion

The HTyr is a phenolic compound present in the EVOO and its consumption in the Mediterranean diet is estimated at 5.6 mg/day (Martínez et al., 2018). Notably, the HTyr is also abundant in olive mill waste-water (OMWW), a waste generated during olive oil production which is considered one of the main environmental problems in the Mediterranean area (Fiorentino et al., 2003).

The HTyr is mainly known for its antioxidant activity, and its beneficial effects on health, such as cardiovascular disease and cancer (Bernini et al., 2013; Marković et al., 2019). Moreover, HTyr can cross the blood-brain-barriers thus reaching the neural tissue, where it has neuroprotective effects (Hornedo-Ortega et al., 2018).

This work is the first study about the effect of HTyr on the generation of new neurons in the dentate gyrus of the hippocampus, a neurogenic niche in the adult mammalian brain.

5.1 Pro-neurogenic effect of HTyr on stem/progenitor cells in adult and aged dentate gyrus and in the aging-like Btg1 knockout model

We observed that HTyr significantly stimulates adult neurogenesis in the neurogenic niche of the DG.

Seeking to identify the functional features of HTyr action, we measured the variation in the expression of specific neural markers, using immunofluorescence assay. We found that in wild-type (2-month-old) adult mice, the treatment with HTyr does not influence the proliferation of the stem and progenitor cells in the dentate gyrus. In fact, we did not observe significant differences in the proliferation of stem cells (Type-1, $Sox2^+/GFAP^+$ Cells) and progenitor cells (Type-2b or Type-3; DCX^+ Cells), as indicated by labelling with Ki67, which marks cycling cells (Scholzen and Gerdes, 2000). Remarkably, however, HTyr is able to induce the proliferation of stem/progenitor cells (Type-1, Type-2, and Type-3 cells) in aged mice (15-month old mice) as well as in the 2-month-old Btg1 knockout mice, in which there is a depletion in neural stem cell pool and represent a model of neural aging (see below).

Moreover, we found that, after the treatment with HTyr, new neurons are generated in greater number, relative to control mice, not only in the aged or in Btg1 knockout DG, but also in 2-month-old wild-type dentate gyrus, where no increase of proliferation of progenitor cells was observed. This was revealed by a protocol aimed at highlighting the survival of new neurons, i.e., by measuring the number of one-month old neurons ($BrdU^+/NeuN^+$), birth dated by incorporation of the thymidine analogue BrdU.

Overall, these data indicate that the treatment with HTyr, at the concentration of 100 mg/kg/day for 30 days, potentiates adult neurogenesis prevalently by enhancing the survival of new neurons. Furthermore, considering that this pro-survival effect was observed both in adult and aged mice, we demonstrated that the beneficial effect of HTyr treatment is age-independent. However, HTyr is also endowed with a pro-proliferative component that becomes detectable only in aged mice and in the adult Btg1 knockout mice, which represent a proliferation-defective aging-like mouse model.

Moreover, in agreement with the pro-survival action displayed by HTyr in the DG, we observed a decrease in the number of apoptotic cells, detected with the expression of the cleaved (activated) caspase-3, in 15-month-old mice DG.

The survival of the new-born cells is an important issue in neurogenesis, both during the development and in the adult brain. Indeed, only a few new-born neurons survive after birth and are incorporated in pre-existing neural circuits, as the majority of new-born cells undergo early apoptosis within 1 to 4 days after the bird, during the transition from amplifying progenitors to neuroblasts (Sierra et al., 2010). A second wave of death in the adult DG occurs when neurons are in the process of attaining terminal differentiation (1-3 weeks after birth), with a decay that can be observed after the first week of life, either by BrdU labelling or by retroviral labelling (Kempermann et al., 2004b; Tashiro et al., 2006). In this second wave of apoptosis, the survival is dictated by different factors, such as the availability of trophic factors, neuronal activity, behavioural/learning stimuli, or the regulation by GABA released from interneurons (Yamaguchi and Miura, 2015). The survival of new-born neurons is an important regulation of hippocampal neurogenesis because in this way the organism regulates the final number of new neurons, checking that the organism does not produce an excess of new cells and it also controls the quality of the newly generated cells, selecting outfit cells. Notably, with aging, it is known that the generation of new neurons in the SGZ of DG decreases (Kuhn et al., 1996), but in parallel the number of neurons undergoing apoptosis also decreases (Sierra et al., 2010), thus preserving a functional balance.

However, in this context, the increased number of new neurons generated by treatment with HTyr produces a functional enhancement of the neural network activity. In fact, in concomitance with the decrease of apoptosis, in aged mice, we observed an increased number of new-born neurons recruitment to neural circuits, as indicated by the increment of one-month-old new neurons positive to the expression of c-fos, and birth dated with BrdU. It is known that new neurons undergo a progressive functional activation within neural circuits, correlated to their age and maturation, as new neurons recruited to circuits become activated and detectable

by c-fos labelling, after the behavioural stimulus that triggers their activation, not earlier than 2-4 weeks after birth (Farioli-Vecchioli et al., 2008; Kee et al., 2007).

Overall, these data suggest that the treatment with HTyr promotes the generation and survival of new neurons, which are integrated into the existing circuits.

Notably is the pro-proliferative effect of HTyr in the Btg1 knockout mouse model, characterized by genetic ablation of gene Btg1. Btg1 is a protein involved in the control of cell proliferation, inducing an arrest of the cell cycle in the G0/G1 phase, mediated by specific inhibition of Btg1 on cyclin D1 expression (Ceccarelli et al., 2015). As shown in Figure 1.14, this gene is expressed in various regions of CNS, including the DG of the hippocampus, and its genetic ablation causes an increase of proliferation of NSCs and NPCs in P7 (P, post-natal day) mice. However, this intensive proliferation of NSCs causes a depletion of NSCs pool in adult age. Therefore, the 2-month-old Btg1 knockout mouse model represents an age-dependent accelerated loss of the proliferative capability of neural cells (Farioli-Vecchioli et al., 2012). In fact, the total cycling cells ($Ki67^+$) are reduced at this age in Btg1 knockout DG with respect to Btg1 wild-type. However, we observed that treatment with HTyr rescues and increases the number of all cycling cells ($Ki67^+$ cells), including the number of proliferating stem cells ($Ki67^+Sox2^+GFAP^+$ cells). These results indicate that in Btg1 knockout mice, the treatment with HTyr is able to reactivate the NSCs pool.

A similar reactivation of NSCs in Btg1 knockout mice was observed following the other two known neurogenic stimuli: voluntary exercise (Farioli-Vecchioli et al., 2014), and chronic treatment with antidepressant Fluoxetine (Micheli et al., 2018). Nevertheless, in the wild-type dentate gyrus, either adult or aged, both these stimuli are unable to induce the proliferation of NSCs; in fact, these neurogenic stimuli strongly activated the proliferation of adult progenitor cells (Type-2 and Type-3 cell) without influencing the proliferation of adult NSCs (Type-1; Encinas et al., 2006; Farioli-Vecchioli et al., 2014; Micheli et al., 2018; Suh et al., 2007). Moreover, voluntary exercise but not Fluoxetine activates progenitor cells in the aged DG (Couillard-Despres et al., 2009; Micheli et al., 2019; Siette et al., 2013). Treatment with HTyr shows some differences respect to voluntary exercise and fluoxetine. In fact, we observed that treatment with HTyr is unable to induce the proliferation of NSCs or NPCs in adult wild-type mice, however in aged mice is able to reactivate the proliferation of these cells.

Therefore, we suggest that treatment with HTyr, although unable to directly stimulate the proliferation of adult wild-type progenitor cells, is nevertheless able to activate the proliferation of stem and progenitor cells in a permissive environment, such as the Btg1-null DG, lacking the inhibitory regulation of the cell cycle exerted by Btg1, or such as the aging (see in Table 5.1 a

summary of the pro-neurogenic effect of HTyr, running and fluoxetine). Given that stem cells are prevalently quiescent (Kempermann et al., 2004b), it is plausible that, in the aged and Btg1-null dentate gyri, HTyr can trigger their exit from this state.

Table 5.1 Proliferative activation of stem and progenitor cells of the dentate gyrus by HTyr compared to other neurogenic stimuli.

System	Neurogenic Stimulus	Stem Cells	Progenitor Cells	References
Adult	Hydroxytyrosol*	NO*	NO	This report
	Running	NO	YES	(Brandt et al., 2010; Farioli-Vecchioli et al., 2014; Kronenberg et al., 2003; Steiner et al., 2008; Suh et al., 2007)
	Fluoxetine	NO	YES	(Encinas et al., 2006; Micheli et al., 2017, 2018)
Aged	Hydroxytyrosol**	YES**	YES	This report
	Running	NO	YES	(Micheli et al., 2019; Siette et al., 2013)
	Fluoxetine	NO	NO	(Mcavoy et al., 2015; Micheli et al., 2018)(Couillard-Despres et al., 2009; Li et al., 2015)
Btg1 KO	Hydroxytyrosol	YES	YES	This report
	Running	YES	YES	(Farioli-Vecchioli et al., 2014)
	Fluoxetine	YES	YES	(Micheli et al., 2018)

*$Ki67^{+}Sox2^{+}GFAP^{+}$

**$Ki67^{+}Sox2^{+}$

5.2 Possible mechanism of increase of survival and anti-aging action by HTyr

We observed that treatment with HTyr potentiates adult neurogenesis prevalently by enhancing the survival of new neurons. This effect was observed also after running and fluoxetine stimuli, which act by increasing the BDNF levels, which in turn leads to an increase of the cytoprotective anti-apoptotic protein Bcl-2 (Manji et al., 2001; Réus et al., 2012; Zhang et al., 2019). Moreover, it has been observed that running induces phosphorylation of Akt, a key gene for cell survival signaling (Chen and Russo-Neustadt, 2009), which is part of the BDNF pathway (Segal, 2003). Also HTyr treatment, for 6 weeks in rats exposed to subarachnoid hemorrhage, enhances the levels of Akt protein in the brain cortex (Fu and Hu, 2016), and prevents the decrease of Akt, Bcl-2 and BDNF elicited by neurotoxic and amyloid protein (Arunsundar et al., 2014), or the decrease of BDNF in the hippocampus by maternal stress (Zheng et al., 2015a).

Therefore, all these pro-survival molecular effects are in common with the other neurogenic stimuli, running, and fluoxetine.

Interestingly, HTyr shows also an anti-inflammatory action in chondrocytes, in consequence of reduced oxidative stress due to the anti-oxidizing potential of the molecule and to autophagy induced by sirtuin 1-dependent mechanism (Cetrullo et al., 2016). A similar mechanism may be at the origin of the strong reduction exerted by HTyr on lipofuscin and Iba1 levels, which we observed.

Lipofuscin is a fluorescent complex mixture composed of highly oxidized cross-linked macromolecules (proteins, lipids, and sugars) with multiple metabolic origins. The nature and structure of lipofuscin appear to vary among the tissue and show temporal heterogeneity in the composition of oxidized proteins (30-70%), lipids (20-50%), metal cations (2%) and sugar residues. Because of its polymeric and highly cross-linked nature, lipofuscin cannot be degraded, nor cleared by exocytosis, thus being accumulated within the lysosome and cell cytoplasm of long-lived post-mitotic and senescent animal cells. For these reasons, the lipofuscin deposits are especially abundant in nerve cells, cardiac muscle cells, and skin. Thus, lipofuscin is considered a consequence of normal aging that gets increasingly deposited as cellular garbage, starting early in life (Moreno-García et al., 2018).

Iba1 is a microglia-specific marker protein, which is up-regulated in activated microglia. Microglia are the resident brain immune cells and have many important roles in the healthy and diseased CNS, and it was observed that in aged mice, there is a significant increase in the density of activated-microglial cells, measured by expression of Iba1 (Gebara et al., 2013). The same results were observed in DG following ischemia (Ito et al., 2001), brain disease, and aging (Cornejo and von Bernhardi, 2016). Besides, in aged mice, it was observed an increase in the expression of inflammatory cytokines and a decrease in the expression of anti-inflammatory cytokines, suggesting a shift of these cells to a more inflammatory phenotype of these cells during aging (Frank et al., 2006; Henry et al., 2009; Sierra et al., 2007). ischemia (Ito et al., 2001), brain disease, and aging (Cornejo and von Bernhardi, 2016). Therefore, activated microglial cells in aged mice create an adverse milieu for neurons, that may favour neurodegenerative diseases (Cornejo and von Bernhardi, 2016).

Therefore, the ability of HTyr to reduce lipofuscin and Iba1 levels, and thus, the number of activated microglia cells, clearly points to an anti-aging action of HTyr. Likewise, running reduces the number of microglia cells, which in turn exert an inhibitory action on the proliferation of stem/progenitor cells (Gebara et al., 2013).

On the other hand, HTyr displays anti-proliferative activity in tumor cells (Imran et al., 2018), as observed in glioblastoma cells, where HTyr represses COS2 and Prostaglandin E2 which has pro-proliferative effects on neural cells and whose synthesis is induced by COX2 (Lamy et al., 2016). However, in normal cells, HTyr exhibits pro-proliferative activity, as, for example, observed in human osteoblasts (García-Martínez et al., 2016), or in endothelial cells, where AKT, mTOR, and Tgfβ were increased (Cheng et al., 2017). This further suggests that the proliferative stimulation by HTyr that we observed in DG cells, either aged or lacking the inhibitory regulation of cell cycle by Btg1, but not in adult cells, is dependent on the cellular conditions.

As mentioned above, running is a powerful inducer of the proliferation of progenitor cells, but not of stem cells (Brandt et al., 2010; Kronenberg et al., 2003). More generally, our data suggest that stem or progenitor cells can be more readily activated by a neurogenic stimulus such as HTyr in the condition where the checkpoint enforcing quiescence is weakened, such as in the aged brain and the Btg1 knockout mice. This effect might be at the origin of the ability displayed by HTyr, in our data, to effectively counteract the decline of neurogenesis during aging. Indeed, activation of stem cells by neurogenic stimulus (i.e., running) also occurs in another model of aged mice, lacking the cell cycle inhibitor $p16^{Ink4a}$; this indicated that DG is endowed with a reserve of proliferative potential during aging and also that their activation by neurogenic stimuli is controlled by a network of inhibitory genes (Micheli et al., 2019).

To conclude, it is worth noting that the human equivalent dose of 100 mg/kg/day HTyr (8.1 mg/Kg/day; Nair and Jacob, 2016) is above the consumption of HTyr within the Mediterranean diet (Martínez et al., 2018), but is well compatible with the use of dietary supplements. Our data represent a proof of principle about the neurogenic effect of HTyr and it will be interesting to test lower doses of the compound and HTyr derivatives.

Considering the correlation between hippocampal neurogenesis with learning and memory processes and the cognitive decline age-related, future studies will be useful to verify the behavioural effects of increased production of hippocampal neurons in adult and aged mice and long-term action of HTyr treatment on neural aging.

5.3 Conclusions and Future Perspectives

HTyr is a phenolic compound assimilated mainly in Mediterranean Diet by olive oil consumption. However, this compound is abundant in OMWW, a waste generated during olive oil production. The HTyr, together with other phenolic compounds, is responsible for the toxicity of the OMWW on the environment (Fiorentino et al., 2003). In fact, the high phenolic nature of OMWW makes it highly resistant to biodegradation and highly phytotoxic (Zirehpour et al.,

2014). However, actually, in Italy the law n° 574/1996 permits the spread of OMWW on agricultural and industry terrain, with the risk of the extensive pollution of the soil and ground and surface water. Therefore, an improvement in the management of this waste is necessary, through a reduction of its production, or its detoxification or recovery and recycling of its high-value components. Valorisation and recycling constitute new conditions that are increasingly necessary worldwide. Typically, by-products, wastes, and effluents from fruit and vegetable processing consist in high amounts of protein, sugars, and lipids along with peculiar aromatic and aliphatic compounds; thus, they could be considered as cheap and abundant raw materials for the synthesis of value-added chemicals and biomaterials (Federici et al., 2009). Considering the various beneficial effects of HTyr on body health, the OMWW could be considered as a source of HTyr to be used as a possible diet supplement, thus valorising this waste and encouraging its recycling.

Our data suggest a potential use of HTyr as a diet supplement to counteract the age- and pathological-related decline in hippocampal neurogenesis, which contributes to cognitive decline, loss of working and episodic memory, impaired learning capacity and motor coordination. Actually, protecting brain functions and preserving cognitive abilities are among the biggest challenges in life science. Therefore, our data valorise the use of HTyr and, considering the great amount of this phenolic compound in OMWW, this data encourage the recovery of HTyr from OMWW, reducing its phenolic content and its environmental risk, representing thus a more sustainable use of olive in the oil production.

As future perspectives, we plan to perform focused behaviour tasks to test the effects of HTyr treatment on learning abilities and on age-related cognitive decline. Furthermore, we intend to analyse the possible effects of HTyr treatment on adult and aged neurogenesis in the Sub-Ventricular Zone (SVZ), another neurogenic niche present in the mammalian brain. In particular, stem and progenitor cells isolated from SVZ can be cultured as neurospheres and easily manipulated, thus representing a useful tool to understand the possible mechanism of action of HTyr on NSCs and NPCs.

In addition, in light of the results obtained, and considering the importance of recovery, recycling and valorisation of phenolic compounds in OMWW, among the future perspectives there is also to test the effects of HTyr recovered from waste-water. Finally, we would like to identify and test the neurogenic potential of other phenolic compounds present in OMWW, thus incentivizing the use of OMWW to recover these high-value compounds and one more sustainable exploitation of olives during the EVOO production.

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