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Hydroxytyrosol stimulates neurogenesis in aged dentate gyrus by enhancing stem and progenitor cell proliferation and neuron survival

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Abstract

The dentate gyrus of the hippocampus is one of two brain areas generating throughout life new neurons, which contribute to the formation of episodic/associative memories. During aging, the production of new neurons decreases and a cognitive decline occurs. Dietary factors influence neuronal function and synaptic plasticity; among them the phenolic compound hydroxytyrosol (HTyr), present in olive oil, displays neuroprotective effects. As age impacts primarily on the hippocampus-dependent cognitive processes, we wondered whether HTyr could stimulate hippocampal neurogenesis in vivo in adult and aged wild-type mice as well as in the B-cell translocation 1 gene (Btg1) knockout 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 survival of new neurons and decreasing apoptosis. Notably, however, in the aged and Btg1-null dentate gyrus, HTyr treatment also stimulates 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 increase of $BrdU^+/c\text{-}fos^+$ neurons. Finally, HTyr treatment also reduces the markers of aging lipofuscin and Iba1. Overall, our findings indicate that HTyr treatment counteracts neurogenesis decline during aging.

KEYWORDS

aging, diet, hippocampus, neural stem cells, neurogenic stimuli

1 | INTRODUCTION

Due to the longer average lifespan of the human population, cognitive impairment associated with age is an increasingly important aspect of health. Maintaining health throughout life is today one of the greatest challenges and the consumption of healthy food is one of the key allies in protecting brain function and preserving cognitive abilities in aging. In fact, similarly to environmental stimulation, exposure to specific dietary factors can influence neuronal function and synaptic

Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BrdU, 5-Bromo-2′-deoxyuridine; Btg1, B-cell translocation 1 gene; DCX, doublecortin; GFAP, glial fibrillary acidic protein; HTyr, hydroxytyrosol; Iba 1, ionized calcium-binding adapter molecule 1; KO, knockout; NeuN, neuronal nuclei; PLSD, protected least significant difference; SEM, standard error of the mean; Sox2, sex-determining region Y-box 2; SVZ, subventricular zone; TRITC, tetramethylrhodamine; WT, wild-type.

plasticity, thus influencing cognitive abilities in particular those which are hippocampus dependent.[1] The hippocampus is a brain structure very sensitive to the environment, which plays a fundamental role in learning processes, as well as in the encoding and retrieval of memories. The main role of the hippocampus is to link separate contextual, spatial, or temporal information, so that the different memories can be easily retrieved and associated.[2,3] Furthermore, the dentate gyrus of the hippocampus is one of the two neurogenic niches—the other being the subventricular zone (SVZ)—where, during all postnatal life, new functional neurons are produced[4,5]; these are generated from radial glia-like stem cells (named type-1 cells), which express glial fibrillary acidic protein (GFAP), nestin and Sox2.[6-9] Dentate gyrus 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.[7,8,10,11] The progenitor cells mature then into early postmitotic cells (stage 5), which express the Ca^{2+}-binding protein Calretinin, and into terminally differentiated neurons (stage 6), positive for Calbindin and for the late differentiation marker NeuN.[12,13]

The new dentate gyrus 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.[3,14,15] Therefore, adult hippocampal neurogenesis contributes to cognition.[16] Remarkably, during aging the generation of progenitor cells and neurons in the dentate gyrus and SVZ undergoes a progressive decrease.[5,17] 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,[18] but also during normal aging.[19]

Environment as well as various factors, such as the diet, can improve neurogenesis and cognition.[1] It is therefore essential to evaluate the effect of dietary elements on the number of stem cells generated and on their differentiation into neurons.

Extra virgin olive oil (EVOO) is a basic component of the Mediterranean diet[20] and its daily intake is related to many beneficial properties for human health. Responsible for these properties are some of the bioactive molecules present in EVOO, such as monounsaturated fatty acids, polyunsaturated fatty acids and phenolic compounds. Among the latter, low-weight molecular compounds such as hydroxytyrosol [2-(3,4-dihydroxyphenyl)ethanol, HTyr], play a particular role. In consideration of its hydrophilic character, HTyr is found also in olive oil by-products (olive oil wastewaters), from which it can be conveniently recovered by innovative technologies to obtain HTyr-enriched extracts exhibiting health beneficial effects.[21,22] The multiple biological properties of HTyr are well known, especially the antioxidant, anticancer, anti-inflammatory, and neuroprotective effects.[23-26] In fact, HTyr reduces oxidative stress in brain slices undergoing such stress, that is, lipid peroxidation, peroxynitrite formation, and production of inflammatory mediators (prostaglandin E2 and interleukin 1beta)[27]; 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.[28-30] Moreover, HTyr is able to cross the blood-brain barrier[31,32] and its treatment in a mouse model of Alzheimer's disease (TgCRND8 mice) caused a significant reduction of the amyloid-β plaque number in hippocampal areas.[33]

However, despite the extensive literature about the modulation of adult neurogenesis by several phenolic compounds (34, 35, for review), little is known about the potential role of HTyr in this process in vivo. In fact, a report by Zheng et al[36] showed that HTyr treatment rescues the mRNA levels of brain-derived neurotrophic factor (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. Moreover, a recent report about treatment with HTyr in vivo after stroke suggested a trend of increase of dentate gyrus total progenitor cells (DCX^+ cells), without, however, attaining a significant difference between treated and untreated conditions after stroke.[37]

Therefore, using HTyr synthesized in our laboratories,[38] we studied here the potential beneficial effect of the oral administration of this natural compound on hippocampal neurogenesis. By means of appropriate experimental paradigms, we analyzed the different stages of production of new neurons in aged and adult mice and in a mouse model which shows impaired neurogenesis, that is, the knockout of the antiproliferative B-cell translocation 1 gene (Btg1). 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 2 months of age.[39] Neurogenic stimuli such as running or an antidepressant (fluoxetine) rescue this defective phenotype.[40,41]

In fact, the present 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.[40-42]

2 | MATERIALS AND METHODS

2.1 | Mouse line, genotyping, husbandry, and hydroxytyrosol (HTyr) treatment

Btg1 knockout and Btg1 wild-type (WT) strains (C57BL/6 background) were generated as previously described.[39]

Briefly, Btg1$^{+/-}$ mice were crossed several times, until an isogenic progeny was obtained, also referred to throughout the paper as Btg1 KO and Btg1 WT for 2-month-old mice, or referred to as WT for 15-month-old WT mice. Genotyping was routinely performed by PCR analysis, using genomic DNA from tail tips, as described.[39]

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 in drinking water).

For 30 days, 100 mg/Kg/day of HTyr (human equivalent dose 8.1 mg/Kg/day)[43] was administered to mice. The daily dosage was selected within a range of antioxidant effects free of cytotoxicity.[44] Moreover, the duration of the treatment ensures an adequate accumulation of HTyr and its metabolites in the brain.[45] The intake of HTyr was calculated considering the weight of mice and the average water consumption per mouse (7 mL/day).[46] The weight was measured before and during the administration of HTyr. Mice were euthanized at the end of the treatment.

Where indicated, mice treated with HTyr or with H2O were randomly placed in cages with a running wheel during the last 7 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.

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). Btg1 knockout mice are available upon request to JP Rouault.

2.2 | Synthesis of HTyr

HTyr was synthesized on a scale of the order of grams using a patented procedure optimized in our laboratories, based on three steps: (a) selective derivatization of the alcoholic group of tyrosol (2-hydroxyphenethyl alcohol) with dimethyl carbonate in acidic medium to produce carboxymethylated tyrosol, (b) oxidation of carboxymethylated tyrosol with 2-iodobenzoic acid and in situ reduction with sodium dithionite to obtain carboxymethylated hydroxytyrosol, and (c) hydrolysis of carboxymethylated hydroxytyrosol under basic conditions to get hydroxytyrosol. The experimental details of each step were already described by us.[38] At the end of each step, the product obtained was purified by chromatography 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.[38]

2.3 | Experimental design, BrdU treatment of mice, and sample preparation for immunohistochemistry

Fifteen-month-old WT mice and 2-month-old Btg1 AT and Btg1 knockout mice were administered with HTyr in their drinking water for 30 days. Control mice received standard drinking water. For the examination of stem and progenitor cell proliferation, mice were euthanized at the end of HTyr or H2O treatment (see scheme of treatment in Figures 2 and 5). To study survival and maturation of newly formed neurons, mice were subjected to one daily injection of 5-bromo-2′-deoxyuridine (BrdU, 95 mg/kg i.p.; Sigma Aldrich, St Louis, MO, USA) during the first 5 days of treatment with HTyr or water and sacrificed at the end of the treatment (see schematic representation in Figures 1 and 6). Alternatively, to analyze the integration of the new neurons in 15-month-old WT mice, these were randomly assigned during the last 7 days of treatment to cages with running wheel and were sacrificed at the end of the treatment (see schematic representation in Figure 4).

Brains were collected after transcardiac perfusion with 4% paraformaldehyde (PFA) in PBS 1× and kept overnight in 4% PFA. Brains were then equilibrated in 30% sucrose and cryopreserved at −80°C.

2.4 | Immunohistochemistry (BrdU labeling, immunofluorescence) 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 Finetek, Torrence, CA, USA). Sections were then processed for multiple labeling immunohistochemistry using fluorescent methods. Sections were previously permeabilized with 0.3% TritonX-100 in PBS, and then incubated with primary antibodies with 3% normal donkey serum in 0.3% TritonX-100 in PBS for 16-18 hours at 4°C.

BrdU incorporation was detected by denaturing DNA through pretreatment of sections with 2N HCl 45 minutes at 37°C, followed by 0.1 M sodium borate buffer pH 8.5 for 10 minutes. Sections were then incubated overnight at 4°C with a rat monoclonal antibody against BrdU (Abcam, Cambridge, UK; AB6326; 1:400). Cell proliferation was detected also through the rabbit monoclonal anti-Ki67 (Biocare Medical, Pacheco, CA, USA; clone SP6, CRM325; 1:100). The antibodies against Ki67 or BrdU were incubated together

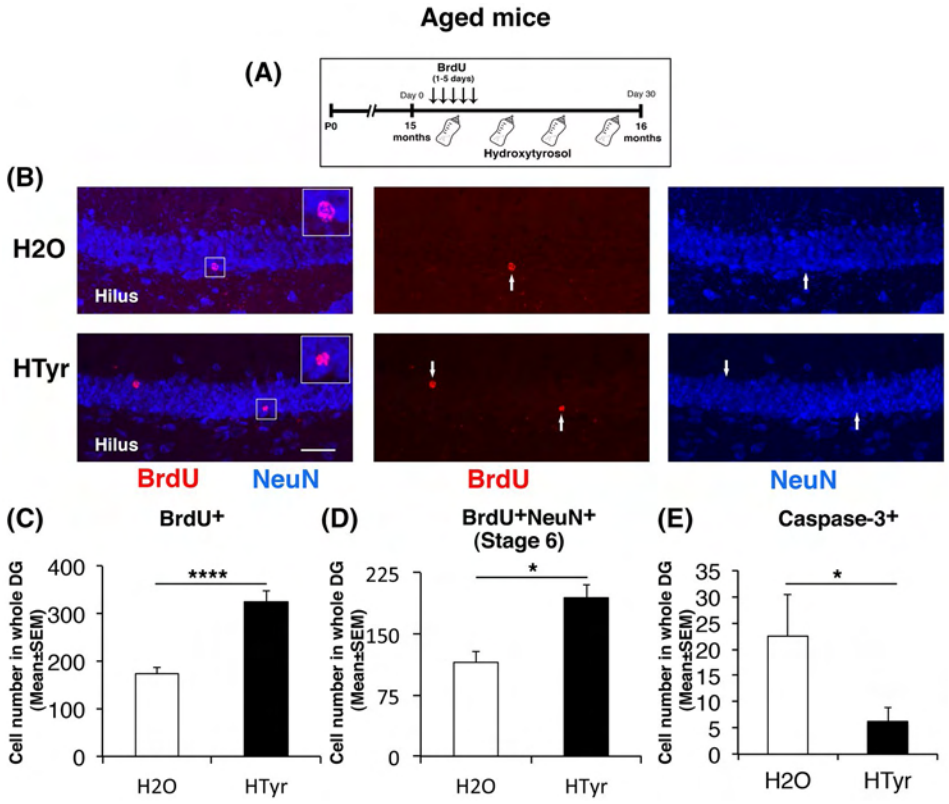

FIGURE 1 HTyr administration enhances the production of new neurons in aged mice by promoting their survival. A, Experimental timeline: 15-month-old WT mice were treated with HTyr in the drinking water for 30 days; mice received 5 daily injections of BrdU (95 mg/kg) during the first days of treatment. 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 2.5× 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 WT mice (*$P < .05$, ****$P < .0001$; 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 (*$P < .05$, Mann-Whitney *U*-test). C-E, The numbers of dentate gyrus cells are means ± SEM; 5 mice per group were used

with other primary antibodies, so as to label the specific subpopulations of dentate gyrus stem, progenitor cells, and neurons: mouse monoclonal antibodies against GFAP (Sigma-Aldrich; G6171; 1:200) or NeuN (Millipore Burlington, MA, USA; MAB377; 1:400); goat polyclonal antibodies against Sox2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; SC-17320; 1:300) or doublecortin (DCX; Santa Cruz Biotechnology; SC-8066; 1:300) or ionized calcium-binding adapter molecule 1 (Iba1) (Abcam; AB5076; 1:600), or rabbit polyclonal antibodies against cleaved (activated) Caspase-3 (Cell Signaling Technology, Danvers, MA,USA; 9661; 1:100) or c-fos (Millipore; Ab-5 PC38; 1:500).

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; 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 1×.

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.[47]

Confocal *Z*-stacks and single-plane images of the immunostained 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.

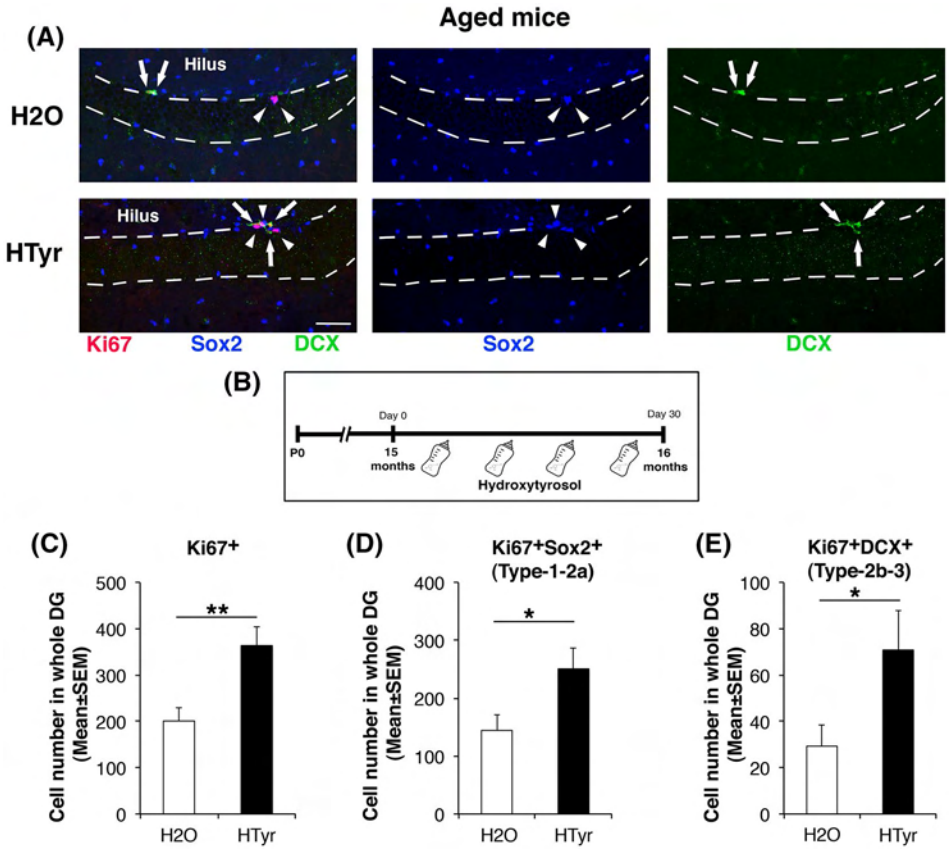

FIGURE 2 HTyr treatment promotes the proliferation of aged dentate gyrus stem/progenitor cells. A, Representative images by confocal microscopy showing that treatment with HTyr, whose timeline is shown in (B), significantly enhances the total number of proliferating progenitor cells (Ki67-positive) and, 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. $^*P < .05$, $^{**}P < .01$, Student's *t*-test

2.5 | 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 rostrocaudal 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.[14,39,48-50] 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, in blinded fashion, using the IAS software to register positive cells (Delta Sistemi, Rome, Italy).

2.6 | Statistical analysis

The effect of HTyr treatment on the cell number of each cell population was statistically analyzed in WT 15-month-old mice treated with HTyr or with water using Student's *t*-test. In experiments where the number of cells was low ($Caspase\text{-}3^+$ cells; Figure 1E) 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 analysis of variance (ANOVA), in order to test the main effects of the two factors, that is, 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 $P < .05$. All data were expressed as mean values ± SEM. The number of mice analyzed per group was either n = 5 (Figures 1 and 6), n = 4 (Figures 2, 3 and 5), or n = 3 (Figure 4).

3 | RESULTS

3.1 | HTyr stimulates hippocampal neurogenesis in aged mice by enhancing the proliferation of stem and progenitor cells and promoting the survival of newly formed neurons

During aging, a profound decline of neurogenesis occurs.[5] Therefore, we sought to examine whether HTyr administration was able to revert the deficit in the production of new neurons in the dentate gyrus of 15-month-old WT mice.

We used the experimental paradigm for HTyr treatment described in Materials and Methods and outlined in Figure 1A, where cells were birth dated by subjecting mice to daily injection of BrdU during the first 5 days of treatment with HTyr (or with water); dentate gyri were then examined after 30 days of HTyr treatment. This protocol allowed monitoring the effect of HTyr on survival and differentiation of 26- to 30-day-old new neurons, by recording the total number of BrdU-positive cells, or also co-labeled with NeuN, which is a marker of stage 6 mature granule cells.[12]

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, $P < .0001$, Student's *t*-test; Figure 1B,C). Moreover, the number of $BrdU^+/NeuN^+$ neurons was expanded by the treatment (WT-HTyr vs WT-H20, 68% increase, $P = .0216$, Student's *t*-test; Figure 1B,D) showing that HTyr was able to revert the physiological decline of neurogenesis observed in the hippocampus of aged mice.

During neurogenesis, at all ages, a fraction of newborn neurons do not survive and undergo apoptosis. 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, $P < .0443$, Mann-Whitney *U*-test; Figures 1E and S1), suggesting that apoptotic cell death was counteracted by HTyr administration.

To investigate 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, we measured the number of total cells labeled by Ki67, which marks cycling cells,[51] in treated and control mice (according to the protocol in Figure 2B). 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, $P = .002$, Student's *t*-test, Figure 2A,C). We also measured the proliferating stem/progenitor cells (type-1-2a; $Ki67^+/Sox2^+$) and proliferating neuroblasts (type-2b-3; $Ki67^+/DCX^+$) to evaluate which cellular population was influenced by HTyr treatment. The cell number of both populations was significantly increased in WT-HTyr mice (WT-HTyr vs WT-H2O, for type-1-2a: 73% increase, $P = .019$; for type-2b-3:141% increase, $P = .04$, Student's *t*-test, Figure 2A,D, and E).

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

Then, we sought to further evaluate whether HTyr had other neural beneficial effects during aging, by analyzing the dentate gyrus for the presence of nonspecific deposits of lipofuscin. Lipofuscin pigments are residues of lipid peroxidation and are considered signs of aging correlated to chronic oxidative stress.[52] After dietary supplementation with HTyr (Figure 3A), 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 the control group (WT-HTyr vs WT-H2O, $P = .042$, Student's *t*-test, Figure 3B,C), demonstrating that HTyr supplementation increased neuronal health, by protecting neurons against degeneration.

Finally, we evaluated the microglia population, which plays 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.[53] Moreover, it is known that microglia reduce proliferation of neural stem/progenitor cells in aged mice and that a neurogenic stimulus, such as voluntary running, counteracts this process by decreasing the number of microglial cells.[54] 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, $P < .0002$, Student's *t*-test, Figure 3D,E). This reduction of microglial cells could be in part responsible for the pro-proliferative effect of HTyr.

Overall, our results revealed that in aged WT 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.

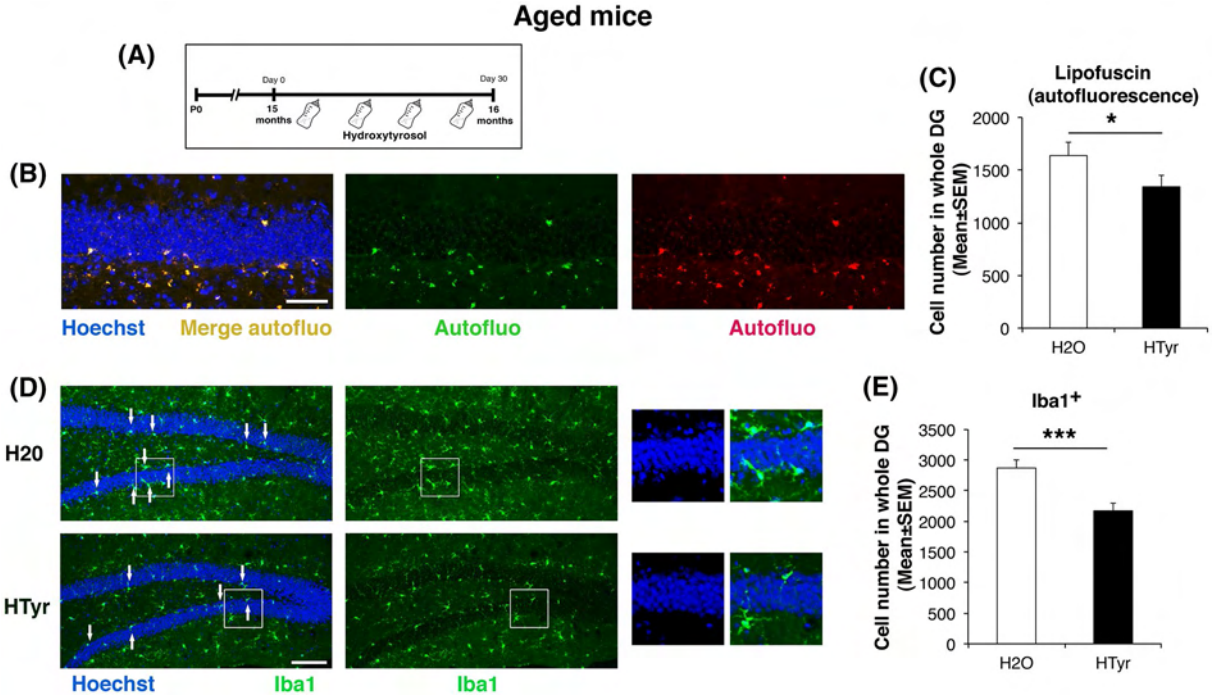

FIGURE 3 HTyr treatment reduces lipofuscin deposits and activated microglia. A, Experimental timeline: 15-month-old WT mice were treated with HTyr for 30 days, followed by immunohistochemistry analysis. B, Fluorescence confocal images representing lipofuscin autofluorescent cells in the dentate gyrus. 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 2.5× 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; *$P < .05$, ***$P < .001$, Student's *t*-test

3.2 | 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 is thus correlated with the recruitment of new neurons into spatial memory circuits.[50] Voluntary exercise also is a neurogenic stimulus able to improve memory and to activate c-fos.[55] 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.[55] Fifteen-month-old mice, treated with HTyr or with 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 4A). Mice were sacrificed 1.5 hours after the end of running, that is, at the moment of highest activation of c-fos.[55] c-fos/NeuN-positive neurons and BrdU$^+$/c-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-fos$^+$/NeuN$^+$, 3.4-fold increase, $P < .0001$, Figure 4B,D; WT-HTyr vs WT-H2O, BrdU$^+$/c-fos$^+$/NeuN$^+$, 2.5-fold increase, $P = .0004$, Figure 4C,E; Student's *t*-test). This demonstrated the efficient integration of newly formed neurons in preexisting 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.

3.3 | In the adult dentate gyrus, HTyr promotes survival of new neurons but not proliferation of stem/progenitor cells, while HTyr has both effects in a neurogenesis-defective mouse model

The observations described above were relative to the condition of neurogenic decline that takes place during aging.

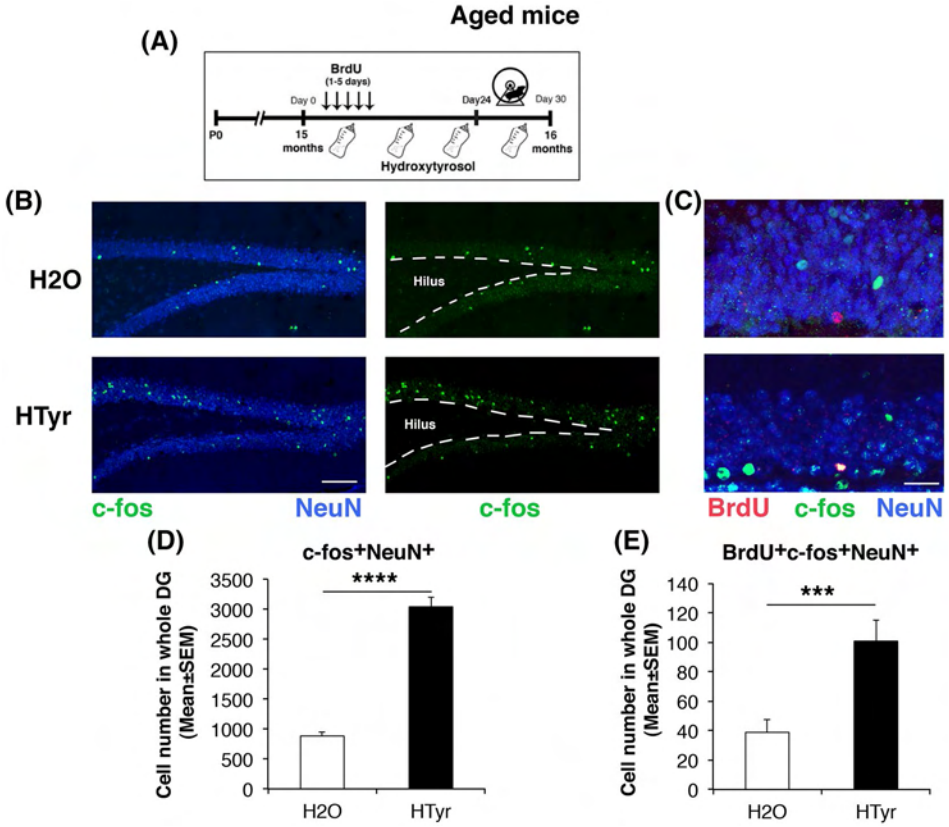

FIGURE 4 HTyr stimulates the activation of new neurons and their incorporation into preexisting circuits of the aged dentate gyrus. A, Experimental timeline: 15-month-old mice received HTyr or water for 30 days. During the first 5 days, mice were injected with BrdU, and the last 7 days of treatment were exposed to a 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 BrdU$^+$/c-fos$^+$/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. ***$P < .001$, *****$P < .0001$, Student's *t*-test

To investigate whether HTyr positively affected also physiological neurogenesis in adult mice or whether it acted only in defective conditions, we tested the compound in 2-month-old WT (Btg1 WT) and in Btg1 knockout (Btg1 KO) mice, the latter presenting a 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.[39]

We examined the effect of HTyr administration on the proliferative state of stem/progenitor cells and neuroblasts and on the number of newly formed neurons, using the same experimental paradigms described above for the aged mice (see timelines in Figures 5B and 6B, respectively).

In 2-month-old Btg1 WT mice treated for 30 days with HTyr (WT-HTyr), no significant changes were observed in the number of total proliferating cells compared to the control group (WT-H2O) (Ki67$^+$ cells: WT-HTyr vs WT-H2O, $P = .80$, Fisher's PLSD ANOVA post hoc test; Figure 5A,C) and of the subpopulations type-1-2a (Ki67$^+$/Sox2$^+$, WT-HTyr vs WT-H2O, $P = .98$; Figure 5D), type-1 (ie, stem cells, Ki67$^+$/Sox2$^+$/GFAP$^+$, WT-HTyr vs WT-H2O, $P = .67$ Fisher's PLSD ANOVA post hoc test; Figure 5E) and type-2b-3 (Ki67$^+$/DCX$^+$, WT-HTyr vs WT-H2O, $P = .29$ Fisher's PLSD ANOVA post hoc test; Figure 5F). As expected, in Btg1 KO-untreated mice (KO-H2O) compared to WT-untreated mice, we observed a significant decrease of total Ki67$^+$ cells (KO-H2O vs WT-H2O, 25% decrease $P = .0139$, 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 $P = .042$ and $P = .0026$, Fisher's PLSD ANOVA post hoc test) (Figure 5C-F).

In contrast to what we observed in WT mice, 2-month-old Btg1 knockout mice treated with HTyr presented a twofold expansion of Ki67 positive, proliferating cells compared to the Btg1 knockout control mice (total Ki67$^+$, KO-HTyr vs KO-H2O, $P < .0001$) (Figure 5A,C). Subpopulations analysis showed a marked increase due to HTyr treatment of proliferating type-1 stem cells number, that is, Ki67$^+$/

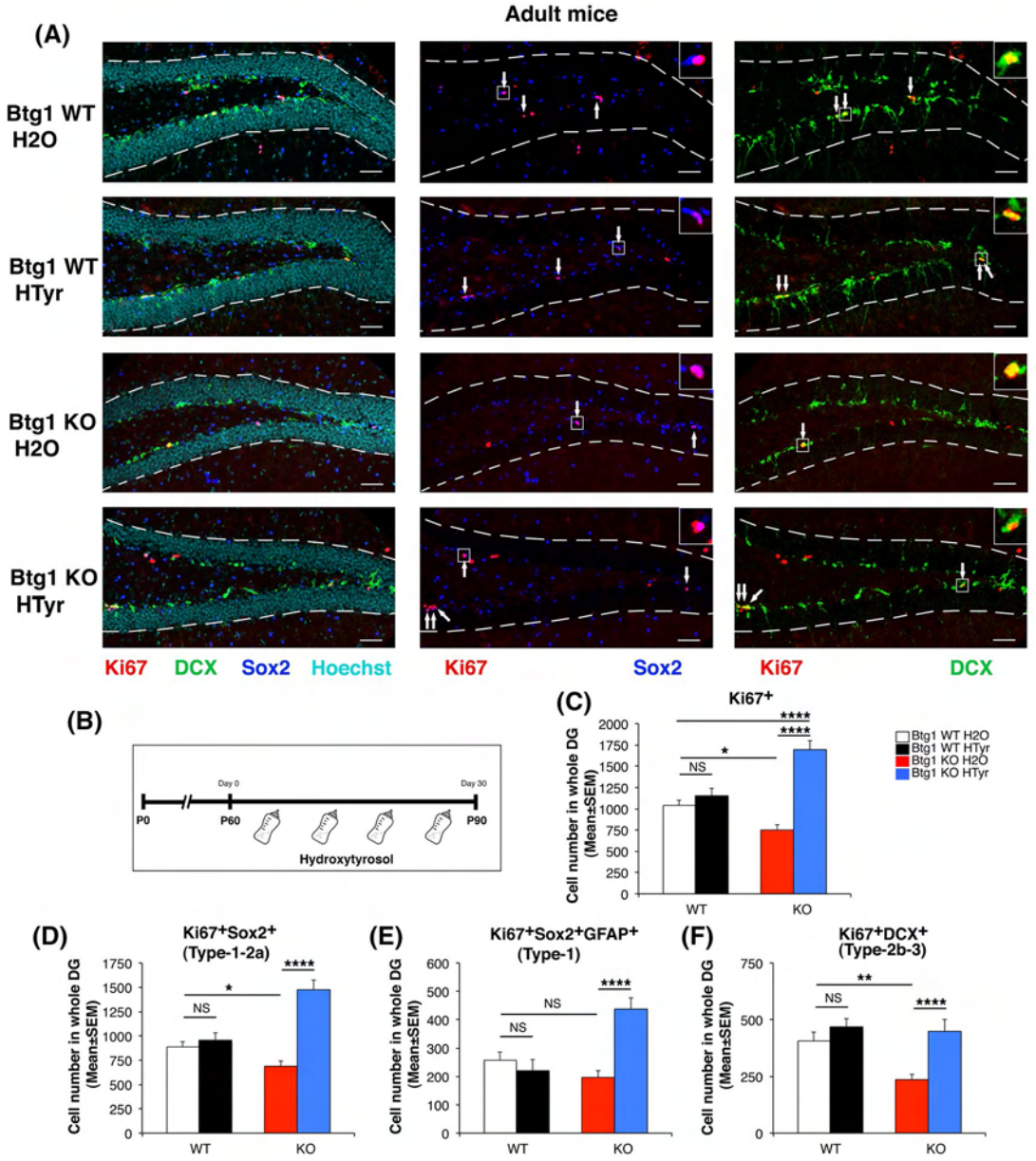

FIGURE 5 HTyr promotes the proliferation of stem/progenitor cells in the dentate gyrus of adult Btg1 knockout mice, a model of defective neurogenesis. A, Representative images by confocal microscopy showing that treatment with HTyr, as outlined in (B), significantly enhances the total number of proliferating cells ($Ki67^+$) and, in particular, the number of type-1-2a 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 WT (Btg1 WT) mice. Arrows indicate double-labeled 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 3× 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: $F_{(1,179)} = 37.003$, $P < .0001$; Genotype effect: $F_{(1,179)} = 4.773$, $P < .032$; Genotype × Treatment effect: $F_{(1.179)} = 33.052$, $P < .0001$. Followed by analysis of simple effects: $^*P < .05$, $^{****}P < .0001$, or NS $P > .05$, 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: $F_{(1.179)} = 20.303$, $P < .0001$; type-2b-3: $F_{(1.175)} = 12.087$, $P = .0006$. Analysis of simple effect: $^*P < .05$, $^{**}P < .01$, $^{****}P < .0001$ 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

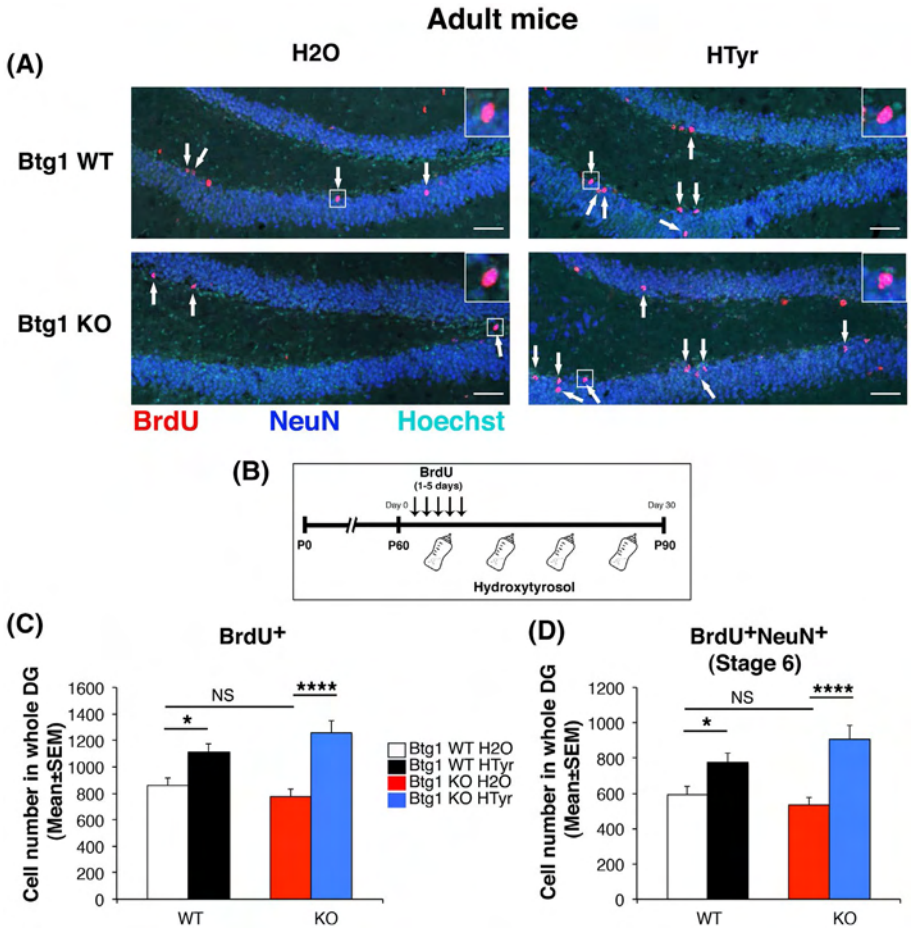

FIGURE 6 HTyr promotes survival of newly-formed neurons in the 2-month-old dentate gyrus. A, Representative images by confocal microscopy showing that HTyr treatment induces the production of 26- to 30-day-old neurons (identified as $BrdU^+$ and $BrdU^+/NeuN^+$, stage 6) both in Btg1 WT and in Btg1 KO 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 3× digital magnification. Scale bar, 50 μm. B, Experimental timeline: 2-month-old WT (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: $F_{(1.197)} = 27.005$, $P < .0001$; $BrdU^+/NeuN^+$: $F_{(1.197)} = 29.974$, $P < .0001$. Analysis of simple effect: $^*P < .05$, $^{****}P < .0001$ 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

$Sox2^+/GFAP^+$, and of type-1-2a progenitor cells, $Ki67^+/Sox2^+$ (respectively, 79% increase and 119% increase in KO-HTyr vs KO-H2O comparison, both with $P < .0001$, Fisher's PLSD ANOVA post hoc test) (Figure 5A and D,E). The proliferating type-2b-3 cells were also amplified by the treatment in the knockout genotype (KO-HTyr vs KO-H2O, 91% increase and $P < .0001$, Fisher's PLSD ANOVA post hoc test) (Figure 5A,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, $P < .0001$) (Figure 5C).

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.

Remarkably, the survival rate of newly-formed neurons was also significantly increased by treatment both in Btg1 WT 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, $P = .0116$; KO-HTyr vs KO-H2O, 62% increase, $P < .0001$, Fisher's PLSD ANOVA post hoc test; Figure 6A,C) and of $BrdU^+/NeuN^+$ 26- to 30-day-old neurons (WT-HTyr vs WT-H2O, 31% increase, $P = .0277$; KO-HTyr vs KO-H2O, 69% increase, $P < .0001$, Fisher's PLSD ANOVA post hoc test; Figure 6A,D).

As a whole, this demonstrated that HTyr is able to activate the proliferation of stem and progenitor cells only in the 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.

4 | DISCUSSION

In this report we show an effect in vivo of HTyr on the survival of new neurons in adult and aged dentate gyrus, as well as on stem/progenitor cell proliferation in aged mice. HTyr is a nutrient whose main ability is to protect cells from oxidation and which crosses the blood brain barrier, thus reaching the neural tissues.[32]

4.1 | Proneurogenic effect in vivo of HTyr on stem/progenitor cells in adult and aged dentate gyrus and on the aging-like Btg1 knockout model

We observed that HTyr does significantly stimulate adult neurogenesis in the neurogenic niche of the dentate gyrus.

Seeking to identify the functional features of HTyr action, we found that in WT adult (2-month old) mice HTyr does not influence the proliferation of dentate gyrus stem cells (type-1, $Sox2^+/GFAP^+$) and progenitor cells (type-2b or type-3, DCX^+), as indicated by colabeling with Ki67, which marks cycling cells. Remarkably, however, HTyr is able to induce the proliferation of stem/progenitor cells (type-1, type-2, and type-3) in the aged dentate gyrus (15-month-old mice) as well as in the 2-month-old Btg1 knockout mice, that represents a model of neural aging (see below). Moreover, we found that, after treatment with HTyr, new neurons are generated in greater number, relative to controls, not only in the aged or in the Btg1 knockout dentate gyrus, but also in the 2-month-old adult dentate gyrus, where no increase of proliferation of progenitor cells was observed. This was revealed by a protocol aimed at highlighting the survival of newborn neurons, that is, by measuring the number of 1-month-old neurons ($BrdU^+NeuN^+$), birth dated by incorporation of the thymidine analog BrdU. Overall, this indicates that HTyr potentiates adult neurogenesis prevalently by enhancing the survival of new neurons, with an age-independent action, being effective both in adult and in old mice; however, HTyr is also endowed with a pro-proliferative component that becomes detectable only in aged mice and in the proliferation-defective aging-like model of Btg1 knockout mice. Moreover, in agreement with the prosurvival action displayed by HTyr in the dentate gyrus, the number of apoptotic cells—detected as Caspase-3^+ cells—is decreased by HTyr, as seen in 15-month-old mice dentate gyrus.

The survival of the newborn cells is an important issue in neurogenesis, both during development and in the adult brain. Indeed, only few newborn neurons survive after birth and are incorporated into memory circuits, as the majority of newborn cells undergo early apoptosis within 1-4 days after birth, during the maturation from amplifying progenitors to neuroblasts.[56] A second wave of death in the adult dentate gyrus 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 labeling or by retroviral labeling.[57,58] In this second wave of apoptosis the survival is dictated by different factors, such as the availability of trophic factors, neuronal activity, behavioral/learning stimuli, or the regulation by GABA released from interneurons.[59,60] With aging, it is known that the generation of new neurons in the subgranular zone of the dentate gyrus decreases,[5] but in parallel the number of neurons undergoing apoptosis also decreases,[56] thus preserving a functional balance.

In this context, the increased number of new neurons generated following HTyr treatment, produces a functional enhancement of the neural network activity. In fact, in concomitance with the decrease of apoptosis, we observed in aged mice an increased number of newborn neurons recruited to neural circuits, as indicated by the increment of c-fos-labeled 1-month-old new neurons, specifically identified by BrdU birth dating. 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 labeling, after the behavioral stimulus that triggers their activation, not earlier than 2-4 weeks after birth.[14,50] Thus, HTyr, by favoring the generation and survival of new neurons, promotes also their integration into existing circuits.

Notably, in the 2-month-old Btg1 knockout mouse model, where an age-dependent accelerated loss of the proliferative capability of neural cells occurs,[39] we observe a strong increase elicited by HTyr of the number of proliferating stem cells in the dentate gyrus ($Ki67^+Sox2^+GFAP^+$ cells). In fact, at this age in the Btg1 knockout dentate gyrus the total cycling cells ($Ki67^+$) are reduced, relative to Btg1 WT (Figure 5A,C), and the treatment with HTyr rescues and increases the number of all cycling cells ($Ki67^+$), including stem cells, above WT levels (Figure 5A,C). A similar reactivation of stem cells in Btg1 knockout mice has been observed following other two neurogenic stimuli, that is, running [40] or fluoxetine.[41] Nevertheless, in the WT dentate gyrus, either adult or aged, both these stimuli are unable, unlike HTyr, to induce the proliferation of stem cells; conversely, in the adult dentate gyrus running and fluoxetine strongly activate the proliferation of progenitor cells (type-2 and type-3; 40, 41, 61, 62). Moreover, in the aged dentate gyrus, running (and HTyr) but not fluoxetine activates progenitor cells.[42,63,64]

This indicates that HTyr, although unable to directly stimulate the proliferation of adult WT progenitor cells, is nevertheless able to activate the proliferation of stem and progenitor cells in a permissive environment, such as the Btg1-null dentate gyrus, lacking the inhibitory regulation of the cell cycle exerted by Btg1, or such as the aging dentate gyrus (see in Table 1 a summary of the proneurogenic effect of HTyr, running and fluoxetine). Given that stem cells are prevalently quiescent,[57] it is plausible that, in the aged and Btg1-null dentate gyri, HTyr can trigger their exit from this state. All that suggests that HTyr stimulates the proliferation of neural cells through pathways that may be, at least partially, common to those activated by running and fluoxetine, either in the adult or aging dentate gyrus.

4.2 | Possible mechanisms of increase of survival and proliferation and antiaging action by HTyr compared to other neurogenic stimuli

Concerning the pro-survival action displayed on neural cells by HTyr, this is common also to running and fluoxetine stimuli. These latter act by increasing BDNF levels, which in turn leads to increases of the cytoprotective antiapoptotic protein Bcl-2.[65-67] Moreover, it has been shown that running induces phosphorylated AKT, a key gene for cell survival signaling,[68] which is part of the BDNF pathway.[69] Also HTyr treatment, for 6 weeks in rats exposed to subarachnoid hemorrhage, enhances the levels of AKT protein in the brain cortex,[70] and prevents the decrease of AKT, Bcl-2 and BDNF elicited by neurotoxic amyloid protein,[71] or the decrease of BDNF in the hippocampus by maternal stress.[36] 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 antioxidizing potential of the molecule and to autophagy induced by sirtuin 1-dependent mechanism.[72] Similar mechanisms may be at the origin of the strong reduction exerted by HTyr on lipofuscin and Iba1 levels, which we have observed. In fact, lipofuscin is a nondegradable aggregate of oxidized unsaturated fatty acids, proteins, oligosaccharides, and metals which accumulates with age in lysosomes of several types of cells, including neurons.[73] Moreover, Iba1 is a microglia-specific marker protein, which is upregulated in activated microglia, following ischemia,[74] brain diseases, and aging.[53] Activated microglia releases inflammatory and immunoregulatory factors and, as the brain ages, microglia acquire an increasingly inflammatory and cytotoxic phenotype, creating an adverse milieu for neurons, that may favor neurodegenerative diseases.[53] Therefore, the ability of HTyr to reduce lipofuscin and Iba1 levels, and thus, the number of activated microglia cells, clearly points to an antiaging 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.[54]

On the other hand, HTyr displays antiproliferative activity in tumor cells [24] as, for instance, observed in glioblastoma cells, where HTyr represses COX2 and Prostaglandin E2 which has pro-proliferative effects on neural cells and whose synthesis is induced by COX2.[75] However, in normal cells HTyr exhibits pro-proliferative activity, as, for example, observed in human osteoblasts,[76] or in endothelial cells, where AKT, mTOR, and Tgf-beta levels were increased.[77] This further suggests that the proliferative stimulation by HTyr that we observed in dentate gyrus cells, either aged or lacking the inhibitory regulation of cell cycle by Btg1, but not in adult cells, is dependent on the cellular condition.

As mentioned above, running is a powerful inducer of the proliferation of progenitor cells, but not of stem cells.[8,78] More generally, our data suggest that stem or progenitor cells can be more readily activated by a neurogenic stimulus such as HTyr in conditions where the checkpoints enforcing quiescence are weakened, such as in the aged and in the Btg1 knockout mice. This effect might be at the origin of the ability displayed by HTyr, in our

TABLE 1 Proliferative activation of stem and progenitor cells of the dentate gyrus by HTyr compared to other neurogenic stimuli. HTyr has the peculiar ability to stimulate stem cells in aged mice, unlike two other neurogenic stimuli such as running and fluoxetine

System	Neurogenic stimulus	Stem cells	Progenitor cells	Reference
Adult	*HTyr*	No[a]	No	This report
	Running	No	Yes	8; 40; 61; 78; 80
	Fluoxetine	No	Yes	41, 62, 81
Aged	*HTyr*	Yes[b]	Yes	This report
	Running	No	Yes	42, 63
	Fluoxetine	No	No	41, 64, 82, 83
Btg1 KO	*HTyr*	Yes	Yes	This report
	Running	Yes	Yes	40
	Fluoxetine	Yes	Yes	41

[a]$Ki67^{+}Sox2^{+}GFAP^{+}$.

[b]$Ki67^{+}Sox2^{+}$ (Type-1-2a).

data, to effectively counteract the decline of neurogenesis during aging. Indeed, activation of stem cells by a neurogenic stimulus (ie, running) occurs also in another model of aged mice, lacking the cell cycle inhibitor p16Ink4a; this indicates that dentate gyrus stem cells are 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.[42]

To conclude, it is worth noting that the human equivalent dose of 100 mg/kg/day HTyr (8.1 mg/Kg/day)[43] is above the consumption of HTyr within the Mediterranean diet,[79] 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.

Future studies will be useful to verify the behavioral effects of increased production of hippocampal neurons in adult and aged mice and the long-term action of HTyr treatment on neural aging.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

G. D'Andrea, L. Micheli, and F. Tirone designed research and analyzed data; G. D'Andrea, M. Ceccarelli, and L. Micheli performed research; L. Micheli and F. Tirone wrote the paper; M. Clemente and R. Bernini synthesized, characterized, and purified hydroxytyrosol; R. Bernini and L. Santi developed the chemistry procedures; C. Caruso participated to the project under study as co-tutor of G. D'Andrea; all authors checked and revised the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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