**INTRODUCTION**

During neurogenesis post-mitotic neurons of distinct types are generated from neural stem/progenitor cell (NSPs), both in the developing brain and in the adult brain niches of the hippocampus dentate gyrus and subventricular zone (SVZ), through progressive steps of cell cycle exit, differentiation, and migration (Gage, 2000; Ming and Song, 2005; Kriegstein and Alvarez-Buylla, 2009). Cell cycle arrest and neurogenesis are highly coordinated and interactive processes, governed by cell cycle genes and neural transcription factors. The expression of proneural genes, which convert undifferentiated precursors into neurons, is also linked to a negative control of the cell cycle (Farah et al., 2000). One molecule coordinating cell cycle exit with differentiation in neural progenitor cells is the transcriptional coregulator PC3/Tis21 (also referred to as Btg2), whose ablation enhances the proliferation of adult hippocampal granule progenitor cells and also impairs their terminal differentiation (Farioli-Vecchioli et al., 2009). Conversely, overexpression of PC3/Tis21 in hippocampal progenitor cells accelerates their differentiation (Farioli-Vecchioli et al., 2008).

Btg1 belongs to a family of cell cycle inhibitory genes. We observed that Btg1 is highly expressed in adult neurogenic niches, i.e., the dentate gyrus and subventricular zone (SVZ). Thus, we generated Btg1 knockout mice to analyze the role of Btg1 in the process of generation of adult new neurons. Ablation of Btg1 causes a transient increase of the proliferating dentate gyrus stem and progenitor cells at post-natal day 7, however, at 2 months of age the number of these proliferating cells, as well as of mature neurons, greatly decreases compared to wild-type controls. Remarkably, adult dentate gyrus stem and progenitor cells of Btg1-null mice exit the cell cycle after completing the S phase, express p53 and p21 at high levels and undergo apoptosis within 5 days. In the SVZ of adult (two-month-old) Btg1-null mice we observed an equivalent decrease, associated to apoptosis, of stem cells, neuroblasts, and neurons; furthermore, neurospheres derived from SVZ stem cells showed an age-dependent decrease of the self-renewal and expansion capacity. We conclude that ablation of Btg1 reduces the pool of dividing adult stem and progenitor cells in the dentate gyrus and SVZ by decreasing their proliferative capacity and inducing apoptosis, probably reflecting impairment of the control of the cell cycle transition from G1 to S phase. As a result, the ability of Btg1-null mice to discriminate among overlapping contextual memories was affected. Btg1 appears, therefore, to be required for maintaining adult stem and progenitor cells quiescence and self-renewal.

**Keywords:** BTG family, differentiation, knock out mice, learning and memory, neural stem cells, neurogenic niches, proliferation

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**Btg1 is required to maintain the pool of stem and progenitor cells of the dentate gyrus and subventricular zone**

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Btg1 belongs to a family of cell cycle inhibitory genes. We observed that Btg1 is highly expressed in adult neurogenic niches, i.e., the dentate gyrus and subventricular zone (SVZ). Thus, we generated Btg1 knockout mice to analyze the role of Btg1 in the process of generation of adult new neurons. Ablation of Btg1 causes a transient increase of the proliferating dentate gyrus stem and progenitor cells at post-natal day 7, however, at 2 months of age the number of these proliferating cells, as well as of mature neurons, greatly decreases compared to wild-type controls. Remarkably, adult dentate gyrus stem and progenitor cells of Btg1-null mice exit the cell cycle after completing the S phase, express p53 and p21 at high levels and undergo apoptosis within 5 days. In the SVZ of adult (two-month-old) Btg1-null mice we observed an equivalent decrease, associated to apoptosis, of stem cells, neuroblasts, and neurons; furthermore, neurospheres derived from SVZ stem cells showed an age-dependent decrease of the self-renewal and expansion capacity. We conclude that ablation of Btg1 reduces the pool of dividing adult stem and progenitor cells in the dentate gyrus and SVZ by decreasing their proliferative capacity and inducing apoptosis, probably reflecting impairment of the control of the cell cycle transition from G1 to S phase. As a result, the ability of Btg1-null mice to discriminate among overlapping contextual memories was affected. Btg1 appears, therefore, to be required for maintaining adult stem and progenitor cells quiescence and self-renewal.

**Keywords:** BTG family, differentiation, knock out mice, learning and memory, neural stem cells, neurogenic niches, proliferation
The hippocampus is known to be required in the formation of spatial and associative memories, a process in which a specific role appears to be played by the new neurons continuously generated during adulthood from progenitor cells (Frankland and Bontempi, 2005; Bird and Burgess, 2008; Deng et al., 2010). In fact, impairment of differentiation of hippocampal progenitor cells in PC3/Tis21-null mice profoundly affects their function in hippocampus-dependent contextual memory circuits and tasks (Farioli-Vecchioli et al., 2008, 2009). Notwithstanding the pan-neuronal expression of PC3/Tis21 and these profound cognitive effects following its ablation, no lethal phenotype is observed, suggesting that other related genes may produce a redundant control of differentiation.

In this regard, B-cell translocation 1 gene (Btg1) belongs to the gene family comprising PC3/Tis21, BTG3, TOB, and TOB2. It was originally identified as a sequence associated to a chromosomal translocation in a lymphoid malignancy (Rouault et al., 1992). Btg1 shares with PC3/Tis21 65% protein identity and the antiproliferative properties (Rouault et al., 1992; Tirone, 2001). Moreover, Btg1 induces avian myoblast differentiation (Marchal et al., 1995; Rodier et al., 1999) and the development of endothelial cells (Iwai et al., 2004) and is also likely involved in the differentiation of spermatogonia cells (Raburn et al., 1995). Btg1 is expressed in the developing and adult brain (Su et al., 2004; Kamaid and Giráldez, 2008), but no information on its function in neural tissues is available. Thus, we generated Btg1 knockout mice and analyzed the functional contribution by Btg1 to the adult neurogenic niches of the hippocampus and SVZ (Zhao et al., 2008). It was turned out that Btg1 is necessary for the maintenance and generation of progenitor cells and new neurons of both regions, because its ablation was associated with a massive apoptosis of stem and progenitor cells, probably a result of the loss in the control by Btg1 of the cell cycle progression from G1 to S phase. Consequently, the number of new dentate gyrus neurons generated was largely reduced in mice lacking Btg1. This decrease of new neurons had a selective effect on hippocampus-dependent memory, as it specifically affected the ability to discriminate between similar contexts (pattern separation).

MATERIALS AND METHODS

CONSTRUCTION OF mBtg1 TARGETING VECTOR

A mouse genomic clone was isolated from 129/Sv mouse library of plasmid 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.

GENERATION OF TARGETED ES CELLS AND OF Btg1-NULL MICE: GENOTYPING

We proceeded as described previously (Berthet et al., 2004), by electroporating embryonic stem cells (ES) with the linearized targeting vector and selecting them with G418 (250 µg/ml) and ganciclovir (0.5 µg/ml). A resistant ES cells 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 5’ of the gene or a fragment of the neomycin sequence (wild-type or knockout alleles generated 5 or 6.1 kb fragments, respectively). Genotyping of mice was routinely performed by PCR, using genomic DNA from tail tips. Three primers were used to identify mice carrying the different genotypes Btg1+/−, Btg1+/+ or Btg1−/−, one complementary to the neo cassette (mBtg1-Neo-R 5’-CGGAGAACCTGCGTGCAATC-3’) and the other two complementary to the targeted exon 1 (mBtg1-F 5’-CCATGCATCCTCTACTACCCC-3’; mBtg1-R 5’- TGCAGGCTCTGCTGAAAGT-3’) and were amplified together in the PCR reaction to obtain 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 1 by mBtg1-F and mBtg1-R primers). Mice were maintained under standard specific-pathogen-free conditions, and underwent behavioral testing during the second half of the light period (between 2:00 and 5:00 p.m.) in sound insulated rooms.

All animal procedures were completed in accordance with the Istituto Superiore di Sanita’ (Italian Ministry of Health) and current European (directive 2010/63/EU) Ethical Committee guidelines. Btg1 knockout mice are available upon request to J.-P. Rouault.

BrdU TREATMENT OF MICE AND SAMPLE PREPARATION FOR IMMUNOHISTOCHEMISTRY

In post-natal day 60 (P60) Btg1+/+ and Btg1−/− mice, 1- to 5-day-old neurons in the dentate gyrus and SVZ were detected by bromodeoxyuridine (BrdU) incorporation, after treatment with five daily injections of BrdU (95 mg/kg i.p.), from P55 to P59, followed by perfusion at P60 (Farioli-Vecchioli et al., 2008; see Figures 3C, 6B, and 7B). Similarly, 28-day-old neurons in the dentate gyrus and olfactory bulb were detected in Btg1+/+ and Btg1−/− mice after treatment with five daily injections of BrdU (95 mg/kg i.p.) from P55 to P59, followed by perfusion at P83 (see Figures 3D and 7C); 28-day-old neurons in the olfactory bulb were also analyzed at an earlier age: treatment with BrdU from P5 to P9, followed by perfusion at P33 (Figure 7C). To detect progenitor cells in the dentate gyrus entering the S phase, P60 or P7 mice were perfused 2 h after treatment with BrdU (a single injection), according to previous protocols (Arguello et al., 2008; Figures 4B,C). To detect progenitor cells in the dentate gyrus that have entered the S phase within the 20–48 h preceding analysis, P60 mice underwent a single injection of BrdU 20 or 48 h before perfusion (Figure 6D). Brains were collected after transcardiac perfusion with 4% paraformaldehyde (PFA) in PBS–DEPC and kept overnight in PFA. Afterward, brains were equilibrated in sucrose 30% and cryopreserved at −80°C.

IMMUNOHISTOCHEMISTRY

Immunochemistry was performed on serial floating sections cut at 40 µm thickness for hippocampus as well as for the SVZ and olfactory bulb, at −25°C in a cryostat from brains embedded in Tissue-Tek OCT (Sakura, Torrence, CA, USA).
Sections were then stained for multiple labeling using fluorescent methods. BrdU incorporation was detected following pretreatment of sections to denature the DNA, with 2N HCl 45 min at 37°C and then with 0.1 M sodium borate buffer pH 8.5 for 10 min.

Primary antibodies used were a rat monoclonal antibody against BrdU (AbD Serotech, Raleigh, NC, USA; MCA2060; 1:400), mouse monoclonal antibodies raised against Nestin (Chemicon International; MAB353; 1:50), NeuN (Chemicon International; MAB377; 1:300), PH3 (Cell Signaling Technology, Danvers, MA, USA; 9706; 1:100), or against p53 (Abcam, Cambridge, UK; ab26; 1:100), a rabbit monoclonal antibody against Ki67 (LabVision Corporation, Fremont, CA, USA; SP6; 1:200), rabbit polyclonal antibodies against cleaved (activated) Caspase-3 (Cell Signaling Technology, Danvers, MA, USA; 9661; 1:100), p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Sc-397; 1:100), or against goat polyclonal antibodies raised against GFAP (Santa Cruz Biotechnology; Sc-6170; 1:300) or DCX (Santa Cruz Biotechnology; Sc-8066; 1:300). Secondary antibodies used to visualize the antigen were either a donkey anti-rat monoclonal antiserum conjugated to Cy2 or TRITC (tetramethylrhodamine isothiocyanate; Jackson ImmunoResearch, West Grove, PA, USA; BrdU), or a donkey anti-mouse antiserum conjugated to Cy2, TRITC, or Alexa 647 (Invitrogen, San Diego, CA, USA; Nestin, NeuN, PH3, p53), or a donkey anti-rabbit antiserum conjugated to TRITC or to Cy2 (Jackson ImmunoResearch; Ki67, Caspase-3, p21), or a donkey anti-goat antiserum conjugated to Cy2, TRITC, or Alexa 647 (Invitrogen, San Diego, CA, USA; GFAP, DCX, p21).

Images of the immunostained sections were obtained by laser scanning confocal microscopy using a TCS SP5 microscope (Leica Microsystems). Analyses were performed in sequential scanning mode to rule out cross-bleeding between channels.

**QUANTIFICATION OF CELL NUMBERS AND VOLUMES**

Stereological analysis of the number of cells was performed by analyzing with confocal microscopy one-in-six series of 40-μm freefloating 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 multiplying the average number of positive cells per section by the total number of 40-μm sections comprising the entire dentate gyrus (about 50–60 sections), as described (Gould et al., 1999; Jessberger et al., 2003; Kee et al., 2007; Farioli-Vecchioli et al., 2008). Three animals per group were analyzed. Cell numbers in the SVZ and in the olfactory bulb were obtained similarly, by analyzing with confocal microscopy one-in-six series of 40-μm sections comprising the whole rostrocaudal extent of these structures in one-in-six series of 40-μm freefloating coronal sections (240 μm apart). Cell number obtained for each SVZ and olfactory bulb section was divided for the corresponding area of the section, as described (Colak et al., 2008), in order to obtain the average number of SVZ or olfactory bulb cells per square millimeter. Areas were obtained by tracing the outline of the whole SVZ, or olfactory bulb, identified by the presence of cell nuclei stained by Hoechst 33258 on a digital picture captured and measured using the I.A.S. software (Delta Sistemi, Rome, Italy). Three animals per group were analyzed. The I.A.S. software was also used to count labeled DCX. The volume of the dentate gyrus and hippocampus was calculated multiplying the average dentate gyrus area by section thickness and by number of sections (one-in-six series of 40-μm coronal sections).

**IN SITU HYBRIDIZATION**

Preparation of sections and hybridization were performed as reported previously (Canzoniere et al., 2004). An antisense ribo-probe detecting Btg1 mRNA was synthesized by SP6 polymerase from the pcDNA3-mBtg1 vector, in whose HindIII 5'-EcoRI 3' sites we cloned the 3' UTR region of mouse Btg1 mRNA (nt 1210–1730). The cloned Btg1 540 bp long sequence, which is part of the second exon of Btg1 and is devoid of cross-homologies, was amplified using genomic mouse DNA as template and was checked by sequencing. Riboprobes were labeled with digoxigenin-UTP (Transcription kit; Roche Products), following the protocol of the manufacturer. No signal was detected by the sense probe.

**DETECTION OF SENESCENT PROGENITOR CELLS AND NEURONS BY β-GALACTOSIDASE STAINING**

Senescent progenitor cells were identified by detecting β-Galactosidase activity at pH 6 as described (Dimri et al., 1995) by means of Senescence staining kit (Cell Signaling Technology, Danvers, MA, USA), following the protocol of the manufacturer.

**NEURAL STEM CELL CULTURES**

Neural stem cells cultures were performed as described by Gritti et al. (2001). Two-month-old mice (wild-type or Btg1-null) were euthanized by cervical dislocation and the brains were removed. SVZ were dissected out and cells were isolated by enzymatic digestion (1.33 mg/ml trypsin, 0.7 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid) for 30 min at 37°C and mechanical dissociation with small-bore Pasteur pipette. Neurospheres were grown in a humidified incubator at 37°C in 5% CO2 and cultured in DMEM/F12 medium supplemented with B27 and EGF and bFGF (20 and 10 ng/ml, respectively). Cells were passaged every 4th day by mechanically dissociating neurospheres into single cells.

**Neurosphere assay**

Cells isolated from SVZ were cultured under clonal conditions, in which it has been reported that neurospheres are generated from single cells and serve as an index of the number of in vivo neural stem cells (Morshed et al., 2003; Kippin et al., 2005). Cells were plated at 10 cells/µl in 24-well (0.5 ml/well) uncoated plates in growth medium. The total number of neurospheres was counted after 7 days in vitro (7 DIV).

**Expansion capacity**

Primary neurospheres were dissociated into single cells and plated at the same clonal density. Then, secondary neurospheres were dissociated and the number of cells was determined and expressed as average expansion from the initial starting population (number of cells from secondary neurospheres at 7 DIV/number of seeded cells). The size of neurospheres was expressed as a volume calculated after measuring their diameter in phase contrast pictures (assuming a spherical shape). For the growth curve, 8000 cells from wild-type and knockout neurospheres at passage 5 were seeded in 24-well plates. At each
subculture passage (every 7 days) the viable cells were counted and totally re-plated under the same conditions.

**Pair cell assay**

The Pair cell assay was performed as described by Bultje et al. (2009). Single cells isolated from SVZ of P7 or 2-month-old mice (wild-type and Btg1-null) were plated at clonal density on poly-d-lysine (Sigma Aldrich; St. Louis, MO, USA) coated coverslips in 24-well plates. After 24 h the cells were fixed in 4% paraformaldehyde and immunostained with goat polyclonal anti-GFAP (Santa Cruz Biotechnology; Sc-6170, 1:200) and mouse monoclonal anti-Tuj1 (Covance, USA; 1:250) antibodies. Secondary antibodies used were donkey anti-mouse TRITC and donkey anti-goat Cy2 (Jackson ImmunoResearch). Nuclei were counterstained with Hoechst. Coverslips were mounted on slides and imaged at confocal microscopy (Leica, TSP). The number of progenitor pairs was determined by counting at least 60 pair cells per mice (at least three mice per genotype).

**Immunofluorescence on neurospheres and microscopy**

For active caspase-3 (Ser-15) immunostaining, neurospheres were plated on matrigel-coated coverslips and then fixed in 4% paraformaldehyde for 10 min at RT. After fixation, neurospheres were permeabilized in 0.1% Triton X-100 in PBS and then incubated with the antibody against active caspase-3 (Cell Signaling Technology).

Immunostained neurospheres were mounted in Aquapoly-mount and analyzed at confocal microscopy (Leica, TSP). Z-stacks images were captured at 1 mm intervals with a 40× objective and a pinhole of 1.0 Airy unit. The numbers of caspase positive cells were counted as a percentage of Hoechst positive-nuclei in four non-adjacent Z-stacks images per neurosphere.

**REVERSE TRANSCRIPTION-PCR; GENOMIC DNA SOUTHERN ANALYSIS**

Total mRNA from neurospheres was extracted and analyzed by semiquantitative reverse transcription (RT-PCR) as described previously, with minor modifications (Canzoniere et al., 2004). Briefly, 10 µg of total RNA were treated with DNase (RQ1; Promega, Madison, WI, USA), denatured at 75°C for 5 min, and added to a final reaction volume of 50 µl. Half of the reaction volume was then incubated for 2 h at 37°C with Moloney murine leukemia virus-RT (Promega). The remaining half of the volume without RT was used as negative control in PCR amplifications for possible contamination by genomic DNA. Two microliters of each RT reaction were then used for PCR amplification, using primers amplifying the region between the end of the first exon and beginning of the second exon of Btg1. The 18S RNA was coamplified to measure the efficiency of the reaction and the RNA amount in each sample. The amplified products were visualized by agarose gel electrophoresis.

To define the structure of the recombined Btg1 locus, Southern blot analysis was performed using genomic DNA extracted from tail tips. Twenty micrograms of genomic DNA was restricted with AcI, gel separated, blotted to a nylon filter, and hybridized with a [32P]-labeled probe, whose sequence encompasses the 5’ region of the Btg1 gene external to the targeting vector.

**BEHAVIORAL TESTS**

Btg1-null (n = 30) and wild-type (n = 30) male mice aged between 2 and 4 months were used for behavioral evaluation. All of them were preliminarily tested in an open field, to assess locomotion and anxiety-related behaviors. Mouse activity was recorded for 15 min and the distance traveled, moving speed, rearing events and relative occupancy of external vs. central sectors of the arena were analyzed. No significant differences between genotypes were observed in any of these variables (data not shown). Additionally, a plus maze test was performed, in which the animals were allowed to explore the apparatus (a cross-shaped maze placed 60 cm from the ground, with four arms 30 cm long) for 5 min and the relative occupancy of closed vs. open arms was evaluated. Again, no significant differences between genotypes were observed (data not shown).

**Water maze**

A delayed-matching-to-place version of the task was conducted as previously described (Chen et al., 2000), with minor modifications. In a pool measuring 130 cm in diameter, mice were trained to navigate to a hidden platform (10 cm in diameter) until reaching a rigorous performance criterion of three consecutive trials with an average escape latency of less than 20 s, or completing a maximum of 24 trials. This was repeated until a total of four different platform locations were learned. The behavior of mice was analyzed by EthoVision software (Noldus Information Technology, Wageningen, NL, USA).

**One-trial contextual fear conditioning**

Conditioning was performed in a training chamber (A) with a single footshock (2 s; 0.7 mA) delivered 180 s after placement of a mouse into the chamber. Mice were left in the chamber for a further period of 20 s and then returned to their home cage. Contextual test (5 min) was performed 24 h after training, in the same chamber. Forty-eight hours after training, mice were tested in a different context (C). The amount of freezing was assessed off-line by an experimenter blind to the genotypes of the animals.

**CONTEXTUAL FEAR-DISCRIMINATION LEARNING**

The test was conducted as previously described (Sahay et al., 2011a), with minor modifications. Conditioning was performed in the training chamber (A). Starting from the next day, mice were exposed, on a daily basis, to both the training context, in which they continued to receive a single footshock, and a similar context (B), in which they were never shocked. Freezing was measured each day in both the contexts, to evaluate continuously the discrimination level.

**RESULTS**

IN ADULT MOUSE BRAIN **Btg1 IS EXPRESSED IN THE DENTATE GYRUS, SVZ, OLFACTOR Y BULB, AND CEREBELLUM**

As we were interested in investigating a possible role of Btg1 in adult neurogenesis, we analyzed the expression of Btg1 mRNA in the brain of 2-month-old mice by in situ hybridization. As shown in Figure 1, high levels of Btg1 are detectable in the adult neurogenic niches, i.e., in the dentate gyrus of the hippocampus and in the SVZ, and also in the olfactory bulb, where neurons from
Btg1 maintains neural stem cells

FIGURE 1 | Expression of Btg1 in the mouse adult brain. A representative sagittal section of the brain from a 2-month-old mice, 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: 500 µm (panel above) or 100 µm (enlargements).

IN THE ABSENCE OF Btg1, ADULT NEUROGENESIS IN THE DENTATE GYRUS IS IMPAIRED

Then, we sought to assess whether the generation of new neurons in the neurogenic adult niches of the hippocampus and SVZ is dependent on Btg1 expression. We first analyzed the maturation of progenitor cells of the dentate gyrus in the adult hippocampus (P60) of Btg1 knockout mice.

We identified new 1- to 5-day-old dentate gyrus progenitors and neurons by treating mice at P55 with five daily injections of BrdU, and analyzing them in the different cell populations of the dentate gyrus (Figures 3A–C). In Btg1-null mice we observed a significant decrease, with respect to control mice, in the number of 1- to 5-day-old type-1 stem and type-2a progenitor cells (BrdU+/nestin+/DCX−; p = 0.004; Figures 3A,C), while type-2b progenitor cells decreased but not significantly (BrdU+/nestin+/DCX+; Figures 3A,C). A significant decrease was evident also for 1- to 5-day-old type-3 progenitor cells (BrdU+/nestin−/DCX+; 22% decrease, p = 0.006; Figures 3A,C), that still express DCX but not nestin (Kronenberg et al., 2003), as well as for 1- to 5-day-old stage 5 terminally differentiated neurons expressing the late differentiation marker NeuN (BrdU+ /DCX+/NeuN+; 20% decrease, p = 0.01; Figures 3B,C). In parallel, in Btg1-null mice a large and significant reduction of the whole population of 1- to 5-day-old new neurons occurred (total BrdU+; p = 0.001; Figure 3C), as well as of nestin+ (33% decrease, p = 0.001; Figures 3A,C) and DCX+ (34%...
Ablation of Btg1 reduces the pool of dividing adult progenitor cells in the dentate gyrus and induces their exit from the cell cycle within a few hours after completing the S phase

First, in the dentate gyrus of P60 mice, we measured the number of proliferating progenitor cells entering S phase, identified by incorporation of a short BrdU pulse of 2h (Arguello et al., 2008), and observed a non-significant decrease in Btg1-null mice (Figure 4B). Moreover, no change was observed in the number of progenitor cells in G2/M-phase, identified by the mitotic marker antiphospho-histone H3, PH3 (99.9 ± 22.5 and 95.3 ± 24.9 average cells ± SEM in whole dentate gyrus of Btg1+/+ and Btg1−/− mice, respectively; Kaitna et al., 2002). This normal rate of BrdU incorporation was an unexpected result, as Btg1 is a known antiproliferative gene (Rouault et al., 1992) whose ablation would be expected to increase the number of dividing cells. Therefore, we verified whether the BrdU incorporation did not change also at an earlier post-natal age, P7, when the proliferation rate is higher, and found that BrdU incorporation increased highly (34%, p = 0.002; Figure 4B). We further analyzed the total number of cycling progenitor cells by means of the proliferation marker Ki67 (Scholzen and Gerdes, 2000) and observed that at P60 Ki67+ cells, quite surprisingly, decreased significantly by 15%, whereas at P7 they increased significantly by about 55% (p = 0.0005 and p = 0.0000, respectively; Figures 4A,B). By analyzing each population of dividing progenitor cells at P60, we found that type-1 stem (Ki67+/GFAP+/nestin+) and type-2ab progenitor cells (Ki67+/GFAP-/nestin+), identified by the presence of nestin and absence of GFAP expression), decreased...
In Btg1-null adult mice the number of new 1- to 5-day-old dentate gyrus progenitor cells and of 28-day-old neurons is reduced. Representative images showing a decrease in the dentate gyrus of Btg1-null mice of (A) new stem and progenitor cells (type-1–2a; BrdU+/nestin+/DCX−, marked by green and red and negative to blue, respectively, indicated by white arrowheads; scale bar, 50 µm) and of (B) post-mitotic 1- to 5-day-old neurons (stage 5; BrdU+/DCX+/NeuN−, indicated within the box by white arrowheads; scale bar, 100 µm), as detected by incorporation of BrdU after five daily injections in P60 Btg1+/+ and Btg1−/− mice, and by the specific markers indicated, through multiple-labeling confocal microscopy. (B) On the left: 3D reconstruction from Z-stack of triple- and double-positive cells shown (Continued)
FIGURE 3 | Continued
in the boxed area (scale bar, 20 µm). In (A) the dentate gyrus is outlined by a broken line. (C) 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 cell, as well as of stage 5 post-mitotic neurons, indicated a significant decrease (except for type-2b) in P60 Btg1-null mice. Also total BrdU-positive, total nestin-positive and total DCX-positive progenitor cells decreased significantly. (D) However, the highest reduction was observed for 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. Cell numbers in dentate gyrus, shown in (C,D), were measured as described in Materials and Methods and are represented as mean ± SEM of the analysis of three animals per group. *p < 0.05, **p < 0.01, or ***p < 0.001 vs. Btg1+/+ dentate gyrus; Student’s t-test.

FIGURE 4 | In Btg1-null adult mice the number of cycling dentate gyrus progenitor cells decreases, after a transient early post-natal increase. (A) Representative confocal images in P60 dentate gyrus Btg1-null mice showing a decrease of dividing stem cells, identified by means of Ki67 (type-1; Ki67+/nestin+/GFAP−, red, green, and blue, respectively, indicated by white arrowheads; scale bar, 50 µm). (B) The quantification of the total number of dentate gyrus cells entering the S phase (total BrdU+ cells after a 2-h pulse) did not show significant differences at P60, whereas at P7 their number increased significantly in Btg1-null mice. Similarly, the total number of cycling cells (total Ki67+) increased at P7, but decreased significantly at P60; such a decrease occurred in dividing type-1 (Ki67+/GFAP+/nestin−), type-2ab (Ki67+/GFAP+/nestin−) and type-2b (Ki67+/nestin+/DCX−) progenitor cells, while type-3 (Ki67+/nestin−/DCX+) did not differ. Cell numbers in the dentate gyrus are mean ± SEM of the analysis of three animals per group. *p < 0.05, **p < 0.01, or ***p < 0.001 vs. Btg1+/+ dentate gyrus; Student’s t-test.
We thus analyzed the survival of dentate gyrus cells. We observed +S phase of BrdU of the pool of progenitor cells. (Caspase-3 mice, while a non-significant decrease appeared in type-2b (Caspase-3) populations showed that type-1 and type-2a progenitor cells of Figures 5A,B). An analysis of the dentate gyrus progenitor pop-ulations showed that type-1 and type-2a progenitor cells of Btg1-null mice underwent apoptosis 3.2-fold more frequently (Caspase-3+/nestin+/DCX−; Figures 5A,B) than in wild-type mice, while a non-significant decrease appeared in type-2b (Caspase-3+/nestin+/DCX−; Figures 5A,B) and in type-3 progen-itor cells (Caspase-3+/nestin−/DCX+).

**ABLATION OF Btg1 DRIVES THE DENTATE GYRUS POOL OF ADULT PROGENITOR CELLS UNDERGOING APOPTOSIS**

We thus analyzed the survival of dentate gyrus cells. We observed that in P60 Btg1-null mice the total number of apoptotic cells underwent a striking 2.4-fold increase with respect to those in wild-type, as detected by positivity to activated Caspase-3, marker of apoptosis (Nicholson et al., 1995; p = 0.001; Figures 5A,B). An analysis of the dentate gyrus progenitor pop-ulations showed that type-1 and type-2a progenitor cells of Btg1-null mice underwent apoptosis 3.2-fold more frequently (Caspase-3+/nestin+/DCX−; Figures 5A,B) than in wild-type mice, while a non-significant decrease appeared in type-2b (Caspase-3+/nestin+/DCX−; Figures 5A,B) and in type-3 progen-itor cells (Caspase-3+/nestin−/DCX+).

**Btg1-NULL ADULT DENTATE GYRUS PROGENITOR CELLS EXIT THE CELL CYCLE EXPRESSING p53 AND p21 AND UNDERGO APOPTOSIS WITHIN 5 DAYS AFTER COMPLETING THE S PHASE**

We further asked whether the striking increase of apoptosis observed for type-1 and type-2a progenitor cells in Btg1-null mice was correlated to the cell cycle progression. Hence, after a 5-day BrdU pulse in P60 mice, we analyzed 1- to 5-day-old BrdU+/Caspase-3+ and BrdU+/Caspase-3+/nestin+ (type-1 and type-2ab) cells, and observed a very large increase (15-fold) of them in Btg1-null mice (p = 0.005 and p = 0.001, respectively; Figures 6A,B). No BrdU+/Caspase-3+ cells were detected with BrdU pulses of shorter duration (data not shown). This demonstrates that Btg1-null type-1 and type-2ab progenitor cells undergo apoptosis within 1–5 days after completing the S phase, and – together with the observed increase of progenitor cells exiting the cell cycle – it raises the question as to whether apoptosis was caused by a defect in cell cycle control consequent to Btg1 deletion. Thus, we analyzed the expression of p53 and p21. p53 is a key regulator of the cell cycle that inhibits cell cycle progression when a cellular stress occurs, such as a misregulation of the cell cycle, acting either directly or through its effector p21, a cyclin-dependent kinase inhibitor that arrests proliferation and leads the cell into a condition of senescence (Brady and Attardi, 2010; Qian and Chen, 2010; Erol, 2011). We observed in the dentate gyrus of P60 Btg1-null mice a major increase of BrdU+/Ki67+/p53+ and BrdU+/Ki67+/p21+ progenitor cells (analyzed after a 20- or 48-h BrdU pulse, respectively; p = 0.001 for p53+ and p = 0.004, for p21+ cells; Figures 6C,D), i.e., of progenitor cells that after the entrance in S phase (BrdU-positive) have then become quiescent or senescent, as indicated by the exit from the cell cycle (being Ki67-negative) concomitant with the expression of p53 or p21. No BrdU+/Ki67+/p21+ progenitor cells were detected after a BrdU pulse shorter than 48 h (data not shown), consistently with the notion that p21 upregulation is effected by p53. We further sought to check which type of progenitor cells expressed p21, and found in Btg1-null mice a significant increase of p21+ type-1 (nestin+/GFAP+/p21+; p = 0.002; Figure 6E) but not of type-2ab progenitor cells (nestin+/GFAP−/p21+; p = 0.53; Figure 6E). We also verified whether the increase of BrdU+/Ki67−/p21+ corresponded to an increase of senescent cells in Btg1-null dentate gyrus, however no difference was found by visualizing β-galactosidase that specifically marks senescent cells (Dimri et al., 1995; data not shown). This indicates that the p21+ (and p53+) progenitor cells entered quiescence without attaining a stable exit from the cell cycle (i.e., a senescent state). As a whole, this indicates that in P60 Btg1-null mice: (i) progenitor cells within 2–20 h after the entrance in S phase undergo a process of transient quiescence (see also Figure 4C), followed by apoptosis; (ii) the ablation of Btg1 impairs neurogenesis in the dentate gyrus probably as a result of the massive apoptosis occurring in type-1/type-2a progenitor cells, which reduces the pool of quiescent and dividing progenitor cells downstream.

**IN THE SVZ ABLATION OF Btg1 REDUCES THE GENERATION OF NEW ADULT NEURONS AND INCREASES THE NUMBER OF ADULT PROGENITOR CELLS UNDERGOING APOPTOSIS**

We were further interested in evaluating whether the ablation of Btg1 affected the generation of the neurons also in the SVZ, the other adult neurogenic niche (Alvarez-Buylla and Lim, 2004). We analyzed the number of dividing stem cells and neuroblasts by the proliferation marker Ki67, and observed that in P60 Btg1-null mice cycling type B astrocytic-like stem cells and type A neuroblasts, identified respectively by GFAP and DCX (Zhao et al., 2008), decreased significantly (about 28% decrease, p = 0.03, for B-cells; 21% decrease, p = 0.02, for A cells; Figures 7A,A′).
FIGURE 5 | Ablation of Btg1 induces a massive apoptosis in adult stem and progenitor cells of the dentate gyrus. (A) Representative confocal images of apoptotic cells in the dentate gyrus of P60 Btg1+/+ and Btg1−/− mice, showing either type-1 stem cells/type-2a progenitor cells (Caspase-3+/nestin+/DCX−; red, green and blue, respectively; white arrowheads) or type-2b progenitor cells (Caspase-3+/nestin+/DCX+; white arrows). Scale bar, 25 µm.

(B) Analysis in the P60 dentate gyrus of the total number of apoptotic cells (Caspase-3+), and of type-1–2a (Caspase-3+/nestin+/DCX−), type-2b (Caspase-3+/nestin+/DCX+) and type-3 (Caspase-3+/nestin+/DCX+) stem and progenitor cell. Apoptosis was significantly higher in type-1–2a stem and progenitor cells. Cell numbers are mean ± SEM of the analysis of three animals per group. **p < 0.01, ***p < 0.001 vs. Btg1+/+ dentate gyrus; Student’s t-test.

Consistently, the total numbers of dividing cells (Ki67+) as well as of type B progenitor cells (GFAP+) and of type A neuroblasts (DCX+) decreased significantly (p = 0.006 for Ki67+ cells; Figures 7A,A′). Thus, the whole population of type B and A cells appeared strongly reduced in Btg1-null P60 mice. Conversely, the total number of cycling cells (Ki67+) in the SVZ of P7 Btg1-null mice increased significantly (p = 0.0001; Figures 7A,A′). In parallel, in SVZ of P60 Btg1-null mice the total number of apoptotic cells increased (2.1-fold; total Caspase-3+, p = 0.0002; Figures 7B,B′), more specifically type B apoptotic cells, within 5 days after birth (2.2-fold; Caspase-3+/GFAP+; p = 0.0001; BrdU+/Caspase-3+/GFAP+, p = 0.03 Figures 7B,B′). Moreover, the 28-day-old
Btg1-null adult stem and progenitor cells of the dentate gyrus undergo quiescence within 20 h and apoptosis within 5 days after entering the S phase. (A) Representative confocal images showing apoptotic type-1-2ab progenitor cells triple-labeled BrdU+/Caspase-3+/nestin+ (indicated by white arrowheads) in the P60 Btg1-null dentate gyrus after a 5-day BrdU pulse. In Btg1 wild-type dentate gyrus only BrdU+/Caspase-3+/nestin+ progenitor cells are detectable (indicated by a white arrow). Scale bars, (Continued)
SVZ neurons at their final migratory destination in olfactory bulb, birth-dated by BrdU with five daily injection from age P55 and identified by the terminal differentiation marker NeuN, were highly reduced in adult Btg1−null mice, by about 40% in the granule cell layer (GCL, \( p = 0.000 \); Figures 7C,C’, see graph on the left and the scheme of BrdU treatment); in the glomerular layer (GL) no significant difference of BrdU+/NeuN+ neurons relative to wild-type was observed \( (p = 0.49; \text{Figures } 7C,C’) \). On the contrary, the 28-day-old SVZ neurons that migrated in the olfactory bulb at an early post-natal age (identified by BrdU labeling from P5), increased significantly in Btg1-null mice \( (GCL, p < 0.0001; \text{Figure } 7C’, \text{graph on the right and BrdU treatment scheme above}) \). No significant difference of 28-day-old SVZ neurons labeled from P5 was observed in the GL, relative to controls (data not shown).

As a whole, this suggests that ablation of Btg1 causes a transient increase in the generation of new neurons at an early post-natal age \( (P7) \), and then impairs neurogenesis in the adult SVZ, similarly to what we observed in the dentate gyrus, probably as a consequence of the massive apoptosis which the SVZ stem B astrocytic-like cells undergo.

**LOSS OF Btg1 IMPAIRS PROLIFERATION AND SURVIVAL OF SVZ NEURAL STEM CELLS**

To examine if the effect of the Btg1 loss on NSPs proliferation and survival observed \textit{in vivo} in this report is an intrinsic property of the cells, we performed proliferation studies on primary NSPs isolated from SVZ and grown in culture as neurospheres. NSPs, derived either from 7-day-old or 2-month-old Btg1 wild-type and knockout mice, were plated at low density in order to perform a clonal neurosphere assay as a measure of the percentage of NSPs in the brain \( (\text{Kippin et al., 2005}) \). By counting the total number of neurospheres formed after 7 days in culture, we observed a significant increase in the neurosphere-forming cell population derived from Btg1-null P7 mice, and a decrease in neurosphere-forming cell population from P60 mice, relative to wild-type \( (p = 0.01 \text{ at } P7, p = 0.04 \text{ at } P60; \text{Figure } 8A) \). This result could either reflect or recapitulate \textit{in vitro} what we had already observed \textit{in vivo}, i.e., an age-dependent reduction in the number of NSP cells in Btg1 knockout vs. wild-type mice, and/or suggest that knockout cells from P60 mice proliferate less than their wild-type counterpart.

To better investigate this aspect, we analyzed the frequency of symmetric and asymmetric cell division occurring within NSP in both mice, by performing secondary neurosphere assays \( (\text{Reynolds and Weiss, 1996; Reynolds and Rietze, 2005}) \). Under normal circumstances, asymmetric division maintains the population of NSPs at the same size; alternatively, symmetric division occurs when NSP generate two NSPs progeny, thereby expanding the population of NSPs. We therefore dissociated primary neurospheres, seeded them at low density, and measured the size (indicative of asymmetric division) of secondary spheres formed after 7 days \textit{in vitro}. We found that in the absence of Btg1 secondary neurospheres from P7 Btg1-null mice were larger, while neurospheres from adult (P60) Btg1-null mice were smaller (reflecting fewer asymmetric divisions), compared to their wild-type counterpart \( (p = 0.000 \text{ at } P7 \text{ and } P60; \text{Figures } 8B,C) \). In addition, we observed that the amplification capacity of Btg1-null neurospheres increased in neurospheres from P7 mice, whereas it decreased in neurospheres from P60 mice \( (p = 0.02 \text{ at } P7, p = 0.04 \text{ at } P60; \text{Figure } 8D) \). Similarly, a decrease was observed in the long term expansion of neurospheres from P60 Btg1-null mice, measured by growth curves \( (\text{Figure } 8E) \). These data suggest that the loss of Btg1 affects both the self-renewal and the proliferative capacity of NSPs, causing a severe depletion of the stem cell compartment in adult mice.

To further analyze the influence of Btg1 ablation on the mode of division of NSPs, we used the clonal pair cell assay that allows one to distinguish the relative changes in symmetric vs. asymmetric division of primary NSPs isolated from SVZ \( (\text{Bultje et al., 2009}) \). We observed that the fraction of NSPs that divided asymmetrically (giving one proliferating GFAP+ cell and one differentiated TuJ1+ neuron) significantly increased (threefold) in cultures from Btg1-null P7 mice relative to wild-type, whereas it significantly decreased (60%) in cultures from Btg1-null P60 mice \( (p = 0.04 \text{ at } P7 \text{ and } P60; \text{Figures } 8F–H) \). No significant difference was observed in symmetric divisions in primary NSPs from P7 or P60 mice. This indicated an age-dependent decrease of asymmetric divisions.

Since stem cells exhaustion could also depend on apoptosis, we also wanted to measure apoptotic cell death in Btg1-null neurospheres by looking at the expression of the apoptotic-specific marker activated caspase-3. We found significantly more active caspase-3-positive cells in Btg1-null neurospheres from 2-month-old mice compared to the wild-type ones \( (56\% \text{ increase, } p = 0.005; \text{Figure } 8I) \). Taken together, our results demonstrate that Btg1 is required for the proper self-renewal of the neural stem cells, since in the absence of Btg1 we observed a decrease in cell proliferation and an increase in apoptotic cell death.

**DEFECTIVE HIPPOCAMPUS-DEPENDENT LEARNING IN Btg1-NULL MICE**

Learning and memory of Btg1-null mice were firstly assessed by a delayed matching-to-place water maze protocol, which has been used by \text{Chen et al. (2000)} and \text{Zeng et al. (2001)} to assess rodents’ ability to perform one-trial learning and episodic-like memory. As training progressed, both Btg1-null \( (n = 12) \) and control (wild-type; \( n = 12 \)) mice retained their ability to locate the
FIGURE 7 | Higher apoptosis frequency and decreased number of cycling stem/progenitor cells of the adult SVZ and of 28-day-old neurons of the adult olfactory bulb. (A) Representative confocal images of coronal sections showing dividing B stem cells in the SVZ of P60 Btg1+/+ and Btg1−/− mice, identified as double-labeled Ki67+/GFAP+ cells (green and red, respectively) and indicated by white arrowheads in the white box area at higher magnification. Scale bars, 100 and 50 µm (enlargement).

(A') Analysis in P60...
hidden platform at each of the four positions it was sequentially moved to [Figure 9A, left to right; effect of trial, for all positions: F(4, 88) = 5.75, p < 0.001; trial × genotype interaction, for all position: F(4, 88) < 1.10, p > 0.361; two-way repeated measures ANOVA]. However, while no effect of genotype was observed for the first and the second platform positions [Figure 9A, positions #1 and #2; effect of genotype, for both positions: F(1, 22) = 2.49, p > 0.129], a statistically significant difference between genotypes emerged for the third and fourth positions, with Btg1-null mice reducing their escape latencies at a slower rate compared to wild-type mice [Figure 9A, positions #3 and #4; effect of genotype, for both positions: F(1, 22) > 10.99, p < 0.003]. Consistently, a statistically significant difference between genotypes was observed in the reduction of escape latencies animals achieved as they passed from the first to the second trial of the last two training sessions (Figure 9B; p = 0.039; Student’s t-test), while maintaining comparable swimming speed (p = 0.617) and thigmotaxis (p = 0.566). Furthermore, Btg1-null mice needed a significantly higher number of trials to reach the performance criterion over the last two training sessions, compared to wild-type mice (Figure 9C; p = 0.003; Student’s t-test).

The ability of Btg1-null mice to differentiate between overlapping contextual representations was further assessed by a contextual fear-discrimination learning task (McHugh et al., 2007; Sahay et al., 2011a). Btg1-null (n = 8) and control (n = 8) mice were preliminarily submitted to a single-trial footshock-context pairing procedure (Figure 9D). Upon re-exposure to the conditioning chamber (A), 24 h after being trained, both groups showed equally increased levels of freezing behavior; conversely, negligible freezing was detected in a distinct context (C), 48 h after training (Figure 9E; effect of genotype: F(1, 14) = 0.50, p = 0.490; effect of context: F(1, 14) = 94.16, p < 0.001; context × genotype interaction: F(1, 14) = 0.01, p = 0.91; two-way repeated measures ANOVA). Independent groups of mice were subsequently tested for contextual fear-discrimination learning by prolonged training in two similar contexts (A and B), with footshock delivered only in one (Figure 9F). Both Btg1-null (n = 10) and wild-type (n = 10) mice showed generalization between the two contexts during the early days of training, and were able to discriminate at the end; however, while wild-type mice gained effective discrimination on day 4, which was stably maintained until day 7 (p < 0.010), Btg1-null mice started discriminating by day 6 (p = 0.074), with difference in freezing behavior evoked by the two contexts reaching statistical significance only by day 7 (p = 0.013) [Figure 9G; training × context interaction: F(6, 108) = 4.90, p < 0.001; Figure 9H; training × context interaction: F(6, 108) = 3.62, p = 0.003; two-way repeated measures ANOVA followed by analysis of simple effects].

Overall, these data indicate that the basic ability to encode contextual features is preserved in Btg1 knockout mice, which makes them able to differentiate among markedly dissimilar contexts; by contrast, their finer mnemonic discrimination appears to be impaired, when challenged by subtle differences in contextual details to be promptly distinguished.

DISCUSSION

Understanding the molecular pathways controlling neural stem cells self-renewal and maintenance may shed light on tissue homeostasis in the neurogenic niches during adult neurogenesis. This study addresses the role of the antiproliferative gene Btg1 in modulating neurogenesis in the adult brain. Our data clearly indicate that Btg1 plays a specific role in regulating neural stem cells proliferation and subsequently their quiescent state as well as their survival. We report that the loss of Btg1 causes an increased proliferation of newborn neurons in an early post-natal age, followed by a decline of neurogenesis in the adult, associated with death by apoptosis. In particular, we observe in the neurogenic niches (subgranular zone of the dentate gyrus and SVZ) of P7 knockout mice that the number of stem cells and progenitors positive for the two proliferation markers Ki67 and BrdU is significantly higher relative to control, whereas in the adult mice the rate of proliferation strongly decreases, especially in the dividing type-1 stem and type-2 progenitor cells of the dentate gyrus.

CONTROL OF CELL CYCLE IN DENTATE GRUYS STEM CELLS BY Btg1: ITS ABLATION IMPACTS ON QUIESCENCE, SURVIVAL, AND PROLIFERATIVE CAPACITY

These effects can be primarily originated by the loss of the antiproliferative action of Btg1, whose deprivation is sufficient to induce an initial expansion of neural stem cells, followed by reduced proliferative capacity and depletion of the population of stem cells, and by the decline of the adult neurogenesis with age.

Given that the cell cycle transition from G1 to S phase is enhanced at P7 in Btg1-null progenitor cells, while the G2/M transition appears not altered (as PH3 labeled cells do not change in Btg1-null mice at either age; data not shown), our findings
Btg1 maintains neural stem cells in vitro, followed by an age-dependent decrease of proliferative capacity, self-renewal, and survival. (A) Number (mean ± SEM) of clonal neurospheres derived from the subependyma of the lateral ventricle from Btg1-null and wild-type P7 or P60 mice (n = 4 and 5, respectively). Relative to control mice, neurospheres generated from P7 Btg1-null mice increased strikingly in number, while those generated from adult P60 mice decreased. (B) Volumes (mean ± SEM) of secondary neurospheres derived from Btg1-null and wild-type mice aged P7 or P60 (n = 4 and 5, respectively). With respect to wild-type mice, the volume of neurospheres from P60 Btg1-null mice was lower, after an initial increase observed in neurospheres from P7 mice.

(C) Representative images of secondary neurospheres derived from Btg1-null and wild-type mice aged P7 or P60. Scale bars, 115 µm. (D) Percentage of cell expansion of primary neurosphere cultures from Btg1-null and wild-type mouse (total number of cells at the end of culture divided by the initial number of cells, represented as mean percentage ± SEM, wild-type set to 100%). Relative to control, a greater expansion occurred in cells from P7 Btg1-null mice, whereas the expansion of cells derived from P60 Btg1-null mice was considerably lower (n = 4 and 5, respectively). (E) Growth curve displaying the amplification of 8000 cells derived from secondary neurospheres plated at t₀, from P60 mice either Btg1-null or wild-type (n = 3). The amplification of (Continued)
suggest that stem and progenitor cells lacking Btg1 are defective in the control of the progression from G1 to S phase. This possibility is consistent with the known ability of Btg1 to arrest the cell cycle in G1 phase (Li et al., 2009). It is well known that the inactivation of molecules regulating the transition from G1 to S phase, for instance pRb, can raise a conflict between ongoing proliferative and proliferation-inhibitory stimuli, leading to cell death (Lee et al., 1994).

Thus, the massive apoptosis of the pool of stem type-1 cells and of transit amplifying progenitor type-2a cells, likely triggered by the absence of the negative control of cell cycle exerted by Btg1, appears to be one cause of the decrease of adult neurogenesis. Apoptosis can in fact account, at least in part, for the reduction in G1 phase (P–P; both labeled by GFAP), one glial astrocytic-like proliferating progenitor cell and one postmitotic neuron (P–N; labeled by GFAP and the neuronal marker TuJ1, respectively), and two postmitotic neurons (N–N; TuJ1/7TuJ1). Scale bar 10 μm. (G,H) Quantification of the percentage of p–p and control mice. Cells counted: n = 198 and 330 for P7 Btg1+/− and Btg1−/− mice, respectively; n = 248 and 192 for P60 Btg1+/− and Btg1−/− mice (at least three mice per age). (i) Percentage of apoptotic cells in secondary neurospheres (mean percent ± SEM), detected as positive to activated Caspase-3. Cells from P60 Btg1−/− null presented a frequency 1.6-fold higher than control. *p < 0.05, **p < 0.01, or ***p < 0.001 vs. Btg1+/−; Student’s t-test.

**FIGURE 8** Continued

Btg1-null cells is reduced in the long term, relative to wild-type cells. (F) Representative images from three different types of daughter-cells originating from individual NSPs from SVZ: two glial astrocytic-like proliferating progenitor cells (P–P; both labeled by GFAP), one glial astrocytic-like proliferating progenitor cell and one postmitotic neuron (P–N; labeled by GFAP and the neuronal marker TuJ1, respectively), and two postmitotic neurons (N–N; TuJ1/7TuJ1). Scale bar 10 μm. (G,H) Quantification of the percentage of P–P and control mice. Cells counted: n = 198 and 330 for P7 Btg1+/− and Btg1−/− mice, respectively; n = 248 and 192 for P60 Btg1+/− and Btg1−/− mice (at least three mice per age). (i) Percentage of apoptotic cells in secondary neurospheres (mean percent ± SEM), detected as positive to activated Caspase-3. Cells from P60 Btg1−/− null presented a frequency 1.6-fold higher than control. *p < 0.05, **p < 0.01, or ***p < 0.001 vs. Btg1+/−; Student’s t-test.

Btg1 ABLATION REDUCES THE SELF-RENEWAL AND PROLIFERATIVE CAPACITY ALSO OF SVZ STEM CELLS

A very similar situation is observed also in the SVZ of adult neurogenic niches associated with the occurrence of a progressive and slow accumulation of apoptotic progenitor cells. However, progenitor cells in Btg1-null adult dentate gyrus exit the cycle as early as 2 h after the completion of the S phase (BrdU+/Ki67− cells) and within 20 h express p53 and within 48 h p21 (BrdU+/Ki67−/p53+ or BrdU+/Ki67−/p21+ cells). Thus, considering that the length of cell cycle in dentate gyrus progenitor cells is about 14 h (Mandyam et al., 2007), we hypothesize that dividing progenitor cells, chiefly type-1 stem and type-2a progenitor cells, enter a quiescent state soon after completing the S phase, followed, within a few days, by apoptosis.

Indeed, the observed increase of cell cycle exit, i.e., the entrance into quiescence, and the increase of apoptosis occurring in progenitor cells within a few days after birth, can be accounted for by the induction in Btg1-null newborn cells of the key negative regulator of cell cycle p53 and its effector p21. In fact, any cellular stress signal, such as a misregulation of cell cycle, activates p53 in a specific manner by post-translational modifications (Qian and Chen, 2010). p53 activation leads to either cell cycle arrest and senescence — through p21, its major effector of growth arrest and senescence — or to apoptosis (Kruse and Gu, 2009; Qian and Chen, 2010). It has been shown that mice lacking p53 display an elevated proliferation rate in the adult neurogenic niches associated with an increase in self-renewal and apoptosis (Meletis et al., 2006), while an overexpression of p53 affects the proliferation of stem and progenitor cells in adult neurogenesis (Medrano et al., 2009).
FIGURE 9 | Learning abilities of Btg1-null mice. Water maze. (A) Escape latencies of the first five trials for each of the four successive platform locations. (B) The reduction of escape latencies (saving) animals achieved as they passed from the first to the second trial of the third and fourth training sessions (averaged). (C) The number of trials animals needed to reach the performance criterion in the third and fourth training sessions (averaged). Contextual fear conditioning. (D) Experimental procedure to test one-trial contextual fear conditioning. (E) Upon re-exposure to the shock-associated context, both Btg1-null and wild-type mice showed equally increased levels of freezing behavior; conversely, negligible freezing was detected in a different context. (F) Experimental procedure to test contextual fear-discrimination learning. (G) Wild-type mice were able to discriminate between the shock-associated context and the similar context by day 4 of testing, which was stably maintained until day 7. (H) Conversely, Btg1-null mice started discriminating by day 6, with difference in freezing behavior reaching statistical significance only by day 7. Results are presented as mean ± SEM. *p < 0.05; **p < 0.01.
We cannot exclude that the observed decrease of the number of dentate gyrus and SVZ progenitor cells and neurons. We cannot conclude that the observed decrease of the number of stage 5 and 6 dentate gyrus post-mitotic neurons might in part depend on a requirement of Btg1 not only for maintenance and survival of the pool of progenitor cells, but also for differentiation, as already observed in knockout mice of the family related gene PC3/Tis21 (Farioli-Vecchioli et al., 2009). It appears, however, that unlike Btg1, the main requirement of PC3/Tis21 in the adult hippocampus is for terminal differentiation of stage 6 neurons, where PC3/Tis21 is expressed (Attardo et al., 2010), rather than for cell cycle progression. In fact, after ablation of PC3/Tis21 the pool remains intact, given that only type-3 progenitor cells show a moderate enhancement of proliferation, probably connected to the control of the asymmetric division preceding terminal differentiation (Farioli-Vecchioli et al., 2009).

REDUCED NEUROGENESIS FOLLOWING Btg1 ABLATION IMPAIRS PATTERN SEPARATION

The loss of progenitor cells and terminally differentiated neurons in the dentate gyrus of Btg1-null mice was associated to impairment in hippocampus-dependent learning and memory, namely, a failure in delayed matching-to-place version of the Morris water maze, designed to assess episodic-like components of memory (Chen et al., 2000; Zeng et al., 2001), and a failure to rapidly distinguish between similar settings when trained in a contextual fear-discrimination learning task (McHugh et al., 2007). This is consistent with the knowledge that adult neurogenesis could ultimately contribute to enhance the extent of information encoded by the dentate gyrus and improve pattern separation, which refers to the ability to discriminate among potentially overlapping experiences (Aimone et al., 2011; Sahay et al., 2011b), identified as intrinsic to episodic memory (Tulving, 2002; Yassa and Stark, 2011).

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