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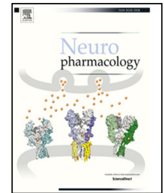
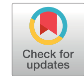


Fluoxetine or Sox2 reactivate proliferation-defective stem and progenitor cells of the adult and aged dentate gyrus



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HIGHLIGHTS

- Fluoxetine activates proliferation-defective stem cells of the adult hippocampus.
- Fluoxetine stimulates the amplification of stem cells also in aged hippocampus.
- The stem cells generated after fluoxetine stimulus mature into new neurons.
- Sox2 rescues the proliferation-defective stem cells of the adult hippocampus.

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ABSTRACT

The dentate gyrus of the hippocampus and the subventricular zone are neurogenic niches where the production of new neurons from glia-like stem cells continues throughout adult life. It is not clear whether the pool of stem cells is fated to be exhausted or is conserved until old age. We observed that the antiproliferative gene Btg1 maintains the quiescence of stem cells, and its ablation causes an increase of stem/progenitor cells proliferation in neonatal mice followed by progressive loss of proliferation during adulthood. Fluoxetine is an antidepressant, which exerts a powerful neurogenic effect on dentate gyrus progenitor cells, but is ineffective on stem cells.

Here we show that adult dentate gyrus stem cells in the Btg1 knockout mice, with reduced self-renewal and proliferative capability, can be reactivated by fluoxetine, which increases their number greatly above the level of control or fluoxetine-treated wild-type mice. The increase of mitotic index above wild-type in Btg1 knockout fluoxetine-treated stem cells indicates that fluoxetine forces quiescent stem cells to enter the cycle. Stem cell proliferation undergoes continuous reactivation until fluoxetine is administered. Remarkably, fluoxetine reactivates proliferation-defective stem cells also in aged Btg1 knockout mice (15-month-old), an effect absent in wild-type aged mice. Moreover, overexpression of Sox2 retrovirally transduced in Btg1 knockout dentate gyrus cells significantly increases the number of neuroblasts, indicating that Sox2 is able to promote the self-renewal of proliferation-defective stem cells.

Overall, the deletion of an antiproliferative gene, such as Btg1, reveals that dentate gyrus stem cells retain a hidden plasticity for self-renewal also in old age, in agreement with a model of permanent self-renewal.

1. Introduction

Neurogenesis remains active throughout adulthood in two main neurogenic areas: the subventricular zone adjacent to the lateral ventricles, which generates olfactory bulb interneurons, and the subgranular zone of the hippocampal dentate gyrus, which gives rise to granular neurons (Imayoshi et al., 2009).

During adult hippocampal neurogenesis putative neural stem cells

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with radial glia-like morphology (type-1 cells, identified by the expression of GFAP in their processes and also of nestin and Sox2; Kempermann et al., 2004; Komitova and Eriksson, 2004; Seri et al., 2001) develop into immature and then into terminally differentiated post-mitotic neurons after completion of successive stages of progenitor cells (type-2ab and type-3 cells; Filippov et al., 2003; Fukuda et al., 2003; Kronenberg et al., 2003). The newborn neurons become morphologically and functionally integrated in the dentate gyrus circuitry within 3 weeks (Ambrogini et al., 2004; Hastings and Gould, 1999; van Praag et al., 2002; Zhao et al., 2006).

Adult neurogenesis is necessary for hippocampus-dependent learning and memory, and especially for pattern separation, which is the ability to finely distinguish among similar memory patterns within the output to the CA3 region; in fact this ability is enhanced by the addition of new neurons (Aimone et al., 2011; Farioli-Vecchioli et al., 2008; Sahay et al., 2011; Tirone et al., 2013).

Some studies suggest that neural stem cells of the adult neurogenic niches are mainly losing proliferative potential during life, but remain able to self-renew (Bonaguidi et al., 2011), while other studies propose that stem cells are determined to be depleted (Encinas et al., 2011; Kempermann, 2011). In fact, when control of the cell cycle is disrupted, this acutely elicits an uncontrolled proliferation of progenitor cells, which after some time ceases, being replaced by a decrease of their proliferative capacity; this latter consists essentially in a greater percentage of stem and progenitor cells entering quiescence, as observed for instance in the knockout of the antiproliferative genes Btg1 and p21 (Farioli-Vecchioli et al., 2012; Kippin et al., 2005). The ablation of Btg1 or of p21 leads to an early postnatal enhancement of proliferation of stem cells, which is however transient; in the adult dentate gyrus and subventricular zone the pool of Btg1-null and p21-null proliferating stem and progenitor cells strongly decreases, with a higher frequency of exit from the cell cycle followed within a few days by apoptosis (Farioli-Vecchioli et al., 2012; Kippin et al., 2005). Thus, after ablation of Btg1 or p21 and following the consequent loss of control of the cell cycle, another component takes place, i.e., an age-dependent decrease of the proliferative capacity of progenitor cells. Consistently, the postnatal increase of proliferation of primary subventricular zone stem cells from young Btg1-null mice is associated to an increase of the asymmetric division, responsible for self-renewal, while stem cells from adult Btg1-null mice show reduced ability to replicate by asymmetric division (Farioli-Vecchioli et al., 2012).

Different systems have been used to induce the amplification of dentate gyrus hippocampal cells, namely, physiological stimuli such as physical activity (van Praag et al., 1999), or artificial manipulations, either by altering corticosterone release through removal of the adrenal glands (Montaron et al., 2006) or by genetically increasing the survival of new neurons (Sahay et al., 2011), or also by treatment with chemical substances impacting on the serotonin (5HT) and GABA pathways (Santarelli et al., 2003; Stachowicz et al., 2009; Wang et al., 2008). In these instances, increased neurogenesis corresponded to improved learning and memory (Sahay et al., 2011). We have recently shown that the enhancement of neurogenesis induced by physical exercise (voluntary running) not only rescues the loss of proliferative potential of the stem cells of dentate gyrus and subventricular zone that occurs in adult Btg1 knockout mice, but it even leads to hyperinduction of the stem cell division above control levels, with a decrease of the cell cycle length (Farioli-Vecchioli et al., 2014). Furthermore, the impaired proliferation of stem cells in Btg1 knockout mice is associated to a behavioral deficit concerning pattern separation, since mice show a reduced ability to finely discriminate between similar contexts, a deficit which is partially rescued by voluntary exercise (Farioli-Vecchioli et al., 2012, 2014).

In this report we aimed to further investigate the model of self-renewal of dentate gyrus stem cells, i.e., whether their pool is fated to be exhausted or conserved until old age, and also to ascertain whether the observed hyperactivation of dentate gyrus stem cells is stimulus-specific and what are its mechanisms.

The importance of exploring the mechanism underlying such hyperactivation of dentate gyrus stem cells devoid of cell cycle control comes from the observations that neither running, nor another powerful neurogenic stimulus such as the antidepressants, are able to induce the expansion of normal stem cells. In fact, several reports indicate that running (Brandt et al., 2010; Kronenberg et al., 2003; Steiner et al., 2008) or fluoxetine (Encinas et al., 2006; Micheli et al., 2017) activate progenitor cells but not glia-like radial stem cells (i.e., GFAP^{+}nestin^{+}Sox2^{+}, type-1 cells).

Thus, we analyzed here the neurogenic action of the antidepressant inhibitor of serotonin reuptake, fluoxetine, as it stimulates neurogenesis efficiently by selective targeting of the serotoninergic neural pathways (Malberg et al., 2000; Santarelli et al., 2003). Moreover, its action appears less dependent from systemic effects implicating general metabolism, vasculogenesis, etc., involved in physical exercise (Falone et al., 2012; Kempermann, 2008).

We observed that the fluoxetine stimulus is able to reactivate the defective proliferation of Btg1-null stem cells, in contrast with its normal effect on adult neurogenesis, which increases the proliferation only of type-2 and type-3 progenitor cells (Encinas et al., 2006). Notably, this reactivation of stem cells occurs also in aged mice, where fluoxetine, unlike running, is devoid of neurogenic action (McAvoy et al., 2015). Furthermore, we observed that also the overexpression of Sox2 in the dentate gyrus of Btg1-null mice is able to overcome, at least in part, the defect of proliferation. Given that Sox2 has been demonstrated to preserve the self-renewal of stem cells (Remboutsika et al., 2011) and to rescue the defective proliferation of RBPJ-null dentate gyrus cells (Ehm et al., 2010), our results suggest that Sox2 and Btg1 may share common pathways. These findings point to the existence of a hidden, residual proliferative potential in stem cells that is brought into evidence by a neurogenic stimulus (fluoxetine or running) after deletion of the quiescence-maintaining gene Btg1.

2. Materials and methods

2.1. Mouse lines and genotyping

The Btg1 knockout mouse line in the C57BL/6 strain was previously generated as described (Farioli-Vecchioli et al., 2012), by inserting the neomycin resistance cassette within exon I of the Btg1 gene. Btg1 knockout and Btg1 wild-type strains were generated crossing Btg1 $^{+/-}$ mice several times, until an isogenic progeny was obtained (also referred to as KO and WT throughout the paper). Genotyping was routinely performed by PCR analysis, using genomic DNA from tail tips as described (Farioli-Vecchioli et al., 2012). Btg1 knockout mice are normal in appearance and fertile. No obvious abnormalities of various organs and tissues were found by histological examinations. Mice were maintained under standard specific-pathogen-free conditions. 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 J.P. Rouault.

2.2. BrdU and fluoxetine treatment of mice and sample preparation for immunohistochemistry

Fluoxetine (10 mg/kg; Tocris Bioscience, Bristol, United Kingdom), or vehicle for control groups (water), were administered daily (by i.p. injection) for 21 days to Btg1 wild-type and knockout mice starting at 56 days or at 15 months of age.

To detect progenitor cells in the dentate gyrus entering the S phase after fluoxetine treatment (Fig. 1 and Fig. S1), mice at postnatal day 77 (P77) were perfused 2 h after treatment with bromodeoxyuridine (BrdU; a single injection, 95 mg/kg i.p.; Sigma–Aldrich, S.Louis, MO,

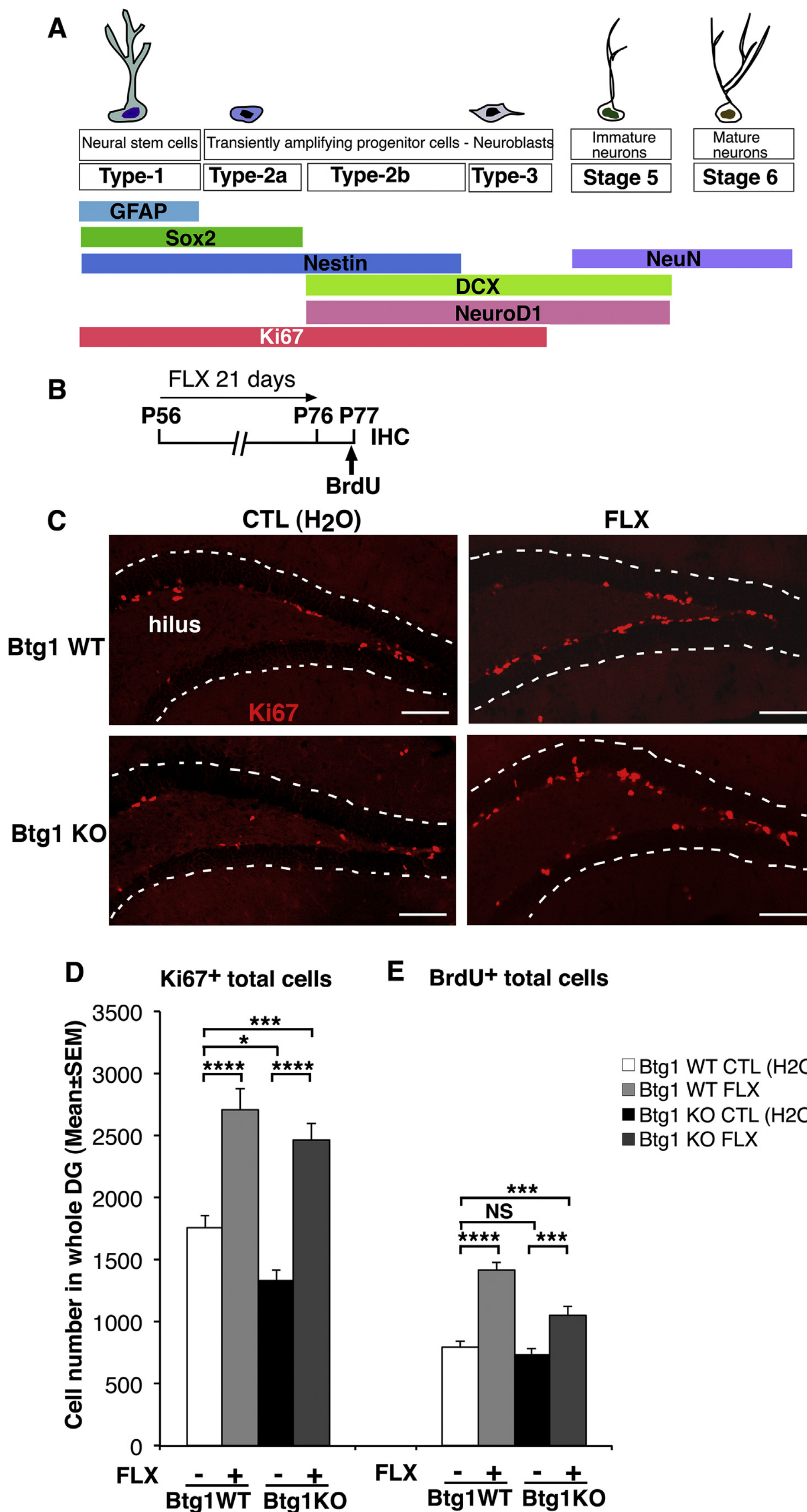

Fig. 1. Treatment with fluoxetine promotes cellular proliferation in the dentate gyrus of wild-type and Btg1 knockout mice. (A) Developmental stages of neural cells in the dentate gyrus and markers. (B) Scheme of treatment of adult (P56) mice for 21 days with daily injections of fluoxetine. BrdU (95 mg/kg) was injected two hours before the sacrifice of mice. (C) Representative images showing the increase of cycling ($Ki67^+$) cells in the dentate gyrus of Btg1 wild-type and knockout mice treated with fluoxetine (WT FLX and KO FLX, respectively), compared to control treatment (water; WT CTL and KO CTL). The white dotted line marks out the dentate gyrus boundaries. Scale bar, 100 μm. (D) Fluoxetine induced a significant increase of dividing ($Ki67^+$) cells in both wild-type and Btg1 knockout genotypes. Analysis of simple effects: * $p < 0.05$, *** $p < 0.001$ or **** $p < 0.0001$, PLSD ANOVA test. (E) Quantification of the absolute number of dentate gyrus cells entering in S phase (total $BrdU^+$ cells after a 2h pulse) showed a significant difference between Btg1 wild-type and knockout mice treated with fluoxetine, relative to their respective controls. Analysis of simple effects: NS $p > 0.05$, *** $p < 0.001$ or **** $p < 0.0001$, PLSD ANOVA test. (D, E) Cell numbers in the dentate gyrus are means ± SEM of the analysis of three animals per group.

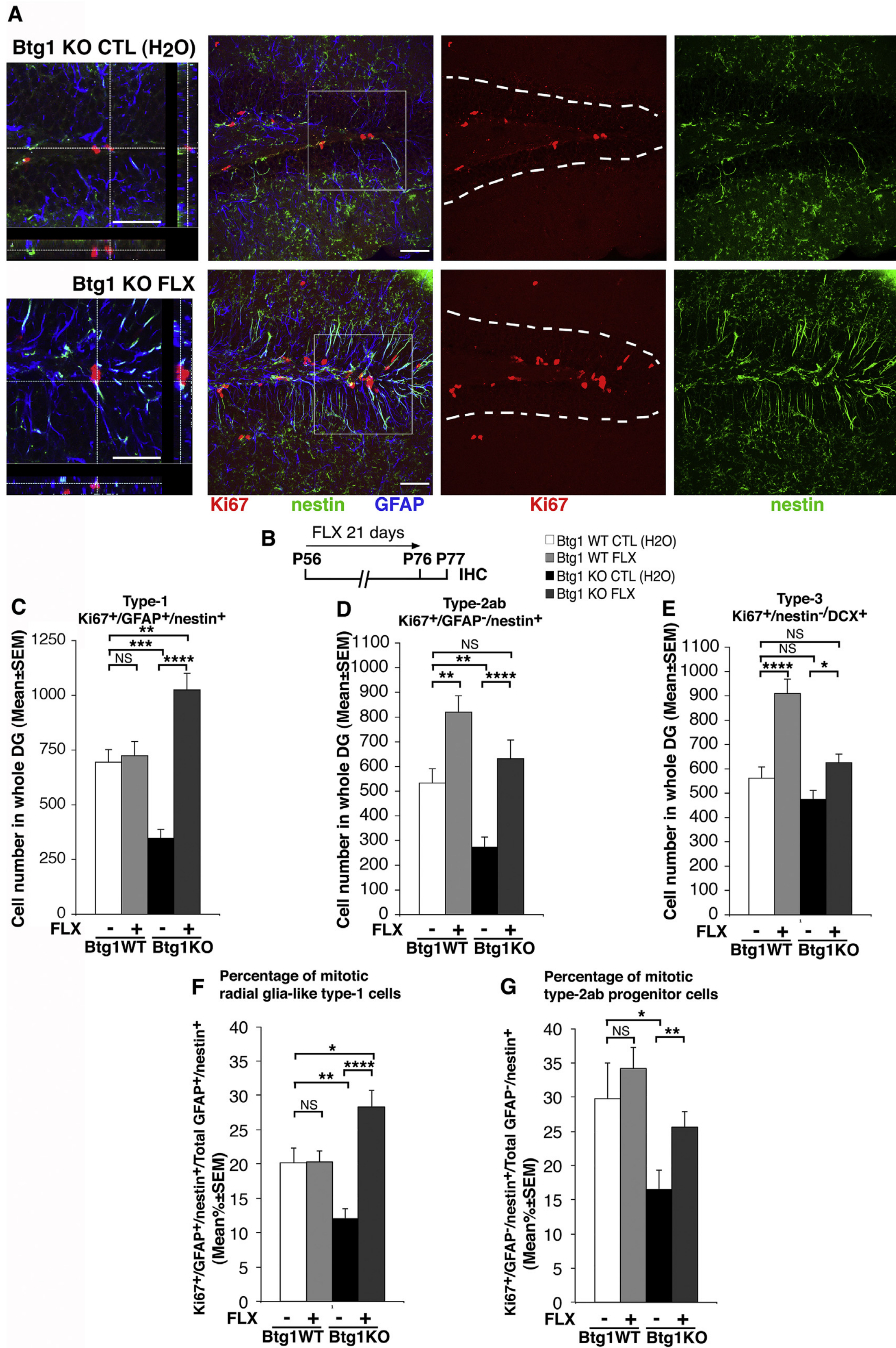

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Fig. 2. Fluoxetine treatment fully reverses the decrease of stem cells occurring in the adult dentate gyrus of Btg1 knockout mice. (A) Representative images by confocal microscopy with 3D reconstruction and orthogonal projections of triple-labeled cells, showing that fluoxetine increases the proliferating type-1 stem cells ($Ki67^+/GFAP^+/nestin^+$) in the dentate gyrus of Btg1 knockout mice, relative to the water-treated Btg1 knockout group (KO CTL). The white dotted line shows the dentate gyrus boundaries. The orthogonal projections correspond to the white box area, with 1.65× magnification. Scale bar, 50 µm. (B) Scheme of treatment of adult (P56) mice for 21 days with daily injections of fluoxetine. (C) Quantification of the absolute number of proliferating dentate gyrus stem cells (type-1; $Ki67^+/GFAP^+/nestin^+$). The significant decrease of Btg1 KO CTL stem cells, relative to Btg1 WT CTL, is reversed above control levels in KO FLX by fluoxetine treatment. (D) The number of type-2ab ($Ki67^+/GFAP^-/nestin^+$) and (E) type-3 progenitor cells ($Ki67^+/nestin^-/DCX^+$), which decreases in Btg1 KO CTL mice relative to WT CTL, is significantly induced by fluoxetine in both Btg1 WT FLX and KO FLX mice. (C–E) Analysis of simple effects: NS $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$, PLSD ANOVA test. Cell numbers in the dentate gyrus are means ± SEM of the analysis of three animals per group. (F) The percentage of stem cells recruited to the cell cycle (mitotic ratio, measured between $Ki67^+/GFAP^+/nestin^+$ and total $GFAP^+/nestin^+$ cells) was significantly increased in KO FLX mice above all other conditions; analysis of simple effects: KO FLX vs all other groups $p \leq 0.02$ Mann-Whitney *U* test. (G) The mitotic ratio of type-2ab progenitor cells (ratio between $Ki67^+/GFAP^-/nestin^+$ and total $GFAP^-/nestin^+$ cells) increased significantly in KO FLX mice only relative to KO CTL. (F, G) Cell percentages in the dentate gyrus are means % ± SEM of the analysis of the same sections of the three animals per group used in (C–E). Analysis of simple effects: NS $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ or < **** $p < 0.0001$, Mann-Whitney *U* test.

USA), according to previous protocols (Arguello et al., 2008). In adult and 15-month-old Btg1 wild-type and knockout mice, 1- to 5- day-old neurons in the dentate gyrus were detected by BrdU incorporation, after treatment with five daily injections of BrdU (95 mg/kg i.p.; Figs. 4 and 5); BrdU was injected during the last 5 days of treatment with fluoxetine and mice were perfused the day after (Farioli-Vecchioli et al., 2008). Alternatively, to analyze 28-day-old mature neurons, adult mice were perfused 28 days later (at P104; Fig. 4). In the kinetics of expansion of the proliferating stem cells (Fig. 3), these were detected either 2, 48 or 72 h after a single BrdU injection (150 mg/kg i.p.) performed at P74.

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

2.3. Immunohistochemistry

Brains were embedded in Tissue-Tek OCT (Sakura, Torrence, CA, USA) and cut with a cryostat at −25 °C in serial free-floating sections of 40 µm thickness. Sections were then processed immunohistochemically for multiple labeling with BrdU and other cellular markers using fluorescent methods. After permeabilization with 0.3% TritonX100 in PBS, sections were incubated with primary antibodies with 3% normal donkey serum in PBS for 16–18 h.

BrdU incorporation was visualized by denaturing DNA through pretreatment of sections with 2N HCl 45 min at 37 °C followed by 0.1 M sodium borate buffer pH 8.5 for 10 min. Sections were then incubated overnight at 4 °C with a rat monoclonal antibody against BrdU (AbD Serotech, Raleigh, NC, USA; MCA2060; 1:400). Proliferation was detected also by using the rabbit monoclonal anti-Ki67 (LabVision Corporation, Fremont, CA; clone SP6; 1:200). The antibodies against Ki67 and BrdU were co-incubated with other primary antibodies, in order to detect the subpopulations of dentate gyrus stem, progenitor cells and neurons: mouse monoclonal antibodies against nestin (Millipore, Temecula, CA; MAB353; 1:100) or against NeuN (Millipore, Temecula, CA; MAB377; 1:300) or GFAP (Sigma Aldrich, St Louis, MO; G6171; 1:200); goat polyclonal antibodies against GFAP (Santa Cruz Biotechnology, Santa Cruz, CA; SC-6170; 1:300) or doublecortin (DCX; Santa Cruz Biotechnology; SC-8066; 1:300) or Sox2 (Santa Cruz Biotechnology; SC-17320; 1:300).

Secondary antibodies used to visualize the antigen were all from Jackson ImmunoResearch (West Grove, PA, USA) as follows: a donkey anti-rabbit antiserum conjugated to Cy3 (Ki67), a donkey anti-rat antiserum TRITC-conjugated (BrdU), a donkey anti-mouse conjugated to Cy2 (nestin) or to Alexa-647 (NeuN, GFAP), a donkey anti-goat conjugated to Alexa-647 (GFAP) or to Cy2 (DCX, Sox2). The green fluorescent protein was directly visualized by microscopy. Nuclei were counterstained by Hoechst 33258 (Sigma–Aldrich; 1 mg/ml in PBS).

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

2.4. Generation of recombinant viruses and infection in vivo

To express the cDNA of Sox2 in dividing neural cells, we used the retroviral vector pCAG-IRES-GFP, kindly provided by Dr. Chichung Lie (Institute of Developmental Genetics, Germany; Jessberger et al., 2008). The pCAG-IRES-GFP-Sox2 sequence was obtained by cloning the full open reading frame of Sox2 mouse cDNA in the sites SfiI-5'/PmeI-3' of pCAG-IRES-GFP. The Sox2 sequence cloned into pCAG-IRES-GFP vector was synthesized by MWG (Ebersberg, Germany) and was checked by DNA sequencing.

The pCAG-IRES-GFP-Sox2 and the pCAG-IRES-GFP-empty (control) retroviruses were propagated and concentrated as described (Farioli-Vecchioli et al., 2008). P70 Btg1 knockout and wild-type mice were anesthetized (10/10/4 mg/kg tiletamine HCL/zolazepam HCL/xylazine, i.p.) and the concentrated virus solution (108 pfu/ml) was infused (1.5 µl at 0.32 µl/min) by stereotaxic surgery into the right and left dentate gyrus (anteroposterior = −2 mm from bregma; lateral = ± 1.5 mm; ventral = 2.0 mm). After surgery mice were administered with 1 ml of normal saline (0.9%) intraperitoneally to prevent dehydration and were monitored post-operatively until they showed undisturbed moving behaviour. Mice were euthanized after 5 days.

2.5. Quantification of cell numbers

The analysis of the number of cells positive for each marker was performed throughout the whole rostro-caudal extent of the dentate gyrus in one-in-eight series of 40 µm free-floating coronal sections (320 µm apart), which were analyzed by confocal microscopy. 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 comprising the entire dentate gyrus (about 50–60 sections), as described (Farioli-Vecchioli et al., 2008; Jessberger et al., 2005; Kee et al., 2007; see also about the cell counting theory: Noori and Fornal, 2011). At least three animals per group were analyzed (see Tables S1 and S2).

In Fig. 2F and G cell numbers were calculated as percentage ratios of Ki67-type-1 or Ki67-type-2ab cells to the total number of type-1 or type-2ab cells, respectively. The Sox2 virus-infected cells (Fig. 6) were calculated as percentage ratios between GFP^+ type-1-2a, -2b or −3 cells and the total number of GFP^+ cells in each section, and then averaged. For each virus-injected mouse at least 20 sections throughout the whole extent of the dentate gyrus were analyzed; the average number of GFP^+ cells identified per mouse was about 30–60. Cell number analyses were performed manually by trained experimenters using the I.A.S. software to record positive cells (Delta Sistemi, Rome, Italy).

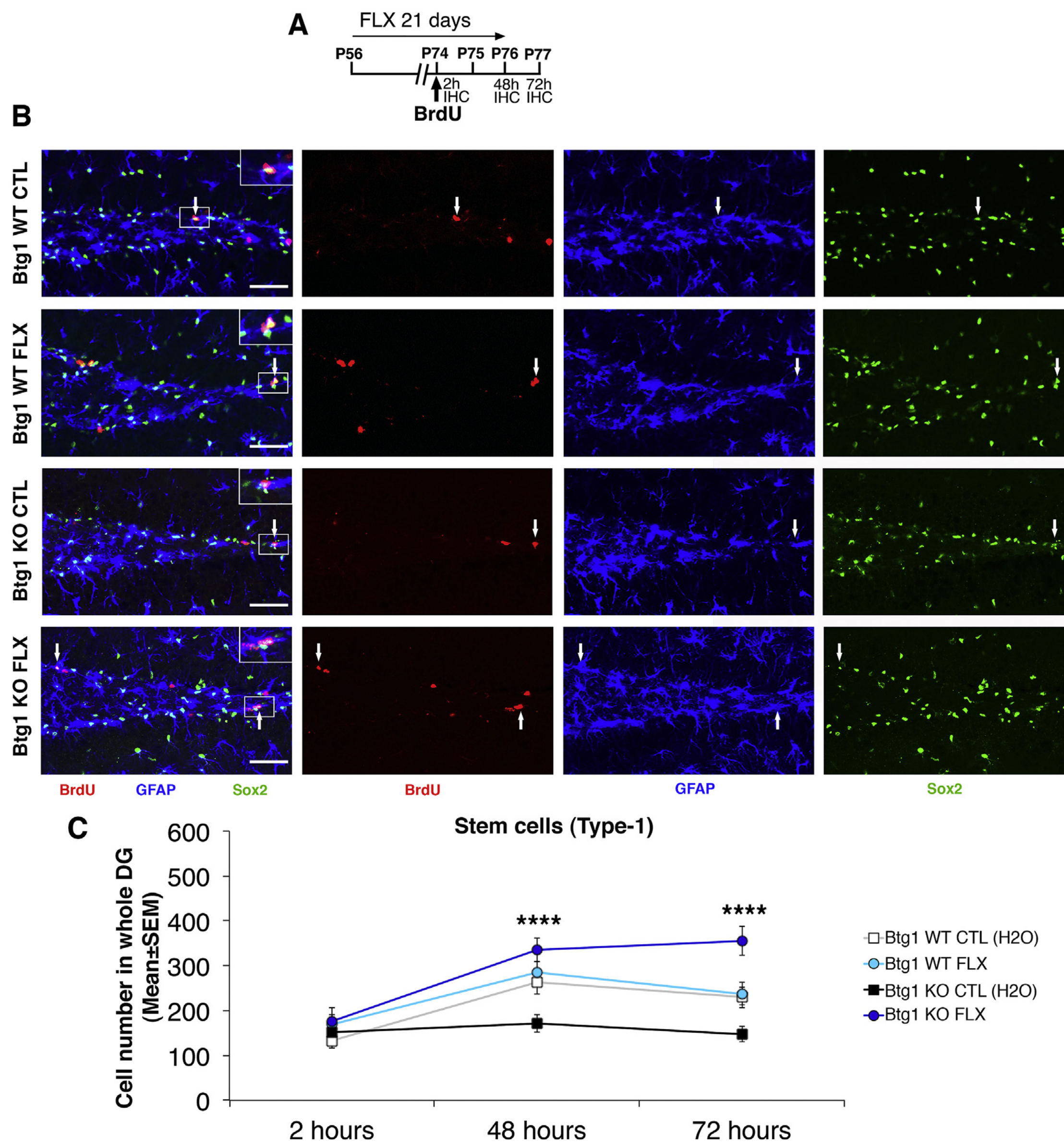

Fig. 3. Btg1-null stem cells are reactivated throughout the whole fluoxetine treatment. (A) Scheme of treatment of adult (P56) mice for 21 days with daily injections of fluoxetine. After 19 days of treatment, mice received a single injection of BrdU (150 mg/kg) and the total number of type-1 cells in the dentate gyrus (identified as $BrdU^{+}/Sox2^{+}/GFAP^{+}$) was measured during the following 2, 48, or 72 h of treatment. (B) Representative images showing how type-1 cells increase in the dentate gyrus of the KO FLX mice relative to the other experimental groups, 72 h after the injection of BrdU, as detected by BrdU incorporation (red) and by Sox2 (green) and GFAP (blue) multiple-labeling confocal microscopy. Arrows indicate cells positive for BrdU, GFAP and Sox2. The white box area is shown with 1.8× digital magnification. Scale bar 50 μm. (C) Measurement of the time-dependent changes in the number of type-1 neural stem cells. In the KO CTL mice the number of type-1 cells remained quite stable at the successive 2, 48 and 72 h time-points, whereas in the KO FLX mice the neural stem cells significantly expanded after 48 h (analysis of simple effects: KO FLX vs KO CTL $p < 0.0001$, PLSD ANOVA test) and after 72 h (analysis of simple effects: KO FLX vs KO CTL $p < 0.0001$, PLSD ANOVA test). In contrast, no significant difference occurred between WT CTL and WT FLX mice at 48 and 72 h. Cell numbers in the dentate gyrus are means ± SEM of the analysis of three animals (2 h and 48 h) or of four animals (72 h) per group. **** $p < 0.0001$, PLSD ANOVA test.

2.6. Statistical analysis

The effect of fluoxetine in wild-type and Btg1 knockout mice was statistically analyzed in all experiments using two-way ANOVA (i.e., to test genotype or fluoxetine main effects). Individual between-group comparisons to test simple effects were carried out by Fisher's PLSD ANOVA post-hoc test. The variance of data in the fluoxetine experiments that were calculated as percentage of proliferating stem and progenitor cells (type-1 or type-2ab) to the total number of type-1 or type-2ab cells (Fig. 2F and G, respectively), was analyzed - after checking the non equality of variances with the Levene's test - using the Kruskall-Wallis test, appropriate for non-normal distribution; individual between-group comparisons were thereafter performed with the non parametric Mann-Whitney *U* test that does not require the assumption of normal distribution. The same non parametric tests were used for Fig. 6C to analyze the percentage ratio between virus-infected (GFP^{+}) type-1-2a, type-2b or type-3 cells and the total number of infected cells.

ANOVA and non-parametric analyses are summarized in Table S1 and Table S2, respectively. Table S2 reports also the analysis performed to test the equality of variances in experiments calculated with a percentage (using Levene's test).

These analyses were performed using the StatView 5.0 software (SAS Institute, Cary, NC, USA). Differences were considered statistically significant at $p < 0.05$. All data were expressed as mean

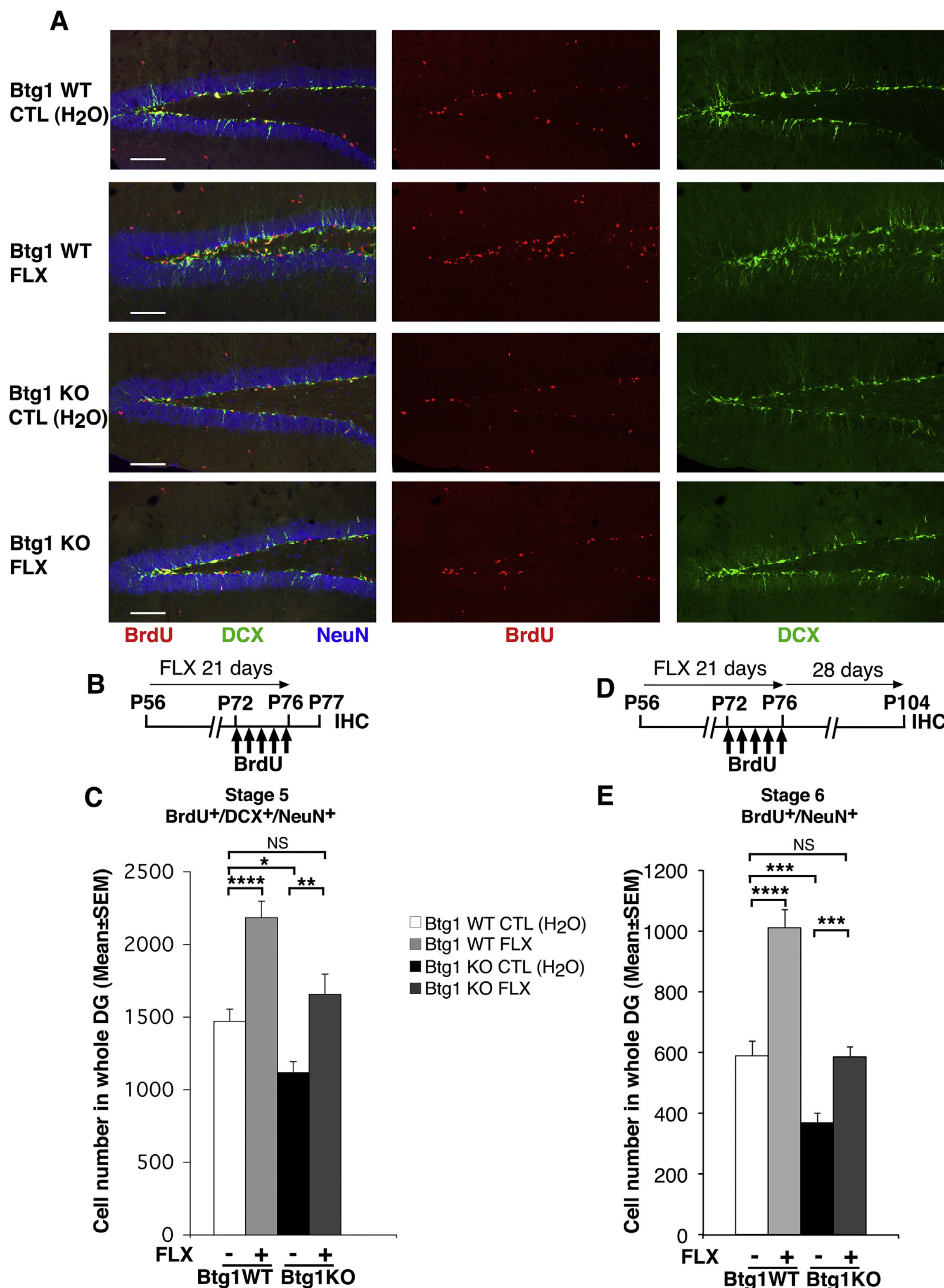

Fig. 4. The Btg1 knockout stem/progenitor cells induced by fluoxetine differentiate into new neurons. (A) Representative images showing that fluoxetine increases the early differentiated (1- to 5-day-old) stage 5 neurons (BrdU$^+$/DCX$^+$/NeuN$^+$) in the dentate gyrus of Btg1 knockout mice, relative to control groups (i.e., KO CTL or WT CTL). Scale bar, 100 µm. (B) Scheme of treatment of adult (P56) mice for 21 days with daily injections of fluoxetine and of BrdU (five injections, 95 mg/kg). (C) The number of stage 5 early differentiated neurons decreases in Btg1 KO CTL mice, relative to WT CTL, and is significantly augmented by fluoxetine in both Btg1 WT FLX and KO FLX mice (stage 5, BrdU$^+$/DCX$^+$/NeuN$^+$; analysis of simple effects: NS $p > 0.05$, * $p < 0.05$, or ** $p < 0.01$, or **** $p < 0.0001$, PLSD ANOVA test). Cell numbers in the dentate gyrus are means ± SEM of the analysis of three animals per group. (D) Scheme of treatment of adult (P56) mice for 21 days with daily injections of fluoxetine and BrdU (five injections, 95 mg/kg), followed by 28 days without treatment. (E) Terminally differentiated neurons decrease in Btg1 KO CTL mice, relative to WT CTL, and are significantly increased by fluoxetine in both Btg1 WT FLX and KO FLX mice (stage 6, BrdU$^+$/NeuN$^+$; analysis of simple effects: NS $p > 0.05$, *** $p < 0.001$, or **** $p < 0.0001$, PLSD ANOVA test). Cell numbers in the dentate gyrus are means ± SEM of the analysis of five animals per group.

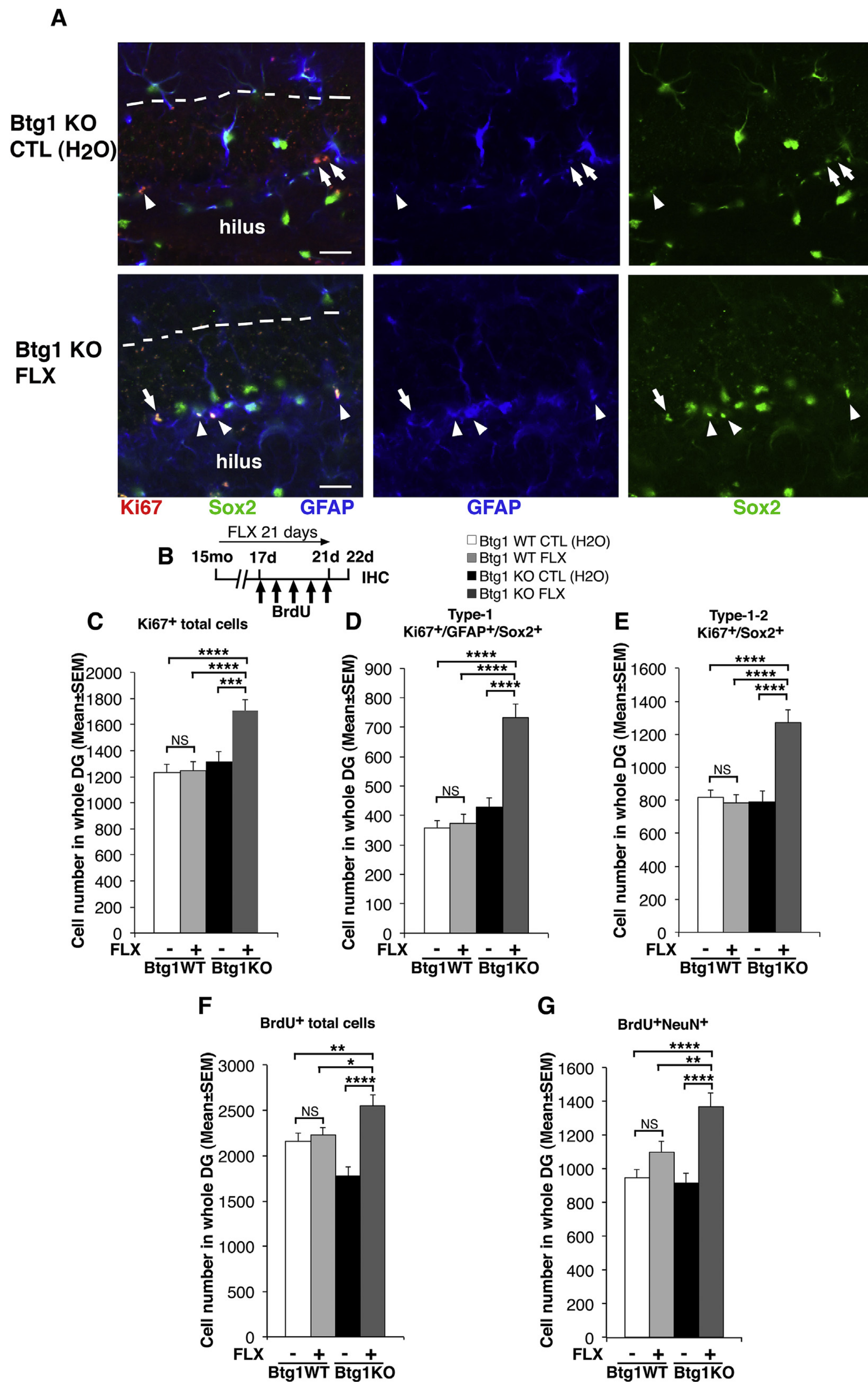

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Fig. 5. Btg1-null stem cells of the dentate gyrus are highly activated by fluoxetine treatment also in aged (15-month-old) mice. (A) Representative images by confocal microscopy showing that fluoxetine increases the proliferating type-1 stem cells ($Ki67^+/GFAP^+/Sox2^+$) in the dentate gyrus of Btg1 knockout mice (KO FLX), relative to control. The white dotted line shows the dentate gyrus dorsal boundary. The white arrowheads indicate cells positive for Ki67, GFAP and Sox2 (type-1), white arrows cells positive only for Ki67 and Sox2 (type-2). Scale bar, 20 μm. (B) Scheme of treatment of 15-month-old (15mo) mice with daily injections of fluoxetine for 21 days and of BrdU for 5 days (95 mg/kg) before the sacrifice. (C) The total number of dividing cells in the dentate gyrus ($Ki67^+$) is increased by fluoxetine only in Btg1 knockout cells. (D, E) The quantification of the number of proliferating dentate gyrus stem cells (type-1; $Ki67^+/GFAP^+/Sox2^+$) (D), or of proliferating type-1 cells analyzed together with type-2 progenitor cells ($Ki67^+/Sox2^+$) (E), shows that both stem and progenitor cells are highly increased by fluoxetine only in Btg1 knockout mice; conversely, no effect by fluoxetine is seen in wild-type dentate gyrus type-1 and type-2 cells. (F, G) The total number of 1- to 5-day-old proliferating cells in the dentate gyrus, detected as $BrdU^+$ cells (F), or of 1- to 5-day-old neurons, detected as $BrdU^+NeuN^+$ (G), are greatly increased only in Btg1 knockout fluoxetine-treated dentate gyrus, indicating that the newly generated stem cells mature into neurons. (C–G) Cell numbers in the dentate gyrus are means ± SEM of the analysis of five animals per group. Analysis of simple effects: NS $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$, PLSD ANOVA test.

values ± SEM.

The minimum sample size required in each experimental group was defined by a priori Power analysis using the G*Power software (Heinrich Heine, Düsseldorf University, Germany).

3. Results

3.1. The neurogenic stimulus by fluoxetine induces stem cells in the dentate gyrus of Btg1 knockout mice to exit from quiescence and proliferate

In this report, our first aim was to assess whether the neurogenic effect of fluoxetine was able to rescue the reduced adult hippocampal proliferation observed in the adult Btg1-null mice (Farioli-Vecchioli et al., 2012).

Thus, we induced the process of neurogenesis by chronically treating two-month-old wild-type and Btg1 knockout mice with fluoxetine for 21 days or with vehicle (control). At the end of treatment we analyzed the proliferating stem and progenitor cells either by labeling all cycling cells by Ki67 (Scholzen and Gerdes, 2000), or by labeling with bromodeoxyuridine (BrdU) the cells that entered in S phase, after treating all groups with a short pulse of BrdU 2 h before the sacrifice (see schemes, Fig. 1A and B).

Immunohistochemical analysis showed that in control Btg1 knockout mice the number of dividing cells, analyzed by measuring $Ki67^+$ cells, was significantly lower than in control wild-type mice, as expected (Farioli-Vecchioli et al., 2014; two-way ANOVA genotype effect $F_{1,101} = 6.4$ $p = 0.012$; KO CTL vs WT CTL $p = 0.042$ PLSD test, 24% decrease; Fig. 1C and D). Fluoxetine stimulated the proliferation rate, as indicated by the 54% increase in the number of Ki67-positive cells in the fluoxetine-treated (FLX) wild-type mice, relative to the wild-type control mice (two-way ANOVA, treatment effect, $F_{1,101} = 57.8$ $p < 0.0001$; WT FLX vs WT CTL $p < 0.0001$ PLSD test; Fig. 1C and D). An even greater increase (85%) of proliferation was observed in the fluoxetine-treated Btg1 knockout mice (KO FLX) compared to the Btg1-null control mice (KO FLX vs KO CTL $p < 0.0001$ PLSD test; Fig. 1C and D). Notably, the number of Ki67-labeled cells in the KO FLX mice resulted significantly greater than in the WT CTL mice ($p = 0.0008$ PLSD test; 40% increase; Fig. 1C and D), reaching a proliferation rate equivalent to that observed in the WT FLX mice.

An equivalent induction of the rate of proliferation by fluoxetine of stem/progenitor cells was detected by analyzing BrdU incorporation 2 h after a single injection of BrdU. In fact, WT FLX as well as KO FLX mice presented a significant increase of $BrdU^+$ cells, relative to their corresponding controls (two-way ANOVA, treatment effect, $F_{1,119} = 71.4$ $p < 0.0001$; WT FLX vs WT CTL $p < 0.0001$ PLSD test, with 77% increase, or KO FLX vs KO CTL $p = 0.0002$ PLSD test, with 42% increase; Figs. S1 and 1E). Moreover, the ablation of Btg1 in itself did not influence the number of cells in S phase, as previously observed (Farioli-Vecchioli et al., 2014; WT CTL vs KO CTL $p = 0.46$ PLSD test; Fig. 1E).

These data indicated that a 5HT-ergic neurogenic stimulus strongly induced the proliferation of Btg1 knockout stem/progenitor cells and was able to reverse their reduced proliferative capability.

We further asked whether this neurogenic stimulus induced a specific subpopulation of dividing stem/progenitor cells in two-month-old mice. We observed that the number of dividing stem cells (type-1 cells), identified by co-expression of GFAP and nestin (Filippov et al., 2003; Fukuda et al., 2003; Seri et al., 2001), was significantly lowered in the KO CTL relative to WT CTL mice, indicating a decreased generation of this cell type ($Ki67^+/GFAP^+/nestin^+$; about 50% decrease; $p = 0.0007$ PLSD ANOVA test; Fig. 2A–C). Moreover, stem cells of wild-type mice were not increased by treatment with fluoxetine, in agreement with the known observation that neural stem cells are not induced by this neurogenic stimulus (Encinas et al., 2006; WT FLX vs WT CTL, $p = 0.77$ PLSD test; Fig. 2C). Fluoxetine treatment, however, strongly increased the number of Btg1 knockout dividing stem cells relative to knockout control, i.e., of about three-fold (two-way ANOVA, treatment effect, $F_{1,101} = 28.0$ $p < 0.0001$; KO FLX vs KO CTL, $p < 0.0001$ PLSD test; Fig. 2C), and of 1.5-fold relative to the number of stem cells in wild-type control mice (KO FLX vs WT CTL; $p = 0.0011$ PLSD test; Fig. 2C).

In parallel, we analyzed the whole population of type-2ab cells (considered as transiently amplifying progenitor cells derived from type-1 cells, identified as $Ki67^+/GFAP^-/nestin^+$; Filippov et al., 2003; Fukuda et al., 2003), or of the neuronally committed type-3 progenitor cells that cease to express nestin but express doublecortin (DCX; $Ki67^+/DCX^+/nestin^-$; Kronenberg et al., 2003). Indeed fluoxetine greatly increased the number of type-2ab as well as of type-3 progenitor cells, in both WT mice and KO mice (type-2ab $Ki67^+/GFAP^-/nestin^+$: two-way ANOVA treatment effect, $F_{1,101} = 28.2$ $p < 0.0001$; WT FLX vs WT CTL, $p = 0.002$ PLSD test; KO FLX vs KO CTL, $p < 0.0001$ PLSD test; Fig. 2D. Type-3 $Ki67^+/DCX^+/nestin^-$: two-way ANOVA treatment effect, $F_{1,129} = 31.0$ $p < 0.0001$; WT FLX vs WT CTL, $p < 0.0001$ PLSD test; KO FLX vs KO CTL, $p = 0.014$ PLSD test; Fig. 2E). Moreover, the number of type-2ab but not type-3 cells is reduced in KO mice, relative to WT mice (as previously observed, Farioli-Vecchioli et al., 2012; KO CTL vs WT CTL: type-2ab $p = 0.004$ Fig. 2D, and type-3 $p = 0.17$ Fig. 2E, PLSD test).

We further analyzed the mitotic ratio of type-1 neural stem and type-2 progenitor cells, in order to have a measurement of cells recruited to the cell cycle (expressed by the percentage of $Ki67^+/GFAP^+/nestin^+$ cells to total $GFAP^+/nestin^+$ for type-1 cells, and by the percentage of $Ki67^+/GFAP^-/nestin^+$ cells to total $GFAP^-/nestin^+$ for type-2ab cells). Interestingly, this mitotic ratio significantly increased in the stem cells of KO FLX mice, relative not only to KO CTL (Kruskall-Wallis [d.f. 3] $H = 36.17$, $p < 0.0001$; $p < 0.0001$ Mann-Whitney U test; Fig. 2F) but also to WT CTL and WT FLX mice (about 40% increase, $p = 0.027$ and $p = 0.009$, respectively, Mann-Whitney U test; Fig. 2F), whereas the mitotic ratio for type-2ab cells of KO FLX mice increased only relative to KO CTL mice (Kruskall-Wallis [d.f. 3] $H = 19.66$, $p = 0.0002$; $p = 0.002$ Mann-Whitney U test; Fig. 2G). In contrast, as expected (Encinas et al., 2006), no significant difference was observed between the mitotic ratios of type-1 stem cells and type 2-ab progenitor cells in WT FLX and in WT CTL mice ($p = 0.83$ Fig. 2F, and $p = 0.18$ Fig. 2G, Mann-Whitney U test). As a whole, this provided the key information that in Btg1-null mice fluoxetine did induce the entry of stem

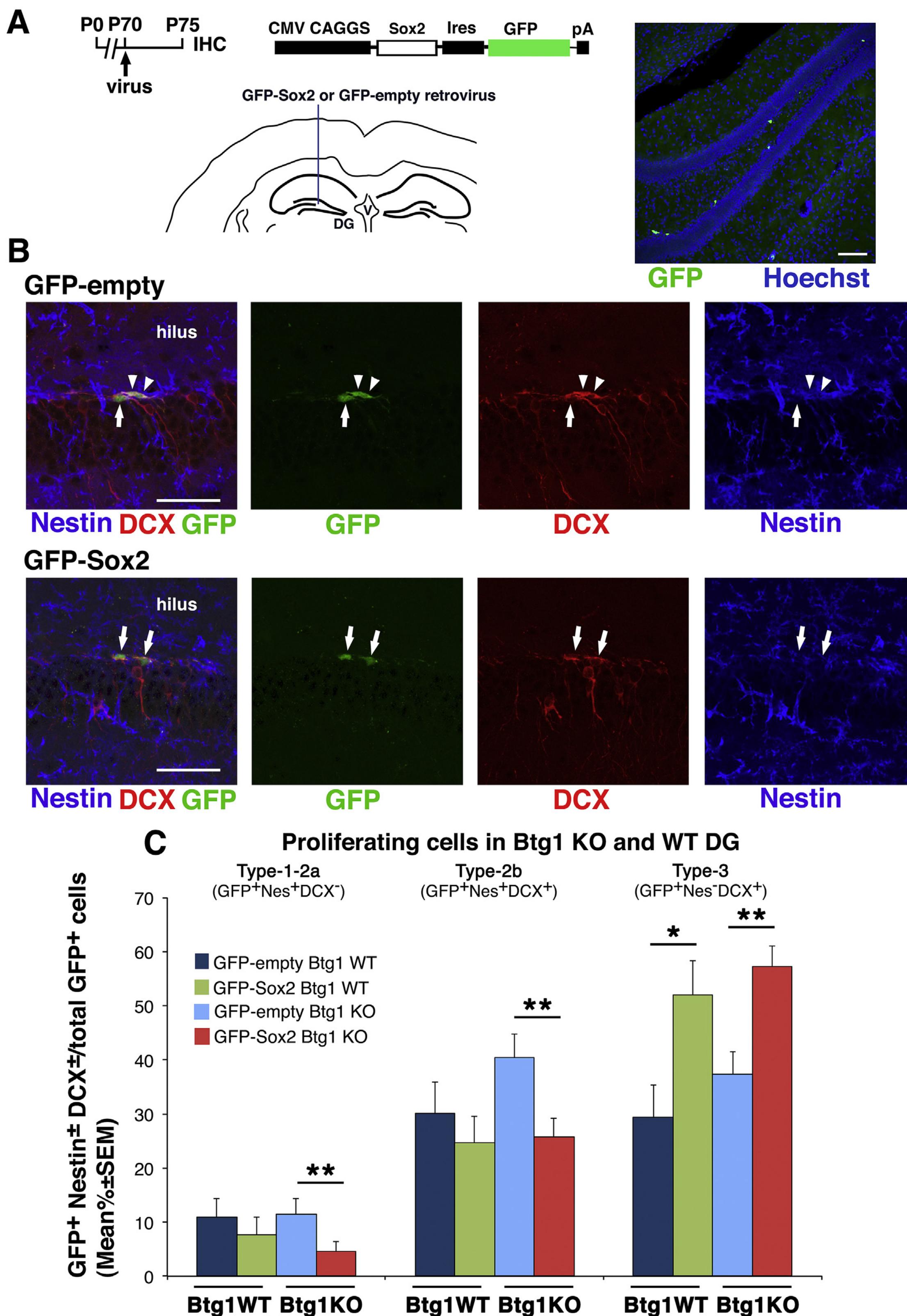

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Fig. 6. Sox2-retrovirus rescues the defective proliferation of Btg1-null progenitor cells. (A) Retrovirus infection protocol, structure, injection area and representative image of the extent of infection (GFP-infected cells are green, nuclei are identified by Hoechst 33258). Scale bar, 100 µm. (B) Representative confocal images (40× magnification) of coronal sections of the dentate gyrus of Btg1 knockout mice, labeled with nestin, DCX and with GFP, 5 days after infection with either GFP-Sox2 or GFP-empty retroviruses. Scale bars, 50 µm. The white arrowheads indicate cells positive for GFP nestin and DCX (type-2b), white arrows cells positive only for GFP and DCX (type-3). (C) Percentage ratio between $GFP^+nestin^+DCX^-$ cells (type-1-2a), $GFP^+nestin^+DCX^+$ cells (type-2b) or $GFP^+nestin^-DCX^+$ cells (type-3), and the total number of infected cells (GFP^+), in Btg1 knockout and wild-type dentate gyrus infected with GFP-Sox2 or GFP-empty retroviruses. The percentage of type-3 cells is significantly increased by the Sox2 virus, relative to control virus infections. Cell ratios in the dentate gyrus are mean% ± SEM of the analysis of three animals per group. GFP-empty cells counted: Btg1 KO n = 174 and Btg1 WT n = 113; GFP-Sox2 cells counted: Btg1 KO n = 189 and Btg1 WT n = 100. * $p < 0.05$ or ** $p < 0.01$ vs cells infected with GFP-empty virus, Mann-Whitney *U* test.

cells into the cycle from a state of quiescence.

3.2. Fluoxetine continuously reactivates the division of a cohort of Btg1 knockout stem cells throughout the whole fluoxetine treatment

We further sought to monitor the kinetics of the expansion of the dividing stem cells, and with this aim we tracked the proliferation of these cells by a pulse-labeling experiment. One cohort of dividing stem/progenitor cells was labeled thanks to a single injection of BrdU, and the number of proliferating type-1 stem cells, identified by BrdU/Sox2/GFAP (Kempermann et al., 2004; Komitova and Eriksson, 2004), was analyzed during the following 72 h (Fig. 3A and B). In fact, the transcription factor Sox2 labels quiescent and proliferating neural stem cells, and also immature progenitor cells (type-2a; Kempermann et al., 2004; Suh et al., 2007).

After 2 h we found no significant change in the number of proliferating type-1 stem cells in the Btg1-null mice (KO CTL) with respect to the WT CTL mice, as previously observed for the $BrdU^+$ total cells. During the following 72 h, however, the stem cell population in KO CTL did not expand, remaining stable in number - which is compatible with the longer S phase demonstrated in adult Btg1 knockout stem/progenitor cells (Farioli-Vecchioli et al., 2014) - whereas the stem cells from the WT FLX or WT CTL groups increased significantly and to the same extent (at 48 h, two-way ANOVA, treatment effect $F_{1,114} = 14.203$ $p = 0.0003$; KO CTL vs WT CTL or vs WT FLX, $p = 0.009$ or $p = 0.0011$, respectively, PLSD test. At 72 h, two-way ANOVA, treatment effect $F_{1,129} = 10.387$ $p = 0.0016$; KO CTL vs WT CTL or vs WT FLX, $p = 0.005$ or $p = 0.006$, respectively, PLSD test; Fig. 3B and C). The lack of difference in stem cell numbers between the WT FLX and WT CTL groups is consistent with the known absence of effect on stem cells by fluoxetine (Encinas et al., 2006). The greatest increase of all was observed in stem cells from Btg1-null mice treated with fluoxetine both at 48 and 72 h (at 48 h, KO FLX vs KO CTL or vs WT CTL or vs WT FLX, $p < 0.0001$ or $p = 0.046$ or $p = 0.16$, respectively, PLSD test; at 72 h, $p < 0.0001$ or $p = 0.0006$ or $p = 0.002$, respectively, PLSD test; Fig. 3B and C). This indicated that the cohort of stem cells (type-1) initially labeled in Btg1-null mice, if challenged with fluoxetine undergoes expansion and accumulation above control (WT CTL) throughout the whole period of treatment. This means that during treatment the proliferation-defective Btg1-null stem cells undergo continuous reactivation, whereas fluoxetine is devoid of effect in wild-type stem cells.

3.3. The stem cells generated in Btg1 knockout dentate gyrus after fluoxetine stimulus mature into new neurons

We then sought to measure whether the increased proliferation of progenitor and stem cells caused by fluoxetine corresponded to an increased production of differentiated neurons. As maturation proceeds, the dentate gyrus progenitor cells become post-mitotic, attaining stage 5 (Kempermann et al., 2004), indicated by expression of the mature neuronal marker NeuN, which coexists with DCX and with calretinin (Brandt et al., 2003; Steiner et al., 2004).

Thus, we measured the 1- to 5-day-old stage 5 early differentiated neurons, identified as $BrdU^+/DCX^+/NeuN^+$ cells after treating P56 wild-type and knockout mice with fluoxetine for 21 days, and with five daily injections of BrdU starting at P72; immunohistochemistry was performed the day following the end of BrdU treatment (P77; Fig. 4A and B). We observed a decrease of stage 5 neurons in Btg1 knockout mice, relative to wild-type mice (two-way ANOVA genotype effect, $F_{1,97} = 14.78$ $p = 0.0002$; KO CTL vs WT CTL, $p = 0.031$ PLSD test; Fig. 4A and C). Moreover, the treatment with fluoxetine induced an increase of the number of both wild-type and knockout stage 5 neurons, relative to their controls (two-way ANOVA treatment effect, $F_{1,97} = 29.9$ $p < 0.0001$; WT FLX vs WT CTL, $p < 0.0001$; KO FLX vs KO CTL, $p = 0.002$ PLSD test; Fig. 4A and C).

This indicated that the stem/progenitor cells amplified by fluoxetine mature within 1–5 days into early differentiated neurons.

We further analyzed the maturation of stem and progenitor cells into terminally differentiated neurons (stage 6; Kempermann et al., 2004), identified as 28-day-old $BrdU^+NeuN^+$ cells, labeled with 5 daily injections of BrdU performed during the last days of fluoxetine treatment; afterwards, neurons were allowed to differentiate throughout 28 days off-treatment (Fig. 4D). We found that the 28-day-old neurons increased significantly in number after treatment with fluoxetine, both in wild-type and Btg1 knockout dentate gyrus, with comparable percent increases (two-way ANOVA treatment effect, $F_{1,163} = 50.7$ $p < 0.0001$; WT FLX vs WT CTL, 71% increase, $p < 0.0001$; KO FLX vs KO CTL, 58% increase, $p = 0.0008$ PLSD test; Fig. 4D and E). Of note, the decrease of Btg1 knockout dentate gyrus neurons was fully rescued by fluoxetine, up to the control level (WT CTL vs KO CTL, $p = 0.0004$ PLSD test; WT CTL vs KO FLX, $p = 0.94$ PLSD test; Fig. 4E).

As a whole, these data provide the information that the total number of immature (stage 5) and terminally differentiated (stage 6) neurons is strongly amplified by fluoxetine treatment in both the wild-type and knockout dentate gyrus, thus indicating that the increase of stem and progenitor cells in Btg1 knockout mice results in the generation of new neurons.

3.4. Fluoxetine induces neurogenesis also in 15-month-old Btg1 knockout mice by promoting the proliferation of stem cells in the dentate gyrus

A large body of evidence indicates that aging inhibits neurogenesis, by reducing both progenitor cells and the neurons generated (Kuhn et al., 1996; van Praag et al., 2005). Moreover, fluoxetine is unable to stimulate the proliferation of progenitor cells in 6–12 month-old mice (Couillard-Despres et al., 2009; Li et al., 2015; McAvoy et al., 2015).

Thus, we wished to test whether this holds true also in aged mice of our Btg1 knockout model, where fluoxetine is able to reactivate stem cells in adult Btg1 knockout mice, a capability not displayed in the normal adult stem cells of the dentate gyrus.

We therefore analyzed the number of proliferating stem cells generated in 15-month-old mice, wild-type and Btg1 knockout, after treatment for 21 days with fluoxetine (Fig. 5A and B).

It turned out that the total number of dividing cells, detected as $Ki67^+$ cells, increased significantly in fluoxetine-treated Btg1 knockout mice, relative to all other conditions (two-way ANOVA, treatment effect, $F_{1,169} = 6.79$ $p = 0.009$; KO FLX vs WT FLX or vs WT CTL: $p < 0.0001$ PLSD test; KO FLX vs KO CTL $p = 0.0006$ PLSD test; Fig. 5A–C). No change was effected by fluoxetine in the wild-type dentate gyrus, as expected. Of note, the number of dividing cells in Btg1 knockout dentate gyrus was not lower than in control wild-type, as

instead observed in young adults (Fig. 1D), suggesting that both wild-type and knockout cells decreased throughout age until a plateau was reached (Fig. 5C).

We then analyzed the number of dividing stem cells (type-1 cells), detected as Ki67$^+$ cells co-labeled also by GFAP and Sox2. We observed an increase, similar although greater than that seen for total Ki67$^+$ cells, of proliferating stem cells (up to two-fold) in fluoxetine-treated Btg1 knockout mice, relative to all other conditions (two-way ANOVA, treatment effect, $F_{1,169} = 17.8$ $p < 0.0001$; KO FLX vs WT FLX or vs WT CTL or vs KO CTL: $p < 0.0001$ in all comparisons, PLSD test; Fig. 5A and D). Again, no change was induced by fluoxetine in the wild-type dentate gyrus.

Also the total number of proliferating Sox2$^+$ cells, which include both type-1 and type-2 cells (since Sox2 co-labels with nestin, see Komitova and Eriksson, 2004), was greatly increased by fluoxetine in Btg1 knockout mice compared to the other conditions (two-way ANOVA, treatment effect, $F_{1,169} = 13.01$ $p = 0.0004$; KO FLX vs WT FLX or vs WT CTL or vs KO CTL: $p < 0.0001$ in all comparisons, PLSD test; Fig. 5A and E), without effect by fluoxetine in the wild-type dentate gyrus.

Overall, these data indicate that fluoxetine elicits a great increase of stem cells in aged mice, but, remarkably, only in the Btg1 knockout dentate gyrus.

Next, we analyzed whether the expansion of stem cells induced by fluoxetine in aged Btg1 knockout dentate gyrus corresponded to an increase in the generation of new neurons. We detected 1- to 5-day-old neurons in the dentate gyrus by BrdU incorporation in the same mice used to analyze dividing stem/progenitor cells, following five daily injections of BrdU performed during the last days of fluoxetine treatment (see scheme Fig. 5B).

First, we measured the total number of 1- to 5-day-old cells in the dentate gyrus (BrdU$^+$ cells), and we observed that fluoxetine induced a significant increase in the Btg1 knockout mice, relative to all other conditions (two-way ANOVA, treatment effect, $F_{1,146} = 16.9$ $p < 0.0001$; KO FLX vs WT CTL: $p < 0.004$ PLSD test, or vs WT FLX: $p < 0.032$ PLSD test, or vs KO CTL: $p < 0.0001$ PLSD test; Fig. 5F).

Then, we analyzed the number of 1- to 5-day-old neurons in the dentate gyrus, detected as BrdU$^+$NeuN$^+$. These new neurons resulted significantly increased by fluoxetine in the Btg1 knockout dentate gyrus, relative to all other conditions (two-way ANOVA, treatment effect, $F_{1,146} = 24.04$ $p < 0.0001$; KO FLX vs WT CTL or vs KO CTL: $p < 0.0001$ PLSD test; KO FLX vs WT FLX $p = 0.0038$ PLSD test; Fig. 5G). No effect by fluoxetine was observed in the wild-type dentate gyrus (WT FLX vs WT CTL $p = 0.076$ PLSD test; Fig. 5G).

We can conclude that in aged mice the Btg1 knockout stem and progenitor cells that are induced to proliferate by fluoxetine do mature into new neurons.

3.5. Sox2-retrovirus rescues the defective proliferation of Btg1-null progenitor cells

It is known that Sox2-positive cells can self-renew and that a single Sox2-positive cell can give rise to a neuron and to an astrocyte (Suh et al., 2007). More specifically, the expression of Sox2 is required for the maintenance of the stemness of adult stem cells (Remboutsika et al., 2011). Consistently, the knockout of Sox2 in adult mice results in the loss of hippocampal neurogenesis (Favaro et al., 2009). Moreover, Sox2 is a major mediator of Notch signaling in the maintenance of stem cells, as the overexpression of Sox2 rescues the loss of self-renewal in RBPJ-deficient neural stem cells, *in vitro* and in vivo (Ehm et al., 2010).

Therefore we reasoned that Sox2 was a candidate gene able to reverse the insufficiency of self-renewal occurring in Btg1 knockout stem cells of the dentate gyrus. To assess this possibility, we sought to rescue the self-renewal by injecting in the dentate gyrus of Btg1 knockout mice - and of Btg1 wild-type as a control - a retrovirus expressing the Sox2 mRNA (Fig. 6A–C).

Only proliferating stem and progenitor cells of the dentate gyrus of adult mice (at P70) were infected with a retrovirus expressing Sox2 and GFP or GFP alone (pCAG-IRES-GFP-Sox2 and pCAG-IRES-GFP-empty, respectively; Fig. 6A–C). The analysis of infected cells (GFP$^+$) was performed 5 days after infection, and we detected all the stem and progenitor cells that continued to proliferate at this time, i.e., the type-1-2-3 cells.

Infected stem cells and type 2a progenitor cells (type-1-2a) were visualized by the expression of nestin and the absence of DCX (GFP$^+$nestin$^+$DCX$^-$), type-2b cells by the expression of both nestin and DCX (GFP$^+$nestin$^+$DCX$^+$; Filippov et al., 2003; Fukuda et al., 2003; Kempermann et al., 2004), and type-3 progenitor cells by the expression of DCX without nestin (GFP$^+$nestin$^-$DCX$^+$; Kronenberg et al., 2003).

We observed in the infected Btg1-null dentate gyrus a pattern of decrease of stem cells and type-2a (Kruskall-Wallis [d.f. 11] $H = 155.699$, $p < 0.0001$; GFP-Sox2$^+$nestin$^+$DCX$^-$/total GFP-Sox2+ vs GFP-empty$^+$nestin$^+$DCX$^-$/total GFP-empty$^+$; $p = 0.004$, Mann-Whitney *U* test, Fig. 6C) as well as of type-2b progenitor cells (GFP-Sox2$^+$nestin$^+$DCX$^+$/total GFP-Sox2$^+$ vs GFP-empty$^+$nestin$^+$DCX$^+$/total GFP-empty$^+$; $p = 0.007$, Mann-Whitney *U* test, Fig. 6B and C), whereas type-3 progenitor cells presented a marked increase (GFP-Sox2$^+$nestin$^-$DCX$^+$/total GFP-Sox2$^+$ vs GFP-empty$^+$nestin$^-$DCX$^+$/total GFP-empty$^+$ 53% increase, $p = 0.003$, Mann-Whitney *U* test, Fig. 6B and C). This denoted that the expression of Sox2 strongly promoted the transition of stem and type-2ab progenitor cells toward the more mature phenotype of type-3 neuroblast cell during the five days post-infection. Similarly, the infection of Btg1 wild-type dentate gyrus cells with the GFP-Sox2 retrovirus stimulated an increase of type-3 progenitor cells (GFP-Sox2$^+$nestin$^-$DCX$^+$/total GFP-Sox2$^+$ vs GFP-empty$^+$nestin$^-$DCX$^+$/total GFP-empty$^+$, $p = 0.044$, Mann-Whitney *U* test, Fig. 6 C); however, the GFP-Sox2 retrovirus did not significantly affect the percentage of type-1-2a and type-2b cells, relative to GFP-empty virus-infected. Given that a considerably greater number of neuroblasts was produced after Sox2 infection, similarly to the effect of the neurogenic stimulus of fluoxetine, we can infer that Sox2 induced a functional rescue, either full or partial, of the defective amplification observed in Btg1-null progenitor cells.

4. Discussion

4.1. Fluoxetine reactivates proliferation-defective stem cells of the adult dentate gyrus; possible mechanisms

In this report we tested whether the potential of stem cell self-renewal is conserved throughout life, even when the proliferative capability is reduced, as it occurs in the aged mouse or in some mouse models missing the control of cell cycle. The knockout of the anti-proliferative quiescence-maintaining gene Btg1 leads, in the dentate gyrus of neonatal mice, to hyperproliferation of stem and progenitor cells, followed in adulthood by a decrease of their proliferative capability and by a decrease of their number (Farioli-Vecchioli et al., 2012). Here we show that in the adult Btg1 knockout dentate gyrus the neurogenic stimulus of fluoxetine reactivates the defective proliferation of stem cells by increasing their number even above that of wild-type stem cells, which are normally not responsive to antidepressants (Encinas et al., 2006). Moreover, we observe that activation of stem cells occurs also in aged Btg1 knockout mice. This finding is noteworthy also considering that fluoxetine is completely devoid of a neurogenic action on aged mice (6–12 month-old), being unable to increase the number of BrdU$^+$ cells (Couillard-Despres et al., 2009; Li et al., 2015; McAvoy et al., 2015). We also observed that, in adult as well as in the aged Btg1 knockout dentate gyrus, the newly generated stem cells induced by fluoxetine mature into viable neurons.

The Btg1 knockout could be considered, during adulthood, as a model mimicking the aging of stem cells: in fact during aging there is a

decrease of the ongoing process of generation of new neurons in the hippocampus from resident neural stem cells (Bizon and Gallagher, 2003; Kuhn et al., 1996). This leads to a decrease during aging of hippocampus-dependent learning and memory tasks (Couillard-Despres et al., 2009). Notably, in aged individuals the need of new neurons for learning is partially compensated by the existence of optimized neuron circuitry (Rapp and Gallagher, 1996), but the ability to cope with new tasks is lower in consequence of the reduced neurogenesis. It has been shown that appropriate stimuli such as an enriched environment and life (Carlson et al., 2008) or also physical exercise (van Praag et al., 2005) can reduce or counteract the cognitive decline in aged individuals by stimulating neurogenesis.

Thus, the activity of a gene that maintains the stem cells in quiescence, such as Btg1 does, is probably exerted according to a program evolutionarily selected in order to preserve existing circuits versus a more dynamic but more expensive condition. We outline here the opposite scenario, showing that ablation of Btg1, after leading to a loss of the proliferative capability of stem/progenitor cells, enables a neurogenic stimulus to reestablish a dynamic condition in stem cell renewal.

Such a reestablishment of a dynamic proliferative condition of stem cells - and by consequence also of type-2ab and type-3 progenitor cells - is triggered, after ablation of Btg1, by fluoxetine, which normally enhances proliferation only of already proliferating dentate gyrus type-2 and type-3 progenitor cells, being ineffective on normal stem cells (Encinas et al., 2006), as we observe also here in wild-type mice. The increase of proliferation by fluoxetine may be a consequence of an increase of BDNF levels (Molteni et al., 2006) and of the increase of 5HT availability on 5HT3,4,6,7 receptors present in the hippocampus, which regulate proliferation (Bolijn and Lucassen, 2015). A key point is that the significant increase induced by fluoxetine of the fraction of Btg1 knockout stem cells shifting from a non mitotic to a mitotic state (Fig. 2F) indicates that the fluoxetine-dependent reactivation of proliferation in Btg1 knockout stem cells occurs from a state of quiescence. Moreover, this reactivation in the cycle of stem cells (type-1) is maintained by fluoxetine until the end of treatment, as the Btg1 knockout stem cells population still expands during the last 72 h of treatment, indicating that stem cells undergo continuous reactivation (Fig. 3C). This is probably due to a reduced (re-)entry into quiescence and therefore to protracted permanence in the proliferative state.

Thus the ablation of Btg1, although apparently reducing the proliferative capability of the stem and progenitor cells as adulthood proceeds, in fact releases them from a constraint to quiescence, enabling stem and progenitor cells to fully respond to a neurogenic stimulus.

4.2. Reactivation of stem cells by fluoxetine also in the aged dentate gyrus and models of self-renewal

Remarkably, we find that such a property of full response to a neurogenic stimulus is intrinsic to stem cells irrespective of age, since it occurs also in aged mice (15-month-old). This finding is interesting not only as fluoxetine does not stimulate neurogenesis at all in the aged dentate gyrus, as mentioned above, but also since the proliferative capability of stem cells is reduced in aged mice to a similar level in both Btg1 knockout and wild-type mice (as observed also in the subventricular zone, see Mastrorilli et al., 2017). This strengthens the indication that this reactivation potential of proliferation-defective stem cells emerging during adulthood is not limited by age-dependent decline.

Ablation of Btg1 coupled to a neurogenic stimulus reveals how stem cells are endowed with hidden self-renewal or amplification capacity. It is worth noting that our findings in the adult and especially in aged mice, not only favor the idea that neural stem cells of adult neurogenic niches suffer a progressive reduction of proliferative potential throughout life, rather than being depleted, as some studies suggest (see for review Kempermann, 2011), but also that this reduction of proliferative potential of stem cells can be fully recovered in specific conditions. In other words, our results do not favor the view that the stem cell pool is disposable and progressively depleted, as proposed by Encinas et al. (2011), but rather suggest that it is carefully and actively maintained until the end of life in a way that may depend on the contingent conditions, such as the expression of quiescence-maintaining genes and/or of proliferation inhibitors. This view probably fits better with the more flexible model of Bonaguidi et al. (2011), proposing a protracted ability to self-renew. This idea is somewhat strengthened by the observation that another neurogenic stimulus, i.e., physical exercise, induces proliferation of stem cells even in the subventricular zone of aged Btg1 knockout mice (Mastrorilli et al., 2017), which suggests this as a general property of adult neural stem cells. Given that Btg1 is a cell cycle inhibitor, it seems plausible that the increase of amplification capacity of the stem cells after neurogenic stimulus is caused by the removal of this cell cycle inhibition. Since the ablation of Btg1 leads in the long term in the adult dentate gyrus to a compensatory increase of p21 (Farioli-Vecchioli et al., 2012) - which might be responsible for the reduced proliferative capacity observed in Btg1 knockout adult stem cells in the absence of a neurogenic stimulus - this would suggest that Btg1 acts as a checkpoint for maintenance of quiescence possibly stronger than p21 itself.

Furthermore, a critical point to be investigated is whether the expansion of Btg1 knockout stem cells triggered by a neurogenic stimulus occurs with asymmetric or symmetric division. In the latter case, each stem cell would generate two progenitor cells, thus leading in the long-term to a reduction of the pool of self-renewing stem cells.

A relevant question emerging is also whether the Btg1 knockout stem cells reactivated by both fluoxetine and the running neurogenic stimuli are a specific sub-pool. Plausibly, the stem cells targeted by the neurogenic stimulus are mainly quiescent, as the percent increase of mitotic stem cells triplicates (Fig. 2F). Moreover, since the extent of reactivation is virtually the same for both the running and the fluoxetine stimuli, it seems reasonable that the population of reactivated stem cell is the same in the two cases.

4.3. Amplification by Sox2 of proliferation-defective stem cells in the adult dentate gyrus

Interestingly, the overexpression of Sox2 in the dentate gyrus appears to increase the population of type-3 progenitor cells. In fact we observe that Sox2, five days after infection with a retrovirus, leads to a significant increase of the Btg1 wild-type and knockout type-3 progenitor cells. Moreover, in Btg1-null dentate gyrus there is also a significant parallel decrease of type-2 and type-1 cells. As a whole, this indicates that Sox2 triggers a process of amplification of wild-type as well as proliferation-defective Btg1 knockout progenitor cells, plausibly starting from the amplification of stem cells, process that after five days can be detected as an increase of type-3 neuroblasts.

The decrease of type-1 and type-2 stem/progenitor cells only in the Btg1 knockout suggests that the kinetics of progenitor cells amplification in the wild-type dentate gyrus is different, being probably slower than in knockout mice, where the effect of Sox2 appears more marked.

It is also worth noting that the basal levels of wild-type and knockout cells infected with GFP-empty virus are not comparable, being expressed as percentage of total infected cells, and thus preventing the possibility to appreciate the differences in cell population size observed between the two genotypes (e.g., see Fig. 2).

Indeed, the expression of Sox2 has been shown to be required for the maintenance of the stemness of adult stem cells, as only Sox2-positive cells are capable of generating secondary neurospheres – this being an indication of the ability to self-renew (Remboutsika et al., 2011) – while Sox2 knockout in adult mice results in the loss of hippocampal neurogenesis (Favaro et al., 2009). Moreover, Sox2 overexpression also reconstitutes the self-renewal ability of stem cells that have lost it, such as the RBPJ-deficient neural stem cells in vivo (Ehm

et al., 2010). This finding and our data indicate that Sox2 has a dual action, being both required and sufficient to promote self-renewal, as seen for Btg1 knockout progenitor cells. This may occur possibly by directly inducing an asymmetric division, i.e., the division mode by which self-renewal of stem cells coupled to generation of new neurons is effected by Sox2 (Suh et al., 2007). Of note, the ablation of Btg1 leads to a decrease of asymmetric divisions in the adult subventricular zone cells in culture (Farioli-Vecchioli et al., 2012), thus suggesting that the cellular mechanisms activated by the ablation of Btg1 are functionally reverted by the pro-self-renewal action of Sox2. Indeed, the actions of Btg1 and Sox2 are opposite, as Btg1 maintains the quiescence of stem cells while Sox2 promotes their self-renewal. Moreover, it is worth noting that p21 controls the expansion of progenitor cells by directly inhibiting the expression of Sox2 (Marqués-Torrejón et al., 2013) and that fluoxetine negatively regulates p21 expression in the subgranular zone of the dentate gyrus in amplifying progenitor cells (Pechnick et al., 2011). This may also further suggest a role of p21 in the Btg1 mechanism of action. Although we cannot directly compare the effect of fluoxetine with that of Sox2 infection, since this latter is a one-hit non-continuous treatment, nevertheless the result at the end-point of our analysis (i.e., type-3 progenitor cells) is an increase of cell number in both treatments, suggesting that both act as a neurogenic stimulus able to counteract the defect of self-renewal in Btg1 knockout dentate gyrus cells.

4.4. Conclusions

The similar positive effect on the amplification of Btg1 knockout stem cells exerted by two different neurogenic stimuli, one non-specific such as running, the other specific such as fluoxetine, an inducer of the serotonin pathway, suggests that the reactivation of self-renewal in defective Btg1 knockout stem and progenitor cells is a process that can be triggered regardless of the stimulus type, by different pathways. The ability of stem cells to be reactivated, displayed after ablation of the cell cycle inhibitor Btg1, warrants further investigations on Btg1 mechanisms of action; we should also determine whether reactivation of stem cells can occur after ablation of other proliferation inhibitors.

Future studies will be necessary to ascertain whether the reactivated type-1 stem cells are in any case a specific subpopulation of the whole pool of stem cells, as this knowledge may reveal new properties of the ability of stem cells for self-renewal.

Furthermore, it will be interesting to evaluate whether fluoxetine treatment is able to rescue the reduced discrimination of memories observed in Btg1 knockout mice, similarly to that observed with running (Farioli-Vecchioli et al., 2014), thus providing indications on the mechanisms by which the two neurogenic stimuli influence the function of stem/progenitor cells and neurons.

Conflicts of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.08.023.

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