EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, pp. 1–9, 2013 doi:10.1111/ejn.12297

Paradoxical increase in survival of newborn neurons in the dentate gyrus of mice with constitutive depletion of serotonin

Silvina L. Diaz,[1,2,3,*,#] Nicolas Narboux-Nême,[1,2,3,*] Sara Trowbridge,[1,2,3] Sophie Scotto-Lomassese,[1,2,3] Felix B. Kleine Borgemann,[4] Sebastian Jessberger,[4] Bruno Giros,[2,5,6] Luc Maroteaux,[1,2,3] Evan Deneris[7] and Patricia Gaspar[1,2,3]

[1]Inserm, UMR-S 839, France
[2]UPMC, Paris, France
[3]Institut du Fer à Moulin, Paris, France
[4]Brain Research Institute, University of Zurich, Zurich, Switzerland
[5]CNRS UMR 7224, Paris, France
[6]Department of Psychiatry, Douglas Hospital, McGill University, Montreal, QC, Canada
[7]Case Western Reserve University, Cleveland, OH, USA

Keywords: 5-hydroxytryptamine depletion, adult neurogenesis, genetic mouse models, hippocampus, para-chlorophenylalanine, pattern separation

Abstract

Increased adult neurogenesis is a major neurobiological correlate of the beneficial effects of antidepressants. Indeed, selective serotonin (5-HT) re-uptake inhibitors, which increase 5-HT transmission, enhance adult neurogenesis in the dentate gyrus (DG) of the hippocampus. However, the consequences of 5-HT depletion are still unclear as studies using neurotoxins that target serotonergic neurons reached contradictory conclusions on the role of 5-HT on DG cell proliferation. Here, we analysed two genetic models of 5-HT depletion, the Pet1$^{-/-}$ and the VMAT2$^{f/f}$; SERT$^{cre/+}$ mice, which have, respectively, 80 and 95% reductions in hippocampal 5-HT. In both models, we found unchanged cell proliferation of the neural precursors in the DG subgranular zone, whereas a significant increase in the survival of newborn neurons was noted 1 and 4 weeks after BrdU injections. This pro-survival trait was phenocopied pharmacologically with 5-HT synthesis inhibitor PCPA treatment in adults, indicating that this effect was not developmental. Furthermore, a 1-week administration of the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT in Pet1$^{-/-}$ and PCPA-treated mice normalised hippocampal cell survival. Overall, our results indicate that constitutive 5-HT depletion does not alter the proliferation of neural precursors in the DG but promotes the survival of newborn cells, an effect which involves activation of postsynaptic 5-HT$_{1A}$ receptors. The role of 5-HT in selective neuronal elimination points to a new facet in its multiple effects in controlling neural circuit maturation.

Introduction

The production of new neurons in the subgranular zone (SGZ) of the dentate gyrus (DG) of adult mammalian brain is a unique form of structural plasticity which enables hippocampal circuits to adapt to changes in the environment (Koehl & Abrous, 2011). The DG SGZ continuously generates cells, but a large proportion (70–90%) of these adult newborn cells are cleared in the first 4 weeks after their generation (Snyder *et al.*, 2009). Numerous molecular signals and processes have been involved in regulating the neurogenesis and survival of newborn cells and their integration in neural circuits (van Praag *et al.*, 1999; Malberg *et al.*, 2000; Warner-Schmidt & Duman, 2006; Fabel & Kempermann, 2008; Kempermann, 2011). Among these factors, neural activity has been shown to promote cell survival; specifically, glutamatergic and GABAergic afferents have been found to play a role in the modulation of neurogenesis (Toni & Sultan, 2011).

Serotoninergic axons originating from the raphe nuclei innervate the DG (Jacobs *et al.*, 1992) and have been involved in the control of adult neurogenesis. This is largely based on studies showing that chronic exposure to the selective serotonin (5-hydroxytryptamine; 5-HT) reuptake inhibitors (SSRIs), a widely used class of antidepressants, increased proliferation of neural progenitors (Malberg *et al.*, 2000; Banasr *et al.*, 2004; Diaz *et al.*, 2012; Petrik *et al.*, 2012) and survival of the newborn cells in the DG (Santarelli *et al.*, 2003; Encinas *et al.*, 2006; Diaz *et al.*, 2012). However, the physiological

Correspondence: Patricia Gaspar, [1]INSERM U839, 17, rue du Fer à Moulin, 75005 Paris, France.
E-mail: patricia.gaspar@inserm.fr

*These two authors contributed equally to this work.

[#]*Present address*: Instituto de Biologia Celular y Neurosciencia, CONICET, Universitad de Buenos Aires, Buenos Aires, Argentina.

Received 23 April 2013, revised 6 June 2013, accepted 6 June 2013

effect of constitutive 5-HT transmission on proliferation and survival remains a matter of debate as pharmacological depletion of 5-HT by the use of the selective 5-HT toxin 5–7 dihydroxytryptamine led either to a reduction (Brezun & Daszuta, 1999, 2000), or to no change (Huang & Herbert, 2005; Jha *et al.*, 2006) in newborn cell numbers.

To evaluate the effects of a constitutive 5-HT depletion, we analysed two genetic models: the Pet1$^{-/-}$ mouse, in which the majority of 5-HT neurons in the raphe fail to differentiate (Hendricks *et al.*, 2003; Kiyasova *et al.*, 2011), and the VMAT2$^{f/f}$; SERT$^{cre/+}$ mouse, which lacks vesicular monoamine storage mechanisms in 5-HT neurons (Narboux-Neme *et al.*, 2011). We report that basal proliferation is undisturbed in these two mouse strains whereas survival of newborn cells is increased. In addition, similar results were obtained using a pharmacological approach for depleting 5-HT in adult WT mice, indicating that this effect is not due to developmental alterations. A normalisation of the increased survival phenotype with 5-HT$_{1A}$ agonists, in both Pet1$^{-/-}$ and pharmacologically 5-HT-depleted mice, revealed an unsuspected role of this receptor in controlling the number of surviving newborn cells in the hippocampus.

Because it has been recently proposed that the immature DG cells are critical for pattern separation (Sahay *et al.*, 2011; Nakashiba *et al.*, 2012), a form of memory that enables encoding of somewhat similar contexts as distinct events, we examined this form of memory in the Pet1$^{-/-}$ mice. However, no change in memory performance in this test could be observed.

Materials and methods

Animals

Procedures involving animals were conducted in accordance with the directives of the European Community (council directive 86/609) and the French Agriculture Ministry (council directive 87–848, 19 October 1987, permissions 00782 to PG).

The Pet1$^{-/-}$ mouse line has been described previously (Hendricks *et al.*, 2003). Heterozygote Pet1$^{+/-}$ females were mated with Pet1$^{+/-}$ or Pet1$^{-/-}$ males to produce mixed litters. Both Pet1$^{+/+}$ and Pet1$^{+/-}$ served as controls, as no differences in 5-HT levels or 5-HT innervation between the two genotypes has been reported (Hendricks *et al.*, 2003; Kiyasova *et al.*, 2011) or noted in the present study.

The VMAT2$^{f/f}$; SERT$^{cre/+}$ mouse line has been described previously (Narboux-Neme *et al.*, 2011). Briefly, these mice result from crossing a floxed VMAT2 mouse line (Slc18a2^{tm1BGi}; B.G., to whom correspondence regarding the mouse line should be addressed) to a SERT^{cre} mouse line (Slc6a4$^{tm1(cre)Xz}$; Zhuang *et al.*, 2005). Male VMAT2$^{flox/+}$; SERT$^{cre/+}$ or VMAT2$^{flox/flox}$; SERT$^{cre/+}$ mice were mated with female VMAT2$^{flox/flox}$ to generate three different genotypes: VMAT2$^{flox/flox}$; SERT$^{cre/+}$ (recombined) and two types of control littermates: VMAT2$^{flox/flox}$ and VMAT2$^{flox/+}$; SERT$^{cre/+}$. The SERT^{cre} strain is a knock-in and thus lacks one allele of the SERT gene. However, we have previously shown that brain monoamine levels do not differ between VMAT2$^{flox/flox}$ and VMAT2$^{flox/+}$; SERT$^{cre/+}$ controls (Narboux-Neme *et al.*, 2011).

The Pet1$^{-/-}$ and VMAT2$^{flox/flox}$; SERT$^{cre/+}$ mouse lines were maintained on a C57BL/6J background, and male littermates were used in all experiments. After weaning, males and females were housed separately (five animals per cage) and maintained under standard laboratory conditions (22 ± 1 °C, 60% relative humidity, 12–12 h light–dark cycle, food and water *ad libitum*).

Pharmacological studies were performed on male wild-type (WT) C57BL/6J mice (purchased from Janvier, France).

Proliferation and survival assays

Dividing cells were labelled with the thymidine analogue 5-bromo-2′deoxyuridine (BrdU; Sigma, B9285) as previously described (Diaz *et al.*, 2012). To study cell survival at 4 and 16 weeks, it is necessary to use relatively high doses of BrdU to ensure that it is not diluted in the process of re-division of daughter cells (Wojtowicz & Kee, 2006). Animals received two injections of BrdU, 150 mg/kg, at a 2-h interval, a dose that has previously been proved to be nontoxic for proliferating and immature neurons (Hancock *et al.*, 2009). BrdU was dissolved in 0.9% NaCl at 50 °C, and the pH was set at 7.4 with NaOH 10 M. Seven-week-old VMAT2$^{f/f}$; SERT$^{cre/+}$, Pet1$^{-/-}$ and their respective controls (n = 3–6 per experimental group) were killed either 24 h or 1, 4 or 16 weeks after BrdU administration, and brains were recovered for cell proliferation and survival studies.

Chronic treatment with para-chlorophenylalanine (PCPA)

To study the effect of chronic 5-HT depletion in adults, 4-week-old WT mice received a daily intraperitoneal (i.p.) injection of PCPA at 300 mg/kg for the first 3 days and at 100 mg/kg for the rest of the treatment (Vitalis *et al.*, 2007). After 4 weeks of PCPA treatment, BrdU was administered as described above. A group of mice was killed 24 h later for proliferation assays and a second group continued receiving PCPA injections for 1 or 4 extra weeks for survival assays.

Treatment with the 5-HT$_{1A}$ agonist, 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)

A group each of Pet1$^{-/-}$ and control mice (7–8-week-old) received PCPA or saline for 4 weeks and then two injections of BrdU as described above (Fig. 2A). Beginning 24 h later, animals received two daily i.p. injections of the 5-HT$_{1A}$ agonist 8-OH-DPAT at 0.5 mg/kg or of vehicle (NaCl 0.9%) for 7 days and were killed on the day of the last injection.

Retrovirus-mediated labeling of new hippocampal granule cells

Three hundred nanolitres of retroviral vector CAG-green-fluorescent protein (GFP; Zhao *et al.*, 2006) was injected into the DG of Pet1$^{-/-}$ and Pet1$^{+/-}$ mice at the following coordinates (in mm): anteroposterior, −1.7; lateral, ±1.7; ventral, −1.75, using the bregma as reference. Mice were perfused 1 week later and 100-μm sections were made with a vibratome (VT1000S; Leica).

Histological methods

Mice were deeply anesthetised (xylazine, 20 mg/kg and pentobarbital, 50 mg/kg), and transcardially perfused with 5 mL of 0.9% NaCl and 50 mL of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (1 × PBS, pH 7.4) for 15 min. Brains were recovered, post-fixed for 24 h at 4 °C and sliced into 50-μm-thick coronal sections with a vibratome. Sections were stored at −20 °C in cryoprotectant (30% ethylene glycol and 30% glycerol in 0.12 M phosphate buffer) if not used immediately. Nonspecific binding was reduced by 1 h incubation in a saturation solution containing 2 g/L gelatin (Merck) and 0.25% triton in PBS prior to primary antibody incubation. In addition, all primary and secondary antibodies were diluted in this saturation solution. The specificity of the BrdU, calbindin and calretinin antibodies has been reported in previous studies (Brandt *et al.*,

2003; Snyder *et al.*, 2009). Omission of one or more of the primary antibodies was used here to check for specificity and cross-reactivity in our immunocytochemical procedures.

Newborn cells were revealed by peroxidase immunostaining of BrdU. Free-floating sections were incubated overnight in 0.1% H_2O_2, and exposed to 2 N HCl for 1 h. Sections were incubated overnight at 4 °C with a rat anti-BrdU antibody (1 : 400; OBT0030, clone BU1/75-ICR1; AbDserotec), which was then detected with a biotinylated goat anti-rat antibody over 2 h (1 : 400; Vector). Revelation steps included 1 h incubation in a streptavidin–horseradish peroxidase complex (1 : 400; Amersham) followed by an exposure to 3,3′-diaminobenzidine (DAB) used as substrate.

To evaluate the maturation of newborn neurons produced in the DG, animals were perfused 2 weeks after BrdU administration, and double-labeling of BrdU and neuronal markers was carried out. Rat anti-BrdU (1 : 400; OBT0030, AbDserotec) was combined with a goat anti-doublecortin (1 : 500; Santa Cruz; immature neuronal marker), an IgM mouse anti-calbindin (1 : 12 000; AbCys; CP331, mature neuronal marker), or a rabbit anti-calretinin (1 : 500; Swant; immature marker) overnight at 4 °C. Sections were then incubated for 2 h at room temperature in the corresponding secondary fluorescent antibodies (1 : 500; Molecular Probes).

To evaluate morphology of retrovirus-infected new neurons, GFP expression was intensified by permeabilising 100-μm-thick sections with proteinase K (10 μg/mL; 2 min), which was subsequently blocked with 2 mg/mL glycine. GFP was then detected with a rabbit anti-GFP antibody at 4 °C overnight (1 : 5000; Molecular Probes) and revealed at room temperature for 2 h with a donkey anti-rabbit antibody (1 : 500; Molecular probes).

Coronal sections from the hippocampus of WT mice were employed for *in situ* hybridisation as described previously (Narboux-Neme *et al.*, 2008). The following cDNAs were used: 5-HT_{1A} (IMAGE:8861702), 5-HT_{2B} (IMAGE:5344328) 5-HT_{3A} (IMAGE: 9053347), 5-HT_4 (IMAGE:9053710), 5-HT_6 (IMAGE:40104889), 5-HT_7 (IMAGE:40126305) dig-UTP-labeled probes were used and detected using an antibody coupled to alkaline phosphatase (1 : 2000; Roche) and NBT/BCIP was used as blue substrate for *in situ* revelation.

Analysis and counting procedure

The number of BrdU-labeled cells was quantified with a bright-field microscope with a 40 × objective, on serial sections through the entire hippocampus. One out of six sections of the series was counted (300-μm intervals, six sections per series). BrdU-labeled cells were counted in the SGZ over the entire dorsal and ventral DG. Cells were considered to be $BrdU^+$ when their nuclei were completely filled with DAB product or showed clear patches of labeling. To facilitate the comparison between the three different mouse models, across different experiments, the numbers of $BrdU^+$ cells were expressed as percentages of control values.

The total surface area of the DG was measured in 2- and 7-week old $VMAT2^{f/f}$; $SERT^{cre/+}$ and control mice. Briefly, images from serial Nissl-stained sections through the entire hippocampus (three mice per experimental condition) were acquired with a 10 × objective and the area covered by the granular cell layer in each section was outlined using Image J software. A mean surface area in μm^2 per animal was then calculated by dividing the sum of the DG surface areas by the number of sections measured.

Double-labeled cells (BrdU with doublecortin, calbindin or calretinin) in the granular cell layer of the DG were imaged with a confocal laser scanning microscope (SP2; Leica, Mannheim, Germany). A total of 45–85 $BrdU^+$ cells per mouse (n = 5 or 6 mice per experimental group) were analysed in their entire z-axis with a 1-μm step to exclude false double-labeling. Double-labeled cells were expressed as percentage of total $BrdU^+$ cells.

In viral labeling experiments, images were acquired with a 40 × objective, with lateral and z-axis resolutions of 385 and 700 nm respectively. Dendritic trees of GFP-expressing new neurons were visualised in 3-D reconstructions using the NeuronStudio software (Rodriguez *et al.*, 2008) to quantify the number of dendritic nodes (n = 3 or 4 mice per experimental group; totals of 193 and 181 GFP^+ cells was analysed in $Pet1^{-/-}$ and $Pet1^{+/-}$ mice, respectively).

Pattern separation

This behavioral test was carried out as previously published (Sahay *et al.*, 2011) using contextual fear-discrimination learning, a type of hippocampal-dependent learning test that captures the animal's ability to discriminate two similar contexts. Briefly, adult (8–10-week-old) $Pet1^{-/-}$ male mice were exposed to two similar contexts, with only one of them associated with a foot-shock. The shock-associated training context A and the similar (no-shock) context B shared many features, such as a stainless steel grid floor and a plastic roof. The differences between contexts were defined in a pilot study. Context B differed from context A in that two colored paper inserts were used to cover the lateral walls and a mild limonene scent was used as an olfactory cue. An alcohol solution was used to clean the grids between runs. The same experimenter manipulated all the animals during all the experiments. Mice were individually brought into the testing room. For discrimination learning, mice were exposed to the training context in which they received a single 2-s foot shock of 0.8 mA at 180 s after placement in the chamber. Mice were taken out of the chamber 15 s after termination of the foot shock and returned to their home cage. After 1 h, each mouse was placed in the similar B context, in which they were left for 180 s and were never shocked. On test days 1, 4, 7, 8 and 10, mice were first exposed to context A before context B, whereas on test days 2, 3, 5, 6 and 9 they were first exposed to context B. Measurement of the freezing levels in both the A training context (3 min pre-shock) and the similar B context (3 min) each day allowed the assessment of discrimination between the two contexts and was computed as a discrimination index: (Freezing $_{Acontext}$ − Freezing $_{Bcontext}$) / (Freezing $_{Acontext}$ + Freezing $_{Bcontext}$). A score of 0 indicates a complete lack of discrimination (equal freezing levels in the two contexts), whereas a score of one indicates perfect discrimination (no freezing in context B).

Statistical analysis

Differences between experimental groups for neurogenic responses were analysed with Student's t-test. Morphological maturation of newborn neurons between $Pet1^{-/-}$ and control mice was analysed with the Mann–Whitney U-test. Behavioral studies were analysed with one-way ANOVA for repeated measures. Two-way ANOVA was employed in the case of the 8-OH-DPAT chronic treatment, with genotype and treatment as main factors. Bonferroni's test was used for *post hoc* comparisons. In all cases, $P < 0.05$ was considered statistically significant.

Results

We examined the consequences of constitutive 5-HT depletion on adult DG neurogenesis in two previously characterised genetic

mouse models of 5-HT depletion, Pet1$^{-/-}$ and VMAT2$^{f/f}$; SERT$^{cre/+}$ mice (Hendricks *et al.*, 2003; Narboux-Neme *et al.*, 2011).

In the Pet1$^{-/-}$ mice, an 80% depletion of brain 5-HT levels is documented (Hendricks *et al.*, 2003). Basal cell proliferation was assessed in the SGZ of the DG in young adults 24 h after BrdU injections and we found that the total number of BrdU-labeled cells was unchanged in Pet1$^{-/-}$ mice ($t_6 = 0.53$, $P = 0.62$; Fig. 1A). However when BrdU-labeled cells in the DG were counted 4 weeks after the BrdU injections, a significant increase was observed in Pet1$^{-/-}$ mice as compared to controls ($t_{16} = 2.6$, $P = 0.02$; Fig. 1B), indicating an increase in the survival rate of newborn cells. It is known that 50–75% of the newly produced cells in the DG undergo cell death between the first and fourth week after their generation (Dayer *et al.*, 2003; Snyder *et al.*, 2009). To determine when a difference in survival rate becomes visible in the Pet1$^{-/-}$ mice, we analysed newborn cell survival 1 week after BrdU injections and found that Pet1$^{-/-}$ mice already presented an increase in BrdU-labeled cells as compared to control ($t_{11} = 2.66$, $P = 0.02$; Fig. 1c). These results suggest that 5-HT may participate in the new neuron elimination process shortly after their production in the DG, as we calculated that in control mice 50% of the newborn cells are lost as early as 1 week after generation, confirming that cell death occurs very rapidly after proliferation (data not shown).

Given these unexpected results and considering that a few residual 5-HT axons still innervate the subgranular layer of the DG in the Pet1$^{-/-}$ mice (Kiyasova *et al.*, 2011; Fernandez & Gaspar, 2012), we performed the same experiments in another mouse model of 5-HT depletion: the VMAT2$^{f/f}$; SERT$^{cre/+}$ mice, in which a complete depletion of 5-HT innervation has been reported (Narboux-Neme *et al.*, 2011). Again, in the proliferation assay, the total number of BrdU-labeled cells was unchanged in VMAT2$^{f/f}$; SERT$^{cre/+}$ mice ($t_{13} = 0.22$, $P = 0.82$) 24 h after BrdU injections (Fig. 1D). In addition, the ratio of BrdU$^+$ cells co-expressing the cell cycle marker Ki67 was not different between VMAT2$^{f/f}$; SERT$^{cre/+}$ and control mice (Fig. 1H), suggesting that, at least during the first 24 h, the rate of rounds of division is similar in the two genotypes. We next evaluated new neuron survival and found that, similarly to observations in Pet1$^{-/-}$ mice, a significant increase in the number of BrdU-

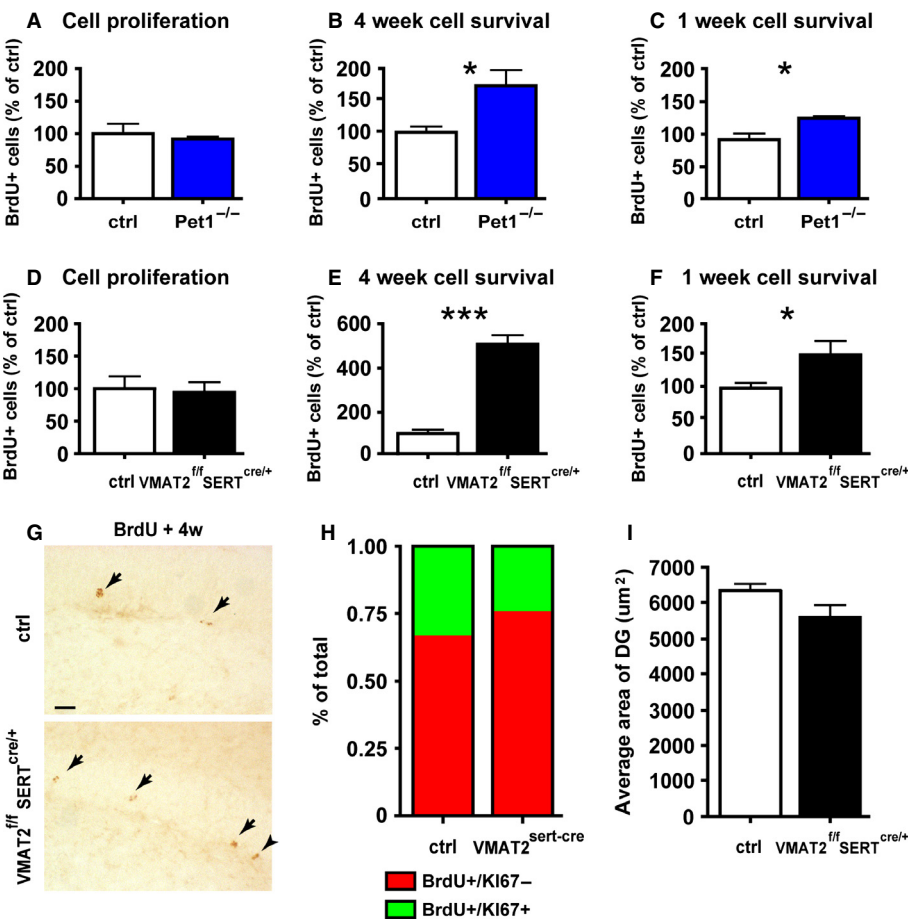

FIG. 1. Hippocampal cell proliferation and survival in two genetic hyposerotoninergic mouse models. Cell proliferation in the SGZ 24 h after BrdU administration was similar in (A) control and Pet1$^{-/-}$ mice ($n = 4$), and (D) control and VMAT2$^{f/f}$; SERT$^{cre/+}$ mice ($n = 7$ or 8). In contrast, cell survival in the cell granular layer, analysed (B and E) 4 weeks or (C and F) 1 week after BrdU administration, was increased in (B and C) the Pet1$^{-/-}$ mice ($n = 8$–10, and $n = 6$ or 7, respectively), and in (E and F) the VMAT2$^{f/f}$; SERT$^{cre/+}$ mice ($n = 5$–7, and $n = 6$, respectively) compared to their respective controls. In the experiments shown in a–c, controls included (in A) four Pet$^{+/+}$ mice; (B) two Pet$^{+/+}$ and six Pet$^{+/-}$ mice; and (C) six Pet$^{+/+}$ mice. Control groups included (D and E) three VMAT2$^{flox/flox}$ and four VMAT2$^{f/f}$; SERT$^{cre/+}$ mice; and (F) three VMAT2$^{flox/flox}$ and three VMAT2$^{f/f}$; SERT$^{cre/+}$ mice. (G) The micrographs correspond to representative BrdU immunolabeling in the DG of VMAT2$^{f/f}$; SERT$^{cre/+}$ 4 weeks after BrdU administration. (h) Proportion of BrdU$^+$ cells co-expressing the cell cycle marker Ki67 (green bars) or only BrdU$^+$ (red bars) is similar in VMAT2$^{f/f}$; SERT$^{cre/+}$ mice and control mice. (i) The area of the DG measured in 7-week-old VMAT2$^{f/f}$; SERT$^{cre/+}$ mice was similar in the two groups ($n = 5$). ***$P < 0.001$; *$P < 0.05$ comparing to control groups. All data are expressed as means ± SEM. The absolute values for the control groups (corresponding to 100%) were as follows: 181.5 ± 27.7 (A); 277.8 ± 30.8 (B); 816 ± 101.4 (C); 350.7 ± 67.1 (D); 100.1 ± 14.7 (E); 376.2 ± 46.3 (F); Scale bar, 20 μm.

labeled cells was observed in VMAT2$^{f/f}$; SERT$^{cre/+}$ mice at 4 weeks (t_{11} = 8.02, P = 0.0016) and 1 week (t_{10} = 2.25, P = 0.048) after BrdU injection (Fig. 1E–G). The area of the DG was measured in 2- and 7-week old VMAT2$^{f/f}$; SERT$^{cre/+}$ and no significant difference was detected compared to control mice (Fig. 1I), suggesting an absolute increase in the number of immature cells in VMAT2$^{f/f}$; SERT$^{cre/+}$ mice. Altogether, these results indicated that in these two constitutive hyposerotonergic mouse models, relying on different mechanisms of 5-HT depletion, newborn cells are generated in normal numbers but survive in greater numbers.

To determine whether the excess surviving neurons are maintained or eventually cleared later when neural networks become consolidated, we counted BrdU-labeled cells 16 weeks after the initial BrdU injection in VMAT2$^{f/f}$; SERT$^{cre/+}$ mice. This experiment showed a four-fold increase in new neuron survival (1335 ± 501 vs. control 342 ± 66, n = 2) similar to the one observed after 4 weeks. Altogether these results indicate that constitutive lack of 5-HT neurotransmission results in an increased survival of newborn cells in the DG, with a cumulative effect between 1 and 4 weeks. These supernumerary cells remain integrated in the hippocampal circuits 4 months after their genesis.

Both Pet1$^{-/-}$ and VMAT2$^{f/f}$; SERT$^{cre/+}$ mice have decreased 5-HT during development, and this could alter the normal maturation of hippocampal circuits (Trowbridge *et al.*, 2011). Indeed, postnatal growth retardation is observed in hyposerotonergic mouse models (Narboux-Neme *et al.*, 2013). To determine whether the observed effects on cell survival may result from defects during development, we analysed mice in which 5-HT depletion was induced in adulthood. PCPA (the inhibitor of TPH, the enzyme responsible of 5-HT synthesis) was administered to 7–8-week-old WT mice over 4 weeks, to induce a pharmacological depletion of 5-HT. At the end of this treatment, BrdU was administered to evaluate both cell proliferation and cell survival as in the other mouse models (Fig. 2A). The chronic 4-week treatment with PCPA in adult WT mice mimicked the effects previously seen in Pet1$^{-/-}$ and VMAT2$^{f/f}$; SERT$^{cre/+}$ mice: no change in cell proliferation (t_5 = 1.11, P = 0.32; Fig. 2b), but a significant increase in cell survival at 4 weeks (t_7 = 4.99, P = 0.002; Fig. 2C) and 1 week (t_4 = 3.17, P = 0.03; Fig. 2D) after BrdU administration. These results demonstrated that inducing a 5-HT depletion in adult life is enough to improve survival of the newborn neurons in the DG.

One possible cause of the increased survival rate of the newborn cells in the DG of hyposerotoninergic mouse models could be a change in the maturation rate of newly produced neurons, as demonstrated after SSRI administration (Wang *et al.*, 2008). We analysed colocalisation of BrdU with calretinin and doublecortin, which are markers of immature neurons, and with calbindin, a marker of more mature neurons. Experiments were performed in the Pet1$^{-/-}$ and control mice, 1 and 2 weeks after BrdU injections. These experiments showed an identical proportion of co-labeled cells in the two genotypes (Fig. 3A–A′ and B–B′), indicating that the surplus surviving newborn cells matured normally. Finally, we analysed the morphological maturation of the newborn neurons, using retroviral vectors that integrate only in the dividing cells. Newborn GFP-labeled cells showed a similar morphological maturation of dendritic trees in Pet1$^{-/-}$ and control mice (P = 0.4; Fig. 3C–C′).

It has recently been proposed that immature DG cells play a role in the pattern separation process (Sahay *et al.*, 2011; Nakashiba *et al.*, 2012). To evaluate the functionality of these supernumerary immature DG cells found in hyposerotonergic mice, Pet1$^{-/-}$ mice were tested in a contextual fear discrimination learning test. In a pilot study, we evaluated the level of freezing in the shock-associated context on day 0 and found negligible levels of freezing for both genotypes (t_8 = 1.5, P = 0.17; Fig. 4A). Similar increased freezing times were found for both genotypes on day 1, i.e. 24 h after the first exposure to context A (t_{22} = 0.20, P = 0.84; Fig. 4A), suggesting that Pet1$^{-/-}$ and control mice acquired and retained contextual fear conditioning at similar rates in our experimental conditions. As expected, control mice showed similar levels of freezing in the two contexts during the first 6 days (Fig. 4B), after which the discrimina-

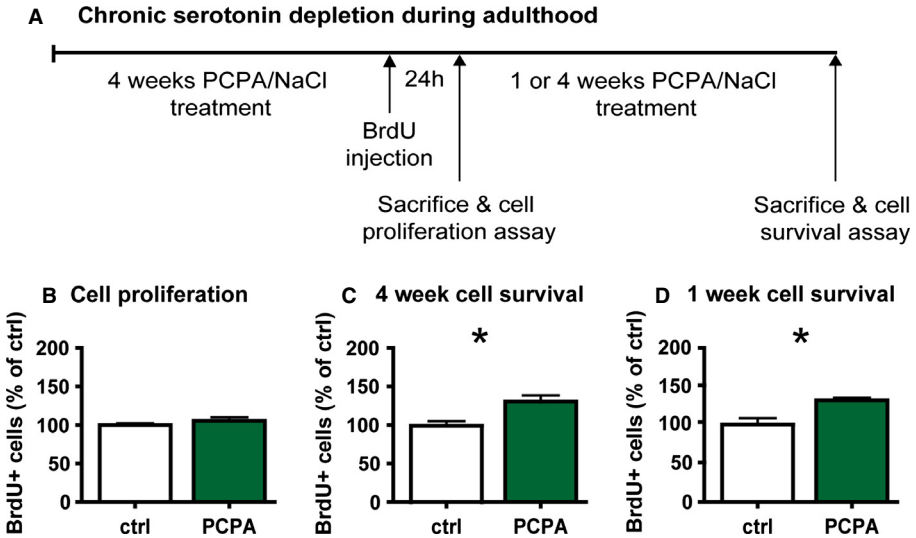

FIG. 2. Hippocampal cell proliferation and survival in a pharmacological hyposerotoninergic PCPA model. (A) Experimental protocol employed for 5-HT depletion during adulthood. PCPA was administered daily for 4 weeks (300 mg/kg for the first 3 days and 100 mg/kg for the rest of the treatment) to WT animals. BrdU (two injections of 150 mg/kg injected 2 h apart) was administered i.p. after the first 4 weeks of PCPA treatment. A group of animals was killed 24 h after BrdU treatment for evaluating cell proliferation whereas two other groups went on receiving PCPA 100 mg/kg and were killed 1 or 4 weeks after BrdU treatment for evaluating cell survival. (B) Cell proliferation in the SGZ 24 h after BrdU administration was similar in control mice and chronically PCPA-treated adult mice. In contrast, cell survival in the cell granular layer, analysed (C) 4 weeks and (D) 1 week after BrdU administration, was increased in the PCPA-chronically treated adult mice compared to their respective controls. * $P < 0.05$ compared to control groups. Data are expressed as mean ± SEM (n = 4–10 mice for each group).

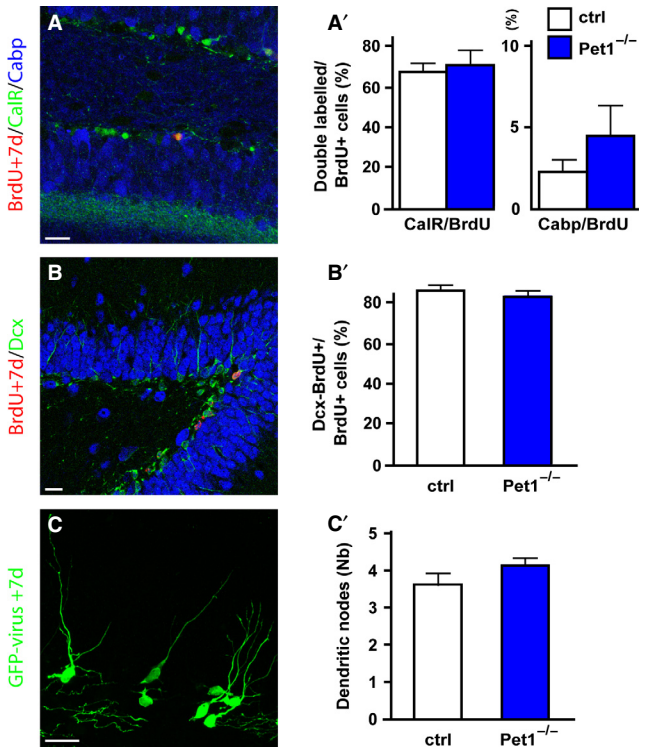

FIG. 3. Normal differentiation of newborn neurons in Pet1$^{-/-}$ mice. (A–B′) Multiple immunohistochemistry was performed on control and Pet1$^{-/-}$ mice 2 weeks after BrdU injection. The proportion of cells expressing a differentiation marker within BrdU-positive cells was measured and compared between genotypes (n = 5 or 6). We checked the expression of the immature neuron markers calretinin (CalR; a, a′) and doublecortin (Dcx; B, B′), as well as the expression of the marker of mature DG neurons calbindin (Cabp; a, a′). No difference was found in the expression of these markers between genotypes. (C, C′) Analysis of the morphology of newborn neurons was performed 1 week after the injection of GFP-expressing retrovirus. The number of nodes was quantified for each labeled cell as a marker of differentiation. No significant difference was found between controls and Pet1$^{-/-}$ mice (n = 3 or 4). Data are expressed as mean + SEM. The control group included five Pet1$^{+/-}$ mice (in experiments 3.A′ and B′), one Pet1$^{+/+}$ and two Pet1$^{+/-}$ mice (in experiment 3.C′). Scale bars, 20 µm.

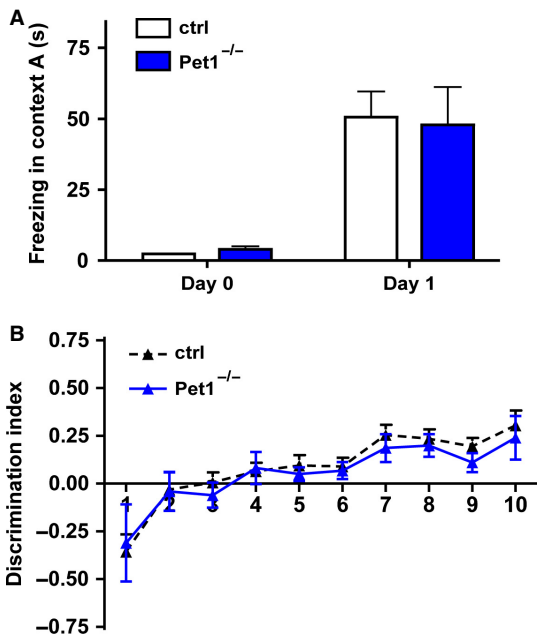

FIG. 4. Increased number of immature cells did not enhance contextual discrimination in Pet1$^{-/-}$ mice. (A) On day 0, Pet1$^{-/-}$ and control mice showed negligible levels of freezing in context A before receiving the foot shock (n = 4–6). On day 1, the two groups showed similar levels of conditioning to training context A (n = 11–13). (B) Freezing levels as indicated by the discrimination ratio, i.e. (Freezing$_{Acontext}$ – Freezing$_{Bcontext}$) / (Freezing$_{Acontext}$ + Freezing$_{Bcontext}$) were similar in Pet1$^{-/-}$ and control mice (n = 6–13). Data are expressed as mean + SEM. Pet1 control groups included four Pet1$^{+/-}$ mice (in experiment 3.a day 0), one Pet1$^{+/+}$ and 12 Pet1$^{+/-}$ mice (in experiment 3.a day 1 and 3.b).

tion index increased progressively, showing their capacity to discriminate between the shock-associated and no-shock contexts. The level of discrimination was unchanged in Pet1$^{-/-}$ mice compared to controls (interaction between time and genotype, F_9 = 0.145, P = 0.99; main effect of genotype, F_1 = 0.2841, P = 0.60), suggesting that the increased number of immature DG cells in the Pet1$^{-/-}$ mice did not modify performance in pattern separation.

The consistency in the increased survival rate of newborn neurons observed in the three different chronic hyposerotonergic models analysed here suggests that a lack of 5-HT during the first weeks after cell generation in the hippocampus hinders the regulatory mechanisms that normally operate to restrict their number before integrating into functional circuits. We hypothesised that 5-HT may act through specific 5-HT receptors to help selected surviving cells and/or to remove surplus neurons. We examined 5-HT receptor expression in the DG using *in situ* hybridisations with a large panel of 5-HT receptor probes. We found expression of six 5-HT receptors in the DG of the hippocampus: the 5-HT$_{1A}$, 5-HT$_{2B}$, 5-HT$_{3A}$ 5-HT$_4$, 5-HT$_6$ and 5-HT$_7$ receptors (Fig. 5). The 5-HT$_{1B}$ and the 5-HT$_{2A–C}$ receptors were expressed in the hippocampus but not in the DG cells. The 5-HT$_5$ probe did not provide an interpretable signal. Among the 5-HT receptors expressed in the granular cells of the DG, the 5-HT$_{1A}$ receptor matched best the localisation of newborn neurons in the SGZ, as also suggested in a recently published work (Klempin *et al.*, 2010). To determine whether the lack of 5-HT$_{1A}$ receptor stimulation in the DG of hyposerotonergic mice may be responsible for the enhanced survival phenotype, we evaluated cell survival in both Pet1$^{-/-}$ and PCPA-treated mice following a 1-week treatment with the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT. As the 5-HT raphe neurons projecting to the hippocampus do not develop in the Pet1$^{-/-}$ mice (Kiyasova *et al.*, 2011), the effects of 8-OH-DPAT in these mutants are essentially postsynaptic. Statistical analysis showed a significant interaction between DPAT treatment and genotype for Pet1 mice (F_1 = 6.58, P = 0.03) but not for DPAT treatment and PCPA treatment (F_1 = 7.28, P = 0.07). Additionally, Bonferroni *post hoc* tests indicated that the number of 1-week-old neurons was significantly higher in Pet1$^{-/-}$ mice ($P < 0.05$) as shown above, and in PCPA-treated mice ($P < 0.01$) compared with their respective control groups (Fig. 6a and b). In control mice, a 1-week treatment with 8-OH-DPAT did not change cell survival. However, the number of newborn cells reverted to control levels after the 1-week treatment with 8-OH-DPAT in the Pet1$^{-/-}$ ($P < 0.05$) and the PCPA-treated mice ($P < 0.01$), suggesting a cell-autonomous regulation of cell survival via the postsynaptic 5-HT$_{1A}$ receptor. Thus, 5-HT$_{1A}$ postsynaptic receptor stimulation can reverse the enhanced survival observed in genetic and pharmacological models of constitutive 5-HT depletion.

Discussion

The present observations demonstrate that 5-HT is: (i) dispensable for neural stem cell and progenitor proliferation in the DG; and (ii)

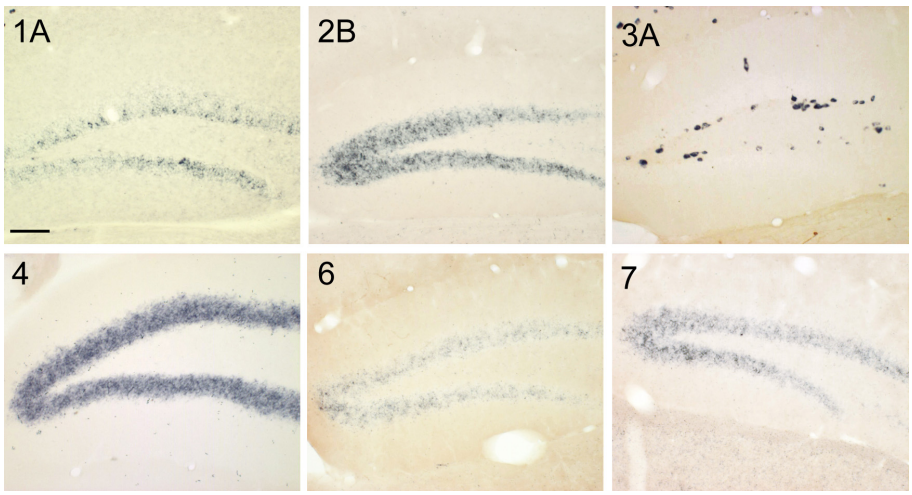

FIG. 5. 5-HT receptor expression in the adult DG. Representative 5-HT$_{1A}$, 5-HT$_{2B}$, 5-HT$_{3A}$, 5-HT$_4$, 5-HT$_6$ and 5-HT$_7$ mRNA labeling in the DG of WT mice determined by *in situ* hybridisation. Scale bar, 100 μm.

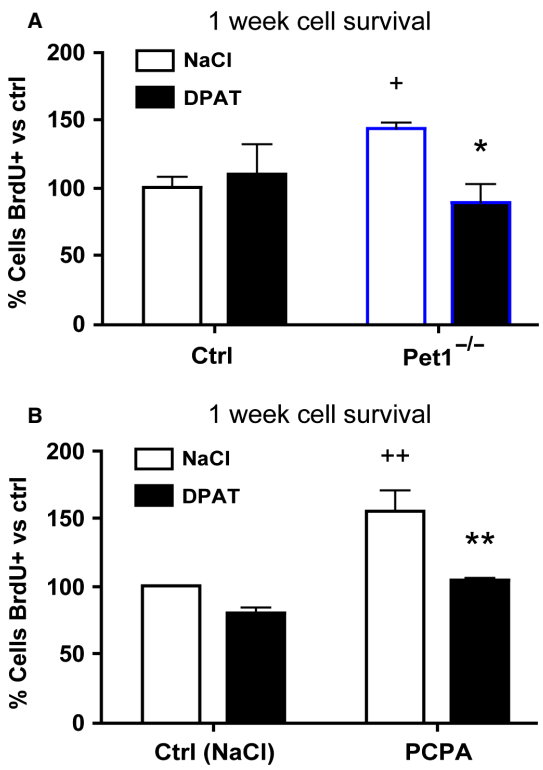

FIG. 6. 5-HT$_{1A}$ agonist administration normalised cell survival in the DG of hyposerotonergic mice. After a 1-week treatment, 8-OH-DPAT (0.5 mg/kg, twice a day) did not affect cell survival in control (Pet1$^{+/+}$) mice, but hindered the increased basal cell survival phenotype described in (A) Pet1$^{-/-}$ mice as well as in (B) 4-week-treated PCPA WT mice. $^{*,+}P < 0.05$; $^{**,++}P < 0.01$; $n = 3$ or 4. Data are expressed as mean + SEM. The absolute values for the control groups (corresponding to 100%) were as follows (mean ± SEM): 686 ± 65 (in experiment 5.a); 1376 ± 11.1 (in experiment 5.b).

necessary for the removal of surplus immature newborn neurons via postsynaptic 5-HT$_{1A}$ receptor stimulation.

A number of studies report that enhanced hippocampal neurogenesis is required for the therapeutic response to the increased 5-HT levels induced by SSRI treatment (Malberg *et al.*, 2000; Santarelli *et al.*, 2003), reviewed in Samuels & Hen (2011) although there is as yet little evidence that impaired neurogenesis has a causal role in the etiology of depression (Pittenger & Duman, 2008); see the meta-analysis in Petrik *et al.* (2012). Furthermore, reduced developmental cell death has been observed in mice knocked out for the serotonin transporter, suggesting an anti-apoptotic effect of 5-HT in the developing brain (Persico *et al.*, 2003). Overall these observations led us to predict that reduced neurogenesis and cell survival should be observed in hyposerotonergic mice. Initial studies in rats by Daszuta and colleagues showed a strong reduction in hippocampal cell proliferation after serotonergic 5,7-DHT lesions in the raphe (Brezun & Daszuta, 1999, 2000). However, subsequent studies using 5,7-DHT lesions by intracerebroventricular injection did not confirm these results and reported no change in cell proliferation (Huang & Herbert, 2005) or in cell survival (Jha *et al.*, 2006). These conflicting reports suggest that 5-HT could play multiple and perhaps opposing roles, via different 5-HT receptors, in the regulation of the different neurogenic stages. Nevertheless, experimental factors, such as the mode of 5-HT depletion or up-regulation, the duration of treatment and timing of observations, could probably also account for these discrepancies (Huang & Herbert, 2005; Jha *et al.*, 2006). Here, we found that there was no change in cell proliferation in the DG of three independent models of 5-HT depletion: complete loss of 5-HT-storage in the VMAT2f/f; SERTcre/+ axons (Narboux-Neme *et al.*, 2011), failure to differentiate raphe neurons in Pet1$^{-/-}$ mice (Hendricks *et al.*, 2003; Kiyasova *et al.*, 2011), and pharmacological inhibition of 5-HT synthesis with PCPA. These results indicate that 5-HT depletion had no effect on neural stem cells or the progenitor cell cycle as demonstrated by the BrdU$^+$-Ki67$^+$/BrdU$^+$-Ki67$^-$ ratio. These observations are in agreement with a recent report on Tph2-deficient mice, in which cell proliferation is unchanged in the hippocampus (Klempin *et al.*, 2013). Overall, these results obtained in different knockout strains support the view that constitutive or chronic hyposerotoninergia is not a negative mirror image of chronic antidepressant administration, at least as far as the control of neurogenesis is concerned.

The majority of the cells generated in the DG of WT mice die during the cell differentiation process (Snyder *et al.*, 2009), and it was even recently demonstrated that this cell death occurs between 1 and 4 days after cell birth (Sierra *et al.*, 2010). In our study, we also found in WT animals that most of the newborn neurons are eliminated within the first week of their maturation. By contrast, all

our three 5-HT depletion models showed a robust increase in newborn neuron survival, an effect that is observed as early as 1 week after their birth and that seems to last at least for 16 weeks, with no change in the DG surface area. Interestingly, the magnitude of increase in the cell survival was inversely correlated with the quantity of 5-HT: the increase in DG cell survival was stronger in the $VMAT2^{f/f}$; $SERT^{cre/+}$ mice, which show the most severe 5-HT depletion (Narboux-Neme *et al.*, 2011), compared to the $Pet1^{-/-}$ mice and PCPA-treated mice in which 5-HT biosynthesis is not totally abolished (Hendricks *et al.*, 2003; Vitalis *et al.*, 2007). These observations indicate that 5-HT is required in the process of eliminating newborn neurons.

Several 5-HT receptors have been implicated in the neurogenic effects of antidepressants (Santarelli *et al.*, 2003; Banasr *et al.*, 2004; Klempin *et al.*, 2010; Soumier *et al.*, 2010; Diaz *et al.*, 2012). Particularly, the down-regulation of presynaptic $5\text{-}HT_{1A}$ receptors induced by chronic SSRI treatment is thought to be required for the expression of some behavioural effects (Popa *et al.*, 2010). To determine which 5-HT receptors are involved in the effects of 5-HT depletion on neuron survival, we performed an *in situ* hybridisation screen and found that the $5\text{-}HT_{1A}$ receptor displays an interesting distribution because it is expressed in the SGZ as shown here with *in situ* hybridisation and previously shown with immunocytochemistry (Klempin *et al.*, 2010), precisely in the DG layer where stem cells and progenitor cells are located (Encinas *et al.*, 2006). However, $5\text{-}HT_{1A}{}^{-/-}$ mice show no change in basal levels of BrdU incorporation (Santarelli *et al.*, 2003), and studies using various 5-HT receptor agonists or antagonists have all concluded that $5\text{-}HT_{1A}$ agonist stimulation from 1 to 2 weeks had no effect in DG $BrdU^+$ cell numbers in WT animals (Banasr *et al.*, 2004; Huang & Herbert, 2005; Klempin *et al.*, 2010; Soumier *et al.*, 2010). Indeed, we confirmed that a 1-week treatment with 8-OH-DPAT did not alter $BrdU^+$ cell numbers in control mice. In contrast, the same treatment normalised the number of newborn cells to control levels in both $Pet1^{-/-}$ and PCPA-treated WT mice. The strong reduction in 5-HT innervation in the hippocampus of $Pet1^{-/-}$ mice allows us to conclude that the results obtained here reflect mostly the involvement of postsynaptic $5\text{-}HT_{1A}$ receptor stimulation in the regulation of cell survival, and suggest a role for 5-HT, via the $5\text{-}HT_{1A}$ receptor, in the differentiation-or-death decision of newborn cells. However because $5\text{-}HT_{1A}$ receptors are expressed in a variety of other hippocampal cell types (our observations; also Klempin *et al.*, 2010) and in the prefrontal cortex, which projects to the hippocampus, it remains unclear whether this pro-survival effect is direct or indirect. Interestingly, the vast majority of DG newborn cells undergo death by apoptosis in the first 4 days of their life, during the transition from amplifying neural progenitors to early post-proliferative neuroblasts (Sierra *et al.*, 2010). The 5-HT survival–apoptosis decision, $5\text{-}HT_{1A}$ receptor-dependant, may correspond to this transition from late-dividing neural progenitors to early postmitotic neuroblasts.

Increased survival of newborn neurons may be expected to have impacts on memory and learning (reviewed in Koehl & Abrous, 2011) and depressive-like phenotypes (reviewed in Petrik *et al.*, 2012), although recent reviews underline the difficulty in establishing a causal role for increased neurogenesis in the control of these behaviours. Cytostatic drugs, irradiation and genetic manipulation have demonstrated that the ablation of newborn neurons impairs several forms of hippocampal-dependent learning and memory. However, controlled elimination of newborn cells by cell death is also crucial, as mice with disruption of the pro-apoptotic factor Bax performed worse than control mice in spatial memory tests, whereas pattern separation was improved (Dupret *et al.*, 2007; Sahay *et al.*, 2011). Likewise, another genetically modified mouse model with an increased population of young DG cells exhibited enhanced pattern separation between similar contexts (Nakashiba *et al.*, 2012). In contrast, we report here that $Pet1^{-/-}$ mice have a normal performance in pattern separation, although they have an increased number of immature DG cells. It is possible that in the hyposerotoninergic mouse lines the functional deficits caused by globally decreased 5-HT neurotransmission prevents the potential beneficial effect of an increased number of immature neurons in the DG. Moreover, despite the apparently normal maturation of newborn neurons at early stages (1–2 week), it is possible that late maturation or integration of these neurons in functional circuits is suboptimal in conditions of hyposerotoninergia. Furthermore, recent observations in Tph2-knockout mice indicate that the normal enhancement of neurogenesis induced by exercise was lacking in the hyposerotoninergic conditions (Klempin *et al.*, 2013), indicating some dysfunctional pro-neurogenic mechanisms. It remains to be determined whether the changes observed in the number of surviving adult-born neurons is responsible for some of the behavioral traits observed in the $Pet1^{-/-}$ mice, such as enhanced conditioned fear responses (Kiyasova *et al.*, 2011). Our results are consistent with a possible relationship between enhanced neurogenesis and reduced depression-like behaviour (Pittenger & Duman, 2008), as the $VMAT2^{f/f}$; $SERT^{cre/+}$ mice displayed enhanced antidepressant-like responses with increased struggling in the tail suspension test (Narboux-Neme *et al.*, 2011) while $Pet1^{-/-}$ mice showed no change in floating time in the forced-swim test (Diaz *et al.*, 2012). Moreover, in both mouse lines anxiolytic-like responses were noted in the novelty-suppressed feeding test (Kiyasova *et al.*, 2011; Narboux-Neme *et al.*, 2011).

The present findings add new insights and a further degree of complexity in the mechanisms and pathways that finely regulate the fate of immature newborn DG cells. More generally, they show that constitutive or long-lasting hyposerotoninergia in adult life does not reflect an inverted mirror image of the long-term effects of antidepressant administration.

Disclosure

The authors have no financial interests or conflict of interest to disclose.

Acknowledgments

This research was funded by Institut National de la Recherche et de la Santé (Inserm), the Université Pierre et Marie Curie (Paris 6), the European Commission (FP7-health-2007-A-201714), the Fondation Jerome Lejeune, the Fondation de la Recherche sur le Cerveau and the Fondation de la Recherche Médicale. S.D. and N.N. were supported by fellowships from the Région Ile de France and S.T. was supported by the Henry Russell Shaw travelling fellowship from Harvard University. The laboratory of P.G. is part of the Ecole des Neurosciences de Paris, training network.

Abbreviations

5-HT, 5-hydroxytryptamine or serotonin; 8-OH-DPAT, 8-Hydroxy-2-(di-n-propylamino)tetralin; BrdU, 5-bromo-2′deoxyuridine; DG, dentate gyrus; GFP, green-fluorescent protein; PCPA, para-chlorophenylalanine; SGZ, subgranular zone; SSRI, selective serotonin reuptake inhibitor; WT, wild-type.

References

Banasr, M., Hery, M., Printemps, R. & Daszuta, A. (2004) Serotonin-induced increases in adult cell proliferation and neurogenesis are

mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone. *Neuropsychopharmacol.*, **29**, 450–460.
Brandt, M.D., Jessberger, S., Steiner, B., Kronenberg, G., Reuter, K., Bick-Sander, A., von der Behrens, W. & Kempermann, G. (2003) Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. *Mol. Cell. Neurosci.*, **24**, 603–613.
Brezun, J.M. & Daszuta, A. (1999) Depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats. *Neuroscience*, **89**, 999–1002.
Brezun, J.M. & Daszuta, A. (2000) Serotonergic reinnervation reverses lesion-induced decreases in PSA–NCAM labeling and proliferation of hippocampal cells in adult rats. *Hippocampus*, **10**, 37–46.
Dayer, A.G., Ford, A.A., Cleaver, K.M., Yassaee, M. & Cameron, H.A. (2003) Short-term and long-term survival of new neurons in the rat dentate gyrus. *J. Comp. Neurol.*, **460**, 563–572.
Diaz, S.L., Doly, S., Narboux-Neme, N., Fernandez, S., Mazot, P., Banas, S.M., Boutourlinsky, K., Moutkine, I., Belmer, A., Roumier, A. & Maroteaux, L. (2012) 5-HT(2B) receptors are required for serotonin-selective antidepressant actions. *Mol. Psychiatr.*, **17**, 154–163.
Dupret, D., Fabre, A., Dobrossy, M.D., Panatier, A., Rodriguez, J.J., Lamarque, S., Lemaire, V., Oliet, S.H., Piazza, P.V. & Abrous, D.N. (2007) Spatial learning depends on both the addition and removal of new hippocampal neurons. *PLoS Biol.*, **5**, e214.
Encinas, J.M., Vaahtokari, A. & Enikolopov, G. (2006) Fluoxetine targets early progenitor cells in the adult brain. *Proc. Natl. Acad. Sci. USA*, **103**, 8233–8238.
Fabel, K. & Kempermann, G. (2008) Physical activity and the regulation of neurogenesis in the adult and aging brain. *Neuromol. Med.*, **10**, 59–66.
Fernandez, S.P. & Gaspar, P. (2012) Investigating anxiety and depressive-like phenotypes in genetic mouse models of serotonin depletion. *Neuropharmacology*, **62**, 144–154.
Hancock, A., Priester, C., Kidder, E. & Keith, J.R. (2009) Does 5-bromo-2'-deoxyuridine (BrdU) disrupt cell proliferation and neuronal maturation in the adult rat hippocampus *in vivo*? *Behav. Brain Res.*, **199**, 218–221.
Hendricks, T.J., Fyodorov, D.V., Wegman, L.J., Lelutiu, N.B., Pehek, E.A., Yamamoto, B., Silver, J., Weeber, E.J., Sweatt, J.D. & Deneris, E.S. (2003) Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. *Neuron*, **37**, 233–247.
Huang, G.J. & Herbert, J. (2005) The role of 5-HT1A receptors in the proliferation and survival of progenitor cells in the dentate gyrus of the adult hippocampus and their regulation by corticoids. *Neuroscience*, **135**, 803–813.
Jacobs, B.L. & Azmitia, E.C. (1992) Structure and function of the brain serotonin system. *Physiol. Rev.*, **72**, 165–229.
Jha, S., Rajendran, R., Davda, J. & Vaidya, V.A. (2006) Selective serotonin depletion does not regulate hippocampal neurogenesis in the adult rat brain: differential effects of p-chlorophenylalanine and 5,7-dihydroxytryptamine. *Brain Res.*, **1075**, 48–59.
Kempermann, G. (2011) Seven principles in the regulation of adult neurogenesis. *Eur. J. Neurosci.*, **33**, 1018–1024.
Kiyasova, V., Fernandez, S.P., Laine, J., Stankovski, L., Muzerelle, A., Doly, S. & Gaspar, P. (2011) A genetically defined morphologically and functionally unique subset of 5-HT neurons in the mouse raphe nuclei. *J. Neurosci.*, **31**, 2756–2768.
Klempin, F., Babu, H., De Pietri Tonelli, D., Alarcon, E., Fabel, K. & Kempermann, G. (2010) Oppositional effects of serotonin receptors 5-HT1a, 2, and 2c in the regulation of adult hippocampal neurogenesis. *Front. Mol. Neurosci.*, **3**, 14.
Klempin, F., Beis, D., Mosienko, V., Kempermann, G., Bader, M. & Alenina, N. (2013) Serotonin is required for exercise-induced adult hippocampal neurogenesis. *J. Neurosci.*, **33**, 8270–8275.
Koehl, M. & Abrous, D.N. (2011) A new chapter in the field of memory: adult hippocampal neurogenesis. *Eur. J. Neurosci.*, **33**, 1101–1114.
Malberg, J.E., Eisch, A.J., Nestler, E.J. & Duman, R.S. (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J. Neurosci.*, **20**, 9104–9110.
Nakashiba, T., Cushman, J.D., Pelkey, K.A., Renaudineau, S., Buhl, D.L., McHugh, T.J., Rodriguez Barrera, V., Chittajallu, R., Iwamoto, K.S., McBain, C.J., Fanselow, M.S. & Tonegawa, S. (2012) Young dentate granule cells mediate pattern separation, whereas old granule cells facilitate pattern completion. *Cell*, **149**, 188–201.
Narboux-Neme, N., Pavone, L.M., Avallone, L., Zhuang, X. & Gaspar, P. (2008) Serotonin transporter transgenic (SERTcre) mouse line reveals developmental targets of serotonin specific reuptake inhibitors (SSRIs). *Neuropharmacology*, **55**, 994–1005.
Narboux-Neme, N., Sagne, C., Doly, S., Diaz, S.L., Martin, C.B., Angenard, G., Martres, M.P., Giros, B., Hamon, M., Lanfumey, L., Gaspar, P. & Mongeau, R. (2011) Severe serotonin depletion after conditional deletion of the vesicular monoamine transporter 2 gene in serotonin neurons: neural and behavioral consequences. *Neuropsychopharmacol.*, **36**, 2538–2550.
Narboux-Neme, N., Angenard, G., Mosienko, V., Klempin, F., Pitychoutis, P.M., Deneris, E., Bader, M., Giros, B., Alenina, N. & Gaspar, P. (2013) Postnatal growth defects in mice with constitutive depletion of central serotonin. *ACS Chem. Neurosci.*, **4**, 171–181.
Persico, A.M., Baldi, A., Dell'Acqua, M.L., Moessner, R., Murphy, D.L., Lesch, K.P. & Keller, F. (2003) Reduced programmed cell death in brains of serotonin transporter knockout mice. *NeuroReport*, **14**, 341–344.
Petrik, D., Lagace, D.C. & Eisch, A.J. (2012) The neurogenesis hypothesis of affective and anxiety disorders: are we mistaking the scaffolding for the building? *Neuropharmacology*, **62**, 21–34.
Pittenger, C. & Duman, R.S. (2008) Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacol.*, **33**, 88–109.
Popa, D., Cerdan, J., Repérant, C., Guiard, B.P., Guilloux, J.P., David, D.J. & Gardier, A.M. (2010) A longitudinal study of 5-HT outflow during chronic fluoxetine treatment using a new technique of chronic microdialysis in a highly emotional mouse strain. *Eur. J. Pharmacol.*, **628**, 83–90.
van Praag, H., Kempermann, G. & Gage, F.H. (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat. Neurosci.*, **2**, 266–270.
Rodriguez, A., Ehlenberger, D.B., Dickstein, D.L., Hof, P.R. & Wearne, S.L. (2008) Automated three-dimensional detection and shape classification of dendritic spines from fluorescence microscopy images. *PLoS ONE*, **3**, e1997.
Sahay, A., Scobie, K.N., Hill, A.S., O'Carroll, C.M., Kheirbek, M.A., Burghardt, N.S., Fenton, A.A., Dranovsky, A. & Hen, R. (2011) Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature*, **472**, 466–470.
Samuels, B.A. & Hen, R. (2011) Neurogenesis and affective disorders. *Eur. J. Neurosci.*, **33**, 1152–1159.
Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C. & Hen, R. (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science*, **301**, 805–809.
Sierra, A., Encinas, J.M., Deudero, J.J., Chancey, J.H., Enikolopov, G., Overstreet-Wadiche, L.S., Tsirka, S.E. & Maletic-Savatic, M. (2010) Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell*, **7**, 483–495.
Snyder, J.S., Choe, J.S., Clifford, M.A., Jeurling, S.I., Hurley, P., Brown, A., Kamhi, J.F. & Cameron, H.A. (2009) Adult-born hippocampal neurons are more numerous, faster maturing, and more involved in behavior in rats than in mice. *J. Neurosci.*, **29**, 14484–14495.
Soumier, A., Banasr, M., Goff, L.K. & Daszuta, A. (2010) Region- and phase-dependent effects of 5-HT(1A) and 5-HT(2C) receptor activation on adult neurogenesis. *Eur. Neuropsychopharm.*, **20**, 336–345.
Toni, N. & Sultan, S. (2011) Synapse formation on adult-born hippocampal neurons. *Eur. J. Neurosci.*, **33**, 1062–1068.
Trowbridge, S., Narboux-Neme, N. & Gaspar, P. (2011) Genetic models of serotonin (5-HT) depletion: what do they tell us about the developmental role of 5-HT? *Anat. Rec. (Hoboken)*, **294**, 1615–1623.
Vitalis, T., Cases, O., Passemard, S., Callebert, J. & Parnavelas, J.G. (2007) Embryonic depletion of serotonin affects cortical development. *Eur. J. Neurosci.*, **26**, 331–344.
Wang, J.W., David, D.J., Monckton, J.E., Battaglia, F. & Hen, R. (2008) Chronic fluoxetine stimulates maturation and synaptic plasticity of adult-born hippocampal granule cells. *J. Neurosci.*, **28**, 1374–1384.
Warner-Schmidt, J.L. & Duman, R.S. (2006) Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment. *Hippocampus*, **16**, 239–249.
Wojtowicz, J.M. & Kee, N. (2006) BrdU assay for neurogenesis in rodents. *Nat. Protoc.*, **1**, 1399–1405.
Zhao, Z.Q., Scott, M., Chiechio, S., Wang, J.S., Renner, K.J., Gereau, R.W. IV, Johnson, R.L., Deneris, E.S. & Chen, Z.F. (2006) Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. *J. Neurosci.*, **26**, 12781–12788.
Zhuang, X., Masson, J., Gingrich, J.A., Rayport, S. & Hen, R. (2005) Targeted gene expression in dopamine and serotonin neurons of the mouse brain. *J. Neurosci. Meth.*, **143**, 27–32.