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Research Report

Selective serotonin depletion does not regulate hippocampal neurogenesis in the adult rat brain: Differential effects of *p*-chlorophenylalanine and 5,7-dihydroxytryptamineShanker Jha^a, Rajeev Rajendran^a, Jasmine Davda^b, Vidita A. Vaidya^{a,*}^aDepartment of Biological Sciences, Tata Institute of Fundamental Research, Mumbai 400005, India^bNicholas Piramal Research Centre, Mumbai 400063, India

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ABSTRACT

Serotonin is suggested to regulate adult hippocampal neurogenesis, and previous studies with serotonin depletion reported either a decrease or no change in adult hippocampal progenitor proliferation. We have addressed the effects of serotonin depletion on distinct aspects of adult hippocampal neurogenesis, namely the proliferation, survival and terminal differentiation of hippocampal progenitors. We used the serotonin synthesis inhibitor *p*-chlorophenylalanine (PCPA) or the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) to deplete serotonin levels. 5,7-DHT selectively decreased hippocampal serotonin levels, while PCPA resulted in a significant decline in both serotonin and norepinephrine levels. We observed a robust decline in the proliferation and survival of adult hippocampal progenitors following PCPA treatment. This was supported by a decrease in the number of doublecortin-positive cells in the neurogenic niche in the hippocampus. In striking contrast, 5,7-DHT did not alter the proliferation or survival of adult hippocampal progenitors and did not alter the number of doublecortin-positive cells. The terminal differentiation of adult hippocampal progenitors was not altered by either PCPA or 5,7-DHT treatment. An acute increase in serotonin levels also did not influence adult hippocampal progenitor proliferation. These results suggest that selective serotonin depletion or an acute induction in serotonin levels does not regulate adult hippocampal neurogenesis, whereas treatment with PCPA that induces a decline in both serotonin and norepinephrine levels results in a significant decrease in adult hippocampal neurogenesis. Our results highlight the need for future studies to examine the role of other monoamines in both the effects of stress and antidepressants on adult hippocampal neurogenesis.

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Abbreviations:

PCPA, *p*-chlorophenylalanine
PCA, *p*-chloroamphetamine
5,7-DHT, 5,7-dihydroxytryptamine
TCP, tranlycypromine
L-Trp, L-Tryptophan
SGZ, subgranular zone
GCL, granule cell layer
BrdU, 5-bromo-2'-deoxyuridine
DG, dentate gyrus
SVZ, subventricular zone
5-HT, 5-hydroxytryptamine
D β H, dopamine β -hydroxylase
DCX, doublecortin
NeuN, neuronal nuclei
GFAP, glial fibrillary acidic protein
SSRI, serotonin selective reuptake inhibitor
BDNF, brain-derived neurotrophic factor
HPLC, high performance liquid chromatography
i.p., intraperitoneal
i.c.v., intracerebroventricular
SEM, standard error of mean
ANOVA, analysis of variance

1. Introduction

In the mammalian brain, progenitor cells residing within the hippocampal dentate gyrus subfield retain the ability to form new neurons throughout adult life (Eriksson et al., 1998; Kempermann and Gage, 2000). These adult hippocampal progenitors are located in the subgranular zone (SGZ), at the border between the hilus and the granule cell layer in the dentate gyrus subfield. Adult hippocampal progenitors undergo mitosis in the SGZ, migrate into the granule cell layer and following terminal differentiation predominantly form granule cell neurons that integrate into hippocampal circuitry (van Praag et al., 2002). The process of adult hippocampal neurogenesis has been implicated to play a role in hippocampal function (Schinder and Gage, 2004) and is regulated by a variety of factors including environmental perturbations like stress (Gould et al., 1997), as well as therapeutic agents such as antidepressants (Malberg et al., 2000). Studies indicate that animal models of depression (Malberg and Duman, 2003), as well as stress (Gould et al., 1997), may reduce the process of adult hippocampal neurogenesis, which in turn can be reversed following antidepressant administration (Czeh et al., 2001). Recent results suggest that hippocampal neurogenesis may be required to mediate some of the behavioral effects of antidepressants in rodent models (Santarelli et al., 2003). This has led to the hypothesis that reduced hippocampal neurogenesis may be associated with depressive disorders, while an enhancement in this process may contribute to the therapeutic effects of antidepressant treatment (Duman et al., 2001). As a consequence, there has been a considerable interest in the neurotransmitter pathways and trophic

factors that regulate adult hippocampal neurogenesis and may contribute to the effects of animal models of depression and antidepressant drugs.

The monoaminergic theory of affective disorders suggests that a reduction in serotonin or norepinephrine levels may contribute to a depressive phenotype, whereas adaptations that result from enhanced monoamines may be critical to the clinical benefits of antidepressants (Heninger et al., 1996; Nutt, 2002). It has been hypothesized that elevated levels of the monoamines, serotonin and norepinephrine may contribute to the antidepressant-mediated increase in hippocampal neurogenesis, while a reduction in these monoamines may underlie the decline in neurogenesis observed in animal models of depression and stress (Duman et al., 2001; Vaidya and Duman, 2001). Reports indicate that both serotonin and norepinephrine may regulate adult hippocampal neurogenesis (Brezun and Daszuta, 1999, 2000; Kulkarni et al., 2002). Norepinephrine depletion is known to reduce hippocampal progenitor proliferation, but not influence progenitor survival and differentiation (Kulkarni et al., 2002). Serotonin has trophic effects during development (Gaspar et al., 2003), and provides a rich innervation to the adult hippocampus (Azmitia and Whitaker-Azmitia, 1995) where it has been suggested to retain a trophic role (Gould, 1999; Djavadian, 2004). The effects of serotonin depletion on adult hippocampal neurogenesis have focused on examining the regulation of hippocampal progenitor proliferation, and there appear to be discrepancies in the reported results with either a decrease or no effect on proliferation (Brezun and Daszuta, 1999; Huang and Herbert, 2005). The influence of serotonin depletion on the survival and differentiation of adult hippocampal progenitors is at present unknown. Given that the process of adult hippocampal

neurogenesis encompasses the proliferation, survival and differentiation of adult hippocampal progenitors, it is important to understand the consequences of decreased serotonergic tone on these distinct aspects of adult neurogenesis. The present study was carried out to examine the influence of serotonin depletion on distinct aspects of adult hippocampal neurogenesis, and to address the consequences of an acute increase in serotonin levels on adult hippocampal progenitor proliferation.

2. Results

2.1. Influence of serotonin depletion on the proliferation of adult hippocampal progenitors

Two different treatment paradigms were followed to induce serotonin depletion, namely treatment with the serotonin synthesis inhibitor *p*-chlorophenylalanine (PCPA) or the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT). The effect of serotonin depletion on the proliferation of adult hippocampal progenitors was examined using the mitotic marker BrdU to label dividing cells (Fig. 1A). BrdU-positive cells

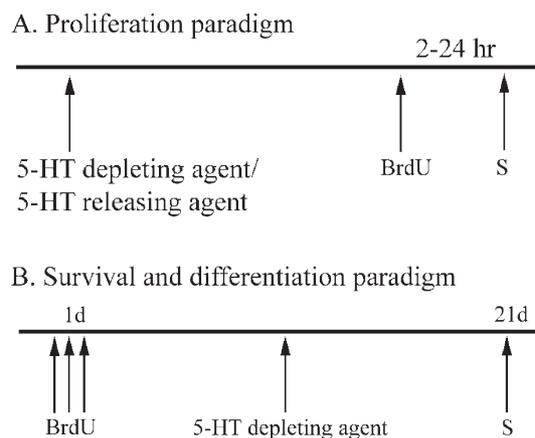


Fig. 1 – BrdU labeling paradigms. (A) Proliferation paradigm; rats were subjected to either serotonin-depleting or -releasing agents. Animals were administered the serotonin depleting agent *p*-chlorophenylalanine (PCPA) or the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT). To induce serotonin release, animals received either *p*-chloroamphetamine (PCA) or a treatment combination of tranlycypromine (TCP) with *L*-Tryptophan (*L*-Trp). To assess the effects of these treatments on the proliferation of adult hippocampal progenitors, animals were treated with the mitotic marker BrdU at distinct time points after the drug treatment regime and sacrificed (S) 2 to 24 h after BrdU treatment. In one experiment with PCPA, animals were also sacrificed 6 h after BrdU treatment. (B) Survival and differentiation paradigms; to address the influence of serotonin depletion on the survival and differentiation of adult hippocampal progenitors, drug-naïve animals were first administered BrdU (3 doses, 2hr apart) followed by treatment with the serotonin depleting drugs PCPA or 5,7-DHT and sacrificed (S) 21 days after BrdU treatment.

observed in the proliferation experiment were localized at the border of the GCL and the hilus within the SGZ and were often seen to be in clusters. Quantitative analysis of the effects of serotonin depletion on the number of BrdU-positive cells in the SGZ and hilus revealed a significant decrease in BrdU-positive cells in the SGZ (40%) and hilus (80%) of PCPA-treated animals compared to the vehicle-treated controls (Figs. 2A–C). In a separate experiment, we also addressed whether such a decline reflected a true decrease in proliferation by sacrificing animals 2 h following BrdU injection. A significant decrease was also observed in the number of BrdU-positive cells in the SGZ and hilus of PCPA-treated animals sacrificed 2 h following a single BrdU injection (SGZ BrdU-positive cell number: Vehicle = 1784 ± 145 , PCPA = $1238 \pm 112^*$; Hilar BrdU-positive cell number: Vehicle = 649 ± 65 , PCPA = $421 \pm 57^*$; results are the mean \pm SEM, $^*P < 0.05$, Student's *t* test). In striking contrast, the number of BrdU-positive cells within the SGZ and hilus of animals subjected to serotonin depletion using the serotonergic neurotoxin 5,7-DHT did not significantly differ from that in the vehicle-treated control group (Figs. 2D–F). The changes in BrdU-positive cell number observed following PCPA treatment are unlikely to be attributable to any changes in hippocampal volume as neither PCPA nor 5,7-DHT treatment led to a significant change in total hippocampal volume or GCL volume in the dentate gyrus (PCPA experiment: Total hippocampal volume: Vehicle = 25.77 ± 0.80 mm³, PCPA = 27.05 ± 0.69 mm³; GCL volume: Vehicle = 1.23 ± 0.09 mm³, PCPA = 1.29 ± 0.12 mm³; 5,7-DHT experiment: Total hippocampal volume: Vehicle = 23.77 ± 1.53 mm³, 5,7-DHT = 24.30 ± 1.66 mm³; GCL volume: Vehicle = 1.04 ± 0.02 mm³, 5,7-DHT = 1.03 ± 0.05 mm³, Results are the mean \pm SEM).

We also determined the number of BrdU-positive cells/mm² in the other major neurogenic region of the adult rat brain, namely the subventricular zone (SVZ). Neither PCPA nor 5,7-DHT treatment resulted in any change in BrdU-positive cell number in the SVZ (PCPA experiment: Vehicle = 4575 ± 231 ; PCPA = 5381 ± 340 ; 5,7-DHT experiment: Vehicle = 5268 ± 561 ; 5,7-DHT = 5248 ± 180 ; Results are expressed as the number of BrdU-positive cells/mm² and are the mean \pm SEM).

2.2. Influence of PCPA and 5,7-DHT treatment on hippocampal serotonergic and noradrenergic innervation, and on hippocampal serotonin and norepinephrine levels

Treatment with PCPA or 5,7-DHT resulted in a marked disruption of the serotonergic fiber innervation to the hippocampus, as determined by immunohistochemical staining for serotonin (Figs. 3A, B). In addition, the noradrenergic terminals in the hippocampus were visualized using immunohistochemical staining for dopamine β -hydroxylase (D β H), a marker for noradrenergic fibers. D β H staining did not reveal any obvious change in the noradrenergic fiber innervation to the hippocampus (Figs. 3A, B). To quantitatively estimate the levels of serotonin and norepinephrine in the hippocampus, we used HPLC analysis. PCPA treatment resulted in a significant decrease in both serotonin (~82%) and norepinephrine (~56%) levels in the hippocampus as compared to vehicle-treated control groups (Table 1). In

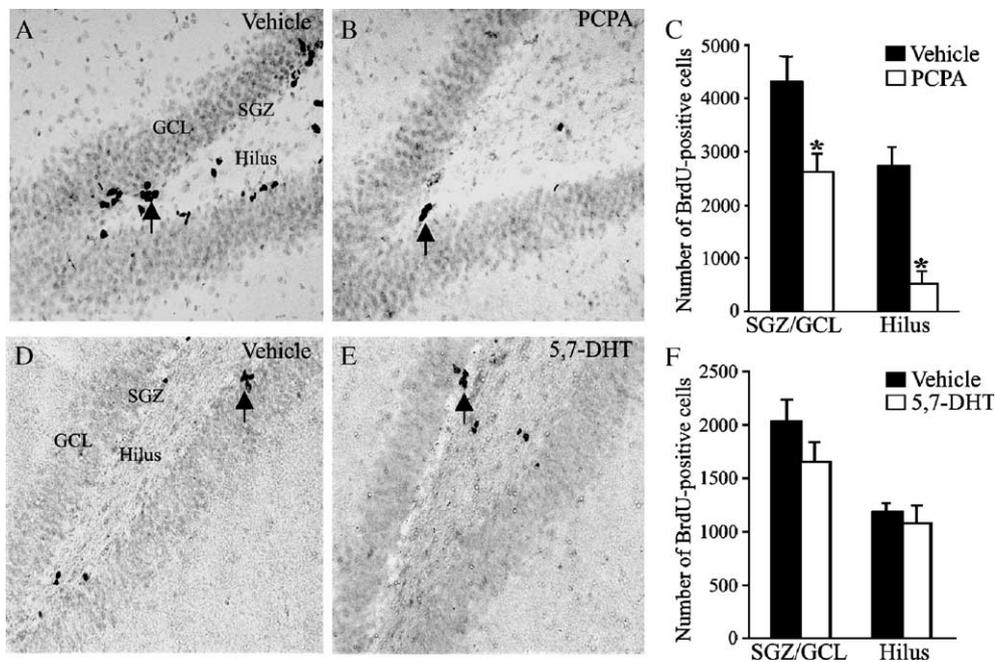


Fig. 2 – Effect of serotonin depletion using PCPA and 5,7-DHT on the proliferation of adult hippocampal progenitors in the dentate gyrus subfield. Shown are representative photomicrographs of BrdU-positive cells from animals treated with vehicle (A, D) and PCPA (B) or 5,7-DHT (E). BrdU-positive cells (arrows) were observed in the subgranular zone (SGZ), at the border of the hilus and the granule cell layer (GCL), and within the hilus. BrdU-positive nuclei were irregularly shaped and mostly observed in clusters. Quantitative analysis of BrdU-positive cells revealed a significant decrease in the number of proliferating cells, both in the SGZ/GCL and the hilus in animals treated with PCPA (C), but no change was observed either in SGZ/GCL or hilus in animals treated with 5,7-DHT (F). Results are expressed as mean \pm SEM of BrdU-positive cells in the dentate gyrus ($n = 6/\text{group}$). * $P < 0.05$ compared to vehicle (Student's *t* test).

contrast, treatment with the serotonergic neurotoxin 5,7-DHT resulted in a selective serotonin loss (~80%) in the hippocampus and no significant change in hippocampal

norepinephrine levels (Table 1). Both PCPA and 5,7-DHT treatment groups had some animals which had severe depletion of serotonin levels (~95%).

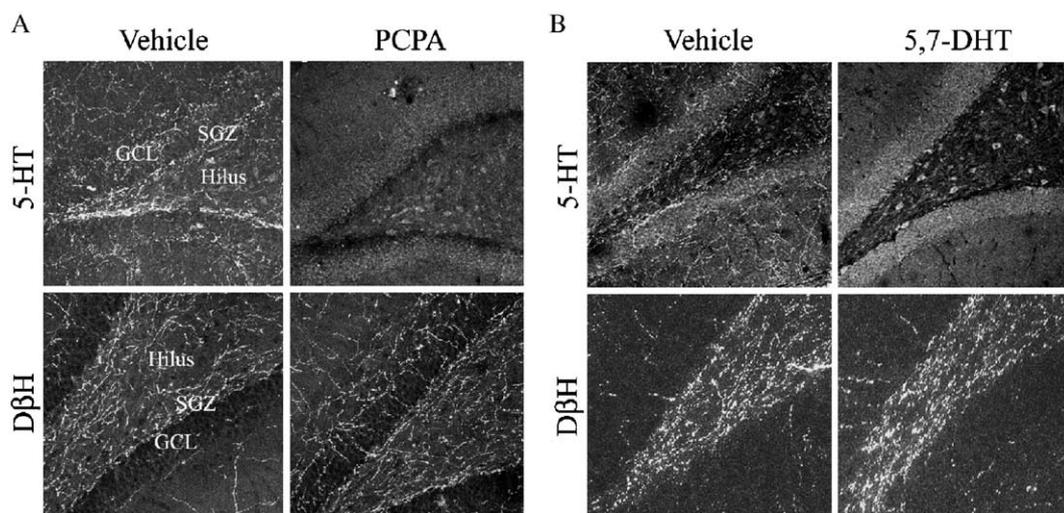


Fig. 3 – Effect of PCPA and 5,7-DHT treatment on serotonergic and noradrenergic terminals in the hippocampus. Representative confocal images of serotonin (5-HT) and dopamine- β -hydroxylase (D β H) immunofluorescence from the dentate gyrus (DG) region of vehicle-treated animals and PCPA or 5,7-DHT-treated animals are shown (A, PCPA experiment; B, 5,7-DHT experiment). Treatment with PCPA (A, upper panel) or 5,7-DHT (B, upper panel) resulted in a reduction in 5-HT immunopositive terminals in the DG. No obvious differences were observed in the D β H immunopositive noradrenergic nerve terminals in the DG following PCPA (A, lower panel) or 5,7-DHT treatment (B, lower panel).

Table 1 – Level (ng/gm tissue weight) of 5-HT and NE in the hippocampus

	Vehicle	PCPA	% of vehicle	Sham	5,7-DHT	% of sham
5-HT	207.6 ± 6.5	35.8 ± 23*	17.2 ± 11.5*	123.23 ± 10	24.80 ± 19*	20.12 ± 15.4*
NE	288.49 ± 36.7	128.4 ± 33.9*	44.5 ± 11.7*	282.15 ± 52	241.76 ± 43	85.59 ± 15

Effect of PCPA and 5,7-DHT treatment on the level of serotonin (5-HT) and norepinephrine (NE) in the hippocampus. Rats were treated with the serotonin synthesis inhibitor PCPA or the serotonergic neurotoxin 5,7-DHT, hippocampi were dissected out and used for HPLC analysis of 5-HT and NE level as described in Experimental procedures. The level of 5-HT and NE is expressed as ng/gm tissue weight. Values are the mean ± SEM (ng/g) and the mean ± SEM percent of vehicle for each treatment ($n = 3-5/\text{group}$).

* $P < 0.05$ compared to vehicle treatment (Student's t test).

2.3. Influence of serotonin depletion on the survival of adult hippocampal progenitors

Drug-naive animals were first administered BrdU prior to either PCPA or 5,7-DHT treatment to determine the influence of serotonin depletion on the survival of a BrdU-labeled cohort of adult hippocampal progenitors (Fig. 1B). The number of surviving BrdU-positive cells in the SGZ and hilus was quantitated in animals sacrificed 21 days after BrdU treatment. The BrdU-positive cells observed in the survival experiment had an ovoid or round shape, were often found within the GCL and were not observed in clusters. The survival of BrdU-positive cells was significantly decreased in the SGZ and hilus of animals treated with PCPA compared to the vehicle-treated group (Figs. 4A-C). In comparison, the number of BrdU-positive cells in animals treated with 5,7-DHT did not differ significantly

from the number of BrdU-positive cells in the vehicle-treated group (Figs. 4D-F).

2.4. Influence of serotonin depletion on the number of doublecortin-positive cells in the adult hippocampus

We also addressed the influence of serotonin depletion by PCPA and 5,7-DHT treatment on the number of doublecortin (DCX)-positive adult hippocampal progenitors. DCX has been reported to be a marker of adult hippocampal progenitors and is expressed within the SGZ (Couillard-Despres et al., 2005). We observed a marked decrease (54%) in the number of DCX-positive hippocampal progenitors following PCPA treatment (Figs. 5A-C). In contrast, 5,7-DHT treatment did not significantly alter the number of DCX-positive cells observed in the SGZ (Figs. 5D-F). In fact, there was a trend towards an increase in DCX-positive cell number in the 5,7-

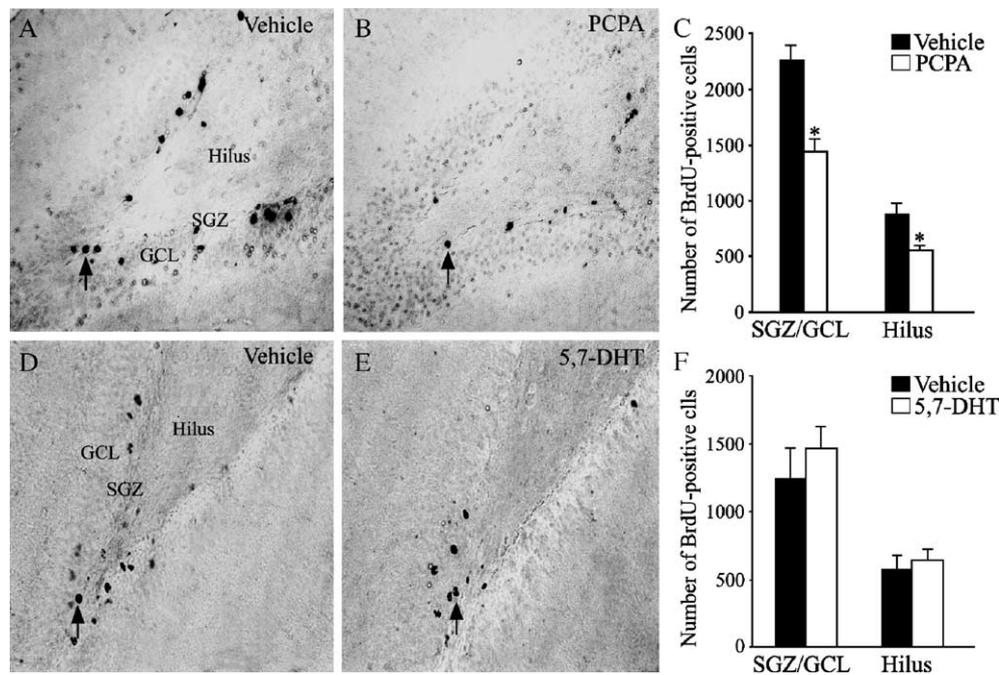


Fig. 4 – Influence of serotonin depletion by PCPA or 5,7-DHT on the survival of adult hippocampal progenitors in the dentate gyrus (DG). Drug-naive rats first received BrdU administration followed by serotonin depletion induced via PCPA or 5,7-DHT treatment as described in Experimental procedures. Shown are representative photomicrographs of BrdU-positive cells from vehicle (A, D) and PCPA (B) or 5,7-DHT (E)-treated animals. The BrdU-positive cells (arrows) had a distinct ovoid morphology and were not observed in clusters. Quantitative analysis of BrdU-positive cells revealed that PCPA (C), but not 5,7-DHT (F), treatment significantly reduced the survival of BrdU-labeled progenitors in the SGZ/GCL and the hilus. Results are expressed as mean ± SEM of BrdU-positive cells in the DG ($n = 5-7/\text{group}$). * $P < 0.05$ compared to control (Student's t test).

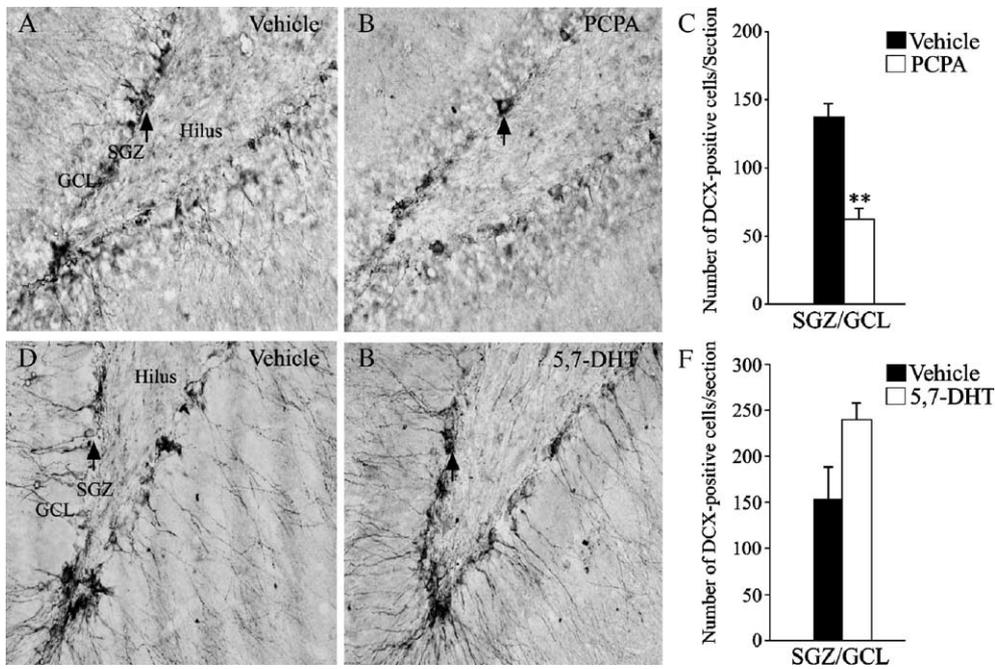


Fig. 5 – Effect of serotonin depletion on doublecortin (DCX)-positive adult hippocampal progenitors within the subgranular zone (SGZ) of the dentate gyrus. Shown are representative photomicrographs of DCX-positive cells from vehicle (A, D) and PCPA (B) or 5,7-DHT (E)-treated animals. The DCX-positive cells (arrows) were clearly observed along the SGZ. Quantitative analysis of DCX-positive cells revealed that PCPA (C), but not 5,7-DHT (F), treatment significantly reduced the number of DCX-positive adult hippocampal progenitors in the SGZ. Results are expressed as mean \pm SEM of DCX-positive cells/section ($n = 5/\text{group}$). ** $P < 0.001$ compared to control (Student's t test).

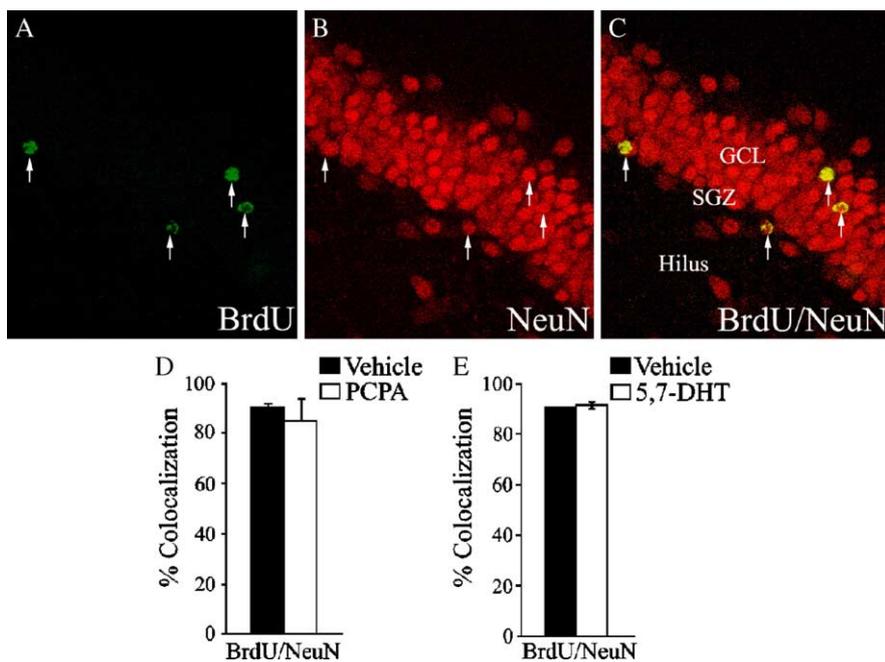


Fig. 6 – Influence of serotonin depletion on the differentiation of adult hippocampal progenitors in the dentate gyrus into NeuN-positive neurons. Colocalization of BrdU-positive cells (A, green) with the neuronal marker NeuN (B, red) is indicated by arrows in the merged image (C, BrdU/NeuN) in a representative confocal image from a vehicle-treated animal. Quantitative analysis showed that neither PCPA (D) nor 5,7-DHT (E) treatment had an effect on the percent colocalization of BrdU-positive cells with the neuronal marker NeuN, in the subgranular zone (SGZ) and granule cell layer (GCL), compared to vehicle-treated controls. Results are expressed as mean \pm SEM ($n = 5/\text{group}$) percent colocalization of BrdU-positive cells with NeuN-positive cells in the SGZ/GCL.

DHT-treated group as compared to the vehicle-treated controls, which, however, did not reach significance ($P = 0.062$).

2.5. Influence of serotonin depletion on the neuronal differentiation of adult hippocampal progenitors

To examine the effect of serotonin depletion on the differentiation of adult hippocampal progenitors in the SGZ/GCL, we examined the percentage of BrdU-positive cells that acquired a neuronal phenotype 21 days after BrdU (Fig. 1B), a time point at which most progenitors have been shown to undergo terminal differentiation (Kempermann et al., 2004). The phenotype of BrdU-positive cells was determined using immunofluorescence to detect the colocalization of BrdU with the neuronal marker NeuN or the glial marker GFAP (Figs. 6A–C). Confocal analysis with Z-plane sectioning revealed that most BrdU-positive cells in the SGZ/GCL acquired a neuronal phenotype (>80%). The percentage of BrdU-positive cells that differentiated into mature neurons did not differ significantly in either the PCPA (Fig. 6D) or 5,7-DHT (Fig. 6E)-treated groups compared to their respective vehicle-treated controls. We did not observe any BrdU-positive cells in the SGZ/GCL that colocalized with the glial marker GFAP. The BrdU-positive cells that did not colocalize with either NeuN or GFAP may represent as yet undifferentiated cells.

2.6. Influence of acute serotonin release on the proliferation of adult hippocampal progenitors

To examine the effects of acute serotonin release on the proliferation of adult hippocampal progenitors in the dentate gyrus, rats were treated with either the monoamine oxidase inhibitor tranylcypromine (TCP) along with the serotonin precursor L-Tryptophan (L-Trp) or *p*-chloroamphetamine (PCA). Animals received a single BrdU injection and were

sacrificed 2 h later (Fig. 1A). Quantitation of the BrdU-positive cell number in the SGZ/GCL showed that neither TCP/L-Trp (Fig. 7A) nor PCA treatment (Fig. 7B) altered the proliferation of adult hippocampal progenitors in the SGZ/GCL.

3. Discussion

The results of this study demonstrate that selective serotonergic depletion, induced by the serotonergic neurotoxin 5,7-DHT, does not alter the proliferation, survival or differentiation of adult hippocampal progenitors. In striking contrast, treatment with the serotonin synthesis inhibitor, PCPA, which was not selective in regulating serotonin and also reduced hippocampal norepinephrine levels, resulted in a significant decline in both the proliferation and survival of adult hippocampal progenitors, but did not affect progenitor differentiation. This decline in proliferation and survival of BrdU-positive cells was observed both in the SGZ and hilar regions of the hippocampal dentate gyrus subfield following PCPA treatment. These results are further supported by our observation of a significant reduction in DCX-positive cell number in the SGZ following PCPA, but not 5,7-DHT, treatment. Our data suggest that selective serotonergic depletion may not regulate adult hippocampal neurogenesis. In addition, we did not observe any change in progenitor proliferation within the other major neurogenic region of the subventricular zone following either PCPA or 5,7-DHT treatment. An acute induction in serotonin levels was found to not alter the proliferation of adult hippocampal progenitors. Taken together, these studies suggest that altered serotonin levels may not per se regulate adult hippocampal neurogenesis.

Our results differ from previous reports (Brezun and Daszuta, 1999, 2000) that indicate a decline in adult hippocampal progenitor proliferation following serotonin depletion. In the previous study by Brezun and Daszuta, both PCPA and

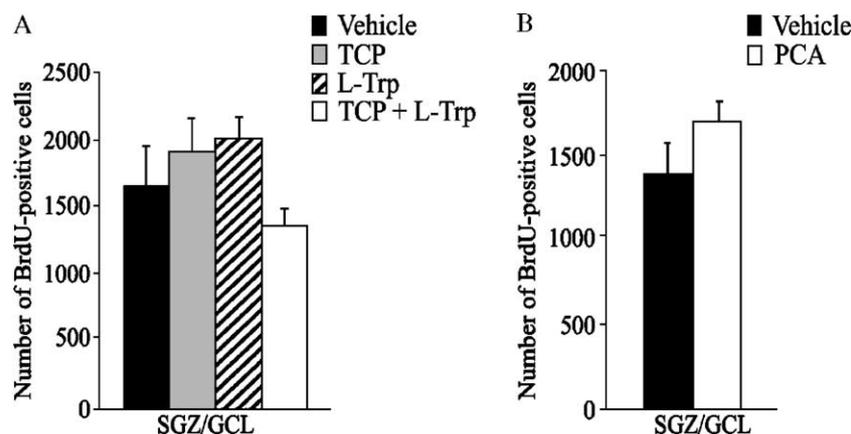


Fig. 7 – Influence of an acute increase in serotonin levels on the proliferation of adult hippocampal progenitors in the dentate gyrus (DG). In two different experiments, serotonin levels were increased using either a combination of tranylcypromine (TCP) and L-Tryptophan (L-Trp) or *p*-chloroamphetamine (PCA) treatment as described in Experimental procedures. Vehicle and drug-treated animals received BrdU and were sacrificed 2 h later. Quantitative analysis of BrdU-positive cells in the subgranular zone (SGZ) at the border of the hilus and granule cell layer (GCL) revealed that an acute increase in serotonin levels does not alter the proliferation of adult hippocampal progenitors (A, TCP/L-Trp experiment; B, PCA experiment). Results are expressed as mean \pm SEM of BrdU-positive cells in the DG ($n = 4$ /group).

5,7-DHT were found to decrease adult hippocampal progenitor proliferation, while effects on survival and differentiation were not addressed. We have used PCPA at the same dose and in a similar dosing paradigm, as previously reported, and find that there is indeed a significant decline in BrdU-positive cell number within the dentate gyrus. However, 5,7-DHT did not result in any change in either BrdU or DCX-positive cell number within the adult dentate gyrus. We observed that while both 5,7-DHT and PCPA decreased serotonergic innervation as well as hippocampal serotonin levels (~80% with both paradigms), PCPA but not 5,7-DHT also caused a significant decline in hippocampal norepinephrine levels (~56%). This decrease in norepinephrine levels was seen in the absence of any obvious change in hippocampal noradrenergic innervation as assessed by $D\beta H$ immunohistochemistry. Our results are in agreement with other reports, that while 5,7-DHT selectively depletes serotonin, PCPA treatment results in a decrease in both serotonin and norepinephrine levels (Reader and Gauthier, 1984; Birthelmer et al., 2003). Given our previous results (Kulkarni et al., 2002) that a specific decrease in norepinephrine levels robustly reduced hippocampal progenitor proliferation, it raises the possibility that the effects of PCPA may involve a possible role for norepinephrine. The previous reports (Brezun and Daszuta, 1999, 2000) addressed only changes in 5-HT innervation and levels and did not examine hippocampal norepinephrine levels or noradrenergic innervation. While our effects with PCPA are in agreement with those of Brezun and Daszuta (1999, 2000), our results do not support the conclusion that it is serotonergic depletion that is responsible for the decline in hippocampal progenitor proliferation, given that the selective serotonin depletion induced by 5,7-DHT did not have any effect on adult hippocampal neurogenesis.

There are several possibilities for the discrepancy in our results with 5,7-DHT treatment, from those previously reported by Brezun and Daszuta. We have carried out all our studies in male rats, while the previous studies were performed using female rats (Brezun and Daszuta, 1999, 2000). One cannot rule out the possibility that the effects of serotonin depletion on adult hippocampal neurogenesis may exhibit some sexual dimorphism. Serotonin depletion is known to suppress preovulatory increases in estrogen (Coen et al., 1980), estrogen itself regulates adult hippocampal neurogenesis (Tanapat et al., 1999) and serotonin has been reported to interact with estrogen to influence hippocampal progenitor proliferation (Banasr et al., 2001). Another difference is in the site of injection of 5,7-DHT, which in our study was done i.c.v. versus the intraraphe injections used by Brezun and Daszuta (1999, 2000). It is possible that this could also contribute to the differences in the results, despite the fact that the extent of the hippocampal serotonin depletion was similar in both studies (~80%). An intraraphe lesion could influence non-serotonergic perikarya and other fiber bundles in the region (Lorens, 1978). In our study, we have also examined the influence of 5,7-DHT on DCX, which has recently been shown to serve as another suitable measure of adult hippocampal neurogenesis (Couillard-Despres et al., 2005). Interestingly, 5,7-DHT appeared to increase the number of DCX-positive hippocampal progenitors, though this did not reach statistical significance ($P = 0.06$), further supporting the

conclusion that adult hippocampal neurogenesis is not decreased following a selective serotonin depletion. A recent report that focused on interactions between serotonin and corticosterone in their effects on adult hippocampal neurogenesis (Huang and Herbert, 2005) also did not find an effect on hippocampal progenitor proliferation following 5,7-DHT treatment. Taken together, this raises the possibility that selective serotonin depletion does not influence adult hippocampal neurogenesis, and that previous studies with PCPA may have resulted in a decline in adult hippocampal progenitor proliferation due to a decrease in hippocampal norepinephrine levels.

At present, the mechanisms that contribute to the effects of PCPA on hippocampal progenitor proliferation and survival are unclear, but several possibilities can be proposed. PCPA depletes both serotonin and norepinephrine, and norepinephrine depletion is known to decrease hippocampal progenitor proliferation. In addition, previous reports suggest that PCPA treatment results in an induction in the norepinephrine transporter mRNA expression, which may further influence noradrenergic turnover (Koed and Linnet, 2000). Our unpublished results support a direct effect of norepinephrine on adult hippocampal progenitors which have been shown to express specific noradrenergic receptor subtypes (Yanpallewar et al., 2004). We also find a clear effect of PCPA treatment on the postmitotic survival of adult hippocampal progenitors. While neither a selective serotonin nor a selective norepinephrine depletion (Kulkarni et al., 2002) regulates hippocampal progenitor survival, depletion of both of these monoamines may contribute to these effects of PCPA. PCPA has also been reported to deplete dopamine levels; however, dopamine regulates SVZ, but not hippocampal, neurogenesis (Kippin et al., 2005) and hence the effects of PCPA on dopamine may not play a major role in the decline in adult hippocampal neurogenesis. The effects of PCPA treatment are unlikely to be due to a change in neurotrophic factors like BDNF, which is known to increase hippocampal neurogenesis (Scharfman et al., 2005). PCPA treatment induces BDNF expression (Zetterstrom et al., 1999) and despite this induction PCPA appears to decrease hippocampal progenitor proliferation and survival. Although PCPA and 5,7-DHT produce similar extensive depletions of central 5-HT levels, there have been previous reports of differential effects that arise from these two different serotonin-depleting paradigms. While PCPA has been reported to induce hyperalgesia, 5,7-DHT treatment had no effect on pain sensitivity (Lorens, 1978). PCPA treatment has been reported to alter the expression of 5-HT_{1A} and 5-HT_{1B/1D} receptors, while 5,7-DHT did not appear to influence the expression of these receptors (Compan et al., 1998). Since specific serotonergic receptors have been reported to regulate adult hippocampal progenitor proliferation (Santarelli et al., 2003; Banasr et al., 2004), in particular the 5-HT_{1A} and 5-HT_{1B/1D} receptors, effects of PCPA and 5,7-DHT on the expression of specific serotonergic receptor subtypes could act to contribute to the differential effects of these two treatments on adult hippocampal neurogenesis.

It has been hypothesized that serotonin may mediate the effects of animal models of depression and stress, as well as antidepressant treatments on adult hippocampal neurogenesis (Santarelli et al., 2003). The decreased hippocampal

progenitor proliferation following stress or in animal models of depression has been suggested to involve decreased serotonin levels (Malberg and Duman, 2003). Our results indicate that it is unlikely that a simple reduction in serotonin levels contributes to the decreased neurogenesis seen in the above behavioral paradigms. Despite a robust reduction in serotonin levels, far more severe than the decreased serotonin turnover seen in these behavioral models (Torres et al., 2002), we observed no change in the number of BrdU- and DCX-positive hippocampal progenitors. Studies indicate that chronic, but not acute, treatment with serotonin selective reuptake inhibitors (SSRIs) enhances adult hippocampal neurogenesis (Malberg et al., 2000). Given that acute SSRI treatment is known to enhance hippocampal serotonin levels (Blier and de Montigny, 1994), it suggests that a simple induction in hippocampal serotonin levels may not be sufficient to influence hippocampal neurogenesis. To specifically address whether an acute increase in serotonin levels altered adult hippocampal progenitor proliferation, we used two different acute pharmacological treatments, namely a combination of tranylcypromine and L-Tryptophan or *p*-chloroamphetamine. Our results indicate that an acute increase in serotonin levels does not regulate adult hippocampal progenitor proliferation.

The effects of antidepressants on adult hippocampal neurogenesis have been suggested to involve a neurogenic role for serotonin (Radley and Jacobs, 2002; Gould, 1999), and this is supported by recent reports that the 5-HT_{1A} receptor may be critical in the increased hippocampal neurogenesis following chronic SSRI treatment (Santarelli et al., 2003). In this context, our results suggest that while an acute change in serotonin levels may not per se increase adult hippocampal neurogenesis, they certainly do not rule out the possibility that the effects of serotonin on specific serotonergic receptors may influence adult hippocampal progenitors. Given that chronic antidepressant treatment regulates the expression of specific serotonin receptor subtypes (Dremencov et al., 2003), this raises the possibility that the neurogenic response to elevated serotonin levels following an acute versus chronic SSRI treatment may be mediated by a different complement of serotonin receptors. Recent studies do indicate that specific serotonergic receptors may indeed have an effect on adult hippocampal progenitor proliferation. While 5-HT_{1A} and 5-HT_{1B} receptor stimulation enhances hippocampal progenitor proliferation, stimulating the 5-HT_{2A/2C} receptor does not appear to alter the proliferation of adult hippocampal progenitors (Banasz et al., 2004). Given that most serotonergic receptor subtypes are expressed within the hippocampus (Dremencov et al., 2003), further studies are required to examine the role of specific serotonin receptors as they may have differential effects on adult hippocampal progenitors. It will be interesting to address the changes that arise following sustained elevation of serotonin that may be of relevance to the effects of antidepressant treatment on adult hippocampal neurogenesis.

In conclusion, our studies indicate that selective serotonin depletion and an acute induction in serotonin levels may not influence adult hippocampal neurogenesis. Our results motivate the need for further studies to examine the role of other monoamines, and of adaptations that arise following altered

monoamine levels in the effects of behavioral models of depression and in the actions of antidepressant treatments on adult hippocampal neurogenesis.

4. Experimental procedures

4.1. Animal treatments and surgical paradigms

Adult male Wistar rats (225–275 g) bred in our animal-breeding colony were used in all experiments. Animals were group housed and maintained on a 12-h light–dark cycle with access to food and water ad libitum. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the TIFR Institutional Animal Ethics Committee and all care was taken to minimize pain or discomfort to the animals. To induce serotonin depletion, animals were treated with either the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (PCPA) or the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT). For the PCPA experiment, animals received intraperitoneal (i.p.) injections of PCPA (300 mg/kg; Sigma, USA) once daily for 2 days followed by PCPA (100 mg/kg) once daily on the third and fifth day. The control group received vehicle treatment (0.9% saline). This dosing paradigm was selected based on previous reports that it induces a decrease in adult hippocampal progenitor proliferation (Brezun and Daszuta, 1999). For the 5,7-DHT experiment, animals were first treated with the noradrenergic reuptake inhibitor desipramine (25 mg/kg; Sigma) 40 min prior to the surgery to protect noradrenergic terminals, and were then deeply anesthetized with chloral hydrate (400 mg/kg; Sigma) prior to placement in a David Kopf stereotaxic apparatus (Stoelting Physiology Instruments, USA). Animals received an intracerebroventricular (i.c.v.) infusion of either the serotonergic neurotoxin 5,7-DHT (200 µg/animal; 20 µg/µl, creatinine sulfate salt, Sigma) or vehicle (0.1% ascorbic acid in 0.9% saline). 5,7-DHT was infused into the lateral ventricle unilaterally at the stereotaxic coordinates: AP, –0.8 mm, ML, –1.4 mm and DV, –4.0 mm from bregma (Paxinos and Watson, 1998). After infusion, the cannula was left in place for another 2 min and withdrawn slowly. The incision was sutured and animals were returned to their home cage following recovery from anesthesia.

To induce serotonin release, two different treatment paradigms were used. In the first paradigm, animals received an i.p. injection of the monoamine oxidase inhibitor tranylcypromine (TCP, 20 mg/kg, Sigma) followed by treatment with L-Tryptophan (L-Trp, 100 mg/kg, Sigma), 10 min later. The animals in the control groups received either vehicle injections (0.9% saline), TCP followed by vehicle treatment or vehicle followed by L-Trp treatment. In the second paradigm, animals received either a single injection of *p*-chloroamphetamine (PCA, 10 mg/kg, Sigma) or vehicle (0.9% saline).

4.2. BrdU labeling

To label dividing cells, the mitotic marker 5-bromo-2'-deoxyuridine (BrdU; 50 mg/kg, Sigma) was administered intraperitoneally. Two different BrdU labeling paradigms were utilized in the study, the first dosing paradigm was

used to study effects on hippocampal progenitor proliferation and the second was used to assess effects on hippocampal progenitor survival and differentiation (Fig. 1). To study the effect of PCPA on hippocampal progenitor proliferation, vehicle and PCPA-treated groups ($n = 6/\text{group}$) received BrdU (50 mg/kg) on the 3rd, 4th and 5th day from the start of treatment. Animals were sacrificed 6 h following the last BrdU administration. This BrdU-labeling paradigm was selected to repeat previous reports that revealed an effect of PCPA treatment on BrdU-positive cell number in the dentate gyrus (Brezun and Daszuta, 1999). We sought to more specifically address the effects of PCPA on progenitor proliferation by sacrificing animals ($n = 5/\text{group}$) 2 h following a single BrdU injection (50 mg/kg) on the last day of PCPA treatment. To study the effect of 5,7-DHT on cell proliferation, BrdU was administered 15 days after the infusion of the drug and animals ($n = 6/\text{group}$) were sacrificed 24 h following BrdU treatment. To examine the effects of serotonin releasing agents on adult hippocampal progenitor proliferation, animals ($n = 4\text{--}6/\text{group}$) were administered a single dose of BrdU (100 mg/kg) one and half hour after TCP + L-Trp treatment or 30 min after PCA treatment and sacrificed 2 h after BrdU treatment.

In the survival labeling paradigm, drug-naive animals first received three injections of BrdU (50 mg/kg) 2 h apart (Fig. 1B). To address the influence of PCPA on hippocampal progenitor survival, animals ($n = 5\text{--}7/\text{group}$) received PCPA treatment as described above 2 days later. To examine the influence of 5,7-DHT treatment on the survival of hippocampal progenitors, animals ($n = 5/\text{group}$) were subjected to 5,7-DHT administration i.c.v. as described above 2 days following BrdU treatment. In both the survival experiments, with PCPA as well as 5,7-DHT, animals were sacrificed 21 days after BrdU administration. Animals were deeply anesthetized with an overdose of chloral hydrate and were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and stored in 0.1 M phosphate buffer prior to being sectioned and processed for immunohistochemistry.

4.3. Immunohistochemistry and immunofluorescence

Serial coronal sections (50 μm) through the rostro-caudal axis of the hippocampus (bregma: -2.56 to -5.80 ; Paxinos and Watson, 1998) were cut on a vibratome (TPI, USA). Every fifth section from the hippocampus was processed for BrdU immunohistochemistry as described previously (Malberg et al., 2000; Kulkarni et al., 2002). In brief, post-DNA-denaturation with 50% formamide/2 \times SSC for 2 h at 65 $^{\circ}\text{C}$ and acid hydrolysis (2 N HCl at 37 $^{\circ}\text{C}$ for 30 min), sections were rinsed in 0.1 M Boric Acid and then blocked with 10% normal horse serum. Sections were incubated overnight at room temperature with Mouse anti-BrdU (1:500; Roche, Switzerland) followed by treatment with biotinylated anti-mouse secondary antibody (1:500; Vector, USA). Signal amplification was performed using a Vectastain Elite Avidin–Biotin system (Vector) and was visualized using diaminobenzidine (Sigma) as a substrate.

For triple labeling immunofluorescence, free floating sections were subjected to DNA denaturation and acid hydrolysis as described above, prior to exposure to the following cocktail:

rat anti-BrdU (1:200, Accurate Biochemicals, USA) along with mouse anti-neuronal nuclei (NeuN; 1:1000; Chemicon, USA) and rabbit anti-gial fibrillary acidic protein (GFAP; 1:500; Sigma) overnight at room temperature. Sections were then incubated with a cocktail of secondary antibodies: biotinylated anti-rat IgG (1:500, Chemicon) along with rhodamine-conjugated anti-mouse IgG (1:500, Chemicon) and Cy-5-conjugated anti-rabbit IgG (1:500, Chemicon). This was followed by incubation with fluorescein-conjugated streptavidin (1:500, Amersham, UK).

To examine the effect of serotonin depletion on the number of doublecortin (DCX)-positive cells in the hippocampus, sections were processed for DCX immunohistochemistry. Sections were incubated with goat anti-doublecortin antibody (1:200, Santa Cruz Biotechnology, USA) overnight followed by incubation with biotinylated rabbit anti-goat secondary for 2 h. Signal amplification was performed using a Vectastain Elite Avidin–Biotin system (Vector) and was visualized using diaminobenzidine (Sigma) as a substrate.

To examine the effect of depleting agents (PCPA and 5,7-DHT) on serotonergic innervation to the hippocampus, immunohistochemistry to label serotonin was performed. Sections were incubated with rabbit anti-serotonin antibody (1:5000, Sigma) for 3 days at room temperature followed by incubation with biotinylated anti-rabbit secondary antibody and fluorescein-conjugated streptavidin. To examine the effect of the serotonin depletion treatments on noradrenergic innervation to the hippocampus, immunohistochemistry was performed to detect dopamine β -hydroxylase ($\text{D}\beta\text{H}$)-positive nerve fibers. Sections were incubated with mouse anti-dopamine β -hydroxylase ($\text{D}\beta\text{H}$) antibody (1:200, Chemicon) overnight, followed by incubation with biotinylated anti-mouse antibody and fluorescein-conjugated streptavidin. Immunofluorescence was visualized on a Biorad Laser Scanning System, Radiance 2100 (Biorad, USA).

4.4. Cell counting

Quantitation of BrdU-positive cells within the dentate gyrus (DG) was performed using a modified, unbiased stereology protocol (Malberg et al., 2000). Sections were coded and quantitation was done by an experimenter blind to the code. Sections spanned the rostro-caudal extent of the hippocampus, and every fifth hippocampal section was processed for quantification (11 sections/animal). BrdU-positive cells were counted as within the SGZ/GCL when they were within the SGZ or directly touching it and were counted as hilar when they were at least two cell depths away from the SGZ. Counting of cells was done at 400 \times using a light microscope (Zeiss Axioskop, Germany) omitting cells in the outermost focal plane. The total number of BrdU-positive cells in the SGZ/GCL and hilus was estimated by multiplying the total number of BrdU-positive cells per SGZ/GCL and hilus counted from every 5th section by the section periodicity (5), and reported as the total number of BrdU-positive cells per region.

To control for differences in bioavailability and to check whether the effect of treatments was specific to the hippocampus, BrdU-positive cells were also quantitated in the subventricular zone (SVZ, bregma: 1.7 to 0.48; Paxinos and Watson, 1998). Every third striatal section was processed for

BrdU immunohistochemistry and BrdU-positive cells in the SVZ along the boundary between the corpus callosum and the striatum were counted (six–eight sections/animal, $n = 4$ /group). The area in which the cells were counted was traced and measured in mm^2 using NIH Image 1.62 software (Scion Image, USA) and the results were expressed as number of BrdU-positive cells per mm^2 .

To determine the influence of serotonin depletion on doublecortin-positive cell number in the hippocampus, the number of DCX-positive cells in the SGZ of the DG was quantitated (six–eight sections/animal, $n = 5$ /group) at 400 \times using a light microscope (Zeiss Axioskop). The results were expressed as the number of DCX-positive cells per section.

To examine the effect of serotonin depletion on the differentiation of hippocampal progenitors into neurons or glia, the percentage of BrdU-positive cells which colocalized with the neuronal marker NeuN or the glial marker GFAP was determined using confocal microscopy. Eight sections (250 μm apart) from each animal were analyzed from the survival experiments. A minimum of 50 BrdU-positive cells/animal ($n = 5$ /group) were analyzed using Z-plane sectioning with 0.5 μm steps on a Biorad MRC 1024 confocal microscope to confirm colocalization with either NeuN or GFAP.

4.5. Volume measurement

Every fourth section (19 sections/brain) of the hippocampus (Bregma -1.60 to -6.30 ; Paxinos and Watson, 1998) was processed for cresyl violet staining. The area of the hippocampus and granule cell layer in each section was measured by outlining the region according to boundary criteria established by Paxinos and Watson (1998) using a Macintosh-based Scion 1.62 image analysis software (Scion, USA). Volume (V) was calculated by using the formula $V = \sum A \times T \times 4$, where $\sum A$ represents sum of all area measurements from a brain, T section thickness and 4 is the section periodicity.

4.6. Biochemical detection of serotonin and norepinephrine

To determine the extent of serotonin and norepinephrine depletion following PCPA and 5,7-DHT treatments, high performance liquid chromatography (HPLC) was used. Rats were sacrificed by decapitation and the hippocampi were rapidly dissected out and frozen in liquid nitrogen. Hippocampi were sonicated in 0.1 M perchloric acid and the homogenates were centrifuged at 15,000 rpm for 10 min at 4 $^{\circ}\text{C}$. Supernatants were processed for determination of serotonin and norepinephrine. Levels of serotonin and norepinephrine were quantitated using a fluorometric detection method for HPLC analysis as described previously (Lakshmana and Raju, 1997) using purified serotonin and norepinephrine (Sigma) as standards. Neurotransmitter content was determined and expressed as ng/g tissue weight.

4.7. Statistical analysis

Statistical analysis was performed using Prism 3.0 software (Graphpad, USA). Differences of means between two groups were subjected to Student's t test. Experiments with more than two groups were analyzed by two-way analysis of

variance (ANOVA) followed by the Bonferroni post hoc test. Statistical significance was determined at $P < 0.05$.

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