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SHORT COMMUNICATION
Depletion of norepinephrine decreases the proliferation, but does not influence the survival and differentiation, of granule cell progenitors in the adult rat hippocampus

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Abstract

The dentate gyrus region retains the ability to generate neurons throughout adulthood. A few studies have examined the neurotransmitter regulation of adult hippocampal neurogenesis and have shown that this process is regulated by serotonin and glutamate. Given the strong noradrenergic innervation of the adult hippocampus and the ability of norepinephrine to influence proliferation during development, we examined the influence of norepinephrine on adult hippocampal neurogenesis. Our study indicates that depletion of norepinephrine by the selective noradrenergic neurotoxin, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride (DSP-4), results in a 63% reduction in the proliferation of dentate gyrus progenitor cells identified through 5-bromo-2′-deoxyuridine (BrdU) labelling. In contrast, the survival of BrdU-positive cells labelled prior to treatment with DSP-4 is not influenced by norepinephrine depletion. The differentiation of BrdU labelled progenitors into neurons or glia was also not sensitive to noradrenergic depletion. These results indicate that the proliferation, but not the survival or differentiation, of adult hippocampal granule cell progenitors is affected by norepinephrine depletion.

Introduction

The adult mammalian brain retains the ability to form new neurons, within discrete regions, throughout the lifetime of the animal. Amongst these regions is the subgranular zone (SGZ) within the dentate gyrus subfield of the hippocampus, which is neurogenic in several mammalian species (reviewed in Gould & Gross, 2002). Progenitors dividing within the SGZ migrate into the granule cell layer, express neuronal markers, receive synaptic input and extend processes to the hippocampal CA3 region (van Praag *et al*., 2002). Taken together these events encapsulate the process of adult hippocampal neurogenesis. Amongst these events the process of proliferation of progenitors, their survival and differentiation into neurons or glia are events whose regulation is amenable to being examined experimentally, thus providing an interesting system in which to study factors that regulate adult neurogenesis.

Previous studies indicate that adult hippocampal neurogenesis is regulated by environmental perturbations like stress, as well as by psychotropic agents such as antidepressants (Gould *et al*., 1997; Malberg *et al*., 2000). However, the neurotransmitter pathways and growth factors that control ongoing adult neurogenesis are not well understood. Proliferation of dentate granule cell progenitors is decreased by excitatory input and by adrenal steroids (Cameron & Gould, 1994; Gould *et al*., 1997). In contrast, serotonin enhances the proliferation of hippocampal progenitors (Brezun & Daszuta, 2000). Studies thus far have not addressed the role of norepinephrine in the regulation of adult hippocampal neurogenesis despite reports of the substantial noradrenergic input to the dentate gyrus (Loy *et al*., 1980) and the influence of norepinephrine on proliferation of neuroepithelial cells during development (Popovik & Haynes, 2000). Furthermore, the norepinephrine system is a major target of stress and antidepressant treatments (Brady, 1994), both of which are known to influence adult hippocampal neurogenesis. Given these associations we hypothesized that adult dentate granule cell progenitors may be sensitive to manipulations of the noradrenergic pathway.

The present study was carried out to examine the influence of norepinephrine depletion on adult hippocampal neurogenesis. Our results indicate that norepinephrine depletion decreases the proliferation, but does not influence the survival and differentiation of dentate granule cell progenitors. These data suggest the possibility that norepinephrine may contribute to the effects of stress and antidepressant treatments on hippocampal neurogenesis.

Materials and methods

Animal treatments

Adult male Wistar rats (250–300 g) were used in all experiments, which were in accordance with NIH guidelines and were approved by the TIFR Animal Ethics Committee. Animals were group housed (12-h light : dark cycle) with access to food and water *ad libitum*. The noradrenergic neurotoxin DSP-4 (10 mg/kg) or vehicle (0.9% NaCl) was administered intraperitoneally (i.p.) once daily for three days. Fluoxetine (5 mg/kg, Sigma) was given i.p. 30 min prior to DSP-4, or vehicle, administration to protect serotonergic neurons.

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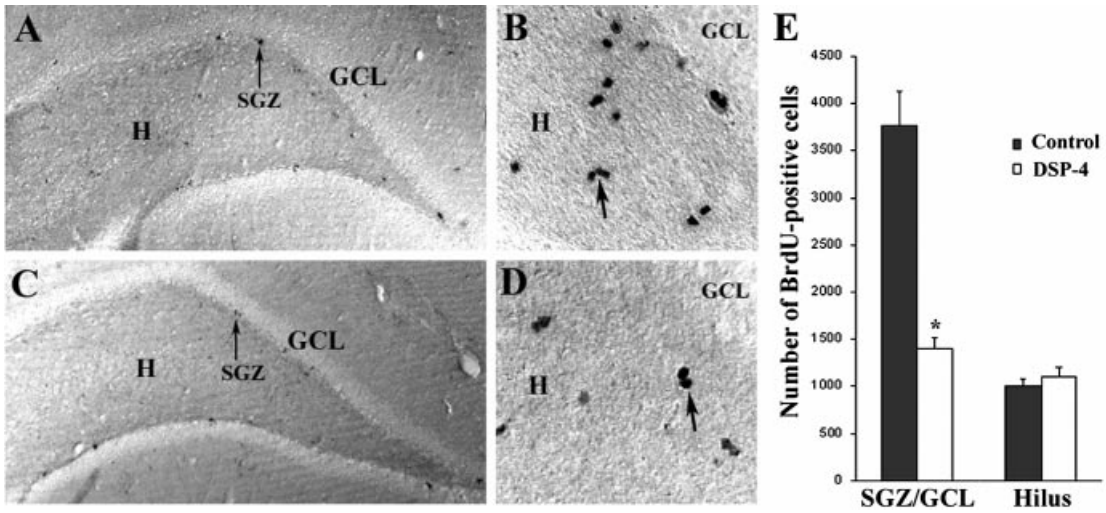

FIG. 1. Norepinephrine depletion significantly decreases the proliferation of hippocampal progenitors in the subgranular zone (SGZ), but not the hilus (H), region of the dentate gyrus in comparison to controls (E). Representative differential interference contrast photomicrographs of BrdU-positive cells from control (A and B) and DSP-4 (C and D) treated animals are shown. BrdU positive cells (arrows B and D) were observed in the SGZ, at the border of the hilus and the granule cell layer (GCL), and within the hilus. Results are expressed as the mean ± SEM (n = 6 per group). *$P < 0.05$ compared to control (Student's t-test).

BrdU labelling

The thymidine analogue 5-bromo-2′-deoxyuridine BrdU (Sigma) was administered intraperitoneally. Vehicle and DSP-4 treated animals (n = 6 per group) received BrdU (100 mg/kg) once daily for two consecutive days starting on the third day after the last DSP-4/vehicle treatment and were killed 24 h following BrdU administration. To examine the effects of DSP-4 treatment specifically on cell proliferation, animals (n = 5 per group) received a single BrdU (100 mg/kg) injection on the third day after the last DSP-4/vehicle treatment and were killed 2 h later. To address the influence of DSP-4 on cell survival, BrdU (200 mg/kg once daily for three days) was administered to drug-naive rats and two days later vehicle/DSP-4 treatment (n = 4 per group) was administered. Animals were killed 24 h (n = 3) or 21 days (n = 4 per group) after BrdU treatment. Animals were killed 24 h (n = 3) or 21 days (n = 4 per group) after BrdU treatment with an overdose of chloral hydrate (400 mg/kg, Sigma).

Immunohistochemistry and immunofluorescence

Animals were transcardially perfused with 4% paraformaldehyde. Serial coronal sections (50 μm) were cut on a vibratome. Sections were processed for BrdU immunohistochemistry as described previously (Malberg *et al*., 2000). In brief, post DNA denaturation and blocking, sections were incubated overnight at 25 °C with mouse anti-BrdU antibody (1 : 500, Boehringer Mannheim, USA). Sections were exposed to secondary antibody (biotinylated antimouse IgG, 1 : 200, Vector Laboratories, USA) and signal was amplified with the Vectastain Elite Avidin-Biotin system and detected with diaminobenzidine as a substrate.

For double labelling immunofluorescence, sections were processed as described above before exposure to one of the following cocktails: rat anti-BrdU (1 : 200, Accurate Biochemicals, USA) with mouse anti-neuronal nuclei (NeuN) 1 : 800, Chemicon, USA or rabbit anti-glial fibrillary acidic protein (GFAP) 1 : 500, Chemicon overnight at 4 °C. Sections were incubated with cocktails of secondary antibodies: biotinylated anti-rat IgG (1 : 500, Chemicon) with either rhodamine-conjugated anti-mouse or rabbit IgG (1 : 500, Chemicon). Sections were then exposed to fluorescein-conjugated streptavidin (1 : 400, Vector Laboratories) for 2 h. To examine the depletion of norepinephrine, sections were incubated with mouse anti-dopamine-β-hydroxylase (DβH) antibody (1 : 200, Chemicon) overnight at 4 °C. To detect serotonergic innervation sections were exposed to rabbit anti-serotonin antibody (1 : 5000, Sigma) for 72 h. Sections were washed, incubated with biotinylated secondary antibodies followed by fluorescein-conjugated streptavidin for 2 h. Immunofluorescence was visualized on a Biorad MRC 1024 confocal microscope.

Cell counting

Quantification of BrdU positive cells within the dentate gyrus was carried out using a modified, unbiased stereology protocol (Malberg *et al*., 2000) on a Zeiss Axioskop-2 Plus microscope. Sections were coded and quantification was performed by an experimenter blind to the code. Sections spanned the rostro-caudal extent of the hippocampus (Bregma –1.80 to –6.04) and every sixth hippocampal section was processed for quantification (11 sections per animal). BrdU positive cells within the subdivisions of the dentate gyrus (SGZ and hilus) were counted omitting those in the outermost focal plane. BrdU positive cells were counted as SGZ when they were directly touching the SGZ or within it. Cells were counted as hilar when they were further than two cell body widths from the SGZ. The total number of BrdU positive cells per SGZ or hilus was estimated by multiplying the total number of BrdU cells counted from every 6th section by six and were reported as total BrdU positive cells per region. To control for differences in bioavailability of BrdU between different treatment groups, striatal sections were processed for BrdU immunohistochemistry to label subventricular zone progenitors. BrdU positive cells present on the lateral face of the lateral ventricle were counted in eight sections per animal (Bregma 1.60–0.20; n = 6 per group) and statistical analyses was performed on the average-number of BrdU positive cells per section.

The percentage colocalization of BrdU labelling with cell-specific markers was determined in the SGZ and granule cell layer in eight

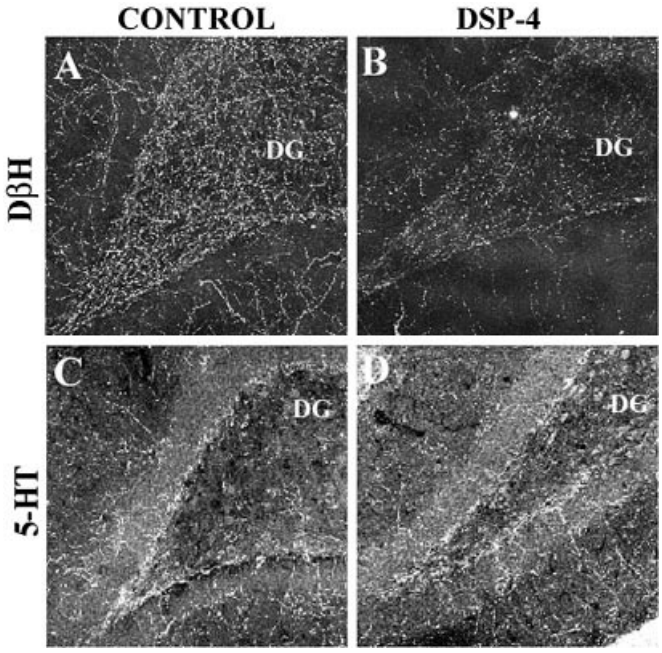

FIG. 2. Representative confocal images of dopamine-β-hydroxylase (DβH) and serotonin (5-HT) immunofluorescence from the dentate gyrus (DG) region of a control and DSP-4 treated animal are shown. DSP-4 treated animals (B) showed a large reduction in DβH-immunopositive fibers within the DG compared to vehicle treated controls (A). There was no difference between the 5-HT innervation in the DG of controls (C) and DSP-4 treated animals (D).

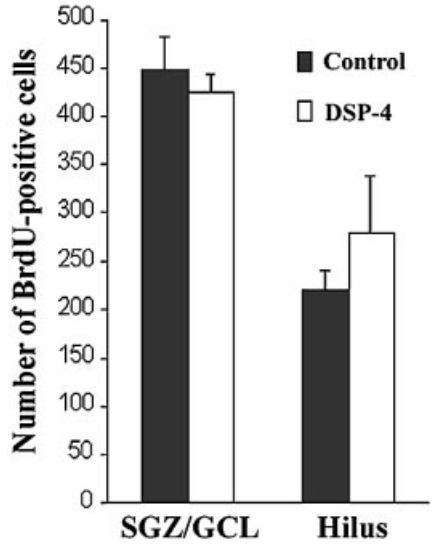

FIG. 3. Norepinephrine depletion does not influence the survival of dentate granule cell progenitors. Rats received BrdU injections two days prior to DSP-4 or vehicle treatment and were killed three weeks later. Quantitative analysis indicated that the number of BrdU-positive cells, in the SGZ/GCL or hilus, that survived in the DSP-4 treated group did not differ from vehicle treated controls. Results are expressed as the mean ± SEM (n = 4 per group). *P > 0.05 compared to control (Student's t-test).

sections (250 μm apart) per animal from the survival experiment paradigm. A minimum of 100 BrdU positive cells were analyzed per animal (n = 3 per group) using Z-plane sectioning with 0.6 μm steps to confirm the colocalization of BrdU with the neuronal marker NeuN or the glial marker GFAP.

Statistical analysis

Results were subjected to statistical analysis. The means of experimental groups were compared using the unpaired Student t-test (Graphpad InStat, USA). Statistical significance was determined at P-values < 0.05.

Results

Norepinephrine depletion decreases the proliferation of dentate granule cell progenitors

The effect of norepinephrine depletion on the proliferation of hippocampal progenitors was assessed using the mitotic marker BrdU to label dividing progenitors. The number of BrdU positive cells within the SGZ region of the granule cell layer (GCL), but not the hilar region, were significantly decreased (63%) in the norepinephrine-depleted animals relative to controls (Fig. 1). BrdU positive cells were found primarily within the SGZ at the border of the hilus and the GCL in both control and DSP-4 treated animals (Fig. 1A–D) and were often detected in clusters. To confirm that the effects of norepinephrine depletion were specific to cell proliferation animals were killed 2 h after a single BrdU injection, which would allow a single round of cell division to be examined. The number of BrdU positive cells in the SGZ/GCL of control animals was 2040 ± 112 in comparison to 1083 ± 132 cells in the SGZ/GCL of DSP-4 treated animals, indicating a 47% decrease (P < 0.05). There was no influence of norepinephrine depletion on the number of BrdU positive cells within the hilus (Control = 688 ± 57; DSP-4 = 663 ± 76, P > 0.05).

The effect of norepinephrine depletion on progenitor proliferation appears specific to the dentate gyrus as the number of BrdU-positive cells per section within the subventricular zone was not significantly different in control and DSP-4 treated animals (Control = 254 ± 13; DSP-4 = 261 ± 24; n = 6 per group, P > 0.05). As reported previously DSP-4 treatment caused a marked disruption of noradrenergic fibre innervation to the hippocampus (Fritschy & Grzanna, 1989) and was assessed by immunohistochemistry for the noradrenergic marker dopamine β-hydroxylase (DβH) (Fig. 2A and B). There was no influence of DSP-4 treatment on the serotonergic fibre innervation to the hippocampus as determined by immunohistochemistry with an anti-serotonin antibody (Fig. 2C and D).

Norepinephrine depletion does not influence the survival and differentiation of BrdU labelled dentate granule cell progenitors

In the survival experiment, BrdU treatment was given to drug-naive animals prior to treatment with vehicle or DSP-4. This paradigm allowed us to assess the survival of a newly born BrdU-labelled pool of progenitors when exposed to norepinephrine depletion. The survival of progenitors three weeks after BrdU labelling was not influenced by norepinephrine depletion in both the SGZ/GCL and hilar regions (Fig. 3; P > 0.05). Although the number of BrdU-positive cells in control and DSP-4 treated animals did not differ from each other they both showed a significant decrease in number at the three-week survival point (Fig. 3) relative to the number of BrdU-positive cells in the SGZ and hilus 24 h after BrdU labelling (SGZ/GCL = 4408 ± 414, Hilus = 1005 ± 115 n = 3; P < 0.05) consistent with previous reports (Malberg *et al*., 2000). The BrdU positive cells at three weeks had a distinct, ovoid morphology, were often localized within the GCL and were never observed in clusters.

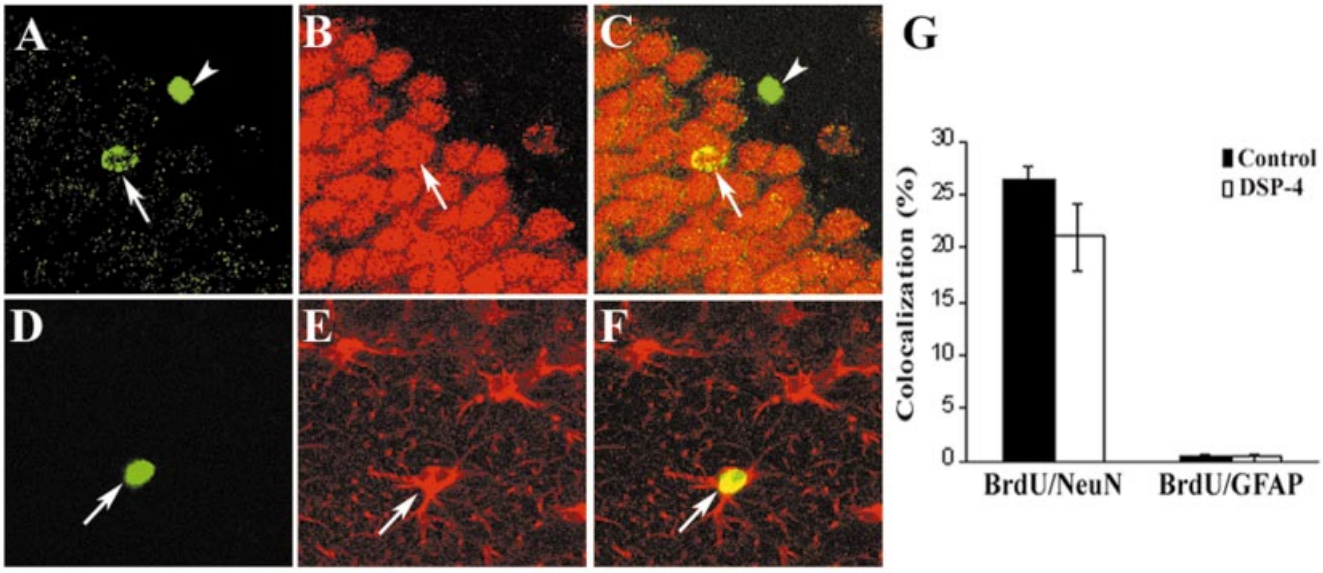

FIG. 4. Colocalization of BrdU positive cells (A and D) with the neuronal marker NeuN (B) or the glial marker GFAP (E) is indicated by arrows in the merged images (C, BrdU/NeuN; F, BrdU/GFAP) in representative confocal images from a control animal. The arrowhead (C) indicates lack of colocalization of another BrdU positive cell. Quantitative analysis revealed that norepinephrine depletion does not influence the percentage colocalization of BrdU positive cells, in the subgranular zone and granule cell layer, with NeuN or GFAP in DSP-4 treated animals relative to controls. Results are expressed as the mean ± SEM ($P > 0.05$, $n = 3$ per group).

The differentiation of surviving BrdU labelled progenitors in the SGZ/GCL was determined by double label immunofluorescence for BrdU and the neuronal marker NeuN (Fig. 4A–C) or the glial marker, GFAP (Fig. 4D–F). The colocalization (%) of BrdU positive cells with neuronal or glial markers did not differ in the control and DSP-4 treated groups (Fig. 4G; $P > 0.05$). In both groups approximately 25% of BrdU positive cells within the SGZ/GCL showed colocalization with NeuN, whereas very few BrdU labelled cells were positive for the glial marker GFAP. The remaining BrdU positive cells did not colocalize with either of the cell specific markers and may represent as yet undifferentiated progenitors. These results indicate that norepinephrine depletion does not influence the differentiation of labelled BrdU progenitors at least at the three-week time point examined in this study.

Discussion

The results of this study demonstrate that norepinephrine depletion significantly reduces the proliferation of hippocampal granule cell progenitors. The decrease in BrdU positive cells following norepinephrine depletion is specific to SGZ progenitors that give rise to granule cell neurons as no change in BrdU labelling was observed in either the subventricular zone or the hilus. A 47% decrease in BrdU labelling is already evident in the DSP-4 treated animals 2 h after a single BrdU dose confirming that norepinephrine depletion exerts a strong influence on SGZ progenitor proliferation. In contrast, the survival of granule cell progenitors and their differentiation into a neuronal or glial phenotype is not affected by norepinephrine depletion. The hippocampus, in particular the hilar and SGZ regions receive dense noradrenergic input from the locus coeruleus. Previous studies have shown that norepinephrine enhances the proliferation of embryonic germinal neuroepithelial cells, as well as non-neuronal cells (Cruise *et al*., 1985; Popovik & Haynes, 2000). Our data indicates that proliferative signals to adult hippocampal progenitors within the SGZ may also be regulated by alterations in norepinephrine levels.

The mechanisms underlying the decrease in granule cell progenitor proliferation following norepinephrine depletion are as yet unknown and require further investigation, but several possibilities can be proposed. Previous reports indicate that norepinephrine increases embryonic neuroepithelial cell division *via* the α_1 adrenergic receptor (Popovik & Haynes, 2000) suggesting the possibility that norepinephrine may influence adult progenitor proliferation directly through adrenoceptor subtypes. Although hippocampal neurons express several adrenoceptor subtypes (Nicholas *et al*., 1996) it is as yet unknown whether any of these are expressed by SGZ progenitors. Alternatively, norepinephrine depletion may influence proliferation indirectly *via* alterations in other factors that regulate hippocampal neurogenesis. Noradrenergic neurons contain fibroblast growth factor-2, which is known to enhance adult hippocampal neurogenesis, and a loss of this factor in depleted animals could contribute to the decrease in proliferation (Chadi *et al*., 1993; Palmer *et al*., 1999). In contrast, norepinephrine depletion enhances dentate gyrus nerve growth factor and brain derived neurotrophic factor mRNA levels (Hutter *et al*., 1996). It is possible that an altered complement of distinct growth factors in the hippocampus may contribute to the effects of norepinephrine depletion on progenitor proliferation. DSP-4 treatment also results in a loss of nitric oxide synthase immunoreactivity in the SGZ and hilus (Zhang & Yu 1995), and given the evidence that nitric oxide donors enhance hippocampal neurogenesis (Zhang *et al*., 2001), this suggests the possibility that norepinephrine depletion induced decreases in nitric oxide synthase may contribute to a reduction in progenitor proliferation.

Previous studies have indicated that excitatory input and adrenal steroids both suppress granule cell progenitor proliferation (Cameron & Gould 1994; Gould *et al*., 1997), whereas serotonin stimulates proliferation (Brezun & Daszuta, 2000). Norepinephrine exerts a mainly inhibitory influence on hippocampal neurons through the

inhibition of glutamate release (Boehm, 1999). Norepinephrine by decreasing excitatory input could act to stimulate progenitor turnover suggesting a scenario where norepinephrine depletion may alter adult neurogenesis through changes in excitatory input. Norepinephrine depletion is unlikely to influence progenitor proliferation *via* altered serotonin or adrenal steroids levels as studies indicate that norepinephrine depletion does not significantly influence hippocampal serotonin innervation (Fig. 2) or basal circulating corticosterone levels (Ziegler *et al*., 1999). It is also possible that the norepinephrine depletion may lead to the death of proliferating SGZ progenitors or their being shifted to a quiescent phase. Indeed, transection of noradrenergic axons innervating the cortex in embryonic explant cocultures results in a robust increase in apoptotic BrdU-positive cells within the germinal zone (Popovik & Haynes, 2000). Similar possibilities may underlie the effects of norepinephrine depletion on adult SGZ progenitors and require further examination.

Although the mechanisms *via* which altered norepinephrine levels regulate adult hippocampal neurogenesis are as yet unknown, it is striking to note that stress and antidepressant treatments which both regulate the noradrenergic pathway (Brady, 1994) also influence adult neurogenesis. Studies have suggested that chronic stress decreases hippocampal neurogenesis and can also result in norepinephrine depletion (Brady, 1994; Gould *et al*., 1997). Dysfunction of the noradrenergic pathway has been linked with depression (Brady, 1994) and recent studies indicate that chronic treatment with several classes of antidepressants, including the norepinephrine selective reuptake inhibitor reboxetine, enhances neurogenesis (Malberg *et al*., 2000). Taken together these results motivate future studies to examine the role of norepinephrine in the basal, as well as stress and antidepressant-mediated, regulation of adult neurogenesis.

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Abbreviations

BrdU, 5-bromo-2′-deoxyuridine, DβH, dopamine-β-hydroxylase; DSP-4, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride; GCL, granule cell layer; GFAP, glial acidic fibrillary protein; i.p., intraperitoneal; NeuN, mouse antineuronal nuclei; SGZ, subgranular zone.

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