

# The $\alpha_2$ -Adrenoceptor Antagonist Dexefaroxan Enhances Hippocampal Neurogenesis by Increasing the Survival and Differentiation of New Granule Cells

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The generation of new neurons in the hippocampus is a dynamic process regulated by environmental, endocrine, and pharmacological factors. Since enhancement of hippocampal neurogenesis has been associated with learning and memory, and the locus coeruleus–noradrenergic system has been shown to modulate these cognitive functions, we hypothesized that activation of noradrenergic neurotransmission might enhance neurogenesis in the adult hippocampus. To test this hypothesis *in vivo*, we induced the release of noradrenaline in the hippocampus by blocking presynaptic inhibitory autoreceptors with the selective  $\alpha_2$ -adrenoceptor antagonist dexefaroxan. Confocal microscopy showed that noradrenergic afferents make contact with proliferating and differentiating cells, suggesting a direct noradrenergic influence on neurogenesis. Chronic systemic treatment of rats with dexefaroxan did not affect cell proliferation *per se* in the dentate gyrus (as monitored by bromodeoxyuridine-labeling), but promoted the long-term survival of newborn neurons by reducing apoptosis. Dexefaroxan treatment also enhanced the number and complexity of the dendritic arborizations of polysialated neural cell adhesion molecule-positive neurons. The trophic effects of dexefaroxan on newborn cells might involve an increase in brain-derived neurotrophic factor, which was upregulated in afferent noradrenergic fiber projection areas and in neurons in the granule cell layer. By promoting the survival of new endogenously formed neurons, dexefaroxan treatment represents a potential therapeutic strategy for maintaining adult neurogenesis in neurodegenerative conditions, such as Alzheimer's disease, that affect the hippocampus.

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## INTRODUCTION

Adult neurogenesis is a dynamic process that occurs in the brains of several different species, including man (Eriksson *et al*, 1998). The two best-established regions in which adult neurogenesis is observed are the subventricular zone and the dentate gyrus (DG) of the hippocampus. In the subventricular zone, proliferating progenitor cells differentiate into neurons that migrate to the olfactory bulb (Doetsch and Alvarez-Buylla, 1996; Lois and Alvarez-Buylla, 1994). In the hippocampus, proliferating progenitors in the subgranular zone (sgz) migrate to the granule cell layer (gcl)

where they form new neurons that innervate the CA3 region (Cameron *et al*, 1993).

Neurogenesis in the hippocampus is of particular interest because of the role this structure plays in learning and memory. It is known that hippocampal neurogenesis is sensitive to environmental, endocrine, and pharmacological factors. It is increased by exposure to an enriched environment, exercise, antidepressants, and hippocampus-dependent learning (Duman *et al*, 2001a,b; Malberg *et al*, 2000) and is decreased with aging, and by environmental or psychosocial stress and glucocorticoids (Cameron and McKay, 1999; Gould *et al*, 1999a; Kuhn *et al*, 1996). However, the endogenous factors (neurotransmitters or neurotrophins) that regulate neurogenesis in the adult hippocampus must be better identified.

The hippocampus has an abundant noradrenergic innervation (Loy *et al*, 1980) that promotes both short- and long-term enhancement of sensory-evoked responses in the DG, consistent with a role in hippocampal learning and memory (Harley, 1991). Recent studies have reported that

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altering noradrenaline levels can change the rate of progenitor proliferation in the hippocampus. Noradrenergic depletion with the selective noradrenergic neurotoxin *N*-(2-chloroethyl)-*N*-ethyl-2-bromo benzylamine hydrochloride (DSP-4) decreased the number of proliferating cells in the DG of the adult rat (Kulkarni *et al*, 2002). Conversely, increasing synaptic levels of noradrenaline with antidepressants, including selective noradrenaline reuptake inhibitors, enhanced neurogenesis (Malberg *et al*, 2000). Furthermore, activation of the noradrenergic system by blockade of presynaptic inhibitory autoreceptors with an  $\alpha$ 2-adrenoceptor antagonist has been reported to increase adult neurogenesis in the olfactory bulb, by preventing the loss of newly formed neurons (Bauer *et al*, 2003).

We therefore hypothesized that antagonism of  $\alpha$ 2-adrenoceptors would enhance neurogenesis in the DG as it does in the olfactory bulb, given that  $\alpha$ 2-adrenoceptor blockade increases noradrenaline synthesis and release in forebrain target areas including the hippocampus (van Veldhuizen *et al*, 1994). To test this hypothesis, we first studied the rate of proliferation and cell death in the DG of the dorsal hippocampus following acute and chronic treatment with the selective  $\alpha$ 2-adrenoceptor antagonist dexefaroxan. We then determined the effects of dexefaroxan treatment on differentiation and on morphological plasticity in this structure and investigated whether this treatment could modify the endogenous levels of brain-derived neurotrophic factor (BDNF), a target-derived survival factor for newborn neurons in the DG.

## MATERIALS AND METHODS

### Animals

Animals were housed, handled, and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (NCR, 1996) and the European Union Council Directive 86/609/EEC, and the experimental protocols were carried out in compliance with institutional ethical committee guidelines for animal research. All efforts were made to minimize the number of animals used and their suffering.

Male Sprague–Dawley rats (IOPS Han, Centre d'Élevage René Janvier, Le Genest St Ile, France), weighing 250–300 g at the beginning of the experiments, were housed two per cage at 22°C under a 12 h light:dark cycle (lights on at 0700 hours AM) with free access to food and water. Five rats were used for each experimental condition.

Acute treatment with dexefaroxan consisted of four intraperitoneal (i.p.) injections of vehicle (3 ml/kg) or dexefaroxan (0.63 mg/kg) at 4-hr intervals. For chronic treatment, a single i.p. injection of vehicle (3 ml/kg) or dexefaroxan (0.63 mg/kg) was administered just before subcutaneous implantation of ALZET<sup>®</sup> osmotic minipumps (model 2ML4) under equithesin anesthesia (2.5 ml/kg i.p.). The pumps delivered vehicle (distilled water) or dexefaroxan (0.63 mg/rat/day) over a 28-day period. To verify that dexefaroxan was released by the minipumps for 28 days, we monitored the inhibition of hypothermia induced by  $\alpha$ 2-adrenoceptor agonist (data not shown). For long-term treatment (70 days), the pumps were changed every 28 days.

At the end of the experiments, the rats were anesthetized with equithesin (2.5 ml/kg i.p.) and perfused transcardially with saline buffer containing 0.1% NaNO<sub>3</sub> and heparin (5 U/ml), followed by a 20-min perfusion with 4% paraformaldehyde (pH 7.4). Brains were then removed, post-fixed in the same fixative for 2 h at 4°C, and cryoprotected in 15% sucrose in distilled H<sub>2</sub>O at 4°C for 2 days. The brains were cut into 25  $\mu$ m coronal sections through their entire length with a sledge freezing microtome at –20°C.

### Determination of Noradrenaline Concentrations by Microdialysis and HPLC

Intracerebral microdialysis was used to assess the effect of dexefaroxan on hippocampal noradrenaline release *in vivo*. Surgery was performed as described previously (Tellez *et al*, 1999) with minor modifications. Rats were anesthetized with a halothane/oxygen mixture and were implanted under stereotaxic control with a microdialysis probe (CMA/12, 3 mm membrane length; CMA/Microdialysis AB, Stockholm, Sweden) terminating in the hippocampus (5.4 mm posterior to bregma, 4.9 mm lateral to the midline at bregma, 6.5 mm ventral from dura). Probes were anchored to the skull with stainless-steel screws and dental acrylic. Immediately after surgery, rats were placed in bowl-shaped Plexiglas cages with free access to food and water. The dialysis probe was immediately connected to a microliter syringe pump and perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 1.3 mM CaCl<sub>2</sub>) at a flow rate of 0.2–0.4  $\mu$ l/min during an overnight recovery period. The following morning, the infusion syringes were refilled with fresh filtered and degassed Ringer's solution, and the pump infusion rate was increased to 2.0  $\mu$ l/min. After a 2.5 h equilibration period, three 20-min dialysate samples were collected in glass microtubes containing 10  $\mu$ l of 1 mM Na<sub>2</sub>EDTA in 0.05 M HCl, for baseline measurements of endogenous noradrenaline. Animals then received an i.p. injection of vehicle (saline) or dexefaroxan (0.63 mg/kg), and dialysate samples were collected continuously for 5 h. At the end of the experiment, the rats were decapitated and the brains removed and fixed in 4% formalin solution. Coronal sections (500  $\mu$ m thick) of fixed brain were prepared using a Vibroslice oscillating microtome (Camden Instruments, London, UK), and probe placement was assessed by visual inspection of the probe tract in the tissue.

Noradrenaline in the dialysates was derivatized and analyzed by HPLC with fluorescence detection as described (Yoshitake *et al*, 2003). This method is based on precolumn derivatization of noradrenaline with benzylamine in the presence of potassium hexacyanoferrate(III), resulting in a highly fluorescent and stable benzoxazole derivative. Derivatized samples were loaded into a refrigerated HPLC system (Waters 2690 Alliance Separation Module) for automated injection and analysis. Chromatographic conditions consisted of a C<sub>18</sub> reverse-phase column (Waters Symmetry Shield, 2.1  $\times$  150 mm, 5  $\mu$ m particle size) maintained at 30°C, and a mobile phase (flow rate 0.4 ml/min) composed of 15 mM sodium acetate, 4 mM octane sulfonic acid, and acetonitrile (25% (vol/vol)), final pH 4.5. Fluorescent derivatives were detected with a Waters Model 474 scanning fluorescence detector ( $\lambda_{\text{ex}}$  = 345 nm,  $\lambda_{\text{em}}$  = 480 nm). Quantification of the chromatographic peak

of noradrenaline was based on the comparison with calibration curves derived from authentic standards of noradrenaline (0.1–10 pg/ $\mu$ l) prepared in Ringer's solution and carried through the derivatization procedure. The level of noradrenaline in the dialysates was expressed as a percentage of the average amount of noradrenaline in the three baseline samples preceding drug or vehicle injection. For the comparison of drug and vehicle treatments between different subjects at corresponding time points, Kruskal–Wallis ANOVA followed by Mann–Whitney *U*-test was used.

### Analysis of Cell Proliferation with Bromodeoxyuridine (BrdU)

BrdU (50 mg/kg, 15 mg/ml in 0.9% NaCl containing 0.007 N NaOH; Sigma, St Louis, MO) that is incorporated into cellular DNA during the S phase of the cell cycle was injected i.p. either once or on 7 consecutive days preceding treatment with dexefaroxan (see Results for experimental designs). BrdU incorporation was determined by immunohistochemistry with an anti-BrdU antibody (see below).

### Immunohistochemistry

Free-floating 25  $\mu$ m sections of fixed brains from the experimental groups were processed in parallel. Immunohistochemistry was performed by standard procedures. Briefly, sections were pretreated with 0.3% H<sub>2</sub>O<sub>2</sub> in 0.1 M PBS and incubated in blocking solution (5% normal horse serum and 0.2% Triton X-100 in PBS), and then with primary antibodies for 48 h at 4°C in PBS. Antibody binding was visualized with appropriate secondary antibodies (incubation, 2 h at room temperature), either coupled directly with fluorescent markers or biotinylated for revelation with horseradish peroxidase (ABC Vectastain kit, Vector laboratories, Burlingame, CA) and diaminobenzidine (DAB, 0.5 mg/mL; Sigma) as chromogen. For BrdU labeling, sections were first denatured in 2 M HCl for 30 min at 37°C and then neutralized in 0.1 M borate buffer, pH 8.5, for 10 min. Multifluorescent labeling was carried out sequentially. To label nuclei, sections were counterstained with Hoechst 33342. Labeled sections were mounted on glass slides with Mowiol medium (Calbiochem, San Diego, CA). Fluorescent images of mounted sections were acquired with an epifluorescent microscope (Axioplan 2, Zeiss, Germany) equipped with FluoUp image analysis system software (Explora Nova, La Rochelle, France).

The primary antibodies used were: rat anti-BrdU (mAb, 1:200; ImmunologicalsDirect, Oxfordshire, UK); mouse anti-polysialated neural cell adhesion molecular (PSA-NCAM) (monoclonal IgM, 1:1000; gift of G Rougon, Centre National de la Recherche Scientifique, Marseille, France); mouse anti-NeuN (1:200; Chemicon, Temecula, CA); mouse anti-GFAP (1:1000; Sigma); mouse anti-proliferating cell nuclear antigen (PCNA) (1:100; DAKO, Denmark); rabbit anti-BDNF (1:1000; gift of Dr Q Yan, Amgen, Thousand Oaks, CA); rabbit anti-noradrenaline transporter (NET) (1:5000; gift of Dr RD Blakely, Vanderbilt University, Nashville, TN); goat anti-doublecortin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), and guinea-pig

anti- $\alpha$ 2c-adrenoceptor (1:300; Neuromics, Bloomington, MN).

The secondary antibodies used were biotinylated anti-rat (1:250; Vector Laboratories) or biotinylated anti-mouse IgM (1:200; Zymed, South San Francisco, CA), Cy3-conjugated anti-rat, Cy3-conjugated anti-rabbit, FITC-conjugated anti-mouse IgM, FITC-conjugated anti-mouse (1:400, 1:400, 1:150, 1:100, respectively; Jackson Laboratories, West Grove, PA), and Cy3-conjugated anti-goat (1:400; Chemicon).

### Terminal Deoxynucleotidyl Transferase (TdT)-Mediated UTP Nick-End Labeling (TUNEL)

The TUNEL assay was performed on free-floating sections using the Apoptag *In Situ* cell death detection kit (Intergene, Purchase, NY) as described previously (Tonchev *et al*, 2003). Briefly, the sections were washed in TBS containing 0.15% Triton X-100, dehydrated in an ascending ethanol/distilled water series (30–70–95%, 5 min each), incubated in 100% ethanol (15 min), and gradually rehydrated. The sections were then placed in the equilibration buffer, followed by incubation in the TdT/reaction buffer mixture (15/85, ApopTag kit) at 37°C for 2 h. The reaction was visualized by overnight incubation at 4°C with sheep anti-digoxigenin–fluorescein antibody in blocking solution (62/68, ApopTag kit). The sections were counterstained with Hoechst 33342 and then mounted on glass slides with Mowiol medium, and visualized as above.

### Stereological Cell Counts

BrdU-labeled nuclei and PSA-NCAM cells were counted by the optical disector method using a microscope (Axioplan 2) equipped with a  $\times 100$  objective (numerical aperture 1.4), a video camera (Retiga, Qimaging, BC, Canada), and an image analysis system (Mercator, ExploraNova, La Rochelle, France) dedicated to stereology (Howard *et al*, 1985). Series of regularly spaced sections at 300  $\mu$ m intervals (every 12 sections, for a total of six sections) covering the dorsal hippocampus were sampled, starting where the DG blades become fully established (approximately 2.6 mm posterior to Bregma). The analysis was performed bilaterally and sampling ended where the hippocampus contained both a dorsal and ventral component, at approximately 4.3 mm posterior to Bregma (Paxinos and Watson, 1986). The gcl always included the sgz, defined here as a two-cell body wide border between the gcl and hilus. The boundaries of the structures in the coronal plane were determined microscopically ( $\times 4$  objective). The average section was initially 25  $\mu$ m thick (with an estimated average shrinkage of 37% taken into account in the final results). The top and bottom 2.5  $\mu$ m were excluded from the analysis. The average number of profiles counted ( $Q^-$ ) per region per animal was 102 for BrdU-labeled nuclei and 134 for PSA-NCAM. The density of the cells ( $N_v$ ) was calculated with the formula  $N_v = \sum Q^- / a \times h$ , where  $a$  is the total area of section examined for the given region and  $h = 300 \mu$ m. The total number of cells in the DG and in the hilus was estimated by multiplying the volume of the region by the corresponding cellular density.

For colocalization studies with NeuN or GFAP, BrdU-labeled cells were first counted using a fluorescent microscope. Confocal microscopy (Leica DM IRBE, Germany) was then used to determine the percentage of colabeled cells in the dorsal hippocampus. For each animal, 15 random fields containing BrdU-labeled cells were examined. Differences between mean values for each treatment group were analyzed by Kruskal–Wallis ANOVA followed by Mann–Whitney *U*-test.

### Quantification of BDNF Immunostaining

For quantitative analysis of BDNF staining, sections from control and experimental animals were processed simultaneously. The density of BDNF immunostaining was assessed using the same computerized image analysis system equipped with software for optical density measurements. BDNF staining was analyzed in five anatomical regions of the hippocampus (hilus, gcl, CA1, CA3 pyramidal cell body layer, and CA3 stratum radiatum), corresponding to the same levels used for the BrdU and PSA-NCAM cell counting. Optical density was determined as gray levels and expressed in arbitrary units. Blank values were measured where no BDNF immunostaining was observed. Differences between mean values for each treatment group were analyzed by Kruskal–Wallis ANOVA followed by Mann–Whitney *U*-test.

## RESULTS

### Dexefaroxan Increases Noradrenaline Release in the Hippocampus *In Vivo*

We first confirmed that the  $\alpha$ 2-adrenoceptor antagonist, dexefaroxan, enhanced noradrenaline release in the

hippocampus by microdialysis in conscious freely moving rats (Figure 1). I.p. injection of the vehicle evoked an immediate 64% increase in noradrenaline outflow that returned to baseline levels by 20 min postinjection. Dexefaroxan (0.63 mg/kg i.p.) also evoked an immediate increase (+74%) in noradrenaline outflow, which remained significantly higher (32–73%) than in vehicle-treated animals for up to 4 h (Figure 1). Upregulation of noradrenaline release by dexefaroxan is therefore potentially able to affect target cells in the DG of the hippocampus.

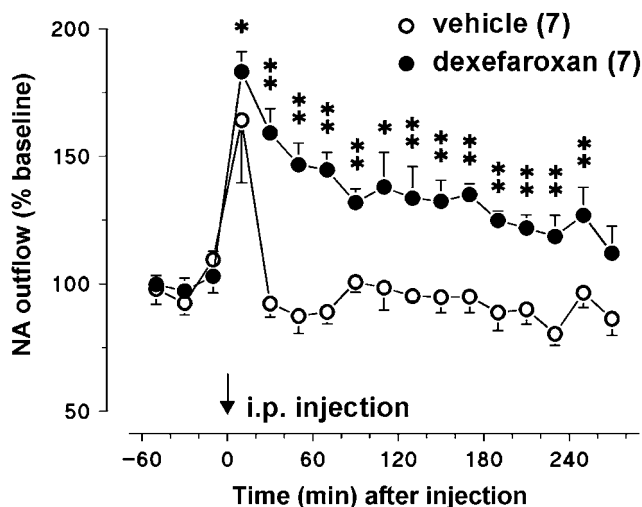
### What Cells in the DG Receive Noradrenergic Innervation?

We next analyzed the noradrenergic innervation of the DG in vehicle-treated rats. Presynaptic  $\alpha$ 2-adrenoceptors were present on fibers innervating the gcl of the dorsal hippocampus (Figure 2a). Noradrenergic fibers identified by immunoreactivity for the NET were found in abundance throughout the dorsal hippocampus, with a high density in the DG (Figure 2b and c). NET-positive fibers were observed in close proximity to cells positive for PCNA (marker of proliferation) (Figure 2d and e) and to cells positive for the membrane-associated polysialated neural cell adhesion molecule (PSA-NCAM) (marker of differentiation) (Figures 2f, g). Confocal microscopy revealed noradrenergic contacts both with PCNA-positive cells (Figure 2e) and with PSA-NCAM-positive cells (Figure 2g). These observations suggested that both newborn PSA-NCAM-positive granule cells and proliferating PCNA-positive precursors were potentially responsive to noradrenaline release induced by dexefaroxan. This hypothesis was examined in the following experiments.

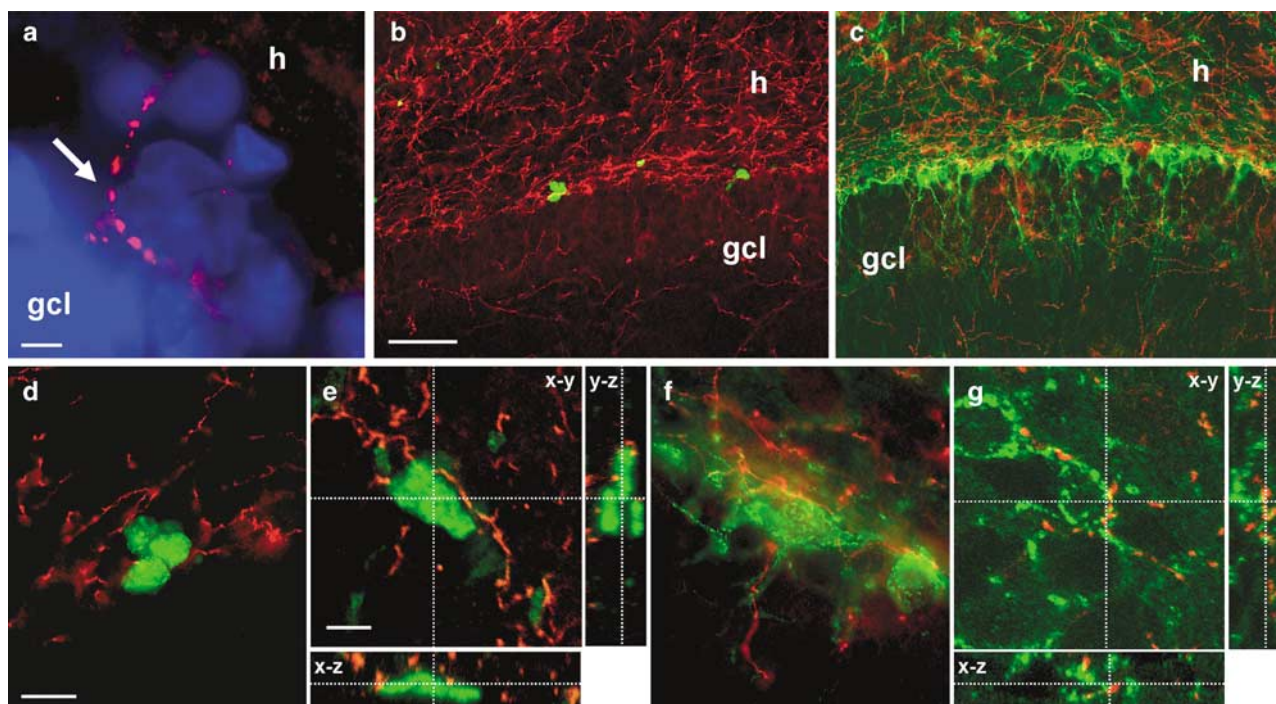
### Dexefaroxan does not Affect the Rate of Cell Proliferation in the Adult DG

To determine how increased noradrenaline release in the hippocampus affects adrenoceptive target cells, we first examined the effects of both acute and chronic dexefaroxan administration on the rate of cell proliferation in the DG of the dorsal hippocampus. For the acute treatment, the rats received a single i.p. injection of BrdU (50 mg/kg) followed immediately by four i.p. injections of vehicle (3 ml/kg) or dexefaroxan (0.63 mg/kg) at 4 h intervals. Rats were killed 24 h later, a time period sufficient for all cells that incorporated BrdU during the S phase to complete mitosis. Effects of chronic treatment were examined in rats that received a single i.p. injection of BrdU after subcutaneous administration of dexefaroxan with a minipump for 20 days and were killed 24 h later. The results are shown in Figure 3. A 21 day survival period was chosen because in that interval around 50% of new cells will have died (Dayer *et al*, 2003).

After a 24 h exposure to both BrdU and dexefaroxan, proliferating cells with BrdU-positive nuclei were observed predominantly in the deeper layers of the gcl (Figure 3a and b). The nuclei were darkly and uniformly stained and varied in shape and the cells frequently appeared in clusters of two or more (Figure 3b). This observation that, at 24 h, BrdU-positive cells were rarely found outside the interface between the gcl and the hilus suggest that incorporation of BrdU occurred during proliferation of progenitor cells



**Figure 1** Effect of a single i.p. injection of saline or dexefaroxan (0.63 mg/kg) on the outflow of endogenous noradrenaline (NA) in the ventral hippocampus of conscious freely moving rats measured by microdialysis. Baseline: the average amount of noradrenaline in three 20-min dialysis samples collected prior to i.p. injection ( $t=0$  min). Significantly different than corresponding samples from vehicle-injected animals (\* $p<0.05$ , \*\* $p<0.01$ ; Kruskal–Wallis ANOVA followed by Mann–Whitney *U*-test,  $n=7$  rats per group).



**Figure 2** Markers of noradrenergic innervations in the DG. (a) Presynaptic  $\alpha_2c$ -adrenoceptors (red, arrow) on fibers in the gcl. Hoechst 33342-stained nuclei are shown in blue. (b and c) NET-positive fibers (red) in the hilus and gcl contiguous to proliferating PCNA-positive cells (b, green) and PSA-NCAM-positive cells (c, green). (d–g) NET-positive fibers (red) were also found in proximity to PCNA-positive (d and e, green) and PSA-NCAM-positive (f and g, green) cells in the sgz. Corresponding orthogonal reconstructions of confocal images are shown in x–y, x–z, and y–z planes in (e) and (g). Scale bars = 5  $\mu$ m (a, e and g), 50  $\mu$ m (b and c), 10  $\mu$ m (d and f).

rather than as a result of a damaged DNA repair process (Cameron and McKay, 2001). The morphology and distribution of nuclei of BrdU cells were similar in the dexefaroxan- and vehicle-treated control animals (not shown).

The numbers of BrdU-labeled nuclei in both the gcl and the hilus, determined by stereological cell counts, were similar after acute injection (24 h) or chronic treatment (21 days) in both dexefaroxan treated animals and vehicle controls (Figure 3c and d). These results show that neither acute nor long-term dexefaroxan treatment up to 21 days affected the rate of cell division over a 24 h period in the DG. Dexefaroxan treatment does not therefore affect the rate of cell proliferation (number of cells entering the S phase in 24 h) in the DG.

#### Does Dexefaroxan Treatment Affect the Number of Newborn Cells in the DG?

To determine whether dexefaroxan treatment increases the number of newborn cells in the DG, rats treated with dexefaroxan or vehicle via osmotic minipumps were injected daily for 7 days with BrdU and were killed 21 or 70 days after the beginning of treatment. The 70-day time point was chosen to study long-term survival because it has been shown previously that the number of BrdU-labeled cells in the gcl remains stable from 1 to 6 months after BrdU administration (Dayer *et al*, 2003).

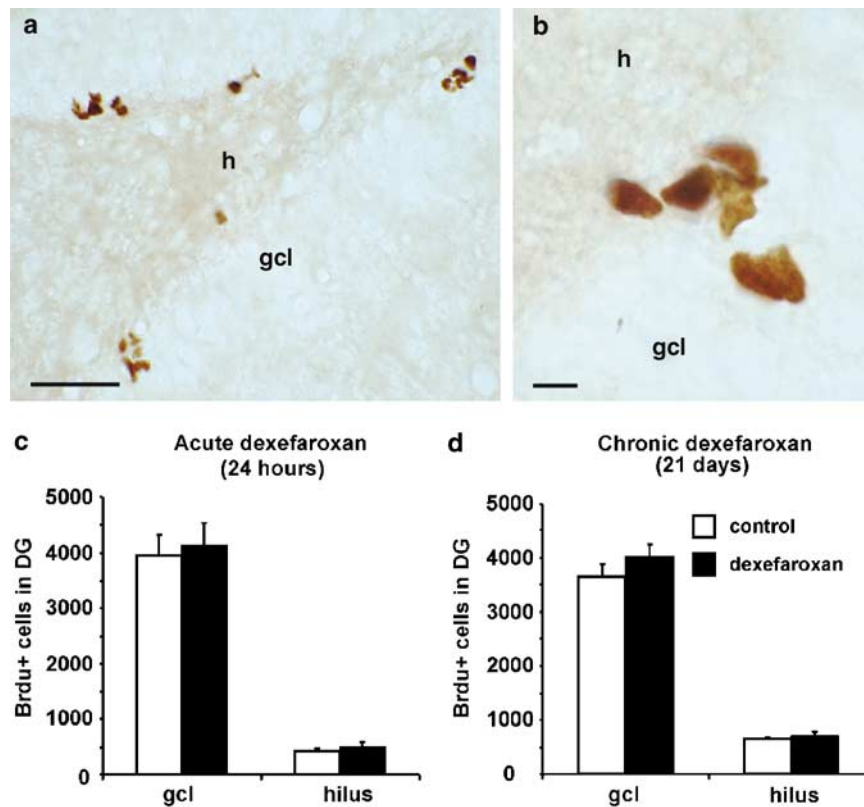
All BrdU-positive cells observed at days 21 or 70 were large and round with punctate or uniformly stained nuclei and were principally confined to the gcl in both vehicle and

dexefaroxan treated animals (Figure 4a and b). Stereological counts in the gcl showed that the number of BrdU-positive cells in the gcl was 43% higher ( $p < 0.05$ ) in rats treated for 21 days with dexefaroxan than in vehicle-treated controls (Figure 4c). When the duration of treatment was extended to 70 days (Figure 4d), BrdU-positive cells in the dexefaroxan group were again significantly more numerous than in the vehicle group (57%,  $p < 0.05$ ). These results show that although there was an increase in the number of new-born cells in the gcl in dexefaroxan-treated animals, there was also considerable attrition over time in both treated and untreated animals (average reductions of 493 and 482 cells, respectively). Although the decrease in the number of BrdU-positive cells over time may be due to dilution of BrdU through successive cell divisions, it is most probably the result of an ongoing cell death process (Dayer *et al*, 2003).

The difference in the absolute number of BrdU-positive cells in the dexefaroxan treatment group, with respect to vehicle, was the same at days 21 and 70 of treatment (average of +840 and +829 cells, respectively). Given the similar rate of BrdU-positive cell attrition in both groups between 21 and 70 days of treatment (average loss of 493 and 482 cells; see above), these results suggest that dexefaroxan critically influenced the generation or survival of cells newly formed during the first 21 days of treatment, but did not affect the process of cell elimination that occurred later.

To examine this hypothesis, we performed a stereological count of cells labeled with BrdU when chronic dexefaroxan treatment was delayed until 21 days after BrdU adminis-





**Figure 3** Effect of acute and chronic dexefaroxan treatments on proliferation of dentate gyrus progenitors. Rats received either a single i.p. injection of BrdU (50 mg/kg) and four i.p. injections of vehicle (3 ml/kg) or dexefaroxan (0.63 mg/kg), or were infused via osmotic minipumps for 21 days with vehicle or dexefaroxan (0.63 mg/rat/day) and received a single i.p. injection of BrdU (50 mg/kg) on day 20. Rats were killed 24 h after BrdU injection. (a) A representative photomicrograph of proliferating BrdU-positive cells in the DG, 24 h after BrdU injection. (b) A progenitor cell cluster at higher magnification. (c and d) The number of BrdU-positive cells was the same in dexefaroxan-treated and control rats in both the acute (c) and chronic (d) treatment paradigms. Values are means  $\pm$  SEM ( $n = 5$  rats per group). Scale bars = 50  $\mu$ m (a) and 5  $\mu$ m (b).

tration and continued to day 70. When dexefaroxan treatment was delayed to day 21, the difference between treated and untreated animals at day 70 was no longer observed (Figure 4e), suggesting that dexefaroxan affected cell survival almost exclusively during the first 21 days.

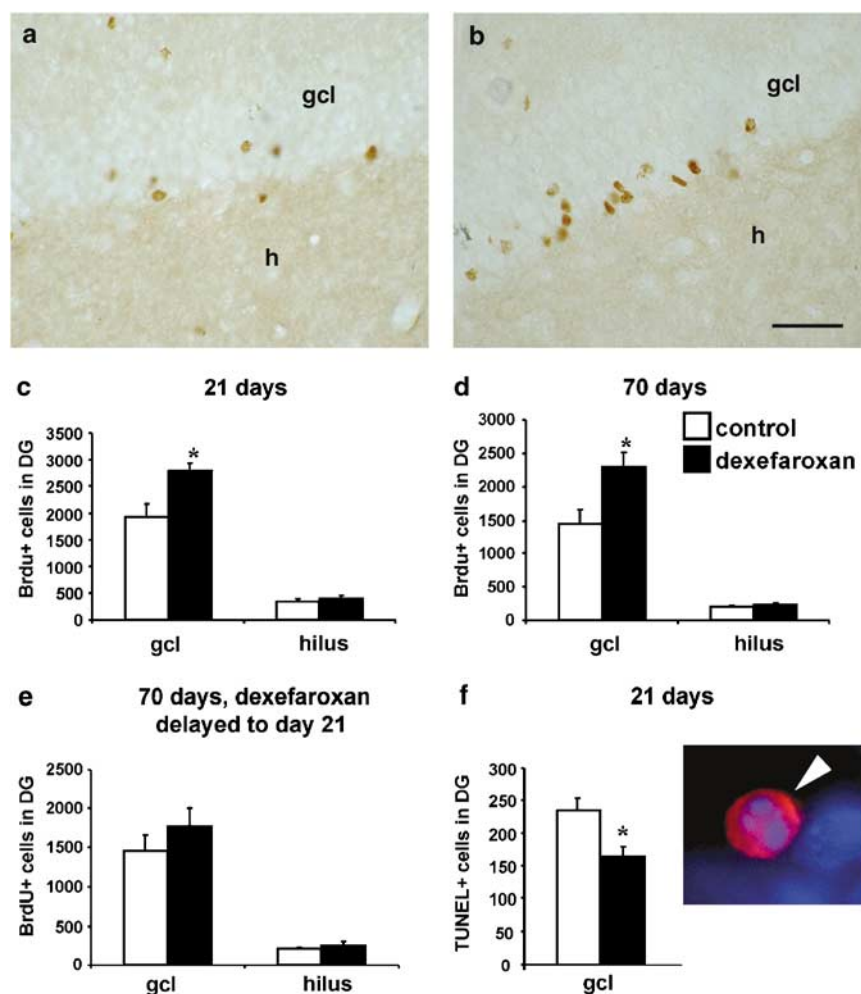
To further examine this hypothesis, we used terminal transfer labeling (TUNEL) to label cells with fragmented chromatin after 21 days of treatment with dexefaroxan or vehicle (Figure 4f). Stereological counts show that dexefaroxan treatment caused a significant decrease ( $-29\%$ ;  $p < 0.05$ ) in the number of TUNEL-labeled cells at day 21 (Figure 4f), confirming that the  $\alpha$ 2-adrenoceptor antagonist promoted cell survival rather than proliferation.

#### Effect of Dexefaroxan Treatments on the Differentiation of Newborn Granule Cells

Finally, we determined whether the BrdU-labeled cells in the DG were neurons or astrocytes, and the effect of dexefaroxan on these two types of neural cells. Double immunolabeling of the BrdU cells was performed with antibodies against the neuronal marker NeuN and the astrocytic marker GFAP (Figure 5), after both 21 and 70 days of chronic treatment with dexefaroxan. Both NeuN/BrdU (Figure 5a and b) and GFAP/BrdU (Figure 5c and d) colabeled cells were detected. The NeuN/BrdU-positive

cells largely out-numbered GFAP/BrdU-positive cells at both time points. After 21 days of treatment,  $81.3 \pm 4.5\%$  of BrdU-positive cells in vehicle-treated animals and  $84.9 \pm 5.0\%$  in dexefaroxan-treated animals were NeuN-positive (Figure 5e). GFAP/BrdU-positive astrocytes represented only  $14.1 \pm 1.7$  and  $11.5 \pm 1.9\%$  in vehicle- and dexefaroxan-treated groups, respectively (Figure 5f). Similar percentages of BrdU-positive neurons and astrocytes were also found after the 70-day treatment protocol. The number of BrdU/NeuN-labeled cells at both time points was significantly higher in dexefaroxan-treated animals than in vehicle-treated controls. There was a significantly greater number of BrdU/NeuN-colabeled cells in dexefaroxan-treated animals than in vehicle-treated controls at day 21 ( $2359 \pm 92$  and  $1570 \pm 117$ , respectively;  $p < 0.05$ , Figure 5e) and day 70 ( $2080 \pm 127$  and  $1252 \pm 99$ ;  $p < 0.05$ ). The effect was the same at both time points ( $+789$  and  $+828$  cells). Similarly, at day 70 there was a significant difference between the number of GFAP/BrdU-labeled cells in vehicle- and dexefaroxan-treated animals (Figure 5f). The large majority of BrdU-positive cells quantified was therefore neurons.

The above results show that dexefaroxan treatment increases the number of newborn granule cells in the DG. To determine whether it also affects their state of differentiation, we evaluated the number of cells expressing



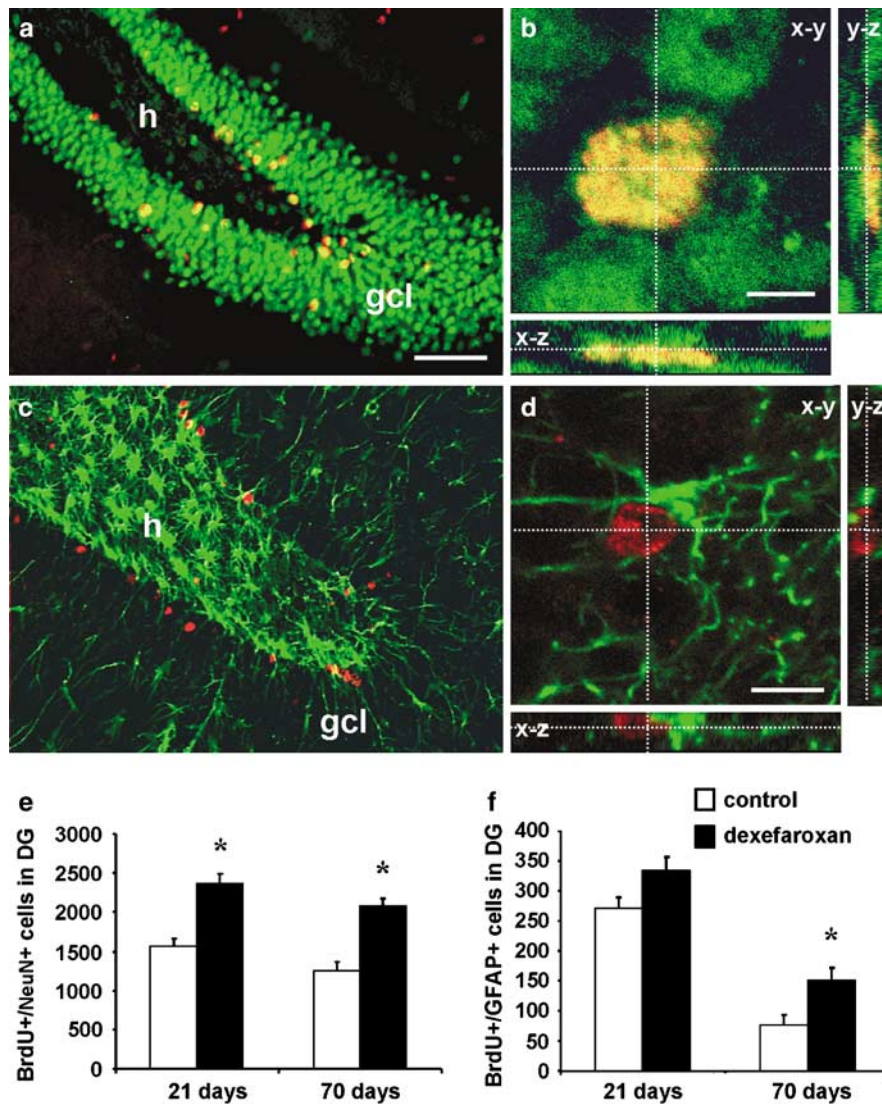
**Figure 4** Effect of chronic dexefaroxan treatment on the survival of newly born granule cells. Cells were examined after 7 consecutive daily injections of BrdU in rats infused with dexefaroxan (0.63 mg/rat/day) or vehicle (distilled water) from day 0 to 21 (a–c and f), 0 to 70 (d), and 21 to 70 (e). (a and b) BrdU-positive cells (DAB staining) in the gcl of rats receiving vehicle (a) and dexefaroxan (b) for 21 days. (c and d) The number of BrdU-positive cells was significantly increased in the gcl but not the hilus after 21 (c) and 70 (d) days of dexefaroxan treatment. (e) The effect was not statistically significant if treatment was delayed by 21 days. (f) The number of TUNEL-positive cells was lower after a 21-day treatment with dexefaroxan than with vehicle; note condensed chromatin in TUNEL-positive cells (photo inset, arrowhead). Values are the mean  $\pm$  SEM ( $n = 5$ ). \*Significantly different from vehicle controls ( $p < 0.05$ ). Scale bar = 50  $\mu$ m (a and b).

PSA-NCAM, a marker of activity-induced synaptic plasticity (Muller *et al*, 1996) after 21 days of vehicle or dexefaroxan treatment (Figure 6). PSA-NCAM-positive cells were located in the deepest region of the gcl at the interface of the hilus (Figure 6a–d). These cells had the morphological characteristics of granule neurons, with apical dendritic trees expanding into the granular layer (Figure 6c and d). However, the dendritic arborization was more complex in dexefaroxan-treated animals (Figure 6d) than in vehicle-treated controls (Figure 6c). This was also seen when doublecortin, a marker for dendritic growth of newly generated neurons (Rao and Shetty, 2004), was immunolabeled in vehicle- (Figure 6e) and dexefaroxan-treated (Figure 6f) animals.

Stereological cell counts showed that dexefaroxan increased the number of PSA-NCAM-positive cells by a mean of 50% in the sgz of the DG compared to vehicle treatment ( $56378 \pm 3723$  vs  $37478 \pm 4123$  cells, respectively;  $p < 0.05$ ) (Figure 6g).

### Dexefaroxan Treatment Increases BDNF Expression in the DG

BDNF is a known proliferation, differentiation, and survival factor for progenitor cells (Katoh-Semba *et al*, 2002; Lee *et al*, 2002; Shetty and Turner, 1998) that has been detected in the noradrenergic neurons of the locus coeruleus (Fawcett *et al*, 1998). We therefore investigated whether the dexefaroxan-induced increase in the survival and differentiation of newborn granule cells was related to a change in BDNF expression in the dorsal hippocampus. In control rats, BDNF immunoreactivity was pronounced in the hilus and CA3 region (Figure 7a). After 21 days of dexefaroxan treatment, BDNF immunoreactivity was markedly enhanced (Figure 7b). The enhancement was still observed after 70 days of treatment (not shown). The increase could already be observed 24 h after i.p. injection of 0.63 mg/kg (not shown), indicating that there was a temporal correlation between the treatment and the levels of BDNF in the DG.



**Figure 5** Dexefaroxan increases the number of surviving new neurons without affecting the neuron/glia ratio. (a–d) BrdU/NeuN-positive neurons (a and b) and BrdU/GFAP-positive glial cells (c and d) in coronal sections of the DG after 21 days of treatment with dexefaroxan. Corresponding orthogonal reconstructions of confocal images in x–y, x–z, and y–z planes are shown in (b) and (d). (e and f) There were significantly more NeuN/BrdU-positive cells in dexefaroxan-treated animals after both 21 and 70 days of treatment (e) and GFAP/BrdU-positive cells at day 70 (f). Values are means  $\pm$  SEM ( $n = 5$  rats per group). \*Significantly different from vehicle controls ( $p < 0.05$ ). Scale bars = 50  $\mu$ m (a and c), 5  $\mu$ m (b), and 10  $\mu$ m (d).

The increase in BDNF immunoreactivity was particularly evident in the gcl, the hilus, and the stratum radiatum of CA3, but not in the CA1 region (Figure 7b). Quantitative analysis of BDNF immunostaining after 21 days of treatment is shown in Figure 7e. A significant increase in BDNF immunostaining was observed in the hilus (276%), in the gcl (181%), in the cell layer of the CA3 (156%), and in the stratum radiatum of CA3 (143%) of dexefaroxan-treated animals compared to controls (Figure 7e). No differences in BDNF staining after treatment were observed in other regions of the brain such as the CA1 of the hippocampus, frontal cortex, or temporal cortex (data not shown), although this could be due to levels of BDNF in these regions undetectable by our method. Several cells in the gcl were intensely stained in their cytoplasm, while those in the sgz were only weakly stained (Figure 7d). Furthermore, enhanced BDNF immunostaining was observed in the mossy fibers in the hilar region and the CA3 (Figure 7b, d).

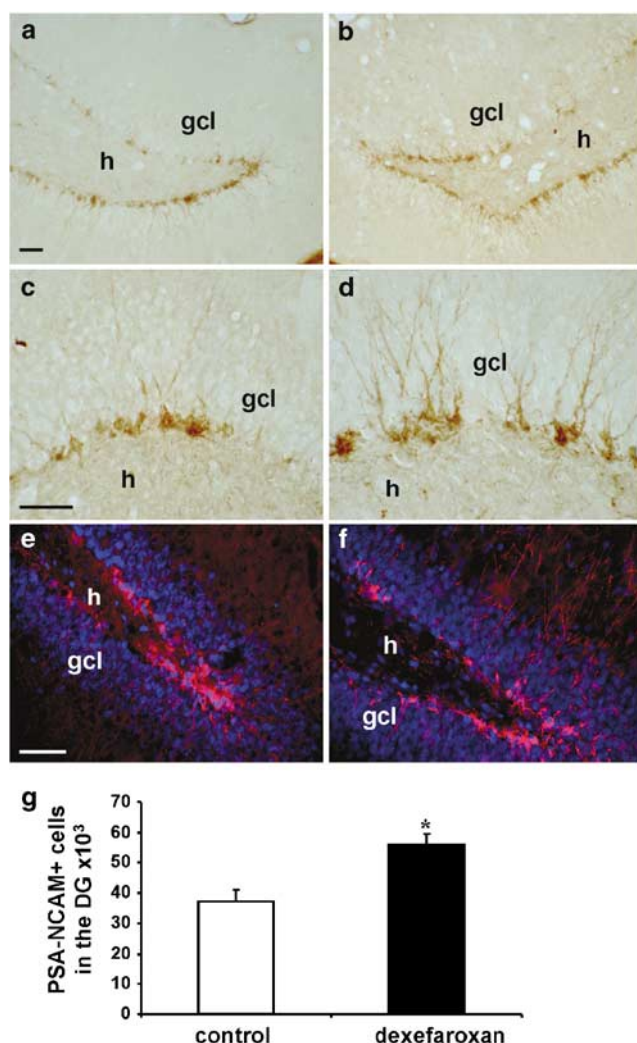
## DISCUSSION

In the present study, neurogenesis in the adult rat dorsal hippocampus *in vivo* was enhanced by chronic systemic treatment with the selective  $\alpha$ 2-adrenoceptor antagonist dexefaroxan. The treatment increased both the long-term survival of new granule cells in the DG and the extent and complexity of their neuritic arborization. Dexefaroxan treatment also increased the expression of BDNF in the hippocampal neuropil and in granule cell neurons, suggesting that it could play a role in the effects of the  $\alpha$ 2-adrenoceptor antagonist on neurogenesis in this brain structure.

### Dexefaroxan Acts at a Critical Period for Cell Survival

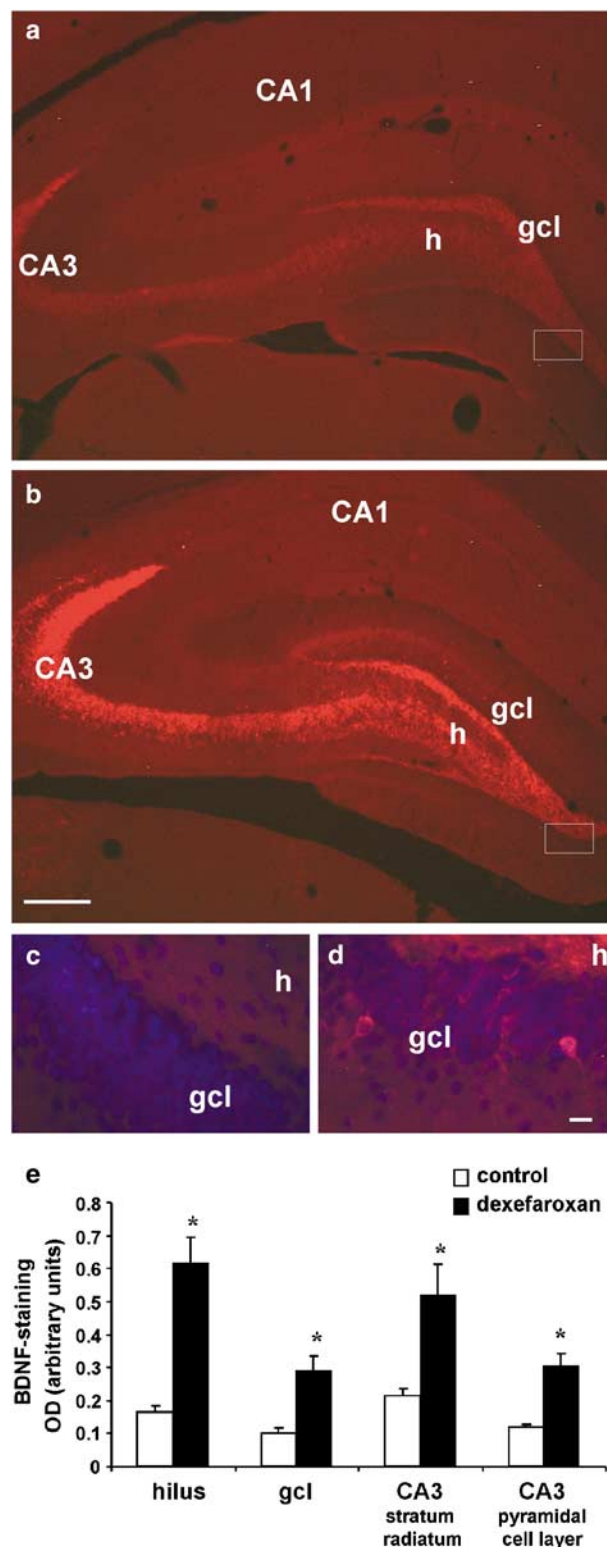
Neurogenesis is the net outcome of cell proliferation and cell death. Chronic dexefaroxan treatment was found to





**Figure 6** Dexefaroxan treatment enhances neural plasticity in the DG. (a–d) Representative light microscopic images of PSA-NCAM-immunoreactive cells in the DG of control (a and c) and dexefaroxan-treated (b and d) rats after 21 days of treatment. Note the higher level of PSA-NCAM expression (b) and the more complex dendritic arborization (d) in dexefaroxan-treated animals. (e and f) Doublecortin immunoreactivity (red) also increased in dexefaroxan-treated rats (f) compared to controls (e) (nuclei stained with Hoechst 33342, blue). (g) The number of PSA-NCAM-positive cells in dexefaroxan-treated rats also increased compared to vehicle controls. \*Significantly greater than controls ( $p < 0.05$ ). Scale bars = 15  $\mu$ m (a and b), 30  $\mu$ m (c and d), and 50  $\mu$ m (e and f).

increase net neurogenesis by promoting long-term cell survival, rather than modifying cell proliferation. Under our experimental conditions, many of the newborn cells in the DG were eliminated within the first 21 days after their generation. This was consistent with a previous study showing a 50% loss of BrdU positive cells between the first and fourth week after BrdU-labeling, and that the new



**Figure 7** Dexefaroxan enhances BDNF expression in the DG. (a and b) BDNF immunoreactivity (red) is greater in the hilus and CA3 region of dexefaroxan-treated rats (b) than in controls (a) after 21 days of treatment. (c and d) Higher magnification shows a homogeneous pattern of immunoreactivity in the control DG (c) and intensely stained granule cells in dexefaroxan-treated animals (d). Nuclei stained with Hoechst 33342 (blue) in (c) and (d). (e) Quantitative analysis of BDNF immunostaining after 21 days of treatment shows an average three-fold increase in BDNF levels in the gcl and CA3 area of dexefaroxan-treated rats compared to vehicle controls. The greatest increase was observed in the hilus. \*Significantly greater than controls ( $p < 0.05$ ). Scale bars = 100  $\mu$ m (a and b) and 10  $\mu$ m (c and d).

granule cells that survived persisted for several more months (Dayer *et al*, 2003). The first 21 days following the birth of the new cells were critical for the observation of a dexefaroxan-induced increase in cell survival. Indeed, when dexefaroxan treatment was delayed for 21 days after BrdU administration, we observed no significant effect on cell survival, reinforcing the notion of a critical window for drug action. What happens in the 7–14 day period following the birth of a new cell must now be determined more precisely. The TUNEL findings indicate that dexefaroxan was protective against apoptotic cell death and could affect new cells during this early phase of cell sensitivity.

### Dexefaroxan may also Enhance Neuronal Plasticity

In addition to increasing the number of new neurons, dexefaroxan apparently enhanced their differentiation, increasing quite spectacularly the extent and the complexity of their dendritic arborization visualized with an antibody against PSA-NCAM, a putative marker of activity-induced plasticity in the hippocampus (Cremer *et al*, 2000; Muller *et al*, 1996). PSA-NCAM is associated with granule neurons and mossy fiber boutons in the adult hippocampus, and is thought to participate in the formation and remodeling of synapses between granule neurons and CA3 pyramidal neurons (Seki and Arai, 1999). The expression of doublecortin, a marker of dendritic growth in newly generated neurons (Rao and Shetty, 2004), also increased in the hippocampus in a pattern similar to that of PSA-NCAM. In both cases, the dendritic arborizations of the granule cell neurons appeared to be more elaborate. Although this may be due to a better visualization of the dendritic arbors in cells expressing higher levels of the markers, these results suggest that dexefaroxan may have an effect on dendritic morphology. It may be hypothesized that the effect on morphology contributes to the reported long-term enhancement of sensory-evoked potentials in the DG (Harley, 1991).

The effect of dexefaroxan on the survival of newly formed granule cells and on PSA-NCAM expression may be indicative of the functional integration of these cells into hippocampal neuronal circuits. The time period during which dexefaroxan affected the survival of newborn granule cells coincided with the period in which these cells can extend axons into the CA3 region (Hastings and Gould, 1999). In a neurotrophin-dependent model of hippocampal progenitor differentiation, granule cell survival appears to be dependent on their integration and subsequent synaptic activity (Castren, 2004).

In apparent contradiction with our study, it was previously reported that noradrenaline depletion reduced hippocampal neurogenesis by decreasing proliferation, without affecting cell survival or differentiation (Kulkarni *et al*, 2002). These findings together with our own indicate that hippocampal neurogenesis is sensitive to the state of activation of the locus coeruleus–noradrenergic system, and suggest that the degree of noradrenergic tone might somehow determine the relative participation of receptor subtypes and their different signaling pathways at different stages of the neurogenetic process. We cannot rule out a direct effect of the *alpha2*-adrenoceptor antagonist dexefaroxan on neurogenesis and the use of other specific adrenoceptor ligands to further identify that the receptor

subtype candidates for receptor mediation of these noradrenergic effects would be useful to better understand the mechanisms involved. However, the results obtained with dexefaroxan in the olfactory bulb (Bauer *et al*, 2003) are similar. Reminiscent of what we find in the hippocampus, the survival-promoting effect of dexefaroxan was concluded by Bauer *et al* (2003) to result from a reduction in apoptotic cell death and not from a modification of progenitor proliferation. The remarkable similarities between the effects of dexefaroxan in these two *in vivo* studies suggest that neurogenesis in these otherwise disparate parts of the brain is regulated by common noradrenergic mechanisms.

### What Mechanism Underlies the Effects of Dexefaroxan on the Survival and Differentiation of New Neurons in the Hippocampus?

The hippocampus is extensively innervated by noradrenergic fibers projecting exclusively from the locus coeruleus (Loy *et al*, 1980), and all major classes of adrenoceptors are found in this structure (Nicholas *et al*, 1996). In the present study, NET-positive fibers were abundant at the interface between the hilus and gcl, and in contact with both proliferating cells (PCNA positive) and immature differentiating neurons (PSA-NCAM positive).

Presynaptic *alpha2*-adrenoceptors are most likely responsible for dexefaroxan-induced noradrenaline release in the hippocampus (Arima *et al*, 1998; Starke, 2001; van Veldhuizen *et al*, 1994). Postsynaptic *alpha2*-adrenoceptors have been reported to enhance apoptosis in a cell culture system when activated and to delay apoptosis when inhibited (Wang and Limbird, 1997), and may therefore be involved in the effects of dexefaroxan on neuronal survival. Other adrenoceptors may also play a role, however. Postsynaptic *alpha1*-adrenoceptors were also reported to have antiapoptotic activity in some cellular systems (Popovik and Haynes, 2000). The role of postsynaptic *beta*-adrenoceptors has not been evaluated. The individual contributions and possible interactions of all these receptors on neurogenesis remain subject to further investigation. A receptor-independent effect of noradrenaline that can act as an antioxidant might also promote neuronal survival (Traver *et al*, 2005; Troadec *et al*, 2001).

Our study also points to a role of BDNF in the noradrenergic regulation of neurogenesis. BDNF is present in locus coeruleus–noradrenergic projections (Aloyz *et al*, 1999) and undergoes anterograde transport to terminal fibers. In our study, BDNF expression was massively upregulated from the beginning of dexefaroxan treatment, in the afferent noradrenergic fibers from the locus coeruleus, in all neurons in the gcl, and in their projections, the mossy fibers, to CA3. *In vitro*, BDNF has been shown to enhance neurogenesis by increasing both the proliferation of neural stem cells and their survival (Katoh-Semba *et al*, 2002; Lee *et al*, 2002; Shetty and Turner, 1998). Its role in dexefaroxan-induced survival and differentiation of new granule cells in the adult hippocampus thus warrants further study, notably with respect to the relationship between BDNF expression and neuronal activity. A strong upregulation of BDNF expression in the hippocampus, comparable to what we have observed following dexefaroxan treatment, has been associated with seizures (Binder

*et al*, 2001), a situation in which neurogenesis is induced (Parent *et al*, 1997). Neurogenesis in response to increased neuronal activity has been reported in a number of models (Bai *et al*, 2003; Derrick *et al*, 2000), and BDNF is reported to increase neurotransmission in the hippocampus (Lu, 2004). Most interestingly, unilateral BDNF infusion in the DG was reported to induce neurogenesis in the contralateral hemisphere, a result which may be explained by BDNF-dependent enhancement of transmission by hippocampal neurons such as the pyramidal cells with commissural projections (Scharfman *et al*, 2005). Alternatively, dexefaroxan treatment could increase BDNF levels by increasing neuronal activity. Alpha2-autoreceptor blockade, which leads to increased activation of the noradrenergic neurons in the locus coeruleus, may result in an increase in the activity-dependent synthesis and anterograde transport of BDNF from the locus coeruleus to forebrain target regions (Fawcett *et al*, 1998).

### The Noradrenergic System as a Target for Therapeutic Manipulation of Adult Neurogenesis in the Hippocampus

In animal studies, hippocampal neurogenesis has been postulated to play a role in learning and memory (Gould *et al*, 1999b). Impaired performance on hippocampus-dependent learning tasks was observed when neurogenesis was reduced in the hippocampus and was restored when neurogenesis resumed (Shors *et al*, 2001, 2002). Conversely, performance of such learning tasks increased neurogenesis and enhanced the survival of new granule cells in the hippocampus (Gould *et al*, 1999a). This occurred on a time scale suggesting that newly formed cells become functional around 1–2 weeks after their generation, as they integrate into the gcl and make synaptic contact with targets in the CA3 area (Hastings and Gould, 1999). These new cells appear to facilitate synaptic plasticity, since the induction of associative long-term potentiation in these cells is more rapid than in mature neurons (Schmidt-Hieber *et al*, 2004). Enhancement of the survival and plasticity of newborn neurons by pharmacological activation of the locus coeruleus–noradrenergic system may therefore provide a way to facilitate certain types of hippocampal learning and memory. Dexefaroxan has indeed been shown to improve the performance of rodents in tests of both short- and long-term memory, and to ameliorate the performance of old rats on the Morris water maze and passive avoidance tasks to the level of young adult animals (Chopin *et al*, 2002). Although the increased survival of newborn neurons may result in the functional improvement reported, acute effects of BDNF on synaptic activity in the hippocampus cannot be excluded.

Can adult neurogenesis be used for treating neurodegenerative diseases? Hippocampal neurogenesis was shown recently to be increased in Alzheimer's disease, possibly as a compensatory attempt to replace lost neurons (Jin *et al*, 2004). Although neurogenesis is reduced with normal aging, the aged brain may retain a capacity to upregulate neurogenesis in response to some pathological or pharmacological challenges (Cameron and McKay, 1999; Seki and Arai, 1995). Enhancing new neuron survival, facilitating structural plasticity, and stimulating the expression of neurotrophins in the hippocampus may constitute poten-

tially effective compensatory strategies in neurodegenerative conditions like Alzheimer's disease, in which hippocampal function is impaired (Tatebayashi *et al*, 2003). Dexefaroxan had such effects in our study, and may therefore be of therapeutic interest for the treatment of these disorders.

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