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Exposure to Fox Odor Inhibits Cell Proliferation in the Hippocampus of Adult Rats via an Adrenal Hormone-Dependent Mechanism

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ABSTRACT

To determine whether exposure to fox odor alters granule neuron production, we examined proliferating cells and their progeny in the dentate gyrus of adult male rats exposed to trimethyl thiazoline, a component of fox feces. Additionally, to determine whether this effect is adrenal hormone-mediated, we examined animals exposed to fox odor after bilateral adrenalectomy and replacement with low levels of the endogenous glucocorticoid corticosterone. Stereologic analyses of the number of 5-bromo-2′deoxyuridine (BrdU) -labeled cells revealed that exposure to fox odor but not other, nonthreatening, odors (mint or orange) rapidly decreased the number of proliferating cells in the dentate gyrus. This effect is dependent on a stress-induced rise in adrenal hormones; exposure to fox odor resulted in an increase in circulating corticosterone levels and prevention of this increase (by means of adrenalectomy plus low-dose corticosterone replacement) eliminated the suppression of cell proliferation. Examination at longer survival times revealed that the decrease in the number of new granule cells in fox odor-exposed animals was transient; a difference was still detectable at 1 week after BrdU labeling but not at 3 weeks. In both fox and sham odor-exposed animals, many new cells acquired morphologic and biochemical characteristics of mature granule neurons. The majority of these cells expressed a marker of immature granule neurons (TuJ1) by 1 week after BrdU labeling and markers of mature granule neurons (calbindin, NeuN) by 3 weeks after labeling. These findings suggest that stressful experiences rapidly diminish cell proliferation by increasing adrenal hormone levels, resulting in a transient decrease in the number of adult-generated immature granule neurons. J. Comp. Neurol. 437:496–504, 2001.

Indexing terms: neurogenesis; dentate gyrus; granule neuron; stress; glucocorticoid

Granule cells in the dentate gyrus are produced throughout life in many mammalian species, including humans (Altman and Das, 1965, 1967; Gould et al., 1997, 1998, 1999a; Kempermann et al., 1997; Eriksson et al., 1998; Hastings et al., 2000). In adulthood, precursor cells located on the border of the granule cell layer (gcl) and hilus, in a region called the subgranular zone (sgz), divide and produce daughter cells that become incorporated into the gcl, extend axons into the CA3 region (Stanfield and Trice, 1988; Markakis and Gage, 1999; Hastings and Gould, 1999), receive synaptic input (Kaplan and Bell, 1983), and exhibit morphologic and biochemical characteristics of mature granule neurons (Cameron et al., 1993a; Gould et al., 1999b; Tanapat et al., 1999). Stereologic analyses have demonstrated that several thousand new hippocampal cells are produced each day in adult rodents (Gould et al., 1999b; Tanapat et al., 1999; Cam-

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eron and McKay 2001). Although the function of adult-generated hippocampal neurons is not known, the substantial number of new cells produced suggests that these cells are biologically significant.

Previous studies have demonstrated that stress inhibits cell proliferation in adult tree shrews and marmosets. In adult tree shrews, a rapid decrease in the number of proliferating cells is observed in the dentate gyrus after a single exposure to subordination stress, a condition sufficient to elevate circulating levels of the glucocorticoid cortisol (Gould et al., 1997). Likewise, a similar decrease in cell proliferation in the dentate gyrus is observed in adult marmosets that are exposed to social stress in a resident-intruder paradigm. The factors that underlie these stress-induced changes in cell proliferation are presently unknown. However, it has been shown that the production of granule neurons in the dentate gyrus of adult rats is regulated by adrenal steroids (Gould et al., 1992; Cameron and Gould, 1994), suggesting that stress decreases cell proliferation via an adrenal steroid-dependent mechanism.

Exposure to predator odor is a natural stressor for rodents that is known to activate the hypothalamic-pituitary-adrenal axis (Perrot-Sinal et al., 1999), resulting in elevated serum glucocorticoid levels (Vernet-Maury et al., 1984) and a characteristic electrophysiological response in the dentate gyrus (Heale et al., 1994). These observations raise the possibility that exposure to fox odor naturally modulates granule cell production during adulthood. To determine whether exposure to fox odor alters granule cell production, we examined the numbers of proliferating cells and their progeny in the dentate gyrus of adult male rats exposed to trimethyl thiazoline, a component of fox feces (Vernet-Maury et al., 1984) as well as two other, nonthreatening, odors (mint and orange). To further determine whether the effect of fox odor on cell production is mediated by adrenal hormones, we examined the number of proliferating cells in the dentate gyrus of animals exposed to fox odor after bilateral adrenalectomy and replacement with low levels of the endogenous glucocorticoid corticosterone.

MATERIALS AND METHODS

Animal treatments

Adult male Sprague-Dawley rats (220–260 g) from Charles River and the breeding colony at Princeton University were used in all experiments. Animals were group-housed and maintained on a 12:12h light/dark cycle (lights on 7:00 AM) and provided with *ad libitum* access to food and water. For perfusion, all of the animals were deeply anesthetized with an overdose of sodium pentobarbital (Nembutal; 100 mg/kg body weight [bwt]) and transcardially perfused with 4.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). For corticosterone assay, rats were rapidly decapitated and trunk blood was collected. Plasma for hormone assay was obtained after low-speed centrifugation of whole blood samples. All animal experimentation was conducted in accordance with University guidelines and with The National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experiment 1. To determine whether exposure to fox odor alters the proliferation of granule cell precursors and, ultimately, the production of new granule neurons in the adult dentate gyrus, rats were placed into clean cages in one of two separate experimental rooms and allowed to acclimate to the novel environment for at least 1 hour. A vial containing gauze and either 150 μl of trimethyl thiazoline (Pherotech, Vancouver, Canada), a component of fox feces (Vernet-Maury et al., 1984), or saline was then placed in the middle of the cage. One hour later, rats from each group were injected with the thymidine analog 5-bromo-2′deoxyuridine (BrdU; i.p.; 200 mg/kg bwt in saline with 0.007 N NaOH; Sigma, St. Louis, MO), a marker of proliferating cells and their progeny (Nowakowski et al., 1989), and then placed back into the experimental cages. Two hours after BrdU injection, groups of these animals were perfused immediately (sham, n = 5; fox odor, n = 5) or moved into clean cages and perfused either 1 (sham, n = 5; fox odor, n = 5) or 3 weeks (sham, n = 10; fox odor, n = 11) later. The 2-hour survival was selected, because it is sufficient for the incorporation of BrdU by cells in S phase but not for the completion of mitosis or migration to occur (Nowakowski et al., 1989). The 1-week survival was chosen because the number of BrdU-labeled cells appears to be maximum at this time (Gould et al., 1999b). The 3-week survival was selected because the majority of adult-generated cells exhibit neuronal characteristics by this time point (Cameron et al., 1993b).

To investigate the possibility that exposure to other, nonthreatening, odors may also alter the proliferation of granule cell precursors, additional animals were exposed to either saline (n = 4), fox odor (n = 5), mint odor (McCormick, Hunt Valley, MD; n = 4), or orange odor (McCormick; n = 5) as described above. One hour later, these animals were injected with BrdU, placed back into the experimental cages, and perfused 2 hours later.

After perfusion, all of the brains were removed and processed for BrdU immunohistochemistry. Additional sections from the 1- and 3-week groups were processed for combined BrdU labeling and immunohistochemistry for neuronal nuclei (NeuN) or the calcium binding protein calbindin, both markers of mature granule neurons (Kempermann et al., 1997; Tanapat et al., 1999), or for glial fibrillary acidic protein (GFAP), a marker of astroglia (Cameron et al., 1993b). Sections from the 1-week group were also processed for combined BrdU labeling and immunohistochemistry for a neuron-specific class III beta-tubulin (TuJ1), a marker of immature and mature granule neurons. The number of single- or double-labeled cells in the dentate gyrus was then compared across groups.

To determine whether exposure to fox odor nonspecifically alters the uptake of proliferation markers (e.g., by altering the rate of blood flow), the number of BrdU-labeled cells in the subventricular zone (svz) of sham and fox odor-exposed animals in the 2-hour group was also compared. In addition, the degree of labeling of individual cells was compared between additional groups of sham and fox odor-exposed animals that were injected with [^{3}H]-thymidine. Specifically, additional animals were exposed to sham (n = 5) or fox odor (n = 5) for 1 hour, injected with [^{3}H]-thymidine (i.p.; 5.0 μCi/g bwt; New England Nuclear, Boston, MA), and perfused after a 2-hour survival. Their brains were then processed for [^{3}H]-thymidine autoradiography, and the number of silver grains over each labeled cell was counted. Because the number of silver grains over a labeled cell is dependent on the amount of [^{3}H]-thymidine that has been incorporated, [^{3}H]-thymidine autoradiography, unlike BrdU immuno-

histochemistry, allows for quantitative comparisons of marker uptake between stressed and unstressed animals.

To establish whether exposure to fox odor increases circulating corticosterone levels, trunk blood was collected from unstressed control rats and from rats exposed to trimethyl thiazoline for 30 minutes. Circulating corticosterone levels were then determined by radioimmunoassay by using a Coat-a-Count Rat Corticosterone Kit (detectability limit, 5.7 ng/ml; cross-reactivity, <3.0% for 11-deoxycorticosterone and <1.0% for all other steroid hormones; Diagnostics Product Corporation, Los Angeles, CA).

Experiment 2. To determine whether a stress-induced increase in circulating adrenal hormone levels is responsible for decreased cell proliferation after fox odor exposure, additional rats were either bilaterally adrenalectomized (ADX) (n = 11) or sham-operated (n = 6). Adrenalectomized animals were given corticosterone replacement in the drinking water (25 μg/ml in 0.9% saline; Sigma) to maintain low baseline levels of corticosterone, plasma electrolyte levels, and baseline dentate gyrus cell proliferation within normal physiologic parameters (Cameron et al., 1998). One week after surgery, animals from both sham-operated and ADX groups were exposed to either trimethyl thiazoline (sham ADX, n = 3; ADX, n = 6) or saline (sham ADX, n = 3; ADX, n = 5) and injected with BrdU 1 hour later. The 1-week time point after adrenalectomy was chosen because it is sufficient to allow the clearance of endogenous corticosterone. Two hours after BrdU injection, the rats were perfused and their brains were processed for BrdU immunohistochemistry. Additionally, trunk blood was collected from ADX and sham-operated animals at the time of perfusion and circulating corticosterone levels were determined by radioimmunoassay to verify efficacy of adrenalectomy.

Histological procedures

For each brain, 40-μm sections through the entire dentate gyrus were cut into a bath of 0.1 M phosphate buffered saline (PBS; pH 7.2) with an oscillating tissue slicer. Unless otherwise noted, all rinses were done in PBS for peroxidase immunolabeling and in 0.1 M Tris-buffered saline (pH 7.6) for fluorescence immunolabeling.

BrdU immunohistochemistry

For peroxidase immunolabeling, sections were mounted onto coated slides, incubated in 0.1 M citric acid for 5 minutes at 90°C, incubated in 0.6% H_2O_2 for 30 minutes, digested in trypsin (0.05% in Tris buffer containing 0.1% $CaCl_2$) for 10 minutes, denatured in 2 N HCl for 30 minutes, rinsed, blocked in 3.0% normal horse serum, and incubated overnight at 4°C in mouse monoclonal antibody raised against BrdU (1:250 + 0.5% Tween-20; Novocastra, Newcastle Upon Tyne, UK). The sections were then rinsed, incubated in biotinylated secondary mouse antisera (1:200; Vector, Burlingame, CA) for 60 minutes, rinsed, incubated in avidin-biotin-horseradish peroxidase (AB; 1:100; Vector) for 60 minutes, rinsed, and reacted for 10 minutes in 0.01% diaminobenzidine (DAB) with 0.003% H_2O_2. The sections were then counterstained with cresyl violet, dehydrated, and cover-slipped under Permount.

For fluorescence immunolabeling, sections were denatured in 2 N HCl for 20 minutes, rinsed, and incubated overnight at 4°C in rat monoclonal antibody raised against BrdU (1:250 + 0.5% Tween-20; Accurate, Westbury, NY). The sections were then rinsed, incubated in biotinylated secondary rat antisera (1:250; Chemicon, Temecula, CA) for 60 minutes, rinsed, incubated in streptavidin Alexa-568 (1:500; Molecular Probes, Eugene, OR), rinsed, and incubated in either (1) mouse anti-TuJ1 (1:500; a gift from Dr. Anthony Frankfurter), (2) mouse anti-NeuN (1:500, Chemicon), (3) rabbit anti-calbindin (1:500, Chemicon), or (4) goat anti-GFAP (1:5,000; Santa Cruz Biotechnologies, Santa Cruz, CA) for 2 days at 4°C. After several rinses, sections were incubated in the appropriate secondary antisera conjugated to Alexa-488 (1:500; Molecular Probes) for 30 minutes. The sections were rinsed, mounted, dried, counterstained with the DNA dye Hoechst 33342, and then cover/slipped under 25.0% glycerol in TBS.

[^{3}H]-thymidine autoradiography

Brain sections for [^{3}H]-thymidine autoradiography were mounted onto glass slides, dried, dipped in NTB-2 photographic emulsion (Eastman Kodak, Rochester, NY), and stored in the dark for 3 weeks. The slides were then developed in Dektol (Eastman Kodak), rinsed, fixed in Ektaflo (Eastman Kodak), and rinsed under running water for 20 minutes. The sections were then counterstained with cresyl violet, dehydrated, and cover-slipped under Permount.

Data analysis

BrdU labeling. The slides were coded before quantitative analysis, and the code was not broken until the analysis was complete. For peroxidase BrdU-immunolabeled tissue, BrdU-labeled cells on every twelfth section through the entire dentate gyrus were counted at 1,000×, excluding cells located in the outermost plane of focus. The number of cells counted in each brain was then multiplied by 12 to obtain a stereological estimate of the total number of BrdU-labeled cells in the entire dentate gyrus. For purposes of comparison, the number of BrdU-labeled cells in the caudal svz was determined on the same sections.

For sections processed for combined immunohistochemistry, a minimum of 40 cells per marker from anatomically matched sections was analyzed per brain. The percentage of BrdU-labeled cells in the gcl and sgz that were immunoreactive or nonimmunoreactive for TuJ1, NeuN, calbindin, or GFAP was determined by using a confocal laser-scanning microscope (Zeiss Axiovert 510 LSM; lasers: Argon 458/488, HeNe 543, UV 351/364). Z-sectioning at 1-μm intervals was performed for analysis and optical stacks of five to six images were obtained for figures.

[^{3}H]-thymidine autoradiography

Because [^{3}H]-thymidine can only be detected in the top 1–3 μm of a tissue section by using autoradiographic techniques, stereological analyses of the total number of labeled cells cannot be performed on thick tissue sections processed for [^{3}H]-thymidine autoradiography. Instead, the number of [^{3}H]-thymidine-labeled cells on neuroanatomically matched sections was counted throughout the entire dentate gyrus. A cell was considered to be [^{3}H]-thymidine labeled if it possessed at least five silver grains over its nucleus, a value 20× background. The cross-sectional area of the gcl was determined, and the data were expressed as numbers of labeled cells per μm^2. To determine whether the availability of [^{3}H]-thymidine differed between groups, the number of silver grains over

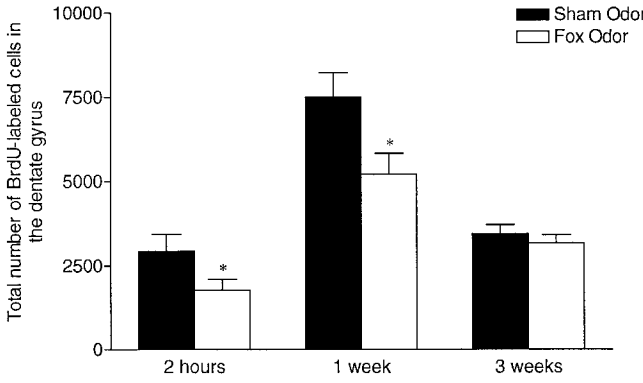

Fig. 1. Stereological estimates of the total numbers of 5-bromo-2′deoxyuridine (BrdU) -labeled cells in the dentate gyrus of adult male rats. Two hours after BrdU injection, rats that were exposed to fox odor exhibited fewer BrdU-labeled cells in the dentate gyrus compared with sham odor-exposed controls. Examination at longer survival times revealed that the decrease in the number of new granule cells in fox odor-exposed animals was transient; a decrease in the number of BrdU-labeled cells in the dentate gyrus of fox odor exposed animals was detectable at 1 week after BrdU labeling but not at 3 weeks after labeling. Bars represent mean + SEM obtained from 5–11 animals. Asterisks indicate significant difference from sham odor control value ($P < 0.05$).

labeled cells from these brains was counted. Bilateral gcl volume estimates were determined by applying Cavalieri's Principle to cross-sectional area measurements of the gcl obtained by means of Image Pro Software (Media Cybernetics, Silver Spring, MD).

Statistical Analysis

Total cell counts, densities, numbers of silver grains, and volumes were analyzed by using Student's *t* tests with two exceptions: (1) because the variance associated with each group was found to be proportional to its mean, the effect of fox odor versus other odors on the total number of BrdU-labeled cells (Experiment 1) was analyzed using the Kruskal-Wallis H test followed by pairwise comparison of means using the Mann-Whitney *U* test; and (2) the total number of BrdU-labeled cells in experiment 2 were analyzed by using a two-way ANOVA followed by Tukey-HSD post hoc comparison. For analysis of combined immunohistochemistry (Experiment 1), the numbers of double-labeled cells were expressed as percentages of the total number of BrdU-labeled cells and analyzed using Student's *t*-tests.

RESULTS

Exposure to fox odor decreases the number of proliferating cells in the dentate gyrus

A single exposure to fox odor resulted in a significant decrease in the number of BrdU-labeled cells in the dentate gyrus of adult male rats. Two hours after BrdU injection, rats exposed to fox odor exhibited fewer BrdU-labeled cells in the dentate gyrus compared with saline controls ($t_{[7]} = 17.560$; $P = 0.004$) (Fig. 1). In both groups, the majority of these cells were clustered in the sgz (Fig. 2A–C). A similar decrease in the density of labeled cells was observed in animals injected with [^{3}H]-thymidine after fox odor exposure ($t_{[8]} = 2.387$; $P = 0.038$). Exposure to fox odor did not affect the number of BrdU-labeled cells in the caudal svz or the number of silver grains over labeled cells in the dentate gyrus. Additionally, although a single exposure to fox odor decreased the number of BrdU-labeled cells in the dentate gyrus ($U_{(1,9)} = 1.50$; $P = 0.037$), the number of labeled cells observed in animals exposed to either mint ($U_{(1,9)} = 7.00$; $P = 0.462$) or orange ($U_{(1,9)} = 5.00$; $P = 0.221$) odor was not different from that observed in saline controls (Fig. 3). Moreover, no differences were detected between the number of labeled cells in animals that were exposed to either mint or orange odor ($U_{(1,10)} = 11.50$; $P = 0.835$).

The diminished number of BrdU-labeled cells in the fox odor-exposed animals remained detectable at the 1-week survival time ($t_{[8]} = 2.408$; $P = 0.0426$) (Fig. 1). At this time point, approximately 90% of BrdU-labeled cells in the gcl and sgz were immunoreactive for TuJ1 ($\bar{x}_{\text{sham odor}} = 89.2 \pm 0.99$, $\bar{x}_{\text{fox odor}} = 90.9 \pm 0.76$, $P > 0.05$, not significant [ns]) (Fig. 2H), whereas only a small percentage were immunoreactive for GFAP ($\bar{x}_{\text{sham odor}} = 9.0 \pm 1.00$, $\bar{x}_{\text{fox odor}} = 13.0 \pm 3.20$, $P > 0.05$, ns) (Fig. 2K). The majority of BrdU-labeled cells were located in the sgz or in the deep aspect of the gcl, and expressed the morphological characteristics of granule neurons, i.e., large, round, or oval nuclei (Fig. 2D). Additionally, both BrdU-labeled and non–BrdU-labeled pyknotic (Fig. 2F) and mitotic (Fig. 2G) cells were also observed in the sgz and deep aspect of the gcl in these animals. Three weeks after BrdU injection, the difference in number of BrdU-labeled cells between groups was no longer detectable ($t_{[10]} = 0.823$; $P = 0.386$) (Fig. 1). By this time point, the majority of BrdU-labeled cells that had been incorporated into the gcl were morphologically indistinguishable from neighboring mature granule neurons (Fig. 2E) and were immunoreactive for NeuN ($\bar{x}_{\text{sham odor}} = 79.8 \pm 3.91$, $\bar{x}_{\text{fox odor}} = 84.8 \pm 2.18$, $P > 0.05$, ns) (Fig. 2I), and calbindin ($\bar{x}_{\text{sham odor}} = 73.1 \pm 0.97$, $\bar{x}_{\text{fox odor}} = 75.9 \pm 1.21$, $P > 0.05$, ns) (Fig. 2J). A small percentage of BrdU-labeled cells expressed GFAP ($\bar{x}_{\text{sham odor}} = 5.2 \pm 3.0$, $\bar{x}_{\text{fox odor}} = 6.8 \pm 4.5$, $P > 0.05$, ns) (Fig. 2K). No differences in the total gcl volume were detected between groups at any time point.

Exposure to fox odor diminishes the number of BrdU-labeled cells by elevating adrenal hormone levels

A single exposure to fox odor resulted in a significant increase in the levels of circulating corticosterone after 30 minutes compared with sham controls ($\bar{x}_{\text{sham odor}} = 11.97 \pm 2.150$ μg/dl; $\bar{x}_{\text{fox odor}} = 26.63 \pm 3.727$ μg/dl; $t_{(1,8)} = 3.407$; $P = 0.0093$). Additionally, the decrease in the number of BrdU-labeled cells in the dentate gyrus after fox odor exposure was eliminated by prevention of a stress-induced rise in adrenal hormone levels ($F_{(1,13)} = 6.69$, $P = 0.022$) (Fig. 4). In ADX rats that had been given low-dose corticosterone replacement, no difference in the number of BrdU-labeled cells was detected between sham odor- and fox odor-exposed animals (Fig. 4). Similar to unoperated control animals (Experiment 1), sham-operated animals showed a decrease in the number of BrdU-labeled cells when exposed to fox odor ($F_{(1,13)} = 10.58$, $P < 0.01$) (Fig. 4). Radioimmunoassay confirmed the efficacy of adrenalectomy in all ADX animals. In addition, sham-operated, sham odor-exposed animals exhibited a comparable number of BrdU-labeled cells to those observed in naïve control

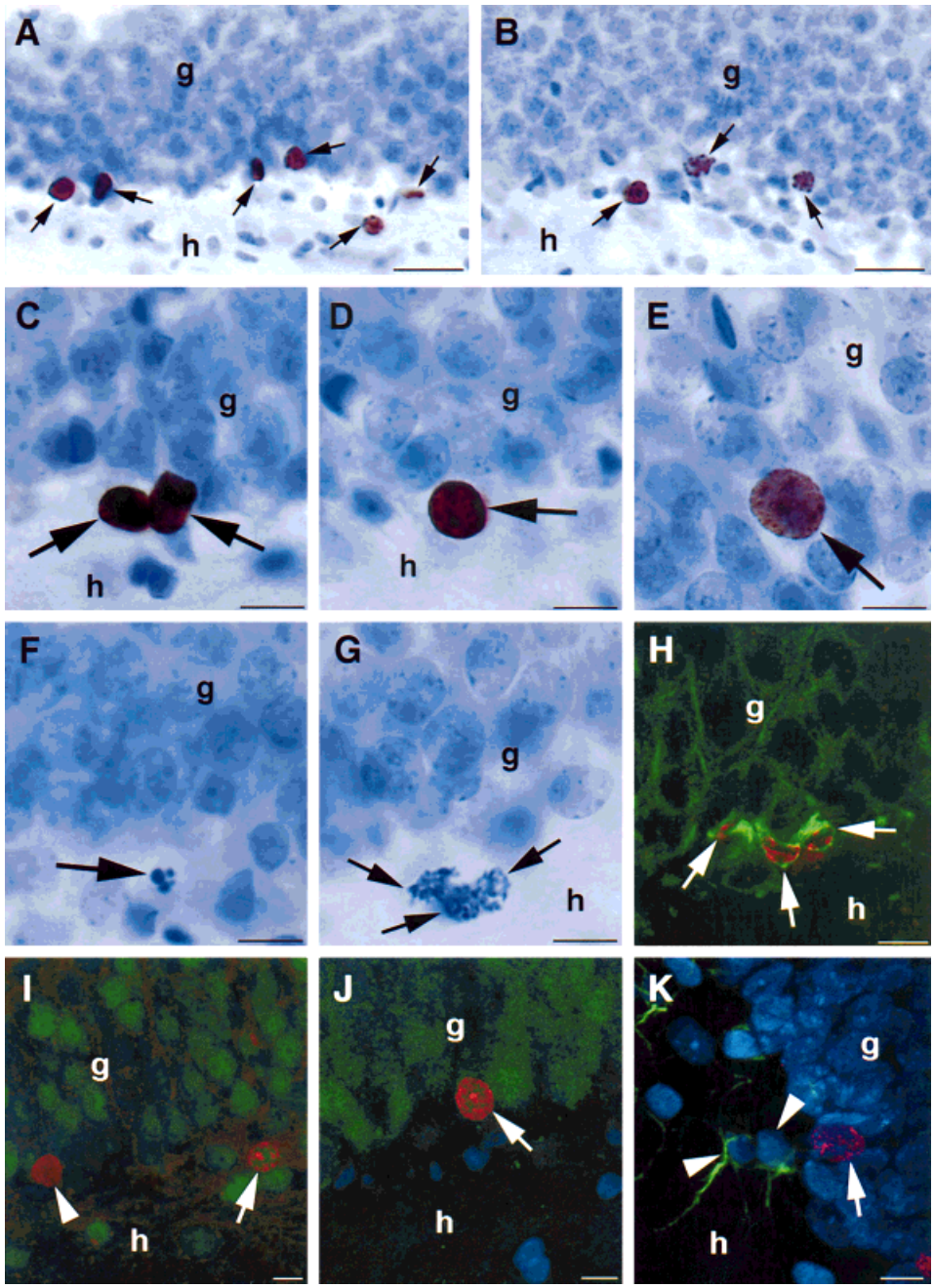

Fig. 2. **A,B:** Confocal images (400×) of 5-bromo-2′deoxyuridine (BrdU) -labeled cells (red) in the dentate gyrus of adult male rats exposed to either sham or fox odor. One week after BrdU injection, animals that were exposed to fox odor (B) exhibited significantly fewer BrdU-labeled cells compared with sham controls (A). **C–E:** Photomicrographs (1,000×) of BrdU-labeled cells in the dentate gyrus of adult male rats exposed to either sham or fox odor. With increasing time after BrdU injection, BrdU-labeled cells became incorporated into the granule cell layer and expressed the morphological characteristics of mature granule neurons. No difference in the morphology or location of BrdU-labeled cells was detected between sham odor- and fox odor-exposed animals at the 2-hour (C), 1-week (D), or 3-week time points (E). **F,G:** Photomicrographs (1,000×) of a pyknotic cell (F, arrow) and mitotic cells (G, arrows) in the dentate gyrus of a rat that was exposed to fox odor and perfused 1 week later. **H:** Confocal image of BrdU-labeled cells colabeled with TuJ1, a marker of immature granule neurons (arrows), in the dentate gyrus of a rat that was exposed to fox odor and perfused after 1 week. I–K: Confocal images of BrdU-labeled cells in the dentate gyrus of rats that were exposed to sham or fox odor and perfused after 3 weeks. **I:** Example of a BrdU-labeled cell that is colabeled with NeuN (arrow), a marker of mature granule neurons. Arrowhead indicates a BrdU-labeled cell that is not colabeled with NeuN. **J:** Example of a BrdU-labeled cell that is colabeled with calbindin (arrow), also a marker of mature granule neurons. **K:** Example of a BrdU-labeled cell (arrow) that is not colabeled with GFAP, a marker of astroglia. Arrowheads indicate GFAP-labeled cells not labeled with BrdU. g, granule cell layer; h, hilus. Figures were produced by using Adobe Photoshop software (Adobe Systems, Seattle, WA). Scale bars = 25 μm in A,B, 12.5 μm in C–G, 10 μm in H–K.

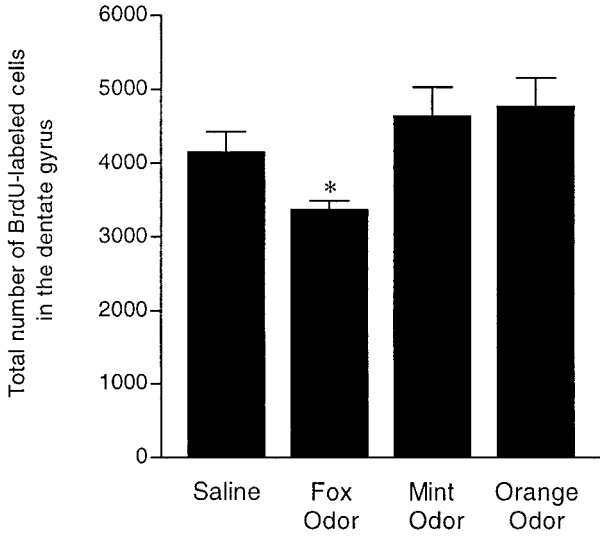

Fig. 3. Analysis of the total numbers of 5-bromo-2′deoxyuridine (BrdU) -labeled cells in the dentate gyrus of adult male rats revealed a significant effect of olfactory experience ($H_{(3)} = 9.953$; $P = 0.019$). Two hours after BrdU injection, rats that were exposed to fox odor, but not those exposed to mint or orange odor, exhibited fewer BrdU-labeled cells in the dentate gyrus compared with sham odor-exposed controls. Bars represent mean + SEM obtained from four to five animals. Asterisk indicates significant difference from sham odor control value ($P < 0.05$).

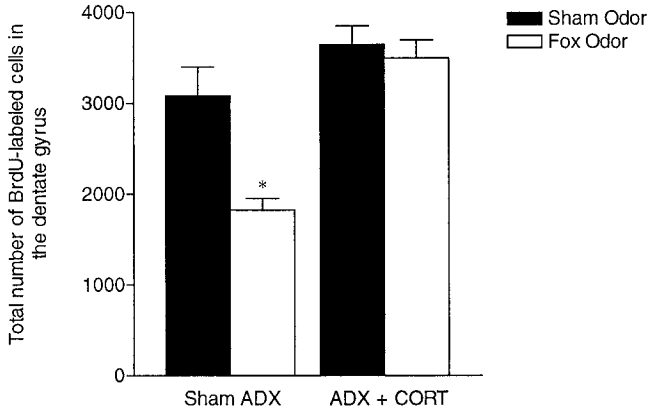

Fig. 4. Stereological estimates of the total numbers of 5-bromo-2′deoxyuridine (BrdU) -labeled cells in the dentate gyrus of adult male rats that were either sham ADX or ADX and replaced with low levels of corticosterone. Two hours after BrdU injection, sham ADX animals exposed to fox odor exhibited fewer BrdU-labeled cells in the dentate gyrus compared with sham odor-exposed controls. In contrast, no difference was detected in the total number of BrdU-labeled cells between animals that were exposed to fox odor versus sham odor in ADX, corticosterone replaced animals. Bars represent mean + SEM obtained from three to six animals. Asterisk indicates significant difference ($P < 0.05$).

animals. Moreover, no difference in the number of BrdU-labeled cells was detected between sham-operated, sham odor-exposed and ADX, sham-odor exposed animals, indicating that low-dose corticosterone replacement was effective in preventing an ADX-induced rise in baseline cell proliferation (Fig. 4) (Cameron et al., 1998).

DISCUSSION

These findings demonstrate that exposure to fox odor (but not other, nonthreatening, odors) rapidly decreases the number of BrdU-labeled cells in the dentate gyrus of adult rats by means of an adrenal hormone-dependent mechanism. No difference was detected between groups in the number of BrdU-labeled cells in the svz or the number of silver grains over [^{3}H]-thymidine-labeled cells in the dentate gyrus, indicating that the decrease in BrdU-labeled cells in the dentate gyrus did not result from differences in marker availability due to changes in blood flow. Although the decrease in the number of BrdU-labeled cells in the dentate gyrus may reflect a shortening of the DNA synthetic (S) phase, this result is unlikely given that S is the most stable phase of the cell cycle (Takahashi et al., 1992). Thus, the decrease in labeled cells most likely reflects a decrease in the proliferation of granule cell precursors.

Exposure to fox odor increased circulating adrenal glucocorticoid levels and diminished the number of BrdU-labeled cells in the dentate gyrus. Moreover, the effect of fox odor exposure on the number of BrdU-labeled cells was not observed in animals rendered incapable of exhibiting a stress-induced rise in glucocorticoid levels, suggesting that adrenal steroids are responsible for mediating this effect. Examination at longer survival times revealed that the decrease in the number of BrdU-labeled cells in fox odor-exposed animals was transient; a difference was detectable at 1 week after BrdU labeling but not at 3 weeks.

In both fox odor- and sham odor-exposed animals, many new cells acquired morphological and biochemical characteristics of mature granule neurons. The majority of these cells expressed the marker of immature granule neurons TuJ1 by 1 week after BrdU labeling and the markers of mature granule neurons calbindin and NeuN by 3 weeks after labeling. Only a small percentage of these cells expressed GFAP, a marker of astroglia, at either time point.

Stress-induced changes in hippocampal cell production

Previous studies have shown that social stress inhibits the proliferation of granule cell precursors in adult tree shrews and marmosets (Gould et al., 1997, 1998). In the present study, we have found that exposure to fox odor, an experience that increases circulating corticosterone to levels comparable to those observed with other paradigms, such as subordination stress and immobilization stress (Albeck et al., 1997; Clement et al., 1998), has a similar effect on cell proliferation in the dentate gyrus of adult rats. This observation suggests that regulation of this process by stress may be a feature common to all mammalian species. Moreover, prior studies have shown that exposure to predator odor, an experience which elevates corticosterone levels during the postnatal stress hyporesponsive period, also decreases cell proliferation in the developing dentate gyrus (Tanapat et al., 1998). Thus, stress-induced suppression of cell proliferation is a phenomenon that is likely to occur throughout life as well.

The decrease in number of BrdU-labeled cells after fox odor exposure was observed 2 hours and 1 week after labeling. The majority of these cells expressed TuJ1 and a significant percentage expressed NeuN and calbindin at the 1-week time point, suggesting that stress-induced inhibition of cell proliferation diminishes the pool of cells

that express a neuronal phenotype. However, the lack of a difference in the number of BrdU-labeled cells at the 3-week time point indicates that this stressor results only in a transient decrease in the number of new neurons.

One possible explanation is that fox odor-exposed animals may experience a rebound in cell proliferation after the initial stress-induced suppression that ultimately compensates for the stress-induced decrease in the number of labeled cells. Although this possibility cannot be ruled out with the present data, the decreased number of new cells persists at least until 1 week after stress, suggesting that any compensatory cell proliferation does not affect the BrdU-labeled population until later. It is more likely that the lack of a detectable difference between fox odor-exposed and control animals at the 3-week time point results from differences in the survival of new cells. Under standard laboratory conditions, control rats experience a significant loss of adult-generated cells between 1 and 3 weeks after mitosis (Cameron et al., 1993b; Gould et al., 1999b). It may be that fewer new cells die during this period in stressed animals compared with controls. This possibility does not necessarily suggest activation of a compensatory mechanism in the brains of stressed animals; more likely, it reflects diminished competition for trophic support among the relatively smaller pool of cells produced after stress.

Potential mechanisms of stress-induced changes in cell proliferation

The physiological stress response is mediated by coordination of both the central and peripheral nervous systems. Although the central neurophysiological response to fox odor exposure may be involved in mediating observed changes in cell proliferation, our present results demonstrating that adrenalectomy prevents the effect indicate that this process requires adrenal hormones. Because the entire adrenal gland was removed, an involvement of adrenal catecholamines cannot be entirely ruled out. However, a vast body of work indicates that adrenal steroids are primarily responsible for conveying information from the adrenal gland to the hippocampal formation. Specifically, both the type I (mineralocorticoid) and type II (glucocorticoid) adrenal steroid receptors, are known to be expressed by hippocampal neurons (McEwen et al., 1968) and have been shown to mediate a wide range of effects on neuronal excitability, neurochemistry, and structural plasticity in the hippocampal formation (reviewed in McEwen, 1999). Moreover, peripherally released epinephrine, the main catecholamine of the adrenal gland, does not cross the blood brain barrier (Weil-Malherbe et al., 1959). Taken together with the fact that adrenal steroids, specifically glucocorticoids, are known to suppress cell proliferation in the dentate gyrus (Cameron and Gould, 1994; Gould et al., 1991), these observations strongly suggest that glucocorticoids are responsible for mediating observed stress-induced decreases in cell proliferation.

Adrenal steroid regulation of cell proliferation, however, is not direct, as most precursor cells do not express either Type I or Type II adrenal steroid receptors (Cameron et al., 1993a). Instead, adrenal steroids act by means of an N-methyl-D-aspartate receptor-dependent excitatory pathway (Cameron et al., 1998). Consistent with this, exposure to fox odor not only elevates levels of circulating glucocorticoids but also elicits a characteristic excitatory electrophysiological response in the dentate gyrus (Heale et al., 1994). Collectively, these results suggest that stress increases glucocorticoids, which activate an excitatory pathway that in turn inhibits cell proliferation. At present, the intermediate events between excitatory input and alterations in cell proliferation remain unknown. One possibility is that decreases in the number of proliferating cells may be the result of a lengthening of G1 such that cells are delayed from entering S phase until after BrdU is no longer available. Alternatively, the decrease in cell proliferation may be due to an inhibition of cells from entering S phase at the G1/S transition.

Function of adult-generated neurons

The precise function of adult-generated neurons is not known. However, the number of new granule neurons produced in the adult dentate gyrus suggests that these cells play an important role in hippocampal function. The hippocampal formation is a region that has been implicated in certain types of learning and memory (Whishaw, 1987; McNaughton et al., 1989; Squire and Zola, 1998). A recent study has demonstrated that decreasing the number of adult-generated hippocampal neurons by means of depletion of the granule cell precursor population prevents acquisition of a hippocampal-dependent learning task (Shors et al., 2001). In addition, several studies have shown that conditions which increase the number of BrdU-labeled cells in the dentate gyrus, such as estrogen treatment (Luine et al., 1998), living in an enriched environment (Kempermann et al., 1997), and running (van Praag et al., 1999), are associated with enhanced performance on hippocampal-dependent learning tasks, whereas conditions that diminish the number of new hippocampal neurons, such as glucocorticoid treatment (Luine et al., 1993; Bodnoff et al., 1995), ovariectomy (Daniel et al., 1999), and stress (Luine et al., 1994, 1996; Krugers et al., 1997), are associated with impaired learning on these tasks.

Functional implications of stress-induced suppression of cell proliferation

At present, the functional significance of a stress-induced decrease in the number of proliferating cells remains unclear. However, it is generally consistent with the catabolic action of glucocorticoids, which enables an organism faced with a potentially life-threatening circumstance to allocate resources away from growth processes toward the execution of an adaptive response (McEwen, 2000; Sapolsky et al., 2000).

Although the stress-induced decrease in the number of new cells after a single exposure to fox odor was not detectable after a 3-week survival, evidence suggests that adult-generated cells may exert a functional impact well before differentiation into mature granule neurons. Adult-generated cells express markers of immature granule neurons (TOAD-64, TuJ1) during the first week after mitosis (Tanapat et al., 1999; present study) and, moreover, extend axons into the mossy fiber pathway as early as 4–10 days after their production (Hastings and Gould, 1999). Collectively, these observations suggest that stress-induced decreases in the number of new immature neurons may be functionally significant.

An alternative possibility is that adult-generated cells may not be important for hippocampal function in control animals living in a laboratory setting. Previous studies have shown that the survival of new cells in the dentate

gyrus is dependent upon experience. Environmental complexity has been shown to enhance the number of new hippocampal cells in birds and mice (Barnea and Nottebohm, 1994; Kempermann et al., 1997). Furthermore, certain types of learning increase the survival of adult-generated cells in the dentate gyrus of rats (Gould et al., 1999b). Taken together, these results indicate that the relatively deprived conditions in which laboratory control animals live are insufficient to rescue new cells from death. In more naturalistic settings where learning opportunities are increased, unstressed animals may maintain more new hippocampal neurons for a longer period of time. Under these conditions, the decrease in adult-generated cells in fox odor-exposed animals relative to controls may persist for longer periods of time, and, therefore, affect hippocampal function.

A third possibility is that the decrease in cell proliferation resulting from a single brief exposure to stress is not sufficient to exert a significant impact on hippocampal neuron production, suggesting that this process may be somewhat resistant to acute episodes of stress. In contrast, however, previous work indicates that chronic exposure to stress decreases cell proliferation as well as neuron production, and is associated with an overall decrease in the volume of the granule cell layer (Fuchs et al., 1997). Thus, chronic stress is likely to persistently inhibit the proliferation of granule cell precursors and, therefore, result in an impairment of hippocampal function. Indeed, previous studies have demonstrated that exposure to chronic stress significantly impairs certain forms of hippocampal-dependent learning (Luine et al., 1994, 1996). However, the extent to which stress-induced inhibition of cell proliferation contributes to this functional deficit has not yet been investigated.

When examining the potential functional implications of stress-induced changes in hippocampal cell proliferation, it should be noted that, although the effect is large, it is not likely to have a generalized impact on all aspects of hippocampal function. Patterns of neuronal integration, preliminary neuroanatomic data, (unpublished observations) and electrophysiological evidence (Wang et al., 2000) collectively suggest that adult-generated cells comprise a subpopulation of granule neurons that is separate from the developmentally derived population. Diminished cell proliferation within the adult dentate gyrus is, therefore, likely to have very specific effects on hippocampal function. Moreover, that stress-induced decreases in hippocampal cell production may negatively affect certain types of hippocampal-dependent learning does not necessarily suggest that other aspects of hippocampal function will be affected in the same way. For example, it has been suggested previously that stress-induced changes in hippocampal neuron production may play an important role in fear-related learning and memory (McEwen, 1999) because of the anatomic and functional connections between the dentate gyrus and the amygdala (Ikegaya et al., 1996). Thus, it may be that certain types of learning are impaired by stress-induced changes in cell proliferation, whereas others may actually be enhanced. Future studies will be necessary to determine how different aspects of hippocampal function are affected by stress-induced changes in cell proliferation.

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