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cAMP Response Element-Binding Protein Deficiency Allows for Increased Neurogenesis and a Rapid Onset of Antidepressant Response

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cAMP response element-binding protein (CREB) has been implicated in the molecular and cellular mechanisms of chronic antidepressant (AD) treatment, although its role in the behavioral response is unclear. CREB-deficient (CREB$^{\alpha\Delta}$ mutant) mice demonstrate an antidepressant phenotype in the tail suspension test (TST) and forced-swim test. Here, we show that, at baseline, CREB$^{\alpha\Delta}$ mutant mice exhibited increased hippocampal cell proliferation and neurogenesis compared with wild-type (WT) controls, effects similar to those observed in WT mice after chronic desipramine (DMI) administration. Neurogenesis was not further augmented by chronic DMI treatment in CREB$^{\alpha\Delta}$ mutant mice. Serotonin depletion decreased neurogenesis in CREB$^{\alpha\Delta}$ mutant mice to WT levels, which correlated with a reversal of the antidepressant phenotype in the TST. This effect was specific for the reversal of the antidepressant phenotype in these mice, because serotonin depletion did not alter a baseline anxiety-like behavior in CREB$^{\alpha\Delta}$ mutant mice. The response to chronic AD treatment in the novelty-induced hypophagia (NIH) test may rely on neurogenesis. Therefore, we used this paradigm to evaluate chronic AD treatment in CREB$^{\alpha\Delta}$ mutant mice to determine whether the increased neurogenesis in these mice alters their response in the NIH paradigm. Whereas both WT and CREB$^{\alpha\Delta}$ mutant mice responded to chronic AD treatment in the NIH paradigm, only CREB$^{\alpha\Delta}$ mutant mice responded to acute AD treatment. However, in the elevated zero maze, DMI did not reverse anxiety behavior in mutant mice. Together, these data show that increased hippocampal neurogenesis allows for an antidepressant phenotype as well as a rapid onset of behavioral responses to AD treatment.

Key words: novelty-induced hypophagia; NIH; CREB; neurogenesis; DMI; mouse; depression

Introduction

Antidepressant (AD) treatment involves amelioration of symptoms over the course of weeks or months (Wong and Licinio, 2001). Chronic exposure to AD is accompanied by transcriptional and cellular modulation, occurring on a later timescale than changes in monoamine levels and consistent with the onset of the relief of symptoms (Frazer and Benmansour, 2002; Nestler et al., 2002; Young et al., 2002; Carlezon et al., 2005; Blendy, 2006). In particular, the transcription factor cAMP response element-binding protein (CREB) is upregulated after chronic AD treatment; increases in CREB mRNA and protein, or phosphorylation of CREB have been shown in the hippocampus after

chronic, but not acute AD administration (Nibuya et al., 1996; Thome et al., 2000; Blom et al., 2002; Tiraboschi et al., 2004).

The significance of CREB regulation in mechanisms through which chronic AD treatment produces symptomatic relief is unknown. Hippocampal atrophy is hypothesized to be one contributing factor to the affective and cognitive impairments observed in depression (Watanabe et al., 1992; Stein-Behrens et al., 1994; Magarinos et al., 1996; Sheline, 1996; McEwen, 1999; Sheline et al., 2003). AD drugs that increase neurogenesis in the dentate gyrus of the adult hippocampus plausibly counteract this pathology of depression (Malberg et al., 2000; Czeh et al., 2001; Manev et al., 2001; Santarelli et al., 2003; Kodama et al., 2004; Sairanen et al., 2005). CREB appears to play a role in neurogenesis, because immature neurons in the adult hippocampus express phosphorylated CREB (Nakagawa et al., 2002b). Furthermore, transgenic mice expressing a dominant-negative CREB mutation in the hippocampus demonstrated a reduction in neurogenesis (Nakagawa et al., 2002a). The concurrent increases of both CREB and neurogenesis in the hippocampus in response to chronic AD treatment suggest that CREB may regulate hippocampal neurogenesis.

Although CREB may mediate molecular and cellular changes induced by AD treatment, its role in modulating behavioral responses to chronic treatment is still unclear. We previously

reported that mice deficient in CREB (CREB$^{\alpha\Delta}$ mutant) (Hummler et al., 1994; Blendy et al., 1996) show a baseline decrease in immobility in the forced-swim test (FST) and tail suspension test (TST), behavioral models commonly used to evaluate AD efficacy (Lucki, 2001; Cryan et al., 2005). However, immobility is further decreased with AD treatment (Conti et al., 2002), suggesting that this acute behavioral response is mediated by a CREB-independent mechanism. We examined neurogenesis in these animals and showed that CREB$^{\alpha\Delta}$ mutant mice had increased levels of hippocampal neurogenesis at baseline. Reductions in neurogenesis achieved by serotonin depletion in mutant animals correlated with a reversal in the baseline antidepressant phenotype of CREB$^{\alpha\Delta}$ mutant mice in the TST, whereas wild-type (WT) mice did not show a change in either parameter. The novelty-induced hypophagia (NIH) and novelty suppressed feeding (NSF) paradigms are two closely related paradigms that are responsive to chronic antidepressant treatment, and this behavioral response has been correlated with neurogenesis. In these paradigms, latency to consume food in a novel environment is measured and chronic but not acute AD treatment, as well as acute benzodiazepine treatment, reduces the latency to feed in a novel environment (Merali et al., 2003; Dulawa and Hen, 2005). This effect of chronic antidepressant treatment, however, is diminished when neurogenesis is impaired (Santarelli et al., 2003). This result suggests that the increase in neurogenesis associated with chronic antidepressant treatment is required for the behavior associated with this paradigm. Therefore, we examined the effect of chronic DMI administration using the NIH paradigm. We found that, whereas both WT and CREB$^{\alpha\Delta}$ mutant mice responded to chronic AD treatment in this paradigm, only CREB$^{\alpha\Delta}$ mutant mice responded to acute treatment. Together, these findings suggest that, in the absence of CREB throughout development, an antidepressant phenotype emerges on both a cellular and behavioral level.

Materials and Methods

Animals. CREB$^{\alpha\Delta}$ mutant mice were backcrossed to the inbred mouse strains 129SvEv and C57BL/6 for >20 generations. For these experiments, WT and CREB$^{\alpha\Delta}$ mutants were F_1 hybrids obtained from crossing mice heterozygous for the CREB mutation from each strain. Mice (3–6 months of age; 20–40 g) were group housed and maintained on a 12 h light/dark cycle with food and water available *ad libitum* in accordance with the University of Pennsylvania Animal Care and Use Committee. For the NIH paradigm, mice were housed in groups of two. All experimental testing sessions were conducted between 9:00 A.M. and 3:00 P.M., with animals randomly assigned to treatment conditions and tested in counterbalanced order.

Drugs. p-Chlorophenylalanine methyl ester hydrochloride (PCPA), a tryptophan hydroxylase inhibitor (250 mg/kg, i.p.) (Sigma, St. Louis, MO) was dissolved in deionized water and injected twice daily for 3 d. The last dose was administered 18 h before behavioral testing or killing for neurogenesis. Desipramine (DMI) (12.5 mg/kg) (Sigma) was prepared immediately before use and injected intraperitoneally using a volume of 10 ml/kg. The dose was calculated as milligrams per kilogram of the salt form and was dissolved in 0.9% saline. For acute treatment, mice were injected with DMI or 0.9% saline in the morning and afternoon of the day before, and morning of the day of testing, for a total of three doses before exposure to the zero maze or NIH paradigm. For chronic treatment, mice were treated with DMI or saline twice daily for 3 weeks.

Tail suspension test. Eighteen hours after the final injection of saline or PCPA, mice were individually suspended by the tail to a horizontal ringstand bar (distance from floor, 35 cm) using adhesive tape affixed 2 cm from the tip of the tail. Mice demonstrated several escape-oriented behaviors interspersed with bouts of immobility as the session progressed. A 6 min test session was videotaped and scored by a trained observer who was blind to the experimental conditions. The behavioral measure scored was the duration of "immobility," defined as the time when mice were judged to cease escape-motivated behaviors.

Elevated zero maze. The zero maze (Stoelting, Wood Dale, IL) was elevated 24 inches from the ground and consisted of two open areas (wall height, 0.5 inches) and two closed areas (wall height, 12 inches). Each mouse was placed in the closed area and the duration of testing was 300 s. The Viewpoint Tracking System (Viewpoint, Champagne au Mont d'Or, France) was used to video record and register the time spent in the open areas and the number of entries into each area. Ethological parameters [rearing, stretch-attend-posture (SAP), and head-dips] were scored manually. The test was performed in dim lighting. Mice were acclimated to the room for 1 h before testing. For the PCPA experiment, mice received the final dose of PCPA 18 h before testing. For the DMI experiment, mice received a total of three doses of DMI before testing (9:00 A.M. and 5:00 P.M. of the day before testing, and 9:00 A.M. on test day) 1–4 h before testing.

Novelty-induced hypophagia. For 1 week before the training period, and for the duration of the experiment, mice were housed in groups of two. Training consisted of daily sessions in which mice were exposed to a highly palatable food (peanut butter chips; Nestle, Glendale, CA) in a clear plastic dish. Plastic dividers were placed inside each cage to separate the mice during the training and home cage testing periods. Mice were acclimated for 1 h before placement of food. Food was placed in the cage for 15 min and latency to consume was measured. By the 12th day, a baseline latency to approach the food dish was reached such that there was <20% variability between mice. For chronic antidepressant treatment, mice were injected for 25 d with 12.5 mg/kg DMI or 0.9% saline in the morning (9:00–10:00 A.M.) and afternoon (4:00–5:00 P.M.). Testing in the home, novel environment, and home environment occurred on the last 3 d of injections.

For testing in the novel environment, mice were removed from the home cage and placed in an empty standard cage with no bedding, which was sprayed with a cleaner (Pine Sol) to emit a novel odor and was placed in a white box with bright light illumination. Latency to consume was recorded. These mice were tested 3–6 h after DMI injection. Mice were tested again in the home cage on the day after novel testing (Home 2), under the same conditions as the first home test day (Home 1). In the acute treatment experiment, training was performed as described above. Mice were injected on the three testing days with 12.5 mg/kg DMI or saline in the morning (9:00–10:00 A.M.) and afternoon (4:00–5:00 P.M.) and testing occurred 3–6 h after the morning injection of DMI. Thus, a total of three doses of DMI were administered before exposure to the novel environment.

Bromodeoxyuridine injections and tissue preparation. For evaluation of the effect of DMI on short-term cell survival and bromodeoxyuridine (BrdU) labeling, animals were administered saline or DMI (12.5 mg/kg, i.p.; Sigma) twice daily for 38 d. Twenty-four hours after the last DMI or saline injection, mice were administered BrdU twice daily (50 mg/kg, i.p.; Zymed, San Francisco, CA) for 4 d. Twenty-four hours after the last BrdU injection, mice were anesthetized and transcardially perfused (0.1 M cold PBS for 5 min followed by 4% cold paraformaldehyde for 15 min). A second set of animals were allowed to survive for 6 weeks after the BrdU injection, at which time they were anesthetized and perfused for evaluation of long-term cell survival and phenotypic labeling. To determine the effect of PCPA depletion on cell proliferation in CREB$^{\alpha\Delta}$ mutant and WT littermates, mice were perfused 2 h after a single BrdU injection (100 mg/kg). All brains were postfixed overnight in paraformaldehyde at 4°C and stored at 4°C in 30% sucrose. Serial sections of the brains were cut (35 μm sections) through the entire hippocampus using a cryostat, and sections were stored in 0.1% NaN$_3$/PBS. This BrdU-labeling paradigm has been successfully used in multiple laboratories to accurately quantify labeled cells. In addition, the use of confocal microscopy with BrdU and neuronal and glial markers allows for additional evaluation of neurogenesis.

Immunohistochemistry. Free-floating sections were used in the determination of BrdU labeling. Series of every ninth section through the hippocampus or subventricular zone of the lateral ventricle were processed for BrdU immunohistochemistry (~10–12 sections per brain).

Using this spacing ensures that the same BrdU-positive cell will not be counted twice. Sections evaluated for proliferation and neurogenesis were heated for 20 min at 75°C in 0.01 M citric acid, pH 6.0, for antigen retrieval. After washing in 0.1 M PBS, sections were permeabilized in 0.1% trypsin with 0.1% $CaCl_2$ for 10 min at room temperature. Sections were rinsed with 0.1 M PBS and denatured with 2N HCl for 30 min at room temperature. After blocking with 3% normal goat serum for 60 min at room temperature, sections were incubated with anti-mouse BrdU (1:150; Becton Dickinson, Franklin Lakes, NJ) with 0.5% Tween 20 overnight at room temperature. Sections were then incubated for 60 min with secondary antibody (1:200 biotinylated goat anti-mouse IgG; Sigma) followed by quenching of endogenous peroxidases with 3% hydrogen peroxide for 15 min at room temperature. Secondary antibody signal was amplified using an avidin–biotin complex (Vector Laboratories, Burlingame, CA) and visualized with DAB (Vector Laboratories).

For analysis of mature cell phenotypes, free-floating sections were incubated in 2N HCl at 40°C for 20 min. Sections were rinsed in 0.02 M potassium PBS (KPBS) and incubated with 0.3% Triton X-100 and 5% normal goat serum in 0.02 M KPBS. Slices were incubated in primary antibodies for 3 d at 4°C [1:100 anti-rat BrdU (Immunologicals Direct, Raleigh, NC); 1:100 anti-mouse neuronal nuclei (NeuN) (Millipore, Temecula, CA); and 1:2500 rabbit anti-S100β (Swant, Bellinzona, Switzerland)]. After rinsing in KPBS, a 1:200 dilution of secondary antibodies Alexa 488 anti-rat IgG (in goat), Alexa 633 anti-rabbit IgG (in goat), and Alexa 568 anti-mouse IgG (in goat; highly cross absorbed) was applied for 2 h at room temperature (Invitrogen, Eugene, OR). After washing, sections were slide-mounted with ProLong antifade solution (Invitrogen) and stored under dark conditions at 4°C.

Immunohistochemical quantitation. All BrdU-labeled cells in the dentate gyrus (granule cell layer) were counted in each section by an experimenter blinded to the study code. To distinguish single cells within clusters, all counts were performed at 40 and 100× under a light microscope, omitting cells in the outermost focal plane. For the short-term survival and proliferation cell counts ($n = 6$–8 per group), a cell was counted as being in the subgranular zone (SGZ) of the dentate gyrus if it was touching, or within the SGZ. Cells that were located more than two cells away from the SGZ were classified as hilar. For long-term survival cell counts ($n = 6$–8 per group), all BrdU-positive cells that were located within the granule layer of the dentate gyrus were scored. Cells that were located more than two cells away from the granule layer were considered hilar. The total number of BrdU-labeled cells per section was determined and multiplied by 9 to obtain the total number of cells per dentate gyrus. For phenotypic analysis ($n = 6$ per group), triple-labeled slices (BrdU, NeuN, S100β) were analyzed using a Leica (Bannockburn, IL) TCS-NT confocal microscope, and images were captured using Leica confocal software. At least 50 BrdU-positive cells per animal were further analyzed using z-plane sectioning in 1 μm steps to confirm the colocalization of both BrdU and either of the markers NeuN or S100β. Cells were analyzed in the *xyz*-plane using Imaris Software (Bitplane, Zurich, Switzerland).

Analysis of serotonin tissue content. Eighteen hours after the last injection of PCPA, mice were killed. Brains were rapidly microdissected, and tissues were flash frozen in liquid nitrogen and stored at −80°C until analysis. Individual brain regions were homogenized in a 100 μM ethylenediamine tetra-acetic acid solution dissolved in 0.1N perchloric acid (15 μl/mg of tissue). Samples were centrifuged at 2–8°C for 15 min at 15,000 rpm and supernatant was filtered using 0.45 μm nylon Costar (Cambridge, MA) Spin-X centrifuge filters. HPLC coupled with electrochemical detection (EC) was used to assay the filtered supernatant for serotonin content.

The HPLC-EC system (Bioanalytical Systems, West Lafayette, IN) consisted of a PM-80 pump, a Sample Sentinel autosampler, and a LC-4C electrochemical detector. Twelve microliters of sample were injected and pumped through a reverse-phase microbore column (ODS; 3 μm; 1 × 100 mm; Bioanalytical Systems) at a flow rate of 0.6 ml/min with electrodetection at +0.6 V. Separation for serotonin was accomplished by using an HPLC mobile phase consisting of 90 mM sodium acetate, 35 mM citric acid, 0.34 mM EDTA, 1.2 mM sodium octyl sulfate, and 9% methanol (v/v) with the addition of 2% dimethlyacetamide (v/v). HCl (12N) was used to adjust the pH of the mobile phase to 4.2.

Statistics. For the BrdU immunohistochemistry, ANOVA and Fisher *post hoc* tests were performed to establish statistical significance. For the PCPA/neurogenesis experiment, we applied a mixed-model analysis (SAS Linux, version 9; Proc Mixed) with genetic group as a between-group factor and saline versus PCPA as a within-group factor. A *post hoc* mixed-model contrast analysis was performed to test the hypothesis that the CREB$^{\alpha\Delta}$ mutant mice show increased neurogenesis in comparison with other groups, comparing the saline-treated CREB$^{\alpha\Delta}$ mutant mice with all other groups. For the TST, we applied a mixed-model analysis with genetic group as a between-group factor and saline versus DMI as a within-group factor. A *post hoc* mixed-model contrast analysis was performed, comparing the saline-treated CREB$^{\alpha\Delta}$ mutant mice with all other groups, to test the hypothesis that the CREB$^{\alpha\Delta}$ mutant mice showed decreased immobility in comparison with other groups. For the zero maze experiments, the ANOVA was performed on two genetic groups (CREB$^{\alpha\Delta}$, WT) by two treatment groups (SAL, DRUG), with time in the open arm as a dependent variable. For the NIH paradigm experiments, latency served as a dependent variable in a two genetic groups (CREB$^{\alpha\Delta}$, WT) by two treatment groups (SAL, DMI) ANOVA, with condition (Home 1, Novel, Home 2) as a repeated-measures (within) factor. The analysis was implemented in SAS (Linux, version 9; Proc GLM).

Results

The antidepressant phenotype is associated with increased cell proliferation in CREB-deficient mice

Chronic antidepressant treatment has been shown to increase hippocampal neurogenesis. CREB$^{\alpha\Delta}$ mutant mice demonstrate antidepressant-like responses in tests such as the FST and TST, which are readouts of antidepressant efficacy. To determine whether altered neurogenesis may underlie the antidepressant phenotype, we examined granule cell proliferation and neurogenesis in wild-type and CREB$^{\alpha\Delta}$ mutant mice.

Animals were administered BrdU, and after different time points of killing, the number of BrdU-positive cells in the adult dentate gyrus was determined using immunohistochemical detection.

At the 4 d time point, CREB$^{\alpha\Delta}$ mutant mice exhibited significantly more BrdU-positive cells than respective WT controls ($F_{(1,23)} = 5.24$; $p = 0.0316$), corresponding to a 52% increase (Fig. 1A). In addition, chronic DMI treatment (5 weeks; 12.5 mg/kg, twice daily, i.p) was also examined. WT mice demonstrate a significantly increased number of BrdU-positive cells in DMI treated compared with saline controls by 51% ($F_{(1,23)} = 4.73$; $p = 0.0402$). However, DMI treatment did not further increase the number of BrdU-positive cells in CREB$^{\alpha\Delta}$ mutant mice compared with saline-treated mutant controls. The number of BrdU-positive cells in saline-treated CREB-deficient mice was comparable with that of DMI-treated WT mice.

The antidepressant phenotype is associated with increased neurogenesis in CREB-deficient mice

To determine whether the newly born cells observed in WT and CREB-deficient mice survive and differentiate into neurons, cells labeled with BrdU were allowed to mature for 6 weeks. Saline-treated CREB$^{\alpha\Delta}$ mutant mice exhibited significantly increased numbers of BrdU-positive cells in the granule cell layer compared with WT controls ($F_{(1,24)} = 12.49$; $p = 0.0017$) (Fig. 1B), suggesting that the many of the newly born cells survived for at least 6 weeks. In a similar manner, in WT mice, DMI treatment significantly increased the number of BrdU-positive cells compared with saline-treated WT controls ($F_{(1,24)} = 5.098$; $p = 0.0033$) (Fig. 1B) at this 6 week time point. However, in CREB$^{\alpha\Delta}$ mutant mice, DMI had no additional effect on the number of
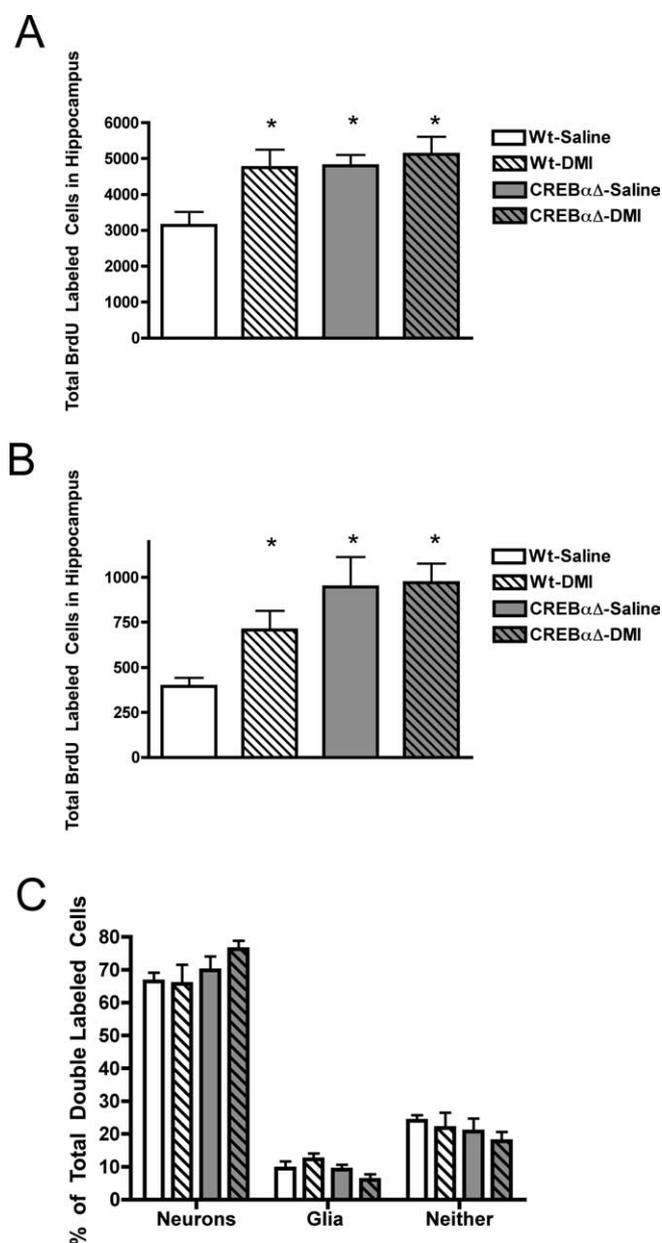
Figure 1. *A–C*, CREB deficiency increases the number of BrdU-positive cells at 4 d (*A*) and 6 weeks (*B*) in the dentate gyrus, with the majority of BrdU-positive cells differentiating into neurons (*C*). Saline-treated CREB$^{\alpha\Delta}$ mutant mice demonstrated significantly increased number of BrdU-positive cells compared with WT controls (*$p < 0.05$; $n = 6–8$). This increase was maintained at the 6 week time point, at which the majority of the BrdU-positive cells matured into neurons, indicating a net increase in neurogenesis in the CREB$^{\alpha\Delta}$ mutant mice. Chronic DMI treatment significantly increased BrdU labeling in the dentate gyrus of WT mice at both time points. The majority of the surviving cells matured into neurons, indicating a DMI-induced increase in neurogenesis in WT mice. In contrast to the DMI-induced increase in neurogenesis seen in WT mice, the CREB$^{\alpha\Delta}$ mutant mice show no additional changes in BrdU labeling or neurogenesis. Saline-treated CREB$^{\alpha\Delta}$ mutant mice demonstrated comparable numbers of BrdU-positive cells and neurogenesis with that of DMI-treated WT controls (*$p < 0.05$; $n = 6–9$). In all groups, the majority of surviving BrdU-positive cells expressed the neuronal marker, NeuN. DMI treatment had no effect on the phenotypic distribution of surviving BrdU-positive cells in WT or CREB$^{\alpha\Delta}$ mutant mice. Error bars indicate SEM.

BrdU-positive cells. Similar to previous reports, the total number of BrdU-positive cells after 6 weeks was lower compared with that at 24 h (Gould et al., 1999; Malberg et al., 2000; Nakagawa et al., 2002a).
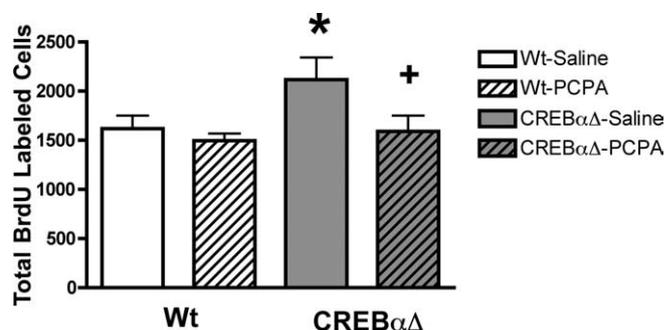
Figure 2. Serotonin depletion reduced proliferation in CREB$^{\alpha\Delta}$ mutant mice to WT levels. CREB$^{\alpha\Delta}$ mutant mice show a significant increase in cell proliferation compared with WT mice ($p = 0.0494$). Treatment with PCPA (250 mg/kg) twice a day for 3 d reverses this increase in cell proliferation in CREB$^{\alpha\Delta}$ mutant mice to WT levels ($p = 0.0340$), whereas PCPA treatment had no effect on cell proliferation in WT mice ($N = 5–6$). Error bars indicate SEM. *$p < 0.05$; [†]$p < 0.05$.

Phenotypic analysis of neurogenesis

To determine whether the BrdU-positive cells at the 6 week time point have matured into neurons or glia, triple labeling with BrdU, NeuN (marker for mature neurons), and S100β (glial marker) was performed (Fig. 1*C*; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Confocal microscopic analysis with *z*-plane sectioning confirmed that the majority of surviving BrdU-positive cells matured into neurons in all groups. Phenotypic analysis revealed that $67 \pm 3\%$ of the surviving BrdU-labeled cells in saline-treated WT mice expressed the neuronal marker, NeuN. After DMI treatment, $66 \pm 6\%$ of surviving BrdU-labeled cells exhibited a neuronal phenotype. CREB$^{\alpha\Delta}$ mutant mice demonstrated similar patterns of neuronal differentiation, with $70 \pm 4\%$ of surviving BrdU-positive cells maturing into neurons in saline controls, compared with $76 \pm 3\%$ after DMI treatment. Fewer BrdU-labeled cells differentiated into glial cells (Fig. 1*C*). In WT mice, saline controls demonstrated $10 \pm 2\%$ of BrdU-positive cells with glial phenotypes as did $12 \pm 2\%$ after DMI treatment. In saline-treated CREB-deficient mice, $9 \pm 2\%$ of BrdU-labeled cells expressed glial markers, compared with $6 \pm 2\%$ after DMI treatment. The remaining 18–24% of BrdU-labeled cells were not labeled with either NeuN or S100β, indicating a population of quiescent, undifferentiated cells, or an existing cell type not labeled with the antibodies used (Fig. 1*C*). BrdU, however, is not without its caveats (Eisch, 2002). BrdU may be taken up not only by proliferating cells but also by cells undergoing repair. Although we cannot rule out this possibility, it has been reported that this low level of DNA incorporation is not easily detected using immunohistochemical methods (Palmer et al., 2000).

Together, these data indicate that CREB deficiency produces an increase in BrdU-positive cells that survive for 6 weeks, and the majority of those mature cells are neurons, indicating a net increase in neurogenesis.

Serotonin depletion decreased cell proliferation in CREB-deficient mice to WT levels

To examine the role of CREB deficiency and serotonin depletion on cell proliferation, mice were treated with saline or PCPA, a specific inhibitor of tryptophan hydroxylase, and killed 2 h after the BrdU injection. This 2 h time point was chosen because it represents one cell cycle of labeling (Cameron and McKay, 2001) and is thought to be an accurate measure of cell proliferation (Malberg et al., 2000). As shown in Figure 2, CREB$^{\alpha\Delta}$ mutant
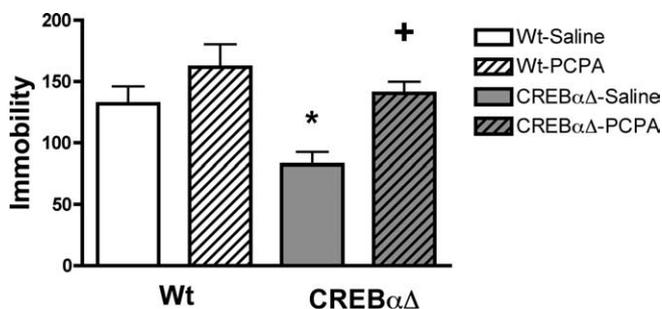
Figure 3. Serotonin depletion reversed the antidepressant phenotype of CREB $^{\alpha\Delta}$ mutant mice in the TST. Mice were treated with PCPA twice a day for 3 d. Immobility in the TST was measured 18 h after the last injection. The TST revealed a significant effect of genotype and treatment, with the PCPA-treated CREB $^{\alpha\Delta}$ mutant mice returning to WT levels ($n = 4$–8). Error bars indicate SEM. $*p < 0.05$; $^{\dagger}p < 0.05$.

Figure 4. Serotonin depletion does not have an effect on anxiety in the elevated zero maze. Mice were treated with PCPA and tested in the elevated zero maze 18 h after the last dose. CREB $^{\alpha\Delta}$ mutant mice spent significantly less time in the open arm of the maze, reflecting a proanxiety phenotype. Treatment with PCPA did not alter anxiety levels in either WT or CREB $^{\alpha\Delta}$ mutant mice ($n = 8$–9). Error bars indicate SEM. $*p < 0.05$.

mice show an increase in BrdU-labeled cells. PCPA treatment led to a reversal of this increase in CREB $^{\alpha\Delta}$ mutant mice, whereas PCPA had no significant effect on WT mice. A mixed model statistical analysis found main effects for genetic group, ($F_{(1,22)} = 4.35$; $p = 0.0494$) and for treatment, ($F_{(1,22)} = 5.14$; $p = 0.0340$). *Post hoc* contrast analysis revealed that the hypothesized (CREB $^{\alpha\Delta}$ saline) group had significantly increased values compared with all other groups, ($F_{(1,23)} = 10.54$; $p = 0.0036$). These data indicate that CREB deletion increases cell proliferation, which can then be decreased to WT levels by serotonin depletion.

Serotonin depletion increased immobility times of CREB-deficient mice to WT levels
To determine whether reversing the baseline increase in neurogenesis would have a functional behavioral correlate in the TST, we administered PCPA to WT and CREB $^{\alpha\Delta}$ mutant mice and measured the effect on immobility. Mixed-model analysis found main effects for genetic group ($F_{(1,22)} = 6.18$; $p = 0.0210$) and for treatment, ($F_{(1,22)} = 9.51$; $p = 0.0054$). As shown in Figure 3, CREB $^{\alpha\Delta}$ mutant mice had significantly reduced values compared with all other groups ($F_{(1,24)} = 10.10$; $p = 0.0041$). This increase demonstrates that the relationship between neurogenesis and behavior in these mice is maintained after serotonin depletion and supports a causal relationship between neurogenesis and behavior in these mice.

Serotonin depletion has no effect on anxiety in the elevated zero maze
In addition to an antidepressant phenotype, CREB $^{\alpha\Delta}$ mutant mice also exhibit increased baseline anxiety (Graves et al., 2002; Hebda-Bauer et al., 2004). To determine whether the reversal of neurogenesis and the antidepressant phenotype is accompanied by a decrease in anxiety, we evaluated mice in the elevated zero maze after serotonin depletion. As shown in Figure 4, treatment with PCPA did not have an effect on time spent in the open arm in either WT or CREB $^{\alpha\Delta}$ mutant mice ($F_{(1,30)} = 0.06$; $p > 0.05$), with the latter once again demonstrating a baseline increase in anxiety ($F_{(1,30)} = 6.76$; $p = 0.0143$).

Serotonin levels are equivalent in WT and CREB $^{\alpha\Delta}$ mutant mice
A possible explanation for the phenotype observed in CREB $^{\alpha\Delta}$ mutant mice would be that the animals have altered serotonin levels. Therefore, we measured tissue serotonin content in these animals. Two-way ANOVAs demonstrate that the baseline serotonin level in CREB $^{\alpha\Delta}$ mutant mice did not differ from that of

WT mice throughout the amygdala, cortex, hippocampus, nucleus accumbens, and raphe (Table 1). 5-Hydroxy-indoleacetic acid (5-HIAA)/5-HT ratios were measured as a marker for turnover, and no significant differences between mutants and WT were found in the amygdala, cortex, hippocampus, nucleus accumbens, and raphe (Table 1). This suggests that baseline alterations in behavior and neurogenesis in CREB $^{\alpha\Delta}$ mice are not attributable to changes in serotonin or its turnover. Stimulated serotonin release was also equivalent in WT and CREB $^{\alpha\Delta}$ mutant mice (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), again demonstrating that CREB deficiency throughout development does not impact serotonin metabolism. The efficacy of PCPA treatment in these experiments was verified by a 72–90% reduction in serotonin levels in comparison with saline-treated animals: $F_{(1,29)} = 213.09$, $p < 0.0001$; $F_{(1,29)} = 217.51$, $p < 0.0001$; $F_{(1,29)} = 148.14$, $p < 0.0001$; $F_{(1,27)} = 24.54$, $p < 0.0001$; $F_{(1,28)} = 122.10$, $p < 0.0001$, measured in the amygdala, cortex, hippocampus, nucleus accumbens, and raphe, respectively. The magnitude of PCPA depletion in discrete brain regions was equivalent across genotypes.

WT and CREB $^{\alpha\Delta}$ mutant mice respond to chronic antidepressant treatment in the NIH paradigm
The NIH paradigm has been reported to be sensitive to chronic, but not acute, antidepressant treatment. Therefore, we used this paradigm to determine whether CREB $^{\alpha\Delta}$ mutant mice respond to chronic AD treatment. As shown in Figure 5, DMI significantly reduced the latency to consume the food in a novel environment for both WT and CREB $^{\alpha\Delta}$ mutant mice. There was a main effect of genotype with mutants showing overall higher latencies ($F_{(1,34)} = 4.78$; $p = 0.0358$), an effect of treatment with treated mice showing lower latencies ($F_{(1,34)} = 11.11$; $p = 0.0021$), and no genotype by treatment interaction ($F_{(1,34)} = 1.35$; $p = 0.2541$). Two-way ANOVA with repeated measures revealed a main effect of condition with longer latencies for the novel compared with home conditions ($F_{(2,68)} = 58.12$; $p < 0.0001$), a genotype by condition interaction with higher latencies for CREB $^{\alpha\Delta}$ mutant mice compared with WT in the novel condition ($F_{(2,68)} = 4.68$; $p = 0.0125$), a treatment by condition interaction with lower latencies for DMI-treated mice in the novel condition ($F_{(2,68)} = 12.21$; $p < 0.000$), but no three-way interaction ($F_{(2,68)} = 1.42$; $p = 0.2476$). In addition, CREB $^{\alpha\Delta}$ mutant mice had generally increased latencies in the novel environment compared with WT mice, consistent with the anxiogenic phenotype observed in the zero maze (Fig. 4). Of the 40 mice tested, 2 were eliminated from

Table 1. Serotonin tissue content (picograms per microliter) and turnover (5-HIAA/5-HT)

Brain region	WT-saline		CREB$^{\alpha\Delta}$-saline		WT-PCPA		CREB$^{\alpha\Delta}$-PCPA	
	Levels	Turnover	Levels	Turnover	Levels	Turnover	Levels	Turnover
Amygdala	367 ± 22	0.52 ± 0.03	338 ± 24	0.53 ± 0.03	62 ± 4	0.24 ± 0.01	57 ± 6	0.25 ± 0.02
Frontal cortex	223 ± 6	0.53 ± 0.02	196 ± 11	0.52 ± 0.02	54 ± 21	0.33 ± 0.13	34 ± 8	0.19 ± 0.03
Hippocampus	653 ± 59	1.07 ± 0.05	690 ± 59	1.14 ± 0.08	82 ± 7	0.36 ± 0.03	64 ± 9	0.32 ± 0.05
Nucleus accumbens	195 ± 11	0.52 ± 0.06	198 ± 35	0.42 ± 0.03	41 ± 7	0.23 ± 0.04	37 ± 9	0.18 ± 0.04
Brainstem	457 ± 36	1.04 ± 0.09	440 ± 38	1.02 ± 0.07	128 ± 22	0.92 ± 0.05	124 ± 16	0.69 ± 0.07

Mice ($n = 8 – 9$ per group) were killed 18 h after the last injection of PCPA. Brains were microdissected and HPLC coupled with EC was used to assay the filtered supernatant for serotonin content. Baseline serotonin levels, turnover, and depletion in CREB$^{\alpha\Delta}$ mutant mice did not differ from that of WT mice throughout the amygdala, cortex, hippocampus, nucleus accumbens, and raphe.
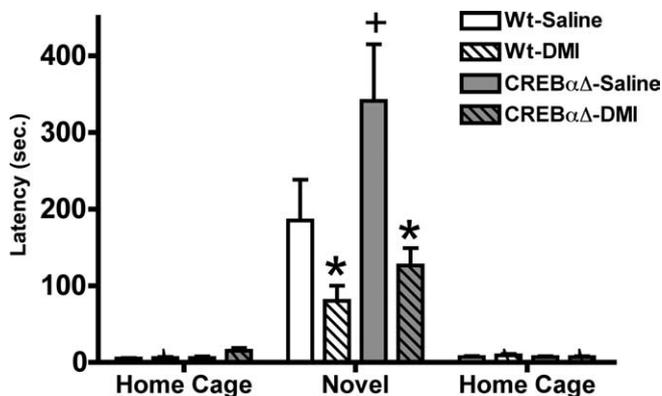
Figure 5. Chronic DMI treatment decreased the latency to consume peanut butter chips in the novel environment in both WT and CREB$^{\alpha\Delta}$ mutant mice. Mean latencies to consume the highly palatable food in the home and novel environment are shown. Mice were treated with DMI (12.5 mg/kg) for 25 d before testing. There was a decreased latency in DMI relative to saline-treated animals (*$p < 0.004$). There was an overall increased latency in mutant relative to WT animals ($^{+}p < 0.04$) ($n = 9$–10). Error bars indicate SEM.
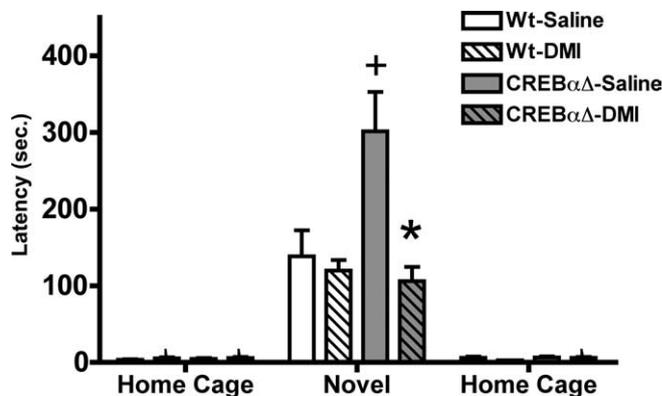
Figure 6. CREB$^{\alpha\Delta}$ mutant mice demonstrated a significant decrease in latency in response to acute DMI treatment, whereas there was no effect of treatment in the WT mice. Mean latencies to consume the highly palatable food in the home and novel environment are shown. Mice ($n = 9$–10 per group) received a dose of DMI (12.5 mg/kg) in the morning and evening of each of the 3 d of testing. A total of three doses of DMI was received before exposure to the novel environment. There was a decreased latency in DMI relative to saline-treated CREB$^{\alpha\Delta}$ mutant mice (*$p < 0.01$). There was also an increased latency in mutant relative to WT animals ($^{+}p < 0.04$) ($n = 7$). Error bars indicate SEM.

analysis for having latency scores less than or greater than 2 SDs from the mean.

CREB$^{\alpha\Delta}$ mutant mice respond to acute antidepressant treatment in the NIH paradigm

To control for the acute effects of AD in this paradigm, mice were trained to consume a highly palatable food, and then injected on the three testing days with 12.5 mg/kg DMI or saline in the morning (9:00–10:00 A.M.) and afternoon (4:00–5:00 P.M.). Testing occurred at exactly the same time point after DMI as described for the chronic experiment. As expected, acute DMI had no effect on altering latency to consume food in a novel environment in WT mice. In contrast, the time required by CREB$^{\alpha\Delta}$ mutant mice to investigate and consume the food was significantly reduced (Fig. 6). The overall main effects of genetic group and treatment group were replicated, with mutants showing overall higher latencies ($F_{(1,24)} = 4.90$; $p = 0.0366$) and treated mice showing lower latencies ($F_{(1,24)} = 9.40$; $p = 0.0053$), but this time the genotype by treatment interaction was also significant ($F_{(1,24)} = 6.18$; $p = 0.0202$). There was no treatment effect in the WT mice. Two-way ANOVA with repeated measures showed a main effect of condition ($F_{(2,48)} = 89.98$; $p < 0.0001$) with longer latencies in the novel condition, a genetic group by condition interaction ($F_{(2,48)} = 4.64$; $p = 0.0143$) with longer latencies in the CREB$^{\alpha\Delta}$ mutant mice for the novel condition, a treatment by condition interaction ($F_{(2,48)} = 9.86$; $p = 0.0003$) with longer latencies in untreated than treated mice, and these were accounted for by a three-way interaction ($F_{(2,48)} = 6.86$; $p = 0.0024$). The three-way interaction indicates an effect of acute treatment in the CREB$^{\alpha\Delta}$ mutant mice relative to the absence of treatment effects in the WT mice. As seen previously (Fig. 5), saline-treated CREB$^{\alpha\Delta}$ mutant

mice demonstrated a significant increase in latency to approach the food and eat over WT mice. Of the 33 mice tested, 5 were eliminated from analysis for having latency scores less than or greater than 2 SDs from the mean, or for failing to reach baseline latency.

Increased baseline anxiety in the elevated zero maze is not reversed in the CREB$^{\alpha\Delta}$ mutant mice by acute antidepressant treatment

To determine whether DMI had an anxiolytic-like effect in the CREB$^{\alpha\Delta}$ mutant mice, we tested this compound in the elevated zero maze in WT and CREB$^{\alpha\Delta}$ mutant mice. CREB$^{\alpha\Delta}$ mutant mice spent significantly less time in the open arm relative to WT mice, reflective of an anxiety-like phenotype. However, this behavior was not mitigated by acute treatment with DMI (Fig. 7A). Two-way ANOVA revealed a main effect of genotype ($F_{(1,34)} = 20.71$; $p < 0.0001$) on time spent in the open arm. Neither treatment nor genotype by treatment interaction approached significance on this parameter. In regard to ethologically relevant parameters and latency to enter the open arm (Fig. 7B,C), two-way ANOVAs demonstrated a significant effect of genotype on stretch-attend-posture ($F_{(1,34)} = 5.93$; $p = 0.02$), head dips ($F_{(1,34)} = 10.904$; $p = 0.0023$), and latency ($F_{(1,34)} = 4.237$; $p = 0.0473$), but not on rearing ($F_{(1,34)} = 0.026$; $p > 0.05$). There was no significant effect of treatment or genotype by treatment interactions on any of these parameters, including head dip, SAP, latency, or rearing. Thus, the acute effect of DMI in CREB$^{\alpha\Delta}$ mutant mice is specific to an antidepressant effect in these mice.
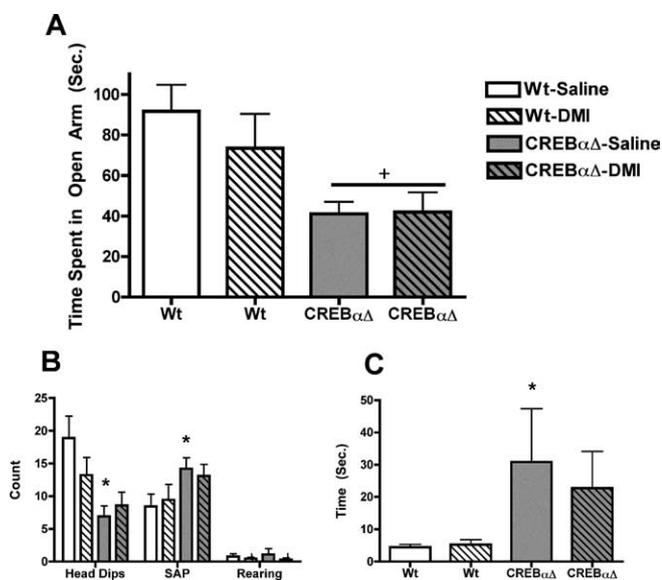
Figure 7. *A*, CREB$^{\alpha\Delta}$ mutant mice showed significantly reduced time spent in the open arm relative to WT ($^+p < 0.0001$) in the elevated zero maze. This reduction was not affected by acute treatment with DMI. Mice ($n = 9$–10 per group) received a total of three doses of DMI (12.5 mg/kg) before testing, with the same schedule used in the NIH. *B*, In regard to ethologically relevant parameters, there is a significant effect of genotype on stretch-attend-posture, and head dips (*$p < 0.05$), but not on rearing. *C*, CREB$^{\alpha\Delta}$ mutant mice demonstrated an increased latency to enter the open arm ($^+p < 0.05$). Error bars indicate SEM.

Discussion

The notion that reduced hippocampal neurogenesis is involved in the pathophysiology of depression has gained traction in recent years (Dranovsky and Hen, 2006; Duman and Monteggia, 2006). AD treatment is thought to ameliorate the symptoms of depression by reversing this process. However, the connection between neurogenesis, its molecular underpinnings, and behavioral consequences are mostly correlative. The FST and the TST are commonly used to assess antidepressant effects of drugs and, more recently, depressive phenotypes in mice (Cryan et al., 2002). CREB$^{\alpha\Delta}$ mutant mice demonstrated decreased immobility in both of these paradigms, which was further reduced by ADs (Conti et al., 2002), suggesting an "antidepressant state" that is still sensitive to drug treatment. Changes in homecage motor activity or swimming behavior were not found (Walters and Blendy, 2001; Graves et al., 2002), and therefore do not account for these findings. To gather information on possible biological substrates responsible for the antidepressant phenotype of CREB$^{\alpha\Delta}$ mutant mice, we examined neurogenesis, a parameter altered by chronic AD treatment.

Our data demonstrate that constitutive reduction in CREB protein enhances BrdU-labeled cell proliferation. Furthermore, the majority of these cells mature into neurons, indicating that CREB-deficient mice have increased proliferation and neurogenesis, comparable with levels observed in WT mice receiving chronic AD treatment (Malberg et al., 2000). However, chronic treatment with DMI did not further increase hippocampal neurogenesis in CREB-deficient mice. This lack of an additional DMI-induced increase in neurogenesis in the CREB mutant mice may be attributable to inherent proliferative limits of the hippocampus. In previous rat studies, treatment with fluoxetine increased the number of BrdU-labeled cells by 20–40%, whereas electroconvulsive seizure caused a ~50% increase (Malberg et al., 2000; Warner-Schmidt and Duman, 2007). Because CREB$^{\alpha\Delta}$ mutant mice demonstrate an ~52% increase in BrdU-positive

cells compared with WT mice, it is possible that the hippocampus has reached the upper limits of its neurogenic capacity. Indeed, in another mouse model showing 20% elevation in baseline levels of hippocampal neurogenesis, chronic treatment with DMI and citalopram failed to further increase neurogenesis (Morcuende et al., 2003).

Our finding of increased levels of hippocampal neurogenesis in the CREB-deficient mice (Fig. 1) differs from the report of decreased levels in a transgenic mouse expressing dominant-negative CREB (mCREB) (Nakagawa et al., 2002a). However by binding to CRE sequences in DNA, mCREB may target genes that are not normally regulated by CREB (Blendy, 2006), thereby altering neurogenesis through other mechanisms. CREB manipulations in other mouse models, and viral vector-mediated gene overexpression, have led to behavioral changes in depression-related behavioral paradigms as well. Acute overexpression of CREB in the nucleus accumbens of rats leads to a pro-depressant response in the FST, and a decrease of CREB mediated by expression of a dominant-negative form of the protein in rats leads to an antidepressant-like effect (Pliakas et al., 2001), with similar effects found in the learned helplessness paradigm in mice and rats (Newton et al., 2002). However, acute overexpression of CREB in the hippocampus leads to an antidepressant effect in the FST (Chen et al., 2001). To date, neither levels of neurogenesis nor behavior in paradigms that are sensitive to chronic AD treatment have been evaluated in these models.

To investigate a more causal relationship between neurogenesis, behavior, and CREB, we depleted mice of serotonin with PCPA. Serotonin is thought to play a role in neurogenesis (Gaspar et al., 2003), and previous studies have reported either no change or a decrease in proliferation in rats treated with PCPA (Brezun and Daszuta, 1999, 2000; Banasr et al., 2001; Huang and Herbert, 2005; Jha et al., 2006). Serotonin has been implicated in both the pathophysiology and treatment of depression. Therefore, we wanted to examine the role of serotonin in maintaining cell proliferation and antidepressant behavior in mice that demonstrate an antidepressant-like phenotype and increased levels of neurogenesis. We found that serotonin depletion with PCPA reversed the antidepressant phenotype of CREB$^{\alpha\Delta}$ mutant mice on both a cellular (Fig. 2) and behavioral (Fig. 3) level, whereas it had no effect on WT mice. Our data support previous findings that PCPA depletion does not have an effect on immobility in WT mice in the TST (O'Leary et al., 2007). Of interest, serotonin levels and turnover did not differ between WT and CREB$^{\alpha\Delta}$ mutant mice (Table 1) and stimulated release of serotonin was also consistent between the two groups (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). However, the serotonergic tone in CREB$^{\alpha\Delta}$ mutant mice, although not different in magnitude, provides a critical stimulus that causes mice to behave at baseline as though they have undergone chronic AD treatment. Additional studies are warranted to investigate the nature of this complex interaction.

In addition to an antidepressant-like phenotype, CREB$^{\alpha\Delta}$ mutant mice demonstrate increased levels of anxiety (Graves et al., 2002; Hebda-Bauer et al., 2004). This anxiety phenotype is also evident in the NIH paradigm with CREBαΔ mutant mice showing increased latency to feed in a novel environment (Figs. 5, 6). Serotonin depletion does not reverse the anxiogenic-like phenotype in the elevated zero maze (Fig. 4), further emphasizing a specific correlation between the antidepressant phenotype and neurogenesis. Whether PCPA treatment reduces the latency to feed in a novel environment in CREB$^{\alpha\Delta}$ mutant remains to be seen. Unfortunately, serotonin depletion produces an anxiolytic,

or decreased hyponeophagia effect in the NIH test in rat (Bechtholt et al., 2007), making it difficult to interpret baseline effects in CREB$^{\alpha\Delta}$ mutant mice.

Overall, the antidepressant-like phenotype may at first seem at odds with the anxiety phenotype. However, 5-HT$_{1A}$ knock-out mice also demonstrate an antidepressant phenotype in combination with an increased level of anxiety (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998), suggesting that endophenotypes related to comorbid neuropsychiatric disorders may be uncoupled in animal models.

To date, the significance of CREB in mediating any behaviors associated with chronic AD treatment have not been investigated in part because of the lack of well validated behavioral paradigms designed to evaluate chronic AD effects. Therefore, to examine the functional significance of increased neurogenesis as well as gain additional insight into possible behavioral implications of this alteration, we tested the CREB$^{\alpha\Delta}$ mutant mice in the NIH paradigm. In this paradigm, exposure to a novel environment elicits an increased latency to consume a highly palatable food. This increase in latency is reversed by chronic, but not short-term, AD treatment (Merali et al., 2003), making it a useful model to understand molecular and cellular changes underlying the behavioral response to chronic AD treatment. It is closely related to the NSF paradigm, with the latter using food deprivation to induce consumption of regular food. A limitation of both the TST and FST is their responsiveness to acute administration of antidepressants. Clinically, weeks of treatment are needed to ameliorate behavioral symptoms, reflecting the need for long-term transcriptional and cellular changes. Using the NIH paradigm, the present data demonstrate that CREB$^{\alpha\Delta}$ mutant mice respond to acute AD treatment, an effect not seen in WT mice.

Acute anxiolytic compounds are effective in the NIH paradigm (Merali et al., 2003); thus the acute effect of DMI in the NIH test in CREB$^{\alpha\Delta}$ mutant mice could be interpreted as an anxiolytic-like response. Caveats associated with constitutive knock-out mouse models range from unexpected compensation, effects on other gene products throughout development, and altered endocrine and neuronal feedback. These types of alterations in the CREB$^{\alpha\Delta}$ mutant mice could result in a modification of pharmacological profiles of classic AD. To determine whether DMI is exerting anxiolytic effects in CREB$^{\alpha\Delta}$ mutant mice, we examined its effects on WT and CREB$^{\alpha\Delta}$ mutant mice in a second test for anxiety, the elevated zero maze. Few studies have examined the response to acute or chronic AD treatment in the elevated plus and elevated zero maze and results have been highly variable. Depending on the test used and the AD administered, many studies have reported no effect or an anxiogenic effect (Borsini et al., 2002). Our results replicate the lack of efficacy of acute DMI treatment in the elevated zero maze in WT mice. Moreover, DMI is not effective in reducing anxiety in CREB$^{\alpha\Delta}$ mutant mice, suggesting the acute action of DMI in the NIH test may be more related to the antidepressant responsive aspects of this paradigm.

AD effects in the NIH paradigm may require cellular changes in response to AD treatment as well. In the NSF paradigm, it has been shown that neurogenesis is necessary for a behavioral response to AD treatment (Santarelli et al., 2003). Thus, the increased levels of neurogenesis in CREB$^{\alpha\Delta}$ mutant mice may provide a mechanism for the rapid onset of response to treatment effects in these mice. Without the usual requirement of increasing neurogenesis, AD drugs may exert behavioral effects more rapidly in an animal that has been "primed" to respond to these compounds. Although CREB activation after AD treatment may

serve to initiate mechanisms that underlie the lengthy time course needed to achieve the behavioral effect in WT mice, under conditions in which CREB is constitutively deleted these mechanisms are already engaged. Our findings have potential clinical implications, because the delayed onset of a behavioral response to AD treatment poses a challenge. Identification of mechanisms through which CREB influences neurogenesis and the behavioral response to AD treatment may identify new targets for faster-acting therapies for depression.

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