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Localization of Phosphorylated cAMP Response Element-Binding Protein in Immature Neurons of Adult Hippocampus

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Neurogenesis continues to occur in the adult hippocampus, although many of the newborn cells degenerate 1–2 weeks after birth. The number and survival of newborn cells are regulated by a variety of environmental stimuli, but very little is known about the intracellular signal transduction pathways that control adult neurogenesis. In the present study, we examine the expression of the phosphorylated cAMP response element-binding protein (pCREB) in immature neurons in adult hippocampus and the role of the cAMP cascade in the survival of new neurons. The results demonstrate that virtually all immature neurons, identified by triple immunohistochemistry for bromodeoxyuridine (BrdU) and polysialic acid-neural cell adhesion molecule (PSA-NCAM), are also positive for pCREB. In addition, upregulation of cAMP (via pharmacological inhibition of cAMP breakdown or by antidepressant treatment) increases the survival of BrdU-positive cells. A possible role for pCREB in the regulation of PSA-NCAM, a marker of immature neurons involved in neuronal remodeling and neurite outgrowth, is supported by cell culture studies demonstrating that the cAMP–CREB pathway regulates the expression of a rate-limiting enzyme responsible for the synthesis of PSA-NCAM. These findings indicate that the cAMP–CREB pathway regulates the survival, and possibly the differentiation and function, of newborn neurons.

Key words: dentate gyrus; neurogenesis; rolipram; PSA-NCAM; TUC-4; polysialyltransferases; antidepressant

The occurrence of neurogenesis has been well established in the adult brain of a variety of animal species, including humans (Gould et al., 1999; Gage, 2000; Duman et al., 2001). Proliferation of neurons is observed most notably in the subventricular zone, which gives rise to new neurons that migrate to the olfactory bulb, and the subgranular zone of the hippocampus, which gives rise to granule cells in adult dentate gryus. Moreover, the proliferation and survival of newborn cells in the hippocampus are highly regulated by a variety of environmental, endocrine, and pharmacological stimuli (Gould et al., 1999; Gage, 2000; Duman et al., 2001). For example, the proliferation of new neurons in the hippocampus is upregulated by exercise, estrogen, and antidepressant treatment and downregulated by stress, aging, glucocorticoids, and opiates (for review, see Duman et al., 2001).

The majority of newborn cells (~80%) in the adult hippocampus differentiate into cells that express neuronal phenotypic markers. The new neurons migrate into the granule cell layer (GCL), extend dendrites and axons, and integrate into the existing hippocampal circuitry (van Praag et al., 2000; Cameron and McKay, 2001). The immature neurons that extend processes during this time express polysialic acid-neural cell adhesion molecule (PSA-NCAM), a marker of immature neurons and of neurons undergoing remodeling and plasticity (Kiss and Rougon, 1997). Although many of the newborn cells survive and become mature granule cells, 50–60% of the cells undergo a process of degeneration by 4–5 weeks after cell division (Gould et al., 1999; Biebl et al., 2000; Cameron and McKay, 2001). The survival of newborn neurons is increased by enriched environment and hippocampal-dependent learning, demonstrating that survival, as well as proliferation, of newborn cells in hippocampus can be regulated by behavioral and environmental factors (Kempermann et al., 1997; Gould et al., 1999). However, the intracellular signaling pathways that control the survival of newborn neurons in adult hippocampus have not been determined.

There is evidence to support a role for the cAMP second-messenger cascade and the cAMP response element-binding protein (CREB) in the differentiation and survival, as well as proliferation, of new neurons in adult hippocampus. First, studies in cultured cells demonstrate that activation of the cAMP second-messenger pathway influences the differentiation of progenitor cells (Herman et al., 1994; Satoh et al., 1994; Palmer et al., 1997). Second, CREB is a target of neurotrophic factor-stimulated signaling, as well as the cAMP and Ca^{2+} signaling pathways, and is required for neurotrophic factor-dependent survival of cultured neurons (Bonni et al., 1999; Riccio et al., 1999). Third, in a recent study, we report that activation of the cAMP–CREB cascade increases the proliferation of newborn cells in the adult hippocampus (Nakagawa et al., 2000).

In the present study, we examine the role of the cAMP–CREB pathway on the survival of newborn cells. First, the relationship between phosphorylated CREB (pCREB), the active form of this transcription factor, and maturing newborn cells is examined by colocalization with bromodeoxyuridine (BrdU) and PSA-NCAM in hippocampal cells. Second, the influence of the cAMP–CREB cascade on the survival of BrdU-labeled cells in adult hippocampus is examined. Rolipram, a selective inhibitor of the high-

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affinity cAMP-selective phosphodiesterases type IV (PDE4), is used to activate the cAMP–CREB cascade (Conti and Jin, 2000). In addition, the influence of another drug reported to upregulate CREB function and expression, the 5-hydroxytryptamine-selective reuptake inhibitor fluoxetine, is also tested (Nibuya et al., 1996; Thome et al., 2000). Finally, the relationship between pCREB and expression of PSA-NCAM is directly examined in cultured cells. The results demonstrate that pCREB and PSA-NCAM are colocalized in newborn cells during neuronal maturation and that activation of the cAMP–CREB pathway increases cell survival.

MATERIALS AND METHODS

Animals and drug treatment. Male C57BL/6 mice, 10 weeks old (Charles River Laboratories, Wilmington, MA), were used for the study. Mice ($n = 6$ per group) were administered BrdU (75 mg/kg, i.p.; Sigma, St. Louis, MO) once and killed 2 hr or 1, 2, 3, or 4 weeks later to determine the expression of pCREB in the developing newly born cells. Five animals were used at each time point. To evaluate the effect of activation of the cAMP–CREB cascade on survival of cells in hippocampus, mice were administered rolipram, an inhibitor of PDE4. Animals were administered BrdU (three times at 2 hr intervals). One week later, the mice were administered either saline containing 2% DMSO as control ($n = 12$) or rolipram (1.25 mg/kg, i. p.; Sigma) in saline containing 2% DMSO ($n = 12$) once daily for 3 weeks. Animals were killed 24 hr after the last treatment, and brains were harvested. To determine the influence of antidepressant treatment, animals were administered a serotonin selective reuptake inhibitor reported to upregulate the cAMP–CREB cascade (Nibuya et al., 1996; Thome et al., 2000). For these studies, male Sprague Dawley rats (150–175 gm) were administered vehicle (saline) or fluoxetine (5 mg/kg, i.p.) once daily for 21 d, and animals were decapitated 24 hr after the last treatment. Rats were used for the fluoxetine study because our previous work with this drug has been in this species (Malberg et al., 2000). All animal procedures were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Yale Animal Care and Use Committee.

Immunohistochemistry. All mice were killed via intracardial perfusion with 4% paraformaldehyde under anesthetic with sodium pentobarbital (100 mg/kg, i.p.). A freezing microtome was used to collect serial coronal 30 μm sections through the hippocampus. For immunoperoxidase staining of CREB or pCREB (see Fig. 1), free-floating sections were incubated in TBS–0.1% Triton X-100–3% normal goat serum (TBS-Tds) for 60 min and then with primary antibodies in TBS-Tds overnight at 4°C. Primary antibodies used were rabbit anti-CREB IgG (1:1000; Upstate Biotechnology, Lake Placid, NY), or rabbit anti-pCREB IgG (1:500; New England Biolabs, Beverly, MA). The sections were incubated in biotinylated rabbit secondary antisera (1:200; Vector Laboratories, Burlingame, CA) for 60 min, incubated in avidin–biotin–horseradish peroxidase (1:50; Vector Laboratories) for 60 min, and reacted in the solution of 3,3′-diaminobentidine containing nickel ammonium sulfate (Vector Laboratories). For immunoperoxidase staining of BrdU (see Figs. 5*a*, 6), every ninth section was slide mounted. The sections were incubated in 0.01 M citric acid at 90°C, digested in trypsin (0.1%) in Tris buffer containing 0.1% CaCl2 for 10 min, denatured in 2N HCl for 30 min, blocked in 3% normal horse serum for 20 min, and incubated overnight at 4°C in mouse monoclonal antibody against BrdU (1:100; Becton Dickinson, San Jose, CA) in PBS containing 3% normal horse serum and 0.1% Tween 20. On the next day, the sections were incubated in biotinylated mouse secondary antisera (1:200; Vector Laboratories) for 60 min following the same steps as described above. The sections were counterstained with cresyl violet.

For immunofluorescence staining (see Figs. 2–5*b*), free-floating sections were used. To denature DNA, sections were incubated in 50% formamide–2× SSC (0.3 M NaCl and 0.03 M sodium citrate) at 65°C, rinsed for 5 min in 2× SSC, incubated for 30 min in 2N HCl at 37°C, and then rinsed for 10 min in 0.1 M boric acid, pH 8.5. Sections were incubated in TBS-Tds for 30 min and then with primary antibodies in TBS-Tds for 1–3 d at 4°C. The primary antibodies used were as follows: rat anti-BrdU IgG, 1:100 (Harlan Sera Lab, Loughborough, UK); rabbit anti-CREB IgG, 1:300 (Upstate Biotechnology); rabbit anti-pCREB IgG, 1:400 (New England Biolabs); mouse anti-PSA-NCAM IgM, 1:400 (gift from G. Rougon, Centre National de la Recherche Scientifique, Paris, France); goat anti-pCREB IgG (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-TOAD-64/Ulip/CRMP-4 (TUC-4) IgG, 1:1000 (gift from S. Hockfield, Yale University); mouse anti-neuronal-specific nuclear protein (NeuN) IgG, 1:100 (Chemicon, Temecula, CA); and rabbit anti-S100β IgG, 1:2500 (Swant, Bellinoza, Switzerland). The fluorescent secondary antibodies used were anti rat FITC or Cy5, anti-rabbit FITC or Cy3, anti-mouse FITC or Cy5, and anti-goat Cy3 (1:200; Jackson ImmunoResearch, West Grove, PA). After drying sections in the dark boxes for 2 hr, they were mounted with Vectashield (Vector Laboratories). Several different titers of each antibody were tested to determine the concentration for optimal signal-to-noise results. The antibodies used in this study have been used extensively in previous work and demonstrated to be selective for the antigens indicated (Muller et al., 1996; Kempermann et al., 1997; Bonni et al., 1999; Gould et al., 1999, 2001; Cameron and McKay, 2001).

Analysis of BrdU, pCREB, and PSA-NCAM colocalization. A one-in-nine series of sections from each animal surviving 2 hr or 1, 2, 3, or 4 weeks ($n = 5$ per time point) after the injection of BrdU were triple labeled for BrdU, pCREB, and PSA-NCAM as described above and analyzed by confocal laser microscopy (LSM 510; Zeiss, Oberkochen, Germany). Fifty BrdU-positive cells were randomly identified from sections throughout the septotemporal axis for each animal and analyzed for coexpression of pCREB and/or PSA-NCAM. The number of cells in each of two categories was determined: BrdU plus pCREB and BrdU plus pCREB plus PSA-NCAM. The total number of cells counted was 50 per animal, five animals per group, for a total of 250 cells at each time point. The number of cells in each category for each animal was determined, and the mean ± SEM was calculated for each group. From these data, the percentage of cells in each category was calculated. This method has been used routinely to analyze the cellular phenotype of BrdU-labeled cells using markers of either neurons or glia (Kempermann et al., 1997; Gould et al., 1999, 2001; Eisch et al., 2000; Malberg et al., 2000; Cameron and McKay, 2001). We adapted this procedure in the present study to determine the number of BrdU-positive cells that have a pCREB and/or PSA-NCAM phenotype at different time points. The results obtained in this study are susceptible to variation inherent in the immunohistochemical technique (e.g., problems with altered thickness of sections attributable to fixation and penetration of antisera). These problems do not allow for absolute measurements of the number of double- or triple-labeled cells per volume of tissue. However, because the tissue sections from the different time points are prepared simultaneously, the comparative results within each experiment are valid, and the interpretation is justified.

Quantitation of BrdU-labeled cells. All BrdU-positive cells in both the rolipram and fluoxetine studies were counted using a modified stereology protocol that has been used previously to quantify BrdU labeling (West et al., 1991; Gould et al., 1999; Eisch et al., 2000, Malberg et al., 2000). The method used takes into account two major considerations in stereological analysis: that no BrdU-labeled cells are counted twice and that the area counted is consistent in each section. In the present study, every ninth section throughout the entire hippocampus was processed for BrdU immunohistochemistry. This spacing ensures that the same cell is not counted twice. All BrdU-labeled cells in the granule cell layer and hilus were counted by an experimenter that was blinded to the code. All BrdU-positive cells regardless of size or shape were counted through a 100× objective to distinguish single cells. Cells were counted as being in the subgranular zone (SGZ) if they were touching or in the SGZ. Cells that were located more than two cells away from the SGZ were counted as in the hilus. The numbers of labeled and counted cells in the granule cell layer or hilus were multiplied by nine and reported as the total number of cells per region. Statistical analysis of the data resulting from the cell counts was conducted by use of the Student t test ($p < 0.05$).

To determine the phenotype of newborn cells, a one-in-nine series of sections from control and rolipram-treated animals surviving 4 weeks after the injection of BrdU were triple labeled for colocalization of BrdU with NeuN and S100β, markers of neurons or glia, respectively. Fifty BrdU-positive cells were randomly identified from sections throughout the septotemporal axis for each of six rolipram-treated and six control mice. Ratios of BrdU-positive cells colabeled with NeuN, S100β, or neither of these phenotypic markers were determined.

Plasmid constructs. Reporter gene constructs were generated for use in transfection experiments by subcloning selected regions of mST8Sia II (STX) promoter upstream of the luciferase reporter in the pPicaGene-Basic II (pPGBII; Tokyo-ink, Tokyo, Japan) (Yoshida et al., 1996). All constructs terminated at base +167 bp from the cDNA start site (a *Nco*I

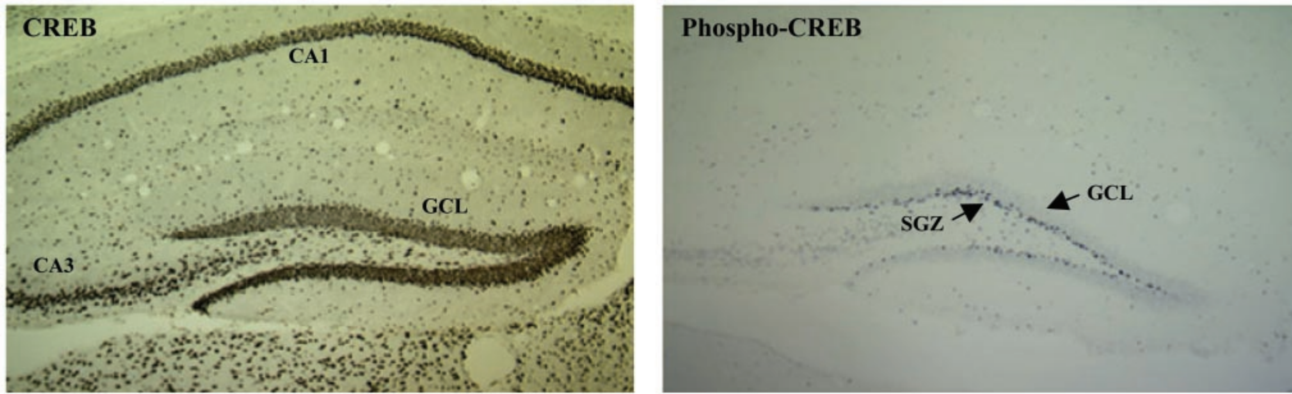

Figure 1. The distribution of CREB (*a*) and phosphorylated CREB (*b*) in the adult mouse dentate gyrus. CREB is phosphorylated constitutively in the cells near the subgranular zone, whereas CREB is expressed in almost all cells of hippocampus, including the CA1 and CA3 pyramidal cell layers and the dentate gyrus granule cell layer.

site). Construction began at −5400 (pBO1-RN5.5), −3400 (pBO1-NhN3.5), −1645 (pBO1-EN1.8), −659 (pBO1-BN0.8), −293 (pBO1-SN0.45), and −9 bp (pBO1-XN0.15) from the transcription start (+1).

Transient transfection assays. PC12 cells were grown in Roswell Park Memorial Institute medium containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 μU/ml penicillin at 37°C in a humidified atmosphere at 5% CO_2. Medium was changed every second day. All reagents for cell culture media were obtained from Invitrogen (Grand Island, NY). Cells were grown to 50–60% confluency for the transfection experiments in 6 × 35 mm plates. The luciferase plasmid (2.5 μg) used as the reporter and the pSRβ-Gal plasmid (1.5 μg), which carried a β-galactosidase gene under the control of the SRα promoter, used as an insertional control for transfection efficiency, were transfected into the cells by means of Lipofectamine (Invitrogen). After 6 hr transfection, the medium was replaced. After 48 hr incubation, forskolin (10 μM) or vehicle was added to the cells. Four hours after the drug treatment, cells were harvested and lysed in 200 μl of lysis buffer (Boehringer Mannheim, Indianapolis, IN). Luciferase assays were performed according to the protocol of the manufacturer (Boehringer Mannheim). Light activity measurements were performed in triplicate with a luminometer. To determine the influence of the dominant negative mutant of CREB (mCREB) on STX promoter activity, the pBO1-BN0.8 construct (2.5 μg) that contained the putative CRE, pSRβ-Gal plasmid (1 μg) and a construct containing mCREB (1 μg) were cotransfected to PC12 cells. The cells were incubated with forskolin or vehicle, and luciferase activity was determined as described above. β-Galactosidase activity was simultaneously measured by the β-gal reporter gene assay system (Roche, Mannheim, Germany), and luciferase activity was normalized to the β-gal activity. The data are expressed as the percentage in enzyme activity compared with transfections using a promoterless luciferase plasmid, pPGBII. Results are the average of three to six separate experiments each performed in duplicate.

RESULTS

Colocalization of pCREB and PSA-NCAM in newborn cells in hippocampus

Previously, we observed that, in unstimulated animals, immunostaining of pCREB is restricted to a specific subregion of the granule cell layer of adult mouse hippocampus (Thome et al., 2000). In the present study, we confirmed this finding, demonstrating that immunoperoxidase staining using a pCREB-specific antibody is localized in cells along the innermost region of the GCL adjacent to the hilus (Fig. 1*b*). In contrast, when using an antibody for total CREB protein, most cells in the GCL of hippocampus are stained, demonstrating that the localized expression of pCREB is not because of restricted expression of CREB (Fig. 1*a*).

The region of the GCL in which the pCREB labeling is observed is also the region in which neurogenesis occurs in adult hippocampus (Gould et al., 1999; Gage, 2000). To examine the relationship between neurogenesis and pCREB, newborn cells were visualized by immunohistochemical analysis of BrdU, a thymidine analog that is incorporated into the nuclei of dividing cells. Expression of pCREB in the newborn cells was examined by immunofluorescence analysis of BrdU and CREB or pCREB at various times (2 hr and 1, 2, or 4 weeks) after BrdU injection. Double labeling for BrdU and CREB 2 hr after BrdU injection demonstrates that the BrdU-positive cells are restricted to the subgranular zone between the GCL and hilus and are lightly stained with the CREB antibody (Fig. 2*a–c*). At the 2 hr time point, none of the BrdU-positive cells are stained with the pCREB antibody (Fig. 2*d, f, g*).

Although newborn cells labeled 2 hr after BrdU administration do not express pCREB, it was clear that the pCREB-positive cells are aligned very close to or in the subgranular zone in which the newborn, BrdU-positive cells are localized. To further assess the types of cells expressing pCREB, sections were also analyzed for levels of PSA-NCAM, a marker of immature neurons, which is found on the surface of cell bodies, dendrites, and axons. PSA-NCAM is expressed by immature neurons during development and by immature neurons in adult brain or by mature neurons undergoing remodeling (Kiss and Rougon, 1997). At the 2 hr time point, the BrdU-positive cells are not labeled with PSA-NCAM (Fig. 2*d, e, g*). However, pCREB and PSA-NCAM double-positive cells are present in the sections taken 2 hr after BrdU administration, but these cells are not positive for BrdU at this time point (Fig. 2*e–g*).

One to 2 weeks after BrdU injection, a different pattern of staining is observed. The BrdU-positive cells are localized to the innermost region of GCL and are colabeled with pCREB, as well as PSA-NCAM (Fig. 2*h–k*). This demonstrates colocalization of pCREB expression in the immature, newborn cells. At the 4 week time point, when the BrdU-positive cells reach a more mature stage, the pattern of staining shifts once again. At this time point, most of the BrdU-positive cells have migrated into the GCL and are no longer stained with either pCREB or PSA-NCAM antibodies (Fig. 2*l–o*). The BrdU-positive cells appear to acquire the phenotype of mature granule cells that do not express pCREB (Fig. 1).

The numbers of pCREB- and/or PSA-NCAM-stained cells colocalized with BrdU-positive cells were analyzed at various time points after BrdU injection by double and triple immuno-

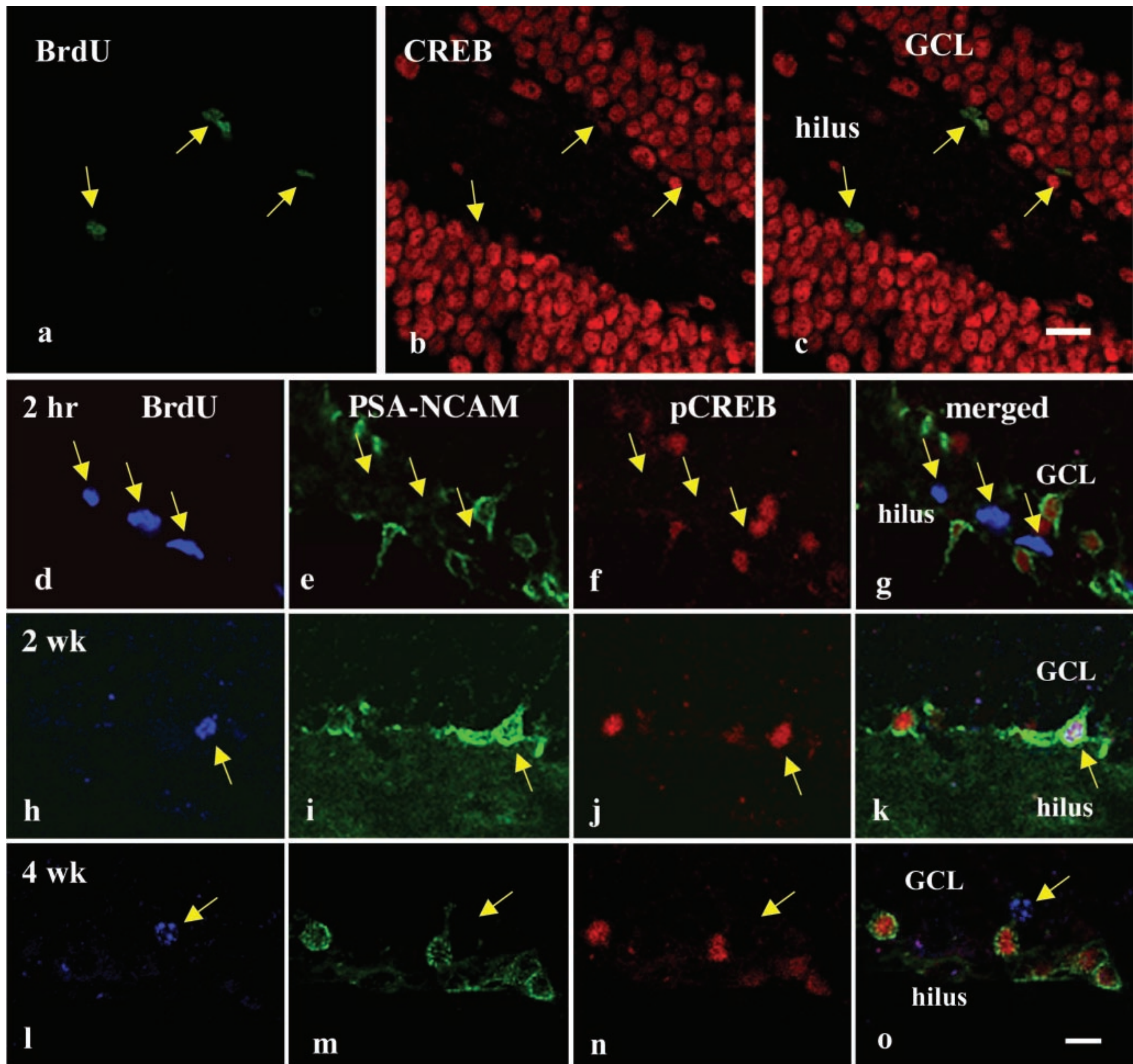

Figure 2. Colocalization of CREB or pCREB and PSA-NCAM in the developing BrdU-positive cells. *a–c*, *Top*, Representative confocal micrographs depicting double immunolabeling for BrdU (*green*) and CREB (*red*) 2 hr after BrdU injection. The *panels* below depict triple immunostaining for BrdU (*blue*), PSA-NCAM (*green*), and pCREB (*red*) at different time points after BrdU injection: 2 hr (*d–g*), 2 weeks (*h–k*), or 4 weeks (*l–o*). *Yellow arrows*, Localization of BrdU-positive cells. Note that BrdU-positive cells migrate from the subgranular zone into granule cell layer at the later time points and transiently express pCREB and PSA-NCAM at the 2 week, but not the 2 hr or 4 week, time point. Scale bars: *c*, *o*, 20 μm.

fluorescence (Fig. 3). For this study, 50 BrdU-positive cells were examined in each animal ($n = 5$ animals per group) for a total of 250 cells analyzed per time point. The results are presented as the percentage of cells out of 250 that are positive for two or three of the markers. Two hours after BrdU injection, there were no double- or triple-labeled cells in any of the sections examined as described in Figure 2. In contrast, at 1, 2, or 3 weeks after BrdU injection, the majority of BrdU-positive cells were costained with pCREB and PSA-NCAM antibodies (61.2 ± 1.5% for 1 week; 66 ± 3.2% for 2 weeks; 59.6 ± 2.1% for 3 weeks; mean ± SEM; $n = 5$ per group), and, 4 weeks later, the ratio of triple-labeled cells decreased (means of 18.8 ± 2.9%). The ratio of cells double labeled with only BrdU and pCREB was very low at all time points examined (0% for 2 hr; 0.8 ± 0.8% for 1 week; 3.6 ± 1.7% for 2 weeks; 3.2 ± 0.8% for 3 weeks; 0.8 ± 0.5% for 4 weeks; mean ± SEM), and there were no cells observed that were double labeled for only BrdU and PSA-NCAM.

To confirm the presence of CREB phosphorylation in newborn cells during an immature stage, triple-labeled immunohistochemistry was conducted for another marker of immature neurons, TUC-4, as well as BrdU and pCREB (Fig. 4). TUC-4 has homology with *unc-33*, a *Caenorhabiditis elegans* gene, and has been implicated in axonal outgrowth and guidance (Quinn et al., 1999). Immunolabeling for TUC-4 is seen in the cytosol of cell bodies, dendrites, and axons as reported previously (Quinn et al., 1999). The time course of the triple-labeling pattern of TUC-4, pCREB,

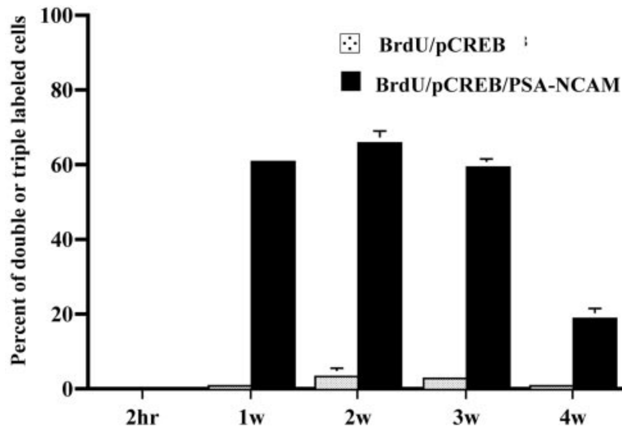

Figure 3. Time course for colocalization of pCREB and/or PSA-NCAM in BrdU-positive cells in the adult hippocampus. Triple immunolabeling was conducted as described in Materials and Methods and Figure 2. The number of cells expressing pCREB and BrdU or pCREB, BrdU, and PSA-NCAM was determined by identifying 50 BrdU-positive cells for each animal ($n = 5$) at each time point. The *x*-axis indicates the time after BrdU injection. The results are presented as percentage of double- or triple-labeled cells and are the mean ± SEM ($n = 250$ at each time point).

and BrdU is similar to that observed when using PSA-NCAM as a marker for immature neurons. Two weeks, but not 2 hr, after BrdU injection, most of the BrdU-positive cells are also positive for TUC-4 and pCREB antibodies. Almost all TUC-4-positive cells also express PSA-NCAM, although there is not a complete overlap of the staining (data not shown).

Influence of the cAMP cascade on the survival of BrdU-labeled cells

We next investigated the effect of pharmacological activation of the cAMP–CREB cascade on the survival of newborn cells. The agent that we used was rolipram, an inhibitor of the high-affinity, cAMP-specific PDE4, a family of enzymes responsible for breakdown of cAMP (Conti and Jin, 2000). We found that rolipram administration increases the phosphorylation of CREB and increases the proliferation of granule cells in adult mouse hippocampus (Nakagawa et al., 2000). In the present study, mice were given BrdU, followed by chronic rolipram administration during the maturation–survival stage of the BrdU-labeled cells (i.e., starting 4 d after BrdU administration and continuing for 4 consecutive weeks). In rolipram-treated animals, the number of BrdU-positive cells in the GCL was 2520 ± 155 (mean ± SEM) cells per bilateral dentate gyri (BDG) compared with 1902 ± 149 cells per BDG in controls, which corresponds to a 32% increase ($p < 0.01$) (Fig. 5*a*). In the hilus, the number of BrdU-positive cells was 750 ± 72 cells per BDG in rolipram-treated animals compared with 581 ± 65 cells per BDG in controls. To examine the phenotype of the BrdU-positive cells, triple labeling for BrdU and markers for either neurons (NeuN) or for glia (S100β) was performed (Fig. 5*b*). Confocal microscopic analysis demonstrates that the majority of the BrdU-positive cells are positive for NeuN (78 ± 3.9 and 73.7 ± 3.7% for control and rolipram-treated animals, respectively; mean ± SEM) compared with those that were positive for S100β (8.7 ± 1.3 and 13.3 ± 2.1% for control and rolipram-treated animals, respectively; mean ± SEM). There

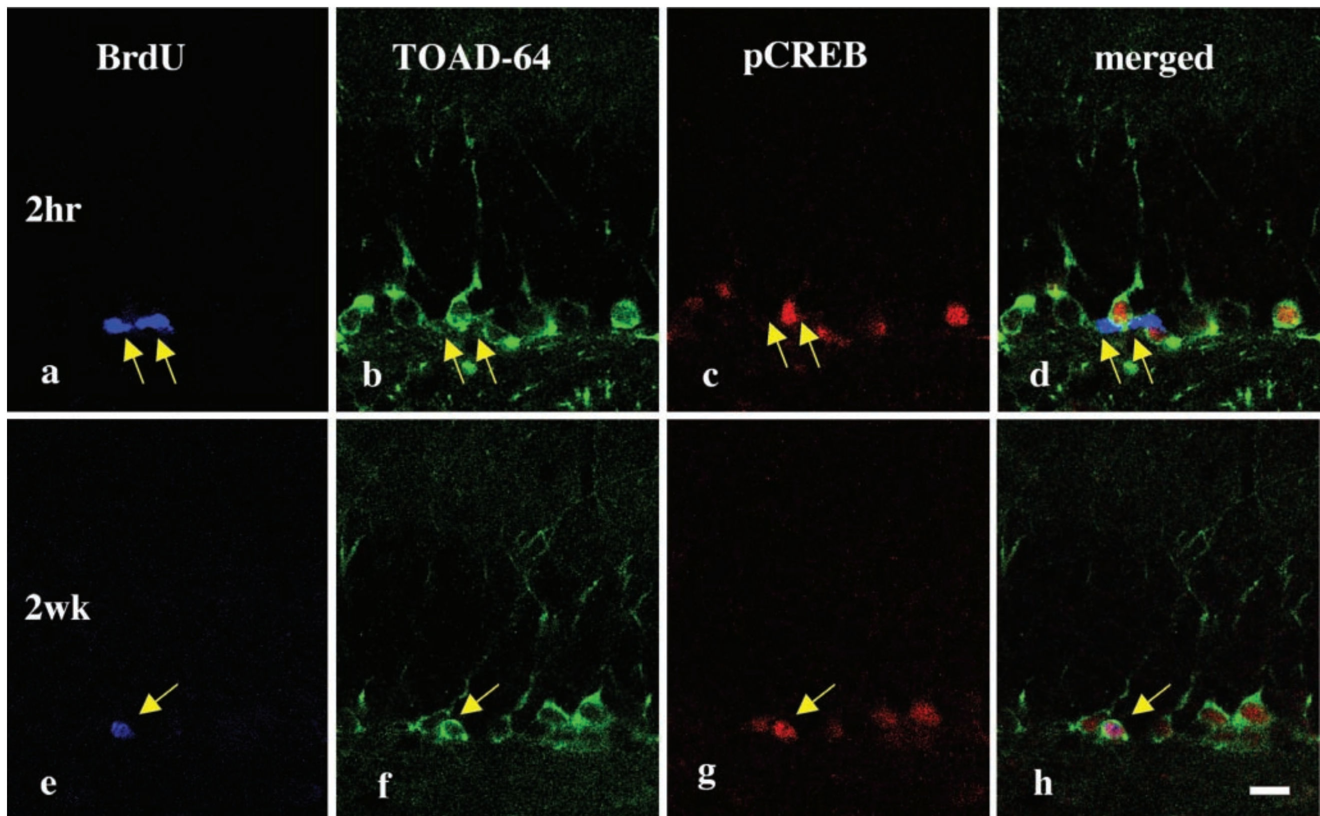

Figure 4. Colocaliation of TUC-4 and pCREB in the developing BrdU-positive cells. Representative confocal micrographs are shown for triple labeling of BrdU (*blue*), TUC-4 (*green*; *TOAD-64*), and pCREB (*red*) at different time points after BrdU injections: 2 hr (*a–d*) or 2 weeks (*e*, *f*). *Yellow arrows*, Localization of BrdU-positive cells. Two weeks after injection, BrdU-positive cells are colabeled with TUC-4 and pCREB. Scale bar, 20 μm.

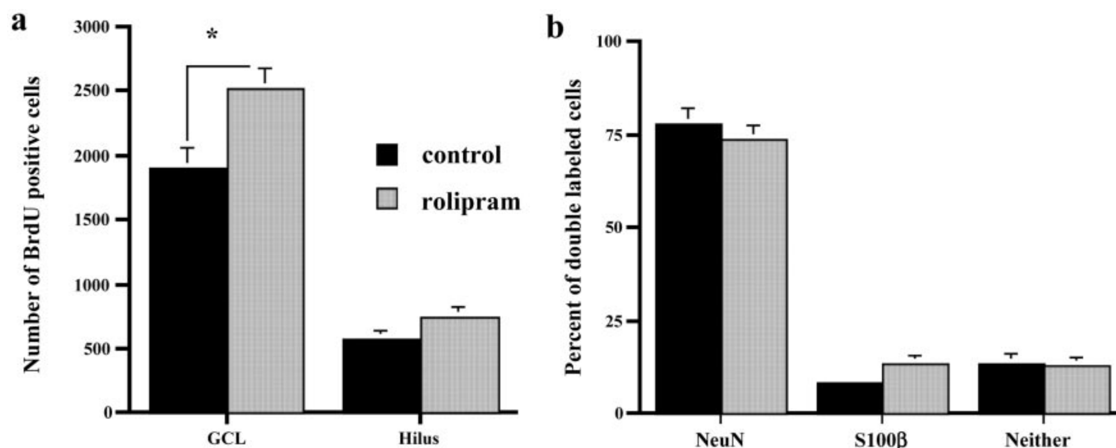

Figure 5. Influence of rolipram administration on the number (*a*) and phenotype (*b*) of surviving BrdU-positive cells in the adult hippocampus. Mice were injected with BrdU on 4 consecutive days to label newborn cells. One week after the beginning of BrdU injections (3 d after the last injection), animals were administered rolipram once daily for 3 weeks as described in Materials and Methods. *a*, The number of BrdU-positive cells throughout the entire hippocampus was determined using a modified unbiased stereological procedure. The results are expressed as the number of BrdU-positive cells in the granule cell layer or hilus of the bilateral dentate gyrus (mean ± SEM). $^*p < 0.01$ compared with vehicle-treated controls (Student's *t* test). *b*, The percentage of surviving BrdU-positive cells stained with NeuN, a marker of mature neurons, or S100β, a glial marker, was also determined. There was no significant difference in the phenotype of the BrdU-positive cells between control and chronic rolipram-treated mice.

was no significant difference between the phenotypic characteristics of the two groups.

We also reported that chronic antidepressant administration increases CREB expression and pCREB immunostaining in the granule cell layer (Nibuya et al., 1996; Thome et al., 2000), suggesting that antidepressant treatment may also increase the survival of BrdU-positive cells. To examine this possibility, animals were injected with BrdU and then administered the serotonin-selective reuptake inhibitor fluoxetine for 4 weeks. Fluoxetine treatment significantly increased the number of BrdU-labeled cells in the granule cell layer, an effect similar to that observed with chronic rolipram administration (Fig. 6). This effect is different from what was observed in a previous study in which we reported that 2 weeks of fluoxetine treatment immediately after BrdU administration, followed by 2 weeks without treatment, were not sufficient to increase survival measured a total of 4 weeks after BrdU (Malberg et al., 2000). We attribute the significant effect observed in the present study to the extended administration of fluoxetine during the 4 week period after cell birth that may be critical to survival.

Influence of the cAMP–CREB cascade on expression of polysialic acid synthase

The colocalization studies suggest that there may be a functional relationship between pCREB and PSA-NCAM. One possibility is that CREB is involved in the expression of enzymes, polysialic acid synthases, responsible for the synthesis of PSA-NCAM. The STX and ST8SiaIV genes encode the two major polysialic acid synthases expressed in brain. To directly examine the potential relationship between CREB and expression of these two polysialic acid synthases, we conducted gene expression studies using reporter assays in transfected cells. Because of the enrichment of STX in cells adjacent to the subgranular layer, we focused our studies on the promoter for this gene (Hildebrandt et al., 1998). In addition, analysis of the STX promoter sequence has revealed a putative cAMP response element in the region proximal to the initiation start site. The sequence of this putative site is TGACCTCA, which only differs from the consensus CRE by one nucleotide substitution (TGACGTCA). We used a series of STX

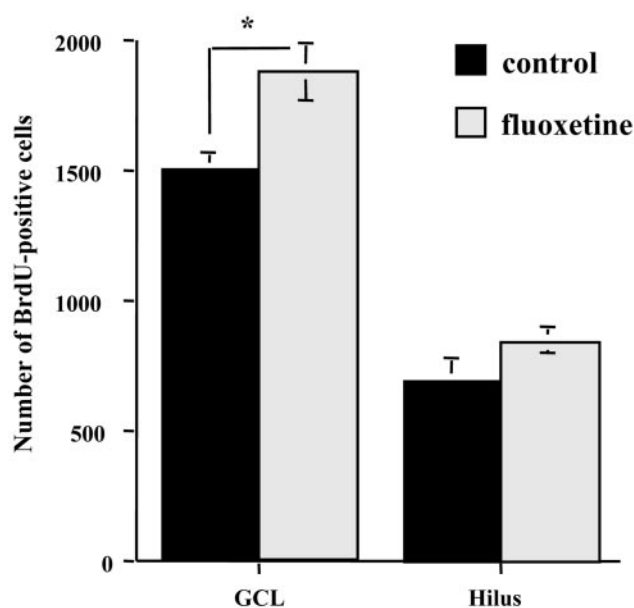

Figure 6. Influence of chronic fluoxetine administration on the number of surviving BrdU-labeled cells in hippocampus. Animals were administered BrdU and were then treated with either vehicle or fluoxetine for 4 weeks as indicated. Analysis of the number of BrdU-positive cells was conducted 4 weeks after the first BrdU administration. The number of BrdU-positive cells throughout the entire hippocampus was determined using a modified stereological procedure as described in Materials and Methods. The results are expressed as the mean ± SEM number of BrdU-positive cells in the granule cell layer or hilus of the entire dentate gyrus. $^*p < 0.01$ compared with vehicle-treated controls (Student's *t* test).

deletion constructs (Yoshida et al., 1996; Takashima et al., 1998), transfected into PC12 cells, to examine the regulation of STX promoter activity by the cAMP–CREB cascade. PC12 cells are commonly used for gene reporter studies and were chosen for the promoter analysis of STX.

The results demonstrate that incubation of cells with forskolin, which stimulates adenylyl cyclase and intracellular levels of

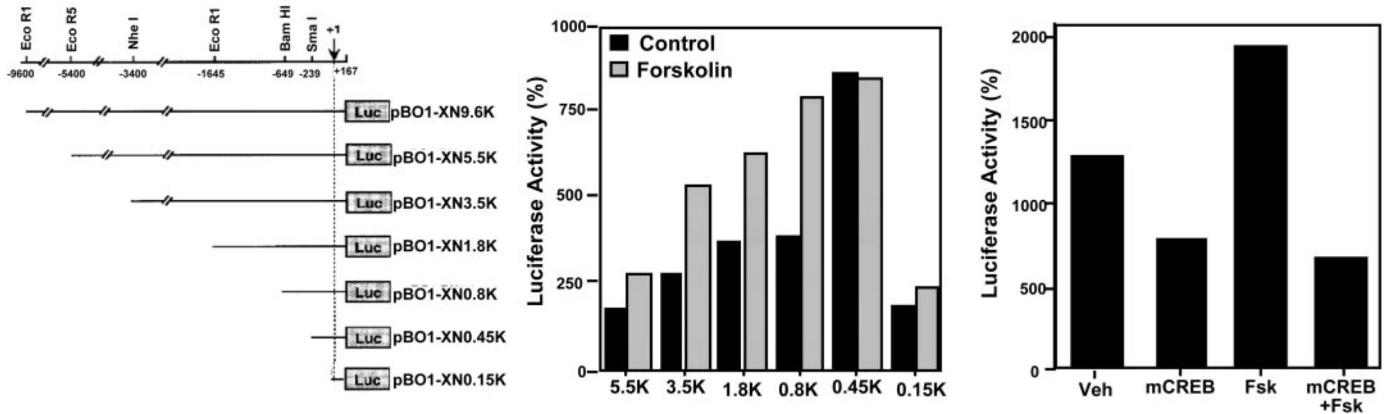

Figure 7. Influence of the cAMP–CREB cascade on STX promoter activity. Deletion mutants of the STX promoter, shown in *a*, were transfected into PC12 cells, which were then incubated with either vehicle (*Control*) or forskolin (10 μM), and levels of luciferase reporter activity were determined (*b*). The influence of mCREB on STX promoter activity was determined (*c*). The 0.8 kb construct, which contains the putative CRE, and a construct containing mCREB were cotransfected into PC12 cells. The cells were incubated with vehicle (*Control*) or forskolin, and levels of luciferase activity were determined. The results (*b*, *c*) are expressed as a percentage of the activity of the control plasmid (lacking insert) and are the mean of three separate experiments, each performed in duplicate.

cAMP, increases STX promoter-induced luciferase activity (Fig. 7). Forskolin activation of the STX promoter is observed in the promoter constructs that contain the putative CRE (5.5, 3.5, 1.8, and 0.8 kb) but not in those lacking this element (0.45 and 0.15 kb). In contrast, preliminary studies indicate that forskolin does not regulate the promoter activity of ST8SiaIV, the other major polysialic acid synthase expressed in adult brain (data not shown). The role of CREB, or other CREB-like transcription factors, was examined by determining the influence of a dominant negative mutant of CREB (mCREB), a Ser^{133} to Ala phosphorylation mutant, on STX promoter activity. We found that overexpression of mCREB in PC12 cells significantly reduces the level of promoter activity in cells that are incubated with vehicle or forskolin (Fig. 7).

DISCUSSION

In a recent study, we observed that pCREB immunostaining is localized in a region close to or in the subgranular zone of the hippocampus (Thome et al., 2000). A similar pattern of pCREB immunostaining has been observed during development and in adult hippocampus (Young et al., 1999; Bender et al., 2001). The purpose of the current investigation was to extend these previous findings on the cAMP–CREB cascade and regulation of neurogenesis in adult hippocampus. The results confirm our previous observations and demonstrate that pCREB immunostaining is colocalized with BrdU in immature neurons during a specific time period of cell maturation and survival. Triple-labeling studies demonstrate that the majority of BrdU- and pCREB-positive cells (60–65%) are also positive for PSA-NCAM when analyzed 1–3 weeks after BrdU administration. In contrast, when analyzed just 2 hr after BrdU administration, none of the BrdU-positive cells are positive for pCREB, and, when analyzed 4 weeks after BrdU, the number of triple-labeled cells is significantly decreased relative to the 3 week time point (15–20%). Similar results are obtained when the triple labeling is conducted with another marker of immature neurons, TUC-4, in place of PSA-NCAM, providing additional support for the localization of pCREB in immature neurons.

The time course for the expression of pCREB and PSA-NCAM or TUC-4 staining in the BrdU-positive cells correlates with the maturation, differentiation, and survival phase of the newborn cells (Gould et al., 1999; Gage, 2000; Cameron and McKay, 2001). Newborn cells in the subgranular zone first appear as irregularly shaped cells, are often found in clumps, and do not express markers of either neurons or glia. Over the next few days, the cells begin to change in shape, and, by 1 week, many of the cells extend processes and express cellular markers characteristic of immature neurons, such as PSA-NCAM and TUC-4. During this time (between 1 and 2 weeks), the total number of BrdU-positive cells is also dramatically reduced by ~50–60% (Gould et al., 1999; Biebl et al., 2000). As the surviving neurons become mature, with full extension of axons and dendrites, the expression of markers of mature neurons such as NeuN are observed in many of the BrdU-positive cells, whereas markers of immature neurons, PSA-NCAM and TUC-4, are no longer expressed (Gould et al., 1999; Cameron and McKay, 2001).

The time course for the expression of pCREB in BrdU-labeled neurons suggests that the activity of this transcription factor plays a role in the maturation, survival, and function of new neurons. The possibility that pCREB influences cell survival is particularly interesting because recent studies have demonstrated that CREB is required for neurotrophic factor-dependent survival of cultured neurons (Bonni et al., 1999; Riccio et al., 1999). In the present study, we used a pharmacological agent known to activate the cAMP–CREB cascade in the hippocampus (Nakagawa et al., 2000) to determine the influence of this second-messenger pathway on the survival of BrdU-labeled cells. The results demonstrate that chronic rolipram treatment significantly increases the number of surviving BrdU-positive cells analyzed 4 weeks after BrdU administration. Rolipram administration did not significantly influence the percentage of BrdU-positive cells that express markers of neurons (NeuN) or glia (S100β). In addition to rolipram, we also found that chronic administration of fluoxetine, a serotonin-selective reuptake inhibitor that increases pCREB in hippocampus (Thome et al., 2000), also increases the survival of BrdU-labeled cells. These findings indicate that activation of the cAMP–CRES pathway during the time when many newborn cells typically die increases the survival of these cells in the adult hippocampus. Combined with the finding that BrdU-positive,

immature neurons also express pCREB, these results also suggest that activation of CREB contributes to increased survival of the BrdU-labeled neurons, although there is no direct evidence to support this conclusion at the present time. We are currently developing inducible transgenic mice that overexpress CREB or a dominant negative mutant of CREB in newborn granule cells to directly test this possibility.

The upregulation of phosphorylated CREB during maturation could also influence the differentiation and function of newborn neurons in the adult hippocampus. Incubation of cultured progenitor cells with forskolin is reported to increase the number of NeuN-positive cells by over fivefold, suggesting that activation of the cAMP–CREB cascade increases the differentiation of newborn cells into neurons (Herman et al., 1994; Satoh et al., 1994; Palmer et al., 1997). In the present study, we found that nearly all of the BrdU-positive, immature neurons (i.e., those cells that express PSA-NCAM) are also positive for pCREB, suggesting that expression of pCREB contributes to the neuronal phenotype. In addition, it is also possible that pCREB influences the function of immature BrdU-positive cells. Activation of the cAMP–CREB cascade contributes to the formation of long-term potentiation and is suggested to play a role in learning and memory (Barad et al., 1998; Silva et al., 1998). In addition, the survival of BrdU-positive cells is upregulated by, and participates in, hippocampal-dependent learning (Gould et al., 1999; Shors et al., 2001). The immature neurons are already in a heightened state of plasticity as processes are being extended, and the expression of pCREB could contribute to an increased sensitivity of these neurons to the molecular events underlying new memory formation. A role for pCREB in the formation of synaptic contacts that are made by newborn neurons during development and in adult brain has also been suggested (Bender et al., 2001).

The expression of PSA-NCAM in immature neurons is consistent with the role of this molecule in mediating dynamic changes in the maturation of newborn neurons during development and in adult hippocampus (Seki and Arai, 1991; Rougon, 1993; Fryer and Hockfield, 1996; Theodosis et al., 1999). PSA-NCAM is expressed at very high levels during development and disappears from most of the CNS after birth or early postnatal development. However, PSA-NCAM continues to be expressed in adult brain regions that undergo synaptic plasticity or remodeling, including the granule cell layer of the hippocampus (Seki and Arai, 1991, 1999; Bonfani et al., 1992). PSA-NCAM is reported to influence long-term potentiation (Becker et al., 1996; Muller et al., 1996; Cremer et al., 1998) and activity-dependent morphological plasticity of hypothalamic neurons (Theodosis et al., 1999; Hoyk et al., 2001).

The colocalization of pCREB and PSA-NCAM in immature neurons suggests a possible functional relationship. The formation of PSA-NCAM is controlled by polysialic acid synthases, the rate-limiting step in PSA-NCAM biosynthesis (Kiss et al., 1997). Moreover, polysialic acid synthase activity is controlled primarily at the level of expression (Eckhart et al., 1995; Kiss and Rougon, 1997). The two major polysialic acid synthases, STX and ST8SiaIV, in brain are expressed at high levels during development, consistent with the high levels of PSA-NCAM that are found during this period (Kiss and Rougon, 1997). Both forms are also expressed in adult brain, including the granule cell layer of hippocampus, although at much lower levels (Hildebrandt et al., 1998). Synthesis of PSA-NCAM has been demonstrated to be activity dependent in several systems (Kiss and Rougon, 1997). In the present study, we demonstrate that gene promoter activity of STX, but not ST8SiaIV, is upregulated by activation of the cAMP–CREB cascade in culture cells. Activation of the STX promoter is observed in constructs containing a putative CRE but not in shorter deletion mutants lacking this site. In addition, expression of a dominant negative mutant of CREB significantly decreased STX promoter activity under both basal conditions, as well as when the cAMP–CREB cascade was activated. These findings indicate that ST8SiaII gene expression is dependent on CREB or a CREB-like transcription factor. The results are also consistent with the hypothesis that regulation of this polysialic acid synthase could contribute to the expression of PSA-NCAM in immature, BrdU-positive neurons in adult hippocampus. This does not rule out the possibility that other polysialic acid synthases, such as ST8SiaIV, are also involved in the synthesis of PSA-NCAM in adult hippocampus or that polysialic acid synthase expression and activity are also regulated by other signal transduction pathways. It is also important to point out that these promoter studies were conducted in a model cultured cell system, and different transcription factors and promoter elements could be used in hippocampal granule cells.

The results of this study demonstrate that immature neurons in adult hippocampus express pCREB during a critical phase of maturation and survival, and studies in cultured cells demonstrate that cAMP and CREB regulate polysialic acid synthase (STX) gene expression. Moreover, the results demonstrate that pharmacological activation of the cAMP–CREB cascade increases the survival of newborn neurons in the hippocampus. These findings also suggest that the cAMP–CREB pathway regulates the differentiation of newborn cells, as well as the function of immature neurons. In addition to the cAMP pathway, it is likely that other signal transduction cascades also regulate the phosphorylation of CREB in immature neurons. This includes glutamate activation of calcium signaling and neurotrophic factor activation of the microtubule-associated protein kinase cascade, systems that are known to influence neural plasticity and survival (Bonni et al., 1999; Riccio et al., 1999). Characterization of neurotrophic factor regulation of pCREB in immature neurons is particularly interesting in light of recent studies demonstrating that CREB is necessary for neurotrophic factor-dependent cell survival. Identification of the specific neurotrophic factors and/or neurotransmitters that are responsible for phosphorylation of CREB in immature neurons will provide a target(s) for direct manipulation of pCREB and additional analysis of the function of this transcription factor in immature neurons in adult hippocampus.

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