Development/Plasticity/Repair

DREAM Mediates cAMP-Dependent, Ca^{2+}-Induced Stimulation of GFAP Gene Expression and Regulates Cortical Astrogliogenesis

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In the developing mouse brain, once the generation of neurons is mostly completed during the prenatal period, precisely coordinated signals act on competent neural precursors to direct their differentiation into astrocytes, which occurs mostly after birth. Among these signals, those provided by neurotrophic cytokines and bone morphogenetic proteins appear to have a key role in triggering the neurogenic to gliogenic switch and in regulating astrocyte numbers. In addition, we have reported previously that the neurotrophic peptide pituitary adenylate cyclase-activating polypeptide (PACAP) is able to promote astrocyte differentiation of cortical precursors via activation of a cAMP-dependent pathway. Signals acting on progenitor cells of the developing cortex to generate astrocytes activate glial fibrillary acidic protein (GFAP) gene expression, but the transcriptional mechanisms that regulate this activation are unclear. Here, we identify the previously known transcriptional repressor downstream regulatory element antagonist modulator (DREAM) as an activator of GFAP gene expression. We found that DREAM occupies specific sites on the GFAP promoter before and after differentiation is initiated by exposure of cortical progenitor cells to PACAP. PACAP raises intracellular calcium concentration via a mechanism that requires cAMP, and DREAM-mediated transactivation of the GFAP gene requires the integrity of calcium-binding domains. Cortical progenitor cells from *dream*$^{-/-}$ mice fail to express GFAP in response to PACAP. Moreover, the neonatal cortex of *dream*$^{-/-}$ mice exhibits a reduced number of astrocytes and increased number of neurons. These results identify the PACAP-cAMP-Ca^{2+}-DREAM cascade as a new pathway to activate GFAP gene expression during astrocyte differentiation.

Key words: **glial fibrillary acidic protein; DREAM; gliogenesis; cAMP; calcium; transcription**

Introduction

In the developing mammalian CNS, the generation of astrocytes from neural progenitor cells occurs mostly during the early postnatal period once neurogenesis is completed (Qian et al., 2000; Sauvageot and Stiles, 2002). This is attributable, in part, to the existence of mechanisms that actively prevent the differentiation of astrocytes during the neurogenic period, even in the presence of astrogliogenic signals (Park et al., 1999; Nieto et al., 2001; Sun et al., 2001; Takizawa et al., 2001; Hermanson et al., 2002; Namihira et al., 2004; Angelastro et al., 2005). The capacity of neural precursors to switch from neurogenesis to gliogenesis is favored by certain extracellular factors (Qian et al., 1997; Viti et al., 2003) as well as by induced chromatin remodeling of gliogenic genes (Song and Ghosh, 2004; Fan et al., 2005).

Whether neural progenitor cells generate neurons or astrocytes also depends on the type of extracellular signals to which they are exposed. Thus, it has been shown that the differentiation of astrocytes is triggered by cytokines such as ciliary neurotrophic factor (CNTF), leukemia inhibitory factor or cardiotrophin-1, by bone morphogenetic proteins (BMPs), or by pituitary adenylate cyclase-activating polypeptide (PACAP) (Gross et al., 1996; Bonni et al., 1997; Rajan and McKay, 1998; Vallejo and Vallejo, 2002; Barnabé-Heider et al., 2005; Cebolla and Vallejo, 2006).

Expression of glial fibrillary acidic protein (GFAP), which provides a phenotypic marker characteristic of astrocytes, is induced by activation of intracellular signaling mechanisms that directly stimulate GFAP gene transcription. Thus, neurotrophic cytokines activate Janus kinases (JAKs) at their receptors, resulting in the phosphorylation and nuclear translocation of signal transducer and activator of transcription (STAT) proteins, which in turn act at specific regulatory sites of the GFAP gene promoter (Johe et al., 1996; Bonni et al., 1997; Koblar et al., 1998; Rajan and McKay, 1998; Park et al., 1999; He et al., 2005). However, the signaling pathways activated by BMP involve the activation of Smad proteins, which then translocate to the nucleus to stimulate

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GFAP gene transcription (Gross et al., 1996; Mehler et al., 1997; Mabie et al., 1999; Nakashima et al., 1999).

Previous work in our laboratory led to the identification of an additional intracellular signaling mechanism for the generation of astrocytes that involves activation of a cAMP-dependent pathway (McManus et al., 1999; Vallejo and Vallejo, 2002). This mechanism is activated by PACAP, which promotes astrocyte differentiation without influencing neuronal or oligodendroglial lineages by acting on specific PACAP type 1 (PAC1) receptors to induce the synthesis of cAMP in cortical precursor cells. In turn, activation of a cAMP-dependent signaling system is required for astrogliogenesis induced by this peptide (Vallejo and Vallejo, 2002). However, the molecular mechanism by which PACAP and cAMP stimulate GFAP gene expression and induce astrogliogenesis *in vivo* are unknown. In the present study, we identified the requirement of transcription factor downstream regulatory element antagonist modulator (DREAM) bound to the GFAP promoter as a major effector of this response, and we demonstrate that DREAM responds to cAMP-dependent increases in intracellular calcium concentration ($[Ca^{2+}]_i$) to stimulate expression of GFAP in cortical progenitor cells.

Materials and Methods

Plasmids. Luciferase reporter plasmids for the rat GFAP gene promoter have been described previously (Bonni et al., 1997; Krohn et al., 1999; Cebolla and Vallejo, 2006). DRE1 and DRE2 sites in GFAP-A7Luc were mutated by oligonucleotide directed mutagenesis using *Pfu Turbo* DNA polymerase from a QuikChange kit (Stratagene) and the following primers: DRE1 mutant, 5′-CAAGTATGCACGCGTAAACCAGGCCT-3′; and DRE2 mutant, 5′-GCCAGGACTGCAGGGGCAGATCCAGT-3′. Expression vectors encoding DREAM (Carrión et al., 1999; Ledo et al., 2000a) were provided by Dr. J. R. Naranjo [Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain].

Cell culture and transfections. Primary cortical cell cultures from the cerebral cortex from fetal brains of embryonic day 17 (E17) Wistar rats or E16 mice were prepared as described previously (Vallejo and Vallejo, 2002; Cebolla and Vallejo, 2006). Cells were seeded in serum-free DMEM containing N1 supplement (Sigma) and 1 mM sodium pyruvate (defined medium), as well as 20 ng/ml basic fibroblast growth factor (bFGF) (PeproTech) at a density of $2–4 \times 10^4$ cells/cm^2, and were expanded at 37°C for 3–4 d. Medium was replaced every 2 d. Differentiation experiments were performed as described (Vallejo and Vallejo, 2002; Cebolla and Vallejo, 2006). Cells (4×10^4 cells/cm^2) were plated into 35 mm dishes and incubated at 37°C. After 24 h, bFGF-containing medium was replaced with bFGF-free defined medium, and PACAP (100 nM) or CNTF (50 ng/ml) was immediately added.

Transfections of primary cortical precursor cells prepared from E17 Wistar rat fetuses have been described in detail (Cebolla and Vallejo, 2006). Cells were incubated with 6 μg of reporter plasmid DNA mixed with 10 μl of FuGENE 6 transfection reagent (Roche) in defined medium for 4 h. Expression vectors encoding DREAM were used at 1 μg unless stated otherwise, and total DNA amount was kept constant in all transfections by adding empty vector when necessary. The medium-DNA mix was removed and substituted with fresh defined medium, or with defined medium containing PACAP (100 nM) or 8Br-cAMP (1 mM). In experiments with the calcium ionophore A23187 (0.02 μM), cells were treated for 15–20 min 24 h after transfections. Luciferase activity was determined with a commercial assay system (Promega) 48 h after transfection. Luciferase activity from a Rous sarcoma virus enhancer reporter plasmid (RSV-Luc) was used as an independent standard for normalization, and efficiencies were corrected by using the *Renilla* luciferase assay system (Promega). All of the values are expressed as mean ± SEM of at least three independent experiments performed in duplicate.

Neurosphere cultures. Pieces from cerebral cortex dissected from E18 mouse fetuses were mechanically triturated in serum-free DMEM and then filtered through a 40 μm nylon cell strainer (BD Biosciences). After centrifugation, the pellet was resuspended in a solution containing 0.25% trypsin with 0.1 mM EDTA (Invitrogen), 1 μg/ml hyaluronidase (Sigma), and 30 μg/ml DNase (Promega), and incubated with agitation at 37°C for 1 h. Cells were then centrifuged and resuspended in Dulbecco's PBS (Invitrogen) containing 0.9 M sacarose, centrifuged again, and washed in Dulbecco's PBS containing 4% BSA. Finally, cells were resuspended in DMEM-F12 mixture (1:1) medium (Invitrogen) containing B27 supplement (Invitrogen) and were seeded in six-well plates (BD Biosciences). Basic FGF and epidermal growth factor (EGF) (PeproTech) were added to a concentration of 10 ng/ml each, and the cultures were maintained in a 37°C incubator. From the second day on, bFGF and EGF were added daily at a concentration of 5 ng/ml each. After 7 d, neurospheres were transferred onto poly-ornithine-coated glass coverslips in 24-well dishes containing DMEM-F12 medium without bFGF or EGF to allow them to attach, and BDNF (PeproTech) was added to a concentration of 50 ng/ml to induce neurogenesis. After an additional period of 5 d in culture, neurospheres were fixed and processed for immunocytochemistry followed by confocal microscopy.

ChIP assays. For chromatin immunoprecipitation (ChIP) assays (Gerrish et al., 2001), cells were treated with 1% formaldehyde for 10 min, pelleted by centrifugation in PBS, and sonicated. Chromatin was diluted in buffer containing 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and protease inhibitors. Immunoprecipitations were performed using an anti-DREAM rabbit polyclonal IgG (FL-214; Santa Cruz Biotechnology), or control normal rabbit IgG (s.c.-2025; Santa Cruz Biotechnology). Antibody-protein-DNA complexes were isolated by incubation with protein A-Sepharose. The DNA was eluted and detected by PCR using oligonucleotide primers that amplify a 270 nucleotide fragment of the proximal promoter region of the GFAP gene. The sequences of the oligonucleotide primers were as follows: forward, 5′-CCCTCTCCTGACCCATTTACCAGAA-3′; reverse, 5′-GCCCCTGACCATCGTCTCGGAGGAG-3′. PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, after which a 5 min incubation at 72°C followed. As a negative control, PCR was performed using oligonucleotide primers that amplify a 430 nucleotide fragment of the promoter of the somatostatin gene. In this case, PCR conditions were 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a 5 min incubation at 72°C. The sequences of the oligonucleotide primers were as follows: forward, 5′-GATTGGACAAAGTGATGCTC-3′; reverse, 5′-AGTGAGGGGAGGCGACAC-3′. PCR products were run on a 1% agarose gel, stained with ethidium bromide, and photographed.

Electrophoretic mobility shift assays. Nuclear protein extracts from primary cortical precursor cells were prepared and electrophoretic mobility shift assays were performed exactly as described (Cebolla and Vallejo, 2006). The sequences of the oligonucleotides used were as follows (sense strand): DRE1, 5′-GATCCAAGTATGCACTGTCAAACCAGGCA-3′; mutated DRE1 (DRE1m), 5′-GATCCAAGTATGCACGCGTAAACCAGGCA-3′; DRE2, 5′-GATCCGCCAGGAAGTCAGGGGCAGA-3′; DRE2m, 5′-GATCCGCCAGGACTGCAGGGGCAGA-3′.

For supershift experiments, nuclear extracts were preincubated with anti-DREAM antiserum Ab 1013 (Link et al., 2004) provided by Dr. J. R. Naranjo (Centro Nacional de Biotecnología, CSIC, Madrid, Spain), before the addition of the labeled probes.

Measurement of $[Ca^{2+}]_i$ variations. Ca^{2+} levels in primary cortical precursor cells were monitored by fluorescence microscopy. Cultures were incubated at 37°C for 20–45 min with the Ca^{2+} indicator Fluo-3-AM (10 μg/ml; Invitrogen). Cells were illuminated with a xenon lamp at 490 nm using a monochromator Polychrome II (T.I.L.L. Photonics) during 200–500 ms, and imaged using a CCD camera (Retiga EX; QImaging) attached to an upright BW50WI Olympus microscope. Images were acquired every 15–30 s. The control of the Polychrome II and the CCD camera, and the quantitative epifluorescence measurements, were made using the IPlab software (Scanalytics). $[Ca^{2+}]_i$ variations were estimated as $\Delta F/F_0$ after background subtraction, and cells were considered to respond to the stimulation when the fluorescence signal increased both five times above the SD of the basal signal and >5% for at least two consecutive images. Data are expressed as mean ± SEM.

Mice. DREAM-deficient mice (Cheng et al., 2002) were provided by

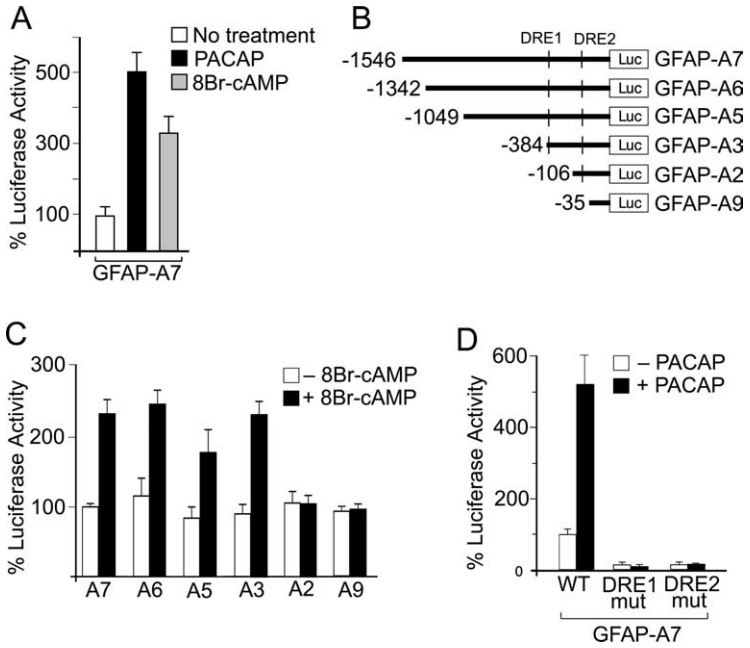

Figure 1. Stimulation of the GFAP promoter by PACAP depends on the integrity of DRE sites. ***A***, Relative levels of luciferase activity elicited in primary cortical precursor cells transfected with the reporter plasmid GFAP-A7Luc. Cells were left untreated, or were treated with PACAP (100 nM) or 8Br-cAMP (1 mM). ***B***, Schematic depiction of the 5′-deletion constructs of the rat GFAP-luciferase fusion gene used in transfection studies for deletional analysis of the promoter. The relative positions of DRE1 and DRE2 are indicated. ***C***, Relative luciferase activities elicited in primary cortical precursor cells transfected with the luciferase reporter plasmids indicated above to map the location of promoter regions required for the response to cAMP. Note that deletion to nucleotide −106 (GFAP-A2) abolishes the response to 8Br-cAMP, even though DRE2 is present. ***D***, Mutations of either DRE1 or DRE2 identical to those depicted in Figure 2*C* abolish basal and PACAP-stimulated luciferase activity in primary cortical precursor cells transfected with the luciferase reporter plasmid GFAP-A7. Values represent the mean ± SEM of three independent experiments performed in duplicate.

Dr. J. M. Penninger (University of Toronto, Toronto, Canada) and were maintained in a C57BL/6 background. Null mutants were produced by mating heterozygote animals, and genotyping was performed by PCR using genomic DNA as described previously (Cheng et al., 2002).

Immunocytochemistry. DAB immunocytochemistry was performed exactly as described previously (Vallejo and Vallejo, 2002), using a GFAP-specific monoclonal antibody (1:500 dilution, clone G-A-5; Sigma) and immunoperoxidase staining with a Vectastain ABC kit (Vector Laboratories). For double antigen immunofluorescence, cells were grown on glass coverslips, and coincubation with anti-GFAP monoclonal antibody and rabbit polyclonal anti-DREAM IgG was followed by incubation with 594-Alexa anti-rabbit and 488-Alexa anti-mouse secondary antibodies (1:1000; Invitrogen). Images were taken using confocal microscopy.

Neurospheres attached to coverslips were incubated with anti-neuronal-specific class III β-tubulin (Tuj1) monoclonal antibody (1:500; Sigma) followed by incubation with a 488-Alexa anti-mouse secondary antibody (1:1000; Invitrogen). Nuclei were counterstained with Hoechst (Invitrogen). Digital images were taken using confocal microscopy. Cell counts were performed on the crown of cells attached to the plate surrounding the neurosphere. The percentage of Tuj1-positive cells relative to the total number of cells was determined. The number of neurospheres analyzed was 17–20 in three different experiments. Data represent the mean ± SEM. Statistical analysis was performed using the Student's *t* test.

Immunohistochemistry. DAB immunohistochemistry (Schwartz et al., 2000) was performed on cryostat sections (14 μm) of brains fixed in 4% paraformaldehyde and incubated at 4°C overnight with anti-GFAP (1:400, Sigma) or anti-neuronal-specific nuclear protein (NeuN; 1:100, Millipore) monoclonal antibodies. Immunodetection was performed with immunoperoxidase staining with a Vectastain ABC kit (Vector Laboratories). For NeuN, staining was performed in the presence of nickel ammonium to yield a dark blue color. Images were taken on a Nikon microscope equipped with an Olympus DP50 digital camera.

For immunofluorescence, sections were incubated with an anti-S100β monoclonal antibody (1:400; Sigma), followed by incubation with 488-Alexa anti-mouse secondary antibodies (1:1000; Invitrogen). Nuclei were counterstained with TO-PRO-3 (Invitrogen). Images were taken using a Leica TCS SP5 confocal microscope.

Cell counts were performed on digital images taken from sections corresponding to identical rostrocaudal levels, using calibrated AnalySIS imaging software (Soft Imaging System). Defined areas from the dorsal and lateral regions of the cortex (see Fig. 6*C*) or the hippocampus were used on both sides. At least three animals per experiment were used, and a total of five sections per animal were analyzed. Quantitative results provided represent the mean ± SEM. Statistical analysis was performed using Student's *t* test.

Western immunoblot. Mouse cerebral cortex or cultured cells were lysed and proteins (20 μg) were resolved by SDS-PAGE and blotted onto a nitrocellulose membrane. GFAP immunoreactivity was detected with a specific monoclonal antibody (clone G-A-5, Sigma; 1:10,000 dilution) followed by incubation with a horse anti-mouse peroxidase-conjugated secondary antibody (1:5000 dilution) (Bio-Rad). Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (GE Healthcare). Membranes were subsequently stripped with stripping buffer (70 mM Tris, pH 6.8, 10% SDS, and 0.7% β-mercaptoethanol) for 20 min at 60°C, washed, and reprocessed using a monoclonal anti-α-tubulin antibody (1:20,000; Sigma). Films were scanned and densitometry measurements of bands were performed using NIH ImageJ 1.37b software.

Results

DREAM binds to specific sites of the promoter of the GFAP gene

To search for the existence of regulatory elements in the GFAP promoter that could mediate transcriptional responses to stimulation by PACAP/cAMP, we performed transient transfections in primary cortical precursor cells obtained from E17 rat fetuses. These are proliferating nestin-positive cells that differentiate into astrocytes in response to treatment with PACAP (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) or with the cAMP analog 8Br-cAMP, a system that we have characterized in detail in previous studies (McManus et al., 1999; Vallejo and Vallejo, 2002; Cebolla and Vallejo, 2006). We observed that the activity of the luciferase reporter plasmid GFAP-A7Luc, spanning 1.5 kb of the GFAP promoter (Bonni et al., 1997), is stimulated in transfected cells treated with either PACAP or 8Br-cAMP (Fig. 1*A*). Because PACAP induces astrogliogenesis via stimulation of a cAMP-dependent signaling pathway (Vallejo and Vallejo, 2002), we then used 8Br-cAMP to map the promoter region responsible for the observed transcriptional effect by deletional analysis. We found that 8Br-cAMP failed to stimulate luciferase activity when a plasmid with a deletion to nucleotide −106 (GFAP-A2Luc) or to nucleotide −35 (GFAP-A9Luc) was used, indicating the location of putative cAMP-responsive regulatory elements downstream from nucleotide −384 (Fig. 1*C*).

This region does not contain a typical cAMP-response element, but we noticed the presence of two sites with GTCA motifs similar to those found in the prodynorphin and *c-fos* genes that bind DREAM (Carrión et al., 1998, 1999). Because DREAM participates in the regulation of transcriptional mechanisms that depend on cAMP stimulation (Ledo et al., 2000a), we termed these sites DRE1 (nucleotides −373 to −367) and DRE2 (nucleotides −62 to −59) and we sought to determine whether they are important for transcriptional transactivation in response to PACAP. Mutations in any one of these sites completely inhibited basal and PACAP-stimulated luciferase activity (Fig. 1*D*), suggesting that both DRE1 and DRE2 are important sites for transactivation of the GFAP gene. Thus, we investigated whether DRE1 and DRE2 are recognized by DREAM in cortical progenitor cells.

DREAM is expressed in primary cortical precursor cells before astrocyte differentiation was induced, and also in GFAP-expressing cells differentiated by PACAP (Fig. 2*A*). To determine whether DREAM binds to the endogenous GFAP gene in the context of native chromatin *in vivo*, we performed ChIP assays. The anti-DREAM antiserum specifically immunoprecipitated a fragment of chromatin corresponding to the proximal region of the GFAP promoter both in undifferentiated primary cortical precursor cells (Fig. 2*B*, left) and in cells treated with PACAP (Fig. 2*B*, right). ChIP specificity was tested on the promoter of the somatostatin gene, which was not amplified in samples obtained after immunoprecipitation with the anti-DREAM antiserum (Fig. 2*B*). Thus, these experiments indicate that DREAM occupies the GFAP promoter before the differentiation response is triggered, and remains bound after GFAP expression has been initiated.

Direct binding of DREAM to DRE1 and/or DRE2 was tested by electrophoretic mobility shift assays (Fig. 2*C–E*). Sequence-specific DNA-protein complexes were observed with nuclear extracts from primary cortical cells from E17 rat fetuses and DRE1 or DRE2 oligonucleotides, and the presence of DREAM in these complexes was detected by adding a specific anti-DREAM antiserum. Consistent with the results from the ChIP experiments mentioned above, treatment of cells with PACAP did not result in loss of binding of DREAM to either DRE1 or DRE2 (Fig. 2*D*,*E*). Thus, DREAM expressed in cortical precursor cells can bind specifically to the DRE1 and DRE2 sites of the GFAP promoter.

Stimulation of the GFAP promoter by DREAM requires the integrity of Ca^{2+}-binding domains

To investigate whether DREAM activates the GFAP gene in response to PACAP, we cotransfected primary cortical precursor cells with the GFAP-A7Luc reporter and with an expression vector encoding DREAM (100 ng to 3 μg). We found that DREAM did not modify basal or PACAP-induced luciferase activity (data not shown), suggesting that DREAM is bound to the GFAP promoter in saturating amounts. Thus, we sought to confirm a possible involvement of DREAM in the regulation of GFAP gene expression by cotransfecting GFAP-A7Luc with expression plasmids encoding dominant negative mutants of DREAM generated by mutations in the functional calcium-binding EF-hand domains, or in the leucine-charged domains (LCD) required for direct interactions with α-cAMP-responsive element modulator (αCREM) in response to increased levels of cAMP (Fig. 3*A*) (Carrión et al., 1999; Ledo et al., 2000a,b; Scsucova et al., 2005).

We found that DREAM-EFm/LCDm, which has both EF-hand and LCD motifs inactive, did not alter basal luciferase activity but completely inhibited the stimulation of reporter activity

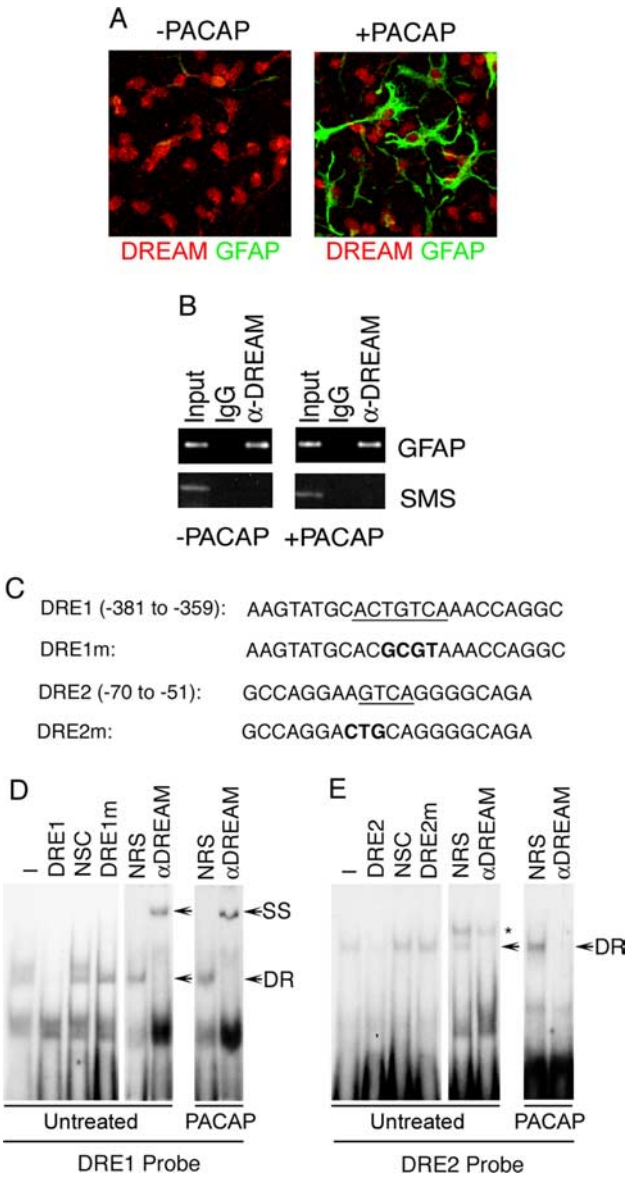

Figure 2. DREAM expressed in cortical neuroepithelial precursors induced to differentiate with PACAP occupies the promoter of the GFAP gene. ***A***, DREAM immunofluorescence (red) in primary cortical neuroepithelial cells unexposed (left) or exposed (right) to PACAP (100 nM) for 2 d. All cells were processed for double-label DREAM (red) and GFAP (green) immunofluorescence. ***B***, Chromatin immunoprecipitation assays indicating that DREAM occupies the promoter of the endogenous GFAP gene in primary cortical neuroepithelial cells. A specific anti-DREAM antiserum (αDREAM) or control normal rabbit IgG were used for immunoprecipitations. Undifferentiated primary cortical precursors (left) or GFAP-expressing cells differentiated with PACAP (100 nM) for 2 d (right) were used. PCR amplifications were not obtained with the control somatostatin (SMS) gene (bottom). ***C***, Sequence of the wild-type or mutated DRE1 and DRE2 oligonucleotides used in the electrophoretic mobility shift assays. The consensus DREAM-binding sites are underlined, and the nucleotides mutated are indicated in bold. The nucleotide numbers relative to the transcription initiation site are indicated in parenthesis. ***D***, Electrophoretic mobility shift assays showing the binding of proteins present in nuclear extracts of cortical precursor cells cultured from E17 rat brains to an oligonucleotide probe corresponding to a region of the rat GFAP promoter that contains the DRE1-binding site (nucleotides −381 to −359). Cells were left untreated or were treated with PACAP (100 nM) for 2 d before nuclear extracts were prepared. Nuclear extracts were incubated in the absence (−) or presence of a competing oligonucleotide of identical probe sequence (DRE1) or in the presence of a nonspecific competing (NSC) oligonucleotide of unrelated sequence, or a mutated DRE1 oligonucleotide (DRE1m), each used in a 100-fold molar excess. In addition, binding reactions were performed in the presence of either nonimmune rabbit serum (NRS) or anti-DREAM antiserum. Arrows indicate bands corresponding to protein-DNA complexes containing DREAM (DR) and to the supershifted (SS) complexes. ***E***, Experiments similar to those described in ***D***, but using a probe corresponding to the DRE2 element. In this case, addition of the anti-DREAM antiserum results in inhibition of binding. The asterisk indicates a nonspecific band.

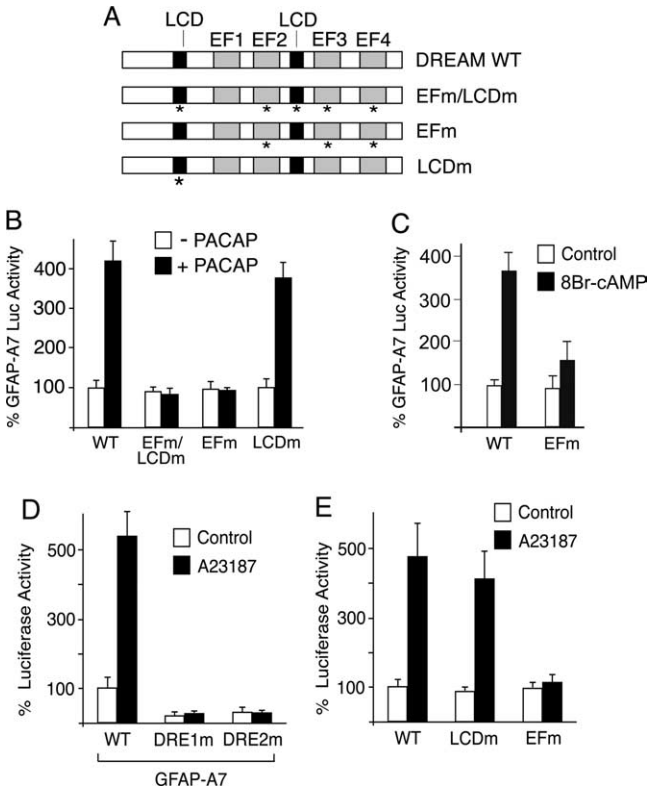

Figure 3. DREAM mediates calcium-dependent stimulation of the GFAP promoter by PACAP and cAMP. ***A***, Schematic depiction of the wild-type and mutated versions of DREAM used in the transfection experiments. The mutated LCD or EF-hand motifs are indicated by asterisks. ***B***, Relative luciferase activities elicited in primary cortical precursor cells cotransfected with the reporter plasmid GFAP-A7Luc, and expression vectors encoding wild-type or mutated versions of DREAM, as indicated schematically in ***A***. Cells were left untreated, or treated with PACAP (100 nM). ***C***, Relative luciferase activities elicited in primary cortical precursor cells cotransfected with GFAP-A7Luc, and expression vectors encoding wild-type DREAM or a version of DREAM in which the EF-hands have been mutated. In this case, cells were treated with 8Br-cAMP (1 mM). ***D***, Mutations of either DRE1 or DRE2 abolish basal and calcium ionophore A23187-stimulated luciferase activity in primary cortical precursor cells transfected with the GFAP-A7Luc reporter. ***E***, Relative luciferase activities elicited in primary cortical precursor cells cotransfected with GFAP-A7Luc, and expression vectors encoding wild-type DREAM or mutated versions of DREAM in which either the LCD domain or the EF-hand domains had been mutated. Cells were left untreated or treated with the calcium ionophore A23187 (0.02 μM). In all cases, values are expressed as percentages of the activities elicited by GFAP-A7Luc, and represent the mean ± SEM of at least three independent experiments performed in duplicate.

elicited by PACAP (Fig. 3*B*), suggesting that DREAM mediates PACAP-induced GFAP promoter activation via mechanisms that involve cAMP- and/or calcium-dependent signaling. Because stimulation of GFAP expression by PACAP requires cAMP (Vallejo and Vallejo, 2002), we hypothesized that PACAP could act by promoting the interaction of phosphorylated αCREM with the LCD domains of DREAM, a mechanism by which DREAM mediates cAMP-dependent responses (Ledo et al., 2000a). However, DREAM-LCDm, which contains intact EF-hands and a double mutation (L47,52V) in the first LCD whose integrity is required for cAMP-dependent DREAM-αCREM interactions (Ledo et al., 2000a), did not alter significantly the stimulation of luciferase activity induced by PACAP (Fig. 3*B*), indicating that interactions with αCREM are not involved in this response. Furthermore, we did not observe alterations in the luciferase activity induced by PACAP when an expression vector encoding αCREM was cotransfected, or when cells were pretreated with the protein kinase A inhibitor H89 (data not shown). In contrast, mutations of the three functional EF-hand motifs completely blocked the capacity of PACAP or 8Br-cAMP to stimulate GFAP-A7Luc activity (Fig. 3*B*,*C*). Thus, these results suggest that the transcriptional activation of the GFAP gene induced by PACAP and cAMP involves a Ca^{2+}-dependent mechanism.

To investigate this, we first determined whether an increase in $[Ca^{2+}]_i$ is sufficient to stimulate transcription from the GFAP promoter. We found that in transfected cells, treatment with the calcium ionophore A23187 stimulated GFAP-A7Luc activity with a magnitude similar to that obtained with PACAP, but this activation was dependent on the integrity of DRE sites (Fig. 3*D*). To test whether DREAM mediates this response, we cotransfected GFAP-A7Luc with expression vectors encoding dominant negative inhibitors. We found that the LCD mutant version of DREAM did not alter the stimulation of luciferase activity induced by A23187. In contrast, an EF-hand DREAM mutant completely inhibited the stimulation induced by A23187 (Fig. 3*E*). Thus, these experiments demonstrate that DREAM mediates the calcium-dependent transcriptional transactivation of the GFAP promoter.

PACAP increases $[Ca^{2+}]_i$ in cortical precursor cells in a cAMP-dependent manner

Astrocyte differentiation of cortical precursors induced by PACAP is blocked by the cAMP antagonist Rp-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS) (Vallejo and Vallejo, 2002). To determine whether treatment of cortical precursor cells with PACAP rises $[Ca^{2+}]_i$ in a cAMP-dependent manner, we monitored changes in the intensity of the fluorescence emitted by cortical precursor cells that had been loaded with the calcium indicator Fluo-3-AM in response to different types of treatments.

Incubation of cells with PACAP (1 μM) resulted in a relatively slow but sustained increase in cell fluorescence values that became evident between 1 and 5 min after the administration of the peptide, and lasted for >15 min (~75% of cells were active 15 min after the onset of treatment) (Fig. 4). These changes were specifically caused by the effect of PACAP, because administration of equimolecular amounts of the related vasoactive intestinal peptide (VIP) did not have any significant effect (Fig. 4*B*), consistent with our previous observation that PACAP acts on cortical precursor cells via activation of specific PAC1 receptors that are not recognized by VIP (Vallejo and Vallejo, 2002). Importantly, pretreatment of cells with the cAMP antagonist Rp-cAMPS completely blocked the effect of PACAP on $[Ca^{2+}]_i$ increase (Fig. 4*A*,*B*). These results indicate that the activation of specific receptors by PACAP induces elevations in $[Ca^{2+}]_i$ in cortical precursor cells via a cAMP-dependent pathway.

Furthermore, we found that exposure of cells to thapsigargin, a Ca^{2+}-ATPase inhibitor that depletes internal Ca^{2+} stores, did not significantly modify the capacity of PACAP to increase $[Ca^{2+}]_i$ (Fig. 4*C*). In contrast, when cells were incubated in Ca^{2+}-free medium PACAP failed to increase $[Ca^{2+}]_i$ (Fig. 4*C*). Thus, these experiments indicate that cAMP-dependent PACAP-induced increase in $[Ca^{2+}]_i$ is caused by calcium influx from the extracellular medium.

DREAM is necessary for PACAP-induced GFAP expression in cortical precursors and regulates astrocyte differentiation

All of the above experiments indicate that PACAP induces the expression of GFAP by increasing $[Ca^{2+}]_i$ in a cAMP-dependent manner, and that calcium acts on DREAM to transactivate the GFAP promoter. Therefore, we tested whether DREAM is implicated in astrocyte differentiation using knock-out mice in which

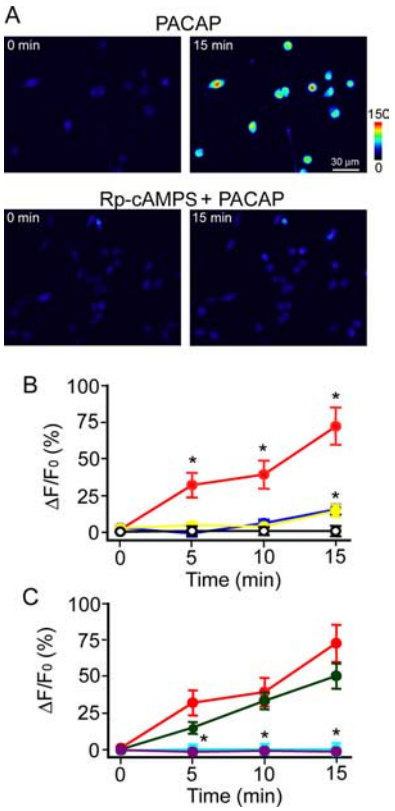

Figure 4. PACAP elicits cAMP-dependent increases in $[Ca^{2+}]_i$ in Fluo-3-AM-loaded primary cortical precursor cells. ***A***, Pseudocolor images representing fluorescence intensities of Fluo-3-AM-loaded cells before (left, 0 min) and after (right, 15 min) exposure to PACAP (1 μM). Cells depicted in the bottom panels were treated with the cAMP antagonist Rp-cAMPS (10 μM) before exposure to PACAP. Scale bar, 30 μm. ***B***, Relative changes in fluorescence intensity observed in cortical precursor cells in the absence (blue circles) or presence of PACAP (red circles). Fluorescence intensity observed in cells pretreated with the cAMP antagonist Rp-cAMPS before exposure to PACAP is depicted by yellow circles. As an additional control for specificity, a group of cells was treated with the PACAP-related peptide VIP (white circles). In each condition, 75–120 cells from at least six coverslips were analyzed. ***C***, Relative changes in fluorescence intensity observed in cells treated with PACAP in the absence (red circles) or presence (green circles) of thapsigargin (1 μM). An additional group of cells was incubated in medium lacking calcium (purple circles), and in that case PACAP did not elicit increases in $[Ca^{2+}]_i$ (light blue circles). In each condition, >45 cells from at least four coverslips were analyzed. Significant differences from control values were established at $^*p < 0.001$.

the DREAM-encoding gene had been deleted by homologous recombination (Cheng et al., 2002). Initially, we tested for the induction of GFAP expression by immunocytochemistry in primary cultures of E16 mouse cortical precursor cells. As observed with rat cells cultured in similar conditions (McManus et al., 1999; Vallejo and Vallejo, 2002; Cebolla and Vallejo, 2006), exposure of cells prepared from wild-type mice to either PACAP or CNTF over a period of 2 d in culture resulted in the expression of GFAP accompanied by the extension of processes (Fig. 5*A*). In contrast, when cells prepared from *dream*$^{-/-}$ mice were used, exposure to CNTF stimulated the expression of GFAP but exposure to PACAP did not (Fig. 5*A*). Western immunoblot confirmed lack of GFAP expression in DREAM-deficient PACAP-treated cells (Fig. 5*B*).

These experiments indicate that stimulation of GFAP expression by PACAP in cortical precursor cells requires the presence of DREAM. Because PACAP has been proposed to act as a neurotrophic signal during cortical development to regulate astrocytogenesis (Vallejo and Vallejo, 2002; Cebolla and Vallejo, 2006), lack of DREAM could be predicted to impair cortical gliogenesis *in vivo*. To test this notion, we measured GFAP levels by Western immunoblot in the cerebral cortex of mice at postnatal day 1 (P1), a time that coincides with active astrogliogenesis. We found that *dream*$^{-/-}$ mice contain GFAP levels that are reduced by >50% relative to those of control wild-type animals (Fig. 6). Reduced levels of GFAP were observed in DREAM-deficient mice for at least up to P3, although the magnitude of the difference at this time was smaller (Fig. 6*A*,*B*).

In addition, we demonstrated that the reduced levels of GFAP in the brains of DREAM-deficient mice are caused by a primary defect in astrocyte differentiation and not to a decrease in the expression of the GFAP gene at the transcriptional level, because the number of cortical astrocytes, as assessed by immunohistochemistry for the astroglial markers GFAP and S100β, was determined to be smaller in *dream*-mutant mice than in control animals (Fig. 6*D–G*). The numbers of immunoreactive cells per square millimeter in wild-type and knock-out animals were 1262 ± 80 and 817 ± 104, respectively, for GFAP, and 1021 ± 66 and 637 ± 109, respectively, for S100β (in both cases, $p < 0.002$, Student's *t* test). Furthermore, this is not a local effect restricted to the cortex, because we also observed reduced numbers of GFAP-stained cells in other areas of the brain including white matter tracts such as the corpus callosum (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). These data indicate that generation of astrocytes in DREAM deficient mice is defective, underscoring the importance of DREAM for astrogliogenesis during the development of the cerebral cortex.

Lack of DREAM delays the timing of the neurogenic to gliogenic switch

Because it is well established that the onset of astrogliogenesis takes place after neurogenesis is mostly completed, and that at least some gliogenic signals coordinately inhibit neurogenesis (Miller and Gauthier, 2007), we examined whether the apparent delay in the onset of gliogenesis was accompanied by an alteration in the number of neurons that could indicate a concomitant delay in the inhibition of neurogenesis. To this end, we performed NeuN immunohistochemistry and counted the number of immunopositive cells in the cortex of P1 mice. We found that in P1 *dream*-deficient mice, the number of NeuN-immunopositive neurons is increased by ~20% relative to wild-type controls (4072 ± 170 vs 3298 ± 172 neurons/mm^2; $p < 0.005$, Student's *t* test) (Fig. 7*A*). When we performed a similar analysis in P7 mice, we found that the number of neurons was still higher in *dream*-deficient than in control animals (2066 ± 122 vs 1740 ± 69 neurons/mm^2; $p < 0.03$, Student's *t* test), indicating that this effect persists and is sustained well beyond the time of completion of neurogenesis.

One possible explanation for this observation is that, apart from its involvement in the mechanisms that promote astrogliogenesis in response to specific signals, DREAM could participate coordinately in restrictive mechanisms opposing the generation of neurons at the end of the period of neurogenesis. Therefore, if DREAM regulates the generation of neurons negatively, a prediction would be that DREAM-deficient neural precursors would show an enhanced capacity to generate neurons. To test this prediction, we analyzed the number of neurons generated from cortical precursors in neurospheres allowed to differentiate in the presence of BDNF. We found that the percentage of Tuj1-immunoreactive cells was 15.3 ± 2.2 in BDNF-treated neurospheres from control animals and 26.2 ± 2.3 BDNF-treated neurospheres from DREAM-null mice ($p < 0.005$, Student's *t* test).

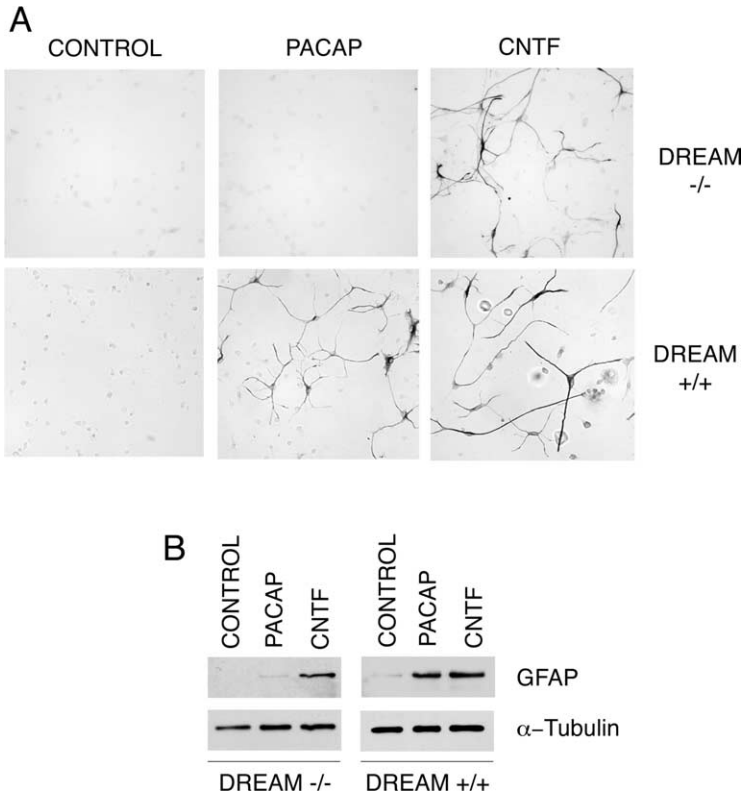

Figure 5. DREAM is required for expression of GFAP induced by PACAP in mouse fetal cortical precursor cells. ***A***, Immunocytochemical staining of GFAP in primary cells prepared from the cerebral cortex of E16 mouse fetuses and cultured in defined medium in the presence of bFGF (control). To induce differentiation, bFGF was withdrawn and cells were treated for 2 d with PACAP (100 nM) or CNTF (50 ng/ml). No GFAP immunoreactivity was detected in untreated control cells, or in cells prepared from DREAM-deficient mice treated with PACAP. ***B***, Western immunoblots showing GFAP expression in extracts of mouse primary cortical cells treated under the same conditions to those shown in ***A***. Note that GFAP is not detectable in PACAP-treated cells prepared from DREAM-deficient mice. α-Tubulin immunoreactivity was used as a control to monitor the even loading of samples.

Thus, neurospheres prepared from DREAM-deficient mice generated a higher number of neurons than those prepared from wild-type animals (Fig. 7*B*), consistent with a restrictive role for DREAM on neurogenesis important for regulating the timing of the neurogenic-to-gliogenic switch.

In light of these results, and given that newly generated neurons appear to have a direct effect on the generation of astrocytes by activating neurotrophic cytokine signaling (Barnabé-Heider et al., 2005), which appears to be unaffected in DREAM-deficient mice (Fig. 5), we sought to determine the GFAP content and the number of astrocytes in older animals once astrogliogenesis has been completed.

We found that GFAP expression in the cerebral cortex of DREAM-deficient mice from P7 onwards is higher than in control animals (Fig. 8*A*,*B*). In adult animals (12 weeks), GFAP immunostaining revealed a higher number of astrocytes in the brains of mice lacking DREAM (86 ± 4.2 $GFAP^+$ cells/mm^2) than in those of age-matched wild-type controls (72 ± 2.3 $GFAP^+$ cells/mm^2; $p < 0.05$, Student's t test) (Fig. 8*C*). This increase in the number of astrocytes is probably not caused by increased astrocyte proliferation, because we did not observe increased number of proliferating GFAP-positive cells in DREAM-deficient mice treated with intraperitoneal injections of bromodeoxyuridine (50 mg/kg) (data not shown). Thus, together, our results indicate that during the early postnatal period lack of DREAM results in increased number of neurons and delayed astrogliogenesis, but that once astrocyte generation has started, compensatory mechanisms appear to be activated to maintain the balance between the number of neurons and the number of astrocytes.

Discussion

We showed previously that PACAP triggers astrocytogenesis by stimulating cAMP synthesis after acting on specific PAC1 receptors present in cortical precursors (Vallejo and Vallejo, 2002). Here, we show that cAMP production induced by PACAP in cortical precursor cells results in a raise in $[Ca^{2+}]_i$, that Ca^{2+} acts on DREAM to stimulate transcription of the GFAP gene, and that DREAM is required for the regulation of normal astrocyte differentiation *in vivo*. As the expression of PACAP and PACAP receptors in the developing cortex is well documented (Skoglösa et al., 1999; Jaworski and Proctor, 2000; Suh et al., 2001), we propose that the PACAP-cAMP-Ca^{2+}-DREAM cascade constitutes a novel signaling pathway to generate astrocytes in the developing brain.

DREAM as a transcriptional transactivator of the GFAP gene

The observations that a dominant negative mutant of DREAM inhibits GFAP promoter stimulation by PACAP, and that DREAM bound to the GFAP promoter in cortical precursor cells remains in place after induction of astrocyte differentiation, indicate that DREAM acts as a transcriptional transactivator. This is an unexpected finding, because DREAM has been shown previously to act as a repressor, allowing transcriptional stimulation after dissociation from regulatory elements located downstream of the transcription initiation site of target genes (Carrión et al., 1999; Ledo et al., 2000a; Campos et al., 2003). However, transcriptional transactivation by DREAM is not unprecedented, because DREAM has been described to act as an activator by binding to sites located upstream of a TATA box (Scsucova et al., 2005).

The mechanism by which DREAM transactivates the GFAP promoter in response to calcium is likely to involve changes in protein conformation (Carrión et al., 1999; Osawa et al., 2001, 2005) that could be stabilized by interactions with other proteins bound in close proximity (Rivas et al., 2004; Gomez-Villafuertes et al., 2005; Scsucova et al., 2005). Of note, the transcription factor nuclear factor-I is important for GFAP expression and occupies a site in the GFAP promoter located adjacent to DRE2 (Cebolla and Vallejo, 2006). The observation that disruption of DREAM binding to DNA by mutation of the DRE sites completely abolishes GFAP promoter activity further indicates that DREAM may be a key component to assemble transcriptionally competent protein complexes.

DREAM stimulates GFAP gene transcription in response to PACAP via a Ca^{2+}-dependent mechanism, as indicated by the observation that integrity of the EF-hands is required for GFAP promoter activation. Furthermore, the antagonist Rp-cAMPS inhibits PACAP-induced Ca^{2+} entry (this study) and astrocyte dif-

ferentiation (Vallejo and Vallejo, 2002). These findings, together with our observations that cortical precursor cells from *dream* $^{-/-}$ mice fail to activate GFAP expression after exposure to PACAP, and that lack of DREAM results in delayed generation of cortical astrocytes *in vivo*, support that cAMP-dependent elevation of $[Ca^{2+}]_i$ induced by PACAP constitutes an important signal to stimulate astrocytogenesis.

It is well established that PACAP increases $[Ca^{2+}]_i$ by mobilizing intracellular pools or by increasing extracellular calcium influx (Chatterjee et al., 1996; Przywara et al., 1996; Osipenko et al., 2000; Morita et al., 2002; Liu et al., 2003; Payet et al., 2003). Our data favor the notion that in cortical precursors PACAP promotes calcium entry from the extracellular milieu, in agreement with previous reports indicating that PACAP-induced Ca^{2+} influx requires the activation of cAMP-dependent signaling (Przywara et al., 1996; Osipenko et al., 2000). Also, this is consistent with our observation that the most abundant PACAP receptor isoform expressed in cortical precursor cells is the short splice variant of the PAC1 receptor (Vallejo and Vallejo, 2002), which couples to stimulation of adenylate cyclase.

Fluctuations in $[Ca^{2+}]_i$ in cortical progenitor cells have been shown to occur as coordinated waves that depend on intracellular stores and modulate cell proliferation or neuronal differentiation (Owens and Kriegstein, 1998; Weissman et al., 2004), or as a consequence of increased influx from the extracellular milieu via activation of membrane channels (D'Ascenzo et al., 2006; Ma et al., 2001; Sah et al., 1997). Thus, calcium can be mobilized dynamically in different ways within neuroepithelial cells to participate in different signaling processes that coordinately regulate proliferation or differentiation into neurons or astrocytes.

Our results show that astrocytes can be generated by CNTF in DREAM-lacking cortical precursor cells, although these do not respond to PACAP. CNTF (and other neurotrophic cytokines) generates astrocytes by activating a gp130-JAK-STAT pathway (Bonni et al., 1997; Rajan and McKay, 1998; Nakashima et al., 1999). Thus, although PACAP and CNTF can synergistically activate GFAP expression (Cebolla and Vallejo, 2006), our results indicate that the CNTF-gp130-JAK-STAT pathway can function independently from the PACAP-cAMP-Ca^{2+}-DREAM pathway.

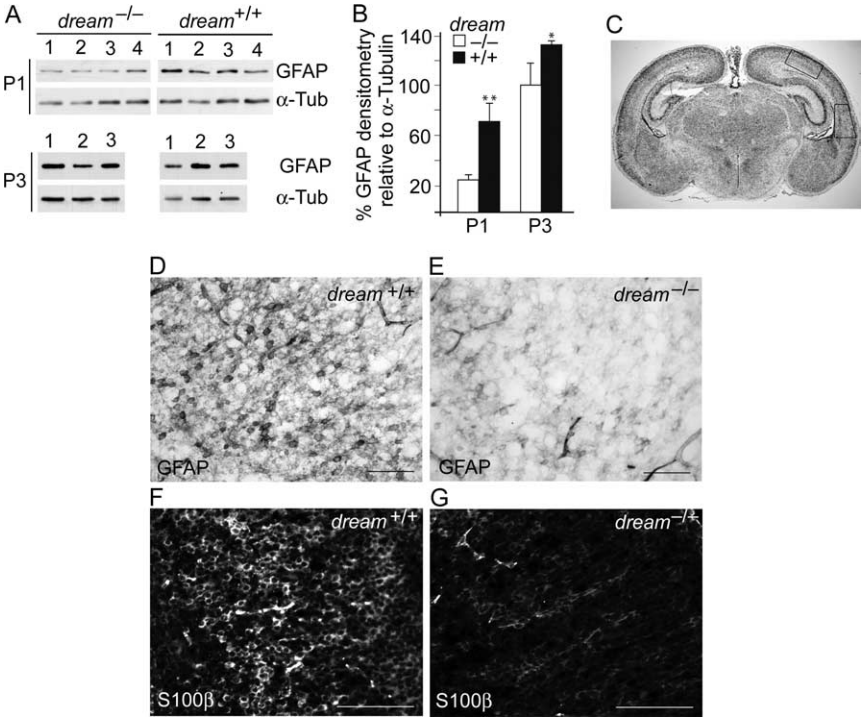

Figure 6. DREAM-deficient mice exhibit reduced number of astrocytes in the cortex during early postnatal period. ***A***, Western immunoblots showing GFAP expression in extracts of cortices isolated from P1 or P3 DREAM-deficient (*dream* $^{-/-}$) or control wild-type (*dream* $^{+/+}$) mice. The numbers on top of each panel indicate individual mice from which samples were obtained. α-Tubulin (α-Tub) immunoreactivity was used as a control to monitor even loading of samples. ***B***, Densitometric analysis performed to quantify the relative intensity of DREAM-immunoreactive bands detected by Western immunoblot. Results are expressed as percentage of increment of densitometry measurements of DREAM bands (in arbitrary units) relative to the intensity of the corresponding α-tubulin bands. $^*p < 0.05$; $^{**}p < 0.02$. This figure represents one of four different experiments with similar results. ***C***, Cresyl violet-stained brain section showing a schematic depiction of the dorsal and lateral regions (boxed areas) of the cortex of P1 mice used to determine the number of astrocytes after immunohistochemical staining. Cells from symmetrical regions on both sides were counted. ***D–G***, Representative examples of sections from the dorsal cortex of P1 wild-type (***D***, ***F***) or *dream*-deficient (***E***, ***G***) mice, processed for GFAP immunoperoxidase staining (***D***, ***E***) or S100β immunofluorescence (***F***, ***G***) used for quantitative analysis. Scale bars, 50 μm.

GFAP expression and astrocyte formation *in vivo*, despite the observation that cells lacking DREAM fail to respond to PACAP *in vitro*. The observed decrease in the number of astrocytes was accompanied by increased number of neurons at a time that coincides approximately with the onset of astrocyte generation after transition from neurogenesis to astrogenesis. This observation is consistent with the possibility that neurogenesis has not been inhibited in time and precursor cells keep on producing neurons instead of astrocytes. One possible interpretation of our results is that in cortical precursors DREAM might be part of a signaling mechanism involved in the coordinated inhibition of neurogenesis at the same time that it stimulates astrogliogenesis.

Signals acting on cortical precursors that stimulate astrogliogenesis and at the same time inhibit neurogenesis are important for regulating the timing of activation of the neurogenesis-to-gliogenesis switch (Miller and Gauthier, 2007). It is possible to predict that if these signals fail, the outcome would be an excess production of neurons at the expense of astrocytes, which would be present in lower numbers at the beginning of the astrogliogenic period. Our observations fit with this model, and thus support the notion that DREAM may be part of the complex mechanisms that operate during the transition from neurogenesis to astrogliogenesis.

Interestingly, the relatively high number of astrocytes generated in *dream* mutant mice after P7 indicates that mechanisms to

Role of DREAM in astrocyte differentiation

The requirement of DREAM for appropriate expression of GFAP during astrocytogenesis is indicated by our observations that cortical precursor cells prepared from *dream* $^{-/-}$ mice fail to differentiate into astrocytes in response to PACAP, and that these mice exhibit lower levels of GFAP and reduced astrocytogenesis during the early postnatal period. These observations are also consistent with the finding that treatment of rats with a PACAP receptor antagonist during late pregnancy significantly reduces the number of cortical astrocytes in the offspring (Zupan et al., 1998).

We found that lack of DREAM does not prevent, but delays,

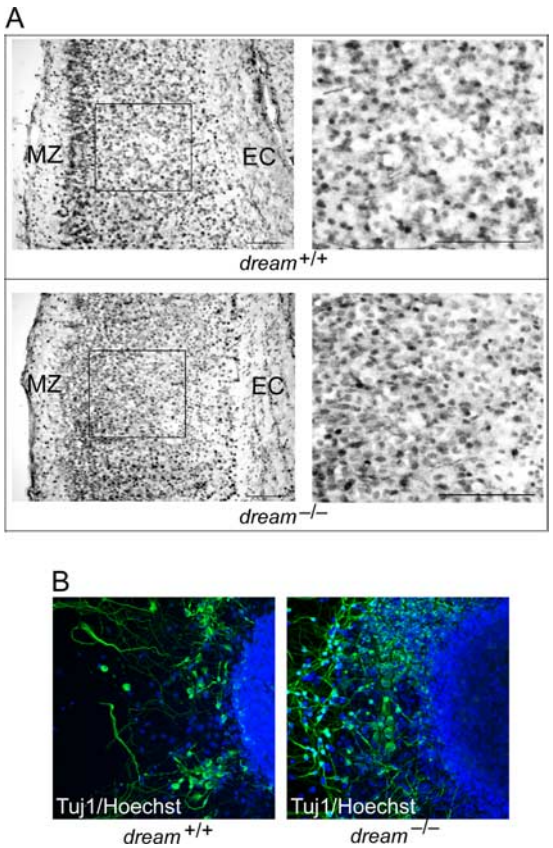

Figure 7. DREAM-deficient mice exhibit increased number of neurons in the cortex during early postnatal period. ***A***, Photomicrograph depicting a lateral region of a coronal section of the brain from postnatal day 1 mice similar to that shown in Figure 6*C*. Sections from wild-type (top) or dream-deficient (bottom) mice were stained for NeuN immunoreactivity. The right panels depict higher-magnification images corresponding to the areas indicated by a rectangle. EC, External capsule; MZ, marginal zone. Scale bars, 100 μm. ***B***, Tuj1 immunoreactivity (green) detected in BDNF-treated attached neurosphere cultures prepared form the brains of wild-type (left) or *dream*-deficient (right) mice. Blue indicates cells counterstained with Hoechst.

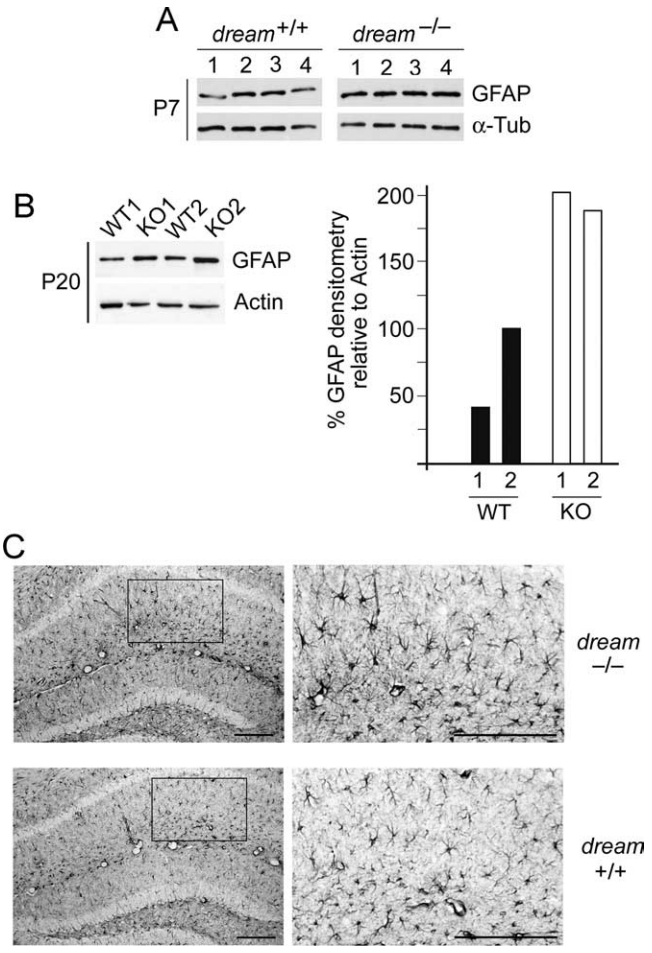

Figure 8. Increased number of astrocytes in the cortex of adult DREAM-deficient mice. ***A***, Western immunoblots showing GFAP expression in extracts of cortices isolated from P7 DREAM-deficient (*dream* $^{-/-}$) or control wild-type (*dream* $^{+/+}$) mice. Numbers on top of each lane indicate individual mice from which samples were obtained. α-Tubulin (α-Tub) immunoreactivity was used as a control to monitor even loading of samples. Results from densitometric analyses performed to quantify the relative intensity of DREAM-immunoreactive bands expressed as a percentage of the increment of densitometry measurements (in arbitrary units) relative to the intensity of the corresponding α-tubulin bands were 84 ± 15 for wild-type mice and 137 ± 13 for DREAM-null mice (mean $\pm$ SEM; $p < 0.002$, Student's *t* test). ***B***, Western immunoblots showing GFAP expression in extracts of cortices isolated from P20 DREAM-deficient (KO) or control wild-type (WT) mice. The numbers on top of each lane indicate individual mice from which samples were obtained. Actin immunoreactivity was used as a control to monitor the even loading of samples. Depicted to the right is the densitometric analyses performed to quantify the relative intensity of DREAM-immunoreactive bands. Results are expressed as a percentage of the increment of densitometry measurements of DREAM bands (in arbitrary units) relative to the intensity of the corresponding actin bands. Individual densitometric values are plotted. ***C***, Representative examples of sections from the hippocampus of *dream*-deficient (top) or wild-type (bottom) mice, processed for GFAP immunostaining used for quantitative analysis. The right panels depict higher-magnification images corresponding to the areas indicated by a rectangle. Scale bar, 100 μm.

compensate for the initial loss of astrocytes are activated to match the number of glial cells to that of neurons. The existence of this type of compensatory mechanisms has been proposed previously (Koblar et al., 1998; Barnabé-Heider et al., 2005; Miller and Gauthier, 2007) and could involve, at least in part, the activity of neuron-derived neurotrophic cytokines (Barnabé-Heider et al., 2005), which, in our case, would be present in higher amounts as a consequence of the initial overproduction of neurons. Therefore, in an environment relatively enriched in neurons, such as the one found in the developing cortex of newborn DREAM-deficient mice, this situation could yield to the delayed generation of a relatively increased number of astrocytes.

Obviously such a situation requires that the signaling pathways used by neurotrophic cytokines in neural precursors have to be intact. Neurotrophic cytokines secreted from newly differentiated neurons, which provide a major signal promoting the generation of astrocytes (Barnabé-Heider et al., 2005), act via the gp130-JAK-STAT pathway (Bonni et al., 1997; Koblar et al., 1998; Rajan and McKay, 1998; Nakashima et al., 1999; Barnabé-Heider et al., 2005). Our experiments with CNTF on cortical precursors indicate that this signaling pathway is not affected by lack of DREAM for the expression of GFAP. Whether the enhanced astrocyte generation observed in mice with defective DREAM-dependent signaling after overproduction of neurons and delayed astrogliogenesis is indeed attributable to the existence of higher amounts of neuron-derived neurotrophic cytokines remains the subject of further investigations.

DREAM has been shown to regulate the expression of different neuronal genes acting as a transcriptional repressor (Carrión et al., 1999; Link et al., 2004; Gomez-Villafuertes et al., 2005). In this study, we present evidence indicating that in cortical precursor cells DREAM can act as a transcriptional transactivator that regulates GFAP gene expression during astrocyte differentiation via cAMP-dependent calcium signaling. Thus, these findings provide new insights into the mechanisms that regulate astrocytogenesis in the developing cortex.

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