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Enhanced CREB phosphorylation in immature dentate gyrus granule cells precedes neurotrophin expression and indicates a specific role of CREB in granule cell differentiation

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Abstract

Differentiation and maturation of dentate gyrus granule cells requires coordinated interactions of numerous processes. These must be regulated by protein factors capable of integrating signals mediated through diverse signalling pathways. Such integrators of inter and intracellular physiological stimuli include the cAMP-response element binding protein (CREB), a leucine-zipper class transcription factor that is activated through phosphorylation. Neuronal activity and neurotrophic factors, known to be involved in granule cell differentiation, are major physiologic regulators of CREB function. To examine whether CREB may play a role in governing coordinated gene transcription during granule cell differentiation, we determined the spatial and temporal profiles of phosphorylated (activated) CREB throughout postnatal development in immature rat hippocampus. We demonstrate that CREB activation is confined to discrete, early stages of granule cell differentiation. In addition, CREB phosphorylation occurs prior to expression of the neurotrophins BDNF and NT-3. These data indicate that in a signal transduction cascade connecting CREB and neurotrophins in the process of granule cell maturation, CREB is located upstream of neurotrophins. Importantly, CREB may be a critical component of the machinery regulating the coordinated transcription of genes contributing to the differentiation of granule cells and their integration into the dentate gyrus network.

Keywords

development; hippocampus; neurogenesis; rat; transcription factor

Introduction

In most regions of mammalian brain, production of neurons is largely confined to discrete developmental periods, and the differentiation of precursors to postmitotic neurons is completed by birth. However, in the dentate gyrus (DG) of the hippocampal formation, neuronal stem cells located at the inner border of the granule cell layer (the subgranular zone) continue to produce new granule cells (GCs) throughout adult life. This special quality of DG (Altman & Das, 1965; Kaplan & Hinds, 1977; Bayer *et al*., 1982), has attracted considerable interest because of its potential implications for brain repair. This attention has been further kindled by the discovery of ongoing production of new GCs, not only in rodent,

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but also in adult primate (Gould *et al*., 1999b) and human hippocampus (Eriksson *et al*., 1998). However, while the understanding of factors influencing the proliferation rate of this neural stem cell population has been evolving (Cameron & Gould, 1994; Cameron *et al*., 1995; Gould *et al*., 1999a; Van Praag *et al*., 1999), the processes that regulate differentiation and maturation of newly produced GCs remain elusive.

In the developing rat hippocampus, production of GCs begins on embryonic day 17 (E17), with proliferation of putative stem cells in a primary dentate neuroepithelium adjacent to the alveus (Altman & Bayer, 1990a). Subsequently, stem cells leave the primary dentate matrix and proceed towards the developing DG, where they form secondary and tertiary proliferation hilar matrices that give rise to the majority of GCs. Some of these stem cells eventually settle in the subgranular zone and retain their proliferative capacity (Altman & Bayer, 1990b). During development, new GCs migrate from the hilus to the GC layer, where they join its deepest portion (Schlessinger *et al*., 1975; Crespo *et al*., 1986; Rickmann *et al*., 1987). GC differentiation requires the interplay of multiple factors. For example, neurotrophic factors have been shown to influence the process (Lowenstein & Arsenault, 1996), and afferent innervation and neuronal activity are essential for GC dendrites to assume their mature morphology (Zafirov *et al*., 1994; Drakew *et al*., 1999). Thus, both inter and intracellular communication is required to activate and coordinate the GC differentiation program.

Cues derived from several intracellular signal transduction pathways may be integrated by transcription factors, including the cAMP-response element binding protein (CREB). CREB is a member of a transcription factor family, belonging to the basic-domain leucine-zipper class, that regulates transcription via a specific DNA target, the cAMP-response element (CRE; Brindle & Montminy, 1992; Sassone-Corsi, 1995). CREB activity requires phosphorylation at Ser-133 in response to diverse physiological signals (Gonzalez & Montminy, 1989). Once phosphorylated, CREB interacts with coactivators to govern the transcriptional machinery (Montminy, 1997). Importantly, neuronal activity (Ghosh & Greenberg, 1995; Bito *et al*., 1996; Deisseroth *et al*., 1996; Impey *et al*., 1996) and neurotrophic factors (Ginty *et al*., 1994; Finkbeiner *et al*., 1997) are major physiologic regulators of CREB function, and CREB activation has been suggested to contribute to GC survival (Walton & Dragunow 2000). These facts led to the supposition that CREB may play a key role in governing coordinated gene transcription during GC differentiation. To examine this hypothesis, we determined the temporal and spatial activation of CREB during postnatal development and its relationship to the expression of markers of discrete GC differentiation stages and of neurotrophins.

Materials and methods

Animals and tissue handling

Offspring of timed-pregnant Sprague-Dawley dams (Zivic-Miller, Zelienople, PA, USA) were used for these experiments. All experiments aimed to minimize pain or discomfort, were approved by the UCI Animal Care Committee and conformed to NIH guidelines. Animals were deeply anaesthesized with sodium pentobarbital and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) on postnatal days (P) 2, 5, 7, 9, 10, 12, 18, 20 or 35 (n = 4 per timepoint). Brains were removed, postfixed in perfusion buffer (4 h), cryoprotected, and frozen in −50 °C isopentane. Coronal sections (30 or 50 μm) were cut using a cryostat and collected in 0.1 M Tris-buffer for immunocytochemistry or diethylpyrocarbonate-treated 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate) for *in situ* hybridization.

Immunocytochemistry

Sections were washed (2 × 5 min) in 0.1 M Tris-buffer and preincubated in the same buffer supplemented with 3% normal goat serum and 0.25% Triton X-100 (Sigma, St Louis, MO; 1 h) before being incubated with primary antisera recognizing the Ser-133-phosphorylated isoform of CREB (pCREB; rabbit polyclonal IgG, Upstate Biotechnology, Lake Placid, NY; 1: 2000) at room temperature (RT; 12 h). Sections were washed (3 × 15 min) and exposed to a secondary antiserum (biotinylated antirabbit IgG; Vectastain ABC-Kit, Vector Laboratories, Burlingame, CA; 1: 250). Antibody binding was visualized by the avidin–biotin–peroxidase reaction with 3, 3′-diaminobenzidine (DAB; Sigma, 0.05%) and hydrogen peroxide (0.002%). Control sections, incubated without the primary antibody, had no immunostaining. For detection of calbindin-D28k (rabbit polyclonal IgG, Chemicon, Temecula, CA; 1: 1000), or polysialylated neural cell adhesion molecule (PSA-NCAM; mouse monoclonal IgG, courtesy Dr Tatsunori Seki; 1: 500), Triton was excluded from the solutions. Some labelled sections were counter-stained with cresyl violet, and all were mounted, dehydrated, cleared and cover-slipped (Permount, Fisher, Pittsburgh, PA). For colocalization purposes, sections were first exposed to the pCREB-antiserum, but signal was visualized using DAB supplemented with 0.01% $CoCl_2$ and 0.01% $NiCl_2$. This produced a black reaction product in pCREB-immunopositive nuclei. After washing, sections were incubated with anti-PSA-NCAM or parvalbumin (monoclonal IgG, Chemicon; 1: 7000). This second immunoreaction was visualized using DAB alone, resulting in a light brown membranous (PSA-NCAM) or cytoplasmic (parvalbumin) reaction product.

BrdU labelling

Rats were injected with 5-bromo-2′-deoxyuridine (BrdU, Roche, Indianapolis, IN; 50 μg/g body weight, i.p.) on P8 and perfused 48 h later. After calbindin- or pCREB-immunocytochemistry, sections were transferred to 2x SSC, washed (2 × 15 min), immersed in 50% formamide/2x SSC to denature DNA (2 h; 65 °C) and incubated in 2 M HCl (30 min at 37 °C). Following neutralization with 0.1 M sodium borate (10 min at RT), sections were incubated with an antibody directed against BrdU (rat monoclonal IgG, Accurate Chemical, Westbury, NY, 1: 400), followed by biotinylated second antibody and the avidin–biotin–peroxidase reaction components. Benzidine dihydrochloride (Sigma, 0.01%), a peroxidase-substrate producing a blue granular staining, was used as chromogen for BrdU-detection, to permit distinction of overlapping nuclear BrdU- and pCREB-labelling.

In situ hybridization

Tissue sections (25 μm) were processed for *in situ* hybridization either slide-mounted (P2, P9) or free-floating (P20) as described in detail previously (Lauterborn *et al*., 1994). Briefly, ^{35}S-labelled cRNA-probe for brain-derived neurotrophic factor (BDNF) exon V was transcribed (Isackson *et al*., 1991), yielding a 540 base length probe. For neurotrophin-3 (NT-3) mRNA localization, a 550 base ^{35}S-cRNA was transcribed from *Pvu*II-digested recombinant plasmid pRNT3–1 (Gall *et al*., 1992). Following proteinase K treatment (for P20 brain only, Sigma; 1 μg/mL), sections were hybridized for 30– 40 h at 60 °C as described (Lauterborn *et al*., 1994). Sections were washed, RNAse treated, and washed to a final stringency of 0.1x SSC at 60 °C for 1 h, then mounted and dipped in emulsion (NTB-2, Kodak, Rochester, NY; 1: 1 with H_2O) for 4–6 weeks at 4 °C.

Results

Time course of CREB activation in the hippocampal formation

Immunostaining for pCREB resulted in the expected nuclear localization of the activated transcription factor (Ginty *et al*., 1993) and revealed a highly reproducible spatial and

temporal pattern of pCREB-immunoreactivity in the postnatal hippocampus. During the first postnatal week, the pyramidal cell layer was well labelled (Fig. 1A). In DG, scattered hilar cells were pCREB-immunoreactive, whereas little signal was observed over the GC layer. This pattern was quite different during the second postnatal week, when pCREB-immunoreactivity was drastically reduced in the pyramidal cell layer while the GC layer labelling was increased (Fig 1B and C). However, immunoreactive cells were not equally distributed throughout the GC layer: nuclei located in the deeper (hilar) half of the layer were strongly immunoreactive for pCREB, but immunoreactivity decreased with increasing distance from the hilus, so that GCs in the superficial half were virtually unlabeled (Figs 1C and 2A). Dense pCREB-immunoreactivity was still observed during the third post-natal week, but the proportion of pCREB-immunoreactive cells was now lower, occupying only the innermost cells of the GC layer (Figs 1D and 2C). By P35, pCREB-immunoreactivity was restricted to scattered cells at the base of the GC layer (Figs 1E and F, and 3C and D) possessing irregular shaped, elongated or ellipsoid nuclei characteristic of immature granule cells (Seki & Arai, 1993).

Selectivity in the distribution of pCREB was evident not only in the superficial–deep axis of the GC layer, but also comparing the suprapyramidal and infrapyramidal blades. In both, pCREB was detected as early as P7. However, whereas during the third postnatal week CREB activity was clearly diminished in the suprapyramidal blade, GCs in the infrapyramidal blade were still intensely labelled (Fig. 1D). This sustained pCREB-immunoreactivity in the infrapyramidal blade may reflect a maturational delay, as both the production of GCs destined to form the infrapyramidal blade (Altman & Bayer, 1990b), and their structural differentiation (Cowan *et al*., 1980; Tamamaki, 1999) lags that of the suprapyramidal blade by several days.

Distribution of immature and mature granule cells in the granule cell layer

If pCREB is involved in differentiation, then its expression should be specific for late proliferative and/or early differentiation phases of immature GCs. To determine the relationship of CREB phosphorylation to proliferation of GCs, we injected rat pups (at P8) with the thymidine-analogue BrdU and perfused them 48 h later (P10). The location of newly formed, BrdU-labelled cells in the GC layer was then determined and BrdU-positive neurons were examined for pCREB-immunoreactivity. BrdU-immunopositive nuclei were abundant in the deeper, intensely pCREB-labelled zone of the GC layer. Indeed, BrdU was frequently located in pCREB-immunoreactive nuclei (Fig. 2E). BrdU-immunopositive neurons were rarely observed in the pCREB-negative, superficial zone, indicating that CREB activation in GCs is restricted to a discrete early period after their birth.

To further pinpoint the relationship of pCREB and differentiation, we immunolabeled sections with antisera directed against established markers for immature and fully differentiated GCs. The polysialylated isoform of the neural cell adhesion molecule (PSA-NCAM; Seki & Arai, 1993) was used to visualize immature GCs, whereas the calcium-binding protein calbindin-D28k (Sloviter, 1989) provided a marker for fully differentiated neurons. Patterns of PSA-NCAM- and pCREB-immunoreactivities were quite similar. Neurons expressing PSA-NCAM on their cell membrane were densely distributed throughout the deeper GC layer at P10 (Fig. 3A), but their number decreased with advancing age until, by P35, only neurons at the base of the GC layer still expressed PSA-NCAM (Fig. 3B). Double-labelling for pCREB and PSA-NCAM revealed that the two molecules were highly colocalized in these neurons (Fig. 3C), thus further supporting the notion that CREB is preferentially activated in immature GCs. The presence of PSA-NCAM-immunostaining in both GC perikarya and in dendrites that were already extending into the molecular layer illustrates that these neurons are relatively advanced in their differentiation and do not represent newborn GCs. Nuclei of parvalbumin-positive basket cells, intermingled with

GCs, were mostly devoid of pCREB-immunoreactivity (Fig. 3D) further indicating the cell-type specificity and/or the differentiation-stage-specific roles for pCREB.

The pattern of calbindin-immunoreactivity was generally the inverse of those observed for pCREB and PSA-NCAM. Calbindin-expressing neurons were confined to the superficial half of the GC layer at P10 (Fig. 2B), and occupied a progressively greater proportion of the layer with increasing age (Fig. 2D). As expected from the data described above, BrdU-labelled nuclei were rarely observed in cells immunoreactive for calbindin (Fig. 2F). Together with the results of the BrdU/pCREB and PSA-NCAM/pCREB colocalization studies, these highly specific distribution patterns of pCREB and calbindin warrant the conclusion that CREB is activated in GCs early after their birth and that this activation is sustained throughout a considerable time period of their differentiation.

Expression of BDNF and NT-3 in the developing granule cell layer

Neurotrophins are a major activator of CREB function (Ginty *et al*., 1994; Finkbeiner *et al*., 1997). BDNF and NT-3 are expressed relatively early during GC layer development (Friedman *et al*., 1991; Dugich-Djordjevic *et al*., 1992; Lauterborn *et al*., 1994; Martinez *et al*., 1998), and both neurotrophins have been suggested to contribute to GC differentiation (Lowenstein & Arsenault, 1996). Previous studies on neurotrophin expression in DG, however, have mainly reported on their ontogeny and general location, but have not described in detail the location of neurotrophins within the GC layer. If CREB and neurotrophins interact to promote differentiation of GCs, their temporal and spatial expression/activation patterns should be similar. Therefore, we studied the spatial distribution of BDNF and NT-3 gene expression in DG during the first, second and third postnatal weeks using *in situ* hybridization. At P2, low levels of both neurotrophin mRNAs were distributed rather homogeneously throughout the barely distinct GC layer. However, by P9 and P20 both BDNF and NT3 (Fig. 4) were highly expressed in the superficial GC layer zones, but not in the deeper zones that consist of immature GCs. This expression pattern is highly consistent with the distribution of mature GCs (Fig. 2B and D). The absence of BDNF and NT-3 expression in immature GCs implies that the synthesis of neurotrophins is a late rather than an early event during GC maturation.

Discussion

The major results of this study are: (i) The transcription factor CREB is highly phosphorylated (activated) in the dentate gyrus GC layer during a discrete developmental period. (ii) Precise timing of CREB phosphorylation, using morphological and spatial criteria as well as proliferation and differentiation markers, indicates that CREB activation is confined to GCs during a late proliferative/early differentiation stage. (iii) Maximal CREB phosphorylation precedes neurotrophin expression in the differentiating GC layer. Therefore, we suggest that CREB may play a critical and specific role, governing transcription of genes involved in the GC differentiation program.

What specific role could CREB fulfill? Potentially, CREB function during GC differentiation may differ from its roles in adult brain. In this scenario, CREB could govern a GC-specific differentiation program by inducing the transcription of an array of stage-specific genes. Alternatively, the function of CREB in developing GCs may be closely related to its proposed role in adult networks. There is considerable evidence that CREB is a crucial player in mediating synaptic plasticity (Martin & Kandel, 1996; Silva *et al*., 1998). As the maturation of a newly formed neuron involves its integration into the surrounding network, a process requiring the formation and honing of neuronal connections, CREB may contribute to functional and morphological synaptogenesis in differentiating GCs.

The first alternative is supported by the demonstration that CREB, while recognizing the CRE-sequence almost exclusively in adult brain, interacts also with the related AP-1 element during the first three postnatal weeks. (Pennypacker *et al*., 1995). In binding AP-1, CREB may act as a hetero rather than homodimer, as the affinity of CREB-homodimers to AP-1 is weak (Foulkes *et al*., 1991; Hai & Curran, 1991). CREB has been shown to form heterodimers with related proteins (Foulkes *et al*., 1991; Hai & Curran, 1991) and such heterodimerization may alter its DNA-binding affinity, enabling induction of genes during development that are completely different from CREB's target genes in adult brain.

The findings in the current study, however, favour the second alternative. GC production starts prior to birth (Altman & Bayer, 1990a), expression of calbindin-D28k, a marker of relatively advanced differentiation, is found in the superficial GC layer already shortly after birth (Goodman *et al*., 1993; Bender, unpublished observations). However, as shown in Fig. 1, CREB activity was minimal in the GC layer during the first postnatal week, and was strongly upregulated specifically during the second and third postnatal weeks. This time course is remarkable, as during the second and third postnatal weeks GCs undergo prominent morphological and physiological changes indicating their functional maturation, including formation of the majority of *afferent* synaptic connections (Crain *et al*., 1973; Cowan *et al*., 1980) and morphological maturation of GC dendrites (Cowan *et al*., 1980; Zafirov *et al*., 1994). Also during this period, GC axons establish giant synapses on CA3 pyramidal cell dendrites indicating maturation of their *efferent* connections (Amaral & Dent, 1981; Gaiarsa *et al*., 1992).

In this developing network, the function of CREB may parallel its proposed role in adult brain, including, for example, contribution to stabilization of functional synaptic contacts that are essential both for maturation and for maintenance of the hippocampal circuit. Thus, the maturation of GC dendrites, including spine formation, requires afferent neuronal activity. A mechanistic role for CREB in dendritic maturation and plasticity has been suggested (Murphy & Segal, 1997), and supported by Crino *et al*. (1998), demonstrating CREB mRNA transcription and CREB phosphorylation in response to external stimuli in developing dendrites. CREB may also participate in the establishment of new connections via presynaptic mechanisms: a close interplay between CREB and the cell adhesion molecule Fasciclin II (FasII) in the formation and honing of new synapses in *Drosophila* larvae neuromuscular junction has been demonstrated (Davis *et al*., 1996). Interestingly, the Fas II mammalian homologue, NCAM, and specifically its isoform PSA-NCAM, is essential for synaptic plasticity in the hippocampal formation (Muller *et al*., 1996). Recent findings providing an intracellular link between NCAM and CREB through the Ras–Mitogen-activated protein kinase/Extracellular-signal Regulated Kinase (MAPK/ERK) cascade (Schmid *et al*., 1999; Davis *et al*. 2000) and our demonstration of colocalization of pCREB and PSA-NCAM in immature GCs (a similar finding was reported by Young *et al*., 1999), further strengthen the assumption of a related functional interaction of these two molecules in the mammalian brain.

As noted, functional differentiation and maturation of DG during the second and third postnatal weeks involve a high degree of synapse formation and plasticity. During this stage, as in adult brain, CREB may be a key transducer of synapse-derived signals to the nucleus (Hatalski & Baram, 1997), thus coordinating the integration of immature GCs into the existing network. This differentiation-promoting role of CREB may not be restricted to GCs. We found that CREB is highly activated in hippocampal pyramidal neurons during the first postnatal week, but not later. Indeed, formation and maturation of pyramidal neurons (Bayer, 1980) and arrival of afferent entorhinal fibers (Supèr & Soriano, 1994) precede the corresponding processes in GCs by several days. Thus, earlier CREB activation in

pyramidal cells supports its role in the integration of both cell types in the evolving hippocampal network.

How does this proposed role of activated CREB in differentiation fit with the established functions of neurotrophins? NT-3 and BDNF mRNA expression have been detected shortly after birth in GCs (Friedman *et al*., 1991; Dugich-Djordjevic *et al*., 1992; Lauterborn *et al*., 1994; Martinez *et al*., 1998) and a differentiation-promoting effect of both neurotrophins on GCs has been demonstrated (Lowenstein & Arsenault, 1996). It is well established that neurotrophins can induce CREB phosphorylation (Finkbeiner *et al*., 1997), and indeed, release of neurotrophins from mature GCs could phosphorylate CREB in immature ones. However, our observation that during the second and third postnatal weeks NT-3 and BDNF are preferentially expressed in more mature GCs, and the discovery that CREB can mediate a calcium (activity)-dependent trans-activation of the BDNF-gene via a CRE-element located in the promoter of BDNF-exon III (Shieh *et al*., 1998; Tao *et al*., 1998), prompt us to consider an alternative sequence of events. In a signal cascade connecting CREB and neurotrophins during GC differentiation, the neurotrophins may be located downstream rather than upstream of CREB. In this scenario, synaptic stimulation of immature GC dendrites induces neurotrophin-transcription via CREB, leading to neurotrophin-induced differentiation of the network, possibly through mechanisms reviewed previously (Marty *et al*., 1997; Klintsova & Greenough, 1999; McAllister *et al*., 1999; Schuman, 1999).

In summary, our results provide the first evidence for a critical role of CREB-mediated gene transcription in the differentiation of DG granule cells. In view of the rapid advances in studies of CREB function, and the large body of accumulated information regarding DG anatomy and physiology, the current findings should stimulate novel and rewarding research avenues into transcription factor regulation of the GC differentiation program.

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Abbreviations

AP-1	activating protein 1
BDNF	brain-derived neurotrophic factor
BrdU	5-bromo-2′-deoxyuridine cAMP, cyclic adenosine 3′, 5′-monophosphate
CRE	cAMP-response element
CREB	cAMP-response element binding protein
DAB	3, 3′-diaminobenzidine
DG	dentate gyrus
ERK	Extracellular-signal Regulated Kinase
FasII	Fasciclin II
GC	granule cell
MAPK	Mitogen-activated protein kinase
NT-3	neurotrophin-3
P	postnatal

pCREB	phosphorylated CREB
PSA-NCAM	polysialylated neural cell adhesion molecule
SSC	saline sodium citrate

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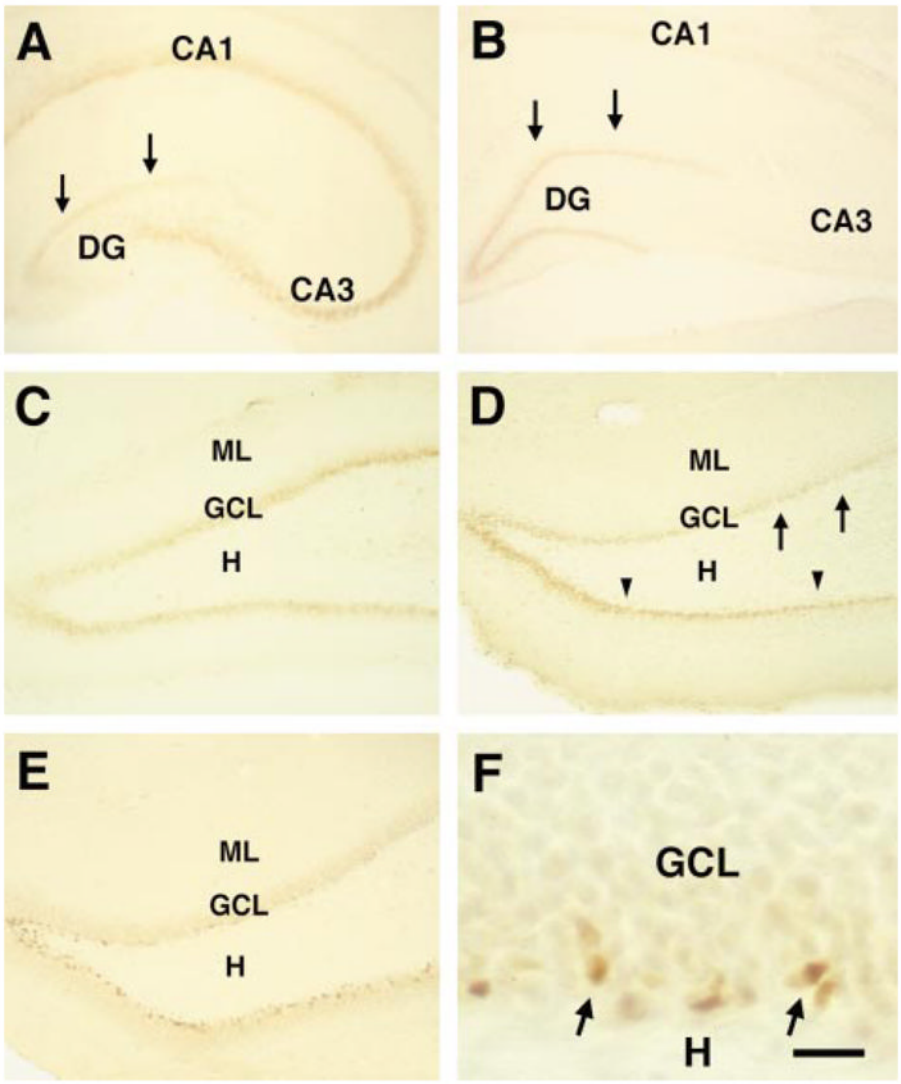

Fig. 1.

Activation pattern of CREB during postnatal hippocampal development. (A) In the hippocampal formation of the five day old rat (P5), phosphorylated CREB (pCREB) is clearly evident in the pyramidal layer (CA1 and CA3) compared with the granule cell (GC) layer (arrows). (B) By P12, hippocampal CREB activation pattern is radically altered; pCREB-immunoreactivity is almost undetectable in the pyramidal cell layer. In contrast, the GC layer (arrows), is now strongly immunoreactive. A higher magnification of the dentate gyrus (C) reveals that during the second postnatal week (P10) CREB is strongly phosphorylated in GCs, forming a 3–4 cells-deep, almost continuous band, along the hilar border of the GC layer. (D) By P18, pCREB-immunoreactive neurons still outline the hilar border, but in the earlier-maturing, lateral portion of the suprapyramidal blade (arrows), the density of immunoreactive cells is already diminished, whereas the hilar border of the infrapyramidal blade (arrowheads) remains intensely stained throughtout its length. (E) By P35, pCREB-immunoreactive neurons are scattered in a discontinuous, thin band at the GC layer hilar border. (F) a higher magnification of (E), shows variable, elongated or oval pCREB-labelled nuclei (typical of immature GCs), confined to the hilar border of the GC

layer (arrows). CA, cornu ammonis; DG, dentate gyrus; GCL, granule cell layer; H, hilus; ML, molecular layer. Scale bar, 250 μm (A and B), 150 μm (C, D and E); 20 μm (F).

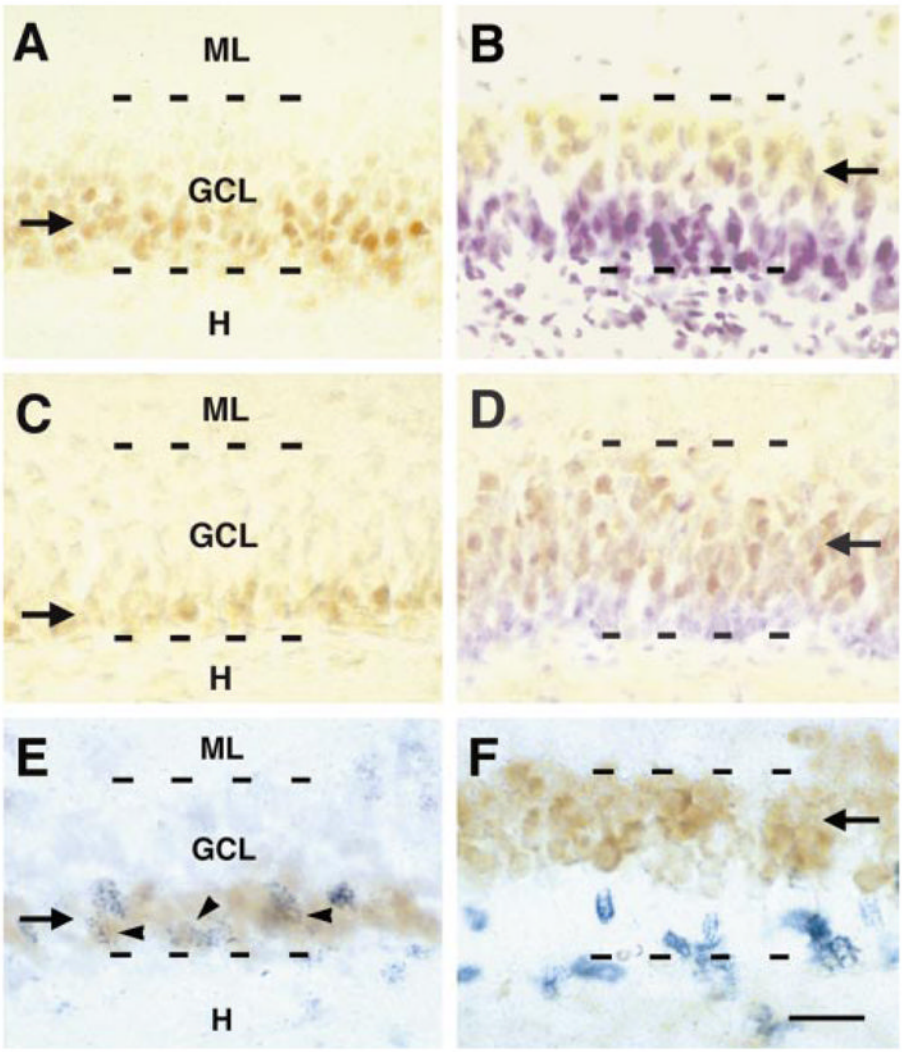

Fig. 2.

Distribution of phosphorylated CREB (pCREB), and its relationship to markers of newly formed and differentiated granule cells (GCs). Panels A–D demonstrate the granule cell layer (GCL; suprapyramidal blade) of a P10 (A and B), and of a P18 rat (C and D). Antisera against pCREB (A and C) and calbindin-D28k (B and D), a marker of differentiated GCs, reveal a virtually inverse labelling pattern, reflecting GCL maturation during the second and third postnatal weeks. The zone occupied by pCREB-immunoreactive neurons (brown reaction product; arrows in A and C) decreases progressively, whereas the zone occupied by calbindin-immunoreactive neurons increases with age (yellowish reaction product, arrows in B and D). Note that B and D are counter-stained with cresyl violet. (E and F) Immature GCs, labelled with 5-bromo-2′-deoxyuridine (BrdU), coexpress pCREB, but not calbindin-D28k. Sections represent the GCL of 10-day-old rats injected with BrdU 48 h earlier and double-labelled for BrdU and either pCREB (E) or calbindin (F). (E) BrdU-positive nuclei (blue granular reaction product), are seen in the deep, hilus-adjacent zone of the GCL, that is immunoreactive for pCREB (brown reaction product, arrow); frequent colocalization of pCREB and BrdU is evident (arrowheads). (F) BrdU-labelled nuclei (in blue) are only rarely found in the superficial, calbindin-immunoreactive zone (brown reaction product, arrow in F). H, hilus; ML, molecular layer. Scale bar, 40 μm (A–D); 20 μm (E and F).

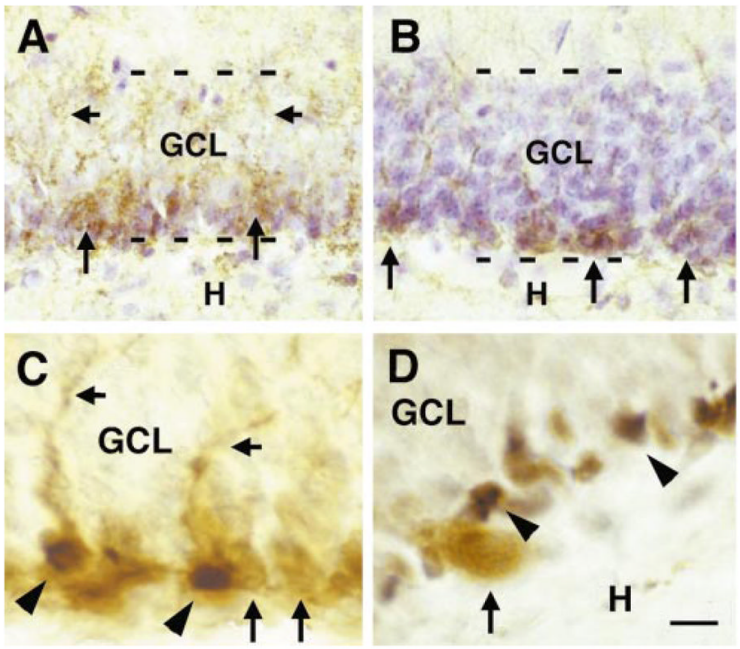

Fig. 3.

The relationship of the polysialylated neuronal cell adhesion molecule (PSA-NCAM), a marker of immature neurons, and phosphorylated CREB (pCREB) in the developing granule cell layer (GCL). (A) On P10, PSA-NCAM-immunopositive perikarya are confined to the deeper half of the GCL (arrows), and are not observed in the superficial half. The PSA-NCAM-labelled dendrites of these neurons are found in the superficial GCL zone, demonstrating that PSA-NCAM-labelled neurons already extend their dendrites towards the molecular layer (short arrows, see also C). (B) By day 35, PSA-NCAM-immunoreactive neurons are restricted to the hilar base of the GCL (arrows). (C) Double-labelling demonstrates colocalization (arrowheads) of PSA-NCAM (brown reaction product) and pCREB (black reaction product) in some but not all PSA-NCAM immunoreactive neurons. Note that neurons expressing PSA-NCAM alone are marked by arrows. (D) In contrast, double-labelling for pCREB and parvalbumin, a protein marker for interneuronal basket cells within the GCL illustrates that pCREB is selectively activated in granule cells (GC) proper. Parvalbumin-positive cells (arrow) are devoid of pCREB-immunoreactivity, whereas many surrounding GC nuclei contain pCREB (arrowheads). H, hilus. Scale bar, 20 μm (A and B); 10 μm (C and D).

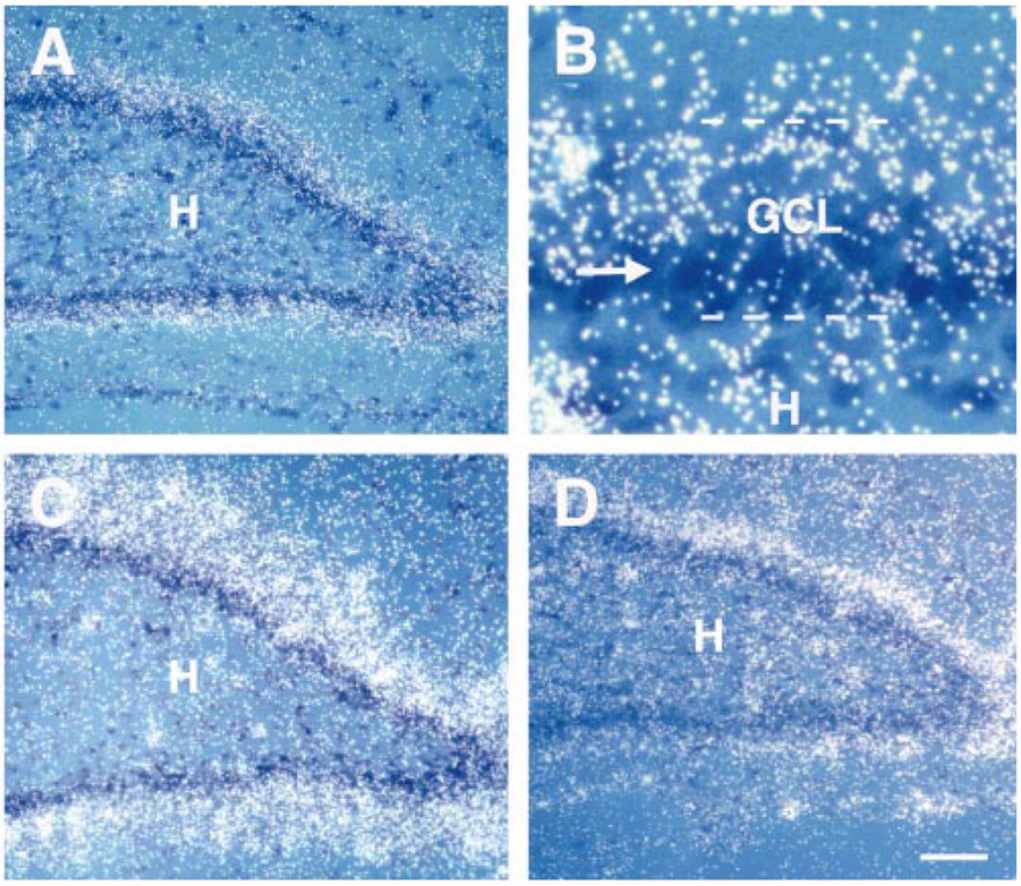

Fig. 4.

Neurotrophin expression in the dentate gyrus (DG) granule cell layer at P9 (A, B and D) and P20 (C). Sections were hybridized with ^{35}S-labelled cRNA-probes directed against BDNF (A, B and C) or NT-3 mRNA (D), and counter-stained with cresyl violet to outline the granule cell layer (GCL). (A–C) At P9 (A and B) and P20 (C) silver grain density, a measure of BDNF mRNA abundance, was greater in the superficial (mature) zones of the GCL compared with deeper (immature) zones (arrow in B). The width of the neurotrophin-expressing, mature zone increased progressively with age (compare A and C). This gradient parallels that of GC maturation (Fig. 2). (D) NT-3 mRNA showed a similar pattern of expression to that of BDNF mRNA. H, hilus. Scale bar, 100 μm (A, C and D); 20 μm (B).