Is CREB a key to neuronal survival?

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A range of molecules control nerve-cell survival in the brain. Many of these molecules might be neuroprotective through activation of the transcription factor cAMP-response-element-binding protein (CREB). Activation of CREB, by phosphorylation of Ser133, occurs in brain-damage-resistant hippocampal dentate granule cells and is triggered by neuroprotective environmental stimulation. In addition, the Akt neuroprotective signaling pathway activates CREB, and CREB synthesis and phosphorylation promote the survival of many cells, including neurons, *in vitro*. Thus, CREB might be responsible for programmed nerve-cell survival. Studies investigating its role in the brain are now required to confirm these *in vitro* results, and the downstream survival genes, whose expression is activated by CREB in neurons, need to be identified.

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SELECTIVE NEURONAL VULNERABILITY is a feature of a number of neurodegenerative diseases, but the processes that target specific neurons for death while allowing others to remain healthy are unclear. The differential activation of an internal death program in vulnerable neurons has been proposed as a mechanism to explain the selective death of neurons[1]. However, it is equally likely that specific neuronal populations contain an intrinsic survival mechanism. The presence or activity, or both, of such a pathway in different cell types could partly explain their varying sensitivities to detrimental brain insults. Research in this cell-survival area has focused on identifying the key mediators in this survival cascade and has concentrated on endogenous neuroprotective messengers, such as neurotrophic factors and various cytokines[2]. Recently, several studies implicated the transcription factor, cAMP-response-element-binding protein (CREB), not only in the signaling pathway activated by these molecules, but also as a possible regulator of a general survival program in neurons.

CREB transcriptional regulation

CREB is a member of a large family of structurally related transcription factors, including activating transcription factor 1 (ATF1), ATF2 (also know as CREBP1), ATF3 and ATF4, which bind to cAMP-response-element (CRE) promoter sites on target genes. The CREB protein can exist in three alternatively spliced isoforms, α, β and Δ, which have different properties and developmental regulation. CREB has been implicated in the transcriptional control of numerous genes, many of which are rapidly expressed in response to an elevation of cytoplasmic cAMP or Ca^{2+} levels. In common with the inducible transcription factors Fos and Jun, the CREB/ATF proteins consist of three functional domains: a leucine-zipper domain that mediates dimerization, a basic DNA-binding domain and the transcriptional activation domain, which contains important phosphorylation sites.

The transcriptional activation of CREB is crucially dependent on phosphorylation of Ser133 by protein kinase A (PKA)[3], Ca^{2+}-activated calmodulin kinases, ribosomal S6 kinase 2, or mitogen-activated protein-kinase-activated protein kinase 2 (Ref. 4). The mechanism by which Ser133 phosphorylation activates CREB remains unclear; however, it has been suggested that this modification induces a conformational change that transforms CREB from an inactive to an active configuration[5,6]. Alternatively, phosphorylated CREB (pCREB) might enhance transcription via the recruitment of co-activators such as CREB-binding protein (CBP)[7,8]. This idea is supported by the finding that microinjection of CBP antibodies inhibits cAMP-stimulated transcriptional responses[9]. Interestingly, in its non-phosphorylated form, CREB can suppress activator protein 1 (AP1) activity by competing with JUN protein for the AP1 site on target genes. As pCREB lacks this ability, its activation might act as a regulatory mechanism to facilitate transcriptional activation of immediate–early genes[10,11].

Selective vulnerability in the hippocampus

In its active form, CREB has been shown to regulate many aspects of neuronal functioning, including neuronal excitation[12], development[13] and long-term synaptic plasticity[14]. Recent evidence suggests that CREB might also be involved in an active process of neuroprotection, which therefore explains the selective vulnerability of the hippocampus to brain injury. The hippocampal subregions show a distinct pattern of cell death in response to a number of insults, in particular after prolonged seizures and hypoxic–ischemic (HI) episodes[1,15]. The CA1 pyramidal cells undergo apoptosis after these types of insult, whereas the dentate granule cells remain resistant to damage[15,16] (Fig. 1). A number of years ago it was speculated that this selective vulnerability was due to differential activation of cell-death (in CA1) and cell-survival (in granule cells) programs. In order to identify these programs, the expression of genes for various transcription factors (*Fos*, *Jun*, *Egr*) were examined after a HI insult[15,17]. The CA1 neurons that undergo apoptosis showed a delayed and prolonged expression of *Jun*, *Fos* and *Nur77* combined with a loss of EGR1. Although the resistant dentate granule cells also expressed *Jun*, as well as *Fos*, *Jund*, *Junb*, *Fosb*, *Nur77* and *Egr1*, the induction was rapid in onset and more transient. On the basis of these results, the now widely accepted hypothesis, that JUN is involved in neuronal death[18] (Fig. 1), was formulated. However, the expression of the genes encoding transcription factors alone does

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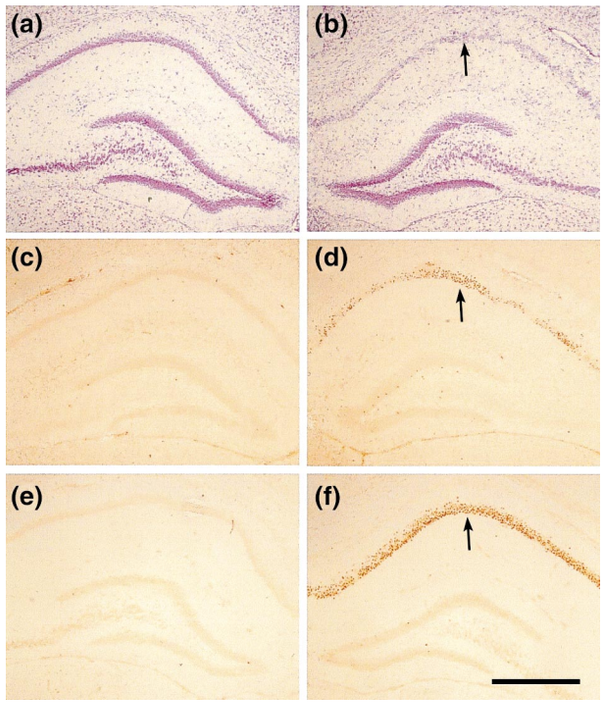

Fig. 1. ***Apoptotic damage to CA1 hippocampal neurons after hypoxia–ischemia.*** *Photomicrographs showing cresyl-violet staining* **(a)** *and* **(b)**, *terminal-deoxynucleotidyl-transferase-mediated dATP biotin nick-end labeling (TUNEL)* **(c)** *and* **(d)**, *and JUN immunoreactivity* **(e)** *and* **(f)** *in the control* (a), (c) *and* (e), *and injured* (b), (d) *and* (f) *hippocampus, 48 h* (e) *and* (f) *and 72 h* (a)–(d) *after a hypoxic–ischemic insult. Note that apoptotic neuronal death in the CA1 pyramidal cells (arrows) is preceded by a dramatic increase in JUN immunoreactivity that possibly activates the cell-death program. In contrast, the dentate granule cells show no evidence of cell death or JUN immunoreactivity at this time point. Scale bar, 720 μm.*

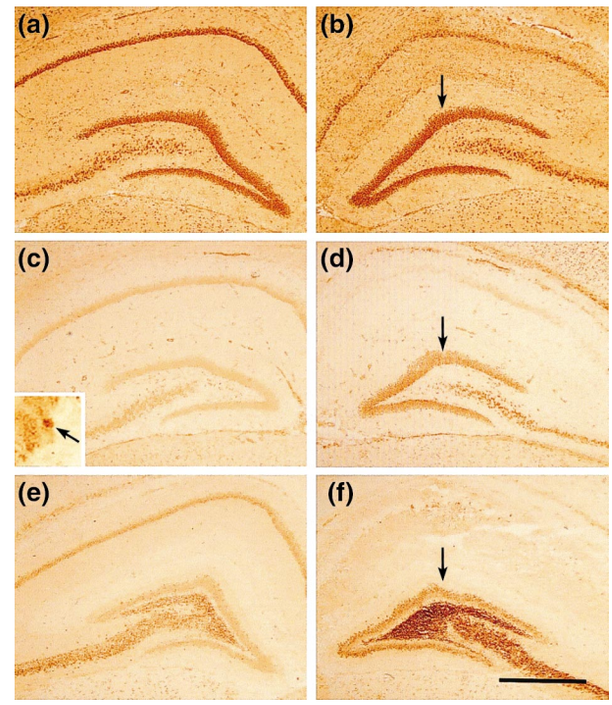

Fig. 2. ***CREB phosphorylation and*** **Bdnf** ***expression in resistant dentate granule cells after hypoxia–ischemia.*** *Photomicrographs showing cAMP-response-element-binding protein (CREB)* **(a)** *and* **(b)**, *phosphorylated CREB* **(c)** *and* **(d)**, *and brain-derived neurotrophic factor (BDNF)* **(e)** *and* **(f)** *immunoreactivity in control* (a), (c) *and* (e), *and injured* (b), (d) *and* (f) *hippocampus 48 h after a hypoxic–ischemic insult. Note the increase in both CREB phosphorylation [arrow in* (d)*] and BDNF immunoreactivity [arrow in* (f)*] in the dentate granule cells that survive this insult. In contrast, levels of CREB* (b), *phosphorylated CREB* (d) *and BDNF* (f) *are reduced in dying CA1 neurons. Insert in* (c) *shows a high-magnification photomicrograph of phosphorylated CREB immunoreactivity in the hilar–granule-cell border of the control hippocampus. The increased levels of phosphorylated CREB in the dividing dentate granule-cell precursors that align this border might be involved in their underlying neurogenic properties or injury resistance. Scale bar, 720 μm.*

not differentiate between sensitive (CA1) and resistant (dentate granule cells) neurons (although their kinetics of induction are vastly different in the two cell populations, which is likely to have functional consequences). This clearly demonstrates that other factors must be involved in neuronal death, one of which might be CREB activation.

After HI, there is a delayed increase in the phosphorylation of CREB within apoptosis-resistant neurons of the dentate granule-cell layer and neocortex, whereas a dramatic loss of CREB (phosphorylated and unphosphorylated) is found in the dying CA1 pyramidal cells[19] (Fig. 2). The prolonged increase in CREB phosphorylation within the dentate granule cells and its loss in the CA1 neurons, which precedes the onset of cell death, are both consistent with the hypothesis that activated CREB is important for survival of hippocampal neurons. Furthermore, this differential distribution of CREB protein and its phosphorylation is not confined to this model system; it has been both replicated and demonstrated in another global ischemic paradigm that displays a comparable pattern of cell death[20], and after a focal ischemic insult that is characterized by widespread neuronal death leading to infarct formation[21]. In the focal-ischemia model, the peri-infarct area shows marked levels of pCREB, while the infarcted core reveals a significant reduction in the number of CREB immunoreactive cells[21].

However, this association does not extend to all members of the CREB/ATF family, as demonstrated by the fact that ischemia-induced ATF2 phosphorylation (at Thr69 and Thr71) is found in the CA1 pyramidal cells undergoing apoptosis but not in the dentate granule cells[20,22]. Interestingly, the peak in ATF2 phosphorylation within the CA1 (48 h post-insult) precedes the appearance of any apoptotic markers and shows a strong temporal relationship with JUN protein synthesis[22]. Phosphorylated ATF2 and JUN share a common expression profile and also act as Jun kinase (JNK) substrates, suggesting that an interaction between these proteins might be important for neuronal apoptosis. Thus, the working hypothesis to account for the selective vulnerability of hippocampal neuronal populations is that dual JUN and ATF2 activation in CA1 neurons leads to their demise, whereas selective CREB phosphorylation in the dentate granule cells activates a survival program (Table 1).

CREB activation and cell survival

The persistent phosphorylation of CREB in response to stressful stimuli is not restricted to the *in vivo* situation, and has been demonstrated in PC12 cells following hypoxia[4] and okadaic-acid treatment[23]. Although, in both cases, increased levels of pCREB are likely to underlie numerous adaptive changes, many of which will be unrelated to cell viability, the delayed timecourse is consistent with the idea that at least part of this induction is involved in a cell-survival mechanism. In support of

TABLE 1. Hippocampal location of various proteins after hypoxic ischemia

	Dentate gyrus		CA1	
	Early[a]	Late[b]	Early[a]	Late[b]
CREB	–	–	–	↓
pCREB	↑	↑↑	↓	↓
pATF2	–	–	–	↑↑
JUN	↑	–	–	↑↑
FOS	↑	–	–	↑
EGR1	↑	–	↓	↓
NUR77[c]	↑	–	↑	↑
BDNF	–	↑	↓	↓

[a]3–12 h post-insult.
[b]48–72 h post-insult.
[c]mRNA only; in this model of hypoxic ischemia the CA1 neurons undergo apoptosis but the dentate granule cells are resistant to damage.
Abbreviations: ATF2, activating transcription factor 2; BDNF, brain-derived neurotrophic factor; CREB, cAMP-response-element-binding protein; EGR1, early growth response 1; pCREB, phosphorylated CREB; pATF, phosphorylated ATF; ↑↑, strong increase; ↑, increase, ↓, decrease, –, no change.

this hypothesis, PC12 cells that overexpress the gene for CREB have a decreased susceptibility to okadaic-acid-induced apoptosis, and a significant proportion of this effect is dependent on prolonged phosphorylation of CREB at Ser133 (Ref. 24). The ability of CREB to promote cell survival has also been clearly demonstrated in other cell systems. The transfection of human melanoma cells with an expression vector containing a dominant-negative CREB that is mutated within its DNA-binding domain, decreased the resistance of these cells towards UV-radiation-induced[25] and thapsigargin-induced apoptosis[26]. Transgenic mice that synthesize this dominant-negative form of CREB have thymocytes and T cells with a profound proliferative defect, and show increased sensitivity to apoptosis[27]. Moreover, suppressed production of CREB has been associated with influenza-A-virus-induced apoptosis of human monocytes[28].

The anti-apoptotic effects of CREB might also extend to the neuroprotection conveyed by environmental enrichment. Recently, it was shown that rats exposed to an enriched environment had reduced spontaneous apoptotic cell death in the hippocampus and were protected against kainate-induced seizures and excitotoxic injury[29]. Some of the resistant cell populations in the hippocampus showed increased CREB phosphorylation, which might account for their increased resistance to damage after environmental stimulation[29]. An interaction between CREB activation (Fig. 2) and the polysialylated form of the neural cell-adhesion molecule (PSA–NCAM) in this system is another possibility, as double immunolabeling showed their co-localization in the apoptotic-resistant precursor cells that align along the hilar–granule-cell border[29]. PSA–NCAM is a cell-to-cell adhesion molecule that is associated with dentate granule-cell neurogenesis, precursor differentiation and migration[30]. In addition, this glycoprotein might activate cell-survival pathways by stimulating CREB phosphorylation in neurons through the RAS–mitogen-activated protein kinase (MAPK) pathway[31]. Indeed, disruption of the NCAM receptor by crosslinking induces programmed cell death in neurons[32].

Survival factors and CREB phosphorylation

While there is growing evidence to support the link between the activation of CREB and programmed cell survival, the identity of the upstream initiators of this signaling pathway in the resistant neurons remains unclear. The endogenous neuroprotective agent, brain derived neurotrophic factor (BDNF) is a potential candidate in this respect, as it is co-localized with phosphorylated CREB in the dentate granule cells following hypoxic ischemia[17] (Fig. 2). This observation supports the idea that CREB is an important regulator of neurotrophin-induced gene expression[33,34]. The exposure of neurons to BDNF, stimulates CREB phosphorylation and activation via at least two kinase-regulated pathways[34]. In addition, CREB is able to regulate the transcription of the *Bdnf* gene directly[35,36], suggesting that a positive-feedback loop might be operating in some cell populations that are resistant to brain injury. Although such a system could function independently, interaction with other survival factors is a distinct possibility. In fact, estrogens might exert their neurotrophic and neuroprotective effects on hippocampal neurons via this mechanism[37,38]. Furthermore, estrogen has been shown to control transcription through the cAMP–PKA–CREB signal-transduction cascade[39], and can also induce the production of BDNF mRNA in the hippocampus[40]. Pituitary adenylate-cyclase-activating polypeptide (PACAP) is another survival factor that might mediate its effects through the phosphorylation of CREB. PACAP or, more specifically, PACAP type-I-receptor activation promotes neuronal survival in a number of model systems through a pathway that involves cAMP, PKA and MAPK (Refs 41–44), all of which activate CREB. Interestingly, the levels of this receptor are related to the selective vulnerability of hippocampal neurons, with the dentate granule cells showing very high amounts of this protein, whereas in the CA1 pyramidal cells it is almost undetectable[45].

In numerous cell types, the protection against apoptosis provided by growth factors, such as insulin-like growth factor 1 (IGF1) and neurotrophins, is thought to involve the signaling pathway from phophoinositide 3′ kinase (PI3′ kinase) to the Ser/Thr protein kinase, Akt (also known as protein kinase B)[46–48]. Although it is unclear exactly how this pathway blocks cellular apoptosis, at least part of this effect might be accounted for by the Akt-mediated phosphorylation of the BCL2 family member, BAD (Refs 49,50) and the protease, caspase 9 (Ref. 51), events that are known to suppress their pro-apoptotic function. However, Akt can also promote cell survival by stimulating the expression of cellular genes via the CREB–CBP nuclear transduction pathway. Du and Montminy showed recently that Akt overproduction in serum-stimulated cells induced Ser133 phosphorylation of CREB and promoted the recruitment of CBP (Ref. 52). There might be considerable convergence in these pathways as Ca^{2+}/calmodulin-dependent protein kinase kinase, an enzyme that is crucial to the Ca^{2+}-mediated transcriptional regulation of CREB, is able to phosphorylate Akt directly and protect cells from apoptosis[53]. Indeed, a wide range of neuromodulators and neurotransmitters could converge on CREB, via various kinase pathways in neurons[54], and regulate neuronal survival and plasticity (Fig. 3). However, although many neurotransmitters can lead to CREB phosphorylation through activation of several second messengers and kinases (for example, protein kinase C, PKA, MAPK)[54]

not all of these are neuroprotective, suggesting that the relationship between CREB phosphorylation and nerve-cell survival is not a simple one. Combinatorial interactions with other induced or constitutive transcription factors (transcriptional crosstalk) and the consequent binding to additional *cis*-acting DNA elements is likely to regulate CREB-mediated functions. In addition, persistent phosphorylation, which occurs in damage-resistant dentate granule cells[19] and in CREB-transfected neuronal cells[24], might be required to activate the genes downstream of CREB that are involved in nerve-cell survival. This hypothesis is supported by a study showing that hypoxia induces a more-robust and persistent phosphorylation of CREB than forskolin or depolarization[4]. Thus, the plasticity genes whose expression is produced by neuronal depolarization and the actions of CREB, might differ from those whose expression is activated by the persistent phosphorylation of CREB that occurs after stressful stimuli (for example, hypoxia), although one target gene, *Bdnf*, is likely to subserve both functions[17].

CREB-induced gene expression

The identities of target genes regulated by CREB in the resistant dentate granule cells after brain injury remain elusive, even though there are likely to be numerous candidates, many of which have not yet been identified. The *Bcl2* gene might be one example, as the phosphorylation of CREB proteins has been shown to have a major role in the induction of its expression during activation of mature B cells and during the rescue of immature B cells from Ca^{2+}-dependent apoptosis[55]. The *Bcl2* gene encodes a membrane-associated protein that can block apoptosis and promote cell survival in many systems, including the nervous system[56]. Moreover, increased BCL2 levels in the dentate granule cells after brief ischemia[57] not only demonstrate a possible involvement of this protein in ischemic tolerance and survival of sub-lethally injured neurons, but also suggests that it might be a downstream target for activated CREB. However, this is unlikely to be the case in all systems as changes in the levels of BCL2 or BCL2-related proteins are not required for CREB-induced survival in human melanoma cells[26]. Another candidate is the immediate–early gene *Mcl1*, which codes for a novel *Bcl2* family member and mediates specific cytokine effects on cell viability[58]. Interleukin 3 induces *Mcl1* expression and anti-apoptotic effects via an Akt–CREB signaling pathway[58]. Future studies using microarray technologies could catalog the array of target genes activated by CREB and identify survival programs in neurons.

Concluding remarks

There is a rapidly growing body of evidence to support the concept that endogenous CREB activation might provide a potent survival signal in times of cellular stress. Not only has CREB phosphorylation been implicated in the resistance of cells to various insults, but a number of well-established neuroprotective agents exert their actions via pathways that converge on the CREB protein. In particular, there is now evidence that the Akt signaling pathway could lead to CREB phosphorylation and that the potent neuroprotective agent, insulin-like growth factor 1 (IGF1), phosphorylates CREB partly through this Akt pathway[59]. These combined results suggest that CREB might be a key player in neuronal survival following injury and therefore responsible, in part, for selective vulnerability within the mammalian brain. Currently, the evidence supporting this hypothesis is correlational *in vivo*[19–21] but causal *in vitro*[24]. It will be vital to establish whether CREB phosphorylation is neuroprotective in the brain, and whether various neuroprotective agents (for example, IGF1) produce neuroprotection by the sequential activation of Akt and CREB. Studies on neurons derived from CREB-knockout mice are now required to resolve these issues. In this regard, one recent study has shown that after middle cerebral artery occlusion, CREB knockout mice do not show altered neurological scores[60]; however, these mice were monitored for only 3 h after the insult, which is before CREB-mediated neuroprotection would be activated. In addition, these mice had a targeted disruption of the α and Δ isoforms of CREB with compensatory increases in other forms[60]. Perhaps the best way of assessing the importance of endogenous CREB phosphorylation to neuronal survival would be to use viral transfection of hippocampal neurons with a dominant-negative CREB construct. If the hypothesis is correct, then this treatment should worsen HI- and seizure-induced

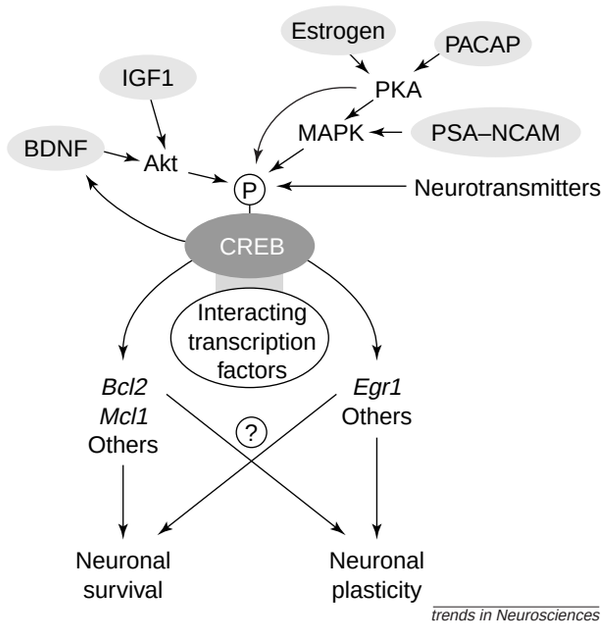

Fig. 3. ***The possible factors involved in CREB-induced survival within the hippocampus.*** *The phosphorylation (P) of cAMP-response-element-binding protein (CREB) at Ser133 has been implicated in the signaling pathway of a number of known neuroprotective factors (IGF1, estrogens, PACAP, BDNF, PSA–NCAM). As this convergence is directed towards CREB, it suggests that it might be an important event in neuroprotection and therefore a key to selective vulnerability in the hippocampus. Exactly how the phosphorylation of CREB leads to neuronal survival is, as yet, unclear, but is likely to involve combinatorial interactions with other transcription factors and to be mediated through the regulation of the expression of downstream target genes, such as* Bcl2, Mcl1 *and* Bdnf, *as well as many other unknown genes. Neurotransmitters (for example, ACh, dopamine, noradrenaline, glutamate) can also lead to CREB phosphorylation at Ser133 via activation of various kinases [for example, MAPK, PKA, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinases, RSK2 (see Ref. 54)], and some of these can activate CREB-mediated neuronal plasticity by the downstream activation of transcription factors such as EGR1. Whether these various downstream targets of CREB activation mediate both neuronal survival and plasticity is presently unclear. Abbreviations: BDNF, brain-derived neurotrophic factor; CREB, cAMP-response-element-binding protein; EGR1, early growth response 1; IGF1, insulin-like growth factor 1; MAPK, mitogen-activated protein kinase; PACAP, pituitary adenylate-cyclase-activating polypeptide; PKA, protein kinase A; PSA–NCAM, polysialylated form of the neural cell-adhesion molecule.*

hippocampal injury, and block the neuroprotective effects of compounds such as IGF1 and BDNF. Conversely, overexpressing the gene for CREB should protect neurons from various insults, as demonstrated recently in primary cerebellar granule cells[61].

In addition to the proposed role for CREB in neuronal survival, there is now extensive data showing that it is crucial for memory formation[14]. Thus, CREB-protein targeting might be an ideal way to achieve neuroprotective cognitive enhancement, a strategy that will have major implications for treating Alzheimer's disease, although, as already discussed, whether genes activated by CREB after learning are the same as those activated during neuronal stress remains to be determined. For example, differences in the degree and kinetics of CREB phosphorylation might lead to different functional outcomes. The challenge for the future is to determine which genes are activated during CREB-mediated neuroprotection (and plasticity), and to develop novel approaches that either mimic the actions of CREB in long-term memory and survival, or enhance its endogenous activity within the brain.

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Dendritic spine formation and pruning: common cellular mechanisms?

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The recent advent of novel high-resolution imaging methods has created a flurry of exciting observations that address a century-old question: what are biological signals that regulate formation and elimination of dendritic spines? Contrary to the traditional belief that the spine is a stable storage site of long-term neuronal memory, the emerging picture is of a dynamic structure that can undergo fast morphological variations. Recent conflicting reports on the regulation of spine morphology lead to the proposal of a unifying hypothesis for a common mechanism involving changes in postsynaptic intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$: a moderate rise in $[Ca^{2+}]_i$ causes elongation of dendritic spines, while a very large increase in $[Ca^{2+}]_i$ causes fast shrinkage and eventual collapse of spines. This hypothesis provides a parsimonious explanation for conflicting reports on activity-dependent changes in dendritic spine morphology, and might link these changes to functional plasticity in central neurons.

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SINCE they were first described over a century ago by Ramón y Cajal, dendritic spines were assumed to constitute the site of neuronal plasticity and long-term memory in central neurons. As such, they have attracted tremendous attention from neuroscientists of all designations who have the desire to formulate common elementary rules that govern the formation, maturation and disappearance of these spines. For example, is the formation of dendritic spines a result of synaptic interaction with afferent inputs, or is it predetermined genetically, much like cell shape and transmitter content? Likewise, is the formation of spines orchestrated centrally via nuclear signals, or locally, in response to synaptic demand? What are the roles of specific plasticity-producing stimuli, hormones and growth factors in the mature neuron? What are the molecular events that underlie long-term changes in spines, and how long do they last? Most importantly, how is a change in spine density, size or shape translated into a change in synaptic function?

Pioneering studies in intact brain attempted to correlate dendritic spine morphology with stimulation of a specific neural pathway, or environmental stimulation[1,2]. Spine morphology was also correlated with cognitive functions in the aging brain[3]. More recently, investigators have used various models of neuronal plasticity for assaying activity-related changes in dendritic spines, ranging from the induction of status epilepticus by kainic acid[4], pilocarpine[5] or kindling[6,7], to deafferentation of the cells of interest. Between the two extremes, more-natural stimuli, such as enriched environment[8,9], learning tasks[10,11] and controlled hormonal states[12,13], are used.

Experiments such as these have reported a wide range of effects on dendritic spines. For example, the type of tetanic stimulation that produces LTP (Ref. 14) can produce changes in spine dimensions[15], and synaptic contact area and shape[16]. Bifurcation of spine head[10] and the formation of novel spines[17] have also been reported. A stronger, seizure-producing synaptic stimulation also gives rise to varied effects on spines, either by forming novel spines in places where they have not been seen before (for example, somatic spines[7]) or by eliminating existing ones[18]. Moreover, the interpretation of these results is complicated when acute brain slices are used, as slicing itself can cause almost a 50% increase in spine density[19].

Developmental studies can provide an important clue as to the mechanisms of spine formation. Neurons are born without dendrites or spines, and develop them slowly over weeks as they become innervated by incoming fibers. It is difficult to discern whether the spines are formed before or after the formation of synapses. Recent *in vivo* electron-microscopy studies[20] indicate that incoming fibers make synapses with dendritic filopodia that then become mature spines, suggesting that spines

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