Ca^{2+}-dependent regulation in neuronal gene expression

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Ca^{2+} is an important signal-transduction molecule that plays a role in many intracellular signaling pathways. Recent advances have indicated that in neurons, Ca^{2+}-controlled signaling mechanisms cooperate in order to discriminate amongst incoming cellular inputs. Ca^{2+}-dependent transcriptional events can thereby be made selectively responsive to bursts of synaptic activity of specific intensity or duration.

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Abbreviations

AP1	activator protein 1
BAPTA	1,2-bis(2-aminophenoxy)ethane-tetraacetic acid
CaM	calmodulin
CaMK	Ca^{2+}/CaM-dependent protein kinase
CaMKK	CaMK kinase
CBP	CREB-binding protein
CRE	cAMP-response element
CREB	CRE-binding protein
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid
GRF	guanine nucleotide releasing factor
JNK	c-Jun N-terminal kinase
L-LTP	late-LTP
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MAPKAP	MAPK-activated protein
NCAM	neural cell adhesion molecule
NFAT	nuclear factors of activated T cell
NMDA	*N*-methyl-D-aspartate
pCREB	phospho-CREB
PKA	protein kinase A
PP1	protein phosphatase 1
SAPK	stress-activated protein kinase
SRE	serum response element
SRF	serum response factor
STAT	signal transducer and activator of transcription

Introduction

Recent studies have helped to delineate some of the mechanisms involved in activity-dependent surface-to-nucleus signaling in neurons [1–8]. The signaling pathways are being mapped, and the points of crosstalk between them are being identified. In some cases, we have even begun to understand the functional significance of these synaptically recruited signaling pathways. In this review, we will summarize briefly recent advances in this rapidly moving field, and then focus specifically on regulatory events that are modulated by Ca^{2+}, a critical messenger in the CNS [9].

Signaling from the synapse to the nucleus: key features of Ca^{2+} as a second messenger

Activity-dependent changes in neuronal structure and synaptic remodeling [1,2], which are so essential for brain function, depend critically on protein synthesis [3]. In considering how these events come about, it is important to understand the relationship between the electrical activity of a CNS neuron and gene expression at its nucleus [4–8]. From its vantage point within the cell body, the nucleus acts as a sensitive information-processing device, receiving inputs derived from surface stimuli that are transferred centrally via diverse cytoplasmic signals.

In some ways, the nucleus may be compared to the axonal action potential initiation zone — both are computers of sorts, stationed downstream of the dendritic tree, with the nucleus deciding on RNA production in the same way that the action potential initiation region decides on spike firing. Both devices are clearly sensitive to the intensity, duration, and temporal pattern of incoming information, and both probably rely on some pre-processing of incoming signals within the dendritic tree, whether the signals be electrical or biochemical.

In several respects, however, the nucleus has a much more complex job to perform. First, whereas the action potential initiation zone takes one type of input (membrane voltage) and generates one type of output (the action potential), the nucleus has many different types of input (multiple converging signal-transduction cascades) and generates many different types of output (multiple genes that can be expressed in different patterns and at different levels). In this sense, the nucleus is the more complicated computer of the two. Second, nuclear signaling may need special engineering in a way that the spike-firing decision does not. The latter takes a fast input (membrane depolarization) and uses a rapid calculator (voltage-gated Na^+ and K^+ channels) to determine a fast output (the action potential). But the nucleus, in generating its slow output of changes in gene expression, needs to consider not only similarly slow inputs, like hourly variations in external hormone, but also fast changes in membrane voltage, on a time-scale of milliseconds. If the nucleus could not respond to fast synaptic depolarizations, it would be throwing away potentially useful information — in modern parlance, wasting bandwidth.

Relying on voltage changes themselves to generate a second messenger for nuclear signaling is part of the solution. As a general strategy, excitable cells achieve fast conversion from electricity to biochemistry through an

intense but local influx of Ca^{2+} [9]. In neurons, voltage-dependent Ca^{2+} channels and postsynaptic NMDA receptor channels are able to respond quickly to millisecond-scale electrical events by generating Ca^{2+} signals, which can have a lasting impact once they are decoded by the appropriate Ca^{2+}-sensitive proteins. This signaling cascade provides a rationale for why Ca^{2+} may be an important mediator of synapse-to-nucleus signaling.

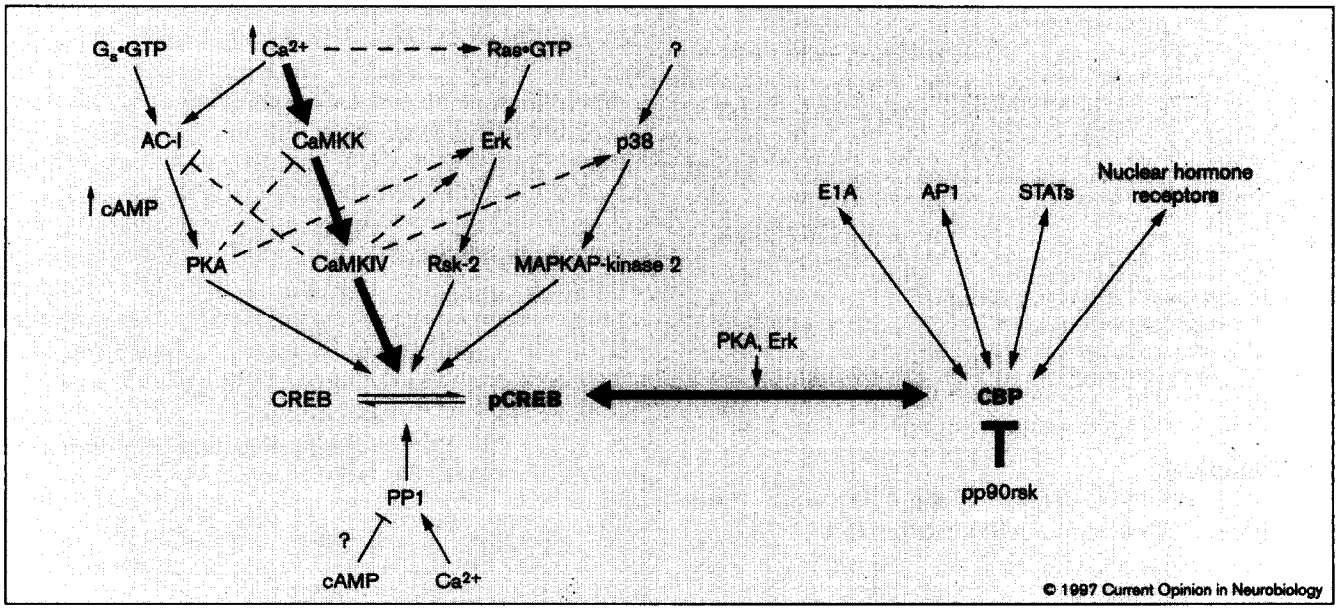
Indeed, extensive studies of stimulus-dependent gene expression in CNS neurons, carried out *in vivo* [5,8,10–13], in acute slices [14••], and in cultured neurons [15••], have generally borne out the importance of Ca^{2+} entry pathways. This has been demonstrated by disrupting signaling to the nucleus with blockers of NMDA receptors [10,11], inhibitors of voltage-gated Ca^{2+} channels [14••], or both [15••]. In these studies, nuclear signaling pathways are able to discriminate between features of the electrical stimuli, such as their frequency, intensity, duration or pattern of repetition. Such discrimination may well involve Ca^{2+} signaling mechanisms. In principle, fast Ca^{2+} influx could link up to Ca^{2+} targets with different Ca^{2+} sensitivity, thereby providing discrimination between Ca^{2+} signals of varying amplitudes, or to Ca^{2+} targets that activate or inactivate over time [9,16•,17••,18], thus representing the duration or temporal pattern of the signals.

Knowledge about the general properties of activity-dependent gene expression has heightened interest in the mechanisms of signaling from the synapse to the nucleus. At least three fundamental questions need to be addressed. First, what are the various molecular pathways that link patterns of synaptic activity with specific downstream genes? Are they all dependent on Ca^{2+}, to some extent, or are some Ca^{2+}-independent? Second, what are the downstream genes and how is their expression modified? How do they give rise to activity-induced changes (or maintenance) of neuronal properties? Third, how does gene expression at the nucleus lead to synapse-specific changes? Does this involve a mechanism of local 'synaptic tagging' [19••]?

Activity-dependent regulation of nuclear transcription factors

Increasing attention has been directed lately toward the signaling pathways that are crucial for the activation of nuclear events. Initial studies carried out in immortalized neuronal cell lines such as phaeochromocytoma PC12 cells highlighted the complexity of the signaling pathways that lead to transcriptional activation (e.g. [4,20–26,27•]). More recently, it has become possible to examine signaling pathways in non-immortalized neurons and to delineate the specific patterns of neuronal input that can lead to transcriptional activation and gene expression [28–33,34••,35••]. Likewise, there has been a progression from gel-shift assays, which measure generic changes in the binding of transcription factors to their cognate regulatory elements (see e.g. [36]), to analysis of

Figure 1



Integration of multiple signaling pathways onto pCREB/CBP. In neuronal cells, phosphorylation of CREB at Ser133 could be mediated by many different serine/threonine protein kinases. Extensive crosstalk amongst these pathways has been reported. Once CREB is phosphorylated to pCREB, it presents a high-affinity binding site to CBP, which is a histone acetyltransferase and which could interact as a co-activator with various other transcriptional activators, such as E1A, AP1, STATs or nuclear hormone receptors. AC-I, type I adenylyl cyclase.

specific transcription factors and their upstream signaling pathways.

A leading example is the Ca^{2+}/cAMP-response element binding protein (CREB), which is activated by phosphorylation of Ser133, an event that can be brought about by a variety of neuronal protein kinases, including protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), CaMKIV, pp90rsk, rsk-2, and MAP kinase-activated protein (MAPKAP) kinase-2 [34••,37–47,48•,49] (Figure 1). The convergence of multiple signaling pathways onto CREB raises the issue of whether and how information from specific neuronal inputs might be preserved. Increased phosphorylation of CREB leads to the formation of a stable complex with the CREB-binding protein (CBP) and, in turn, to recruitment of the RNA polymerase II holoenzyme [50]. CBP is itself a remarkable signal integrator [51–53,54•,55•]. When phosphorylated by PKA and mitogen-activated protein kinase (MAPK), CBP responds in distinct ways to stimulate c-*fos* transcription [56]. Furthermore, pp90rsk, by binding to CBP, seems to act as an inhibitory regulator of CREB-mediated transcription [55•], despite the fact that pp90rsk and rsk-2 are known CREB kinases [35••,48•].

Finally, and most dramatically, CBP has been shown to be a co-activator of transcription not only for CREB, but also for a number of other transcription factors, including (but clearly not restricted to) activator protein 1 (AP1), nuclear hormone receptors, and STATs (signal transducers and activators of transcription) [57••–60••,61–70]. The convergence of signals onto CBP is all the more remarkable in light of evidence suggesting an additional role for CBP (and for P/CAF, a CBP-binding protein) as a histone acetyltransferase critical for transcriptional initiation [71,72••,73••]. Taken together, the multiplicity of signaling mechanisms acting on CREB and CBP provide a rich array of possibilities for input-specific patterns of gene expression.

Interest in the CREB/CBP system has been intensified by rapidly growing evidence for its importance in memory storage. The first analyses of specific transcriptional events in synaptically connected neurons were carried out in *Aplysia* by Kandel's group [3,74–76,77••], who established that PKA-dependent regulation of the CREB system was essential in the long-term sensitization of the gill-withdrawal reflex, a classic example of implicit learning. In *Drosophila*, a dramatic dependence on CREB signaling has been found for protein-synthesis-dependent, long-lasting components of olfactory learning [78,79,80••]. Mutant mice lacking α- and δ-isoforms of CREB display intact short-term memory but deficient long-term memory in three independent learning tasks [81,82••]; concomitantly, late long-term potentiation (L-LTP) in hippocampal CA1 is also impaired [81].

In contrast to the striking phenotype of the CREB-deficient mice, knockouts of immediate early transcription factor *zif*/268 (also known as NGFI-A) [83], NFAT (nuclear factors of activated T cell) [84] and cAMP-response element modulator (CREM) (JA Blendy, JH Kogan, G Schutz, AJ Silva, *Soc Neurosci Abstr* 1996, 22:1391) have not yet yielded remarkable behavioral changes. Deletions of c-*fos* [85] and *fosB* [86] cause behavioral abnormalities, but their neurobiological basis is not understood. Therefore, the pronounced but specific defects arising from elimination of only two of the many CREB isoforms is particularly interesting. Taken together, these results have placed the CREB/CBP system at the forefront of current thinking about the role of the nucleus in controlling long-term changes in the properties of neurons.

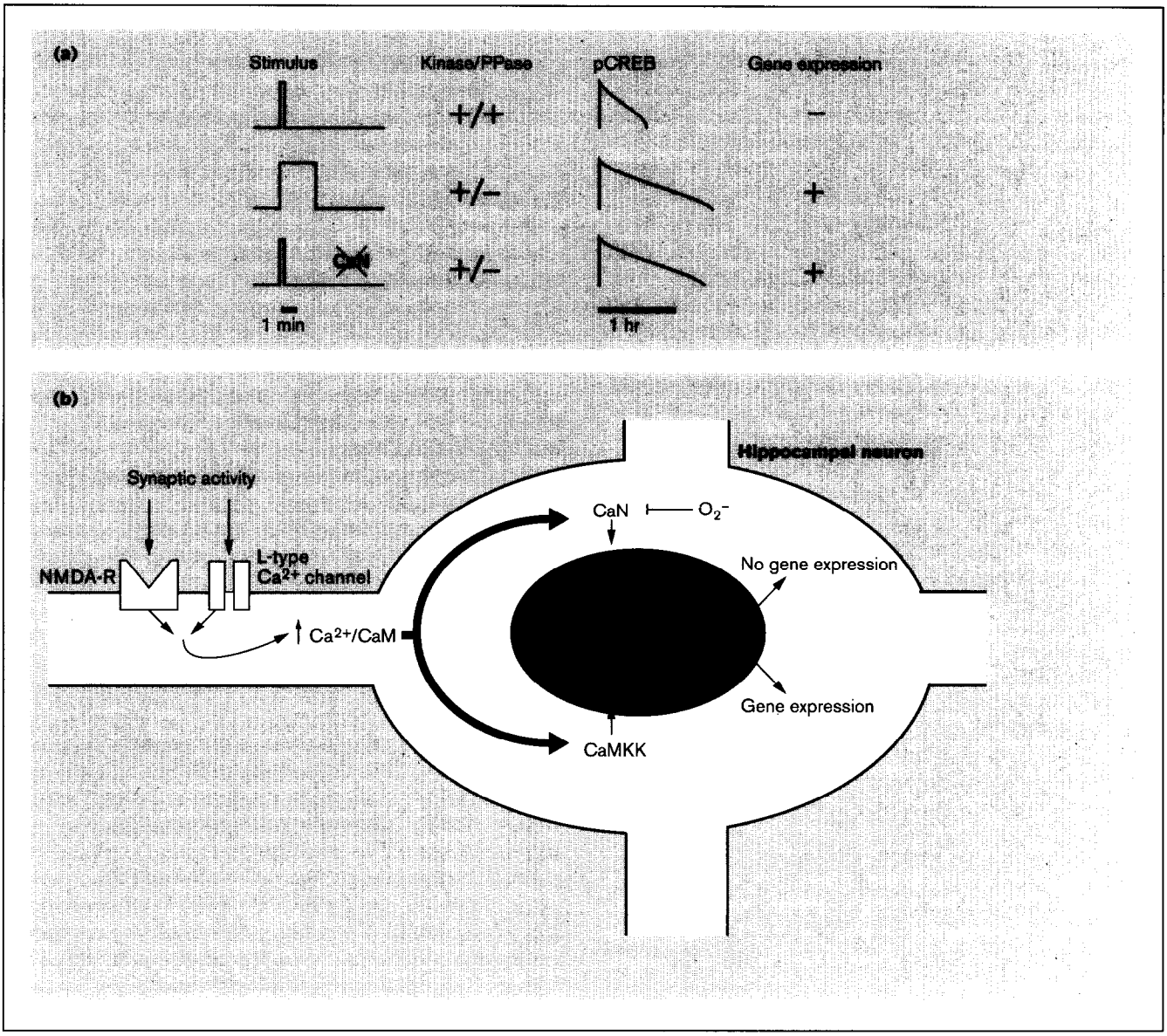
On the basis of studies in *Aplysia* and *Drosophila*, PKA has been assumed to be important in CREB-dependent learning in mammals. PKA clearly is required for learning and L-LTP [87], and its catalytic subunit shuttles to the nucleus in forskolin-stimulated PC12 cells [88]. However, it remains to be established that PKA is involved directly in CREB phosphorylation. PKA may also be important for phosphorylating other components of the transcriptional apparatus, such as CBP, or, alternatively, PKA could be important for the local (e.g. dendritic) implementation of a change directed by the nucleus.

Signaling from the synapse to the nucleus: an example of Ca^{2+}-dependent CREB regulation

A central issue is how synaptic activity leads to activation of the CREB pathway. Several groups have examined the patterns of synaptic activity required for triggering phosphorylation of nuclear CREB at Ser133 and have tried to relate this phosphorylation to activation of downstream genes such as c-*fos* [14••,15••,34••,35••]. In hippocampal cultures, rapid, Ca^{2+}-dependent CREB phosphorylation can be evoked in postsynaptic neurons when they receive synaptic input at frequencies that induce either increases or decreases in synaptic strength; however, in the absence of synaptic transmission, high-frequency action potential firing is not able to induce CREB phosphorylation ([15••]; see also [89]). Thus, CREB activation seems to depend critically on postsynaptic Ca^{2+} entry—the substantial amount of Ca^{2+} influx (and nuclear Ca^{2+} elevation) that occurs during action potential firing is present in either the wrong place or the wrong quantity to give rise to CREB phosphorylation [15••]. This is an interesting and potentially useful distinction for a neuron to make, as discriminating against action potentials allows synaptic potentials to have a much greater relative effect on nuclear signaling.

In hippocampal neurons, both the phosphorylation and dephosphorylation of CREB have been found to utilize Ca^{2+}/calmodulin (CaM)-regulated mechanisms. Positive regulation occurs through a CaMK cascade involving nuclear CaMKIV [34••]. CaMK cascades have been studied intensively *in vitro* [90–96,97••], and it has been

Figure 2



Regulation of CREB by Ca^{2+} in hippocampal neurons. **(a)** Schematic diagram illustrating the net effect on gene expression of a dual Ca^{2+}/CaM-dependent regulation. When a short stimulus is applied, the combined activation of both kinase and phosphatase activities leads to a transient pCREB state in the nucleus, which is presumably insufficient to trigger a significant amount of transcriptional activity. When the synaptic activity is long-lasting, an inactivation of the phosphatase pathway enables a more sustained pCREB state in the nucleus, leading to a detectable amount of CRE-mediated transcription. This state could be mimicked *in vitro* by coupling a short stimulus with inhibition of calcineurin. **(b)** Synaptic activity induces Ca^{2+} influx through glutamate receptor channels of the NMDA-type (NMDA-R), as well as through L-type voltage-gated Ca^{2+} channels. This influx leads to a build-up of Ca^{2+}/CaM near the plasma membrane, which activates two Ca^{2+}/CaM-dependent mechanisms: a CaMK cascade, which culminates in the stimulation of nuclear CaMKIV via CaMKK; and calcineurin (CaN)-mediated regulation of nuclear PP1 activity, presumably by a change in the phosphorylation state of a PP1 regulatory subunit. Both mechanisms are stimulated simultaneously when synaptic stimuli are applied; however, the CaN-regulated mechanism is inhibited when the stimulus duration is increased substantially by a superoxide-sensitive mechanism. Phospho-CREB (pCREB) can then stably associate with CBP in the nucleus (not shown) to induce a variety of CRE-regulated genes. Adapted with permission from [34••]. PPase, protein phosphatase.

established that CaMKIV can be strongly activated by trans-phosphorylation via an upstream CaMK [98–101]. Together, the distinct brain localization of CaMK kinase (CaMKK)-α or CaMKK-β [102] and the differing efficiencies of CaMKK action on various CaMKs [103•] offer interesting possibilities for subtle fine-tuning of Ca^{2+}-dependent CREB phosphorylation in different cell types.

Negative regulation of CREB in hippocampal neurons has been found to occur through calcineurin-dependent regulation of nuclear protein phosphatase 1 (PP1) activity

[34••,35••] (Figure 2). An interesting parallel has been uncovered in organotypic slice cultures of the striatum; Liu and Graybiel [35••] suggest that a calcineurin-controlled phosphatase gate may provide a mechanism for activity-dependent regulation of CREB phosphorylation and striatal compartment formation.

In hippocampal cultures, increasing stimulus duration has been found to block the effect of calcineurin and thereby allow phospho-CREB (pCREB) to persist for a much longer time. This turns out to be significant for gene expression, as sustained, but not transient, elevation of nuclear CREB phosphorylation is required for efficient stimulus–transcription coupling in both hippocampal [34••] and striatal [35••] neurons. This discrimination appears to work through an activity-dependent inactivation of calcineurin [34••]. As first described by Klee and colleagues [16•,17••] *in vitro*, such inactivation requires Ca^{2+}/CaM activation of calcineurin, but it also depends on the action of superoxide. There is evidence for activity-dependent reactive oxygen production in hippocampal neurons, as well as evidence for the involvement of superoxide in controlling the rate of CREB dephosphorylation [34••].

It is tempting to speculate on the information-processing utility of these different Ca^{2+}-dependent control steps. For example, consider Ca^{2+}-dependent activation of CREB phosphorylation coupled with Ca^{2+}-dependent inactivation of CREB dephosphorylation. If activation of both Ca^{2+}-dependent pathways is required to give rise to stable pCREB levels in the nucleus, there will probably exist some degree of cooperativity in the Ca^{2+}-dependent control of CREB-dependent gene expression. Such cooperativity could well allow for non-linear or switch-like behavior in the synaptic control of nuclear gene expression. Whether this intriguing control mechanism is involved in learning and memory remains to be established.

The importance of other signaling pathways and crosstalk

Even though the Ca^{2+}/CaM-dependent component of Ca^{2+}/cAMP-response element (CRE) regulation appears to play a critical role in many types of neurons [15••,20,27•, 31,33,34••,104], several groups (see [15••,104]) have noted a PKA-dependent component in neurotransmitter-activated regulation of CRE, raising the possibility of an additional regulatory phosphorylation event. Without a doubt, CREB phosphorylation is itself strongly dominated by PKA in certain systems (such as dopaminergic neurons in the CNS) [6,35••], even though the dephosphorylation of CREB may still lie under the control of calcineurin in these cells (such as in striosomal neurons [35••]). Furthermore, *in vitro* studies have suggested that PKA may exert its effects by increasing the transcriptional potential of CBP [51,52,54•], as does MAPK [53]. Whether PKA regulates CBP in neurons remains to be determined. PKA may also have an impact on the activation of the Ca^{2+}-effector pathway: PKA-dependent phosphorylation of CaMKK has been found to inhibit its activation of CaMKIV (GA Wayman, H Tokumitsu, TR Soderling, *Soc Neurosci Abstr* 1996, 22:372), and PKA is known to act as a kinase to the phosphorylation site targeted by calcineurin during the calcineurin-mediated inactivation of the inhibitory subunits for PP1, such as inhibitor-1 or DARPP-32 [105]. Conversely, CaMKIV can phosphorylate and inactivate the enzymatic activity of Ca^{2+}/CaM-sensitive type I adenylyl cyclase [106•] (Figure 1, dashed arrows), thus suppressing the PKA pathway.

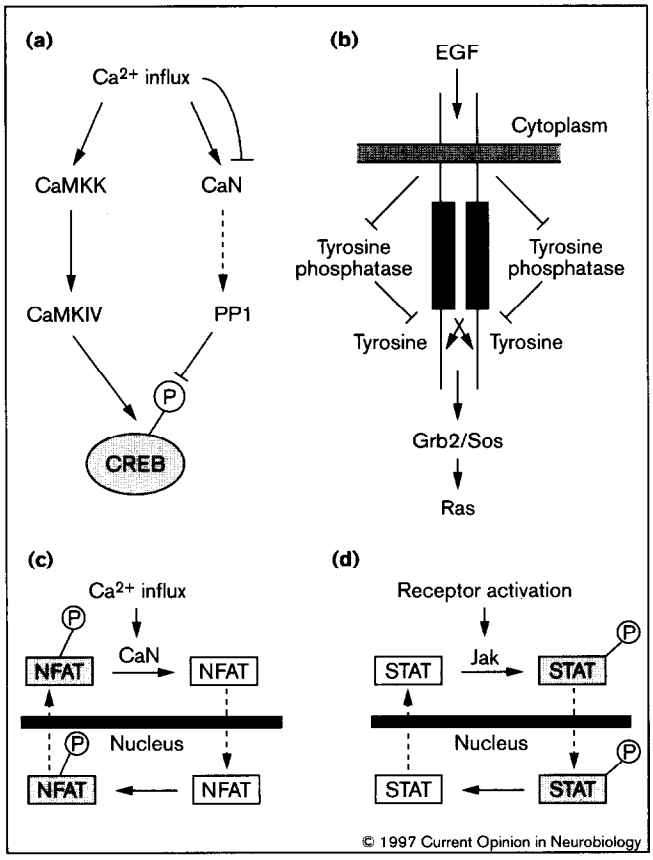
The role of Ca^{2+} in CRE-mediated gene expression may not be limited solely to CaMKIV-induced CREB phosphorylation. In the immortalized AtT20 cell line, CRE-dependent transcription depends more on nuclear Ca^{2+} than on cytoplasmic Ca^{2+} [27•]. This is in contrast to hippocampal neurons, in which highly local rises in Ca^{2+}, but not bulk cytoplasmic or bulk nuclear Ca^{2+}, are the critical signals in synaptic activation of CREB phosphorylation [15••]. In fact, in hippocampal neurons, generalized elevations in nuclear Ca^{2+} are not only unnecessary for CREB phosphorylation but also insufficient [15••]. It is easy to imagine, however, why such different cell types might use different signaling pathways. Neurons, with vast and spatially complex dendritic trees, would perhaps be best designed if they could make good use of local Ca^{2+} signals in synapse-to-nucleus signaling; whereas the much more compact AtT20 cells (which have no neuronal processes) may have no need for such clever engineering, as the whole surface plasma membrane is close to the nucleus. Alternatively, a nuclear Ca^{2+} pool, although not critical for CREB phosphorylation *per se*, could influence the long-term stability of pCREB. If so, this would profoundly affect CRE-dependent gene expression [34••]. Though speculative, a model in which two separate cellular pools of Ca^{2+} are used to control distinct steps in the same signaling pathway is interesting computationally, implying that synaptically generated Ca^{2+} is a variable that may be used in two or more terms within the same equation.

Are the effects of Ca^{2+} restricted to regulation of CRE-like elements? Clearly not. There is strong evidence for a Ca^{2+}-dependent pathway leading to Ras/MAPK activation, which is critical in neuronal serum response element (SRE)-mediated transcription [27•,107,108•]. The links between Ca^{2+} and Ras have not been outlined clearly in neurons, though possible candidates for a Ca^{2+} sensor include protein kinase C (PKC) [109], pyk2 [110••], and Ras-GRF (guanine nucleotide releasing factor) [111••]. Furthermore, overexpression of a constitutively active form of CaMKIV in PC12 cells either phosphorylates serum response factor (SRF) directly [112] or leads to increased basal activity of various MAPK pathways, including the ERK, JNK/SAPK, and p38 pathways [113•] (Figure 1, dashed arrows), all of which have been

implicated upstream of SRE via phosphorylation of either elk-1 or SRF. Other regulators of small GTP-binding proteins such as IQGAP1 and IQGAP2 have also been reported to bind Ca^{2+}/CaM, suggesting an alternate small GTPase route by which Ca^{2+} might regulate SRE through JNK/p38, downstream of Rac/Cdc42 pathways [114–117]. The JNK/SAPK pathways could also be modulated by Ca^{2+} via pyk2 [118].

Taken together, these studies suggest that a wide variety of possibilities must be considered when approaching CREB signaling in specific neuronal systems. In addition, of course, the CREB/CBP system will not stand alone in the induction of target genes. For example, maximal activation of c-*fos* *in vivo* requires cooperation among multiple regulatory elements on the c-*fos* promoter, including SRE, the *sis*-inducible element, the AP1 binding element, and CRE [119••].

Figure 3



Regulation of gene expression by the opposing actions of kinases and phosphatases. Activation of a kinase cascade is associated with inhibition of its opposing phosphatase **(a)** in CREB signaling in hippocampal neurons, as well as **(b)** in EGF signaling in A431 cells. Transcription factor shuttling is regulated by the balance of kinase and phosphatase activities on each side of the nuclear membrane **(c)** in NFAT signaling and **(d)** in the Jak/STAT system.

Common features of phosphorylation/dephosphorylation-mediated regulation of gene transcription

It is interesting to compare Ca^{2+} regulation of gene expression in neurons to that found in other cell types, particularly T lymphocytes (Figure 3a,c). Stimulation of T cells with antigen initiates a sustained Ca^{2+} influx that, in turn, leads to transcription of the interleukin-2 gene (Figure 3c). The targets of Ca^{2+}-dependent regulation are NFATs, transcription factors that are activated when dephosphorylated by calcineurin. Dephospho-NFAT shuttles into the nucleus [120••–122••], where it binds to a co-activator complex such as AP1, thereby activating transcription. A constitutive nuclear protein kinase activity rephosphorylates NFAT, leading to its rapid export from the nucleus [120••,122••]. Nuclear CaMK, such as CaMKIV [123] or a nuclear isoform of CaMKII [124], may also play a role in NFAT-dependent transcription. As in the case of neuronal CREB, activation of NFAT in lymphocytes requires Ca^{2+}/CaM-dependent enzymatic activity, but it is a phosphatase rather than a kinase that acts as the initial trigger.

From an even more general perspective, it is useful to recognize that bidirectional regulation of transcription factor complexes by opposing kinases and phosphatases also exists outside of the specific context of Ca^{2+}-mediated nuclear signaling. A classic example is the activation of the tyrosine kinase cascade by growth factors (Figure 3b,d). As shown recently, autophosphorylation and activation of the epidermal growth factor (EGF) receptor tyrosine kinase is associated with an EGF-induced, hydrogen-peroxide-dependent inactivation of a critical tyrosine phosphatase [125••] (Figure 3b). Two other groups [126•,127•] have found that STAT-dependent transcription is negatively regulated by a nuclear tyrosine phosphatase that promotes export of STAT from the nucleus by dephosphorylating tyrosine residue(s), identical to the Jak kinase phosphorylation site(s), that are critical for STAT's nuclear entry (Figure 3d). Again, as in the case of NFAT, the dynamic shuttling of the activated transcription factor is regulated by the opposing kinase (or phosphatase) activity in the nucleus (Figure 3). Thus, both Ca^{2+}-influx-mediated nuclear signaling and ligand–receptor-interaction-induced surface-to-nucleus signaling may control the timing of nuclear events by using similar signaling principles.

Next steps: what are the target genes and what are their functions?

Despite the considerable effort invested in elucidating the molecular mechanisms involved in activity-induced gene transcription, surprisingly little is known about how changes in gene expression lead to long-term biological consequences, such as synaptic remodeling.

In *Aplysia* neurons in culture, where CREB signaling seems important for enduring changes in the efficacy

and morphology of synapses [3,77••,128], one of the important downstream genes is an adhesion molecule, ApCAM, an *Aplysia* homolog of mammalian neural cell adhesion molecule (NCAM) [77••,129,130]. In mammalian neurons, NCAM is involved in activity-induced synapse plasticity [131,132] and learning [133], but the molecular linkage between activity and NCAM is less clear in the mammalian system than in *Aplysia*. Expression of another neural adhesion molecule, L1, can be induced by restricted patterns of impulse activity [29]. Other gene products whose expression changes in association with synaptic plasticity include tissue plasminogen activator [134•,135], β-A-activin [136], Narp [137], Arc [138•], and cyclooxygenase-2 [139].

As more activity-dependent genes are uncovered, critical attention must be focused on their relationship to the implementation of long-lasting modifications. The elegant study by Frey and Morris [19••] brings to the fore additional questions regarding the interaction between short-term, synapse-specific changes, probably involving post-translational effects, and long-term changes in synaptic number or shape that require transcriptional activation. Many mysteries lie ahead, but there is little question that Ca^{2+} signaling will play a significant role in these events.

Acknowledgements

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