

Genetic Approaches to Investigate the Role of CREB in Neuronal Plasticity and Memory

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Abstract In neurons, the convergence of multiple intracellular signaling cascades leading to cAMP-responsive element-binding protein (CREB) activation suggests that this transcription factor plays a critical role in integrating different inputs and mediating appropriate neuronal responses. The nature of this transcriptional response depends on both the type and strength of the stimulus and the cellular context. CREB-dependent gene expression has been involved in many different aspects of nervous system function, from embryonic development to neuronal survival, and synaptic, structural, and intrinsic plasticity. Here, we first review the different methodological approaches used to genetically manipulate CREB activity and levels in neurons *in vivo* in the adult brain, including recombinant viral vectors, mouse transgenesis, and gene-targeting techniques. We then discuss the impact of these approaches on our understanding of CREB's roles in neuronal plasticity and memory in rodents. Studies combining these genetic approaches with electrophysiology and behavior provide strong evidence that CREB is critically involved in the regulation of synaptic plasticity, intrinsic excitability, and long-term memory formation. These findings pave the way for the development of novel therapeutic strategies to treat memory disorders.

Keywords CREB · Neuronal plasticity · Memory · Transgenic mice · Virus-mediated expression

Introduction

Diverse long-lasting forms of neuronal plasticity, from changes in the number and strength of synaptic connections to the modulation of the intrinsic properties of neurons, are thought to rely on activity-driven gene expression. Although neuronal activity regulates the activation and/or expression of many transcription factors, the cAMP-responsive element (CRE)-binding protein (CREB) is arguably the most studied in the context of the adult nervous system [1, 2]. The activation of CREB by phosphorylation is triggered in neurons by a wide variety of signaling processes, from increases in intracellular Ca^{2+} through activation of voltage- or ligand-gated channels to changes in cAMP levels after activation of G protein-coupled receptors or receptor tyrosine kinases. Signaling upstream of CREB is very complex, and overall, more than 300 different stimuli have been reported to activate CREB [2]. Downstream effects may be even more complex since hundreds of genes have been reported to be regulated by CREB in neurons. The number and identity of those CREB target genes will depend on both the nature of the stimulus and the cellular context [3]. A number of articles have reviewed different aspects of CREB's brain functions (e.g., [1, 2, 4–12]). In this review, we will describe the different methods used to genetically manipulate CREB activity and levels in neurons *in vivo*, discussing their individual advantages and limitations, as well as the large body of knowledge that has emerged from application of these complementary techniques to understand the role of CREB in neuronal plasticity, learning, and memory.

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Genetic Manipulation of CREB Activity

CREB's Structure and Activation

CREB belongs to a family of transcription factors characterized by a highly conserved basic region/leucine zipper (bZIP) domain that binds to a specific DNA sequence called cAMP-responsive element (CRE) found in one or several copies in the promoters of many genes (Mayr and Montminy 2001). Transcriptional activation is mediated by two types of transactivation domains: the central kinase-inducible domain (KID) and the glutamine-rich domains (Fig. 1). The KID contains several sites recognized by protein kinases and its phosphorylation state determines the binding of the transcriptional co-activator CREB-binding protein (CBP), which enables transcription initiation by bringing the RNA polymerase II complex to the promoter. The glutamine-rich domains contribute to basal transactivation activity by interacting with the transcription machinery and stabilizing the interaction with CRE sites.

CREB has a complex gene structure. Alternative splicing generates transcripts encoding both repressors and activators

(Bartsch et al. 1998; Habener et al. 1995; Mayr and Montminy 2001). The repressors are shorter variants with reduced or null transactivation capability that compete for CRE sites.

Although CREB is thought to be constitutively bound to CRE sites in the promoters of cAMP-responsive genes, the transcription of CREB-regulated promoters increases several folds when CREB is phosphorylated at serine 133 (S133) by activity-dependent kinases. The phosphorylated form of CREB can then recruit CBP to the promoter. Another important mechanism of regulation of CREB activity depends on the transducers of regulated CREB activity (TORC). These transcriptional co-activators, contrary to CBP, enhance CRE-dependent transcription through phosphorylation-independent interaction with the bZIP domain of CREB. This interaction favors the interaction of CREB with the TAF(II)130 component of the RNA polymerase II complex [13, 14]. Although TORC proteins interact with CREB in the absence of phosphorylation, they are themselves substrate of kinase transduction cascades. The molecular knowledge described above has been used to generate CREB variants in which this transduction cascade is either enhanced or blocked.

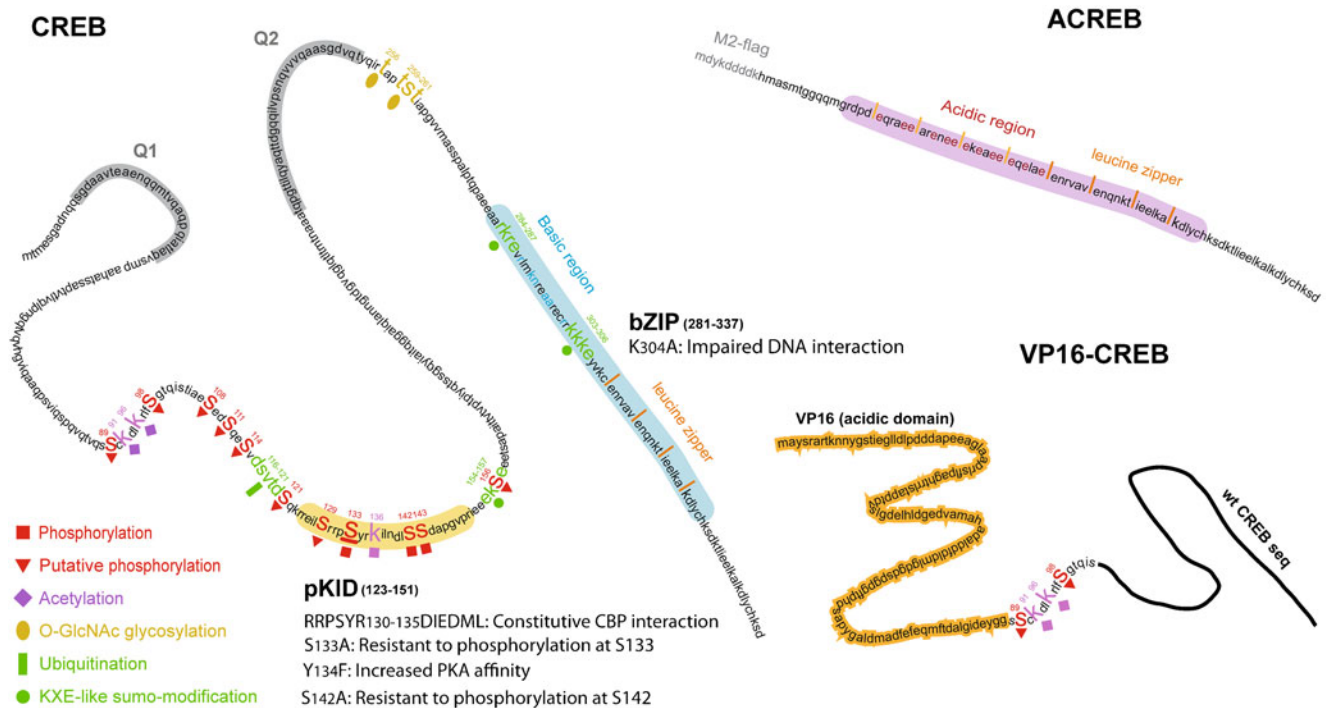


Fig. 1 CREB structure and relevant residues. *Left* CREB structure. CREB has a highly conserved leucine zipper and adjacent basic region responsible for DNA binding, a regulatory kinase inducible domain (KID), and two glutamine-rich regions (Q1 and Q2). CREB is substrate of various posttranslational modifications that affect its activity, the position of residues potentially affected is indicated. The loss- and gain-of-function point mutations described in the text are

also shown (adapted from [9]). *Right* Schematic representation of the dominant negative and constitutively active CREB variants ACREB (*up*) and VP16-CREB (*down*). In the case of VP16-CREB, different groups have produced different versions of the chimeric protein with the VP16 domain located either in position N- or C-terminal. In the protein presented here, the VP16 domain replaces the Q1 domain [44]

Gain-of-Function Approaches

Four different genetic strategies have been used to increase CREB activity in neurons (Fig. 1):

- CREB overexpression: A number of experiments indicate that endogenous levels of CREB are not saturating and consequently the overexpression of wild-type CREB can cause an enhancement of CREB-dependent signaling.
- CREB^{Y134F}: This point mutation next to S133 increases the affinity of CREB for protein kinase A (PKA) (and maybe also with other activity-regulated kinases) and therefore leads to a reduction of the threshold for activation [15].
- CREB^{DIEDML}: The mutation of six amino acids in the kinase-inducible domain (KID) of CREB allows the interaction with CBP in the absence of phosphorylation [16]. This mutant can therefore interact constitutively with CBP, although CBP activity would still be modulated by activity-dependent kinases [17, 18].
- VP16-CREB and CREB-VP16: The fusion between CREB or the DNA binding domain of CREB with the strong acidic transactivation domain of the herpes simplex virus (HSV) protein VP16 produces a chimeric protein that drives transcription from CRE-driven promoters in a constitutive manner [19]. In contrast to the other approaches described above, this manipulation can effectively decouple CREB-dependent transcription from upstream kinase cascades.

Loss-of-Function Approaches

Studies investigating the consequences of reduced or absent CREB activity in neuronal plasticity are based in animals in which either the *creb1* gene has been disrupted or dominant negative CREB variants are expressed (Fig. 1). Three dominant negative mutants have been used:

- CREB^{S133A} (also referred as mCREB or CREB-M1): This point mutation affects the main residue controlling the interaction of CREB with its co-activator CBP, therefore rendering the protein insensitive to most activity-dependent kinase cascades converging on CREB. Importantly, CREB dimers, in which only one subunit is phosphorylated, can still activate transcription [20], which limits the dominant negative effect of the expression of this variant.
- KCREB: This mutant contains a point mutation in human CREB at K304. The K304 residue mediates interaction with Mg²⁺ and is critical for high-affinity DNA binding [21]. The heterodimerization of KCREB with wild-type CREB prevents binding to DNA. In

addition, KCREB can also quench other transcription factors of the CREB family.

- ACREB: This strong dominant negative variant was constructed by fusing an acidic amphipathic extension onto the N-terminus of the CREB leucine zipper region. As a result of this manipulation, the protein binds with very high affinity and specificity to all members of the CREB family (CREB, CREM, and ATF1), preventing dimerization and blocking their binding to CRE sites [22, 23].

Methodological Approaches for Genetic Manipulation in the Brain

The development of techniques to manipulate the genetic content of mammalian embryos has allowed the generation of transgenic and knockout mice and revolutionized biomedical research. Further progress resulting in anatomically restricted conditional promoters and inducible constructs has helped addressing challenging questions concerning the role of specific genes in complex brain functions, such as learning and memory. In parallel, the development and improvement of safe neurotropic viral vectors have provided an alternative method for genetic manipulation of the adult brain.

These two general approaches for genetic manipulation *in vivo* are complementary and present distinct advantages and caveats. Mouse genetics approaches are time consuming in their initial steps, but once the novel mouse strain is generated and the pattern of expression is determined, researchers have continuous access to a reliable and very powerful tool to investigate gene function *in vivo*. By comparison, viral vectors can be developed more rapidly, but the experiments using virus are more technically demanding since it is necessary to precisely deliver the virus and perform post-mortem injection site analysis for each experimental animal. One important limitation of traditional transgenic and gene-targeting approaches is the limited degree of temporal and spatial control of transgene expression. Although this limitation can be overcome with the use of sophisticated mouse genetics strategies (inducible and tissue-specific mutant strains), the stereotaxic delivery of viral vectors can also effectively address these two issues and allows a narrow control of both the location and timing of the genetic manipulation.

Genetically modified mice with altered levels of CREB function, generated by gene targeting, transgenesis, or viral transduction, have been investigated using a combination of biochemical, anatomical, physiological, and behavioral assays. Tables 1 and 2 show, respectively, the different CREB mutant strains and recombinant viruses generated to investigate the function of CREB in the brain. In the next sections, we summarize

Table 1 CREB mutant strains

Mouse strain	Phenotype	References
CREB ^{-/-} (null mutation)	http://www.informatics.jax.org/searches/allele.cgi?7148 Perinatal death	[27, 129]
CREB $\alpha\Delta$ = CREB ^{$\alpha\delta$-/$\alpha\delta$-} (hypomorphic mutation)	Axonal growth defects and degeneration of peripheral neurons http://www.informatics.jax.org/searches/allele.cgi?3853 Upregulation of CREB β and CREM Modest or no effects on CRE-driven gene expression Controversial LTP and memory phenotypes Complex addiction phenotype	[24–26, 89, 90, 95, 111, 113, 134, 152–156]
CREB _{comp} = CREB ^{$\alpha\delta$-/-} (hypomorphic/null mutation)	Normal hippocampal LTP More severe behavioral defects than CREB $\alpha\Delta$	[89, 90]
CREB ^{ff} (Schültz lab) (floxed CREB)	http://www.informatics.jax.org/searches/allele.cgi?7157 Neurodegeneration (CREM ^{-/-} double mutants)	[28]
CREB ^{CaMKCre7} (postnatal forebrain restricted knockout)	Upregulation of CREM Normal hippocampal LTP and LTD No effect in some hippocampus-dependent tasks	[89]
CREB ^{NesCre} (CNS restricted knockout)	Dwarf phenotype Upregulation of CREM Normal hippocampal LTP and LTD No effect in some hippocampus-dependent tasks, enhanced thigmotaxis, impaired CTA	[89]
CREB ^{ff} (Nestler lab) (floxed CREB)	http://www.informatics.jax.org/searches/allele.cgi?617152 Altered opiate addiction	[33]
CREB(S142A) (knock-in point mutation)	http://www.informatics.jax.org/searches/allele.cgi?7824 Altered circadian rhythms and inflammatory nociception	[34, 36]
CREB(S133A) (knock-in point mutation)	http://www.informatics.jax.org/searches/allele.cgi?63712 No neural phenotype has been investigated	[35]
PcP2-CREB (overexpression of wt protein)	Unaltered LTP Impaired habituation to Rotarod	[37]
CaMKII-CREB _{A133} (dominant negative transgene)	Normal LTP in amygdala and hippocampus Mild fear conditioning impairment in one out of three lines	[38]
CaMKII-CREB _{Y134F} (dominant active transgene)	Enhanced LTP in hippocampus Enhanced social recognition, contextual fear, and passive avoidance memory	[39]
CaMKII-CREB _{DIEDML} (dominant active transgene)	Enhanced social recognition and contextual fear memory	[39]
NSE-tTA/tetO-CREB (inducible overexpression of wt protein)	Depressant-like effect	[157–159]
CaMKII-tTA/tetO-CREB (inducible overexpression of wt protein)	Altered response to cocaine administration	[43]
NSE-tTA/tetO-CREB-M1 (inducible dominant negative transgene)	Anti-depressant like effect Inhibition of the differentiation and maturation of newborn neurons	[43, 159, 160]
CaMKII-tTA/tetO-KCREB (inducible dominant negative transgene)	Impaired spatial learning and memory Deficits in some, but not all, forms of LTP	[47, 91, 161, 162]
CaMKII-tTA/tetO-ACREB (inducible dominant negative transgene)	Neurodegeneration and impaired memory Deficits in some forms of LTP	[46, 48, 49, 163, 164]
CaMKII-tTA/tetO-VP16CREB (inducible constitutively active transgene)	Lower threshold for L-LTP in hippocampus Altered learning and memory Enhanced ocular dominance plasticity in visual cortex	[44, 46, 92, 93, 102, 115, 135]
CaMKII-CREB ^{IR} (tamoxifen inducible repressor transgene)	http://www.informatics.jax.org/searches/allele.cgi?32759 Impaired consolidation of fear memories	[50, 51, 112, 117]

Different mouse strains generated to manipulate CREB levels or activity in vivo. The phenotypes observed and the publications related to each strain are indicated

Table 2 CREB recombinant viruses

Virus name	Insert(s)	Virus type	References
HSV-CREB	Wild-type CREB	Herpes simplex virus type 1 (HSV-PrpUC/helper 5dl1.2)	[116, 119, 120, 123, 165–169]
HSV-mCREB	mCREB (S133A) (dominant negative)	Herpes simplex virus type 1 (HSV-PrpUC/helper 5dl1.2)	[116, 119, 120, 123, 165–169]
HSV-GFP-CREB	Wild-type CREB fused to GFP	Herpes simplex virus type 1 (HSV-PrpUC/helper 5dl1.2)	[54, 55, 121, 122, 124, 167]
HSV-GFP-CREB ^{S133A}	mCREB (S133A) fused to GFP (dominant negative)	Herpes simplex virus type 1 (HSV-PrpUC/helper 5dl1.2)	[54, 55, 121, 122, 124, 167]
HSV-GFP-VP16-CREB	CREB (fused to VP16) fused to GFP (constitutively active)	Herpes simplex virus type 1 (HSV-PrpUC/helper 5dl1.2)	[55]
HSV-GFP-CREB ^{Y134F}	CREB (Y134F) fused to GFP (constitutively active)	Herpes simplex virus type 1 (HSV-PrpUC/helper 5dl1.2)	[124]
HSV-FLAG-ACREB-GFP	FLAG-tagged ACREB (dominant negative) and GFP (independent promoters)	Herpes simplex virus type 1 (HSV-PrpUC/helper 5dl1.2)	[56]
HSV-GFP-CREB-cre	Wild-type CREB fused to GFP followed by IRES-cre recombinase	Herpes simplex virus type 1 (HSV-PrpUC/helper 5dl1.2)	[57]
HSV-CREB-AlstR	Wild-type CREB fused to GFP and Allatostatin receptor (independent promoters)	Herpes simplex virus type 1 (HSV-PrpUC/helper 5dl1.2)	[58]
SFV-CREB-GFP	Wild-type CREB and GFP (independent promoters)	Low toxicity Semliki Forest virus (pSFVpd/pSFVhelper2)	[62]
SFV-CREB ^{S133A} -GFP	mCREB (S133A; dominant negative) and GFP (independent promoters)	Low toxicity Semliki Forest virus (pSFVpd/pSFVhelper2)	[62]
SFV-CREB ^{R287L} -GFP	KCREB (R287L; dominant negative) and GFP (independent promoters)	Low toxicity Semliki Forest virus (pSFVpd/pSFVhelper2)	[62]
SFV-VP16-CREB-GFP	VP16-CREB fusion protein (constitutively active) and GFP (independent promoters)	Low toxicity Semliki Forest virus (pSFVpd/pSFVhelper2)	[62]
Sind-FLAG-CREB ^{Y134F} -IRES-GFP	FLAG-tagged CREB (Y134F; constitutively active) followed by IRES-GFP	Sindbis virus (pSINrep-5/helper DH(26S))	[63–65]
Sind-FLAG-CREB ^{S133A} -IRES-GFP	FLAG-tagged mCREB (S133A; dominant negative) followed by IRES-GFP	Sindbis virus (pSINrep-5/helper DH(26S))	[64, 65]
Sind(nsp2s)-FLAG-CREB ^{Y134F} -IRES-GFP	FLAG-tagged CREB (Y134F; constitutively active) followed by IRES-GFP	Low toxicity Sindbis virus (pSINrep-nsP2S ⁷²⁶ /helper DH/BB(tRNA/TE12))	[66–68]
Ad5-CREB-IRES-GFP	Wild-type CREB followed by IRES-GFP	E1-deleted human adenovirus type 5 (pXCXCMV)	[70, 71]
Ad5-ACREB-IRES-GFP	ACREB (dominant negative) followed by IRES-GFP	E1-deleted human adenovirus type 5 (pXCXCMV)	[70, 71]
Ad5-VP16-CREB-GFP (tTA/TetO)	VP16-GFP (constitutively active) and GFP (independent promoters)	E1/E3-deleted human adenovirus type 5	[72]
rAAV2-HA-CREB	HA-tagged wild-type CREB	Recombinant adeno-associated virus (rAAV-2/helpers ACG2/pXX6)	[75]
CAG-CREB ^{Y134F} -IRES-DSRED	CREB (Y134F; constitutively active) followed by IRES-DSRED	MoMLV-derived retrovirus (CAG vector/CMV-VsVg/CMV-gag/pol)	[78]
CAG-ACREB-IRES-GFP	ACREB (dominant negative) followed by IRES-GFP	MoMLV-derived retrovirus (CAG vector/CMV-VsVg/CMV-gag/pol)	[78, 101]

The viruses engineered to modify CREB function are described in this table together with the publications that used them in vivo. Details of the virus backbone and the helper DNA used for viral production are reported in the “virus type” column, whenever this information was available in the publications

the most important results obtained concerning its role in plasticity, learning, and memory. These experiments have enabled both the testing of pre-existing hypothesis about the role of CREB and the discovery of novel and unsuspected CREB functions.

Manipulating CREB Function Through Mouse Transgenesis and Gene Targeting

Different strategies have been used to generate genetically modified mouse strains in which the expression level or the

activity of CREB is directly manipulated, either to enhance it or to reduce it.

Conventional Knockout Mice

First generation gene-targeting techniques were developed in the late 1980s and allow for the selective inactivation of a specific *locus* in all the cells of an organism. The first CREB knockout mouse was produced as early as 1994 by Gunter Schütz and colleagues and showed some flaws associated to this incipient technology. A promoter-less neomycin resistance gene was inserted in frame into exon 2 [24, 25], but this insertion did not cause the loss of CREB. Instead, it resulted in the generation of a hypomorphic mutant in which the *creb1* locus did not produce the major isoforms of CREB α and δ , but overexpressed isoform β [26]. These mice are now referred to as CREB $\alpha\delta$ mice and have been investigated in dozens of publications.

A few years later, the same group successfully generated the intended CREB knockout by knocking out exons 10 and 11, which encode part of the DNA binding domain and the leucine zipper domain. CREB null mice (CREB^{-/-}) were smaller than their littermates and died immediately after birth from respiratory distress [27]. Furthermore, CREB null mice have impaired fetal T cell development of the alpha beta lineage and structural brain abnormalities involving the corpus callosum and anterior commissures [27].

Conditional Knockout Mice

Considering the phenotype of the full knockout, the best-suited strain to explore the role of CREB in the adult mouse brain would be one in which CREB is eliminated in adulthood. The groups of Gunter Schütz and Eric Nestler have independently generated two strains bearing CREB floxed alleles (*creb1*^{f/f} mice). The strain generated by Schütz's lab, first described in Mantamadiotis et al., bears a floxed allele around exon 10 in the *creb1* gene and has been crossed with several Cre recombinase expressing lines to investigate the consequences of CREB ablation in different neuronal types [28–31]. *Creb1*^{f/f} mice have been also crossed with a line expressing a variant Cre recombinase regulated by tamoxifen (creERT2) under the control of the full CaMKII α promoter, in which the ablation of floxed alleles is both spatially restricted and temporarily regulated [32]. The potential of these animals to dissect the requirement of CREB in different memory phases has not been explored. Overall, the characterization of this strain of CREB-floxed mice has resulted in more than a dozen publications; several of them exploring the specific role of CREB in long-term potentiation (LTP) and memory (see “CREB Functions in the Nervous System”). The second

strain of *creb1*^{f/f} mice has been presented more recently and its generation was not described in detail; these mice have only been used to investigate the role of CREB in opiate-induced homeostatic adaptations of locus coeruleus neurons [33].

Other Knock-in Mice

Gene-targeting techniques also allow for more sophisticated manipulation of CREB function. Thus, two point mutations have been introduced by gene-targeting techniques in the *creb1* locus that alter the manner in which this transcription factor is activated by kinases. In CREB^{S142A} mice, a serine to alanine substitution at amino acid 142 (S142A) was introduced on exon 8 and a floxed TK-neo cassette was inserted into the upstream intron via homologous recombination, making this residue resistant to phosphorylation. Immunohistochemistry of brain sections from homozygous mutant animals demonstrates the absence of phosphorylation at S142, whereas the gene expression pattern of homozygous mutants was similar to wild-type CREB [34]. More recently, a knock-in CREB^{S133A} strain has been generated using a similar strategy [35]. Neither one of these mutants has been investigated in the context of plasticity and memory. The first one, however, has been used to demonstrate the role of phosphorylation of S142 in the regulation of circadian rhythms [34] and inflammatory nociception [36].

First Generation Transgenics

Classical transgenic mice express the gene-of-interest under ubiquitous or tissue-specific promoters. The use of brain-specific promoters has allowed the overexpression of CREB or CREB mutant variants in specific neuronal populations. The Pcp2 promoter has been used to produce transgenic mice that overexpress CREB in Purkinje cells and dentate gyrus granule cells [37]. The CaMKII α promoter has been used to drive the expression of the dominant negative mutant mCREB [38] and, more recently, of two different dominant active variants: CREB^{Y134F} and CREB^{DIEDML} [39] in principal neurons of the forebrain. These three strains have been used to investigate the consequences of CREB inhibition or activation in neuronal plasticity and behavior (see “CREB Functions in the Nervous System”).

Second Generation Transgenics

In addition to spatial specificity, temporal control is a highly desirable feature for transgenic mice. To address the limitations associated with constitutive transgene expression, great effort has been put in the development of inducible transgenic systems. A widely used binary system

for regulated transgene expression is based on the bacterial tetracycline repressor developed by Gossen and Bujard [40]. There are two versions of this system: in the first version, the binding of the chimeric tetracycline-controlled transactivator tTA (resulting of the fusion of the viral transactivation domain VP16 and the DNA binding domain of the TetR bacterial repressor) is blocked by tetracycline (tet). This antibiotic is frequently replaced by doxycycline (dox), which also efficiently binds tTA and exhibits lower toxicity. This system is referred to as Tet-Off. In a second version, point mutations in tTA reversed the effects of tet/dox binding, and this variant (rtTA) binds to DNA only when tet or dox is present [41]. The system is referred to as Tet-On. In both systems, the generation of transgenic lines in which the transgene of interest is placed downstream of the tTA/rtTA regulated promoter, referred to as tetO, enables its expression in a restricted and regulated manner. Only the tTA system has been used in the case of CREB.

Double mutants between tetO lines and CaMKII α -tTA mice [42] should express the transgene of interest in post-mitotic forebrain principal neurons. The expression can be temporally regulated by the addition (transgene Off) or removal (transgene On) of dox to the mouse diet. This approach has been used in gain-of-function studies in which wild-type CREB is overexpressed [43], or a constitutively active CREB variant is expressed (tetO-VP16-CREB mice, several lines first described in [44–46]), as well as loss-of-function studies based on the expression of dominant negative CREB variants, such as mCREB [43], KCREB (tetO-KCREB mice first described in [47]), and ACREB (tetO-ACREB mice which have been independently generated by two research groups [48, 49]). Figure 2 depicts two of these inducible strains.

A second, very useful approach to gain temporal control over transgene activity is the use of chimeric constructs between the protein of interest and the ligand-binding domain (LBD) of the estrogen receptor. The fusion protein is retained in the cytoplasm until the administration of tamoxifen, an estrogen receptor antagonist that binds to the LBD domain and causes the translocation of the protein to the nucleus. This approach is particularly powerful for controlling the activity of nuclear proteins such as the cre recombinase or transcription factors. In the case of CREB, Kida and colleagues generated a transgenic strain in which the dominant negative CREB variant mCREB was fused to the LBD domain and cloned under the control of the CaMKII α promoter to achieve inducible repression of CREB activity in principal neurons of the forebrain. The rapid temporal control afforded by the tamoxifen-regulated system, as compared to the tet system (induction in minutes as opposed to hours-days), allowed the investigators to use this approach to test the requirement for CREB in distinct phases of learning and memory [50, 51].

Manipulating CREB Function Using Viral-Mediated In Vivo Protein Expression

CREB function and level have been also manipulated using viral-mediated in vivo expression to achieve sophisticated control of memory-encoding neuronal circuits. The use of viruses enables high temporal specificity and locally restricted expression of CREB and mutant forms of the protein. Five viral systems—the HSV, alphaviruses, adenovirus, adeno-associated virus, and retroviruses—have been used in this context. Table 2 summarizes the different viruses that have been used to study the role of CREB. The currently available viral systems have been engineered for safe use, relying on replication-defective recombinant viruses, i.e., these viruses can only infect cells once but cannot replicate in the infected host cell and therefore cannot propagate to other cells after infection. This is generally achieved by deleting essential genes from the viral genome necessary for replication and/or packaging. During the initial viral production, these genes are provided in *trans* (by helper DNAs or viruses). Below, we will briefly discuss key advantages and disadvantages of each system (see [52] for a recent review on this topic).

Herpes Simplex Virus

HSV type 1 is a 150-kb double-stranded enveloped DNA virus that carries over 75 genes. The recombinant viral amplicon backbone (PrpUC) contains only the minimal HSV-1 sequences, which allows it to be packaged into virus particles with the aid of a helper virus. The HSV-1 viral backbone can host large inserts of interest with good packaging efficiency. This virus is capable of infecting most cell lines and types of mammalian cells. HSV has a particular tropism for neurons. A main advantage of this virus is the possibility of expressing large inserts (up to 150 kb). Transgene expression can be detected within hours in vitro. In vivo expression of the insert can be detected within days of infection, with the highest transgene expression around 3 days after surgery. HSV is less suited for long-term expression because the viral expression is unstable. A limitation of this viral system, however, is that production of the viral particles requires a co-propagated HSV-1 helper virus, resulting in viral stocks that are a mixture of helper and virus of interest leading to cytotoxic effects. Efforts have been made to reduce this cytotoxicity, both in vitro and in vivo, with some success by engineering new generations of HSV vectors. To completely circumvent this problem, a helper virus free system has also been developed but yields relatively low titers of virus, which makes its use difficult for in vivo expression. More information on the HSV viral systems is provided in [53].

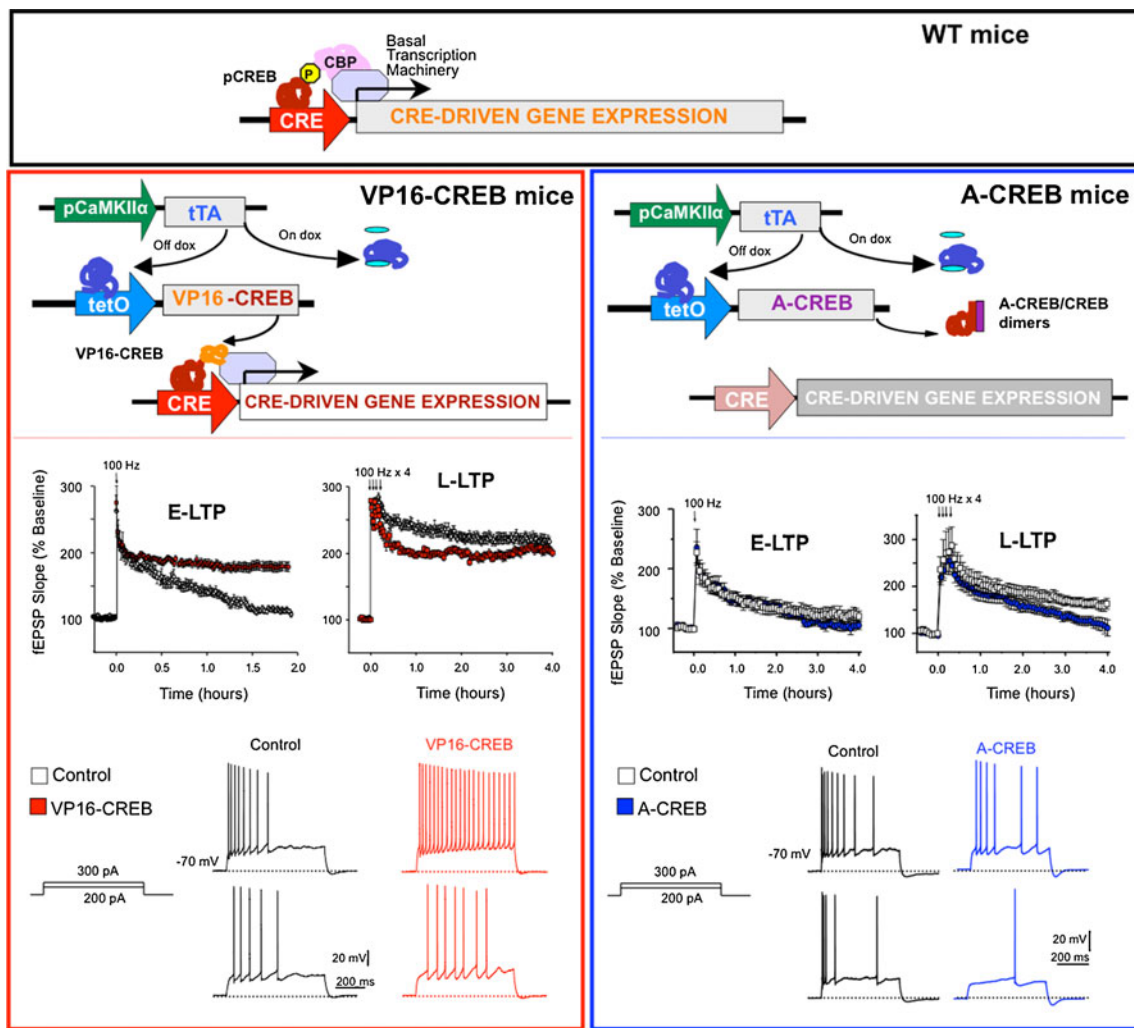


Fig. 2 Evaluation of CREB function in synaptic plasticity and neuronal excitability using CREB transgenics. Schematic representation of CRE-driven gene expression in wild-type mice (upon phosphorylation and recruitment of CBP, *upper scheme*), and in transgenic mice expressing a constitutively active CREB variant (VP16-CREB mice, *left scheme*) or a dominant negative inhibitor

(ACREB mice, *right scheme*). Under the corresponding schemes, we also present the results of the analyses of E-LTP and L-LTP in the Schaffer collateral pathway (data from [44] and [48], respectively) and excitability of CA1 pyramidal neurons (data from [45] and [48], respectively)

Neve and colleagues developed HSV viral vectors for use in neuroscience and successfully implemented them to study the role of CREB in neuronal plasticity and memory formation. Nine CREB-expressing viral vectors have been used in this context as detailed in Table 2. The first generation of vectors, first reported in Carlezon and colleagues [165], contained wild-type CREB or the dominant negative mCREB mutant, but there was no marker of infection. A second generation of vectors, that expressed either wild-type CREB, CREB^{S133A}, CREB^{Y134F}, or VP16-CREB fused to green fluorescent protein (GFP), allowed for live detection of the infected neurons [54, 55]. An HSV vector with two transcriptional units driving the dominant negative mutant ACREB and GFP independently was also engineered by Suzuki and colleagues [56]. These first and

second generation HSV viruses were used in vivo in several studies to evaluate the role of CREB in memory formation, drug addiction, homeostatic spine plasticity, and ocular dominance plasticity (see Table 2 for details and references). More recently, CREB expressing HSVs has been used as a tool for sophisticated manipulation of neuronal circuits. The co-expression of CREB and the diphtheria-toxin [57] or the allatostatin receptor [58] has enabled, respectively, the specific erasure or reversible inactivation of recently acquired memories whose allocation was driven by CREB overexpression (see “CREB and Memory” for further details). These two studies represent particularly good examples of how the use of viral vectors, with the possibility of co-expressing several proteins, provides a unique opportunity to modulate the function of specific

neurons in vivo in a highly temporally and spatially restricted manner.

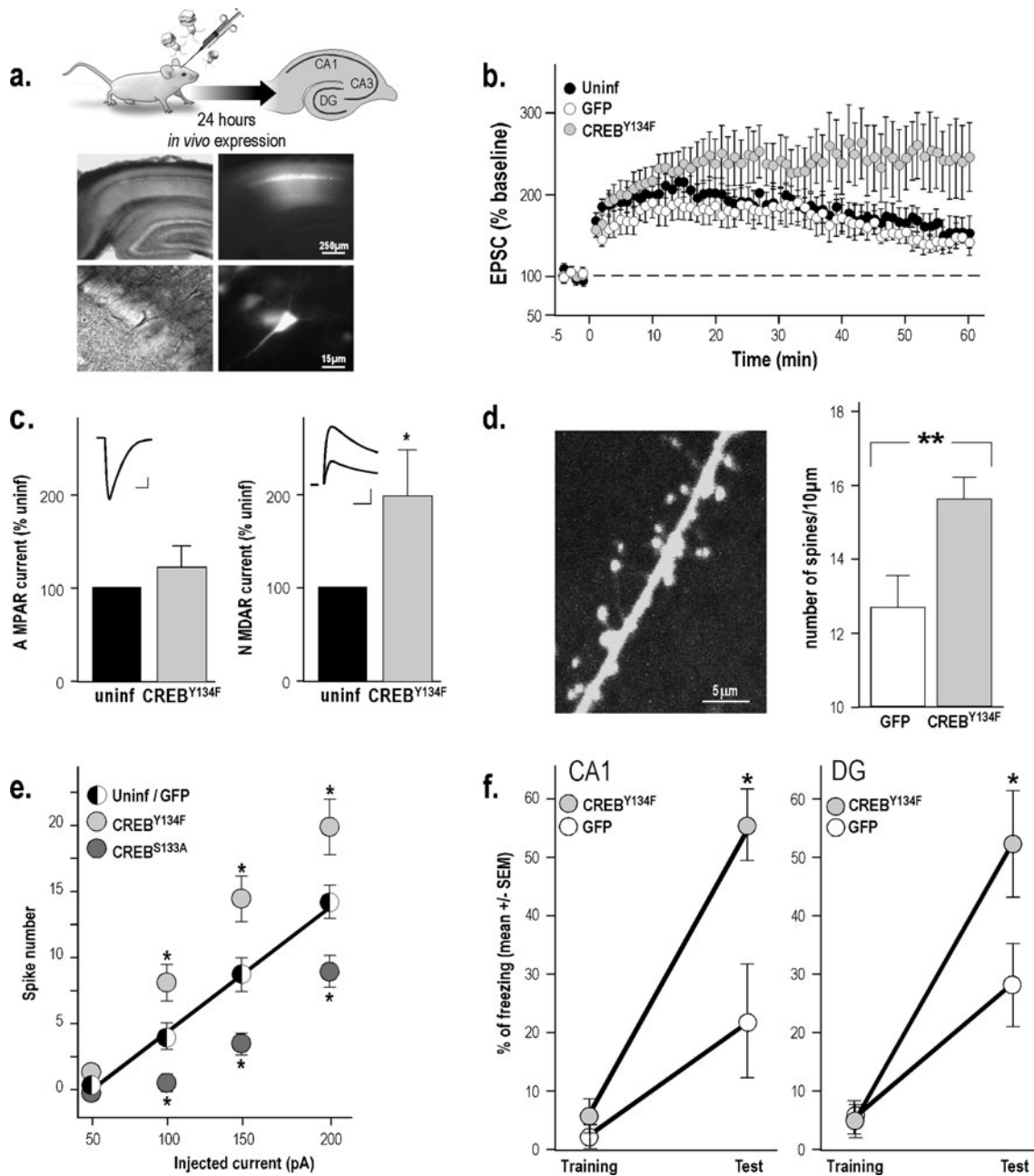
Alphaviruses: Semliki Forest and Sindbis Viruses

The Semliki forest and Sindbis viruses are members of the alphavirus family. These viruses are enveloped viruses with small single-stranded RNA genomes. The first generation of recombinant viral backbones (pSFV, pSINRep-5), in which the insert of interest is cloned, also contains the nonstructural genes, but lacks the structural viral proteins normally necessary to package the RNA into viral particles. These DNA constructs are used to make genome-length RNA transcripts (recombinant RNA) in vitro. Production of replication-deficient infectious viruses is accomplished by transfecting cells with the capped recombinant RNA and a helper RNA that provides the structural proteins in *trans* but does not contain a packaging sequence. Expression of the transgene is detected within a day both in vitro and in vivo. More information on the Semliki forest and Sindbis viral systems can be found in [59]. The main advantages of this type of virus for use in neuroscience are that it is highly neurotropic (targeting preferentially glutamatergic neurons), that it allows for a strong and rapid expression of the transgene, and that it has a good diffusion in vivo. It, however, leads to cytotoxicity within a few days of infection and is therefore not suitable for long-term expression studies. This is due to the fact that the recombinant RNA, once transfected into cells, promptly recruits most of the host translational machinery for its own use, resulting in high levels of the desired protein, but at the expenses of the cell's well-being. Also, transgene size is limited as packaging becomes problematic if the insert size is more than 4 kb. In vivo investigations using this virus have used a time frame of expression of up to 3 days with success. A new generation of viral backbone vectors was designed to reduce this toxicity [60, 61]. These low toxicity vectors (pSFVpd and pSINRep-nsp2^{S726}) contain point mutations in the second nonstructural protein (nsp2), which delay the inhibition of host protein synthesis. For Sindbis virus production using this low toxicity vector (pSINRep-nsp2^{S726}), Kim et al. also constructed an optimized helper vector for production of particles with low levels of helper RNA packaging and high neuro-specificity of infection [61].

Zhu et al. engineered four Semliki forest viruses, using the low toxicity pSFV(pd) vector, to investigate the function of CREB in ischemia-induced neurogenesis in the dentate gyrus (Table 2) [62]. These viruses co-expressed GFP with either wild-type CREB, dominant negative mutants of CREB (mCREB or KCREB), or the constitutively active mutant VP16-CREB. These viruses have not yet been used to study CREB in the context of learning and memory.

Investigation of the function of CREB in neuronal plasticity and memory using in vivo expression of Sindbis viruses was reported by Marie and colleagues (see Table 2 and Fig. 3a). CREB^{Y134F} and CREB^{S133A}, containing the FLAG tag, were cloned into the first generation Sindbis vector which also included the coding sequence of GFP downstream of an internal ribosomal entry site (IRES). This permitted both the transgene and GFP to be translated from a single bicistronic messenger RNA in the same neurons without requiring the use of a fusion protein, which could disrupt normal activity of the transgene being tested. These first viruses were used in electrophysiological studies to identify the role of CREB in the regulation of neuronal physiology [63–65]. More recently, lower toxicity Sindbis variant viruses (pSINRep-nsP2S⁷²⁶), which allow for slightly longer in vivo manipulations (up to 7 days), have been used to evaluate the effects of increasing CREB activity on dentate gyrus synaptic plasticity and on

Fig. 3 Evaluation of CREB function in neuronal plasticity and hippocampal memory formation using Sindbis virus mediated in vivo expression of CREB mutants. **a** Recombinant Sindbis viruses expressing mutants of CREB and GFP were injected in vivo in the hippocampus (**a–d, f**) or nucleus accumbens (**e**). Photos show strong GFP expression detected in CA1 pyramidal neurons in slices from an infected young adult rat 24 h after in vivo injection with a GFP-expressing Sindbis virus: low-resolution ($\times 4$; *top panels*) and high-resolution ($\times 40$; *bottom panels*) images of hippocampal slices (*left panels* show DIC images; *right panels* show GFP fluorescence). **b** In vivo expression of CREB^{Y134F} enhances LTP in CA1 pyramidal neurons. CA1 neurons of young adult rats were infected with either GFP or CREB^{Y134F}-IRES-GFP and LTP (induced at time 0 by 100 Hz/1 s protocol) whole-cell experiments were performed on acute slices from these infected rats 24 h after infection. *Uninf* uninfected control neurons in infected slices. **c** In vivo expression of CREB^{Y134F} enhances NMDAR-mediated but not AMPAR-mediated synaptic transmission in CA1 pyramidal neurons. Average percent change of AMPA receptor (*left*) and NMDA receptor (*right*) currents of CREB^{Y134F}-expressing neurons of in vivo infected young adult rats relative to neighboring uninfected control neurons (sequential paired whole-cell recordings). Overlay of sample currents of pairs are shown *above bar graphs* (scale bars, 20 ms/20 pA). **d** In vivo expression of CREB^{Y134F} enhances spine density in CA1 pyramidal neurons. Confocal micrograph of Alexa 568-filled secondary dendrite from a GFP-expressing neuron after in vivo infection. Spine density was estimated in GFP and CREB^{Y134F}-IRES-GFP-infected neurons using confocal microscopy and 3D reconstruction of dendritic segments. **e** In vivo CREB^{Y134F} and CREB^{S133A} expression increases and lowers, respectively, the intrinsic excitability of nucleus accumbens medium spiny neurons, as measured by the number of spikes elicited by a given injected current. **f** In vivo expression of CREB^{Y134F} in CA1 pyramidal neurons or dentate gyrus (DG) granule cells enhances memory formation in the contextual fear conditioning (CFC) task. Mice were injected with either GFP or CREB^{Y134F}-IRES-GFP viruses bilaterally in the CA1 or in the DG and submitted to CFC training and testing (24 and 48 h after infection, respectively). Freezing behavior was monitored during the training and test sessions and is reported in these graphs. Enhanced freezing during the test session, reflecting better conditioning, was evident in both CA1- and DG-CREB^{Y134F}-IRES-GFP-infected mice compared to GFP-infected mice. *Graphs and pictures* are adapted from [63, 64, 66]



hippocampus-dependent memory formation and extinction [66–68].

Adenovirus

Adenoviruses (Ad) are medium-sized non-enveloped (without an outer lipid bilayer) viruses composed of a nucleocapsid and a double-stranded linear DNA genome. Human Ad serotype 5 is generally used for gene transfer as its biology is best characterized. The first generation of adenoviral vectors was based on this serotype by removing the E1 early genes. This system has, however, been associated with in vivo toxicity due to innate immune responses and inflammation.

The next generation of Ad vectors, referred to as “gutless” or “high-capacity”, have all of the viral genome removed providing greater transgene capacity. However, the active Ad infection still displays some toxicity. One of the main advantages of Ad viruses is that it can carry large inserts (up to 30 kb for “high-capacity” vectors). Expression of the transgene is slow at first requiring several days for detection in vivo, but can be used for long-term expression in neurons albeit the possibility of increased toxicity. Also, there is a lack of neurotropism as these viruses infect neurons and glia equally, which can be circumvented with the use of cell-specific promoters. More information on this viral system can be found in [69].

Ad viruses expressing wild-type CREB and the dominant negative ACREB, together with IRES GFP, have been used in studies to evaluate the role of CREB in neuronal death [70], in perirhinal cortex plasticity, and in recognition memory [71]. Also, Gao et al. designed an Ad virus harboring both GFP and VP16-CREB, in which expression of the latter transgene is regulated by the Tet-Off system (see “Second Generation Transgenics”) [72]. In this study, tTA was provided by another tTA-expressing adenovirus and VP16-CREB expression was turned on when doxycycline was removed from the diet of infected mice or the media of infected neuronal cultures. This virus was only used as yet to evaluate the role of CREB in axon regeneration in vivo [72], but this study proves the feasibility of combining in vivo viral expression with the inducible tetracycline repressor system.

Adeno-associated Virus

Adeno-associated viruses (AAV) are small replication-deficient parvoviruses, which have traditionally required co-infection with a helper adenovirus or herpes virus for productive infection. For safer use of this viral system, helper-free systems have been developed in which most of the adenovirus gene products required for the production of infective AAV particles are supplied by helper plasmids. The recombinant AAV (rAAV-2) serotype 2 is generally used in neuroscience because this serotype shows good neurotropism. One advantage of the AAV-2 virus is its lack of toxicity for in vivo expression studies because it does not generate an immune response nor inflammation at the site of injection. Its main limitation is that packaging capacity is limited to transgenic inserts smaller than 5 kb. Expression of the transgene takes several days to be detected in vivo but, due to the lack of toxicity, this virus is highly suitable for in vivo long-term expression and it is one of the vectors of choice for human gene therapy. More information on this viral system can be found in [73, 74]. Mouravlev and colleagues engineered a rAAV-2 expressing wild-type HA-tagged CREB to study the relationship of CREB and memory impairment during aging in rats (Table 2) [75].

Moloney Murine Leukemia Retrovirus

This retroviral vector is derived from the Moloney murine leukemia oncoretrovirus (MLV). MLVs are lipid-enveloped viruses containing two identical copies of a linear single-stranded RNA genome. These retroviruses have a relatively simple genome (around 10 kb) and structure, and they integrate into the genome, permitting long-term transgene expression. They have been used for several decades for stable transfer into mammalian cells and for gene therapy.

More information on this type of virus can be found in [76]. These viruses have not been exploited much for in vivo expression of transgenes in the brain. Indeed, their main limitation is that they can only infect dividing cells and, thus, are not good for transduction of neurons. Once inside a cell, they retro-transcribe their genome into DNA, which is then used to make more viral RNA and new viruses. However, during this process, the DNA has to be moved to the nucleus and this can be achieved only when cells undergo a mitotic cycle. This feature has been successfully exploited to specifically transduce neuroprogenitor cells in the adult brain, which makes this type of viral vector a powerful research tool to investigate neurogenesis and neuronal lineage [77]. Thus, a recent study described the use of MLV-derived retroviruses expressing either ACREB or $CREB^{Y134F}$ together with IRES-GFP or IRES-DsRed, to study the role of CREB in the maturation of adult newborn neurons of the dentate gyrus [78].

Lentivirus

Lentiviruses (Lv) belong to a different subclass of retrovirus than the MLV. The early Lv vectors were based largely on the human immunodeficiency virus-1 (HIV-1). This type of retrovirus has the major advantage that it can infect both growth-arrested and dividing cells, including neurons and glia. Lv vectors have been extensively developed over the last decades for efficient and safe research tools. The newest generations contain only few sequences of the HIV-1 genome and provide a transgene capacity of about 10 kb. The viral particles are generated from three separate plasmids to ensure that only replication-defective viruses are produced. They generally integrate into the host genome, making them suitable for long-term expression studies, but some types of vectors lack the integrase, which prevent genomic integration if desired. More information on this type of viral vectors can be found in [79]. Lentiviruses have been used extensively in the last decade for neuroscience applications. We are, however, not aware of its use yet for expression of CREB or CREB mutants. Investigators have engineered lentiviruses to modulate the function of the CREB pathway by downregulating TORCs or overexpressing CBP [80, 81].

CREB Functions in the Nervous System

CREB participates in the regulation of neuronal responses to a variety of stimuli. For example, numerous neurotrophins and cytokines activate CREB, as do a host of other cellular perturbations that ultimately increase levels of cAMP or calcium. A large body of work establishes CREB as a critical component of the molecular switch that

controls different forms of neuronal plasticity by regulating the expression of genes necessary to strengthen existing synaptic connections, to promote the formation of new ones, and to modulate the intrinsic properties of the neurons. All these phenomena are thought to underlie learning and memory processes in the brain [11]. Below, we will focus on these functions and discuss the role of CREB in different forms of neuronal plasticity and memory that emerged from studies in which the activity of CREB has been genetically modified by transgenesis, gene targeting, or virus transduction techniques.

CREB and Synaptic Plasticity

Studies in the sea snail *Aplysia*, three decades ago, first established the critical role of the cAMP signaling pathway and CREB in long-term facilitation (LTF), the long-term strengthening of synaptic connections that takes place during simple forms of learning and memory in this animal [82]. Most of the upstream signaling cascade leading to CREB activation appears to be conserved through evolution, and many aspects of the role of CREB in synaptic plasticity described in invertebrates have been also observed in LTP, which is the mammalian equivalent to LTF [83]. Pharmacological experiments distinguish two distinct phases of LTP: an early phase (E-LTP) that is resistant to inhibitors of transcription and translation and a late phase (L-LTP) that is blocked by such compounds. It is thought that E-LTP and L-LTP are the cellular correlates of short-term and long-term memory, respectively [84]. In hippocampal neurons, both CREB phosphorylation and the induction of a CRE-driven *lacZ* reporter construct are triggered in CA1 pyramidal neurons by electrical stimuli that induce L-LTP [85–88]. Although seminal studies in CREB $\alpha\delta$ mice revealed severe L-LTP impairments [25], this deficit appeared to be sensitive to different factors, such as gene dosage and genetic background. A comprehensive study using four different strains of CREB-deficient mice, including CREB $\alpha\delta$ hypomorphic mutants and neuron-restricted knockouts, failed to demonstrate any deficit in both LTP and long-term depression (LTD) in the Schaffer collateral pathway when robust induction protocols were used [89]. Other studies in CREB knockouts and transgenics have also failed to demonstrate deficits in LTP experiments in the hippocampus [90] and the amygdala [38], respectively. As discussed above, knocking out CREB α/δ isoforms causes the overexpression of other CRE-binding proteins, such as CREM and the CREB β isoform [24, 26] that may compensate for the deficiency in CRE-dependent activity and lead to an impaired LTP phenotype.

The use of transgenic strategies that cause a broader inhibition of CRE-driven gene expression has allowed

studies of the role of the CREB pathway in synaptic plasticity without the associated problems of compensation by other CRE-binding proteins. For example, transgenic mice expressing KCREB, a dominant negative form of CREB that prevents its binding to DNA and that can also quench other factors capable of associating with CREB, showed clear deficits in different forms of L-LTP [47, 91]. Similarly, transgenic mice expressing the strong dominant inhibitor ACREB also impaired L-LTP, but spared E-LTP [48] (Fig. 2).

Gain-of-function studies have consistently demonstrated that CREB activity (or more precisely, CRE-binding activity) is sufficient to enhance LTP. Transgenic mice [39, 44, 92] and Sindbis virus-transduced rats [63] that express dominant active CREB variants show enhanced CRE-driven expression in CA1 pyramidal neurons and stronger LTP (e.g., Figs. 2 and 3b). Interestingly, Marie and colleagues found that the expression of CREB^{Y134F} also enhanced synaptic transmission of NMDA receptors, but not of AMPA receptors (Fig. 3c) by increasing the number of silent synapses in CA1 pyramidal neurons [63], a change that can explain the facilitation of LTP observed in those animals. The overexpression of effector molecules downstream of CREB, such as the neurotrophin BDNF that promotes synaptic growth, can also contribute to the enhancement of LTP [93]. More recently, Marie and colleagues demonstrated that increasing CREB activity in granule cells of the dentate gyrus by viral in vivo expression of CREB^{Y134F} is also sufficient to enhance LTP in this structure [68]. Consistent with what has been described for LTF in *Aplysia* neurons [94], these studies suggest that the products generated after activation of the CREB pathway provide the required support for synaptic strengthening.

Overall, these data provide strong evidence supporting a key role for CREB and CRE-driven transcription in synaptic plasticity in rodents. However, some discrepancies between the results of groups using loss-of-function approaches may need additional clarification. The weak LTP phenotype observed in the forebrain-restricted knockout mice is particularly surprising [89]. This, together with the modest transcriptional alterations observed in different loss-of-function studies, suggests that other transcription factors may compensate for the lack of CREB [48, 95, 96]. Although CREB may be sufficient to trigger a transcriptional program able to sustain L-LTP [93], it is not always necessary.

CREB and Structural Plasticity

The concept of structural plasticity in memory storage was first described by Ramon y Cajal in the 1890s, when he suggested that a memory is stored in the growth of new

synaptic connections. In agreement with this hypothesis, LTF in *Aplysia*, which is thought to be a cellular correlate of long-term memory formation, is accompanied by growth of new synaptic contacts [97]. Notably, this form of plasticity involves PKA-dependent CREB phosphorylation. Studies in mammals have also shown that spines are highly dynamic structures during memory formation and the cellular processes of LTP and LTD [98]. The definition of the exact role of CREB in this context is, however, still under investigation. Evidence supports the notion that, like in invertebrates, CREB activation is intimately linked to spine formation in mammals. Work on cultured neurons has demonstrated that phosphorylation of CREB is necessary for estradiol-evoked spine formation [99]. Marie and colleagues demonstrated that in vivo viral-mediated expression of CREB^{Y134F} is sufficient to lead to an increase in spine density in CA1 pyramidal neurons of young adult rats (Fig. 3d; [63]). The importance of CREB in homeostatic spine plasticity was also shown in a recent study on pyramidal neurons of the visual cortex of adult rats infected with an HSV vector expressing ACREB [56]. Suzuki and colleagues observed that CREB inhibition reduced spine head volume, but did not affect spine length or density. They also demonstrated that CREB plays an active role in homeostatic responses to activity suppression (by application of TTX) by controlling enlargement of spine heads and shortening of spine length. These observations suggest that CREB is a positive regulator of spine number and size.

CREB is also involved in another form of structural plasticity in the mature brain: adult neurogenesis. CREB seems to regulate different phenomena during neurogenesis, both during development and in the adult. To date, CREB has been implicated in newborn neuron survival, maturation, and circuit integration [62, 100]. Recent experiments with MLV vectors have demonstrated that loss of CREB in a cell-autonomous manner decreases expression of the critical neurogenic factors (NeuroD and doublecortin) and compromises the survival of newborn neurons. These effects demonstrate that CREB signaling is a central component of adult hippocampal neurogenesis [78]. Similar experiments in the subventricular zone (SVZ) indicate that CREB signaling also plays an essential role in the early stages of SVZ neurogenesis and the maturation of newborn neurons in the olfactory bulb [101].

CREB and Intrinsic Plasticity

A number of recent studies have revealed a novel role for CREB and downstream gene expression in neural plasticity: the control of intrinsic excitability (i.e., the propensity of the neuron to fire action potentials in response to input signals) (see [11] for a recent and detailed review). CREB was first found to regulate neuronal firing in medium spiny

neurons infected with recombinant Sindbis viruses [64]. The expression of the constitutively active CREB^{Y134F} variant enhanced intrinsic excitability, whereas the expression of the dominant negative CREB^{S133A} mutant reduced it (Fig. 3e). Similar results were observed in noradrenergic neurons of the *locus coeruleus* infected with recombinant HSV expressing either the constitutively active VP16-CREB variant, which increased intrinsic excitability, or the dominant negative CREB^{S133A} mutant that caused the opposite effect [55]. Studies in the hippocampus of bitransgenic mice expressing either VP16-CREB [45] or the dominant negative ACREB mutant [48] demonstrated that the excitability of CA1 pyramidal neurons, in particular their post-burst after-hyperpolarization (AHP), was also severely affected by the genetic manipulation of CREB function (Fig. 2). Recent studies in VP16-CREB bitransgenic mice or virus-transduced animals showed that the enhancement of CREB activity also produced a reduction of AHP in pyramidal neurons of the amygdala [58, 102]. Overall, these results suggest that the modulation of intrinsic neuronal properties is a well-conserved CREB function.

CREB and Memory

Substantial evidence in experimental systems ranging from mollusks to humans indicates that the CREB pathway is a core component of the molecular switch that converts short- to long-term memory. Studies in the sea snail *Aplysia* [82, 103] and in the *Drosophila* fly [104–106] first established decades ago the importance of the cAMP and CREB signaling pathway in simple forms of learning and memory. In the mammalian brain, CREB is phosphorylated and CREB-dependent transcription is induced in glutamatergic neurons after training in hippocampus-dependent and amygdala-dependent memory tasks [107–109].

This correlative evidence is complemented by genetic and pharmacological studies demonstrating that activation of the CREB pathway is not just a consequence of training, but plays an active role in learning and memory. A large number of behavioral studies have explored the learning and memory phenotype of CREB mutant strains and CREB virally transduced animals. CREB $\alpha\delta$ mice have a specific deficit in long-term memory revealed in several memory tasks [25]. This seminal study was soon replicated in rats, in which the intra-hippocampal infusion of CREB antisense oligos caused deficits in spatial learning [110]. However, other studies in CREB hypomorphic mutants indicated that the memory defect was sensitive to gene dosage and genetic background [90, 111]. Moreover, as described before for LTP, the parallel behavioral analysis of four different strains of CREB-deficient mice by Balschun and colleagues failed to demonstrate any specific deficit in

classical hippocampus-dependent tasks, including contextual fear conditioning and spatial learning in the water maze [89]. The apparent deficits in the Morris water maze found in some CREB mutants were better explained by an increase in thigmotaxis behavior rather than impaired spatial learning. A controversy regarding the role of CREB in memory is also seen in other behavioral tasks. For example, some fear conditioning studies have shown that CREB-deficient mutants exhibited impaired fear conditioning [25, 90, 111], whereas others failed to reveal significant deficiencies [38, 89]. These discrepancies suggest that, like for L-LTP, the loss of CREB may be compensated by the action of other CRE-binding transcription factors. As a consequence, the genetic approaches designed to overcome the obscuring effects of compensation have been more successful in revealing a role for CREB in learning and memory. Thus, transgenic mice expressing the broad dominant negative mutant KCREB in the dorsal hippocampus showed spatial memory deficits that were reversed after turning off the transgene [47]. Similarly, transgenics expressing another broad dominant negative mutant ACREB also presented severe learning and memory deficits, although in this case the observation of concomitant hippocampal neurodegeneration prevented reliable conclusions concerning a specific role of CREB in memory [48]. Both compensatory and pleiotropic effects were successfully addressed by Kida and colleagues using the tamoxifen-regulated CREB variant described in “[Second Generation Transgenics](#)”. Inducible and transient repression of CREB function specifically blocked the consolidation [50] and reconsolidation [51] of long-term fear memory and spatial memory in the water maze [112]. Behavioral studies on mutant mice have also shown that inhibition of CREB leads to deficits in object recognition [47], socially transmitted food preferences [113], social memory [114], and conditional taste aversion [89].

Gain-of-function transgenic approaches have been also successful for demonstrating a role for CREB in memory. Work by Viosca and colleagues in VP16-CREB transgenic mice demonstrated that constitutive CREB activity in fear memory circuits can bypass the requirement for de novo gene expression associated with long-term fear memory formation [102]. However, their experiments have also shown that the chronic and strong increase of CREB activity can have detrimental effects in memory performance since it interfered with the retrieval of spatial information in the water maze [115]. More recently, the analysis of several transgenic lines exhibiting more moderate upregulation of CREB activity in the forebrain, CaMKII-CREB^{Y134F} and CaMKII-CREB^{D1EDML} mice (two lines per strain), demonstrated that enhanced CREB improved long-term memory in different tasks, including social recognition memory, passive avoidance, contextual

fear conditioning, and spatial navigation [39]. Interestingly, some of these lines also exhibited enhanced short-term memory in contextual fear conditioning and social recognition tasks [39].

Studies using recombinant viruses also allow for acute genetic manipulation of CREB activity and have clearly supported a role for CREB in memory formation. Using recombinant HSVs, Josselyn and colleagues first demonstrated that the acute overexpression of CREB in amygdala facilitated the formation of long term memory [116], whereas the expression of a dominant negative CREB mutant inhibited it [117]. Later, the inhibition of CREB through the expression of dominant negative variants led to deficits in social transmission of food preferences [118] and striatal-dependent procedural learning [119], whereas gain-of-function approaches targeted to the hippocampus have successfully confirmed the enhancement of fear conditioning memory (Fig. 3f; [66]) and supported a role for CREB in spatial memory [120, 121]. Furthermore, Vetere et al. most recently demonstrated that increasing contextual fear memory by increasing CREB activity in the dentate gyrus does not prevent normal extinction of this memory [67]. Somatic gene transfer of CREB has been also shown to attenuate memory impairment in aging rats [75].

As in the case of transgenic studies, experiments with viral vectors have also raised some concern regarding the timing, location, and duration of CREB manipulation. Increasing CREB in the auditory thalamus enhanced formation of an auditory-conditioned fear memory, but caused broader auditory fear generalization [122]. Also, the overexpression of CREB in the basolateral amygdala decreased the number of escape failures in the learned helplessness model of depression when the virus was injected after training, but increased escape failures and other depressive effects when injected before training [123]. Expression of CREB in the basolateral amygdala also increased diverse behavioral measures of anxiety [123]. This variety of effects can be explained considering the duration and strength of the perturbation of CREB pathway achieved in each of these studies.

Josselyn and colleagues elegantly demonstrated that the neurons overexpressing CREB, via HSV viral vectors, were preferentially recruited to form a new fear memory [124], suggesting the existence of a competitive model underlying memory formation, in which eligible neurons are selected to participate in a memory trace as a function of their relative CREB activity at the time of learning (see the recent reviews on this topic by [125, 126]). As a continuation of these experiments, Josselyn’s group showed that the ablation of CREB-overexpressing neurons led to complete loss of the memory allocated in the infected neurons [57]. To achieve this, they engineered an HSV vector that expresses both GFP-CREB and cre recombinase

(GFP-CREB-cre). They injected this virus into transgenic mice expressing simian diphtheria toxin receptor (DTR) in a cre recombinase-inducible manner. Upon infection of neurons with GFP-CREB-cre, these neurons expressed CREB, but also cre recombinase, which excised the loxP-flanked STOP cassette that silenced DTR expression, thereby allowing DTR expression. Injection of diphtheria toxin any time thereafter induced apoptosis only in virus-expressing cells. This innovative approach demonstrates how coupling the use of viruses with that of transgenic mice can provide unique and powerful strategies to selectively target and modify neurons *in vivo* and demonstrated a causal link between a molecularly defined neuronal population in the mammalian brain and the expression of a specific memory.

In agreement with this view, Zhou and colleagues have demonstrated that temporarily silencing CREB-transduced amygdala neurons during tone conditioning prevented memory formation [58]. To achieve this, they co-expressed CREB and the *Drosophila* allatostatin receptor (AlstR) using an HSV vector, which turns on endogenous mammalian G protein-coupled inwardly rectifying potassium (GIRK) channels. Upon binding of allatostatin, the AlstR/GIRK complexes cause membrane hyper-polarization and, consequently, a decrease in neuronal excitability. They co-expressed AlstR with GFP-CREB in the same HSV but driven by two independent promoters. By *in vivo* infection of this virus in the amygdala, they could evaluate the effects of increased CREB-dependent transcription, but also how inactivation of these same neurons (by stereotaxic *in vivo* infusion of allatostatin at the site of viral infection) could perturb memory processes. Again these data demonstrate that CREB drives the allocation of fear memory to specific cells. The impairments observed in most loss-of-function studies and the various effects of overactivation of the CREB pathway, from detrimental to beneficial, highlight the importance of proper and timely activation of the CREB pathway in learning and memory processes.

Other Aspects of CREB Function in the Nervous System

When interpreting the behavioral and plasticity phenotype of mice with genetically altered levels of CREB activity, we should not forget that CREB plays important roles in neuronal physiology that may not be directly related with its function in plasticity. Particularly relevant is the strong evidence supporting a critical role for the CREB pathway in the development of the nervous system and neuronal survival.

Diverse developmental processes in the nervous system have been associated with CREB function. CREB plays an important role in controlling proliferation, differentiation, and survival of newborn neurons [127–129]. CREB activity

promotes the formation of dendrites and growth cones in cultures of embryonic neurons or neuroblastoma cells [72, 130] and probably also during development of the nervous system [129, 131]. CREB also participates in different aspects of developmental plasticity, such as ocular dominance in the visual cortex or the formation of anatomical maps in the barrel cortex [132–135].

Regarding neuronal survival, experiments in neuronal cultures and CREB mutant mice indicate that some neuronal types have a complete requirement for CREB for survival, whereas others, particularly in the central nervous system, are less compromised after the elimination of CREB [19, 28–31, 48, 129, 136, 137]. CREB is not only required for neuronal survival, but may also participate in the defensive response to injury [28, 138]. A variety of studies have demonstrated that overexpression of CREB or transient expression of a constitutively active CREB variant protected different types of neurons from apoptotic death, whereas dominant negative CREB mutants have the opposite effect [70, 130, 139]. Several studies indicate that CREB may also play a role in axonal repair [140–142]. However, the strong chronic activation of CREB in transgenic mice caused sporadic epileptic seizures and loss of hippocampal neurons, indicating that a fine-tuned regulation of CREB's function is required for neuronal survival and function [143].

Given the involvement of CREB in diverse critical aspects of neuronal function, it is not surprising that the consequences of malfunction in its pathway are severe. Thus, great effort has been put to understand the role of CREB in drug addiction [144, 145], mental retardation syndromes caused by mutations of genes in the CREB signaling pathway [146, 147], and neurodegenerative diseases in which the CREB pathway appears affected [12, 148]. The genetic manipulation approaches described above should be, therefore, also very useful to explore the role of CREB in these pathological conditions, as demonstrated, for example, by the investigation of the role of CREB in cocaine addiction [64, 65, 149].

Concluding Remarks

Technical advances in mouse genetics and viral expression systems have allowed the generation of new tools to alter CREB function *in vivo*. The anatomical and temporal restriction of the genetic manipulations combined with multidisciplinary approaches has allowed addressing fundamental biological questions related to CREB function unapproachable by previous efforts, such as its role in memory allocation and consolidation. Some significant discrepancies between studies still need to be clarified, and recent findings have opened numerous novel questions

concerning the role of CREB in the regulation of neuronal excitability and the allocation of new memories. Another important area for future research is to identify the particular gene programs that CREB activates in distinct neuronal contexts. Such studies will likely require the use of emerging techniques for genome-wide analysis of gene expression and genome occupancy. With the challenges ahead in mind, the effort of several dozens research groups during the last 15 years has greatly strengthened and refined our understanding of the role of the CREB-dependent transcription in learning and memory and has consolidated the position of the CREB pathway as one of the most attractive target for drugs aimed at restoring or protecting memory abilities under pathological situations and also possibly to improve memory in the normal brain [150, 151].

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