Papel del factor de transcripción CREB en supervivencia y plasticidad de neuronas hipocampales

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Role of CREB-dependent transcription in the control of hippocampal neurons survival and plasticity

Memoria presentada por Dragana Jančić para optar al Título de Doctor por la Universidad Miguel Hernández

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INFORMA:

Que el presente trabajo de investigación titulado "**Papel del factor de transcripción CREB en supervivencia y plasticidad de neuronas hipocampales**", presentado por **Dragana Jancic**, para optar al grado de Doctor, ha sido realizado bajo la supervisión del Dr. Angel L. Barco Guerrero, y da su conformidad para que sea presentado ante la Comisión de Doctorado.

Y para que conste a los efectos oportunos, firma el presente certificado en San Juan de Alicante, a 26 de Septiembre de 2008.

Fdo.: Juan Lerma Gómez

Director del Instituto de Neurociencias de Alicante

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CERTIFICA

Que el presente trabajo de investigación titulado **"Papel del factor de transcripción CREB en supervivencia y plasticidad de neuronas hipocampales"**, presentado por **Dragana Jancic**, para optar al grado de Doctor, ha sido realizado bajo su dirección en el Instituto de Neurosciencias de Alicante (Universidad Miguel Hernandez – Consejo Superior de Investigaciones Científicas).

Y para que conste a los efectos oportunos, se expide el presente certificado en San Juan de Alicante, a 26 de Septiembre de 2008.

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Resumen

Usando el sistema de expresión prosencefálico regulado por tetraciclina (sistema tetO/CaMKII-tTA) para producir ratones transgénicos dobles, hemos investigado los efectos en supervivencia y plasticidad neural de un aumento o redución de la expresión génica dependiente de CREB. Los ratones que expresan una forma constitutivamente activa de CREB (VP16-CREB) son más propensos a la activación propagada (kindling) y a desarrollar ataques epilépticos espontáneos. Por el contrario, ratones con expresión dependiente de CREB reducida (A-CREB) son más resistentes a drogas pro-convulsivas y requerían una mayor dosis de estas drogas para inducir genes de expresión inmediatamente temprana. En concordancia con este resultado, estudios electrofisiológicos han demostrado un incremento de la excitabilidad de neuronas de la subregión hipocámpica CA1 en ratones VP16-CREB, mientras la excitabilidad de estas neuronas en ratones A-CREB está reducida. Interesantemente, ambas líneas de ratones muestran tras la activación prolongada del transgen una pérdida significativa de neuronas en el área CA1. Sin embargo, los procesos neurodegenerativos disparados en cada caso difieren en el mecanismo y en el curso temporal de la muerte celular. Nuestros estudios revelan que tanto un aumento como una reducción de la transcripción dependiente de CREB pueden resultar en la pérdida de neuronas en la región CA1, aunque dicha manipulación tenga effectos opuestos en excitabilidad neuronal. Lo cual sugiere que tan sólo una precisa regulación de la expresión génica dependiente de CREB puede sustentar la supervivencia de neuronas piramidales de CA1.

Summary

Using the forebrain-specific tetracycline-regulated gene expression system (tetO/CaMKII-tTA system) to produce double-transgenic mice, we compared the effects of enhanced versus reduced CREB-dependent gene expression in the survival and plasticity of CA1 pyramidal neurons. Mice expressing a constitutively active form of CREB (VP16-CREB) are more prone to kindling and spontaneously develop epileptic attacks. On the contrary, mice with reduced CREB-dependent gene expression (A-CREB) were more resistant to proconvulsive drugs and needed higher doses of these drugs to induce immediately early gene expression. In agreement with this result, electrophysiological studies showed increased excitability of VP16-CREB neurons of the CA1 hippocampal subfield, whereas the excitability of these neurons was decreased in A-CREB mice. Interestingly, both strains show significant loss of pyramidal neurons in the CA1 area when transgene expression was turn on for a prolonged period of time. However, the neurodegenerative process triggered by each of these CREB variants differed in the mechanism and the time course of cell death. Our study describes how enhanced versus reduced CREB-driven transcription lead to opposite levels of neuronal excitability, but both can eventually result in CA1 neuronal loss. This suggests that CREB–dependent gene expression can regulate the responsiveness of the hippocampal circuit to external stimuli. Moreover, our results indicate that only fine-tuned regulation of CREB-dependent gene expression can sustain the survival of CA1 pyramidal neurons.

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Abbreviations

AC = adenylyl cyclases

AC(95) = A-CREB (line 95)

AHP = after-hyperpolarization

Arc = activity-regulated cytoskeleton-associated protein

ATF1 = activating transcription factor 1

BCIP = 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt)

Bcl-2 = B-cell CLL/lymphoma 2

BDNF = brain-derived neurotrophic factor

bZIP = basic leucine zipper domain

CaM = calmodulin

CaMKII = Ca2+/calmodulin dependent protein kinase type II

CaMKIV = Ca2+/calmodulin dependent protein kinase type IV

CBP = CREB-binding protein

CRE = cAMP-responsive-element

CREB = cAMP-responsive-element-binding protein

CREM = cAMP responsive element modulator

Cx = cerebral cortex

DAB = 3,3´-diaminobenzidine

DAPI = 4',6-diamidino-2-phenylindole

DG = dentate gyrus

Dox = Doxycycline

E+M (or EPM) = Elevated Plus Maze

Egr1 = Early growth factor 1

Egr2= Early growth factor 2

E-LTP = early phase of long-term potentiation

ERK = extracellular signal-regulated protein kinase

FC = Fear Conditioning

G = Gauge

GAPDH = glyceraldehyde 3-phosphate dehydrogenase

GPCRs = G protein-coupled receptors

HATs = histone acetyltransferases

HC = hippocampus

HD = Huntington´s Disease

HDACs = histone deacetylases

IEGs = immediately early response genes

KA = kainic acid

KID = central kinase-inducible domain

L-LTP = late phase of long-term potentiation

LTM = long-term memory

LTP = long-term potentiation

MAP-2 = microtubule-associated protein 2

MAPK = Ras/mitogen-activated protein kinase

MWM = Morris Watermaze

NA = nucleus accumbebs

NBF = neutral buffered formalin

NBT = nitro-Blue Tetrazolium Chloride

NCS = normal calf serum

NGF = nerve growth factor

NMDA = N-methyl-D-aspartic acid

NMDAR = N-methyl-d-aspartate glutamate receptor

o/n = overnight

OF = Open Field

PBS = phosphate buffer solution

PCR = polymerase chain reaction

PFA = paraformaldehyde

PKA = protein kinase A

PTZ = pentylenetetrazole

qPCR = quantitative PCR

Scn4b = sodium channel, voltage-gated, type IV, subunit β4

SEM = standard error mean

Str = striatum

TetO = tetracycline operator

TGF-β1 = Transforming growth factor-type beta1

Trat1 = T-cell receptor associated transmembrane adaptor 1

tTA = tetracycline-controlled transcriptional activator

TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick and labelling

VC19 = VP16-CREB^{low} line

VC27 = VP16-CREB^{high} line

VSCC = voltage-sensitive calcium channels

To my mamichen and the memory of my dad...

A. Introduction

A1. Molecular and cellular bases of memory

Memory refers to the storage, retention and recall of information, which may include past experiences, skills, knowledge or thoughts. According to the type of information stored, memory can be subdivided into two main forms. Whereas memory as facts and events is referred as explicit or declarative memory, skills, such as motor abilities or simple associations of cues with reward or punishment, are referred as implicit. Both explicit and implicit memories share common phases for their formation. The perception of a new experience triggers a phenomenon of acquisition. After that, the experience is stored for few minutes in the form of short-term memory (STM). The storage as STM is temporary and dependent on attention; the stored information may become unavailable in a few seconds after an eventual distraction (Brown, 1958, Peterson, 1959). Under appropriate conditions, short-term memory may capacitate the storage of the information in the form of long-term memory (LTM), which lasts for hours, days or longer. It is assumed that this storage in declarative type of LTM happens primarily through associative mechanisms, i.e. relations amongst the different items and other attributes of the current context need to be created during the process.

Most types of long-term declarative memory appear to be stored in the cerebral cortex, although different areas of the cortex are specialized in different kinds of information. Therefore, damage to specific areas of cortex can produce specific memory deficits (Kandel, 2000). There are other brain structures involved in memory acquisition and retrieval. For example, formation of new declarative memories mostly depends on the hippocampus and related structures in the medial temporal lobe of telencephalon. The amygdala is as well an important structure, since it is critically involved in emotional memory.

A1.1 The hippocampus as anatomical substrate for declarative memory storage

H.M., also known as "Henry M." was born in 1926 in Connecticut, U.S.A. Since he was 10 years old, the patient H.M. suffered from increasing epileptic seizures, which eventually became so intense and frequent that by the age of 27, he underwent a bilateral medial temporal lobectomy in order to eliminate the epileptic focus responsible for the seizures. During the operation, two-thirds of his hippocampal formation, parahippocampal gyrus and amygdala were removed. As a consequence of the resection, the patient suffered from severe anterograde amnesia due to impairment in the formation of long-term memory. Since the publication of his case (Scoville and Milner, 1957), the inability of H.M. for correct memory acquisition has been under a detailed study, revolutionizing the knowledge of the organization of human memory and, in particular, the neural structures involved. Numerous other studies of patients with lesions of their medial temporal lobe structure confirmed the central role of the hippocampus in memory.

Anatomically, the human hippocampus is a curve shaped structure located in the medial temporal lobe. It can be grossly divided in two regions, the dentate gyrus (DG) and the Cornu Ammonis (CA), which is itself subdivided in humans into four areas (CA1 to CA4). Whereas the dentate gyrus contains mostly small granule cells, the CA areas are densely packed with neurons resembling those of the cortex, the pyramidal cells. Interestingly, the shape of the hippocampus of other mammals, such as rodents, is banana-like and only the CA1 and CA3 Cornu Ammonis subfields can be easily identified.

The connections among cells of the different hippocampal regions associate themselves to form a circuit. Initially, the most external input is established between the adjacent entorhinal cortex, and DG and CA3, via the axons of the perforant path. Additionally, the granulle cells of the DG send their axons to CA3, forming the so-called mossy fibers. The pyramidal neurons from CA3 in turn send connections to CA1 via the Schaffer collateral pathway. Finally, connections from CA1 are sent, directly or via subiculum, back to entorhinal cortex, closing the loop.

There are numerous evidences that hippocampus is essential for rapid storage of new information. In order to resolve the molecular and cellular basis of the memory formation and the role of different anatomical structures, experiments in animal models

are indispensable. Studies in rats showed that blocking hippocampal activity impairs one-trial learning (Morris, 1996, Izquierdo et al, 1997). Furthermore, hippocampal lesions in rats made immediately after learning led to a striking deficit in contextual fear conditioning (Kim, 1992). Similar results were obtained by pharmacologically blocking of hippocampal plasticity (Jerusalinsky et al, 1992, Izquierdo et al, 1997). Thus, hippocampus is able to reorganize its pattern of activity rapidly in response to new information (Wirth et al, 2000, Frank et al, 2006).

A1.2 Synaptic and intrinsic plasticity as cellular substrates for memory

Santiago Ramon y Cajal was first to postulate that the nervous system is made up of billions of separate neurons which receive information at their cell bodies and dendrites, and transmit it unidirectionally towards other sites via their axons. In 1894, he proposed that memories are formed by strengthening the connections between existing neurons to improve the effectiveness of their communication. About half a century was needed that Donald O. Hebb writes: “When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased”. This so-called Hebb's Law was first to explain some types of associative learning in which simultaneous activation of cells leads to pronounced increases in synaptic strength.

Since Bliss and Lømo discovered long-term potentiation (LTP) in the rabbit hippocampus in 1973, a causal link between LTP and behavioral learning is under extensive study until present days. Long-term potentiation (LTP) is a long-lasting increase in synaptic efficacy that follows high-frequency stimulation of afferent fibers. It shares many features with long-term memory (LTM). LTP and long-term memory are both rapidly induced, each depends upon the synthesis of new proteins, each has properties of associativity, and each can potentially last for a long time. Only recently it was shown that learning actually induces LTP in CA1 hippocampal subfield using one-trial inhibitory avoidance learning in rats (Whitlock et al, 2006), but the link between LTP and LTM is still not well understood.

Previously described features in hippocampal neurons involve changes at the synapses between two neurons rather than changes in the electrical properties within a single neuron. In recent years, the persistent modifications of a neuron’s intrinsic

electrical properties by neuronal or synaptic activity were suggested to be an important regulator of synaptic plasticity underlying learning and memory (Zhang and Linden, 2003). A number of experiments were performed in invertebrates and mammals showing that intrinsic excitability in their neurons was increased after associative learning (Daoudal and Debanne, 2003, Saar et al, 19978, Oh et al, 2003).

A1.3. Gene expression and the consolidation of memory process

It was known for a long time that protein synthesis is essential for the formation of long-term memory (Flexner, 1963, Barondes, 1975, Davis, 1984). A genetic screen on fruit-fly *Drosophila* using simple behavior tasks resulted in finding the so-called *dunce* mutant with exhibited specific learning impairment (Dudai, 1976). Subsequent studies revealed three more memory mutants, *rutagaba*, *amnesiac* and *linotte*. These four mutants resulted to be associated to cAMP signaling (Dubnau, 1998). Therefore, in the following years, the cAMP pathway became the focus of many studies in different animal models.

Particularly interesting were the experiments performed in a marine snail *Aplysia*. A brief noxious stimulus to the *Aplysia* tail results in withdrawing of animal's gill and siphon. Testing a memory for this reflex revealed that a single stimulus gave rise to a short-term memory of the event that lasted minutes; spaced training led to long-term memory that lasted for days; whereas mass training produced the long-term memory preserved for weeks (Castelucci, 1970). Interestingly, when the stimulus was applied to the animal, a specific biochemical cascade was activated resulting in elevated level of cAMP, and this was followed by a temporary increase in the sensory-motor neuron connections and by an enhancement of serotonin release. Repetition of the stimulus prolonged the duration of cAMP increase. In addition, during this process, different enzymes were recruited, such as PKA (protein-kinase A) or MAPK (mitogen-activated protein kinase). Based on this evidence, different studies aimed to relating specific animal response to external stimulation with the expression of particular genes were performed. Extensive research in *Aplysia* identified the cAMP-responsive element binding protein (CREB) as one of the key factors in the conversion of short-term to long-term memory (Dash et al, 1990, Bailey et al, 1994). In parallel studies in *Drosophila*, it was demonstrated that blocking normal CREB activity affected acquisition of long-term memory (Yin et al, 1994).

Most of these findings were supported by *in vitro* investigations of synaptic plasticity phenomena, mainly long-term facilitation (LTF). Electrophysiological data in *Aplysia* were one of the first to demonstrate that memory storage is represented at the level of individual neurons by changes in the strength and structure of synaptic connections (Bailey et al, 1988). Moreover, it was shown that short-term cellular changes differ from long-term cellular changes. Short-term changes involve only modification of pre existing proteins, and transcription or translation inhibitors do not block them; whereas long-term changes are associated with the growth of new synaptic connections, activated by altered gene expression and can be blocked by inhibitors of transcription or translation. In mid-90s, Kandel and colleagues showed that blocking the expression of CREB activator CREB-1a, LTF was selectively impaired (Bartsch et al, 1997). By blocking CREB repressors, CREB-2 or CREB-1b, LTF was enhanced and the acquisition of long lasting changes facilitated.

Long-term facilitation in *Aplysia* is similar to long-term potentiation (LTP) in mammalian hippocampus. LTP is commonly associated with memory storage, especially in the Hebbian description of memory formation, since memory is likely to involve a strengthening of synaptic activity. A number of studies have found evidence that LTP-like increases in synaptic potency occur in the hippocampus during learning. LTP is generally divided into short-term potentiation, early LTP (E-LTP) and late LTP (L-LTP). Analogously to long-term memory, L-LTP requires gene transcription and protein synthesis in the postsynaptic cell.

The work in mammals also related changes in LTP, memory storage and the cAMP signaling pathway (Silva et al, 1992, Bliss et al, 1993). Inhibition of specific kinases or transcription factors involved in the cAMP pathway resulted in impairments of L-LTP whereas E-LTP remained intact (Abel et al, 1997, Bourtschuladze et al, 1994). Evidence from mice and rats confirmed the findings in invertebrates, suggesting that the cAMP pathway and, especially, its key protein CREB are required for the cellular events underlying long-term, but not short-term memory (Bourtchuladze et al, 1994). Understanding how CREB participates in the control of memory formation remains to be one of the exciting chapters in modern neuroscience.

A2. CREB signaling cascade

A2.1. CREB is a transcriptional factor belonging to the basic leucine-zipper family

cAMP-responsive-element-binding protein, commonly abbreviated as CREB, is an ubiquitously expressed transcription factor. In general, transcription factors are proteins that regulate gene expression by binding to promoter elements upstream of genes, producing either facilitation or inhibition of their transcription. They are composed of two functional regions: at least one activator or repressor domains and a DNA-binding domain. The DNA-binding domain recognizes specific DNA sequences near the start of transcription. Based on the type of the domain, there are different classes of transcription factors, such as zinc fingers, helix-turn-helix, leucine zipper, helix-loop-helix and others.

CREB and other proteins that share high structural homology with it belong to a subfamily of basic leucine-zipper transcription factors. The leucine zipper (bZIP) is a highly conserved basic DNA-binding domain, which was found in many DNA regulatory proteins. It contains a common pattern of leucines every seven amino acids, which forms the hydrophobic core of a coiled coil and mediates sequence specific DNA binding by facilitating the dimerization of two DNA binding regions.

The transcriptional activation of CREB is mediated through the glutamine-rich domains named Q1 and Q2 that flank the central kinase-inducible domain (KID). The phosphorylation of the KID by various kinases enables the binding of the transcriptional co-activators, such as CBP/p300. Adjacent Q1 and Q2 domains are important in stabilizing binding to DNA and recruiting other proteins that can influence transcriptional activity. The KID and the bZIP domains show a remarkable degree of conservation through evolution (figure 1).

The main feature of the CREB family of leucine-zipper transcription factors is their ability to bind the cis regulatory element cAMP-responsive-element (CRE) and so regulate the transcription of downstream genes. CRE sites are found in at least one copy in the promoter of many genes. The prototypical target sequence for CREB is the palindromic CRE (5'-TGACGTCA-3'), first identified in the neuropeptide somatostatin gene (Montminy, 1986). This sequence has been identified in hundreds of cellular and

viral genes. Its last five bases are highly conserved. The affinity by which CREB binds as a dimer to the CRE is in the range of ~1-2 nM (Richards et al, 1996).

Figure 1: Amino-acid sequence structure of CREB protein. Grey shaded regions show identities or conservative changes between invertebrate and vertebrate sequences. Boxes contain highly conserved regions that contain the target phosphorylation sites (KID), the DNA-binding region (basic region) and the dimerization domain (leucine zipper). The picture is taken from Galliot et al, 1995.

The CREB family of transcriptional factors family includes in addition to CREB, the cAMP responsive element modulator (CREM) and activating transcription factor 1 (ATF1) (figure 2). These proteins may form selective heterodimers with each other, but also with other bZIP transcription factors. CREB and ATF1 are ubiquitously expressed, whereas CREM is mostly expressed in the neuroendocrine system and in testis (Delmas et al, 1994, Monaco et al, 1995, Struthers et al, 1991). *Creb* and *crem*, but not *atf-1* gene encodes many different isoforms, some of which have repressive functions. These products of alternative splicing increase the variety of possible heterodimers and subsequently the complexity of transcriptional regulation of downstream genes (Casadio et al, 1999; Mayr and Montminy, 2001). CRE binding proteins includes other factors, such as some other ATFs, and members of the CAAT/enhancer-binding protein gene family, which cannot heterodimerize with CREB

(Yun et al, 1990).

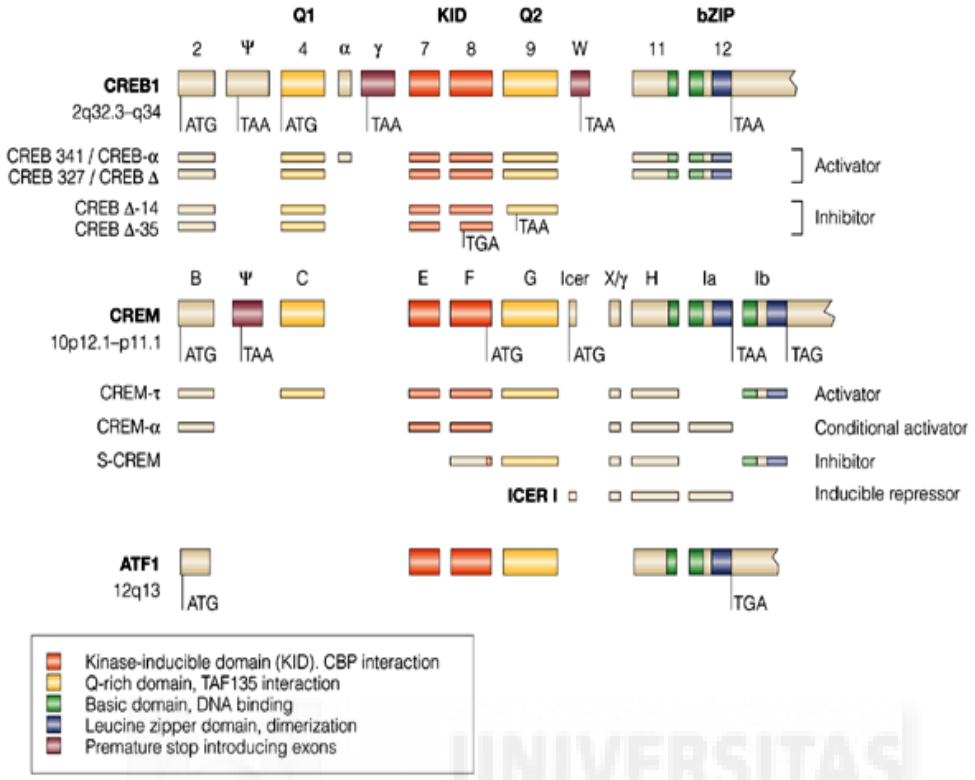

Figure 2: Structure and homology between members of CREB protein family (abbreviations equal as in the text; taken from Mayr, 2001)

A2.2. CREB is activated by phosphorylation

Some studies showed that CREB can bind to CRE sites even in an unphosphorylated inactive state, but its phosphorylation is required in order to promote transcription of downstream genes (Cha-Molstad et al, 2004). Several different protein cascades and many co-activators and modulators of CREB activity (figure 3) are involved in the phosphorylation of CREB. Additionally, CREB phosphorylation enables the recruitment of CREB-binding protein (CBP), which is required in order to bring the basal transcriptional machinery, such as the RNA polymerase II complex and the TATA-box-binding protein, to the promoter. Furthermore, CBP and its homologue p300 are histone acetyltransferases (HAT) that can transfer acetyl groups onto the lysines residues in the N-termini of histones. This acetylation of histones favors transcription.

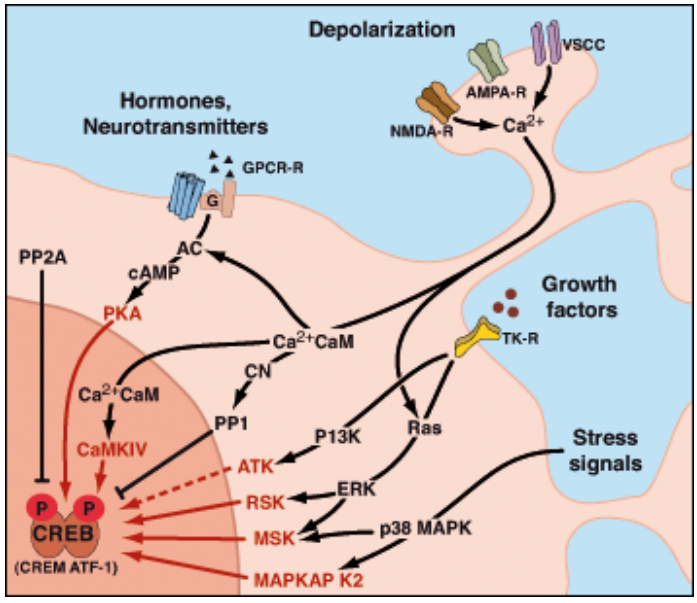

Figure 3: Overview of the CREB signaling pathway. CREB can be activated by various external stimuli that all converge on the phosphorylation at Ser133 (from Barco, Jancic and Kandel, 2007).

A2.3. Various stimuli activate CREB

Phosphorylation of serine 133 (Ser133) at the KID domain of CREB is the most important step for CREB activation. This modification also provides stabilization of the subsequent interaction with the KIX domain of CBP. However, serine residues at different sites may be also activated. For example, phosphorylation of Ser142 is related to the control of the circadian rhythm (Johannessen et al, 2004), whereas Ser143 is specifically activated by casein kinase II (Kornhauser et al, 2002). In addition, the function of CREB as transcription factor is also affected by other covalent post-translational modifications of CREB (whose sites are indicated at the figure 4).

Up to date, more than 300 stimuli have been described as capable to promote the activation of CREB-dependent transcription *in vivo*. Protein kinase A (PKA) was the first protein kinase shown to target Ser133 of CREB (Gonzalez et al, 1989). PKA is an enzyme regulated by intracellular levels of cAMP and it can phosphorylate the major isoforms of CREB, CREM and ATF1.

The most studied signaling cascade activating CREB is initiated by activation of G protein-coupled receptors (GPCRs). Activated GPCRs provoke the increase in intracellular Ca^{2+} that in turn increase cAMP and stimulate the activity of adenylyl cyclases (AC). Adenylyl cyclases convert ATP into cAMP. The increase in cAMP level

leads to CREB phosphorylation through PKA activation (Johannessen, 2004).

Phosphorylation of CREB can be also triggered by the local increment of Ca^{2+} produced by neuronal activity, through, for example, NMDA receptor mediated glutamatergic synaptic transmission (Deisseroth et al, 1996) or Ca^{2+} influx through voltage-sensitive calcium channels (VSCC).

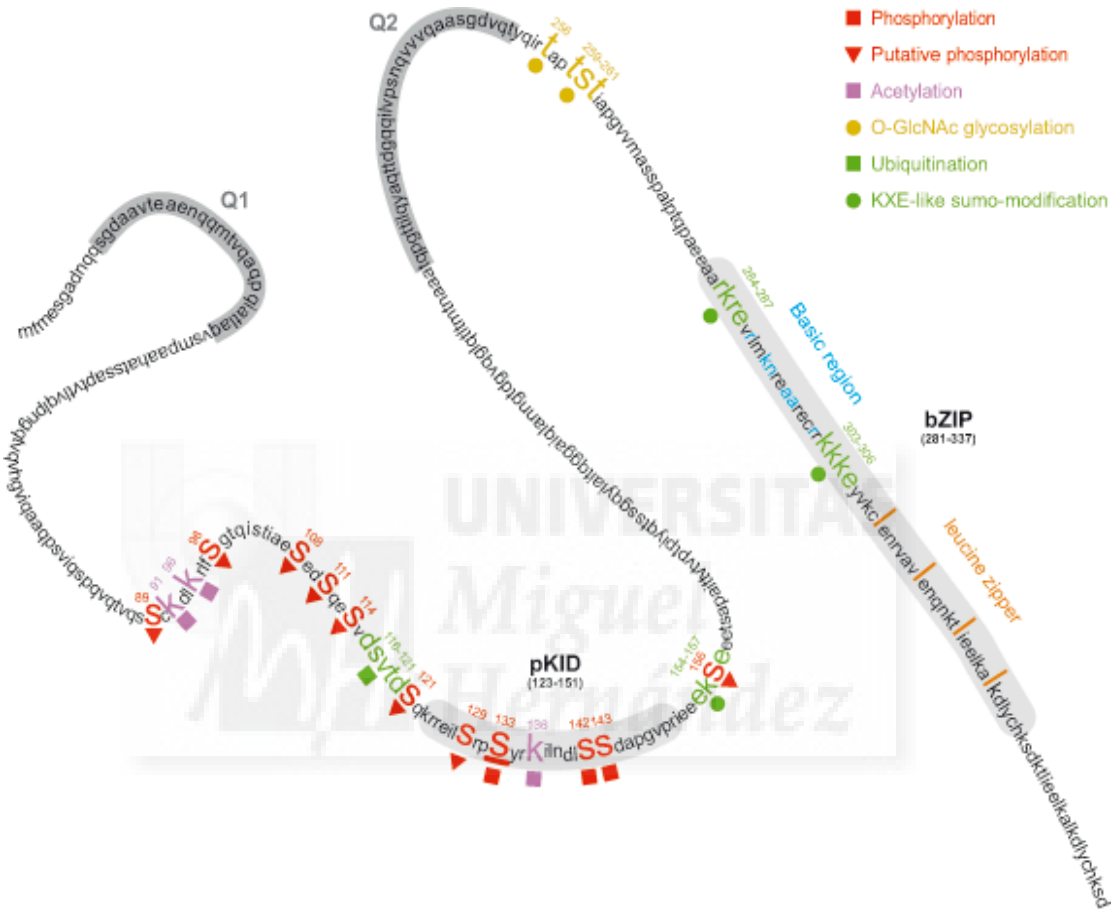

Figure 4: CREB structure and its residues (from Barco, Jancic et Kandel, 2008). Red square stands for phosphorylation site, red triangle putative phosphorylation, pink square acetylation, yellow circle glycosilation, green square ubiquitination and green circle sumo-modification. Underlayed "S" stands for classical Ser133 phosphosite.

Some other important mediators of CREB activation are: calmodulin (CaM), calcium-calmodulin dependent protein kinase IV (CaMKIV), I (CaMKI) and II (CaMKII) (Dash et al, 1991; Deisseroth et al, 1996; Sheng et al, 1991). CaMKIV is the most important CaMK and causes the phosphorylation of CREB by synaptic stimulation *in vivo* (Ho, 2000). Calmodulin plays a critical role on both the activation and inactivation of the CREB pathway, first through its modulation of CaMKs, and second

through interaction with the phosphatase calcineurin (Deisseroth, 1996).

Nuclear kinases can also activate CREB (Hardingham et al, 2001). Furthermore, the Ras/mitogen-activated protein kinase (MAPK), the extracellular signal-regulated protein kinase (ERK) (Thomas and Huganir, 2004) and the PI3-kinase/Akt pathway (Lin et al, 2001; Perkinton et al, 2002) are also involved in CREB phosphorylation and subsequent activation. The number of protein kinases found to phosphorylate CREB *in vitro* is even larger, but the relevance of some of these activities in neuronal function is not clear (Johannessen et al, 2004; Lonze and Ginty, 2002).

The phosphorylation of CREB is also regulated by protein phosphatases. Either they act directly on CREB, or they may control the enzymatic activity of CREB kinases that in turn activate CREB.

The impressive variety of the stimuli that are able to phosphorylate CREB highlights the importance of precise and specific regulation of CREB activity. Different signaling cascades are recruited depending on the cellular context, timing or type of extra cellular stimulus.

A2.4. CREB transcriptome

The genetic program regulated by CREB activity differs among different cells and tissues, and depends on the external stimuli that lead to CREB phosphorylation and subsequent binding of CREB to CRE motif in DNA. Different proteins may bind to CRE and change the availability of these sites. In addition, epigenetic modifications may influence the interaction of CREB with CRE sites. CREB-binding protein (CBP) and p300 function as histone acetyltransferases (HATs). Acetylation and methylation of histones determine open/close state of chromatin and influence the availability of CRE sites.

The list of potential CREB targets includes several hundred genes with very different functions, like transcriptional/nuclear factors, growth factors, signaling molecules, neuronal genes, metabolic factors, opioid receptors, neurotransmitter receptor subunits, molecules important for transport, structural proteins and factors of immune response (Barco et al, 2005; Impey et al, 2004; Lonze and Ginty, 2002; Zhang et al, 2005). The control of the expression of subsets of target genes in a given context or time point is still not well described. Events downstream of CREB activation can also promote transcription and complicate the identification of CREB´s direct targets.

A2.5. CREB and the transcriptional control of immediately early response genes

External stimulation of neurons rapidly induces the expression of immediate-early genes (IEGs) (figure 5). Most of these primary response genes are maximally induced within 30 minutes of stimulation. In fact, these genes show transient transcription even in the absence of *de novo* protein synthesis (Kaczmarek et al, 1997, Pinaud et al, 2004). It is thought that CREB is one of the factors involved in their expression, because many IEGs contain one of more copies of CRE sequence in their promoter.

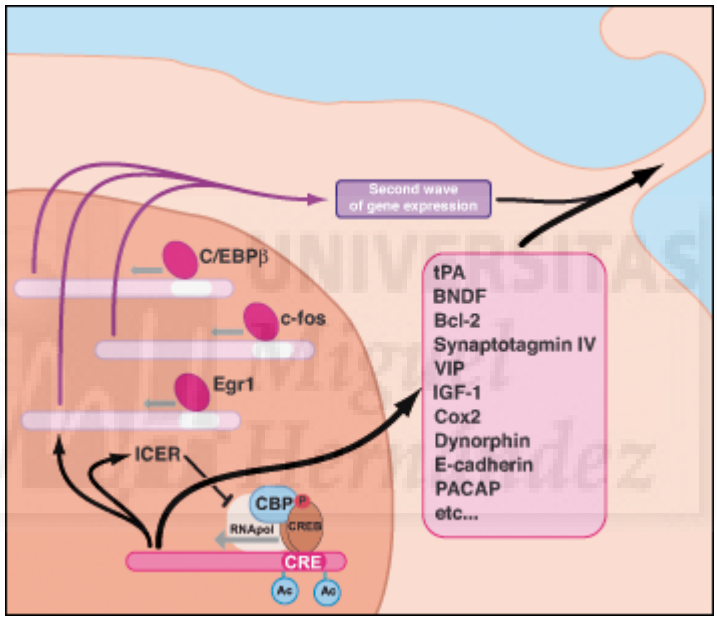

Figure 5 CREB-dependent gene expression is involved in regulation of synaptic function- CREB downstream targets either serve as promoters of short-term synaptic processes or are transcription factors which induce second wave of gene expression (from Barco, Jancic and Kandel, 2008)

Approximately 100 genes are known to belong to the class of immediately early response genes (IEGs). Some of the best described IEGs are c-*fos, c-myc, c-jun, arc, zif268* (*egr1*) etc. For example, c-fos, which is also leucine-zipper transcription factor, has three copies of CRE sites in its promoter and is regulated by CREB (Hipskind et al, 1991). Moreover, mice lacking c-fos have impairment in long-term memory (Fleischmann et al, 2003). Similarly, zif268 is required for expression of late-LTP and contains one CRE site (Jones et al, 2001).

A few examples of early response genes that are induced with slower kinetics

have been described, such as BDNF. BDNF stands for brain-derived neurotrophic factor and it is an important promoter of neuronal survival and differentiation. BDNF is a direct target of CREB and critical for maintenance of LTP (Barco et al, 2005). Many other IEGs are also well-known regulators of cell growth and differentiation and are involved in regulation of synaptic plasticity. Furthermore, many immediately early genes encode transcription factors, which in turn regulate a second wave of response genes as a part of a larger transcriptional program.

According to the stimuli that trigger their induction, their expression kinetics and the information provided by knock-out mice models, it is likely that IEGs play a critical functional role in neuronal plasticity.

A3. Mouse genetics approaches to elucidate CREB roles in hippocampal physiology

Since CREB is one of the most cited proteins in biomedicine, it is not surprising that a number of genetically modified mouse strains were generated with the aim of studying the consequences of altered CREB activity on different cellular processes. Here, I will focus only in the description of those strains used for exploring the role of CREB in the central nervous system, predominantly in the forebrain.

A3.1. Gene targeting approaches

In order to study the role of CREB in the central nervous system of mammals, it is very useful to generate genetically modified mice in which CREB activity is altered. By monitoring animal's phenotype, it is possible to examine the relevance of the protein's function in various processes. However, evaluating the consequences of depleting CREB in the murine brain is not simple, since CREB serves as a transcription factor in a plethora of cellular events and it is not always possible to attribute the observed phenotype to a particular function. Another difficulty is the functional mutual compensation among CREB family members. Thus, it was needed the development of gene targeting approaches for investigating the consequences of the lack of one or more of these proteins in whole organism or in specific tissues (Lonze and Ginty, 2002), as shown in the tables 1, 2 and 3.

In 1994, Hummler and colleagues inserted a neomicine resistance cassette into CREB exon 2. This site was chosen since that is location of the translation initiation site for isoforms α and δ (at that time, the researchers were not aware of other CREB isoforms). This manipulation produced a hypomorphic mutant instead of a null mutant. Detailed analyses of these mutants showed a significant upregulation of CREM and CREB isoform β (Blendy et al, 1996). On the other hand, the level of ATF-1 was not elevated, possibly due to its lower expression in neural tissues.

CREB is expressed during development in all tissues. Therefore, it is not surprising that when a real null mutants for CREB ($CREB^{-/-}$) was generated, it suffered from perinatal lethality due to atelectasis of the lung and exhibited a defect in T cell development (Rudolph et al, 1998). The severe defects during embryonic development in null mutants forced the generation of mice with restricted deletion of CREB in specific cell types. Crossing $Creb^{\alpha\delta}$ mice with $Creb^{+/-}$ mice results in mice that carry one copy of β isoform (so-called $CREB^{comp}$ mice or $CREB^{\alpha\delta/-}$) (Gass et al, 1998).

Table 1: Knock-out approaches on the CREB family members

Name of the strain	The genetical modification	First report in the scientific journals	Phenotype
$CREM^{-/-}$	CREM null mutation	Blendy et al, 1996	Mild behavioral phenotype and impairments outside the CNS
$ATF^{-/-}$	ATF null mutation	Bleckmann et al, 2002	No changes in the CNS
$CREB^{-/-}$	CREB null mutation	Rudolph et al, 1998	Perinatal death

One of the most used gene targeting technique is the Cre/loxP recombination system. This site-specific DNA recombination system is a valuable tool in addressing a variety of biomedical problems and is widely used for generating animals models. Conditional knock-outs of CREB were generated by Cre/loxP technique with Cre recombinase under different promoters with the aim of abolishing its expression selectively in forebrain neurons. $Creb^{loxP/loxP}$ mice were generated by flanking *Creb* exon

10 with loxP sites (Mantamadiotis et al, 2002). These *Creb*$^{loxP/loxP}$ mice were crossed with mutants expressing Cre-recombinase under the control of the CaMKIIα promoter (CREB^{CaMKCre7}). This promoter drives the recombination between loxP sites during the last days of embryonic development and the first postanatal days, specifically in postmitotic neurons. In order to obtain mice with the deletion of CREB in neurons of the entire brain, *Creb*$^{loxP/loxP}$ mice were crossed with mouse strain containing Cre recombinase under the nestin promoter (CREB^{NesCre}). As we will discuss in detail later, these modifications resulted in surprisingly mild phenotype (Balschun et al, 2003). The authors proposed that a possible reason was again compensation by CREM or CREB isoforms. Therefore, the researchers tried to target simultaneously different CREB family members and verify that the upregulation of CREM was the main reason underlying these mild neurological alterations in mutant mice.

ATF1 and CREB, but not CREM, are strongly co-expressed during early mouse development. Complete inactivation of both proteins, ATF1 and CREB, resulted in developmental arrest and embryonic death before implantation (Bleckmann et al, 2002), when CREM is still not expressed. The finding of higher CREM mRNA and protein level in CREB deficient mutants encouraged the generation of double knockouts for CREB and CREM. The repression of both of these genes in the forebrain resulted in progressive neurodegeneration (Mantamadiotis et al, 2002), that demonstrated the importance of CREB in the survival of forebrain neurons. However, neither electrophysiological nor behavioral analyses of synaptic plasticity were performed on these animals. Interestingly, conversely to the neurons of the CNS, the survival of PNS neurons is compromised even if only CREB is removed (Lonze and Ginty, 2002).

Table 2: Different knock-out approaches for manipulation of the CREB expression in the CNS

Name of the strain	The genetical modification	First report in the scientific journals	The most striking phenotype
CREB $^{\alpha\delta-/\alpha\delta-}$	Hypomorphic mutation	Bourtchuladze et all, 1994	Upregulation of CREB and CREM; Controversy about results on higher cognitive functions and synaptic plasticity
CREB $^{\alpha\delta-/-}$	Hypomorphic/null mutation	Gass et al, 1998	Behavioral defects, but normal hippocampal LTP
CREB^{CaMKCre7}	Postnatal forebrain-restricted knock-out	Balschun et al, 2003	Upregulation of CREM Normal synaptic plasticity
CREB^{NesCre}	CNS-restricted knock-out	Balschun et al, 2003	Dwarf phenotype Similar results as CREB^{CaMKCre7}
CREB^{CaMKCre7}/CREM$^{-/-}$	Postnatal forebrain-restricted knock-out/CREM null mutation	Mantamadiotis et al, 2002	Dramatic cell loss in the dorsal striatum and hippocampus
CREB^{NesCre}/ CREM$^{-/-}$	CNS-restricted knock-out/CREM null mutation	Mantamadiotis et al, 2002	Perinatal death
CREB $^{-/-}$/ATF$^{-/-}$	CREB null mutation/ ATF homozygote null mutation	Bleckmann et al, 2002	Embryonic death
CREB $^{-/-}$/ATF$^{+/-}$	CREB null mutation/ ATF heterozygote null mutation	Bleckmann et al, 2002	Embryonic death

A3.2 Transgenic approaches

Genetic modifications of mice by knocking down a gene may lead to unwanted physiological or toxic effects. Inducible systems were developed to control expression of the gene of interest with precise temporal and spatial specificity. In the ideal controlled system, researchers might rapidly turn on and off the expression of the

transgene, exclusively in the cells or tissues of interest at any time point during animal pre- or postnatal development.

In order to regulate expression of CREB and CREB activation in neural tissues, several transgenic mouse models were created. Mice expressing a CREB variant generated by mutagenesis of the main phosphorylation site of CREB, serine 133, into alanine, under control of CaMKIIα promoter, the so-called M-CREB mice were created by Rammes and colleagues. These conventional transgenics exhibited certain difficulties to form contextual fear memories (Rammes et al, 2000). This encouraged the use of inducible systems in order to restrict temporally and spatially the physiological alterations produced by changes in CREB activity or in its ability to occupy CRE sites and suppress endogenous CREB functions.

One of the available regulatable systems used for generation of the transgenic mice is the tamoxifen system. This system is often combined with Cre/loxP system by putting Cre recombinase under tamoxifen regulation in order to gain its precise temporal control (Brocard et al, 1997). Tamoxifen is an estrogen antagonist. The expression of a fusion protein between the nuclear protein of interest and the tamoxifen-binding domain of the estrogen receptor allows rapid activation of the fusion protein by nuclear translocation. Importantly, this activation does not depend on transcription. This makes this system especially adequate in studying temporal requirements of transcription factors. In the study by Kida (Kida et al, 2000) $CREB^{S133A}$, the variant used for generation of M-CREB mice, was fused with a ligand-binding domain of a human estrogen receptor with a point-mutation and expressed under the CaMKIIα promoter. After adding tamoxifen to the mouse diet, inducible CREB repressor ($CREB^{IR}$) was activated. This repressor competes with endogenous CREB and therefore lowers the CREB-driven gene expression exclusively in the murine forebrain.

The best-described inducible and easily reversible systems is tetracycline-controlled transcriptional activator (tTA) system (Gossen and Bujard, 1992, Mills et al, 2001). There are two elements in this system: so-called tetO and tTA. TetO stands for the minimal operator sequence of the bacterial resistance tetracycline system and it serves as a docking site for the tetracycline regulated transactivator tTA (Bujard, 1999). The tTA protein is introduced in the mouse genome under the control of a chosen promoter. If this promoter is CaMKIIα, the expression of the tTA protein takes place in the murine forebrain including the hippocampus, cortex, amygdala and striatum (Mayford et al, 1996). The binding of tTA to its operator sequence, tetO, can be

prevented by tetracycline or its analog Doxycycline (Dox). When provided in the food or drinking water, doxycycline efficiently binds to tTA, preventing binding to tetO and shutting off all transcriptional activity at the tetO-donor operon. This system is usually named *Tet-off*. Conversely, the *Tet-on* system is based on a protein variant originally produced by random mutagenesis of *E. coli* TetR (rtTA). rtTA binds to *tetO* sequences only in the presence of tetracycline, but, unfortunately, this system is not as useful as tTA system for rapid and efficient induction of the transgene in the brain of adult mice. The efficiency of doxycycline administration to the neural tissue is low because of the low permeability of the blood-brain barrier to this drug. Moreover, complete shut-off of tetO during development frequently causes its irreversible inactivation, an event that is avoided or ameliorated when the tTA approach is applied (Zhu et al, 2007).

Table 3: Different transgenetic approaches for manipulation of CREB-dependent gene expression in the CNS

Name of the strain	The genetical modification	First report in the scientific journals	Phenotype
CaMKII-CREB$_{A133}$	Dominant negative transgene	Rammes et al, 2000	Mild Fear Conditioning deficits Normal LTP
CaMKII-tTA/tetO-KCREB	Inducible dominant negative transgene	Pittinger et al, 2002	Some deficits in LTP Impaired spatial learning and memory
CaMKII-CREB^{IR}	Tamoxifen inducible repressor transgene	Kida et al, 2002	Impaired FC (memory consolidation)
CaMKII-tTA/tetO-VP16CREB	Inducible constitutively active transgene	Barco et al, 2002	Lower threshold for eliciting L-LTP, behavioral deficits
NSE-tTA/tetO-CREB	Inducible overexpression of WT protein	Chen et al, 1998	Different responses to cocaine
NSE-tTA/tetO-mCREB	Inducible dominant negative transgene	Chen et al, 1998	Reduced neurogenesis
PcP2-CREB	Overexpression of WT protein	Brodie et al, 2004	Impaired habituation to rotarod

Thus, usually, two single transgenic strains, one carrying the transgene of interest under the control of the Tet operator, and the second the transactivator tTA, are

generated and crossed in order to gain regulation of the desired gene through the administration of doxycycline in the mice diet or water (Ryding, 2001). Altogether, the tTA/tetO system represents an efficient and easily regulatable system. Three to five days are needed for complete induction or suppression of transgene expression. There are many advantages of this method. The specificity of the promoter driving tTA expression provides the regional distribution of the transgene of interest. Moreover, the time and duration of expression is regulated by the presence or absence of Dox in the system. There are many advantages of this system. For example, it can be used in order to answer some very specific questions, such as the induction of the desired transgene exclusively in post-synaptic structures or during exact days of embryonic development.

There are continuous improvements of this model as well. For example, *in vitro* studies pointed the dangers of silencing the transgene (Pikaart et al, 1998) and the potential toxicity of the tTA protein (Gallia et al, 1998) which may lead to loss of expression of the transfected gene construct. Therefore, regulatory systems in which the transactivator expression is also under tetracycline control were developed and so far presented positive results (Strathdee et al, 1999).

In general, this method is widely used in neuroscience and it, up to date, showed positive results. However, there are important caveats. Contrary to knockouts, the exact location of the transgene incorporation into the mouse genome cannot be entirely predicted. Whereas in the gene targeting method by knocking out a gene, incorporation of the altered gene into the mouse genome depends upon replacement of the endogenous gene by homologous recombination through both of the arms of the altered gene into one allele of genomic DNA; in the transgenic mice this locus could be anywhere in the genome. In some cases, that locus can be not optimal for the transcription of the transgene or would interfere with the expression of the gene within whose sequence it incorporated. Therefore, it is convenient to produce a larger number of founder strains to select the one with the most adequate pattern and level of expression. In addition, if the transgene is induced for the first time in the adult animal, its expression level is usually lower than in animals expressing it throughout life (Rennel et al, 2002, Zhu et al, 2007). This may be a problem especially if the activity of the promoter is not optimal or the transgene is expressed in a limited cellular population.

Despite those, the tetO/tTA inducible system has been used in a variety of different studies such as stable mammalian cell lines, transgenic mice, tissue specific

gene expression and adenoviral or retroviral regulatable gene delivery and still is a method of choice for studying the roles of certain genes *in vivo*.

In recent years, this system was widely used in order to generate transgenic mouse lines expressing CREB variants. One of them, K-CREB transgenic mice, contained a point mutation in human CREB at Lys304, which mediates an interaction with Mg^{2+} that is critical for high-affinity DNA binding (Craig et al, 2001). Since it can heterodimerize with wild-type CREB, CREM and ATF1 and prevents DNA binding (Walton et al, 2000), mice expressing K-CREB have putatively strong dominant negative effect (Pittenger et al, 2002). The induction of K-CREB transgene was regulated by the tTA/tetO system under CaMKIIα promoter. Two different mouse lines were obtained: dCA1-KCREB and str-KCREB. dCA1-KCREB expressed the transgene in the CA1 region of the hippocampus, striatum and pyriform cortex, whereas str-KCREB in striatum and pyriform cortex, but not in the hippocampus (Pittenger et al, 2002).

In addition, bitransgenic strains with the inducible expression of a constitutively active CREB were generated in order to investigate the consequences of permanently active CREB. The so-called VP16-CREB mice express a fusion protein between CREB and the transactivation domain of herpes simplex virus VP16. This CREB variant is constitutively active and there is no need of phosphorylation for its activation. Moreover, *in vitro* experiments revealed that it is about 25 times more active than a wildtype CREB (Barco et al, 2002).

A3.3 Other approaches for modifying CREB activation in rodents

In order to reduce CREB driven transcription, researchers also use wild type animals in which antisense oligonucleotides for CREB were deliberated in specific brain areas (Wahlestedt et al, 1994). This acute intervention prevented side effect of chronic alteration of CREB activity, such as compensation by other CREB isoforms or family members that may happen under prolonged alteration of CREB activity. Acute intracerebral injections of synthetic antisense of CREB mRNA showed significant reduction of CREB protein expression (Lamprecht et al, 1997, Guzowski et al, 1997, Zhang et al, 2003). Moreover, experiments using viral vectors to infuse the dominant-negative CREB variants or wildtype CREB into the murine brain changed the level of CREB and of its direct downstream targets, such as dynorphin (Barrot et al, 2002,

Carlezon et al, 1998, Marie et al, 2005). This strategy also has precise temporal and spatial control, but lack cell type specificity and it is much more invasive than transgenesis.

A4. Functions of CREB in the CNS

A4.1. CREB activity is implicated in synaptic plasticity, learning & memory

The ability of neurons to change the strength of their synaptic connections, which is known as synaptic plasticity, supports higher cognitive processes such as learning and memory. Synaptic plasticity plays a crucial role in the etiology of a variety of neuropsychiatric disorders, such addiction, depression and anxiety. Furthermore, some of these mechanisms seem to be dependent on the transcription factor CREB, as concluded from a variety of studies in animal models.

The first study that correlated CREB transcription factor and memory formation dated from 1990 (Dash et al, 1990), in which it was examined the well-described sensitization reflex in the snail Aplysia. When an external tactile stimulus is applied to the siphon of Aplysia, the animal withdraws the siphon between the parapodia. Injection of the CRE antisense sequence into the nucleus of sensory neurons of this marine snail produced the blockage of long-term facilitation, but did not affect short-term sensitization. Further studies revealed that in this invertebrate serotonin activates the transcription of CREB-dependent genes (Kaang et al, 1993). Injection of phosphorylated CREB1 into cultured Aplysia sensory neurons promoted long-term synaptic changes (Bartsch et al, 1998, Casadio et al, 1999). This finding suggested that the CREB signaling pathway is recruited after memory induction upon stimulation. (reviewed in Kandel, 2001). This view was confirmed by experiments in the fruit fly Drosophila. Using this model, Tully et al. (1991) showed that mutants for adenylate cyclase (named *Dunce* mutant) and phosphodiesterase (*Rutagaba* mutant), both involved in CRE-driven transcription, had deficits in olfactory learning and memory. Moreover, acutely disrupted CRE-mediated gene expression by transgenic overexpression of a CREB transcriptional repressor (dCREB2b) resulted in blocking of long-term memory (Yin et al, 1995). Interestingly, short-term memory remained

unchanged, analogously to the conclusions of electrophysiological studies in Aplysia (Kaang et al, 1993).

The experiments in invertebrates provided a fundamental scaffold to explain the molecular basis in memory and guided posterior experiments in mammalian systems. Seminal studies in mice confirmed the involvement of several elements of the CREB signaling pathway in synaptic plasticity and cognitive functions (Bourtchuladze et al, 1994, Abel et al, 1997, Pham et al, 1999). However, the role of CREB protein itself in these processes remains controversial. The first behavioral study on mice with reduced CREB function was done on CREB hypomorphic mutants ($CREB^{\alpha\delta-/\alpha\delta-}$) from a mixed background (129xC57BL/6). Using Morris watermaze, fear conditioning, food preference and object and social recognition tasks, it was shown a deficit in late phase of LTP and in higher cognitive functions (Bourtchuladze et al, 1994). Gass and colleagues (1998) tried to confirm some of these phenotypes in another genetic background (FVB/NxC57BL/6), and observed normal short-term memory and milder deficits in long-term fear conditioning tasks. $CREB^{comp}$ mice have only one allele of CREBβ isoform and they showed stronger phenotype than previous mouse lines with two copies of that CREB variant, leading to conclusion that all isoforms of CREB need to be downregulated in order to observe alterations in behavioural tasks. Further studies provided more evidence for the important role of CREB in memory formation in mammals. The inducible expression of dominant-negative CREB mutant ($CREB^{IR}$) in mice blocked the memory consolidation in forebrain (Kida et al, 2002), revealing for the first time that the stability of new and reactivated fear memories required CREB. Moreover, studies on the K-CREB transgenic mice, which express a CREB repressor in the dorsal hippocampus, using the Morris watermaze demonstrated reversible deficits in spatial navigation (Pittenger et al, 2002), although these mice showed normal contextual fear memory. The examination of $CREB^{NesCre}$ and $CREB^{CaMKCre7}$ mice revealed that conditioned taste aversion learning, a putatively hippocampus-independent memory test, was markedly impaired, although mutant and wildtype mice did not differ in a fear conditioning and watermaze tasks (Balschun et al, 2003).

Although it seems clear the critical importance of CREB-dependent gene expression in the regulation of synaptic plasticity and memory formation, the precise role of CREB itself is often veiled by compensation produced by other family members of CREB isoforms. Thus, the phenotypes observed in the analyses of some CREB

deficient strains have been surprisingly mild, even though the majority of experiments support a role for CREB dependent gene expression.

These contradictory results could be also a consequence of chronically impaired CREB activity in these mice. Therefore, recently it was proposed that the more straightforward results could be obtained from acute injections of viral vectors or antisense oligonucleotides into specific brain areas (Guzowski et al, 1995, Josselyn et al, 2001, 2004, Marie et al, 2005).

Complementary to loss-of-function studies, the contribution of CREB activity to the late phase of long-term potentiation (L-LTP) has been also investigated by experiments on mice expressing the constitutively active CREB (VP16-CREB mice) in gain-of-function approach. The neurons expressing VP16-CREB transgene in the hippocampus stimulated the transcription of a set of downstream target genes that are important for maintenance of late phase of LTP and memory formation. The threshold for the induction of L-LTP is significantly reduced in these animals. Applying a single 100 Hz train to the hippocampal sections is usual protocol for inducing only the early phase of LTP (E-LTP). However, in these mutants a single train evoked L-LTP. This special type of L-LTP differed from the conventional one in not being dependent on new transcription. Moreover, by analyzing electrophysiological properties of VP16-CREB animals, it was suggested that CREB-driven transcription might be sufficient for the consolidation of synaptic changes (Barco et al, 2002). How constitutively active CREB contributes to this enhanced eliciting of L-LTP and what genes are its main targets are some interesting questions still to be addressed, although genetic studies indicate that the neurotrophin BDNF plays an important role (Barco et al, 2005). Similar results were obtained using recombinant viruses (Marie et al, 2005).

A4.2. CREB activity contributes to the neuronal excitability

Neurons process and transmit information in the form of electrical signals. The presence of voltage-sensitive ion channels in the neuronal plasma membrane determines electrical excitability. An increase in a neuron's excitability increases the probability that it will be active during a learning event or in response to another external stimulus. Recent studies showed that activation of CREB can also regulate intrinsic neuronal excitability in forebrain neurons (Han et al, 2006, Dong et al, 2006, Lopez de Armentia

et al, 2007, Huang et al, 2008). Nucleus accumbens medium spiny neurons are a good model of studying neuronal excitability in the central nervous system since they cycle between a functionally inactive downstate and a functionally active upstate. It was shown that CREB increases duration of the upstate and firing of the action potential during this phase. This effect of CREB is mediated by N-methyl-d-aspartate glutamate receptor (NMDAR) function (Huang et al, 2008). Expression of active CREB in rat medium spiny neurons increased their excitability, whereas dominant-negative CREB had the opposite effect (Dong et al, 2006). Moreover, it seems that increased neuronal excitability caused by CREB helped to limit behavioral sensitivity to cocaine. CREB also plays an important role in controlling the electrical excitability of neurons in the locus coeruleus (Han et al, 2006).

Sustained increase in neural excitability may have deleterious effect on the cell survival and neural network function. Abnormal neuronal excitability can lead to abnormal synchronization of a group of neurons. This in turn may result in the development and propagation of epileptic seizures. Interestingly, one of the major CREB downstream targets, BDNF, is also linked to epilepsy. It was shown that the limbic seizures increase the level of mRNAs for BDNF and NGF in the rodent hippocampus (Gall et al, 1991).

Despite well-known contribution of the CREB in the synaptic plasticity, its role in the physiology of the hippocampal neuronal networks was still poorly described. In particular, there was not information concerning the control of neuronal excitability in the CA1 pyramidal layer by CREB activity. Exploring these questions in the hippocampus and cerebral cortex may be helpful in order to link CREB´s role at the cellular and at the system level.

A4.3. CREB promotes neuronal survival

As shown earlier, there was indirect evidence for a role of CREB in neuronal survival, mostly based on the study of neuronal tissues after brain damage. *In vitro* experiments also confirmed a role of CREB in survival of various neuronal subtypes (Bonni et al, 1999; Riccio et al, 1999; Walton and Dragunow, 2000, Papadia et al, 2005). Most of these reports demonstrated that transient expression of dominant negative CREB led to neuronal death, whereas the overexpression of CREB protected

these cells from apoptotic death. Studies *in vivo* confirmed some of these findings, but the ability of CREB family members to compensate each other again veiled the effect of disruption of the *creb* gene (Hummler et al, 1994, Blendy et al, 1996). CREB-/- mutant mice suffer massive loss of neurons in the peripheral nervous system and die shortly after birth. Enhanced apoptosis and neuronal degeneration in both central and peripheral nervous system were reported in the embryos of *creb* knockout mice. Later studies with conditional knockouts and transgenic animals demonstrated a complete requirement of CREB for survival of neurons in the peripheral nervous system. This is not the case for neurons of the central nervous system (CNS), which are in general resistant to CREB depletion (Lonze and Ginty, 2002). However, deletion of CREB in the dopaminergic system caused a partial loss of dopaminergic neurons in the cortex (Parlato et al, 2006), suggesting different susceptibility to abolition of CREB in different neuronal subtypes also in the CNS.

Massive neurodegeneration was observed in forebrain neurons of knockout mice in which both CREB and CREM were eliminated in the same cell (Mantamadiotis et al, 2002). This study demonstrated that CREM can compensate the lack of CREB protein and showed the importance of CRE-driven gene expression in the survival of neurons. CREB participates in many different cellular processes, and likely regulates an extensive set of downstream genes, some of which are involved in the regulation of neuronal survival. For example, CREB is upstream of pro-survival factors, such as BDNF (brain-derived neurotrophic factor) and the anti-apoptotic protein Bcl-2 (Wilson et al, 1996, Shieh et al, 1998). Nevertheless, studies in double CREB/CREM knockout in dopaminergic neurons, which were supposed to have reduced level of CREB-dependent prosurvival factors, revealed that lack of CREB and CREM in these neurons is not sufficient to lead to massive neuronal loss (Parlato et al, 2006). One of the possible explanations is that BDNF and similar proteins, which are easily diffusible among cells, may enter to affected neurons from healthy cells and promote their survival (Alemida et al, 2005, Parlato et al, 2006).

A4.4. Other functions of CREB

CREB is required in development of non-neural and neural cells. In the nervous system, its role in the cell proliferation, differentiation and survival of newborn cells is not negligible. Indeed, recent studies suggest that CREB plays important role in adult neurogenesis in the dentate gyrus of hippocampus (Zhu et al, 2004), although broader investigation should be done. Increase in adult neurogenesis is also linked to stress, and to response to some drugs (as reviewed in Zhao et al, 2008).

During embryonic development, CREB supports the growth of cortical dendrites and axonal projections. This effect is also beneficial later in postnatal life, especially in axonal regeneration after lesions of neural tissues. Studies *in vivo* showed that activated CREB can promote axonal growth by inducing expression of arginase I enzyme, which belongs to the CREB transcriptome (Cai et al, 2002, Gao et al, 2004). In addition, it was shown that CREB is selectively translated in axons in response to nerve growth factor (NGF) and retrogradely trafficked to the cell body. Selective abolition of axonal CREB mRNA resulted in failure of NGF to promote neuronal outgrowth (Vo et al, 2005). Furthermore, recent studies link CREB to synaptogenesis induced by changes in synaptic plasticity and by synaptic vesicle accumulation in axon terminals (Tojima et al, 2003, Yoshida et al, 2005).

For complete understanding of the role of CREB and CREB-dependent gene expression in the CNS, it is essential to have a comprehensive understanding of its complex functions. Thus, CREB´s role in the survival in hippocampal cells cannot be easily dissected from its involvement in synaptic plasticity.

A4.5. Implications of CREB in neurodegenerative diseases

Knowing the importance of CREB pathway in neuronal survival, it is logical that its malfunction produces severe consequences in the central nervous system. Indeed, several neurodegenerative diseases with high prevalence in the western world are linked to CREB-dependent gene expression. The main feature of polygluthamine repeats disorders are expanded repeat sequences of glutamine residues. Huntington's disease, the best described of these disorders, is a dominantly inherited, late-onset,

neurodegenerative process characterized by different motor dysfunctions, such as chorea, and impairment in cognitive performance. Although there are many theories of its ethiopathogenesis, many recent studies brought attention to alterations in the CREB-dependent neuronal activity. It was shown that CBP, CREB´s major co-activator, might be sequestrated into aggregates of mutant huntingtin and reducing the potential for transcriptional regulation of the CREB pathway (Higgins et al, 1999). Moreover, the neurodegeneration observed in mutant mice lacking the CREB family members resembles those generated to model Huntington's disease (Mantamadiotis et al, 2002). It is not clear yet what is the relative relevance of reduction of CBP or malfunction of CREB-driven transcription in neuronal loss in Huntington's disease. Since CBP functions as histone-acetyltransferase (HAT) enzyme, drugs designed to inhibit histone deacetylation were applied to Huntington's disease mouse models to compensate for this deficiency. Results were positive, showing that neurodegeneration was delayed (Ferrante et al, 2002, Hockly et al, 2003).

Alzheimer's disease is the most common cause of dementia. Almost two-thirds of dementia cases are due to Alzheimer's disease, but there is not efficient therapeutics so far. Its main symptom is cognitive impairment that may worsen rapidly, and the main pathological features are amyloid plaques and neurofibrillary tangles in the brain tissue. It is characterized by loss of neurons and synapses in the cerebral cortex and in certain subcortical regions, which leads to gross atrophy of the affected areas. CREB and one of its main kinases, PKA, were shown to be involved at early onset of this disease (Vitolo et al, 2002). Moreover, the stimulators of CREB pathway activation, such as forskolin, seem to be neuroprotective, as shown in *in vitro* experiments (Gong et al, 2004). Currently, CREB and its transcriptome are focus of many studies exploring their potential to prevent neuronal death. Pioneer experiments showed positive effects of enhanced CREB-dependent gene expression on cognitive function of mouse model for Alzheimer's disease (Gong et al, 2006).

Experimenting with drugs known to enhance CREB-driven transcription, as rolipram, provided good basis to consider this approach beneficial for some neurodegenerative disorders. In addition, systemic administration of drugs that act upstream of CREB and stimulate cAMP signaling, like PDE4 inhibitors, have been shown to relieve certain models of depression and they are being already tested in early clinical trials. Finally, administration of CREB pathway-based memory enhancers to patients after stroke has a positive effect during rehabilitation (Tully et al, 2003).

However, due its contribution to various cellular processes, CREB has not been found to be a safe and effective basis for designing the cognitive enhancers and, for that reason, studies have rather focused to its target genes (Tully et al, 2003). Since the correlation between the pathways involved in CREB activation and its effect to specific downstream targets is still not well understood, precise description of the CREB transcriptome at specific cellular context would help in explaining how specific genetic programs are determined and how it can be manipulated, which in turn may benefit designing drugs without severe side effects.

B. Objectives

The main goal of this study is to understand the role of CREB-dependent genes in the survival, plasticity and excitability of hippocampal neurons. For that, we compared the phenotypes of transgenic mice expressing a permanently active modified form of CREB, VP16-CREB, mice expressing a potent dominant negative inhibitor of CREB-dependent transcription, A-CREB and their wildtype littermates. Both transgenic mouse lines were generated using the forebrain-specific tetracycline-regulated gene expression system (tetO/CaMKII-tTA system).

Although the role of CREB in the central nervous system has been explored in a number of studies, there is still controversy regarding the relevance of some CREB functions. The mild or absent phenotype in hippocampal LTP, memory tasks and neuronal survival in different CREB deficient strains has raised questions regarding the relevance of CREB in plasticity and survival (Gass et al, 1998, Rammes et al, 2000, Balschun et al, 2003). Here, I aim at reevaluating the consequences of altering CREB function in forebrain neurons using A-CREB and VP16-CREB mouse strains.

I will focus this analysis in the CA1 hippocampal region, which is involved in higher cognitive functions, such as formation of spatial memory. Using different morphohistological and molecular biology techniques, I aim to answer how altered CREB activity modifies the following processes:

1) Neuronal survival, by investigation time onset, course and nature of cell death in hippocampal neurons of transgenic mice with altered CREB-driven transcription

2) Neuronal excitability, by exploring the response of neurons and neuronal networks to external stimuli, both *in vivo* and in the hippocampal slices of these mice

3) Gene expression, by performing microarray analyses of hippocampal samples obtained from transgenic animals, and carrying out more detailed analyses of candidate genes.

4) Behavior, by providing first and complete behavioral characterization of A-CREB transgenic animals.

C. Results

C1. cAMP Response Element-Binding Protein-Mediated Gene Expression Increases the Intrinsic Excitability of CA1 Pyramidal Neurons

In order to determine the role of the CREB signaling cascade in the control of neuronal excitability, we examined the effect of enhancing the CREB activity in CA1 neurons using a bitransgenic mouse strain that expresses VP16-CREB, a constitutively active CREB, exclusively in forebrain. Additionally, this transgenic mouse allowed us to study in detail the consequences of chronic enhancement of CREB activity. We demonstrated that enhanced CREB-dependent gene expression positively regulates the neuronal excitability of CA1 pyramidal neurons. In addition, we observed that the chronic enhancement of CREB-driven transcription for more than 3 weeks provoked the occurrence of spontaneous epileptic attacks in VP16-CREB^{high} mice, which dramatically shortened their lifespan. This phenomenon had a histological correlate in the severe loss of neurons in the CA1 and DG subfields of the hippocampus.

These observations were presented in the manuscript **"**cAMP Response Element-Binding Protein-Mediated Gene Expression Increases the Intrinsic Excitability of CA1 Pyramidal Neurons" published in the *Journal of Neuroscience* in December of 2007. In that study, I was responsible for histomorphological, molecular biological and biochemical studies, by performing immunohistochemistry, Timm´s and Nissl stainings and quantitative PCR experiments. Mikel López de Armentia and Juan Marcos Alarcon were responsible for electrophysiological analyses, Angel Barco designed and coordinated the project, Roman Olivares maintained and genotyped the mouse colony, and Eric R. Kandel hosted the production of the transgenic strains. The final version of this manuscript is presented below.

Development/Plasticity/Repair

cAMP Response Element-Binding Protein-Mediated Gene Expression Increases the Intrinsic Excitability of CA1 Pyramidal Neurons

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To investigate the role of CREB-mediated gene expression on the excitability of CA1 pyramidal neurons, we obtained intracellular recordings from pyramidal neurons of transgenic mice expressing a constitutively active form of CREB, VP16–CREB, in a regulated and restricted manner. We found that transgene expression increased the neuronal excitability and inhibited the slow and medium afterhyperpolarization currents. These changes may contribute to the reduced threshold for LTP observed in these mice. When strong transgene expression was turned on for prolonged period of time, these mice also showed a significant loss of hippocampal neurons and sporadic epileptic seizures. These deleterious effects were dose dependent and could be halted, but not reversed by turning off transgene expression. Our experiments reveal a new role for hippocampal CREB-mediated gene expression, identify the slow afterhyperpolarization as a primary target of CREB action, provide a new mouse model to investigate temporal lobe epilepsy and associated neurodegeneration, and illustrate the risks of cell death associated to a sustained manipulation of this pathway. As a result, our study has important implications for both the understanding of the cellular bases of learning and memory and the consideration of therapies targeted to the CREB pathway.

Key words: AHP; CREB; excitability; excitotoxicity; learning and memory; synaptic plasticity

Introduction

Studies in different model systems have established a critical role for the cAMP signaling pathway and cAMP-responsive element binding protein (CREB)-mediated gene expression in different forms of synaptic plasticity related to learning (Lonze and Ginty, 2002; Barco et al., 2003; Josselyn and Nguyen, 2005). In particular, it has been found that the late phase of long-term potentiation (LTP) in the Schaffer collateral pathway correlates with the phosphorylation of CREB in CA1 pyramidal neurons and the induction of CRE-driven gene expression (Bito et al., 1996; Deisseroth et al., 1996; Impey et al., 1996; Lu et al., 1999). Furthermore, the enhanced expression of CRE-driven genes favors the formation and stability of LTP in this pathway (Barco et al., 2002, 2005; Marie et al., 2005), whereas the inhibition of CRE-driven expression by deleting specific CREB isoforms (Bourtchuladze et al., 1994) or by overexpressing a dominant negative form of CREB caused deficits in some forms of LTP (Pittenger et al., 2002; Huang et al., 2004). Knocking out a specific CREB isoform, however, may cause the overexpression of other CRE-binding proteins (Hummler et al., 1994; Blendy et al., 1996), which may compensate the deficiency in CREB activity and reduce the impact of the mutation in the phenotype (Gass et al., 1998; Balschun et al., 2003).

Recent studies have demonstrated that CREB activity can also directly control neuronal excitability. The expression of the constitutively active CREB variant, VP16–CREB, using viral vectors increased the firing frequency and reduced the resting potential of neurons in the locus ceruleus (Han et al., 2006) and the excitability of spiny neurons in the nucleus accumbens (Dong et al., 2006). The long-term effects of these modifications in neuronal physiology and survival have not been investigated. Neither is it known what the relationship is between the regulation of long-term forms of synaptic plasticity by CREB and the changes in neuronal excitability.

To explore the role of CREB-mediated gene expression in controlling the excitability of CA1 pyramidal neurons, we performed intracellular recordings in mice that express VP16–CREB in a regulated manner. Here we report that transgene expression caused a rapid and reversible change of the firing properties of CA1 pyramidal neurons by reducing the slow (sAHP) and me-

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Disclosure of financial interest: E.R.K. is one of four founders of Memory Pharmaceuticals and Chairman of its Scientific Advisory Board. Memory Pharmaceuticals is concerned with developing drugs for age-related memory loss. Some of these drugs are also potentially useful in depression and schizophrenia.

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Table 1. Electrophysiological properties of CA1 pyramidal neurons in VP16–CREB^{high} mice

Group of mice (n = neurons, mice)	E_m (mV)	R_{in} (MΩ)	τ (ms)	$AP_{amplitude}$ (mV)	AP_{50} (ms)	dV/dt_{max} (V/s)	Threshold (mV)	Medium I_{AHP} (pA)	Slow I_{AHP} (pA)
VP16–CREB^{high} On ($n = 22, 7$)	−56 ± 1.1	210 ± 20.7	21 ± 1.6	112 ± 1.6	0.99 ± 0.05	251 ± 10.4	−33 ± 1.2	120 ± 13.1	15 ± 2.5
Wild-type On ($n = 16, 5$)	−58 ± 2.1	181 ± 17.3	25 ± 2.7	119 ± 1.3	1.01 ± 0.03	309 ± 9.8	−39 ± 0.7	251 ± 19.8	41 ± 6.7
p	0.40	0.29	0.13	0.004	0.71	<0.001	<0.001	<0.001	<0.001
VP16–CREB^{high} On/Off ($n = 22, 5$)	−60 ± 1.6	168 ± 12.5	23 ± 1.9	120 ± 1.3	0.99 ± 0.04	316 ± 9.9	−39 ± 0.5	224 ± 15.9	36 ± 3.3
Wild-type On/Off ($n = 19, 4$)	−61 ± 1.7	157 ± 10.4	23 ± 1.0	120 ± 1.2	1.01 ± 0.03	306 ± 6.0	−39 ± 0.8	231 ± 15.6	44 ± 3.5
p	0.77	0.51	0.91	0.93	0.74	0.40	0.84	0.27	0.11
VP16–CREB^{high} Off ($n = 13, 2$)	−61 ± 2.1	137 ± 10.0	18 ± 1.5	116 ± 2.2	0.89 ± 0.04	310 ± 14.7	−40 ± 1.1	211 ± 16.6	45 ± 8.6
Wild-type Off ($n = 19, 3$)	−57 ± 1.4	134 ± 10.3	19 ± 1.2	119 ± 1.2	0.86 ± 0.03	321 ± 8.4	−39 ± 1.0	243 ± 13.2	47 ± 7.6
p	0.13	0.95	0.61	0.14	0.43	0.40	0.45	0.12	0.88

τ, Membrane time constant; AP_{50}, AP duration at 50% amplitude; dV/dt_{max}, maximum rate of depolarization. In VP16–CREB^{high} On mice, the recordings were performed 10 d after transgene expression. In VP16–CREB^{high} On/Off mice, the transgene was expressed for 10 d and then silenced for 10 additional days. In both cases, transgene expression was turned on when the mice were 4 weeks old. VP16–CREB^{high} Off mice were 10 weeks old when the experiments were performed. In all these experiments, wild-type mice were housed and maintained in the same conditions than their mutant littermates.

dium (mAHP) afterhyperpolarization. Furthermore, we found that the sustained activation of CREB-mediated activity can trigger epileptic seizures once a given threshold is reached, and cause the excitotoxic cell death of pyramidal and granular neurons in the hippocampus. The comparison of transgenic strains with low and high levels of constitutive CREB activity allowed us to separate these two processes.

Materials and Methods

Generation and maintenance of transgenic mice. Several lines of transgenic mice expressing VP16–CREB under the control of *tetO* promoter were generated by microinjection of the linear construct as previously described (Barco et al., 2002). The founder mice were backcrossed to C57BL6 F1/J mice more than 15 times to generate the transgenic lines used in our study. We have previously described in detail the mice now referred as VP16–CREB^{high} (Barco et al., 2002, 2005). Those bitransgenic animals resulted of the crossing of *pCaMKII*-tTA mice (line B) (Mayford et al., 1996) and *tetO*–VP16–CREB line VC27, the line that provided highest level of expression in the hippocampus. In this study we also investigate the phenotype of bitransgenic animals resulted of the crossing of *pCaMKII*-tTA mice (line B) and *tetO*–VP16–CREB line VC19, which express the transgene at significantly lower level. We refer to this bitransgenic strain as VP16–CREB^{low} mice. In all our experiments, we used as control littermate mice carrying either *pCaMKII*-tTA, *tetO*–VP16–CREB or none transgene. For VP16–CREB^{high} and control littermates, dox was administrated at 40 mg/kg food and removed or added at the indicated times before experimentation. VP16–CREB^{low} mice were raised without dox. Experiments were performed in adult mice (2-4 month old), at least otherwise indicated in figure or table legends. All mice were maintained and bred under standard conditions, consistent with national guidelines and approved by the Institutional Animal Care and Use Committees.

Electrophysiology. Whole cell recordings were performed in acute slices maintained *in vitro*. Mice of either sex were anesthetized with isoflurane, decapitated, and coronal slices that included the dorsal hippocampus (300 μm) were cut in oxygenated (95% O_2/5% CO_2) ice-cold artificial CSF (ACSF) containing (in mM): NaCl 118, KCl 2.5, $NaHCO_3$ 25, NaH_2PO_4 1.2, $MgCl_2$, 1.3, $CaCl_2$ 2.5 and glucose 10. After a recovery period of 30 min at 32°C, slices were maintained at room temperature in ACSF. For recordings, slices were transferred to the recording chamber and superfused with ACSF at 32−34°C. Whole cell recordings were made from CA1 pyramidal neurons using infrared differential interference video microscopy (E600FN, Nikon, Tokyo, Japan). Patch-clamp pipettes were filled with intracellular solution containing (in mM): $KMeSO_4$ 135, NaCl 8, HEPES 10, Mg_2ATP 2, Na_3GTP 0.3 (pH 7.2 osmolarity 290 mOsm/kg). Access resistance was 7-20 MΩ and was monitored throughout the experiment. Experiments were discarded if the series resistance changed by more than 10% during the course of the experiment. Signals were recorded using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) filtered at 10 kHz and digitized at 20 kHz (Digidata 1320A, Molecular Devices). All cells described in this study had a membrane potential more negative than −50 mV. Electrical activity was recorded in current and voltage clamp and analyzed using AxoGraph X 1.0 (AxoGraph Scientific, Sydney, Australia). No series resistance compensation was used and membrane potentials were corrected for junction potentials (-4 mV). To investigate the firing properties of neurons, 15 current injection steps (600 ms) were applied from −200 to + 500 pA in

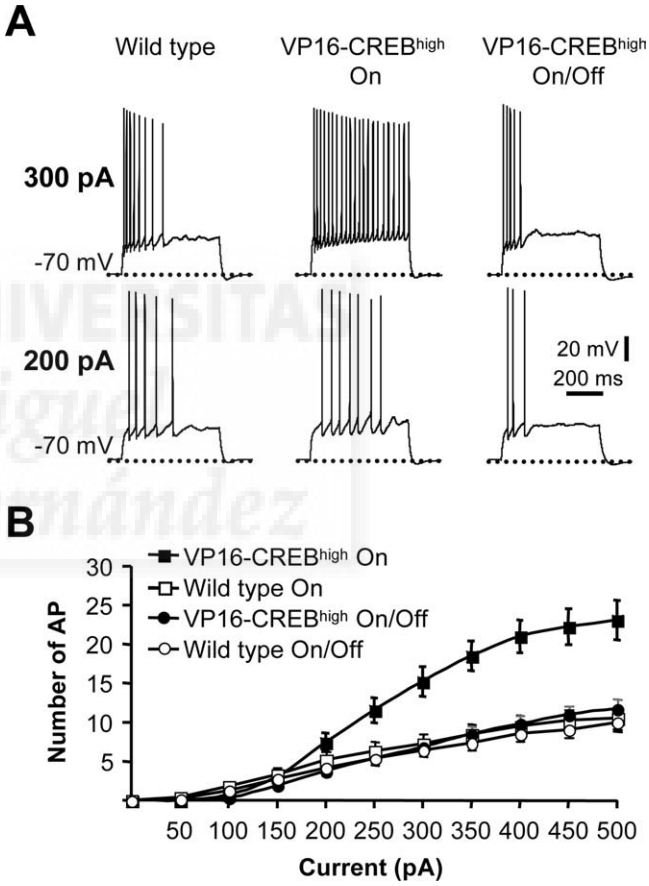

Figure 1. Neuronal excitability is increased in VP16–CREB-expressing neurons. ***A***, Representative CA1 pyramidal neuron response to 200 and 300 pA depolarizing pulse in a wild-type mouse (left), in a VP16–CREB^{high} mouse (middle), and in a VP16–CREB^{high} mouse that expressed the transgene for 10 d before turning off transgene expression for 10 additional days (right). In both experiments, transgene expression was turned on when the mice were 4 weeks old. Note the lack of spike frequency adaptation in CA1 VP16–CREB-expressing neurons. ***B***, Average number of AP triggered in response to increasing depolarizing currents in neurons from VP16–CREB^{high} ($n = 22$) and from wild-type littermate ($n = 16$) mice 10 d after inducing transgene expression (VP16-CREB On). This effect was completely reversed 10 d after turning off again transgene expression with dox (VP16–CREB On/Off; $p = 0.22$; $n = 22$).

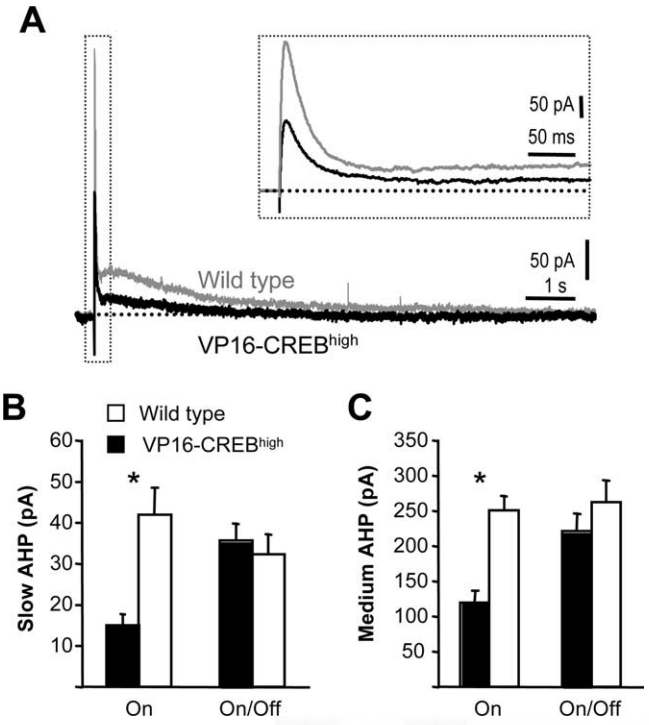

Figure 2. Inhibition of AHP in VP16–CREB-expressing neurons. ***A***, Currents underlying AHP in a CA1 pyramidal neuron of a VP16–CREB^{high} mouse (black) and a wild-type littermate (gray). Medium (inset) and slow component of the I_{AHP} were reduced in VP16–CREB^{high} mice. Neurons were held at −50 mV. Averaged amplitude of slow (***B***) and medium (***C***) component of the I_{AHP} in the same neurons presented in Figure 1*B* (VP16–CREB^{high}, black; WT, white). Inhibition of the I_{sAHP} and I_{mAHP} were reversed by silencing again transgene expression for 10 d with dox (VP16-CREB On/Off). *$p < 0.001$.

50 pA increments from a holding potential of −70 mV. Passive membrane properties were measured at resting membrane potential. I_{AHP} was evoked by a 50 ms depolarizing voltage step to 0 mV from a holding potential of −50 mV. I_{mAHP} and I_{sAHP} amplitudes were measured at the peak of the current and 1 s after the end of the depolarizing pulse, respectively. Because unclamped APs during the depolarizing voltage step could enhance Ca^{2+} currents and consequently I_{AHP}, we confirmed that there was not difference in the number of unclamped AP between different groups. Only a small proportion of neurons in both VP16–CREB strains and wild-type mice groups showed more than one unclamped AP (usually a second small spike) but these neurons did not exhibit bigger I_{sAHP} or I_{mAHP}, suggesting that most of the Ca^{2+} current is evoked by the command step and the contribution of the unclamped APs is not significant. For extracellular recordings transverse hippocampal slices (400 μm) were prepared, incubated in an interface chamber at 32° with oxygenated ACSF and allowed to equilibrate for at least 2 h. For recording of fEPSP in CA1 region both stimulating and recording electrodes were placed in the stratum radiatum of CA1 area. Extracellular activity was recorded simultaneously from stratum pyramidale of the CA1 and CA3 subregions with 1 MΩ pipettes filled with ACSF, filtered at 3 KHz and digitalized at 10 KHz. Power spectrum was calculated from a 250 s period and its area was measured between 0 and 1 KHz as an index of neuronal activity. LTP experiments were performed as previously described (Barco et al., 2002). In all electrophysiological experiments, 'n' indicates the number of cells or slices tested. The number of mice is also indicated in figure legends and Table 1. Two-way ANOVA and Student's *t* test were used for data analysis. Experimenters were blind to mice genotype.

Histological techniques. Nissl staining was realized as previously described (Mayford et al., 1996). For Timm's staining, mice were anesthetized with ketamine/xylazine, transcardially perfused with buffered 0.37% Na_2S solution, pH 7.2, for 5 min followed by 10% neutral buffered formalin (NBF) for 5 min. The brains were removed and postfixed overnight in 10% NBF. 50 μm thick sections were cut with a Leica vibration microtome, collected in 0.1M PB and mounted on the slides. Developer solutions were freshly prepared and mixed in darkness as described (Sloviter, 1982). The slides were incubated in developing solutions in total darkness for 60 min and then rinsed in dH2O and cleared with ethanol/xylene. For immunohistochemistry, mice were anesthetized, perfused with 4% paraformaldehyde, postfixed overnight, and 50 μm sections were obtained. The following primary antibodies were used: α-VP16 (Santa Cruz), α-Synaptophysin, α-MAP-2, α-Calbindin and α-GAP-43 (Sigma). Secondary biotinylated antibodies, streptavidin-peroxidase conjugate and DAB substrate were obtained from Sigma.

Quantitative RT-PCR. qPCR was performed in an Applied Biosystems 7300 real-time PCR unit using the *SYBR* mix (Invitrogen) and primers specific for VP16 (forward: 5′ cctacggcgctctggatatg 3′, reverse: 5′cggtaaacatctgctcaaactcg 3′) and GAPDH (forward: 5′ cttcaccaccatggagaaggc 3′, reverse: 5′catggactgtggtcatgagcc 3′) sequences. Each independent sample was assayed in duplicate and VP16 levels were normalized using GAPDH.

Microarray analysis. U74Av2 genechips were used to analyze changes in gene expression in the hippocampus of VP16–CREB^{high} at different times postinduction, data were processed, normalized and statistically analyzed using GCOS 1.2 software as previously described (Barco et al., 2005). This dataset is accessible at the GEO database (accession no. GSE3965). I (increase), D (decrease), MI (mild increase), MD (mild decrease) and NC (no change) calls were obtained according to the statistical thresholds defined by the software. We filtered and sorted the list of genes using the change *p* value, change call, and Log Ratio Signal. For early time, we requested that the change call for wild-type samples or for those samples in which VP16–CREB expression was turned-off were NC, whereas the change call for samples in which VP16–CREB expression was turned-on for a week (2 samples) indicated a statistically significant change in expression. For late time, we used the same criteria applied to arrays corresponding to 3 or 5 weeks after induction (3 arrays). The two lists of genes so obtained were then analyzed for pathway building and GO group classification using Pathway Studio 5.0 software (Ariadne Genomic Inc.). Similar results were obtained when microarray data were normalized, modeled and filtered using DNA-Chip Analyzer (dChip) software instead of GCOS. Although there were significant differences in the composition of the gene lists generated with each one of these approaches, the results of Pathway Studio analysis were equivalent.

Results

Membrane properties and excitability of VP16–CREB expressing neurons

Our previous research demonstrated that enhanced CREB-mediated gene expression in the bitransgenic strain VP16–CREB reduced the threshold for obtaining the late phase of LTP in the Schaffer collateral pathway (Barco et al., 2002), whereas recent studies indicate that CREB may regulate neuronal excitability in striatal and brainstem neurons (Dong et al., 2006; Han et al., 2006). To investigate whether similar changes in excitability may also occur in the hippocampus, we examined the firing pattern and membrane properties of CA1 pyramidal neurons in VP16–CREB mice. From here on, we will refer to this bitransgenic strain as VP16–CREB^{high} to facilitate its comparison with VP16–CREB^{low}, a new bitransgenic strain which we first describe in a later phase of this study.

The tTA/*tetO* system of double transgenic mice enabled us to obtain precise temporal regulation of transgene expression through the ability of doxycycline (dox) to block tTA binding to DNA. To avoid possible developmental problems attributable to early expression of CREB-activity, we raised VP16–CREB^{high} animals in the presence of dox and induced the expression of the transgene at specific times by removing the drug from the mouse diet. Animals bred under these circumstances were indistinguishable from their wild-type littermates. Intracellular recordings in CA1 pyramidal neurons 10 d after dox removal revealed no dif-

ferences in passive membrane properties between transgenic mice and their littermates (Table 1). However, the expression of VP16–CREB profoundly affected the firing properties of neurons, increasing the number of action potentials (APs) elicited by depolarizing current injections (Fig. 1*A*). Thus, CA1 pyramidal neurons from VP16–CREB^{high} mice (VP16–CREB On) showed a reduced spike frequency adaptation during a depolarizing pulse and triggered on average more APs than their wild-type littermates ($p < 0.001$) (Fig. 1*B*). We also observed a significant increase in the threshold to elicit an AP and decreased AP amplitude and maximum rate of depolarization in CA1 pyramidal neurons from VP16–CREB^{high} mice (Table 1). These later changes suggest reduced Na^+ currents that could balance the increase in excitability observed in these neurons (Davis, 2006).

These changes in firing pattern and membrane properties were reversed when VP16–CREB expression was repressed for 10 d with dox (Table 1, VP16–CREB^{high} On/Off; Fig. 1*A*,*B*). We also recorded from CA1 pyramidal neurons of VP16–CREB^{high} mice that were kept always in the presence of dox and never expressed the transgene. In these mice, we did not detect any difference between those transgenics and their littermates (Table 1, VP16–CREB Off). Therefore, the reduced spike frequency adaptation observed in neurons of VP16–CREB^{high} mice results from the acute expression of this chimeric protein.

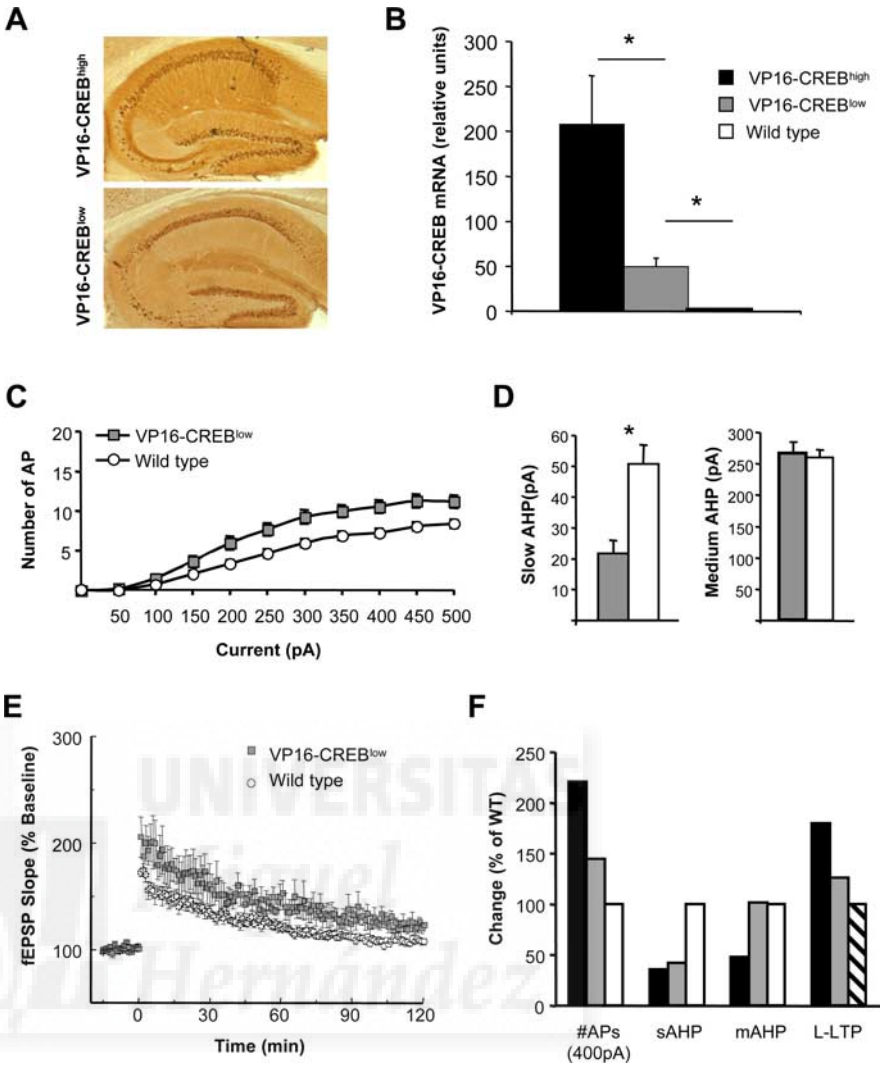

Figure 3. The increase in neuronal excitability and inhibition of I_{AHP} is dose dependent. ***A***, Comparison of the pattern of transgene expression in brain sagittal sections from VP16–CREB^{high} and VP16–CREB^{low} mice using an antibody against the VP16 domain. DAB reaction was longer in the case of VP16–CREB^{low} to facilitate the identification of positive neurons; therefore, expression levels are not comparable between these two panels. ***B***, Real-time quantitative PCR was used to compare the expression of VP16–CREB transcripts in the hippocampus of VP16–CREB^{high} (3 weeks after induction) and VP16–CREB^{low} mice (VP16–CREB^{high} vs pulled WT, $p = 0.01$; VP16–CREB^{low} vs pulled WT, $p = 0.006$; 3 mice per group). ***C***, Average number of AP triggered in response to increasing depolarizing currents in VP16–CREB^{low} ($n = 42$, 5 mice) and wild-type littermate ($n = 38$, 5 mice) mice. ***D***, Averaged amplitude of the I_{sAHP} and I_{mAHP} of the same neurons represented in ***C***. Only I_{sAHP} was significantly affected. *$p < 0.001$. ***E***, A single 100 Hz train (1 s) evoked E-LTP in hippocampal slices from wild-type mice but a longer-lasting form of LTP in VP16–CREB^{low} mice. ***F***, Comparison of electrophysiological phenotypes in the low and high expressing strains. Data are taken from Figures 1*B* and 3*C* (#APs 400 mA), from Figures 2*B* and 3*D* (I_{sAHP}), from Figures 2*C* and 3*D* (I_{mAHP}), and from Figure 3*C* by Barco et al. (2002) and 3*E* in this article (L-LTP, corresponding to the average amplitude response 90-120 min after LTP induction). Values are normalized to those obtained for wild-type littermates, except for L-LTP in which values are expressed as percentage of baseline response (dashed bar).

The expression of VP16–CREB reduces AHP in CA1 pyramidal neurons

What alterations in membrane properties underlie the reduction in spike frequency adaptation? Because firing frequency is importantly modulated by the amplitude and duration of the AHP (Madison and Nicoll, 1982; Peters et al., 2005), we investigated the AHP in CA1 pyramidal neurons of VP16–CREB^{high} mice. Slow and medium AHP has been proposed to reduce temporal summation and prevent afterdepolarization and multiple action potential firing during intense synaptic events (Wu et al., 2004). We performed voltage-clamp recordings in CA1 pyramidal neurons of VP16–CREB^{high} mice and found that the sAHP current were severely reduced (Table 1; Fig. 2*A*,*B*). I_{sAHP} in neurons of VP16–CREB^{high} mice was 36% of that observed in neurons of control littermates. This change was accompanied by a 52% reduction also in the I_{mAHP} (Table 1; Fig. 2*A*,*C*). Both components of the AHP recovered when the expression of the transgene was turned off with dox (Fig. 2*B*,*C*, On/Off; Table 1, On/Off), suggesting that modulatory mechanisms for mAHP and sAHP can be downstream of CREB activation.

The effect of VP16–CREB in AHP current is dose-dependent

To investigate whether the alterations in neuronal excitability and synaptic plasticity were dependent of the level of enhancement of CREB-activity, we compared our results in VP16–CREB^{high} mice with those obtained in a lower-expressing strain, referred as VP16–CREB^{low} (for additional detail, see Materials and Methods). As with VP16–CREB^{high} mice, VP16–CREB^{low} mice expressed the transgene in the hippocampus selectively in granular cells in the dentate gyrus (DG) and CA1 pyramidal neurons, excluding CA3 neurons (Fig. 3*A*). The quantification of VP16–CREB mRNA levels by real-time RT-PCR revealed that the hippocampal expression of this construct was fivefold higher in

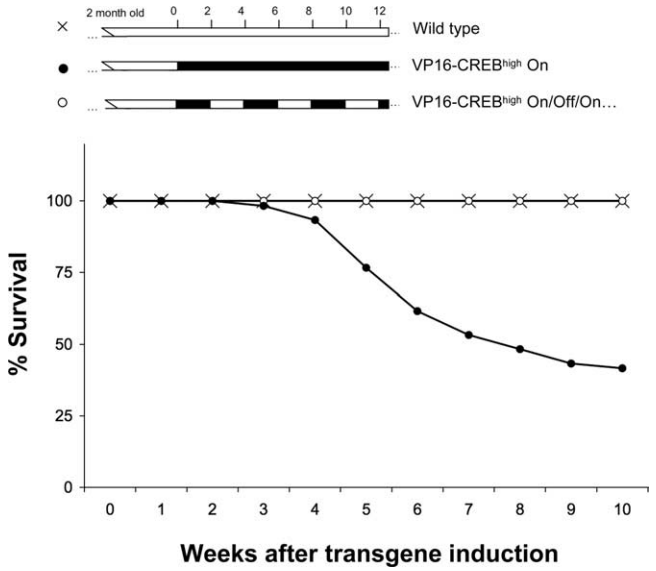

Figure 4. Survival of VP16–CREB^{high} mice. The survival of VP16–CREB^{high} mice (black circles) is compromised after 3-4 weeks of transgene expression. The reduction in the lifespan and the occasional seizures were not observed in wild-type littermates (crosses), in mice maintained in the presence of dox (data not shown), or in mice with periodic induction of VP16–CREB expression (white circles). The upper schemes indicate the transgene induction protocol used for VP16–CREB^{high} On (dox removal after 2 months of age) and for mice with periodic induction of VP16–CREB expression (VP16-CREB^{high} On/Off/On. . .), whose diet alternated every 2 weeks between regular food and food supplemented with dox.

VP16–CREB^{high} than in VP16–CREB^{low} mice (Fig. 3*B*) ($p = 0.04$). We found that, despite the reduced level of expression, these mice also exhibited a significant increase in neuronal excitability (Fig. 3*C*) ($p < 0.001$), although smaller than that observed in VP16–CREB^{high} mice. Moreover, VP16–CREB^{low} mice also presented reduced I_{sAHP} (Fig. 3*D*) (I_{sAHP} in VP16–CREB^{low} = 21 ± 4.1 pA, $n = 38$, 5 mice; I_{sAHP} in WT = 50 ± 6.2 pA, $n = 42$, 5 mice; $p < 0.001$). Interestingly, mAHP was, however, not affected in these mutants (Fig. 3*D*) (I_{mAHP} in VP16–CREB^{low} = 265 ± 18.9 pA, $n = 38$, 5 mice; I_{mAHP} in WT = 260 ± 11.7 pA, $n = 42$, 5 mice; $p = 0.81$).

The reduction in I_{sAHP} correlated with a reduced threshold for the induction of L-LTP in both VP16–CREB^{high} and VP16–CREB^{low} mice. Like VP16–CREB^{high} mice (Barco et al., 2002), VP16–CREB^{low} animals showed an enhanced response to one standard 100 Hz tetanus train of 1 s duration. This stimulation evoked a LTP with an enhanced and sustained long-lasting phase in mutant mice, but only produced a nonsaturating shorter-lasting LTP (E-LTP) in wild-type littermates (Fig. 3*E*) (90-120 min: VP16–CREB^{low}: 126 ± 11%, $n = 4$; WT: 109 ± 6%, $n = 4$; $p = 0.034$).

The comparison of the results obtained in both bitransgenic strains (Fig. 3*F*) shows that the magnitude of the alterations in neuronal excitability and synaptic plasticity correlates well with the level of expression of VP16–CREB.

Long-term consequences of enhanced CREB-mediated gene expression and excitability

We next turned to examine the long-term consequences of enhanced CREB-mediated gene expression and found that the continuous expression of this chimeric transcription factor for several weeks could cause the premature death of the mouse. Indeed, mice expressing VP16–CREB for more than 3-4 weeks occasionally showed spontaneous seizures during handling. These seizures frequently preceded the death of the mouse by 24-48 h (Fig. 4), although ~50% of the animals survived for more than 8 weeks. In contrast, the low-expressing strain VP16–CREB^{low} showed a normal life span even when the transgene was expressed through embryonic development and the whole life of the animal (results not shown).

The premature death of VP16–CREB^{high} mice could be completely prevented by turning off transgene expression with dox during the first month of its induction (data not shown). Even when transgene expression was repeatedly induced several times during the life of the animal by switching between regular food and food supplemented with dox, the deleterious effects were only manifested when the expression was sustained for more than 3-4 weeks (Fig. 4). Conversely, when VP16–CREB^{high} mice were kept off dox during their whole life, including embryonic development, most mice died before reaching the third moth of age. As described for mice in which we induced VP16–CREB expression during adulthood, we occasionally observed severe seizures that preceded the premature death of the animal.

The sustained activation of CRE-driven gene expression causes the loss of CA1 pyramidal neurons

At the time in which we performed the electrophysiological characterization of these mice (one-two weeks after transgene induction) no gross anatomical difference was observed in the brain of transgenic and control littermates (see time course in Fig. 5). In contrast, VP16–CREB^{high} transgenic mice expressing this chimeric transcription factor for several weeks showed progressive cell loss in the CA1 and DG regions. This neurodegenerative process could be easily visualized by Nissl staining (Fig. 5*A*) and by immunostaining with a number of neuronal markers (Fig. 5*B*). In accordance with these results, the neurodegenerative process in the DG severely reduced the mossy fiber projection after 6 weeks of transgene expression as revealed by Timm's staining (Fig. 5*C*).

Although we observed significant variability in the onset of neurodegeneration (from 3 to 5 weeks), all VP16–CREB^{high} mice investigated exhibited severe cell loss 6 weeks after dox removal. The onset of cell loss in the CA1 pyramidal layer seemed to coincide with the occasional observation of epileptic seizures, suggesting that these two events might be related. Mice in which we alternated 2 weeks of regular mouse diet with 2 weeks of food supplemented with dox never showed epileptic attacks (Fig. 4) and exhibited normal hippocampal anatomy (data not shown) despite accumulating several months of transgene expression. Indeed, the degenerative process could be stopped at any time by turning transgene expression off, although this did not cause a reversal of the lesion produced during the expression of the transgene (Fig. 5*D*). Interestingly, no cell loss was observed in VP16–CREB^{low} mice even after one year of transgene expression, indicating that a milder increase in neuronal excitability was compatible with neuronal survival (Fig. 5*A*, VP16–CREB^{low}).

Physiological and pathological regulation of neuronal excitability and gene expression by VP16–CREB

What are the consequences of the early increase in excitability and the late cellular loss in the activity of hippocampal circuits? To investigate this issue we performed field recordings in acute hippocampal slices from VP16–CREB^{high} mice 1 week after dox removal. We found that basal synaptic transmission in the Schaffer collateral pathway was normal (Fig. 6*A*), but there was a clear increase in spontaneous activity at the CA1 subfield (Fig. 6*B*) ($p = 0.01$), as estimated by the power spectrum analysis, that

correlated with the increased neuronal excitability observed in CA1 pyramidal neurons. In contrast, activity in the CA3 subfield, in which the transgene is not expressed, was unaltered (Fig. 6*B*).

When the expression of VP16–CREB was sustained for more than 3 weeks, we observed a significant reduction in the response of CA1 pyramidal neurons to stimulation of afferent CA3 axons (Fig. 6*C*), probably reflecting the incipient loss of synapses in the CA1 subfield. This reduction may compensate the previously observed shifting toward an increase in spontaneous activity of CA1 pyramidal neurons and, consequently, no increase in spontaneous activity in the CA1 subfield was observed after 3 weeks (Fig. 6*D*). Strikingly, we observed, however, an increase in spontaneous activity in the CA3 subfield (Fig. 6*D*) ($p = 0.002$), suggesting the existence of readjustments in the hippocampal circuit to compensate the ongoing neuronal loss.

Because sAHP can be inhibited by glutamate through kainate receptors activation (Melyan et al., 2002), we investigated whether an increase in extracellular glutamate concentration resulting from the neurodegenerative process initiated in VP16–CREB^{high} mice could underlie the reduction of the sAHP. We measured I_{sAHP} of neurons from slices of VP16–CREB^{high} mice that were either incubated for 2 h in the presence of 5 μM NBQX, an AMPA/kainate antagonist, or maintained in normal ACSF. No differences were found between the two groups of neurons (NBQX: $I_{sAHP} = 26 \pm 6.2$pA, $n = 15$; veh: $I_{sAHP} = 26 \pm 3.6$ pA, $n = 17$; $p = 0.99$).Together, these results indicate that the increase in excitability precedes the onset and perhaps contributes to the neurodegenerative process observed in the hippocampus of VP16–CREB^{high} mice.

The gene profiling analysis performed in VP16–CREB^{high} mice (Barco et al., 2005) also supports a dynamic scenario in which a physiological CREB-mediated response out of context eventually triggers a neurodegenerative process. The reanalysis of our microarray data using Pathway Studio software comparing early and late times (1 week vs >3 weeks) after transgene induction revealed clear differences between these two stages. At early times, the groups more represented in the list of significantly altered genes were signal transduction, transport, transcriptional regulation and synaptic transmission. In contrast, at late times, there was a overrepresentation of gene groups related to pathological responses (immune and defense response, proteolysis, apoptosis, inflammation) (Fig. 7*A*). Moreover, we also used Pathway Studio software for pathway building and visualization and found that the overall connectivity of altered genes to CREB decreased significantly at later times (Fig. 7*B*), suggesting that these late changes result from indirect effects that could be mediated by the altered expression of some early target genes.

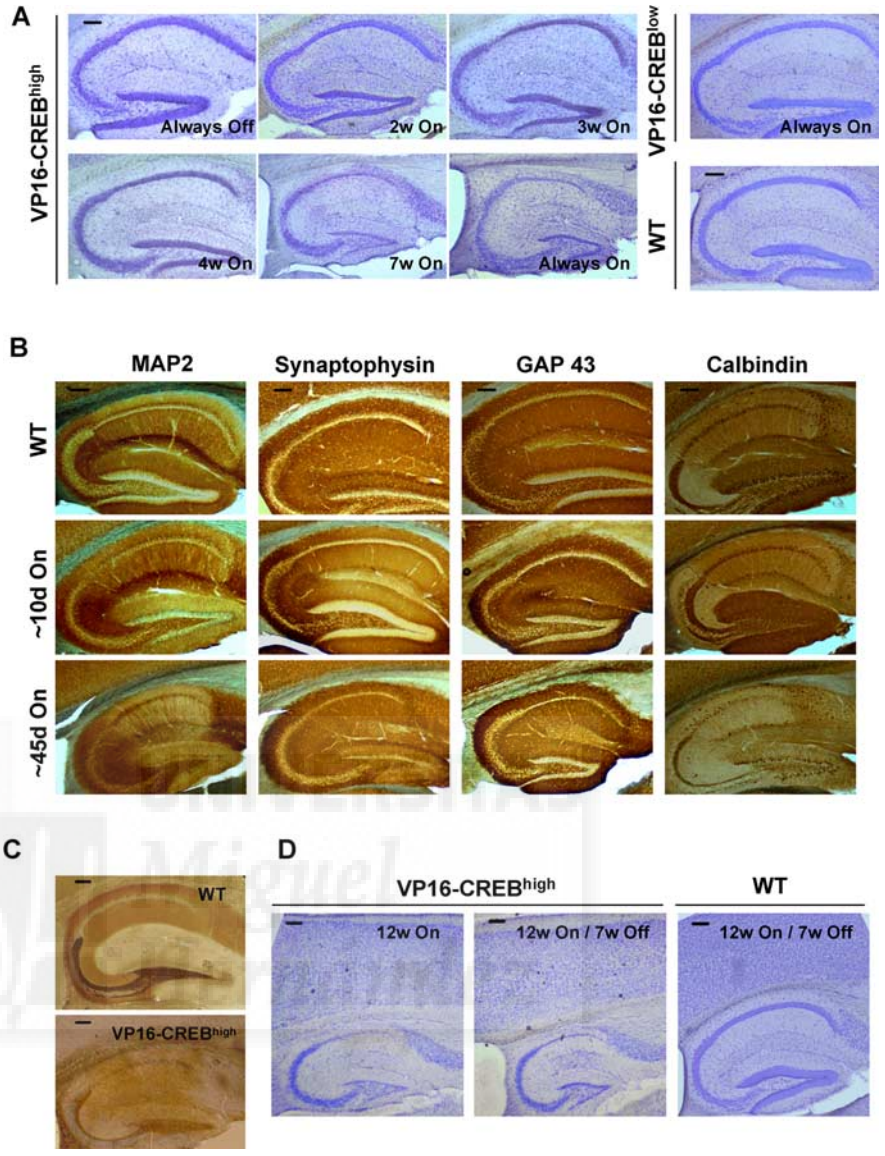

Figure 5. Sustained transgene expression causes cell loss in the hippocampus of VP16–CREB^{high} mice. ***A***, Nissl staining of the hippocampus of VP16–CREB^{high} mice at different times after transgene induction expressed in weeks. Always On indicates that the animal was kept off dox through its whole life, including embryonic development. Panel VP16–CREB^{low} shows the hippocampus of a 1-year-old transgenic mouse. ***B***, Immunohistochemical analysis of the hippocampus of transgenics (VC) and control mice. Floating vibratome sections (50 μm) were stained with antibodies against calbindin, GAP-43, synaptophysin, and MAP2. No significant differences were found in either hippocampus or other brain regions at early times (~10d On), but significant neuronal loss was observed after more than 6 weeks of transgene expression (~45d On). ***C***, Timm's staining of brain vibratome sagittal sections of a wild-type and a VP16–CREB^{high} mouse 6 weeks after dox removal. ***D***, Nissl staining of the hippocampus of VP16–CREB^{high} mice at different times after transgene induction and repression. All the experiments were realized in adult mice. Transgene expression was turned on when the animals were at least 2 month old. Scale bars, 140 μm.

Discussion

Modulation of intrinsic excitability of CA1 pyramidal neurons by CREB-mediated gene expression: Implications for learning and memory

Our results indicate that the enhanced expression of CRE-driven genes increases the neuronal excitability of CA1 pyramidal neurons and reduces the threshold for LTP in the Schaffer collateral pathway. These two effects might be at least partially mediated by a reduction of AHP currents. However, when a high level of

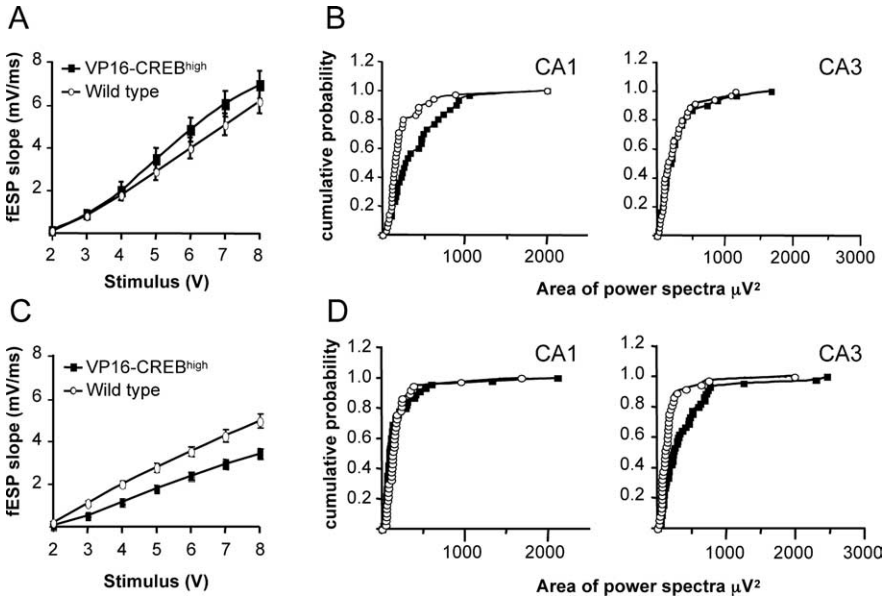

Figure 6. Increased spontaneous activity in the hippocampal circuit. ***A***, Input/output curve of fEPSP slope (millivolts per milliseconds) versus stimulus at the Schaffer collateral pathway of hippocampal slices from VP16–CREB^{high} ($n = 31$) and wild-type ($n = 36$) littermates 1 week after induction ($p = 0.27$). ***B***, Cumulative probability versus area of the power spectra calculated from 250 s recordings at the CA1 (left) and CA3 (right) subfields in the same slices used in ***A***. ***C***, Input/output curve of hippocampal slices from VP16–CREB^{high} ($n = 45$) and wild-type ($n = 37$) littermates 3 weeks after induction ($p < 0.001$). ***D***, Cumulative probability versus area of the power spectra calculated from 250 s recordings in the CA1 (left) and CA3 (right) subfields in the same slices used in ***C***. In all cases, transgene expression was turned on when the mice were 4 weeks old.

CREB activity is maintained for a long period of time and a given threshold is reached, epileptic activity develops in the hippocampal circuit and causes neuronal loss. These deleterious effects are dose-dependent and were not observed in a transgenic strain that express VP16–CREB at lower levels, although these mice also showed increased excitability.

Two recent studies have found that a similar CREB variant, when expressed using viral vectors, increased the excitability of neurons in the locus ceruleus (Han et al., 2006) and the nucleus accumbens (Dong et al., 2006), suggesting that the modulation of intrinsic neuronal properties is a well conserved CREB function through different neuronal types in the CNS. However, the specific changes in gene expression underlying these increments in excitability remain unknown. We describe here that depression of AHP might underlie the changes in neuronal excitability triggered by enhanced CREB function in CA1 pyramidal neurons. In particular, sAHP was affected both in the low and in the high expression bitransgenic strains, whereas mAHP was reduced only in mice expressing high levels of CREB activity. AHP controls the responsiveness of neurons (Sah, 1996) and its modulation can be a powerful mechanism for regulating NMDA-R-mediated plasticity related to learning (Wu et al., 2004; Faber et al., 2005). Blockade of the sAHP or mAHP converts short-lasting forms of potentiation into L-LTP (Sah and Bekkers, 1996; Cohen et al., 1999; Haug and Storm, 2000; Faber et al., 2005), whereas the activation of the AHP increases the threshold for induction of LTP (Sah and Bekkers, 1996). Interestingly, learning hippocampus-dependent tasks, such as eye blinking conditioning or navigation in the Morris water maze, causes a reduction of sAHP in CA1 pyramidal neurons (Moyer et al., 2000; Oh et al., 2003), a phenomenon that may favor learning by reducing the threshold for late forms of LTP.

Although our knowledge of the molecular underpinnings of the plasticity of membrane properties is still limited, the prime candidates to regulate these changes primarily overlap with those thought to regulate LTP. These include adenylyl cyclase and a number of kinases known to phosphorylate CREB (Zhang and Linden, 2003). In particular, variations in cAMP levels and PKA activity, a signaling cascade directly linked to CREB activation in neurons, play a major role in the regulation of the sAHP current. Thus, forskolin inhibits the current underlying sAHP by 90%, whereas the PKA inhibitor Rp-cAMPS increased the current, suggesting that PKA maintains sAHP channels in the closed state (Vogalis et al., 2003). Indeed, suppression of sAHP seems to be mainly mediated by PKA in CA1 pyramidal neurons (Pedarzani and Storm, 1993, 1995; Haug and Storm, 2000). In the case of mAHP, the inhibition observed in VP16–CREB-^{high} mice might result from modulation of the SK current (Sah and Faber, 2002) or M current (Gu et al., 2005), whose suppression in transgenic animals also increased the excitability of CA1 pyramidal neurons (Peters et al., 2005). Although neither CREB nor *de novo* gene expression has been so far directly involved in the modulation of AHP, the persistence of the changes in neuronal excitability and sAHP observed during behavioral training suggests the participation of molecular mechanisms that can support long-lasting changes, such as *de novo* gene expression and protein synthesis. We therefore propose that CREB-mediated changes in excitability of different neuronal subtypes may play a relevant role regulating physiological neuronal responses during addiction and learning and memory. Indeed, a reemerging view in the learning and memory field proposes that although synaptic changes, such as LTP or LTD, are likely to represent the most important cellular mechanism for memory storage given its computational properties, other forms of plasticity, such as changes in neuronal intrinsic excitability, may also importantly contribute to memory storage (Kandel, 1967; Zhang and Linden, 2003). Although the encoding capacity of those mechanisms is significantly lower than synaptic alterations, intrinsic changes might function as a trigger for consolidation or adaptative generalization of memories. Thus, AHP, in addition to its established role in spike frequency adaptation, may work as an adjustable gain control (Sah and Bekkers, 1996). Ascending monoaminergic fibers may facilitate hippocampal LTP induction and LTP-dependent learning processes in a global manner during arousal and attention by suppressing this current (Deng et al., 2007; Reymann and Frey, 2007). This process might be mediated by CREB activation, which is known to be downstream of such inputs (Berke and Hyman, 2000).

We have proposed that the sustained activation of CREB-mediated gene expression causes a cell-wide facilitation that prime the synapses for subsequent induction of L-LTP by a single tetanus (Barco et al., 2002; Alarcon et al., 2006). Now, we found that in the same mice shortly after transgene induction there is a significant reduction in the value of the I_{sAHP}, a current that has been related to learning and memory as much as has L-LTP. Are these two phenomena two manifestation of the same molecular process? The inhibition of sAHP may well represent the cellular

mechanism underlying cell-wide facilitation in VP16–CREB mice, although it cannot explain the resistance to protein and RNA synthesis inhibitors of L-LTP in these mutants, suggesting that other processes also contribute to this phenotype. The specific CREB target genes controlling these changes are still unknown, but it has been suggested that BDNF, a well known CREB downstream gene and the main effector molecule that emerged from our gene profiling analysis of VP16–CREB^{high} mice (Barco et al., 2005), contributes to control the coordinated regulation of synaptic and intrinsic properties aimed at allowing neuronal networks to adapt to long-lasting changes in activity (Desai et al., 1999).

Deleterious effects of the prolonged activation of CREB-mediated gene expression

During normal activity of the brain, the neurons receive transient signals and initiate transcriptional responses that are important for learning and memory. However, more intense or sustained stimulation (for example those used in the kindling model) may initiate inappropriate gene expression response and lead to the formation of epileptic neuronal circuits and disorders of neuronal excitability (McEachern and Shaw, 1999). A physiological process can thereby become pathological. The deleterious effects observed at late times in VP16–CREB^{high} mice could be the undesired consequence of a physiological function of CREB missing regulatory negative feedback mechanisms. VP16–CREB^{high} mice may, therefore, represent a useful model to investigate the cellular and molecular alterations associated with epileptogenic activity in the temporal lobe, as well as to assay possible therapeutic approaches. The reversibility and possibilities for regulation provided by the tTA/*tetO* system of double transgenics would be very useful in such studies.

The genechip analysis performed in VP16–CREB^{high} mice provides some additional clues for understanding this neurodegenerative process. The list of genes upregulated by VP16–CREB^{high} included BDNF, a neurotrophin that, as CREB itself, is known to play an important role in neuroprotection. Genetic and pharmacological studies have shown that increased expression of BDNF leads to hyperexcitability and epileptiform activities, whereas suppression of BDNF function inhibits the development of seizures (Kokaia et al., 1995; Elmer et al., 1997; Binder et al., 1999; Croll et al., 1999; Tandon et al., 1999; Lahteinen et al., 2002). These findings suggest that although BDNF may have a neuroprotective role, too much BDNF may also have adverse effects. VP16–CREB^{high} mice also overexpress diverse MHC I molecules, which have also been involved in adult synaptic plasticity (Huh et al., 2000; Boulanger and Shatz, 2004; Goddard et al., 2007). Although under normal circumstances, the

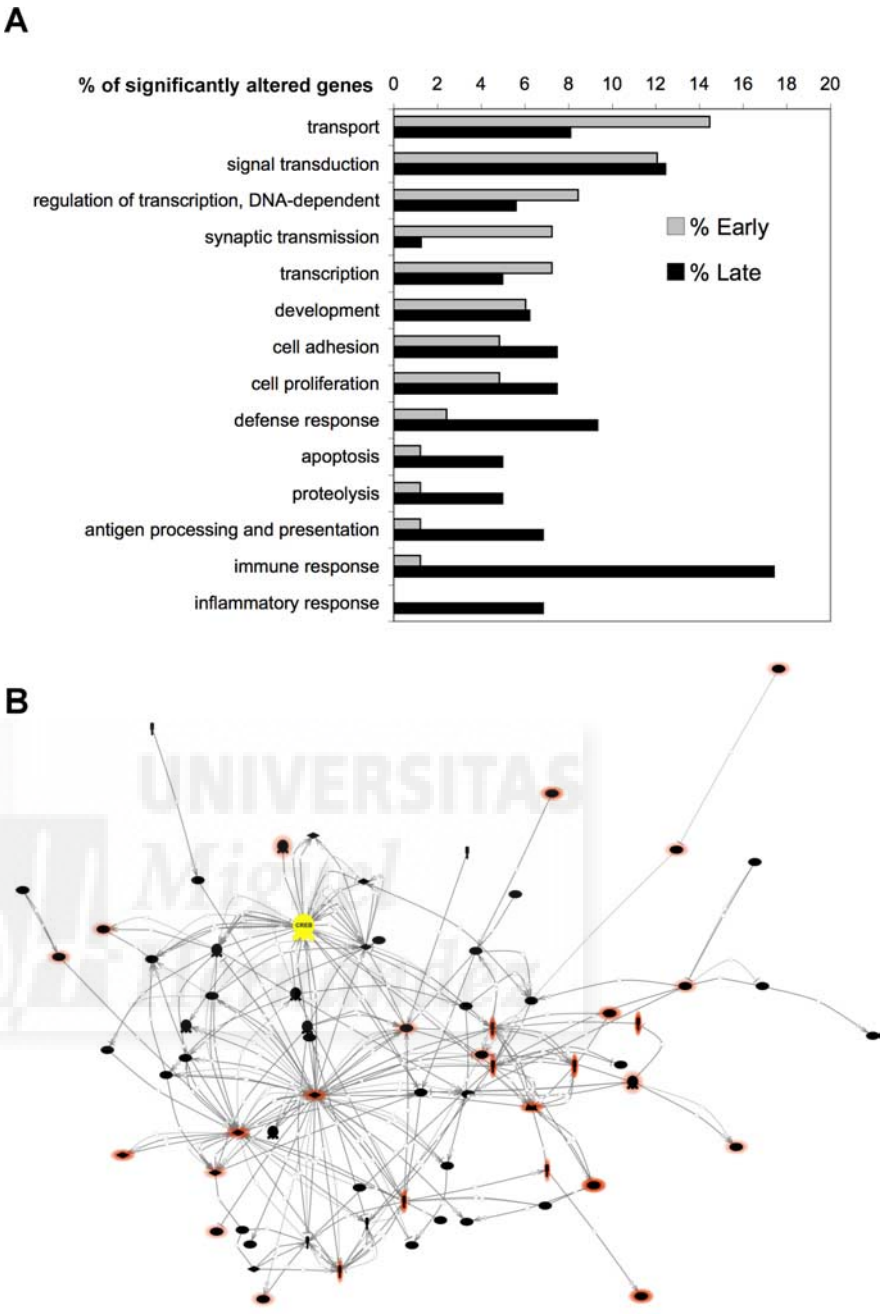

Figure 7. Altered gene expression in VP16–CREB^{high} mice. ***A***, Main cellular processes, as defined in gene ontology (GO), affected early (1 week) or late (>3 weeks) after transgene induction. We compared those groups that represent at least a 5% of significantly altered genes either early or late after expression. The main cellular process groups among the 84 probe sets significantly altered during the first week after dox removal included transport, transcription regulation, signal transduction, and synaptic transmission. However, the main GO groups represented in the 161 probe sets significantly altered at late times were related to defense and inflammatory responses, apoptosis, and proteolysis. ***B***, Pathway created with the 161 probe sets significantly altered at late times using Pathways Studio 5.0 software; only direct interactions were considered and unlinked entities were excluded. CREB is labeled in yellow, whereas genes related to pathological response are highlighted in red (red intensities reflect the overlapping of pathology-related groups). Note that most red nodes are not directly connected to CREB. The comparison of this pathway with the pathway obtained for early changes (data not shown) revealed a reduced connectivity with CREB (CREB participates in 48% of early interactions but only in 17% of late interactions) and a much larger presence of genes related with pathological situations (for additional information, see supplemental Fig. S1, available at www.jneurosci.org as supplemental material).

blood brain barrier prevents entry of leukocytes, antibodies, complement factors and cytokines into the brain parenchyma, a significant increase in MHCI expression, such as in chronic epilepsy or after prolonged VP16–CREB expression, might make the expressing neurons more susceptible to destruction by invading cells of the immune system. The prolonged overexpression of both BDNF and MHC I molecules, individually or in combination with other molecules, could trigger the neurodegenerative process and complex molecular changes observed in mice that expressed VP16–CREB for several weeks or months.

Therapeutic enhancement of the CREB pathway

CREB-dependent gene expression is necessary to maintain the survival of different neuronal subtypes both *in vitro* and *in vivo* (Bonni et al., 1999; Riccio et al., 1999; Lonze et al., 2002; Mantamadiotis et al., 2002; Papadia et al., 2005; Parlato et al., 2006). Moreover, the transient expression of the constitutively active CREB variant VP16–CREB has been shown to promote axon regeneration (Gao et al., 2004), neurogenesis (Zhu et al., 2004) and neuronal survival *in vitro* (Andreatta et al., 2004; Lee et al., 2005; Deng et al., 2006). Furthermore, transcriptional dysregulation in the CREB pathway has been proposed to play a central role in the pathogenesis of various neurodegenerative disorders, including Alzheimer's and Huntington's diseases. Based in these results, CREB activation has been proposed as a possible target of therapeutic approaches for neurodegenerative disorders (Barco et al., 2003; Tully et al., 2003).

Our results in CREB mutant mice support the critical role of CREB promoting neuronal survival and controlling learning-related plasticity, but also highlight the dangers that may be associated to the manipulation of a high level of activity in this pathway. This knowledge, however, does not reduce the promise of drugs targeted to this pathway for treating memory or neurodegenerative disorders. A better understanding of the molecular mechanisms underlying CREB activation and function should make possible the design and development of pharmaceuticals that enhance CREB-mediated gene induction at more physiological levels without excessively elevating basal activity. This type of enhancement should have a beneficial impact in situations in which this signaling cascade is impaired.

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C2. Inhibition of cAMP-response element binding protein reduces neuronal excitability and plasticity and triggers degeneration of CA1 pyramidal neurons

The uses of transgenic and knock-out mice for the analysis of CREB loss-off-function in the mouse have given some controversial results. To obtain a definitive conclusion about the precise role of CREB dependent gene expression in the control of synaptic plasticity and neurosurvival, we blocked the hippocampal CRE-driven gene expression through the expression of a strong dominant negative variant of CREB, known as A-CREB. We found that the expression of A-CREB transgene led to a reduced late phase of long-term potentiation (L-LTP) and caused alterations in spike frequency activation in CA1 pyramidal neurons by increasing the M potassium current. This change in excitability caused delayed kindling and reduced the susceptibility to seizures in A-CREB mice. The histomorphological analysis revealed that sustained inhibition of CREB function by A-CREB caused dramatic neurodegeneration of CA1 pyramidal neurons. Microarray analyses of A-CREB revealed a relatively modest effect in activity driven gene expression.

These findings are presented on the manuscript "cAMP-response element binding protein reduces neuronal excitability and plasticity and triggers degeneration of CA1 pyramidal neurons" currently under revision. In that study, I performed all histomorphological and biochemical assays, and tested the animals in different behavioral tasks. Also, kindling experiment was designed and done by me. Mikel Lopez de Armentia was responsible for electrophysiological analyses, Roman Olivares maintained and genotyped the mouse colonies, and Luis Miguel Valor, Angel Barco and I participated in the gene profiling analyses. The submitted draft is presented below.

Title: Inhibition of cAMP response element binding protein reduces neuronal excitability and plasticity, and triggers neurodegeneration

Abbreviated title: CREB control of neuronal survival and plasticity

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Abstract

The activation of the cAMP responsive element binding protein (CREB) pathway has been involved in two major cascades of gene expression regulating neuronal function. The first one relates CREB to neuronal survival and protection. The second one presents CREB as a critical component of the molecular switch that control long-lasting forms of neuronal plasticity and learning. Although the role of CREB in both genetic programs has been explored in a number of studies, there is still controversy regarding the relevance of some CREB functions. To investigate the role of CRE-dependent gene expression in neuronal plasticity and survival *in vivo*, we generated transgenic mice expressing A-CREB, an artificial peptide with strong and broad inhibitory effect on the CREB family. The expression of A-CREB in hippocampal neurons impaired L-LTP, reduced intrinsic excitability and the susceptibility to induced seizures, and altered both basal and activity-driven gene expression. In the long-term, the chronic inhibition of CREB function caused severe loss of neurons in the CA1 subfield as well as in other brain regions through apoptotic mechanisms. Our experiments confirmed previous findings in CREB deficient mutants and revealed new aspects of CRE-dependent gene expression in the hippocampus supporting a dual role for CRE-dependent gene expression regulating intrinsic and synaptic plasticity and promoting neuronal survival.

Introduction

The activation of the cAMP responsive element binding protein (CREB) pathway has been involved in two major cascades of gene expression regulating neuronal function. The first one presents CREB as a critical component of the molecular switch that control neuronal plasticity by regulating the expression of genes necessary for the formation of new synapses and the strengthening of existing synaptic connections (Kandel, 2001; Lonze and Ginty, 2002; Josselyn and Nguyen, 2005). However, the LTP and memory deficits originally reported for CREB hypomorphic mutants (mice homozygous for a deletion of the a and d isoforms (Bourtchuladze et al., 1994)) has been found to be sensitive to gene dosage and genetic background, and the mild or absent phenotypes in hippocampal LTP and hippocampus-dependent memory observed in other CREB deficient strains have raised questions regarding the relevance of CREB in plasticity and memory (Gass et al., 1998; Rammes et al., 2000; Balschun et al., 2003). More recently, CREB has been also involved in the regulation of intrinsic plasticity in different neuronal types (Dong et al., 2006; Han et al., 2006; Lopez de Armentia et al., 2007; Huang et al., 2008).

The second gene expression cascade relates CREB to neuronal survival and protection through the transcriptional control of neurotrophins and antiapoptotic genes (Riccio et al., 1999; Lonze and Ginty, 2002; Papadia et al., 2005). Studies on $CREB^{-/-}$ mice revealed massive loss of neurons in the peripheral nervous system that caused the death of the newborn shortly after birth, whereas most neurons in the central nervous system were not affected by CREB depletion (Lonze et al., 2002; Parlato et al., 2006). In contrast, double mutants for CREB and CREM (cAMP response element modulator) exhibited a marked cell loss in specific brain structures, such as cortex, hippocampus and striatum, even when these activities were selectively eliminated in the adult brain (Mantamadiotis et al., 2002). Strikingly, this loss of neurons had not been observed in any of the transgenic lines expressing CREB dominant negative mutants reported so far (Rammes et al., 2000; Kida et al., 2002; Pittenger et al., 2002). This may suggest that there was either unobserved compensation by other CREB family factors in the CNS of these animals, or that CREB´s different physiological roles could be dissected in these mice.

The comparison of different CREB-deficient mouse strains has therefore left

important open questions concerning the role of CREB on neuronal plasticity and survival. Can the role of CREB in synaptic plasticity be effectively assessed using transgenic strains that failed to reveal the critical requirement of CREB-dependent gene expression in neuronal survival demonstrated on gene targeting studies? Is the partial inhibition of CREB function and the compensation by CREM the cause of the controversy arising from the comparison of loss-of-function studies (Balschun et al., 2003)? Can the roles of CREB-dependent gene expression in neuronal plasticity and survival be dissected? These questions drove the generation of a new mouse strain in which to reevaluate the consequences of blocking CREB function in forebrain neurons and investigate new aspects of CREB function.

We describe here a novel transgenic strain in which it is possible to repress in a regulated manner CREB-dependent gene expression through expression of a strong dominant negative variant of CREB known as A-CREB. This variant, which was constructed by fusing an acidic amphipathic extension onto the N-terminus of the CREB leucine zipper region, binds with very high affinity and specificity to different members of the CREB family blocking their binding to CRE sites (Olive et al., 1997; Ahn et al., 1998). We investigated the consequences of interfering with CREB family function on neuronal survival and physiology and found that, at early times, the inhibition of this genetic cascade impaired L-LTP, reduced intrinsic neuronal excitability and the susceptibility to induced seizures, altered basal transcription, and had a relatively modest effect on activity driven gene expression. In the long-term, the sustained expression of A-CREB caused neuronal loss in the CA1 subfield of the hippocampus and other brain regions.

Material and Methods

Generation and maintenance of transgenic mice

The DNA fragment encoding A-CREB (Ahn et al., 1998) was subcloned in the plasmid pMM400 (Mayford et al., 1996) and the NotI fragment containing A-CREB downstream of the *tetO* promoter was injected into mouse oocytes. We referred as A-CREB mice those bitransgenic animals that result of the crossing of *pCaMKII*-tTA mice (line B, (Mayford et al., 1996) and line *tetO*-A-CREB-95. *TetO*-A-CREB-95 mice were

backcrossed to C57BL6 F1/J mice more than eight times. A-CREB mice were usually raised without dox. Transgene repression was achieved by dox administration (40 mg/Kg of food) for at least one week. VP16-CREB^{high} mice have been described before (Barco et al., 2002). Mice were genotyped by PCR using the oligonucleotides pMM400-3404: AGCTCGTTTAGTGAACCGTCAGAT; pMM400-3548r: CCTCGCAGACAGCGAATTCTA; and CamKII3´end2: TTGTGGACTAAGTTTGTTCGCATC. The PCR reaction starts at 94ºC for 2min and has 35 cycles: 94ºC for 45 sec, 60.5ºC for 25 sec and 72ºC for 3min. This reaction allows the simultaneous identification of both the *pCaMKIIa*-tTA transgene (450 bp band) and the *tetO*-A-CREB transgene (150 bp band). In all our experiments, we used as control littermates mice carrying either no transgene or the tTA or *tetO* transgene alone. Mice were maintained and bred under standard conditions consistent with national guidelines and approved by the Institutional Animal Care and Use Committee.

Electrophysiology

Extracellular activity and whole cell recordings were made from acute hippocampal slices as described (Lopez de Armentia et al., 2007). XE 991 dihydrochloride (Tocris) 10 mM was bath applied for 5 minutes to ensure a complete block of I_M current. LTP experiments were performed as previously described (Barco et al., 2002).

Kindling

Five weeks old A-CREB and control littermates (n=6 in both groups) were intraperitoneally injected with subconvulsive dosage of pentylentetrazole (50mg/kg, dissolved in saline) for several consecutive days. In the case of VP16-CREB^{high} mice, dox was removed from mouse diet one week prior to the start of the kindling experiment. The behavioural responses to the drug were classified according to the modified Racine scale (Pavlova et al., 2006): 0: no response; 1: facial automatism, with twitching of the ears and whiskers; 2: convulsive waves propagating along the axis of the trunk; 3: myoclonic convulsions with rearing; 4: clonic convulsions with loss of posture; and 5: repeated, forceful, clonic-tonic or lethal convulsions. All subjects were monitored for at least 20 minutes after the injection and scored.

Histological techniques

Nissl and immunohistochemistry stainings were performed as previously described (Lopez de Armentia et al., 2007). In cell counting experiments, cerebral cortex, CA1 pyramidal layer and DG granular cells layer thickness were counted from 50-µm coronal brain sections from ≥6 months old A-CREB (n=7) and their wildtype littermates (n=6) in a Leica microscope. For each animal, 3 sections were Nissl-stained and cells were counted in 5 defined regions and analyzed using Image-J software. a-M2-flag, a-Synaptophysin, a-MAP-2, a-Calbindin, a-GAP-43 and secondary antibodies were obtained from Sigma; a-Cleaved Caspase-3 and a-CREB antibodies were purchased to Cell Signaling; and a-CREM antiserum was a gift from Günther Schütz's lab. *In situ* hybridizations were performed as previously described using appropriate cRNA probes labelled with digoxigenin (Shumyatsky et al., 2002). Silver staining was carried out using the FD Neurosilver^{TM} kit (FD NeuroTechnologies Inc). For TUNEL reaction, serial brain sagittal cryosections of 12 mm thickness were stained using the in situ Cell Death Detection Kit (Roche Applied Science, Germany).

Electron microscopy

For electron microscopy mice were anesthetized and perfused with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M PB (pH 7.4) at the indicated times after dox removal. Then, coronal sections were cut at a thickness of 60 mm using a Leica vibration microtome through the level of the dorsal hippocampus. After several washes in PB, sections were postfixed with osmium tetraoxide (1% in 0.1 M PB) and block-stained with uranyl acetate (1% in distilled water). Sections were then dehydrated in ascending series of ethanol to 100% followed by propylene oxide and flat-embedded on glass slides in Durcupan (Fluka). The CA1 region of the hippocampus was cut at 70-90 nm on an ultramicrotome (Reichert Ultracut E; Leica, Austria) and collected on 200-mesh nickel grids. Staining was performed on drops of 1% aqueous uranyl acetate followed by Reynolds's lead citrate. Ultrastructural analyses were performed in a Jeol-1010 electron microscope.

Quantitative RT-PCR

qPCR was carried out in an Applied Biosystems 7300 real-time PCR unit using *SYBR* mix (Invitrogen) and primers specific for Arc, BDNF, c-Fos, CREM, the N-terminus of CREB and GADPH. Each independent sample was assayed in duplicate and

normalized using GAPDH levels.

Microarray analysis

RNA was extracted from dissected hippocampi. Mouse Genome 430 2.0 genechips were hybridized, stained, washed and screened for quality according to the manufacturer's protocol. The Affymetrix GeneChip® data were processed, normalized and statistically analyzed using GCOS (Affymetrix), GeneSpring GX (Agilent Technologies) and dChip softwares (Li and Hung Wong, 2001). This dataset will be accessible at the GEO database. See additional details in Supplementary Methods.

Results

Regulated expression of A-CREB in forebrain neurons

To investigate the consequences of impaired CREB-dependent gene expression in neuronal survival and function, we generated transgenic mice expressing the strong repressor of CRE-binding activity A-CREB. We used the CamKIIa-tTA system of inducible transgenics to restrict the expression to forebrain neurons (Mayford et al., 1996) (Fig. 1A). We focused our research on the role of CREB-dependent gene expression in hippocampal function in the bitransgenic strain CamKIIa-tTA/AC95, from now on referred as A-CREB mice, which showed the strongest expression in this brain region. The expression of A-CREB mRNA in this strain was restricted to specific layers of the cerebral cortex, the striatum and the hippocampus, preferentially in the CA1 field, although scattered positive cells were also detected in the dentate gyrus (Fig. 1B-C and Supplementary Fig. S1). Western-blot and immunohistochemistry analyses using anti-M2 Flag antibody, which recognizes A-CREB, demonstrated the efficient translation of the transgene (Fig. 1D and Supplementary Fig. S1B). The expression of A-CREB did not affect the level of CREB mRNA (Fig. 1E). However, in agreement with recent studies *in vitro* (Mouravlev et al., 2007), dimerization with A-CREB promoted CREB degradation as evidenced by the decrease of CREB immunoreactivity in the CA1 subfield (Fig. 1F).

We assayed the efficacy of doxycycline (dox) to regulate transgene expression. As expected, we found that addition of dox to the mouse diet turned off transgene

expression in less than two weeks (Fig. 1G). In contrast, the opposite manipulation, turning on transgene expression in mice in which it was turned off during embryonic and early postnatal development, failed to show efficient transgene induction (Fig. 1H). This result is in agreement with the recent report by Zhu and colleagues showing that tetO constructs, when turned off during embryonic development, are some times irreversibly silenced (Zhu et al., 2007).

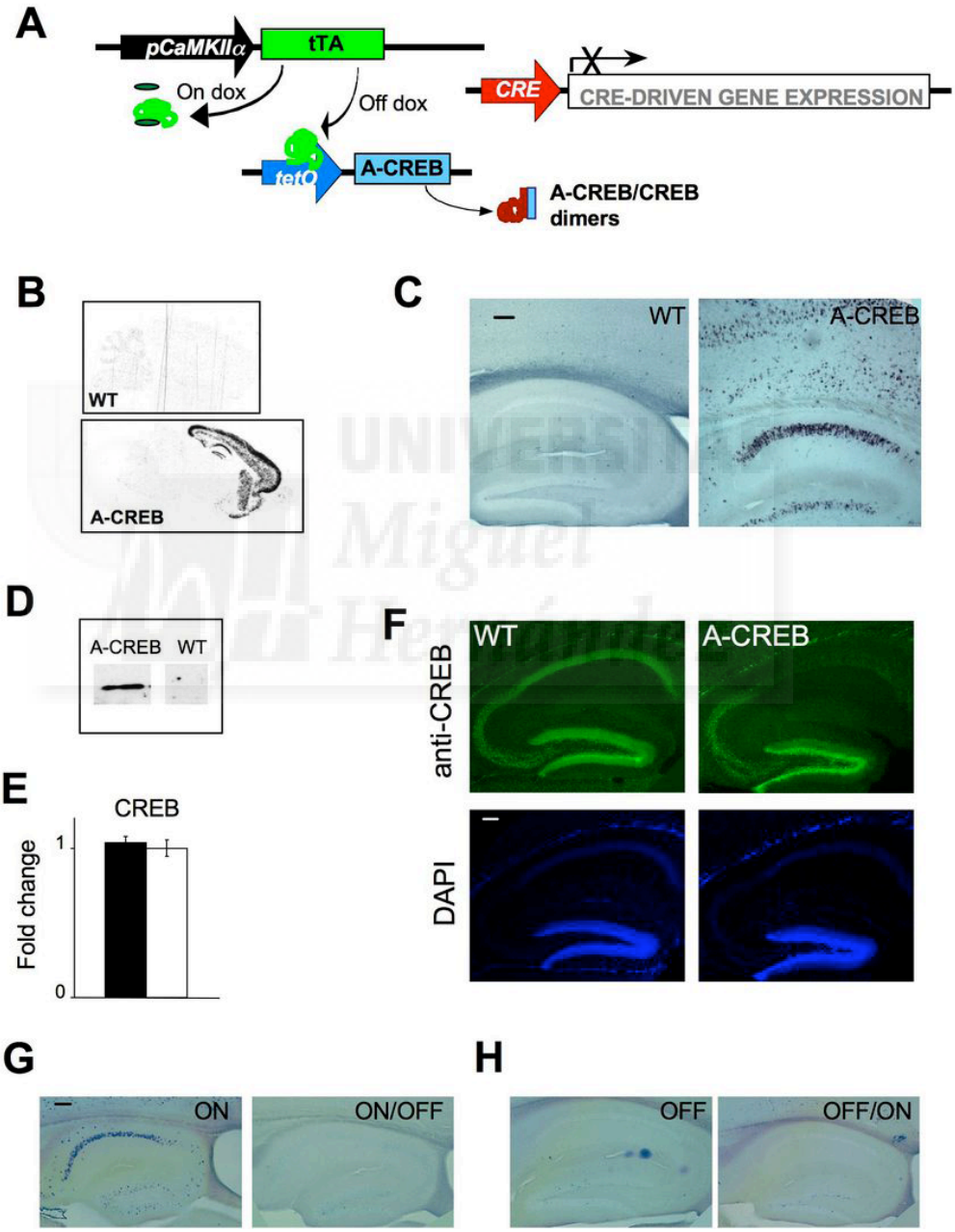

Figure 1. Regulated expression of A-CREB in the brain of transgenic mice: **A.** Scheme presenting the inhibition of CREB-mediated gene expression achieved in our transgenic approach. **B.** *In situ* hybridization on brain sagittal sections from CaMKII-tTA/tetO-A-CREB-95 bitransgenic mice (A-

CREB) and a wild-type littermate (WT) using an oligonucleotide probe specific for A-CREB. **C.** Transgene expression detected by DIG *in situ* hybridization with a probe specific for A-CREB transgene in 2-week old mice. **D.** Western-blot using anti-M2 Flag antibody detected A-CREB expression in hippocampal protein extracts. **E.** qRT-PCR quantification of CREB mRNA in the hippocampus of A-CREB (black bar) mice and control littermates (white bar) (3 mice per group, p=0.66). **F.** Immunostaining of brain sections of 14 days old A-CREB mice showed that CREB immunoreactivity was reduced in those areas with higher transgene expression, such as the CA1 subfield. **G.** DIG *in situ* hybridization showing repression of transgene expression in 5 weeks old A-CREB mice fed with dox food for 2 weeks (ON/OFF). Strong expression was detected in 3 weeks old mice maintained off dox (ON). **H.** DIG *in situ* hybridization showing defective transgene induction in A-CREB mice receiving dox during embryonic and postnatal development. No expression was detected in 8 weeks old mice maintained on dox (OFF). Transgene induction was assessed in 8 weeks old mice after having removed dox for four weeks (OFF/ON). Scale bar: 140 μm.

Chronic expression of A-CREB causes neuronal loss in hippocampus and cortex

A-CREB and control littermates were undistinguishable during the first postnatal weeks and had a normal life span. However, mutant mice did not gain as much weight as their control littermates suggesting some deleterious effect of A-CREB expression (Fig. 2A). *In situ* hybridization analysis of transgene expression at different ages revealed a progressive reduction on the level of expression of A-CREB mRNA in the hippocampus (Fig. 2B). The analysis of hippocampal anatomy in these sections suggested that this reduction on transgene expression was largely due to the death of neurons that expressed the transgene (Fig. 2C and Supplementary Figures S2 and S3). The neurodegenerative process progressed during several weeks and halted at later times, in which we could not longer detect the expression of the transgene in the CA1 area. The thickness of both the CA1 *stratum pyramidale* and cortex were severely reduced in adult A-CREB mice (Supplementary Fig. S2). Mice that expressed the transgene for several months still showed strong expression in cortical layers, indicating that those neurons may be more resistant to the chronic inhibition of CREB family function than CA1 neurons (Supplementary Figs. S1C and S2C). Interestingly, it was possible to stop and reinitiate the degenerative process by turning off and on transgene expression in adult animals (Fig. 2D). A-CREB animals raised in the presence of dox did not express the transgene and therefore did not show cell loss (results not shown).

Severe loss of CA1 neurons was also observed in CREB/CREM double knockout mice (Mantamadiotis et al., 2002). In contrast, no degeneration was

detected in several strains of transgenic mice expressing CREB dominant negative mutants. This might suggest that the disruption of CREB function in A-CREB mice was comparable to that in CREB/CREM double knockouts and likely superior than that achieved in previous transgenic approaches. We then proceeded to re-evaluate in this strain some of the open questions concerning the role of CREB signaling cascade on hippocampal L-LTP and neuronal survival, as well as to assess novel aspects of CREB function, such as regulation of intrinsic excitability in CA1 pyramidal neurons, hippocampal activity-driven gene expression and susceptibility to seizure. We will divide the characterization of A-CREB mice in three phases: (1) Early effects of CREB inhibition on hippocampal physiology and gene expression prior to neuronal damage; (2) characterization of the cell death process triggered in CA1 neurons; and (3) late effects of the sustained inhibition of CREB function and the severe cell loss.

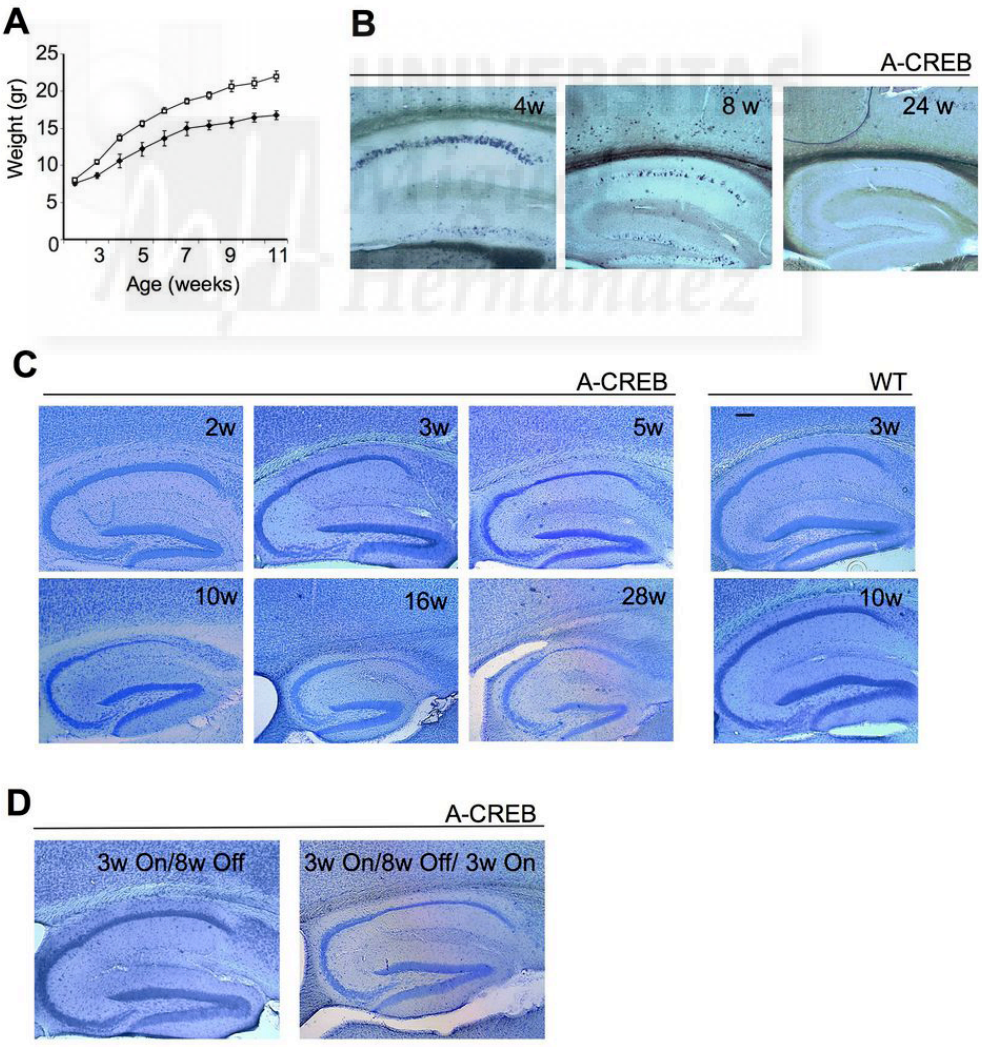

Figure 2. Neuronal loss in the hippocampus of A-CREB mice. **A.** A-CREB mice (◆, n=7) did not gain weight in the same progression that control littermates (□, n=18). **B.** Time course of the reduction of transgene expression detected by DIG *in situ* hybridization. Compare with the result obtained at 2 weeks (Fig. 1C, see also Supplementary Figure S1C). **C.** Nissl staining of the hippocampus of A-CREB mice at different times after transgene induction. Hippocampus morphology in 3 weeks old A-CREB mice (A-CREB 3w) and control littermates are undistinguishable. However, seven weeks later (A-CREB 10w), massive loss of neurons was observed in the CA1 subfield. At least two mice were analyzed per time point. The age of onset of neurodegeneration was between 4 and 8 weeks. Severe cell loss was reliably detected in mice older than 10 weeks (see also quantitative analysis in Supplemental Figure S2). Scale bar: 140 μm. **D.** Interestingly, neuronal loss was prevented by feeding the mice with dox before the onset of cellular death (A-CREB 3w On/8w Off) and could be triggered in adulthood by removing dox from the mouse diet (A-CREB 3w On/8w Off/3w On).

Inhibition of CREB activity impairs L-LTP

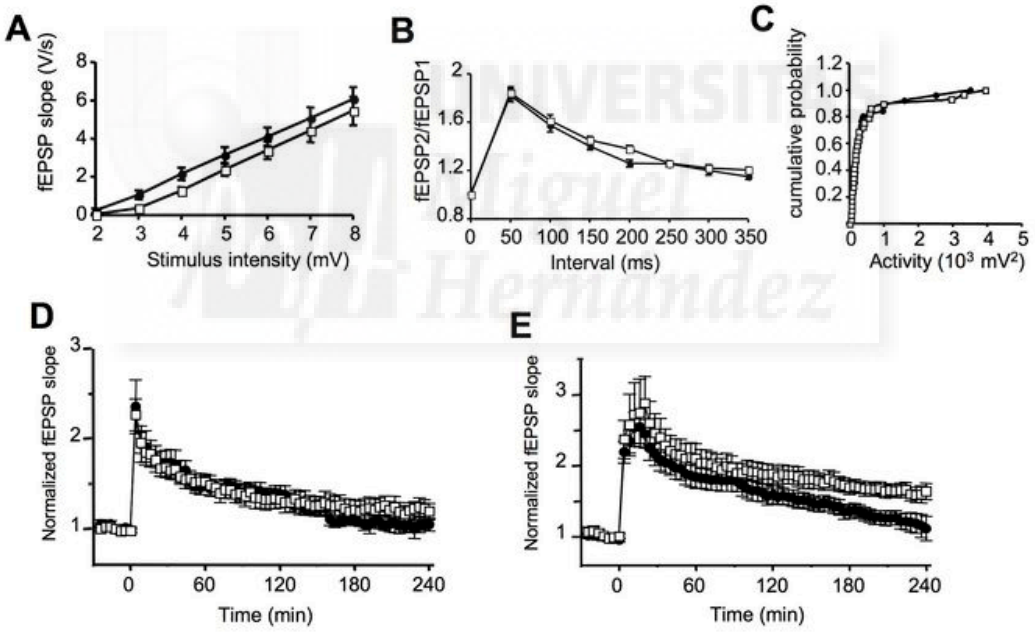

Figure 3. Impaired plasticity in the hippocampus of A-CREB mice. Input-output curve of fEPSP slope (V/s) versus stimulus (V) at the Schaffer collateral pathway of hippocampal slices from 3-week old A-CREB mice (●, n= 24) and control littermates (□, n=28) (p=0.18). **B.** Comparison of pair-pulse facilitation in 3-weeks old A-CREB mice and control littermates. Data are presented as the mean±SEM of the facilitation of the second response relative to the first response **C**. Cumulative probability versus area of the power spectra calculated from 250 s recordings at the CA1 pyramidal layer in hippocampal slices from 3-weeks old control (n=29) and A-CREB mice (n=26, p=0.70). **D.** A single 100 Hz train (1 sec) evoked E-LTP in hippocampal slices of 3-weeks old control and A-CREB mice. **E.** Four 100 Hz trains evoked L-LTP was impaired in 3-weeks old A-CREB mice.

Loss and gain-of-function studies have suggested a role for CREB in the late phase of LTP in the Schaffer collateral pathway. However, the absence of a clear phenotype in LTP studies on some CREB deficient strains has raised questions regarding the relevance of CREB in hippocampal plasticity. These discrepancies may be due to compensatory effects between different CRE-binding proteins. Unfortunately, L-LTP has not been assessed in CREB/CREM double knockout mice. To clarify this issue, we examined synaptic plasticity in the Schaffer collateral pathway of 3 weeks old A-CREB mice, a time at which no neuronal damage was detected. Field recordings in acute hippocampal slices from A-CREB mice did not reveal abnormalities in basal synaptic transmission (Fig. 3A and 3B) or alterations in spontaneous activity at the CA1 subfield (Fig. 3C). E-LTP in response to one standard 100 Hz tetanus train of 1 sec duration was also normal (Fig. 3D). However, in agreement with previous studies in CREB deficient mutants (Bourtchuladze et al., 1994), L-LTP in response to four tetani was impaired after 2 hours (Fig. 3E, 200-240 min: A-CREB: 122±1 %, n=10 (8); WT: 163±1 %, n=12 (8); $p<0.001$).

Inhibition of CREB activity reduces neuronal excitability and delays kindling

Enhanced CREB activity in CA1 pyramidal neurons increased intrinsic excitability and the spontaneous activity of hippocampal circuits (Lopez de Armentia et al., 2007). To investigate whether opposite changes occurred after inhibition of CREB function, we examined the intrinsic properties of CA1 neurons in juvenile A-CREB mice. Intracellular recordings in CA1 pyramidal neurons of three weeks old mutant mice revealed that the expression of A-CREB significantly reduced the number of action potentials (APs) elicited by depolarizing current injections (Fig. 4A and 4B; $p< 0.001$). We also observed that rehobase current to elicit an AP was bigger in A-CREB mice due to a reduction in membrane resistance (Supplementary Table 1 and Fig. 4C). Since we observed differences in the amplitude of the fast but not in the slow component of the AHP, we tested whether the decrease of membrane resistance was produced by an increase of the M potassium current (Storm, 1989). The selective M-channel blocker XE-991 (Wang et al., 1998) reversed the membrane resistance and rehobase differences between A-CREB and control mice (Fig. 4D and Supplementary Table 1) suggesting that an enhancement in the M current may underlay the decrease in intrinsic excitability. The reduction of intrinsic excitability (Fig. 4B; $p=0.30$) and the

differences in resistance and rehobase (Fig. 4E, Supplementary Table 1) were also reversed when transgene expression was repressed for 10 days with dox.

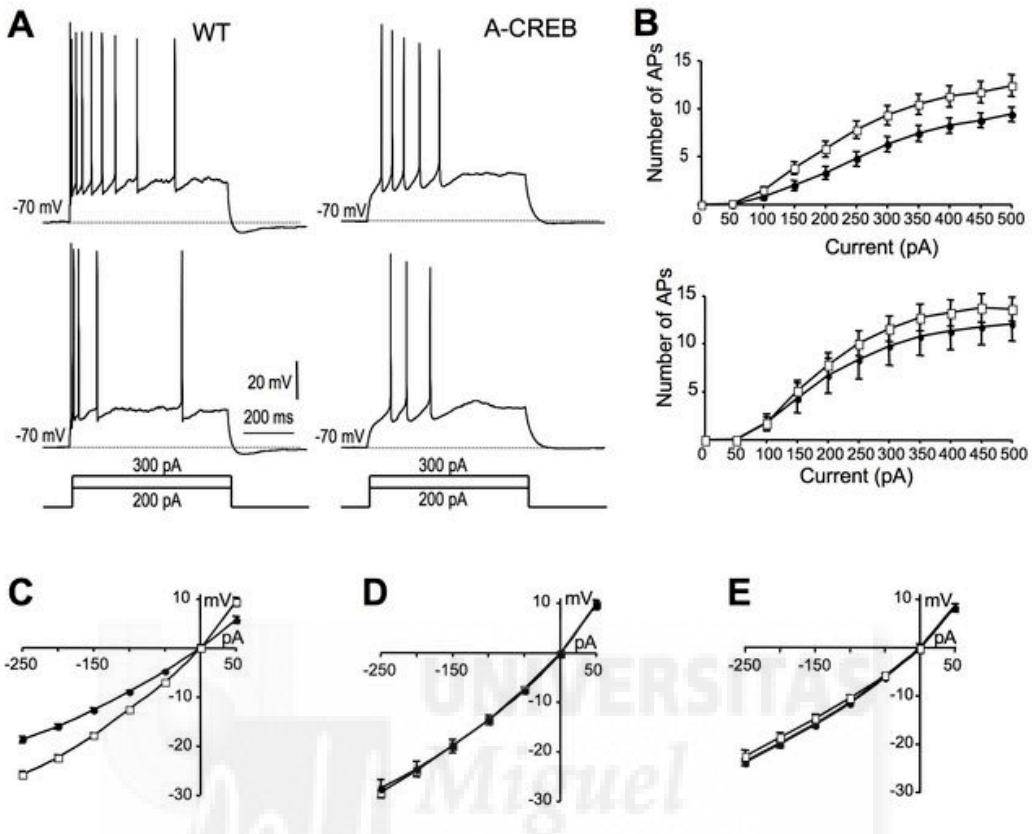

Figure 4. Neuronal excitability is reduced in CA1 neurons expressing A-CREB. A. Representative CA1 neuron response to 200 and 300 pA depolarizing pulses in a 3-weeks old control (left panel) and an A-CREB mouse (right panel). **B.** Average of APs triggered in response to increasing depolarizing currents in CA1 neurons from 3-weeks old A-CREB mice (●) and control littermates (□, upper panel $p<0.001$ ANOVA). This effect was reversed ten days after turning off A-CREB expression with dox (lower panel, $p=0.33$). **C.** Voltage-current relationship in CA1 pyramidal neurons holding at -70 mV in 3-weeks old A-CREB mice and control littermates. The alterations in A-CREB mice were reversed in the presence of the M-current blocker XE-991 (10 mM) (**D),** and after transgene repression by dox for ten days (**E**)

We recently reported that strong chronic increase of CREB activity, when sustained for several weeks, triggered the occurrence of sporadic seizures that often caused the animal death (Lopez de Armentia et al., 2007). In contrast, A-CREB mice had a normal life span and we never observed spontaneous epileptic seizures. In fact, our findings in CA1 neurons physiology suggested that A-CREB mice could be resistant to induced epilepsy.

To assess this hypothesis, we repeatedly injected mice with the pro-epileptic drug pentylenetetrazol (PTZ) at subconvulsive concentration. In control mice, daily injection for 10 days was sufficient to induce kindling in all individuals. In contrast, A-CREB mice exhibited delayed kindling and needed several additional subconvulsive injections of PTZ to show seizure (Fig. 5A). Interestingly, we carried out the same experiment in the transgenic strain with chronic enhancement of CREB function (VP16-CREB^{high} mice) and obtained the opposite result: whereas reduced CREB activity delayed kindling, enhanced CREB activity accelerated it (Fig. 5B). These results indicate that CREB can control neuronal responsiveness in both directions promoting and attenuating intrinsic excitability and plasticity.

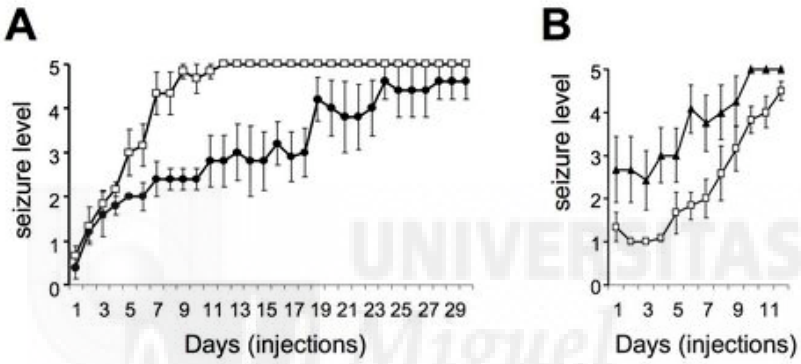

Figure 5. Seizure susceptibility in mice with enhanced or reduced CREB activity in neurons. A. Five-weeks old A-CREB mice (●) and control (□) littermates were intraperitoneally injected with 50 mg/Kg of PTZ every day until seizure stage 5 was consistently observed in both groups (n=6 for both groups). **B.** The same protocol was used in five-week old VP16-CREB^{high} mice (▲) and their littermates (□) (n=6 for both groups). The first PTZ injection was administered one week after transgene induction by dox removal. Note the stronger response to the drug in mutant mice observed already after the first injection. The average scaling is presented as mean ± SEM.

A-CREB expression causes transcriptional alterations

To evaluate the early transcriptional effects of CREB inhibition by A-CREB, we compared the profiles of gene expression in the hippocampus of three weeks old transgenic and control mice using *Affymetrix* microarrays MouseArray 430 2.0. Since we were interested in activity driven gene expression, we compared both genotypes in the basal condition and 2 hours after kainate-induced seizures. We observed that, in agreement with previous observations during kindling experiments, A-CREB mice were

more resistant to seizures than their control siblings. The same dose of kainate elicited less severe seizures in mutant mice, as determined by forelimbs clonus, rearing and falling, and death (Fig. 6A). The reduced susceptibility to KA can obviously interfere with our analysis of activity driven gene expression. For this reason, we obtained samples corresponding to five different conditions: control mice (WT), control mice injected with 14 mg/Kg of KA (seizure >4), A-CREB mice, A-CREB mice injected with 14 mg/Kg of KA (seizure <4), and A-CREB mice injected with 18 mg/Kg of KA (seizure >4).

The screen for genes specifically affected by A-CREB expression in the basal state revealed both downregulated and upregulated probe sets (Fig. 6B and Supplementary Table 2). The largest fold changes (FC) in the short list of genes consistently downregulated in A-CREB mutants, both in mice injected with saline or with kainate, corresponded to *scn4b* and *penk1* probe sets. *Scn4b* encodes the sodium channel subunit b4, which has been recently identified as significantly downregulated in Huntington's disease patients and in pre- and post symptomatic mouse model for this condition (Oyama et al., 2006). *Penk1* encodes proenkephalin, an important neuropeptide previously identified as a direct target of CREB in striatal neurons *in vivo* (Konradi et al., 1993; Pittenger et al., 2002). For upregulated genes, the biggest change corresponded to two probe sets targeted to *Trat1* encoding the T cell receptor associated transmembrane adaptor 1, whose role in neurons remains unexplored. As expected, we also observed strong increase of the signal of the two probes complementary to A-CREB sequence (Fig. 6C). However, we cannot exclude the existence of additional changes in gene expression. For example, some target genes may be misrepresented in the MouseArray 430 2.0. Also, A-CREB is strongly express in CA1 neurons, but it is only expressed in scattered granular cells at the dentate gyrus, and it is not expressed in glial cells or CA3 neurons; since we used mRNA extracted from the whole hippocampus, we are likely missing the possible changes restricted to expressing neurons in the DG and diluting the changes in CA1 neurons.

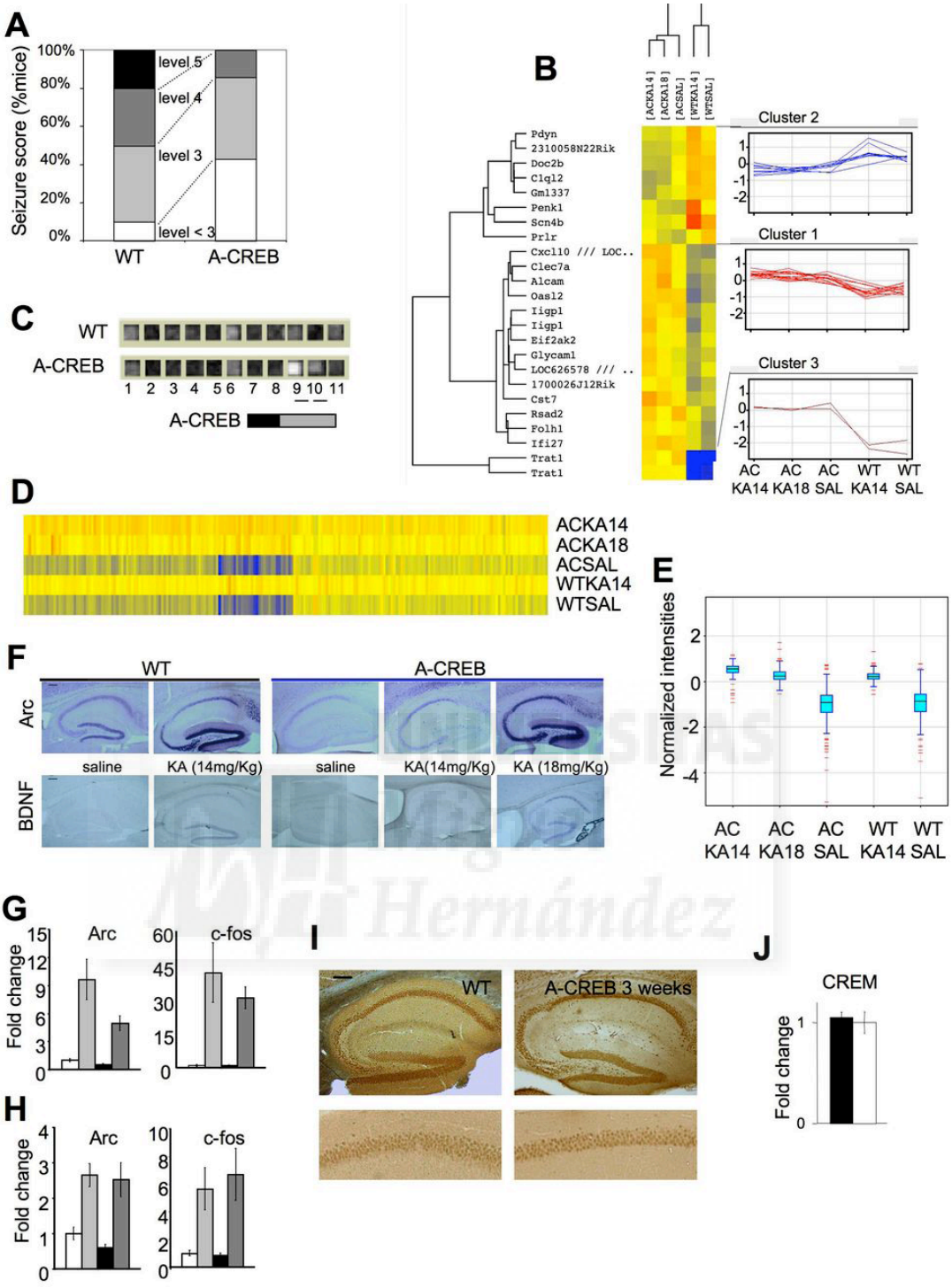

Figure 6. Gene expression analysis of early transcriptional changes in A-CREB mice. A. Three-weeks old A-CREB mice (n=7) showed milder seizures than their control littermates (n=10) in response to 14 mg/Kg of kainic acid. **B.** Two-dimensional hierarchical clustering of 24 probe sets significantly affected by genotype in the two-way ANOVA analysis and showing at least 2 fold change in the comparison between genotypes in animals injected either with saline or kainate. The three right panels show clusters obtained by *K*-mean clustering. WTSAL, wild-type saline; WTKA14, wild-type in 14 mg/Kg kainate; ACSAL, A-CREB saline; ACKA14 and ACKA18, A-CREB in 14 mg/Kg and 18 mg/Kg kainate, respectively (see also Supplementary Table 2). **C.** Results of hybridization for perfect match

(PM) oligonucleotide probes in the probe set 1452529_a_at. The direct observation of the hybridization signals revealed a large increase in the signal corresponding to the only two oligonucleotides complementary to A-CREB sequence. **D.** *K*-means clustering of the 210 probe sets significantly affected by drug treatment in the two-way ANOVA analysis showing a fold change equal or bigger than 2 in the comparison between drug treatments for at least one of the two genotypes. A number of genes in this list, specially those presenting largest changes (blue traces in saline samples) have been previously identified as IEGs, including *arc*, *egr2*, *egr3*, *c-fos*, *fosB*, *crem* and *junB* (see Table 1 and Supplementary Table 3 for additional details). **E.** Whisker box representation of expression changes in the group of 210 probe sets showed in panel 3E. **F.** *In situ* hybridization using DIG-RNA probes specific for Arc (upper panels) and BDNF (lower panels) in sagittal brain sections of 3 weeks old A-CREB mice and control littermates injected with vehicle or with 14 or 18 mg/Kg of kainic acid. Correlating with seizure strength, some A-CREB mice injected with 14 mg/Kg of kainic acid showed very weak induction of IEGs (see example in central panels), whereas other mice showed an induction similar to that observed in control littermates or in A-CREB mice injected with 18 mg/Kg of kainic acid (right panels). Similar results were obtained by immunohistochemistry using antibodies against c-Fos and Egr1 (results not shown). **G.** Quantitative RT-PCR of Arc and c-Fos levels in the hippocampus of 3-weeks old A-CREB mice and control siblings two hours after injection with 14 mg/Kg of kainate (KA). From left to right: WT saline (n=3): white bars; WT KA (n=3): light gray bars; A-CREB saline (n=3): black bars; A-CREB KA (n=3): dark gray bars. We observed a significant reduction in the basal level of Arc expression (p=0.04), but not for c-Fos (p=0.38). **H.** Quantitative RT-PCR of Arc and c-Fos levels in the hippocampus of 3-weeks old A-CREB and control siblings after exploration of a novel environment for 1 hour. From left to right: WT homecage (n=5): white bars; WT novelty (n=6): light gray bars; A-CREB homecage (n=5): black bars; A-CREB novelty (n=5): dark gray bars. There is a significant reduction in the basal level of Arc expression in A-CREB mice (p=0.04). **I.** CREM immunostaining of the hippocampus of a 3-weeks old A-CREB mouse did not reveal an increase in CREM immunoreactivity (3 mice per group). **J.** qRT-PCR quantification of CREM mRNA in the hippocampus of 3-weeks old A-CREB (black bar) mice and control littermates (white bar) (3 mice per group, p=0.69).

The screen for genes specifically affected by seizure revealed a large number of strongly upregulated genes (FC>4) and a few modestly downregulated genes (FC<2). The group of upregulated genes included a number of previously identified immediate early genes (IEGs), such as those encoding the transcription factors c-Fos, FosB, c-Jun, Egr-1, Egr-2, Egr-3, the neurotrophin BDNF, the cytoskeletal protein Arc and others, which represent the initial nuclear response to the activation of intracellular signaling cascades by synaptic activity and may play important roles on neuronal survival and synaptic plasticity (Tischmeyer and Grimm, 1999). Many IEGs have CRE sites in their promoters and are thought to be regulated by CREB. In fact, many IEGs were found upregulated in mice with chronic enhancement of CREB function (Barco et al., 2005).

Strikingly, we found that the induction of most IEGs was not affected by A-CREB expression (Fig. 6D-E, Table 1 and Supplementary Table 3), indicating that, although CREB activity is sufficient for the expression of many IEGs, it was not necessary for their induction in response to kainate. More detailed analyses revealed mild deficiencies in the basal expression of some important activity-dependent genes, such as *egr1*, *egr2*, a possible isoform of Homer 1 (*C330006P03Rik*) and *arc* (Supplementary Table 4), that were not initially detected, probably due to the stringency of the two-way ANOVA analysis (see Supplementary Methods for further details). However, these IEGs where strongly upregulated by kainate in the hippocampus of A-CREB mice. The list of genes significantly altered in A-CREB mice and differentially upregulated in response to kainate in A-CREB mice was surprisingly short, (Supplementary Table 2) and included *penk1* and *pdyn*, which encode for two precursors of opioid neuropeptides previously identified as CREB targets. Interestingly, *pdyn* was the gene that showed the strongest upregulation after chronic enhancement of CREB function (Barco et al., 2005).

To confirm our microarray results indicating that the induction of IEGs was not affected by A-CREB expression, we examined the expression of four representative IEGs, *fos*, *egr1*, *arc* and *bdnf*, in the hippocampus of transgenic mice using well known paradigms that trigger activity-dependent gene expression: induction of epileptic seizure by kainic acid and exploration of a novel environment. Although overall we confirmed the array results, we observed larger individual differences in the response to seizure in A-CREB mice than in control littermates (Fig. 6A). Induction of these four IEGs correlated well with seizure intensity in the lower range of the Racine scale (1-3). As a consequence, activity-dependent upregulation of these genes was apparently impaired in some mutant mice (Fig. 6F). This difference was likely not observed in the microarray analysis because we pooled together the hippocampi of several mice in each sample. When we used a higher dose of kainate (18 mg/Kg) strong upregulation of IEGs was consistently observed in the hippocampus of A-CREB animals (Fig. 6F, right panels). In agreement with our microarray analysis, Arc mRNA was slightly but significantly reduced in the basal condition (Fig. 6G). Similar results were obtained in response to novelty exploration (Fig. 6H and results not shown).

Table 1. The induction of immediate early genes related to synaptic plasticity is largely unimpaired in A-CREB mice. See full list of activity-driven genes in Supplemental Table

Gene	Probe Set ID	Gene Title	Unigene ID	FCWT	FCAC KA14	FCAC KA18	*P*	CRE sites
Arc	1418687_at	activity regulated cytoskeletal-associated protein	Mm.25405	3.58	6.41	5.76	0.002	2
Atf3	1449363_at	activating transcription factor 3	Mm.2706	13.22	13.34	9.70	0.000	3
Bdnf	1422169_a_at	brain derived	Mm.1442	3.29	3.39	2.35	0.001	1
	1422168_a_at	neurotrophic factor		4.50	4.57	3.60	0.000	
Btg2	1448272_at	B-cell translocation gene 2, anti-proliferative	Mm.392646	4.30	4.30	6.02	0.000	5
	1416250_at			8.58	7.38	9.05	0.000	
Crem	1449037_at	cAMP responsive element modulator	Mm.5244	1.90	2.35	2.31	0.002	1
	1418322_at			2.34	3.50	3.02	0.005	
Dusp1	1448830_at	dual specificity phosphatase 1	Mm.239041	3.18	4.31	4.78	0.000	2
Dusp4	1428834_at	dual specificity phosphatase 1	Mm.392187	2.69	2.75	1.88	0.003	3
Egr1	1417065_at	early growth response 1	Mm.181959	1.84	3.27	3.10	0.001	5
Egr2	1427682_a_at	early growth response 2	Mm.290421	5.36	12.91	12.96	0.000	3
	1427683_at			5.60	14.71	12.51	0.000	
Egr3	1436329_at	early growth response 3	Mm.103737	2.18	3.16	2.50	0.006	5
Egr4	1449977_at	early growth response 4	Mm.44137	3.99	4.64	3.64	0.000	4
Fos	1423100_at	FBJ osteosarcoma oncogene	Mm.246513	9.57	12.74	12.09	0.000	9
Fosb	1422134_at	FBJ osteosarcoma oncogene B	Mm.248335	15.43	24.64	19.06	0.000	7
Fosl2	1422931_at	fos-like antigen 2	Mm.24684	1.68	2.28	2.17	0.001	5
	1437247_at			2.47	5.08	3.63	0.005	
Gadd45 b	1449773_s_at	growth arrest and DNA-damage-inducible 45 beta	Mm.1360	5.68	7.29	5.58	0.000	3
	1450971_at			5.85	6.35	4.60	0.000	
Homer1	1425671_at	homer homolog 1 (Drosophila)	Mm.37533	8.84	10.30	6.14	0.002	4
Ier2	1416442_at	immediate early response 2 (Etr101)	Mm.399	4.04	5.69	6.31	0.002	4
Jun	1448694_at	Jun oncogene	Mm.275071	1.60	2.12	2.03	0.011	4
	1417409_at			2.43	2.60	2.50	0.002	
Junb	1415899_at	Jun-B oncogene	Mm.1167	4.80	5.39	4.63	0.001	6
Klf4	1417394_at	Kruppel-like factor 4 (gut)	Mm.4325	2.50	4.63	3.95	0.001	2
	1417395_at			3.00	5.41	5.03	0.001	
Nr4a1	1416505_at	nuclear receptor subfamily 4, group A, member 1 (Nur77)	Mm.119	3.05	3.93	3.81	0.001	6
Nr4a2	1450749_a_at	nuclear receptor subfamily 4, group A, member 1 (Nurr-1)	Mm.3507	2.91	2.94	2.15	0.002	6
	1455034_at			2.98	3.52	2.81	0.000	
	1450750_a_at			3.06	3.88	3.13	0.001	
	1447863_s_at			3.43	3.81	2.88	0.001	
Pim1	1435458_at	proviral integration site 1	Mm.328931	2.88	3.02	2.06	0.000	4
	1435872_at			5.03	5.96	4.62	0.000	
Rgs2	1419248_at	regulator of G-protein signaling 2	Mm.28262	3.68	4.90	4.27	0.000	4
	1447830_s_at			3.98	5.59	4.82	0.000	
	1419247_at			4.74	6.11	5.25	0.000	
Slc2a1	1426599_a_at	solute carrier family 2 member 1 (Glut-1)	Mm.21002	1.60	2.04	1.61	0.001	4

FC WT = fold change wild-type saline vs. wild-type 14 mg/Kg kainate; FC AC KA14 = fold change A-CREB saline vs. A-CREB 14 mg/Kg kainate; FC AC KA18 = fold change A-CREB saline vs. A-CREB 18 mg/Kg kainate. CRE sites = number of CRE predicted in murine promoters (3 Kb upstream and 200 bp downstream of the transcription start site, see Zhang et al. (2005), *PNAS* 102:4459-64, for further details). P Values correspond to two-way ANOVA analysis described in Supplementary Methods.

These results together with the microarray analysis suggest that other transcription factors can compensate the inhibition of CREB function in the control of some forms of activity-driven gene expression. Previous analyses of CREB knockout mice suggested that this compensation could be caused by the upregulation of the cAMP response element modulator (CREM) (Hummler et al., 1994; Blendy et al., 1996; Mantamadiotis et al., 2002). Neither microarray analysis (Supplementary Table 5),

immunostaining using an antiserum against CREM (Fig. 6I), nor qRT-PCR (Fig. 6J) revealed significant changes of CREM expression in the hippocampus of A-CREB mice at the basal stage. Since A-CREB can bind and block the activity of both CREB and CREM (Ahn et al., 1998), these results suggest that the upregulation of CREM observed in CREB knockout mice could be mediated by CREM itself.

Chronic inhibition of CREB function causes apoptotic neuronal death

After investigating the early consequences of disrupting CREB function with A-CREB, we used electron microscopy analysis to investigate the mechanism of cell death induced in CA1 pyramidal neurons by prolonged inhibition of CREB function. We found that the stratum cellular of the CA1 subfield in the hippocampus of six weeks old A-CREB mice contained a large number of shrunken pyramidal cells with highly osmiophilic cytoplasm (Figure 7A). These heteropycnotic cells, which represented up to 20% of pyramidal neurons in the dorsal hippocampus of A-CREB mice, showed apparent nuclear and cytoplasmic condensation, clumping of chromatin and their nuclear and plasma membranes were ruffled. Although most subcellular organelles appeared to be normal, some cells exhibited swelling of mitochondria and the Golgi network. There was no fragmentation of nucleus or cytoplasm and adjacent neurons had a normal appearance. Neurons with a similar aspect have been observed in neurodegenerative diseases, such as Huntington neurodegeneration, in both human and animal models, and the process has been referred as dark cell or dark neuron degeneration (Turmaine et al., 2000; Leist and Jaattela, 2001). TUNEL staining of brain sections in A-CREB mice revealed the presence of positive neurons in the CA1 subfield that preceded the severe cell lost in this area (Figure 7B). In agreement with this result, we also found neurons positive for active caspase-3 (Figure 7C). Brain sections of A-CREB mice were also positive for a reduced-silver staining that label degenerating neurons (Figure 7D). Therefore, the activation of an apoptotic program seems to underlay the loss of CA1 neurons in A-CREB mice.

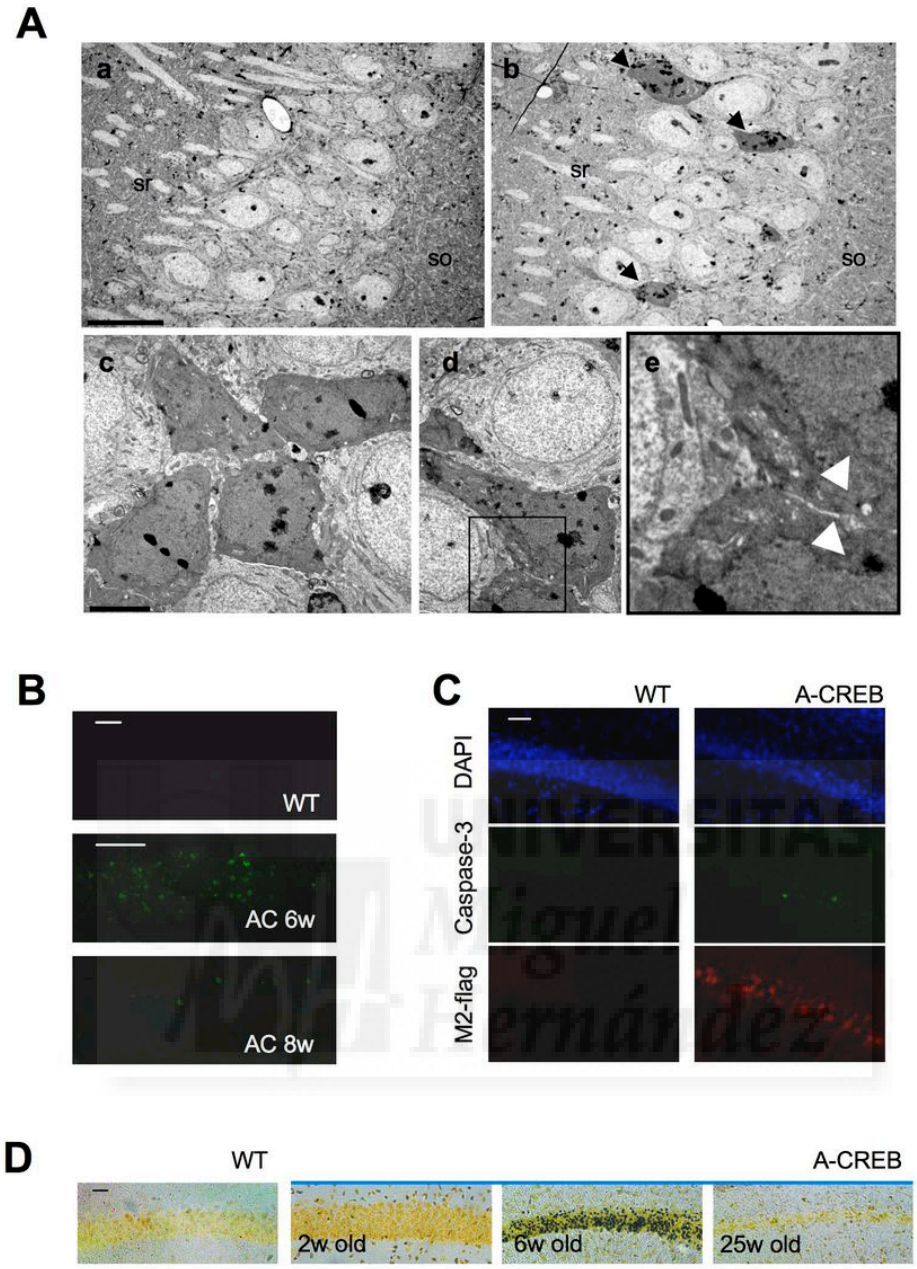

Figure 7. Cell death in the hippocampus of A-CREB mice. A. Micrographs of the CA1 subfield in 6 weeks old A-CREB mice revealed the presence of heteropycnotic pyramidal cells (**b-e**) not observed in sections from wild type animals (**a**). These neurons show early signs of apoptotic-like degeneration, consisting of cell bodies markedly reduced in size and increased electron density as a result of mildly condensation of the nucleus and the cytoplasm, and morphologically injured mitochondria. The nuclear membrane of this neuron is convoluted and chromatin clumps start to appear around the inner membrane of the nucleus (**e,** white arrows). **B.** TUNEL reaction in the hippocampus of 6 and 8 weeks old A-CREB mice and control littermates. Scale: 70 µm. **C.** Anti-caspase-3 immunostaining of CA1 neurons in the hippocampus of 6 weeks old A-CREB mice and control littermates. **D.** Neurosilver staining of brain sections of A-CREB mice and control littermates at indicated ages expressed in weeks.

Late consequences of chronic inhibition of CREB function

The neurodegeneration observed after sustained inhibition of CREB function by A-CREB had important consequences in neuronal physiology and brain function that were independent of the earlier effects of CREB inhibition described above. Thus, whereas 3 weeks old A-CREB mice did not show abnormalities in basal synaptic transmission, one-year old A-CREB mice showed a significant reduction in the response of CA1 pyramidal neurons to stimulation of afferent CA3 axons reflecting the severe loss of neurons in the CA1 subfield (Supplementary Figures S1-3 and S4A). We also tested adult A-CREB mice in an extensive battery of behavioral tasks and detected alterations in some basal behaviors (Supplementary Table 6), such as hyperactivity in an open field (Supplementary Fig. S4B), and strong impairments in spatial navigation (Supplementary Fig. S4C and S4D). These behavioral abnormalities are consistent with the wide spread degeneration detected at this stage.

Discussion

We described here the consequences in neuronal gene expression, plasticity and survival of blocking CREB-dependent gene expression. We showed for the first time that the inhibition of CREB reduced the intrinsic excitability of CA1 neurons through modulation of the I_M current, an alteration that can underlay the reduced seizure susceptibility observed in A-CREB mice. In agreement with previous studies on CREB deficient mutants, we found that the chronic inhibition of CREB function reduced synaptic plasticity in the Schaffer collateral pathway and compromised neuronal viability. Moreover, we demonstrated changes in both basal and activity-induced gene expression that, despite being milder than anticipated, contributed to clarify the genetic program regulated *in vivo* by this family of transcription factors.

Bidirectional modulation of intrinsic and synaptic plasticity by CREB-mediated gene expression

Gain-of-function approaches have demonstrated that the expression of constitutively active CREB facilitates the consolidation of LTP in the Schaffer collateral

pathway (Barco et al., 2002; Marie et al., 2005), suggesting that CREB activity is sufficient to sustain this process. In contrast, loss-of-function studies aimed to investigate the requirement of CREB in L-LTP produced mixed results. Whereas CREB hypomorphic mutants and transgenic mice expressing the dominant negative CREB variant K-CREB showed deficits in some forms of L-LTP (Bourtchuladze et al., 1994; Pittenger et al., 2002; Huang et al., 2004), no deficits were found in mice in which CREB was specifically depleted in forebrain neurons and in transgenic mice expressing the dominant negative CREB variant CREB-M1. These negative results might be caused by insufficient CREB inhibition (Rammes et al., 2000) or by CREM compensation (Balschun et al., 2003). A possible explanation to reconcile these studies would be that CRE-driven gene expression, but not CREB itself, is required for L-LTP in the Schaffer collateral pathway. Testing this hypothesis would require the analysis of animals in which both CREB and CREM activities are simultaneously repressed (Balschun et al., 2003). This seems to be the case in A-CREB mice. Notably, LTP analysis of 3-weeks old A-CREB mice revealed significant deficits in the late phase of LTP, supporting a role for CREB-dependent gene expression in the consolidation of some forms of LTP.

This study also provided first evidence of negative regulation of intrinsic excitability in CA1 neurons by CREB inhibition. Our analysis suggested that modulation of M potassium current, which contributes to spike frequency activation in CA1 pyramidal neurons (Peters et al., 2005), produced a decrement in excitability, a finding that would also contribute to explain the resistance to seizure observed in A-CREB mice. The increase in I_M current observed in CA1 neurons expressing A-CREB resembled that produced by retigabine, an M channel opener that reduces both kindled and epileptic seizures (Rostock et al., 1996). Kindling experiments in bitransgenic mice with chronic inhibition or enhancement of CREB function highlighted the relevance of accurate regulation of neuronal excitability by CREB family in epilepsy. This novel, but now well-established, CREB function suggests that the activation of CREB-dependent gene expression does not only contribute to the stabilization of ongoing reinforcements of synaptic connections (consolidation), but can also facilitate future neuronal responses in a given time range (sensitization).

CREB is sufficient, but not always necessary for activity-driven gene expression

Biochemical and molecular studies have demonstrated the participation of CREB in the regulation of the expression of more than one hundred genes. The availability of complete genome sequences and the widespread application of genome-wide transcriptional profiling and binding mapping techniques have recently allowed the identification of even more potential targets (Euskirchen et al., 2004; Impey et al., 2004; Zhang et al., 2005; Tanis et al., 2008). Complementary to these assays, gene profiling of CREB mutant mice can also contribute to our understanding of the complex gene programs triggered by CREB (McClung and Nestler, 2003; Barco et al., 2005).

The transcriptional response to seizure in the hippocampus of A-CREB and control mice was remarkably similar despite the relatively weaker limbic seizures induced by the drug in A-CREB mice and the clear effects of A-CREB expression in neuronal survival and physiology. Our analysis revealed that the presence of CRE sites in a promoter was not a good predictor of CREB requirement for its seizure-driven transcription (see Table 1 and Supplementary Tables 2-4, column 'CRE sites'), although we cannot discard that these sites could bind CREB under other circumstances. This result is in agreement with a previous study on CREB hypomorphic mice (Blendy et al., 1995) and very recent microarray analyses of activity driven gene expression in CREB/CREM double mutants (Lemberger et al., 2008). However, this result limits our understanding of the molecular mechanisms linking CREB deficiency and observed physiological phenotypes.

Extensive evidence identified the CREB family of transcription factors as a major regulator of activity-dependent gene expression (Lonze and Ginty, 2002; Josselyn and Nguyen, 2005). The relatively modest transcriptional alterations observed in A-CREB mice after kainate injection might be explained by partial or insufficient inhibition of CREB activity by A-CREB. However, the progressive neurodegeneration of CA1 neurons suggested that inhibition of CREB activity in the hippocampus of A-CREB mice was as robust as in CREB/CREM double deficient mutants, which, notably, also showed normal activity-driven gene expression in response to kainate (Lemberger et al., 2008). Another possible explanation would be the compensation by other members of the CREB family, but again the results in CREB/CREM double deficient mutants and the absence of changes in CREM or ATF1 expression in A-CREB mice (Supplementary Table 5) suggested that this is not likely the case. A third explanation

would be that the induction of IEGs by kainate is not mediated solely by CREB and CREs. The promoter region of many IEGs contain binding site for other activity-dependent transcription factors. Mice deficient in the serum response factor (SRF), the main transcription factor binding to the SRE sites also located in the promoter of many IEGs, showed a profound defect in activity-dependent IEG expression, indicating that activity-dependent gene expression in response to epileptic activity may be primarily regulated by this transcription factor rather than by CREB (Ramanan et al., 2005). This does not mean that CREB signaling cascade does not contribute to activity-driven gene expression. On the contrary, CREB/CREM activity seems to be required for most of the cocaine-induced expression changes in the striatum (Lemberger et al., 2008). Moreover, previous studies have shown that CREB contributes to the regulation of important IEGs, such as *bdnf*, *c-fos*, and *JunB*, also in the hippocampus. CREB can even be sufficient for their expression (Barco et al., 2005), but it appears to be not always necessary.

The microarray analysis revealed transcriptional changes that can be highly relevant to explain the phenotype of A-CREB mice. Some genes, such as *arc*, *egr1* or *egr2*, were reduced in the basal condition, and the induction in response to kainate of other genes, such as *penk* and *pdyn*, was impaired. These target genes are known to play critical roles in epileptogenesis, excitability and plasticity. Furthermore, the confluence of diverse subtle changes in gene expression may promote a cascade of summatory events that led to robust phenotypical effects. Further research should determine the precise molecular links between the altered expression patterns and the reduced neuronal excitability and impaired plasticity observed at early times, and the neuronal loss observed at later times.

CREB-dependent gene expression is required for the survival of CA1 neurons

The expression of A-CREB led to massive loss of neurons in the CA1 subfield. Strikingly, loss of CA1 neurons was not observed in any other transgenic line expressing CREB dominant negative mutants (Rammes et al., 2000; Kida et al., 2002; Pittenger et al., 2002). Therefore, it could be suggested that the observed phenotype in A-CREB mice is not due to the block of activity of solely CREB, but also of other CREB family members, which was not the case in the previous transgenic CREB deficiency models. CREB and A-CREB heterodimerize with an affinity 3.3 orders of magnitude greater than CREB homodimers (Ahn et al., 1998). Therefore, as shown in

cell culture (Ching et al., 2004), A-CREB is likely to have a stronger dominant negative effect on CREB than CREB-M1 or K-CREB, the dominant negative variants used in previously described transgenic lines. The level of blockade of CRE-driven gene expression in A-CREB mice may be comparable or higher than that described for CREB/CREM double knockouts. In fact, the neurodegenerative process observed in A-CREB was similar to that described for *Creb1*^{Camkcre4}*Crem*$^{-/-}$ double mutants (Mantamadiotis et al., 2002), suggesting that both CREB and CREM function were effectively blocked by A-CREB expression.

Degenerating neurons in A-CREB mice characteristically exhibited intracellular inclusions, condensation of both the cytoplasm and the nucleus, and ruffling of the plasma membrane while the ultrastructure of cellular organelles was largely preserved. There was no major inflammatory response accompanying neurodegeneration. As in classical apoptosis we observed minimal damage in the surrounding cells. Similar features have been observed in neurons both in the brain of patients who died with HD and in transgenic mouse model for this disease (Leist and Jaattela, 2001), reinforcing the parallelism between CREB deficiency by A-CREB and HD pathology outlined by previous studies on CREB/CREM double mutants (Mantamadiotis et al., 2002).

Gene profiling analysis of A-CREB mice did not reveal significant downregulation of some CREB target genes involved in promoting neuronal survival, such as *bcl-2* and *bdnf*, but identified other genes whose upregulation or downregulation in the basal state may contribute to explain the neurodegenerative process. For instance, *Scn4b*, the cell death-related genes *Cst7* and *Cxcl10* and several genes induced by interferon. These changes may represent an early transcriptional signature preceding neurodegeneration. There may also exist a causal relationship between the early physiological alterations – reduced neuronal excitability and impaired plasticity – and the late pathological events. Lower than normal neuronal activity can, in the long-term, cause the loss of neurons in hippocampal circuits, especially in juvenile animals (Kaindl et al., 2006).

The ability to pause and reactivate neurodegeneration using doxycycline makes the transgenic strain described here a powerful animal model to assay therapies aimed to compensate deficiencies in the cAMP signaling pathway, which has been critically involved in Hungtinton's and Alzheimer's disease neurodegeneration (Vitolo et al., 2002; Sugars et al., 2004).

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Supplemetary material:

Supplementary Methods

Microarray analysis

Each sample contained total RNA from the hippocampi of a group of 3-4 three weeks old mice. We obtained duplicate samples for each experimental condition (in total 14 WT and 20 A-CREB mice where used in this experiment). Mouse Genome 430 2.0 genechips were hybridized, stained, washed and screened for quality according to the manufacturer's protocol. The Affymetrix GeneChip® data were processed, normalized and statistically analyzed using GCOS (Affymetrix), GeneSpring GX (Agilent Technologies) and dChip softwares (Li & Hung Wong, 2001). After the normalization by the median intensities of the arrays, linearity of the signal intensities between arrays was confirmed and Principal Component Analysis (PCA) was performed to check the similarities of the arrays. GeneSpring and dChip softwares were used in parallel and produced highly overlapping lists of significantly changed probe sets after the following filtering: fold change >1.5, signal intensities >20% of the maximal expression (GeneSpring) and PM-MM difference model, >50 (dChip), $P < 0.05$ in Student's t test. The lists presented in Supplemental Tables 2-4 were generated primarily using GeneSpring software because it permitted additional graphical representations and further statistical analyses, such as the *k*-means and hierarchical clustering showed in Figure 5. In the analysis of 3-weeks old mice, we considered two parameters: genotype (wild-type or A-CREB) and drug (three conditions, saline, kainate 14 mg/Kg and kainate 18 mg/Kg). Given this experimental design, two-way ANOVA statistical analysis was performed after filtering the data by expression level. We found 2025 genes significantly affected by drug treatment, 830 genes significantly affected by genotype and 439 in the genotype-drug interaction group (See Supplementary Tables 2-4 for full lists of probe sets showing a fold change equal or higher than 2 in relevant comparisons). We also conducted a complementary pair-wise comparison analysis in GCOS to retrieve additional genes in the non-stimulated condition (wild-type saline vs. A-CREB saline). Probe sets retrieved in both pair-wise comparison replicates were compared to the list generated in the GeneSpring analysis to produce Supplemental Table 4.

Behavioral analysis

For all behavioral tasks, we used adult male mutant and control littermates. The battery of behavioral tasks was initiated when the animals were 2 moths old and finished when they were 4 months old. The experimenter was blind to genotypes in all studies. *SHIRPA primary screen:* Mice were evaluated using a modification of Irwin procedure (Irwin *et al*., 1968). *Open field:* Mice were placed in 50 × 50 cm^2 open-field chambers and monitored throughout the test session (30 min) by a video-tracking system (SMART, Panlab S.L.), which records the position of each animal every 0.5 sec. *Water Maze:* The visible and hidden platform tasks were carried out in a 170 cm pool using SMART software (Panlab S.L.). Four training trials, 120 s maximum and 30-100 min ITI (inter-trial interval) were given daily. Probe trials (60 s) were performed to assess retention of the previously acquired information.

Supplementary tables:

Supplementary Table 1. Electrophysiological properties of CA1 pyramidal neurons in A-CREB mice.

Supplementary Table 1. Electrophysiological properties of CA1 pyramidal neurons in A-CREB mice

Group of mice (n=neurons, mice)	R_{in} (MΩ)	Rehobase (pA)	Medium I_{AHP} (pA)	slow I_{AHP} (pA)
A-CREB On (n=42, 6)	131±9.6	179±9.7	291±13.7	50±5.3
Control On (n=40, 5)	182±19.5	136±8.1	234±12.8	42±4.7
P	***0.01***	***0.001***	***0.003***	*0.26*
A-CREB On/Off (n=32, 4)	151±8.1	142±9.7	200±11.9	27±3.4
Control On/Off (n=17, 2)	159±23.7	144±15.2	143±12.1	31±6.1
P	*0.67*	*0.9*	***0.003***	*0.56*
A-CREB XE 991 (n=14, 3)	256±42	117±16.8	170±19.0	33±6.3
Control XE 991 (n=14, 2)	229±20	121±15.1	131±14.2	21±3.7
P	*0.56*	*0.87*	*0.11*	*0.11*

In A-CREB On mice and control littermates, the recordings were performed in 3-week old animals. In A-CREB On/Off mice and control littermates, the transgene was expressed for 3 weeks and then silenced for 10 additional days. Recording in neurons treated with XE 991 were performed in 3-week old mice.

Supplementary Table 2. Genes altered in the hippocampus of A-CREB mice (GENOTYPE significant genes).

Supplementary Table 2

Genes altered in the hippocampus of A-CREB mice (GENOTYPE significant genes, p<0,05)

Two-way ANOVA analysis: 24 genes out of 830 genes significantly affected by genotype exhibited a fold change larger than 2 in the comparison between genotypes in animals injected either with saline or kainate

Some genes, such as Scn4b and Penk1, are reduced both in animals injected with saline and kainate, whereas the genotype effect in other genes, such as Pdyn and Doc2b, is only evidenced in response to kainate

FC saline = fold change wild-type saline vs. A-CREB saline
FC KA14 = fold change wild-type 14 mg/Kg kainate vs. A-CREB 14 mg/Kg kainate
FC KA18 = fold change wild-type 14 mg/Kg kainate vs. A-CREB 18 mg/Kg kainate
CRE sites = number of CRE predicted in murine promoters (3 Kb upstream and 200 bp dowstream of the transcription start site, see Zhang et al. (2005), PNAS 102:4459-64, for further details)
n.d. = not determined / not found
Fold changes < 1.5 are indicated in gray

Genes downregulated in A-CREB mice

Probe Set ID	Gene Symbol	Gene Title	Unigene ID	FC saline	FC KA14	FC KA18	*P* value	CRE sites
1437397_at	Prlr	prolactin receptor	Mm.442298	-2,02	-1,10	-1,25	0,031	4
1434008_at	Scn4b	sodium channel, type IV, beta	Mm.335112	-1,80	-3,23	-3,54	0,035	n.d.
1427038_at	Penk1	preproenkephalin 1	Mm.2899	-1,54	-2,69	-3,34	0,027	n.d.
1443287_at	Gm1337	gene model 1337, (NCBI)	Mm.35758	-1,42	-2,21	-1,72	0,006	n.d.
1429215_at	2310058N22Rik	RIKEN cDNA 2310058N22 gene	Mm.440654	-1,40	-2,08	-2,03	0,007	n.d.
1444687_at	C1ql2	complement component 1, q subcomponent-like 2	Mm.337409	-1,30	-2,55	-2,31	0,003	n.d.
1420666_at	Doc2b	double C2, beta	Mm.5137	-1,29	-1,99	-2,04	0,022	4
1416266_at	Pdyn	prodynorphin	Mm.6239	-1,21	-1,92	-2,01	0,013	2

Genes upregulated in A-CREB mice

Probe Set ID	Gene Symbol	Gene Title	Unigene ID	FC saline	FC KA14	FC KA18	*P* value	CRE sites
1427532_at	Trat1	T cell receptor associated transmembrane adaptor 1	Mm.167298	8,42	5,95	5,16	0,000	n.d.
1437561_at	Trat1	T cell receptor associated transmembrane adaptor 1	Mm.167298	3,72	4,73	4,58	0,000	n.d.
1421009_at	Rsad2	radical S-adenosyl methionine domain containing 2	Mm.24045	2,29	1,42	1,12	0,045	n.d.
1426278_at	Ifi27	interferon, alpha-inducible protein 27	Mm.271275	2,16	1,46	1,65	0,032	n.d.
1453196_a_at	Oasl2	2'-5' oligoadenylate synthetase-like 2	Mm.228363	2,05	2,79	2,92	0,002	2
1450154_at	Folh1	folate hydrolase	Mm.269137	2,00	1,37	1,16	0,037	0
1418930_at	Cxcl10	chemokine (C-X-C motif) ligand 10	Mm.877	2,00	2,50	2,47	0,003	4
1419042_at	Iigp1	interferon inducible GTPase 1	Mm.440723	1,63	2,28	2,02	0,020	1
1420699_at	Clec7a	C-type lectin domain family 7, member a	Mm.239516	1,55	2,09	2,48	0,005	n.d.
1443086_at	Alcam	activated leukocyte cell adhesion molecule	Mm.288282	1,53	2,49	3,29	0,015	4
1419202_at	Cst7	cystatin F (leukocystatin)	Mm.12965	1,43	2,44	1,91	0,028	0
1419043_a_at	Iigp1	interferon inducible GTPase 1	Mm.440723	1,38	2,99	2,20	0,003	1
1453793_at	1700026J12Rik	RIKEN cDNA 1700026J12 gene	Mm.307720	1,33	1,96	2,17	0,029	n.d.
1440866_at	Eif2ak2	eukaryotic translation initiation factor 2-alpha kinase 2	Mm.378990	1,24	2,03	1,56	0,050	2
1424825_a_at	Glycam1	glycosylation dependent cell adhesion molecule 1	Mm.219621	1,15	2,13	1,74	0,042	0
1447927_at	Mpa2l	macrophage activation 2 like LOC626578	Mm.458491	1,00	2,07	1,78	0,050	n.d.

Supplementary Table 3. Genes altered in the hippocampus of A-CREB mice and control littermates after kainate treatment (DRUG significant genes).

Supplementary Table 3

Genes altered in the hippocampus of A-CREB mice and control littermates after kainate treatment (DRUG significant genes, p>0.05)
Two-way ANOVA analysis: 210 genes out of 2025 genes significantly affected by drug treatment exhibited a fold change larger than 2 in the comparison between drug treatments in animals of either genotype
FC WT = fold change wild-type saline vs. wild-type 14 mg/Kg kainate
FC AC KA14 = fold change A-CREB saline vs. A-CREB 14 mg/Kg kainate
FC AC KA18 = fold change A-CREB saline vs. A-CREB 18 mg/Kg kainate
CRE sites = number of CRE predicted in murine promoters (3 Kb upstream and 200 bp downstream of the transcription start site, see Zhang et al. (2005), PNAS 102:4459-64, for further details)
n.d. = not determined
Fold changes < 2 are indicated in grey

Probe Set ID	Gene Symbol	Gene Title	Unigene ID	FC WT	FC AC KA14	FC AC KA18	*P* value	CRE sites
1459372_at	Npas4	neuronal PAS domain protein 4	Mm.287867	41,88	64,30	53,63	0,000	5
1422134_at	Fosb	FBJ osteosarcoma oncogene B	Mm.248335	15,43	24,64	19,06	0,000	7
1449363_at	Atf3	activating transcription factor 3	Mm.2706	13,23	13,34	9,70	0,000	3
1417262_at	Ptgs2	prostaglandin-endoperoxide synthase 2	Mm.292547	9,70	11,17	8,61	0,000	2
1423100_at	Fos	FBJ osteosarcoma oncogene	Mm.246513	9,57	12,74	12,09	0,000	9
1417263_at	Ptgs2	prostaglandin-endoperoxide synthase 2	Mm.292547	9,39	10,63	8,87	0,000	2
1425671_at	Homer1	homer homolog 1 (Drosophila)	Mm.37533	8,84	10,30	6,14	0,002	4
1416250_at	Btg2	B-cell translocation gene 2, anti-proliferative	Mm.392646	8,58	7,38	9,05	0,000	5
1450842_a_at	Cenpa	centromere protein A	Mm.290563	8,50	6,46	3,78	0,000	3
1422053_at	Inhba	inhibin beta-A	Mm.8042	6,57	8,88	7,43	0,000	2
1450971_at	Gadd45b	growth arrest and DNA-damage-inducible 45 beta	Mm.1360	5,85	6,35	4,60	0,000	3
1438133_a_at	Cyr61	cysteine rich protein 61	Mm.1231	5,73	11,14	11,46	0,000	8
1449773_s_at	Gadd45b	growth arrest and DNA-damage-inducible 45 beta	Mm.1360	5,68	7,29	5,58	0,000	3
1427683_at	Egr2	early growth response 2	Mm.290421	5,60	14,71	12,51	0,000	3
1427682_a_at	Egr2	early growth response 2	Mm.290421	5,36	12,91	12,96	0,000	3
1426063_a_at	Gem	GTP binding protein (gene overexpressed in skeletal muscle)	Mm.247486	5,27	9,99	8,16	0,000	5
1453851_a_at	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	Mm.281298	5,05	7,66	8,73	0,000	3
1435872_at	Pim1	Proviral integration site 1	Mm.328931	5,03	5,96	4,62	0,000	4
1417483_at	Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Mm.247272	4,84	6,53	4,89	0,000	n.d.
1415899_at	Junb	Jun-B oncogene	Mm.1167	4,80	5,39	4,63	0,001	6
1419816_s_at	Errfi1	ERBB receptor feedback inhibitor 1	Mm.318841	4,79	4,63	3,84	0,000	n.d.
1421811_at	Thbs1	thrombospondin 1	Mm.4159	4,78	7,02	4,11	0,000	1
1419247_at	Rgs2	regulator of G-protein signaling 2	Mm.28262	4,74	6,11	5,25	0,000	4
1429444_at	Rasl11a	RAS-like, family 11, member A	Mm.266978	4,72	3,79	3,05	0,000	n.d.
1422168_a_at	Bdnf	brain derived neurotrophic factor	Mm.1442	4,50	4,57	3,60	0,000	1
1416129_at	Errfi1	ERBB receptor feedback inhibitor 1	Mm.318841	4,42	5,03	4,27	0,000	n.d.
1448272_at	Btg2	B-cell translocation gene 2, anti-proliferative	Mm.392646	4,30	4,93	6,02	0,000	5
1436387_at	C330006P03Rik ///	homer homolog 1 (Drosophila) intron	Mm.37533	4,28	10,25	6,95	0,001	n.d.
1418250_at	Arl4d	ADP-ribosylation factor-like 4D	Mm.266840	4,21	4,81	4,69	0,001	n.d.
1416039_x_at	Cyr61	cysteine rich protein 61	Mm.1231	4,14	10,60	11,62	0,000	8
1416442_at	Ier2	immediate early response 2	Mm.399	4,04	5,69	6,31	0,002	4
1420720_at	Nptx2	neuronal pentraxin 2 (Narp)	Mm.10099	4,02	4,79	3,08	0,006	3
1449977_at	Egr4	early growth response 4	Mm.44137	3,99	4,64	3,64	0,000	4
1447830_s_at	Rgs2	regulator of G-protein signaling 2	Mm.28262	3,98	5,59	4,82	0,000	4
1449960_at	Nptx2	neuronal pentraxin 2	Mm.10099	3,82	4,83	3,44	0,000	3
1452160_at	Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	Mm.246398	3,76	4,63	4,10	0,000	n.d.
1419248_at	Rgs2	regulator of G-protein signaling 2	Mm.28262	3,68	4,90	4,27	0,000	4
1437884_at	Arl5b	ADP-ribosylation factor-like 5B	Mm.174068	3,66	4,74	3,27	0,001	n.d.
1418687_at	Arc	activity regulated cytoskeletal-associated protein	Mm.25405	3,58	6,41	5,76	0,002	2
1418932_at	Nfil3	nuclear factor, interleukin 3, regulated	Mm.136604	3,46	4,10	2,93	0,003	6
1452161_at	Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	Mm.246398	3,45	3,77	3,40	0,002	n.d.
1447863_s_at	Nr4a2	nuclear receptor subfamily 4, group A, member 2	Mm.3507	3,43	3,81	2,88	0,001	6
1416067_at	Ifrd1	interferon-related developmental regulator 1	Mm.168	3,43	4,97	4,29	0,000	5
1455899_x_at	Socs3	suppressor of cytokine signaling 3	Mm.3468	3,34	6,32	4,23	0,000	2
1455085_at	1700086L19Rik	RIKEN cDNA 1700086L19 gene	Mm.287421	3,32	2,58	2,44	0,000	n.d.
1422169_a_at	Bdnf	brain derived neurotrophic factor	Mm.1442	3,29	3,39	2,35	0,001	1
1427747_a_at	Lcn2	lipocalin 2	Mm.9537	3,22	2,64	1,66	0,004	1
1455166_at	Arl5b	ADP-ribosylation factor-like 5B	Mm.174068	3,22	4,23	3,12	0,000	n.d.
1448830_at	Dusp1	dual specificity phosphatase 1	Mm.239041	3,18	4,31	4,78	0,000	2
1423294_at	Mest	mesoderm specific transcript	Mm.335639	3,12	4,47	3,41	0,000	1
1448285_at	Rgs4	regulator of G-protein signaling 4	Mm.41642	3,07	3,46	3,02	0,000	3
1450750_a_at	Nr4a2	nuclear receptor subfamily 4, group A, member 2	Mm.3507	3,06	3,88	3,13	0,001	6
1416505_at	Nr4a1	nuclear receptor subfamily 4, group A, member 1	Mm.119	3,05	3,93	3,81	0,001	6
1417051_at	Pcdh8	protocadherin 8	Mm.390715	3,05	2,76	2,25	0,001	3
1455271_at	LOC620695	hypothetical protein LOC620695	Mm.425110	3,04	2,68	2,47	0,002	n.d.
1417394_at	Klf4	Kruppel-like factor 4 (gut)	Mm.4325	3,00	5,41	5,03	0,001	2
1447825_x_at	Pcdh8	protocadherin 8	Mm.390715	2,99	3,18	2,74	0,001	3
1455034_at	Nr4a2	Nuclear receptor subfamily 4, group A, member 2	Mm.3507	2,98	3,52	2,81	0,000	6
1450749_a_at	Nr4a2	nuclear receptor subfamily 4, group A, member 2	Mm.3507	2,91	2,94	2,15	0,002	6
1418666_at	Ptx3	pentraxin related gene	Mm.276776	2,89	1,84	1,30	0,006	1
1435458_at	Pim1	proviral integration site 1	Mm.328931	2,88	3,02	2,06	0,000	4
1441228_at	Apold1	apolipoprotein L domain containing 1	Mm.296104	2,82	4,64	4,16	0,000	n.d.
1444681_at	Erc2	ELKS/RAB6-interacting/CAST family member 2	Mm.318004	2,75	2,45	1,70	0,002	n.d.
1429863_at	Lonrf3	LON peptidase N-terminal domain and ring finger 3	Mm.327654	2,74	2,34	1,81	0,001	n.d.
1427540_at	Zwint	ZW10 interactor	Mm.62676	2,72	3,84	2,84	0,004	3
1418936_at	Maff	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	Mm.86646	2,70	4,23	2,91	0,000	3
1428834_at	Dusp4	dual specificity phosphatase 4	Mm.392187	2,69	2,75	1,88	0,003	6
1417406_at	Sertad1	SERTA domain containing 1	Mm.153684	2,68	3,06	2,19	0,003	n.d.
1438796_at	Nr4a3	nuclear receptor subfamily 4, group A, member 3	Mm.247261	2,64	3,24	2,62	0,000	2
1426870_at	Fbxo33	F-box protein 33	Mm.311026	2,63	3,17	2,80	0,000	n.d.
1426721_s_at	Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	Mm.246398	2,57	3,86	3,68	0,000	n.d.
1437696_at	BC049807	cDNA sequence BC049807	Mm.441097	2,56	2,73	2,42	0,006	n.d.
1456212_x_at	Socs3	suppressor of cytokine signaling 3	Mm.3468	2,54	4,48	3,13	0,000	2
1426871_at	Fbxo33	F-box protein 33	Mm.311026	2,53	3,57	2,98	0,001	n.d.
1460302_at	Thbs1	thrombospondin 1	Mm.4159	2,53	2,97	2,37	0,000	1
1436305_at	Ibrdc1	IBR domain containing 1	Mm.295212	2,53	2,47	1,69	0,002	n.d.
1440179_x_at	Ibrdc1	IBR domain containing 1	Mm.295212	2,53	2,70	1,91	0,002	n.d.
1452352_at	Ctla2b	cytotoxic T lymphocyte-associated protein 2 beta	Mm.439734	2,52	3,60	2,28	0,005	n.d.
1417395_at	Klf4	Kruppel-like factor 4 (gut)	Mm.4325	2,50	4,63	3,95	0,001	2
1451280_at	Arpp21	cyclic AMP-regulated phosphoprotein, 21	Mm.297444	2,50	2,53	2,31	0,012	n.d.
1453590_at	Arl5b	ADP-ribosylation factor-like 5B	Mm.174068	2,49	3,93	2,82	0,001	n.d.
1434350_at	Axud1	AXIN1 up-regulated 1	Mm.125196	2,48	2,44	1,99	0,000	7
1437247_at	Fost2	fos-like antigen 2	Mm.24684	2,47	5,08	3,63	0,001	5
1416756_at	Dnajb1	DnaJ (Hsp40) homolog, subfamily B, member 1	Mm.282092	2,46	2,82	2,64	0,003	6
1459941_at	Rlbp1l1	retinaldehyde binding protein 1-like 1	Mm.458809	2,45	2,47	1,50	0,004	n.d.
1417409_at	Jun	Jun oncogene	Mm.275071	2,43	2,60	2,50	0,002	4
1416811_s_at	Ctla2a / Ctla2b	cytotoxic T lymphocyte-associated protein 2 alpha / beta	Mm.439734	2,43	2,33	1,58	0,011	3 / n.d.
1448728_a_at	Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Mm.247272	2,41	3,55	2,39	0,001	n.d.
1442340_x_at	Cyr61	cysteine rich protein 61	Mm.1231	2,37	3,66	7,81	0,005	8
1416287_at	Rgs4	regulator of G-protein signaling 4	Mm.41642	2,35	3,30	2,80	0,000	3
1457823_at	Cyr61	cysteine rich protein 61	Mm.1231	2,34	2,91	5,88	0,002	8
1419209_at	Cxcl1	chemokine (C-X-C motif) ligand 1	Mm.21013	2,34	3,58	3,19	0,002	2
1418322_at	Crem	cAMP responsive element modulator	Mm.5244	2,34	3,50	3,02	0,002	1
1451680_at	Snx1	sulfiredoxin 1 homolog (S. cerevisiae)	Mm.218639	2,32	1,82	1,50	0,013	n.d.
1420499_at	Gch1	GTP cyclohydrolase 1	Mm.10651	2,29	2,02	1,64	0,002	n.d.
1421396_at	Pcsk1	proprotein convertase subtilisin/kexin type 1	Mm.1333	2,28	2,74	2,34	0,000	3
1448471_a_at	Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha	Mm.30144	2,28	2,31	1,53	0,013	3
1421679_a_at	Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	Mm.195663	2,27	1,74	1,34	0,021	3
1416431_at	Tubb6	tubulin, beta 6	Mm.181860	2,26	2,43	1,81	0,000	n.d.

1416953_at	Ctgf	connective tissue growth factor	Mm.393058	1,71	2,23	1,69	0,006	2
1424107_at	Kif18a	kinesin family member 18A	Mm.274086	1,70	2,44	1,83	0,026	5
1450690_at	Ranbp2	RAN binding protein 2	Mm.431695	1,70	2,03	1,56	0,006	7
1451289_at	Dclk1	doublecortin-like kinase 1	Mm.295263	1,70	2,17	2,03	0,009	n.d.
1435248_a_at	Btaf1	BTAF1 RNA polymerase II, B-TFIID transcription factor-associated, (Mot1 homolog, S. cerevisiae)	Mm.295062	1,69	2,49	1,93	0,015	n.d.
1416892_s_at	3110001A13Rik	RIKEN cDNA 3110001A13 gene	Mm.277864	1,69	2,15	1,78	0,001	1
1446840_at		11 days embryo head RIKEN cDNA, clone:6230412O08	Mm.437560	1,68	2,29	1,56	0,003	n.d.
1451340_at	Arid5a	AT rich interactive domain 5A (Mrf1 like)	Mm.34316	1,68	2,09	1,72	0,002	n.d.
1422931_at	Fosl2	fos-like antigen 2	Mm.24684	1,68	2,28	2,17	0,005	5
1436659_at		Adult male olfactory brain RIKEN cDNA, 6430555I10	Mm.443080	1,67	2,35	2,07	0,002	n.d.
1417612_at	Ier5	immediate early response 5	Mm.12246	1,66	2,42	1,81	0,008	3
1435137_s_at	1200015M12Rik //	Too many locations	Mm.332931	1,66	2,12	1,54	0,002	n.d.
1457651_x_at	Rem2	rad and gem related GTP binding protein 2	Mm.274727	1,66	2,04	1,52	0,004	2
1434967_at	Zswim6	zinc finger, SWIM domain containing 6	Mm.433838	1,66	2,09	1,54	0,004	n.d.
1430535_at	Tsc22d2	TSC22 domain family 2	Mm.218409	1,65	2,12	1,75	0,001	n.d.
1441894_s_at	Grasp	GRP1 (general receptor for phosphoinositides 1)-associated scaffold protein	Mm.276573	1,64	2,06	1,69	0,002	3
1422821_at	Ranbp2	RAN binding protein 2	Mm.401648	1,62	2,01	1,71	0,009	7
1416755_at	Dnajb1	DnaJ (Hsp40) homolog, subfamily B, member 1	Mm.282092	1,62	2,05	2,31	0,009	6
1456216_at		casein kinase I (CKI) alpha intron		1,61	2,25	2,43	0,003	n.d.
1449475_at	Atp12a	ATPase, H+/K+ transporting, nongastric, alpha polypeptide	Mm.273271	1,61	2,60	1,93	0,029	3
1435595_at	1810011O10Rik	RIKEN cDNA 1810011O10 gene	Mm.25775	1,60	2,48	1,84	0,010	3
1426599_a_at	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1	Mm.21002	1,60	2,04	1,61	0,003	1
1448694_at	Jun	Jun oncogene	Mm.275071	1,60	2,12	2,03	0,001	4
1457167_at	Med14	mediator complex subunit 14		1,59	2,38	2,10	0,011	n.d.
1450767_at	Nedd9	neural precursor cell expressed, developmentally down-regulated gene 9	Mm.288980	1,59	2,03	1,34	0,048	3
1448117_at	Kitl	kit ligand	Mm.45124	1,58	2,00	1,74	0,014	3
1423852_at	Tmem46	transmembrane protein 46	Mm.275409	1,55	2,26	1,80	0,005	n.d.
1451415_at	1810011O10Rik	RIKEN cDNA 1810011O10 gene	Mm.25775	1,53	2,31	1,88	0,013	3
1446981_at	A830010M20Rik	RIKEN cDNA A830010M20 gene	Mm.261891	1,53	2,09	1,40	0,050	n.d.
1436202_at		mapped to genomic region in chromosome 19		1,53	1,84	2,89	0,034	n.d.
1430352_at	Adamts9	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 9	Mm.257557	1,50	2,50	2,43	0,003	n.d.
1441536_at	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	Mm.61526	1,49	2,19	2,29	0,030	1
1455872_at	BC065085	cDNA sequence BC065085	Mm.37882	1,49	2,30	1,55	0,006	n.d.
1437100_x_at	Pim3	proviral integration site 3	Mm.400129	1,49	2,01	1,89	0,001	2
1452521_a_at	Plaur	plasminogen activator, urokinase receptor	Mm.1359	1,48	2,33	1,92	0,002	0
1418681_at	Alg13	asparagine-linked glycosylation 13 homolog (S. cerevisiae)	Mm.249084	1,48	2,37	1,72	0,012	n.d.
1433581_at	1190002N15Rik	RIKEN cDNA 1190002N15 gene	Mm.258746	1,47	2,16	1,91	0,004	n.d.
1422609_at	Arpp19	cAMP-regulated phosphoprotein 19	Mm.247837	1,42	2,21	2,09	0,005	2
1425964_x_at	Hspb1	heat shock protein 1	Mm.13849	1,42	2,00	1,94	0,001	4
1442700_at	Pde4b	phosphodiesterase 4B, cAMP specific	Mm.20181	1,39	2,32	2,53	0,007	2
1424893_at	Ndel1	nuclear distribution gene E-like homolog 1 (A. nidulans)	Mm.31979	1,39	1,71	2,09	0,033	5
1457404_at	Nfkbiz	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Mm.247272	1,36	1,72	2,25	0,008	n.d.
1455182_at	Kif1b	kinesin family member 1B	Mm.402393	1,28	2,12	1,77	0,014	n.d.
1418937_at	Dio2	deiodinase, iodothyronine, type II	Mm.21389	1,28	2,20	1,67	0,012	3
1423422_at	Asb4	ankyrin repeat and SOCS box-containing protein 4	Mm.51340	1,21	2,05	1,40	0,044	1
1457984_at	Crh	corticotropin releasing hormone	Mm.290689	1,08	3,41	3,46	0,045	n.d.
1429905_at	Lhx9	LIM homeobox protein 9	Mm.446030	-1,05	-2,09	-1,79	0,025	1
1423635_at	Bmp2	bone morphogenetic protein 2	Mm.103205	-1,13	2,21	1,87	0,045	1
1426514_at	4631426J05Rik	RIKEN cDNA 4631426J05 gene	Mm.213582	-1,44	-1,80	-2,02	0,014	4
1460625_at	Gm1568	gene model 1568, (NCBI)	Mm.29367	-1,48	-2,13	-1,52	0,011	n.d.
1428682_at	Zc3h6	zinc finger CCCH type containing 6	Mm.26377	-1,56	-2,33	-1,98	0,002	n.d.
1423146_at	Hes5	hairy and enhancer of split 5 (Drosophila)	Mm.137268	-1,64	-2,37	-1,82	0,035	3
1456010_x_at	Hes5	hairy and enhancer of split 5 (Drosophila)	Mm.137268	-1,74	-2,54	-1,77	0,033	3
1455147_at		genomic region in chromosome 17	Mm.440042	-1,80	-2,24	-1,94	0,017	n.d.
1455865_at	Insm1	insulinoma-associated 1	Mm.379070	-1,90	-2,15	-1,94	0,004	0

1450377_at	LOC640441	similar to thrombospondin 1		2,24	2,06	1,80	0,001	n.d.
1440104_at	Ranbp2	RAN binding protein 2	Mm.401648	2,24	2,86	4,14	0,021	7
1451264_at	Frmd6	FERM domain containing 6	Mm.2962	2,23	3,43	2,27	0,002	n.d.
1427005_at	Plk2	polo-like kinase 2 (Drosophila)	Mm.380	2,23	1,94	1,72	0,002	4
1453287_at	5730557B15Rik	RIKEN cDNA 5730557B15 gene	Mm.102470	2,23	3,72	2,50	0,004	3
1453326_at	3300001A09Rik	RIKEN cDNA 3300001A09 gene	Mm.106353	2,23	1,55	1,45	0,000	n.d.
1436329_at	Egr3	early growth response 3	Mm.103737	2,18	3,16	2,50	0,006	5
1417357_at	Emd	emerin	Mm.18892	2,18	2,71	2,21	0,000	4
1421756_a_at	Gpr19	G protein-coupled receptor 19	Mm.4787	2,17	2,04	1,92	0,000	2
1452418_at	1200016E24Rik //	Too many locations	Mm.332931	2,15	2,23	1,51	0,003	n.d.
1444402_at	Zc3h12c	zinc finger CCCH type containing 12C	Mm.390172	2,15	1,99	1,40	0,024	n.d.
1424248_at	Arpp21	cyclic AMP-regulated phosphoprotein, 21	Mm.297444	2,14	2,64	2,57	0,002	n.d.
1453238_s_at	3930401B19Rik //	Too many locations	Mm.440795	2,14	2,62	1,74	0,003	n.d.
1422562_at	Rrad	Ras-related associated with diabetes	Mm.29467	2,12	2,61	1,94	0,001	3
1427539_a_at	Zwint	ZW10 interactor	Mm.62876	2,09	4,67	3,76	0,024	3
1450716_at	Adamts1	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	Mm.1421	2,07	3,33	3,14	0,004	3
1422324_a_at	Pthlh	Parathyroid hormone-related protein precursor	Mm.28440	2,07	1,81	1,34	0,003	3
1415834_at	Dusp6	dual specificity phosphatase 6	Mm.1791	2,07	2,36	2,34	0,012	2
1435119_at		Adult male medulla oblongata RIKEN cDNA, clone:6330578N16	Mm.396027	2,05	1,71	1,26	0,038	n.d.
1429856_at	Tspan18	tetraspanin 18	Mm.274159	2,04	1,10	1,21	0,016	n.d.
1455130_at	Spty2d1	SPT2, Suppressor of Ty, domain containing 1 (S. cerevisiae)	Mm.155687	2,04	3,17	2,92	0,001	n.d.
1460510_a_at	Coq10b	coenzyme Q10 homolog B (S. cerevisiae)	Mm.281019	2,04	2,44	2,15	0,000	n.d.
1423619_at	Rasd1	RAS, dexamethasone-induced 1	Mm.3903	2,03	1,94	1,77	0,003	6
1437199_at	Dusp5	dual specificity phosphatase 5	Mm.52043	2,03	2,60	2,24	0,003	n.d.
1416529_at	Emp1	epithelial membrane protein 1	Mm.182785	2,03	3,26	2,78	0,001	3
1422554_at	Ndnl2	necdin-like 2	Mm.19944	2,00	1,53	1,23	0,039	1
1428562_at	2210403K04Rik	RIKEN cDNA 2210403K04 gene	Mm.458401	2,00	1,61	1,29	0,031	n.d.
1450708_at	Scg2	secretogranin II	Mm.5038	2,00	2,06	1,82	0,001	5
1442014_at		interferon-related developmental regulator 1 (hierve growth factor inducible protein PC4) intron	Mm.441586	1,99	3,66	3,76	0,005	n.d.
1416286_at	Rgs4	regulator of G-protein signaling 4	Mm.41642	1,99	2,93	2,44	0,000	3
1436871_at	Sfrs7	splicing factor, arginine/serine-rich 7	Mm.292016	1,97	2,05	1,90	0,029	1
1419706_a_at	Akap12	A kinase (PRKA) anchor protein (gravin) 12	Mm.27481	1,96	3,72	2,49	0,000	3
1449227_at	Ch25h	cholesterol 25-hydroxylase	Mm.30824	1,95	2,41	1,60	0,007	3
1428923_at	Ppp1r3g	protein phosphatase 1, regulatory (inhibitor) subunit 3G	Mm.44745	1,92	2,96	2,42	0,018	n.d.
1452519_a_at	Zfp36	zinc finger protein 36	Mm.389856	1,91	2,37	2,41	0,000	4
1424880_at	Trib1	tribbles homolog 1 (Drosophila)	Mm.40298	1,91	2,26	2,13	0,002	n.d.
1434885_at	Spty2d1	SPT2, Suppressor of Ty, domain containing 1 (S. cerevisiae)	Mm.155687	1,91	2,42	2,21	0,010	n.d.
1428083_at	2310043N10Rik	RIKEN cDNA 2310043N10 gene		1,90	3,43	2,81	0,010	n.d.
1449037_at	Crem	cAMP responsive element modulator	Mm.5244	1,90	2,35	2,31	0,005	1
1448325_at	Myd116	myeloid differentiation primary response gene 116	Mm.4048	1,90	2,13	2,25	0,002	3
1418572_x_at	Tnfrsf12a	tumor necrosis factor receptor superfamily, member 12a	Mm.28518	1,89	2,27	1,75	0,006	4
1443196_at		13 days embryo heart RIKEN cDNA, D330001J12	Mm.373919	1,88	2,39	1,56	0,010	n.d.
1428487_s_at	Coq10b	coenzyme Q10 homolog B (S. cerevisiae)	Mm.281019	1,85	2,54	2,23	0,000	n.d.
1434585_at	2210038L17Rik	RIKEN cDNA 2210038L17 gene	Mm.440133	1,85	2,10	1,67	0,010	n.d.
1417065_at	Egr1	early growth response 1	Mm.181959	1,84	3,27	3,10	0,001	5
1451612_at	Mt1	metallothionein 1	Mm.192991	1,83	1,76	2,13	0,006	6
1457644_s_at	Cxcl1	chemokine (C-X-C motif) ligand 1	Mm.21013	1,81	2,35	1,90	0,017	2
1435249_at	Btaf1	BTAF1 RNA polymerase II, B-TFIID transcription factor-associated, (Mot1 homolog, S. cerevisiae)	Mm.295062	1,80	2,29	1,87	0,003	n.d.
1460275_at	Gpr3	G-protein coupled receptor 3	Mm.4721	1,80	2,33	1,71	0,001	8
1426081_a_at	Dio2	deiodinase, iodothyronine, type II	Mm.21389	1,79	2,12	1,82	0,047	3
1448509_at	3110001A13Rik	RIKEN cDNA 3110001A13 gene	Mm.277864	1,78	2,08	1,85	0,006	1
1428758_s_at	Ccdc49	coiled-coil domain containing 49	Mm.33206	1,77	2,62	2,41	0,000	n.d.
1439826_at	Hspa14	heat shock protein 14	Mm.89341	1,74	2,08	1,94	0,001	n.d.
1437481_at	Spdya	Speedy protein A		1,73	2,58	2,27	0,000	n.d.
1433582_at	1190002N15Rik	RIKEN cDNA 1190002N15 gene	Mm.258746	1,73	2,04	1,71	0,004	n.d.
1424271_at	Dclk1	doublecortin-like kinase 1	Mm.295263	1,72	2,22	2,04	0,003	n.d.

Supplementary Table 4. Kainate-induced genes altered in the hippocampus of A-CREB mice at the basal state (Pair-wise significant genes).

Supplementary Table 4

Kainate-induced genes altered in the hippocampus of A-CREB mice at the basal state (Pair-wise significant genes)

FC GCOS = average of fold change control saline vs. A-CREB saline in the two pairs of samples (Batch analysis, GCOS, Affymetrix)

Probe Set ID	Gene Symbol	Gene Title	Unigene ID	FC GCOS	CRE sites
1436387_at	C330006P03Rik	homer homolog 1 (Drosophila) intron	Mm.37533	-1,93	n.d.
1451264_at	Frmd6	FERM domain containing 6	Mm.2962	-1,67	n.d.
1427683_at	Egr2	early growth response 2	Mm.290421	-1,61	3
1418687_at	Arc	activity regulated cytoskeletal-associated protein	Mm.25405	-1,56	2
1422609_at	Arpp19	cAMP-regulated phosphoprotein 19	Mm.247837	-1,56	2
1417065_at	Egr1	early growth response 1	Mm.181959	-1,41	5
1416286_at	Rgs4	regulator of G-protein signaling 4	Mm.41642	-1,32	3
1416287_at	Rgs4	regulator of G-protein signaling 4	Mm.41642	-1,32	3
1422554_at	Ndnl2	necdin-like 2	Mm.19944	1,43	1

Supplementary Table 5. Probe sets targeted to members of the CREB family of transcription factors.

Supplementary Table 5. Probe sets targeted to members of the CREB family of transcription factors

	Fold change			p-value					
Probe Set ID	wt vs A-CREB	SAL vs KA14 (WT)	SAL vs KA14 (AC)	Genotype	Treatment	Both	Unigene ID	Gene Symbol	Gene Description
1417296_at	1.03	-1.18	-1.02	0.53	0.57	0.64	Mm.676	Atf1	activating transcription factor 1
1421582_a_at	1.00	-1.43	1.05	0.53	0.64	0.54	Mm.466618	Creb1	cAMP responsive element binding protein 1
1423402_at	1.50	-1.05	-1.22	0.07	0.41	0.62			
1452529_a_at [1]	1.39	-1.16	-1.27	0.39	0.56	0.89			
1428755_at	1.16	-1.29	-1.25	0.38	0.22	0.93			
1452901_at	1.09	-1.19	-1.13	0.34	0.23	0.82			
1418322_at [2]	-1.00	2.33	3.51	0.45	**0.01***	0.45	Mm.5244	Crem	cAMP responsive element modulator
1449037_at [2]	1.19	1.91	2.35	0.28	**0.03***	0.66			
1430847_a_at [2]	1.15	1.12	1.11	0.20	0.30	0.98			

[1] Probe set 1452529_a_at contains two probes that recognize a sequence common to A-CREB and wild type CREB (Figure 6C). This abnormal pattern of upregulation, affecting only two probes out of 11 escaped conventional screening using GCOS and GeneSpring, but dChip revealed a fold change > 10 (the largest change between wild type and A-CREB mice), when no correction for outliers probes were considered.
[2] Probe set 1430847_a_at is targeted to the 2nd Q-rich domain specific of CREMτ, whereas probe sets 1418322_at and 1449037_at are targeted to the 3'UTR and can therefore recognize the inducible ICER repressor.
* Significant changes, $p<0.05$.

Supplementary Table 6: Basal behavior analysis of A-CREB mice.

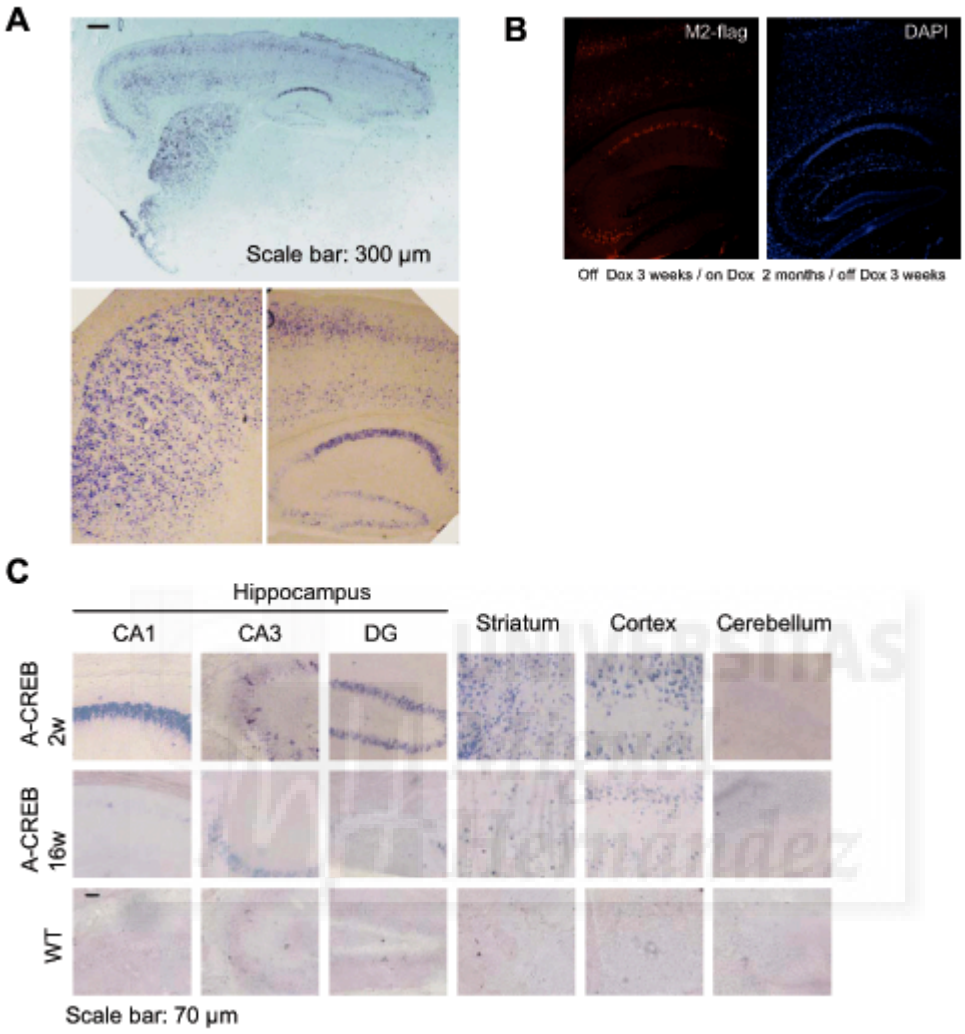
Supplementary Table 6: Basal behavior analysis of A-CREB mice

Parameter	Control	A-CREB	p-value
Abdominal Tone	1 (1-1)	1 (1-1)	1
Aggression	0 (0-0)	0 (0-0)	1
Barbering	0 (0-0)	0 (0-0)	1
Body Length (mm)	93.67 ± 1.59	79.17 ± 1.56	**<0.001**
Body Position	2 (2-3)	2 (2-3)	0.27
Contact Righting Reflex	1 (1-1)	1 (1-1)	1
Corneal Reflex	1 (1-1)	1 (1-1)	1
Defecation	2 (1-3)	2 (1-3)	0.84
Fear	0 (0-1)	0 (0-1)	0.58
Gait	0 (0-0)	0 (0-0)	1
Grip Strength	2 (2-3)	2 (2-3)	1
Irritability	0 (0-0)	0 (0-1)	0.32
Lacrimation	0 (0-0)	0 (0-0)	1
Limb Grasping	1 (1-1)	1 (1-1)	1
Limb Tone	1 (1-1)	1 (1-1)	1
Locomotor Activity	12.83 ± 2.12	14 ± 2.09	0.47
Negative Geotaxis	0 (0-0)	1 (1-2)	**0.02**
Palpebral Closure	0 (0-0)	0 (0-0)	1
Pelvic elevation	3 (3-3)	3 (3-3)	1
Piloerection	0 (0-0)	0 (0-0)	1
Pinna Reflex	1 (1-1)	1 (1-1)	1
Provoked Biting	1 (1-1)	1 (1-1)	1
Respiration Rate	2 (2-2)	2 (2-2)	1
Righting Reflex	0 (0-0)	0 (0-0)	1
Seizures	0 (0-0)	0 (0-0)	1
Skin Color	2 (2-2)	2 (2-2)	1
Spontaneous Activity	2 (1-2)	1 (1-2)	0.06
Tail elevation	1 (1-1)	1 (1-1)	1
Toe Pinch	2 (2-2)	2 (2-3)	0.32
Touch Escape	3 (3-3)	2 (1-2)	**0.02**
Transfer Arousal	5 (4-5)	5 (4-5)	0.58
Tremor	0 (0-0)	0 (0-0)	1
Trunk Curl	0 (0-0)	0 (0-0)	1
Urination	0 (0-1)	0 (0-1)	0.26
Vibrissae	1 (1-1)	1 (0-1)	0.32
Visual Placing	2 (1-2)	2 (1-2)	0.52
Vocalization	0 (0-1)	0 (0-1)	1
Weight (gr)	27.8 ± 1.48	18.1 ± 0.58	**<0.001**
Wire Maneuver	1 (0-1)	1 (0-1)	0.92

p-values are calculated using T-test for data expressed as Mean ± S.E.M, and Mann-Whitney test for data expressed as Median followed by Interquartile Range.
Bolded values indicate parameters with significant differences ($p<0.05$)

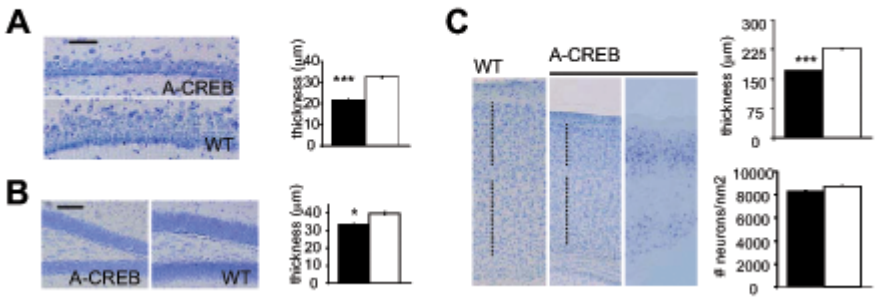
Supplementary Figures

Supplementary Figure S1



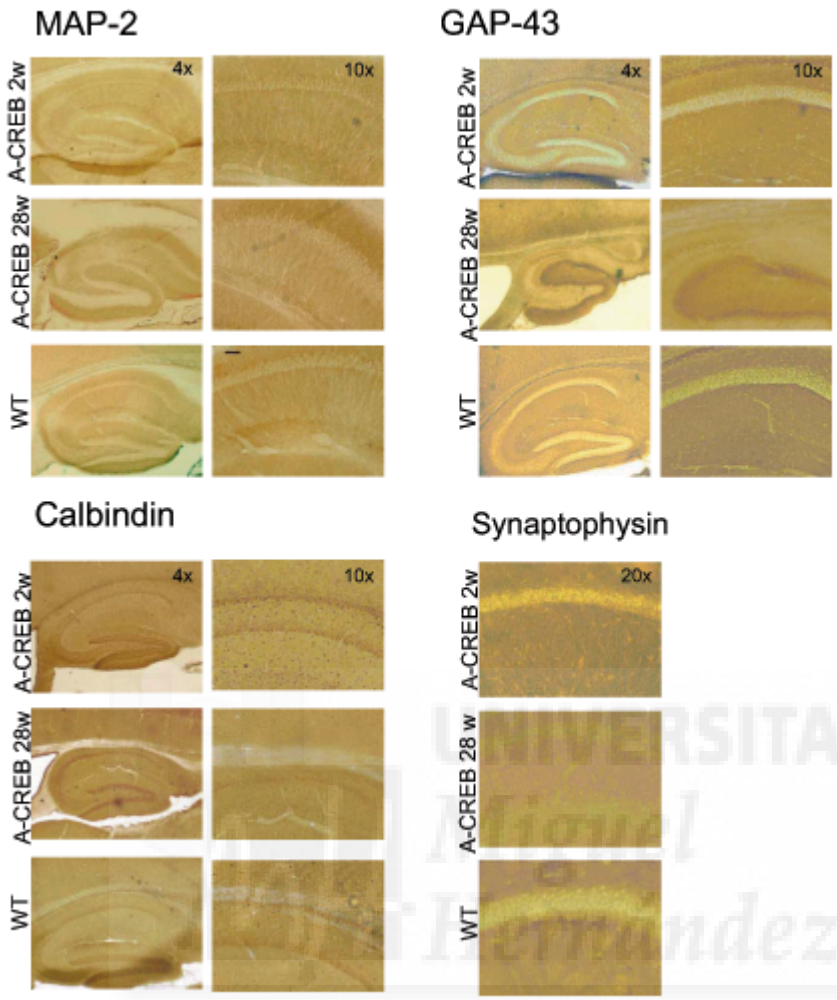
Supplementary Figure S1. Pattern of transgene expression in line AC95. **A**. DIG *in situ* hybridization (ISH) with a probe specific for A-CREB transgene in brain sagittal sections from a 2-week old CaMKII-tTA/tetO-A-CREB-95 bitransgenic mouse (A-CREB). The lower panel show higher magnification images of striatum (left) and hippocampus and cortex (right). **B**. Transgene expression can also be detected by immunohistochemistry (IHC) using an antibody that recognizes the M2-Flag sequence in the N-terminus of A-CREB (red). The sections were counterstaining using DAPI (blue). As expected, the pattern of transgene expression using IHC and ISH was the same. **C**. Details of DIG-ISH using a probe specific for A-CREB. Note the dramatic reduction of transgene expression in 16-week old A-CREB mice when compared to 2-week old mice.

Supplementary Figure S2



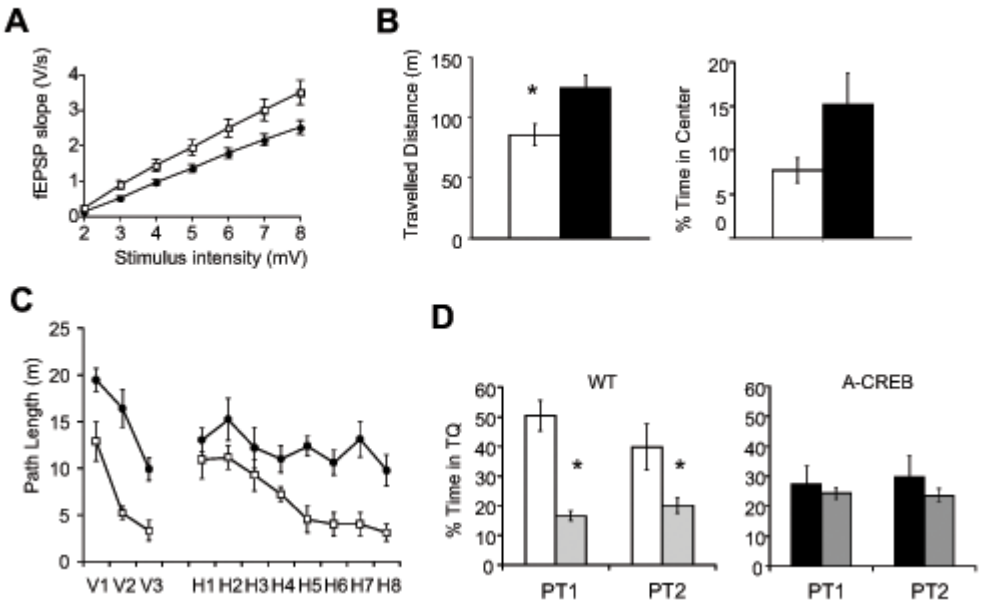
Supplementary Figure S2. Loss of forebrain neurons in A-CREB mice. We quantified the loss of neurons in different brain region in adult mice (≥6 months old; WT: n=6; A-CREB: n=7) **A.** The thickness of the CA1 cellular layer was significantly reduced in A-CREB mice ($p<0.001$). **B.** The thickness of the dentate gyrus upper and lower blades was slightly reduced in A-CREB mice ($p<0.001$). **C.** The thickness of the cortex was significantly reduced in A-CREB mice ($p<0.001$), but we did not detect changes in cell density ($p=0.14$). Layers 2 and 3, where transgene expression is stronger (DIG in situ at the right panel and Fig. 1), were particularly affected (layer 2/3: $p<0.001$; layer 5: $p=0.04$), but still showed clear transgene expression. Scale bar: 140 µm.

Supplementary Figure S3



Supplementary Figure S3. Immunohistochemical analysis of the hippocampus of A-CREB mice. The neurodegenerative process was also visualized by immunostaining with a number of neuronal markers. Floating vibratome sections (50 µM) were stained with antibodies against MAP2, GAP43, calbindin and synaptophysin. No significant differences were found in either hippocampus or other brain regions at early times (2 weeks old mice), but significant neuronal loss was observed after sustained CREB inhibition (28 weeks old mice).

Supplementary Figure S4



Supplementary Figure S4. Long-term consequences of chronic inhibition of CREB function by A-CREB. A. Input/output curve of fEPSP slope (V/s) versus stimulus at the Schaffer collateral pathway of hippocampal slices from one year old A-CREB mice (●, n= 23) and control littermates (□, n=26, p=0.01). **B.** Ten-weeks old A-CREB mice (black) show hyperlocomotion (left panel, ambulatory distance during a 30 min period: wild type, n = 11; A-CREB; n=9; p=0.01) and a trend towards reduced anxiety behavior in an open field (right panel, percentage of time spent in the center of the arena, p=0.14). **C.** Spatial navigation in the Morris water maze in adult A-CREB mice (A-CREB: n=9; WT: n=11). Path length analysis revealed deficits in both the visible platform and the hidden platform tasks associated to chronic A-CREB expression (ANOVA repeated measures, genotype effect: visible platform, p=0.09; hidden platform, p=0.01). Similar deficits were also observed in escape latencies (ANOVA repeated measures, genotype effect: visible platform, p=0.01; hidden platform, p=0.001). Swimming speed and tygmotaxis were not significantly affected. **D** Spatial memory was assessed in two probe trials that revealed strong deficits in A-CREB mice. Values represent percentage of time in the target quadrant compared to the average of other quadrants. Asterisks indicate $p<0.05$.

C3. Bidirectional control of CA1 pyramidal neuron survival by cAMP response element binding protein dependent gene expression

Our studies in inducible bitransgenic strains expressing either a strong dominant negative inhibitor of CREB or a constitutively active CREB variant have revealed that both the chronic inhibition and the chronic enhancement of CREB function compromise the survival of CA pyramidal neurons. We compared the neurodegenerative processes triggered by these two genetic manipulations of CREB function and found that the sustained blockade of CREB function led to reduced neuronal responsiveness and atrophy, whereas its strong chronic activation caused inflammation and excitotoxic cell death. The comparison of transgenic animals with loss versus gain-of-function demonstrates the importance of fine-tuned regulation of CREB-dependent gene expression for CA1 neuron survival and function, and revealed the ultrastructural and transcriptional signatures associated to these two forms of neurodegeneration.

In this study, currently under preparation, my contribution was to perform all histomorphological and biochemical and assays. I performed immunohistochemistry, quantitative PCR, death assays, Neurosilver and Nissl stainings. In addition, I prepared samples for microarray analyses and electron microscopy. Luis Miguel Valor and Angel Barco participated in the gene profiling analyses. Rafael Lujan performed and analysed the brain samples collected by myself using electron microscopy facilities at Universidad de la Castilla y la Mancha. Roman Olivares maintained and genotyped the mouse colonies.

Title: Bidirectional control of CA1 pyramidal neuron survival by cAMP response element binding protein dependent gene expression

Abbreviated title: Bidirectional control of neuronal survival by CREB

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Abstract

Recent studies in inducible bitransgenic strains expressing either a strong dominant negative inhibitor of CREB or a constitutively active CREB variant revealed that both the chronic inhibition and the chronic enhancement of CREB function compromise the survival of CA1 pyramidal neurons. We compared here the neurodegenerative processes triggered by these two genetic manipulations of CREB function and found that the sustained blockade of CREB function led to reduced neuronal responsiveness and atrophy, whereas its strong chronic activation caused inflammation and excitotoxic cell death. The comparison of transgenic animals with loss versus gain-of-function demonstrates the importance of fine-tuned regulation of CREB-dependent gene expression for CA1 neuron survival and function, and revealed the ultrastructural and transcriptional signatures associated to these two forms of neurodegeneration.

Introduction

cAMP response element binding protein (CREB)-mediated gene expression is necessary for the survival of several neuronal subtypes both in vitro and in vivo (Riccio et al., 1999; Papadia et al., 2005). Experiments in neuronal cultures have revealed that transient expression of dominant negative CREB variants in different neuronal types caused cell death, whereas the overexpression of CREB or the expression of constitutively active variants protected from apoptotic death (Andreatta et al., 2004; Glover et al., 2004; Lee et al., 2005). The picture that emerges from in vivo studies is more complicated. Studies on CREB-/- mice revealed a complete requirement of CREB for survival of dorsal root ganglia sensory neurons (Lonze et al., 2002), whereas only an specific population of dopaminergic cortical neurons were sensitive to the deletion of CREB in the central nervous system (CNS) (Parlato et al., 2006). When both CREM and CREB were eliminated, the survival of CNS neurons was also compromised and severe neuronal loss was observed in cortex, hippocampus and striatum (Mantamadiotis et al., 2002). Similar effects were observed after chronic inhibition of CREB function by A-CREB (Jancic, 2008, submitted), an artificial peptide with strong inhibitory effect on the CREB family of transcription factors. On the other hand, the characterization of a line of transgenic mice with inducible expression of a constitutively active CREB variant, VP16-CREB, demonstrated that strong chronic enhancement of CREB function also caused severe loss of hippocampal neurons. These deleterious effects were dose dependent and could be prevented by turning off transgene expression (Lopez de Armentia et al., 2007).

Despite the similarities on the long-term consequences for the survival of CA1 pyramidal neurons of chronic inhibition or enhancement of CREB function, these two genetic manipulations had different and even opposite effects in the physiology of CA1 neurons. Whereas chronic inhibition of CREB activity tuned down intrinsic excitability, impaired L-LTP and reduced the susceptibility to induced seizures (Jancic et al, 2008, submitted), chronic enhancement of CREB increased the excitability of CA1 neurons (Lopez de Armentia et al., 2007), reduced the threshold for obtaining the late phase of LTP (Barco et al., 2002; Marie et al., 2005) and caused spontaneous epileptic seizures (Lopez de Armentia et al., 2007). Gene profiling in the hippocampus of both strains of mutant mice shortly after transgene induction also revealed important differences. A

number of immediate early genes were upregulated in VP16-CREB^{high} mice (Barco et al, 2005), but only a modest effect in the basal expression of few IEGs was detected in A-CREB mice (Jancic et al, 2008, submitted), indicating that CREB may be sufficient for activity-dependent gene expression, but is not always necessary.

How these initially divergent phenotypes lead to similar final effects on neuronal survival? Do both, the blockade and the enhancement of CREB function, trigger the same death-signaling cascade? To answer these questions we compared gene profiling, histological and cell death assays in brain sections from A-CREB (Jancic et al, 2008, submitted, the reuls presented in the previous chapter) and VP16-CREB^{high} mice. We show that the sustained blockade of CREB function leads to reduced neuronal responsiveness and atrophy, whereas its strong chronic activation causes inflammation and excitotoxic cell death. We also define the ultrastructural and transcriptional signatures associated to these two forms of neurodegeneration.

Results

Chronic enhancement of CREB function causes inflammation and strong gliosis response

We have shown in the previous chapter that chronic inhibition of CREB function causes apoptotic neuronal death. In contrast, the electron microscopy analysis of VP16-CREB^{high} mice showed that the pyramidal cell layer maintained a normal appearance during the first two weeks after transgene induction (Figure 1). However, three weeks after transgene induction, some signs of neurodegeneration were observed. These consisted in cytoplasmic and mitochondrial swelling, membrane rupture, and tigroid-type chromatin fragmentation. A few infiltrated glial cells, and some cell debris and vacuolated neurons were observed between the cell bodies of apparently intact CA1 pyramidal neurons (Figures 1b, d and f). Six weeks after dox removal, the number of pyramidal neurons was severely reduced and massive astrogliosis was apparent in both the cellular and the dendritic layer (Figures 1c and f). In terms of number of synapses in the stratum radiatum, we did not observe significant differences two weeks after induction neither in inhibitory (Figures 1g-h) or excitatory synapses (Figures 1i-j). However, there was a pronounced reduction of glutamatergic synapses in mice expressing the transgene for 6-weeks (wt: 166±4 syn/100mm3; VP16-CREB^{high}: 27±2

syn/100mm3; p<0.001). At these late times, we observed ultrastructural abnormalities both at postsynaptic and presynaptic sites. Dendritic spines appeared smaller and elongated, whereas axonic terminal were enlarged and contained fewer synaptic vesicles and mitochondria (Figures 1k-m).

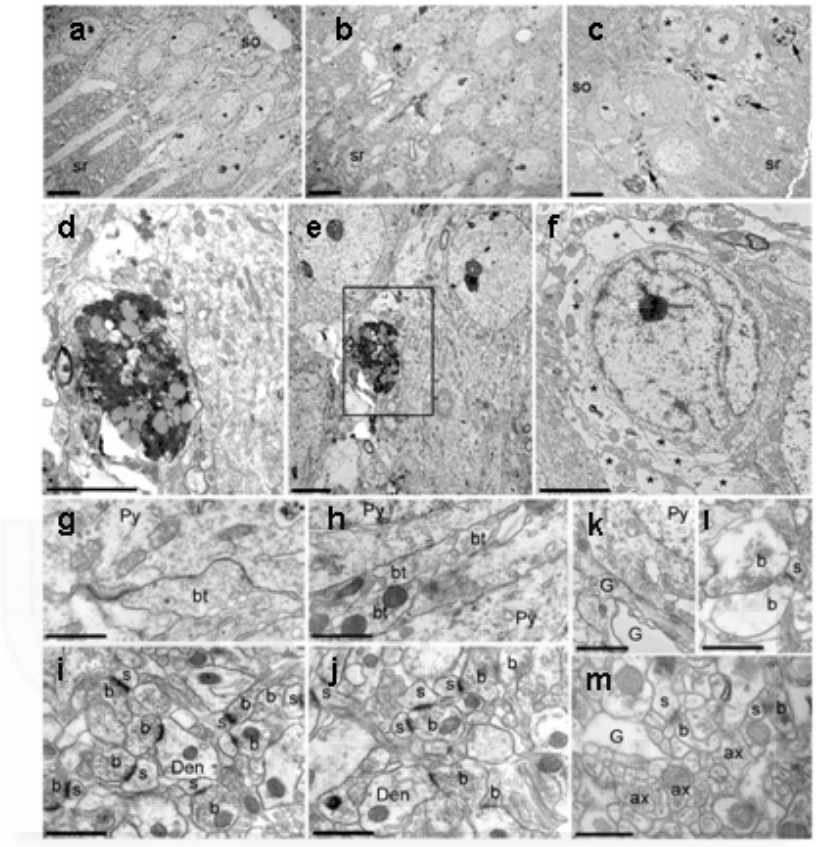

Figure 1. Electron micrographs of the pyramidal neurons in the CA1 region of the dorsal hippocampus of VP16-CREB^{high} mice. Electron micrographs of the pyramidal neurons in the CA1 region of the dorsal hippocampus of VP16-CREB^{high} show progressive neurodegeneration after transgene induction. Micrographs of the CA1 subfield in wild type animals (**a**, **g** and **i**) were undistinguishable of those of VP16-CREB^{high} one (results not shown) or two weeks after transgene induction (**h** and **j**). Three weeks after transgene induction (**b**, **d**, **e**, **k** and **l**) a number of ultrastructural changes are appreciable both in the cellular layer (**b**, **d** and **e**) and the *stratum radiatum* (**k** and **l**). Six weeks after transgene expression the desorganization of the hippocampus is evident (**c** and **m**), and a large number of astrocytes appear in the proximity of pyramidal cells, in some cases, phagocytosing those cells (**f**). Both inhibitory (**g**, **h** and **k**) and excitatory synapses (**i**, **j**, **l** and **m**) are normal at two weeks, but appear affected at later times after transgene induction. See text for additional details. Abbreviations: Py, piramidal cell; sr, stratum radiatum; so, stratum orines; bt, basket cell terminal; s, spine; b, bouton or axon terminal; Den, dendritic shaft; ax, axons; G and asterisk, glial cell process; black arrow, glial cell nucleus. Scale bars: **a-c** = 5 microns; **d-f** = 2 microns; **g-m** = 0.5 microns.

Immunohistological analysis using anti-GFAP antibody confirmed the active gliosis observed in the electron microscopy images. GFAP reactivity in sections of VP16-CREB^{high} mice was spreader and stronger than that observed in the hippocampus of A-CREB mice (Figure 2A). Furthermore, using the microglia marker F4/80 we detected reactive microglia in the hippocampus of VP16-CREB^{high} mice, but not in A-CREB mice (Figure 2B).

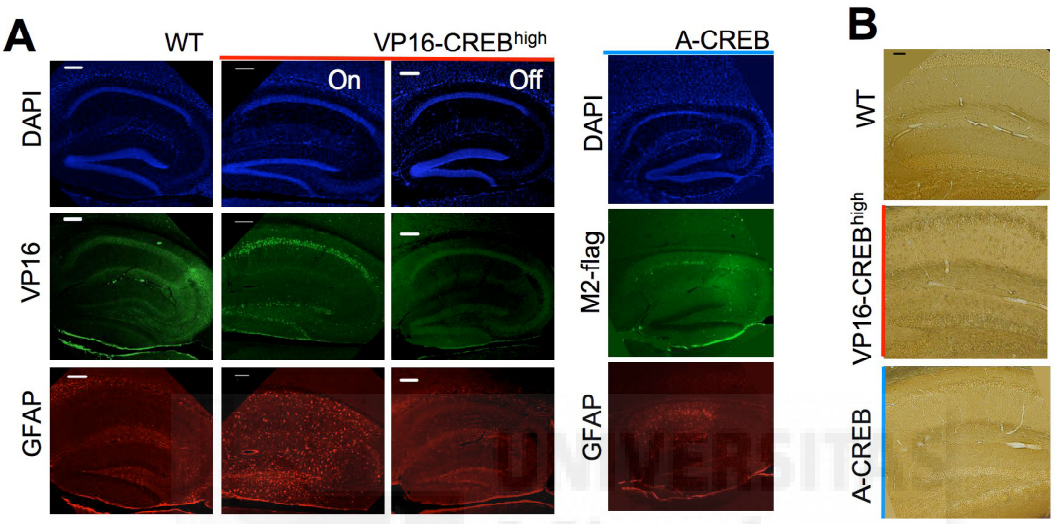

Figure 2. Gliosis in A-CREB and VP16-CREB^{high} mice. A. GFAP immunostaining of the hippocampus of 1.5 month old A-CREB and VP16-CREB^{high} mice expressing (1 month On) or not the transgene (Off, animals fed with dox food). Sections were double stained with antibodies against GFAP (red) and VP16 for VP16-CREB (green) or with antibodies against GFAP (red) and M2-Flag, which recognizes A-CREB (green). Sections were also counterstained with DAPI (blue). Scale: 140 µm. The result for A-CREB wild type littermates (not shown) was equivalent to that of VP16-CREB^{high} littermates. **B.** Immunostaining using antibodies against the microglia marker F4/80 revealed the presence of reactive microglia only in the hippocampus of VP16-CREB^{high}, A-CREB mice did not show enhanced signal for this marker. Scale bar: 140 µm.

These signs of inflammation support an excitotoxicity scenario in the case of VP16-CREB mice. Although it is now believed that seizure-induced neuronal death is primarily morphologically necrotic, excitotoxic insults may result in mixed apoptotic-necrotic features depending of their intensity (Ishimaru et al., 1999; Leist and Jaattela, 2001). In agreement with this view, we found scattered TUNEL positive cells in the hippocampus of VP16-CREB^{high} mice one month after induction (Figure 3A). A even lower percentage of neurons were also positive for active caspase 3 staining at that time

(Figure 3B). Brain sections obtained at either earlier (1-2 weeks) or later (2 or more months) times after transgene induction were negative for both assays (results not shown). The sections of VP16-CREB^{high} mice were also positive for silver staining, (Figure 3C), which has been associated to different pathometabolic conditions, including status epilepticus, ischemia and NMDA or glutamate toxicity (Gallyas et al., 2005). In addition, previous studies showed severely damaged the mossy fiber projections in VP16-CREB^{high} mice (Lopez de Armentia et al, 2007) that may be the consequence of dramatic shrunkening of the dentate gyrus in these animlas. However, as reveled by Timm´s staining, the mossy fibers pathways appeared intact in old A-CREB animals with a severely degenerated hippocampus (figure 4).

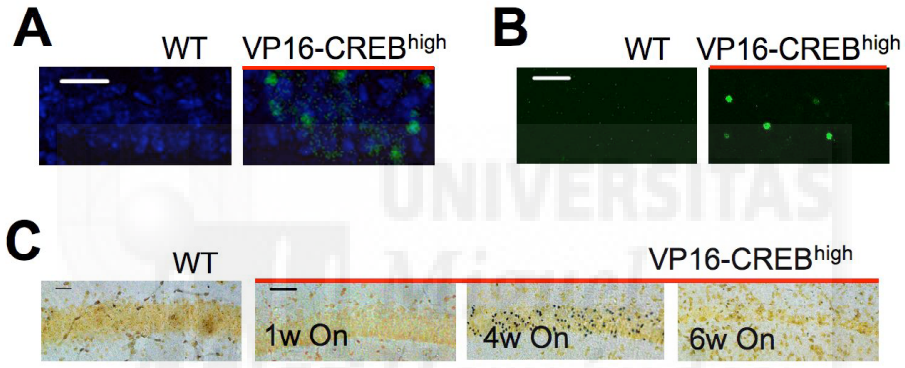

Figure 3. Cell death markers in VP16-CREB^{high} mice. A. TUNEL reaction (green) in the hippocampus of VP16-CREB^{high} mice and control littermates. Sections were counterstained with DAPI (blue). Scale: 50 µm. **B.** Anti-caspase-3 immunostaining of CA1 neurons in the hippocampus of VP16-CREB^{high} mice and control littermates (time after induction expressed in weeks). Scale: 50 µm. **C.** Neurosilver staining of brain sections of VP16-CREB^{high} mice and control littermates at indicated time after induction expressed in weeks. Scale: 50 µm.

Importantly, neither cell loss nor caspase, TUNEL or neurosilver positive neurons were observed in mice expressing lower levels of VP16-CREB protein (VP16-CREB^{low} mice, (Lopez de Armentia et al., 2007)) even after one year of transgene expression (results not shown). The features of degenerating neurons in VP16-CREB^{high} mice, together with the enhanced excitability of VP16-CREB expressing neurons and the spontaneous epileptic activity (Lopez de Armentia et al, 2007), indicate that cellular loss in the hippocampus of these mice is primarily caused by excitotoxic neuronal death

and includes at least a percentage of degenerating neurons undergoing apoptotic cell death.

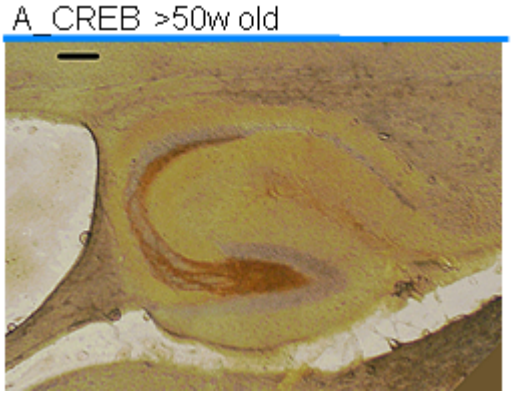

Figure 4. Intact mossy fiber in A-CREB mice. Timm's staining of brain vibratome sagittal sections of an A-CREB mouse one year old revealed an intact mossy fiber projection, despite the massive loss of neurons in the CA1 area.

Gene profiling comparison of hippocampal degeneration by chronic enhancement or inhibition of CREB function

In previous chapter we provided detailed gene profiling analyses of gene expression changes in A-CREB mice. It should be noted that A-CREB is strong inhibitor of activity of the other CREB family members as well, so the observed lack of gross effect on IEGs expression, as described in the chapter C2, cannot be attributed solely to CREB. This suggests that transcription factors, other than CREB family, may bind to CRE and promote the transcription of downstream genes, or that these downstream genes have other DNA domains, for example SRE and others, in their regulatory regions.

Interestingly, the comparison of gene ontology groups associated to late changes in VP16-CREB^{high} (Lopez de Armentia et al., 2007) and A-CREB mice confirmed the similarities and differences between the two neurodegenerative processes outlined in our histological analyses. In particular, markers of lymphocytes activation, such as multiple histocompatibility genes, Aif-1 and L-plastin (Lcp1), were largely present in the VP16-CREB^{high} samples indicating that inflammation processes were specifically associated with the chronic enhancement of CREB function (Figure 5). In an independent analysis using eGOn 2.0 software, immune response-related terms

appeared in both genotypes but only antigen presentation terms were found in VP16-CREB^{high}, reinforcing the idea that active inflammation was taken place in this genotype.

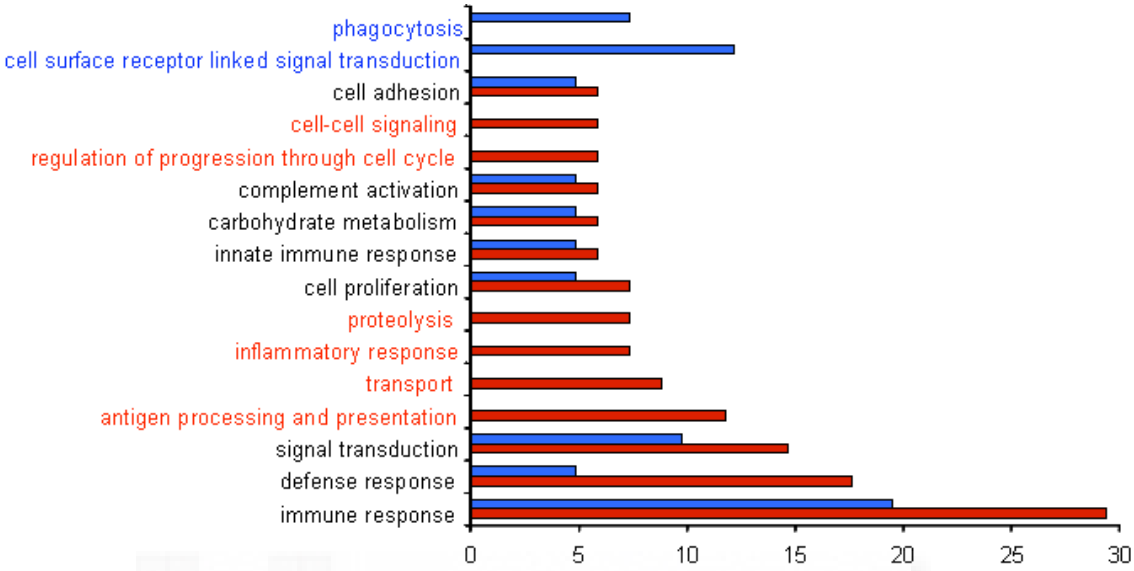

Figure 5. Gene profiling comparison of the neurodegenerative processes undergoing in the hippocampus of A-CREB and VP16-CREB^{high} mice. Main biological processes, as defined in gene ontology (GO), affected after sustained chronic inhibition (in blue, 6 weeks old A-CREB mice) or enhancement (in red, VP16-CREB^{high} mice more than 3 weeks after transgene induction) of CREB function. We compared those processes significantly ($p<0.05$) overrepresented in the lists of altered genes in either one of these two situations. Only those processes that represented at least 5% of significantly altered genes in one of the two lists were considered. The percentage of genes with altered expression at late times in A-CREB mice (dChip analysis of 3 sets of paired samples, fold change>1.5, signal difference>50, p value<0.05, provided a list of 41 genes) that are represented by these biological processes are indicated in blue bars. Those processes affected only in A-CREB mice are indicated in blue characters. The main biological process groups among the 68 probe sets significantly altered at late times in VP16-CREB^{high} mice (dChip analysis of 3 sets of paired samples, fold change>1.5, signal difference>50, p value<0.05) are indicated in red. Processes that were found altered only in VP16-CREB^{high} mice are indicated in red characters.

Discussion

We have recently demonstrated that both the chronic enhancement and the chronic inhibition of CREB function can have deleterious consequences for the survival of neurons in the CA1 subfield and in other forebrain regions (Lopez de Armentia et al.,

2007; Jancic et al., 2008). We revealed here the ultrastructural and transcriptional signatures associated to these two forms of neurodegeneration and showed that the death of CA1 pyramidal neurons in transgenic mice with either chronic enhancement or inhibition of CREB function shared a number of features, but also differed in important aspects.

The analysis of different cellular markers suggested that the cellular and molecular mechanisms underlying neurodegeneration in these two mutant strains were different. As shown earlier, in A-CREB mice we found neurons that characteristically exhibit intracellular inclusions, condensation of both the cytoplasm and the nucleus, and ruffling of the plasma membrane while maintaining ultrastructural preservation of cellular organelles. There was no major inflammatory response accompanying neurodegeneration. As in classical apoptosis we observed minimal damage in the surrounding cells. Similar features have been observed in neurons both in the brain of patients who died with Huntington's disease and in transgenic mouse model for this disease (Leist and Jaattela, 2001). As described earlier (in the chapter C2), A-CREB and CREB/CREM knockouts share some histomorphological aspects (Mantamadiotis et al, 2002), so this phenotype is likely due to efficient inhibition of CREB family activity .

In VP16-CREB mice, we observed active gliosis and microglia as well as other ultrastructural features that suggest an excitotoxic death. It is now believe that seizure-induced neuronal death is primarily morphologically necrotic and does not depend on the activation of apoptotic cascade (Ishimaru et al., 1999; Fujikawa, 2002; Olney, 2003), although, depending of the intensity of insult and the region of the brain affected, it may result in mixed apoptotic-necrotic features (Leist and Jaattela, 2001). In excitotoxic cell death, cellular contents are liberated into the intercellular space, often damaging neighboring neurons and inducing an inflammatory response. As a consequence, inflammation seems to be much more severe in the case of VP16-CREB^{high} mice than in A-CREB mice. Moreover, whereas the progression of degeneration in A-CREB mice seemed to progress slowly and continuously after transgene induction; neurodegeneration in VP16-CREB^{high} mice progressed quickly after the detection of spontaneous seizures. Consistently with these observations, gene profiling also identified inflammation as a pathological process specifically associated to the sustained chronic enhancement of CREB activity, whereas other pathological markers were observed in both mutant strains.

Granular cells in the dentate gyrus field were more affected in VP16-CREB^{high} than in A-CREB mice. In accordance with this result, the mossy fiber projection did not seemed to be impaired in A-CREB animals, which at that age already suffered a severe neurodegeneration, but almost disappeared after six weeks of transgene expression in VP16-CREB^{high} mice (Lopez de Armentia et al., 2007). At the moment, it is not possible to distinguish whether this is attributable to differences in the pattern of expression of both transgenes (transgene expression in granule cells is weaker in A-CREB mice) or to reduced susceptibility of granular cells to inhibition of CREB-mediated gene expression.

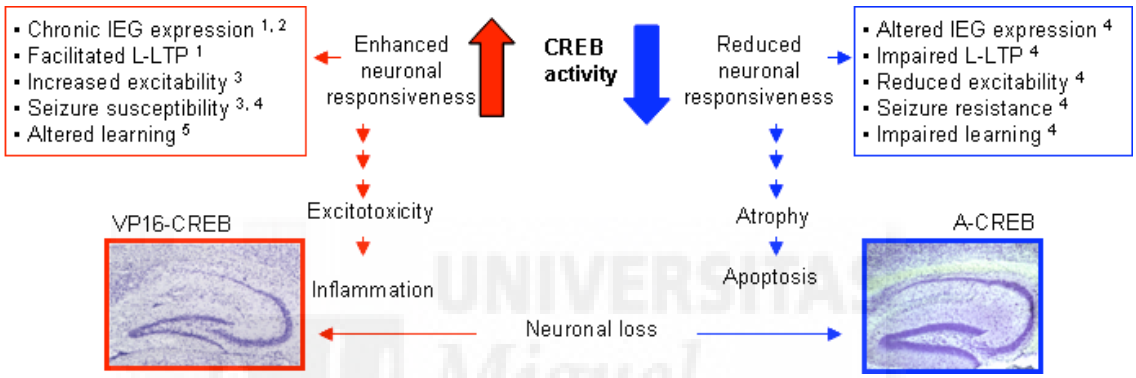

Figure 6. Bidirectional control of CA1 neuronal survival and plasticity by CREB: Summary of opposite and coincidental alterations detected by the characterization of transgenic strains with regulatable enhancement or inhibition of CREB function. [1](Barco et al., 2002), [2](Barco et al., 2005), [3](Lopez de Armentia et al., 2007), [4](Jancic et al., 2008) and [5](Viosca et al., 2008).

The picture that emerges from our analysis of CREB mutants with gain (VP16-CREB) and loss-of-function (A-CREB) together with previous studies in CREB deficient mice indicates that the precise control of CREB-dependent gene expression is a critical requirement for the viability of CA1 pyramidal neurons. Overall, this comparison demonstrated that the mechanisms of cell death in A-CREB and VP16-CREB mice differ and suggested that any unbalance in the activity of the CREB mediated gene expression cascade can have a negative impact in the survival and function of CA1 pyramidal neurons, likely through regulation of neuronal responsiveness (Figure 6). In agreement with this view, we observed spontaneous epileptic seizures and premature death in VP16-CREB^{high} mice (Lopez de Armentia,

2007), but not in A-CREB mice (Jancic et al, 2008, submitted). In fact, A-CREB mice were more resistant to induced epilepsy in response to two different proepileptic drugs. The capability to initiate, pause and reactivate neurodegeneration using doxycycline makes the transgenic strains described here powerful animal models to assay therapies aimed to recover after neurodegeneration triggered by different or even opposite mechanisms.

Material and methods

Generation and maintenance of transgenic mice

A-CREB, VP16-CREB^{high} and VP16-CREB^{low} mice have been described before (Barco et al., 2002; Lopez de Armentia et al., 2007; Jancic et al., 2008). In all our experiments, we used as control littermate mice carrying either no transgene ot the tTA or tetO transgene alone. For VP16-CREBhigh and control littermates, dox was administrated at 40 mg/Kg of food and removed or added at the indicated times before experimentation. A-CREB and VP16-CREBlow mice raised without dox. All mice were maintained and bred under standard conditions, consistent with national guidelines and approved by the Institutional Animal Care and Use Committees.

Histological techniques

Nissl and Timm's staining were performed as previously described (Lopez de Armentia et al., 2007). Silver staining was carried out using the FD NeurosilverTM kit (FD NeuroTechnologies Inc). For immunohistochemistry, mice were anesthetized, perfused with 4% paraformaldehyde, postfixed overnight, and 50 μm sections were obtained using a Leica vibration microtome. The following primary antibodies were used: ά-Cleaved Caspase-3 antibodies (Cell Signalling), ά-VP16 (Santa Cruz), and ά-M2-flag, ά-Synaptophysin, ά-MAP-2, ά-Calbindin, ά-GAP-43, ά-F4/80 and ά-GFAP (Sigma). Secondary biotinylated antibodies, streptividin-peroxidase conjugate and DAB substrate were obtained from Sigma and fluorescent secondary antibodies from Invitrogen. For TUNEL reaction, serial brain sagittal cryosections of 12 μm thickness were stained using the in situ Cell Death Detection Kit (Roche Applied Science, Germany).

Electron microscopy

For electron microscopy mice were anesthetized and perfused with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M PB (pH 7.4) at the indicated times after dox removal. Then, coronal sections were cut at a thickness of 60 µm using a Leica vibration microtome through the level of the dorsal hippocampus. After several washes in PB, sections were postfixed with osmium tetraoxide (1% in 0.1 M PB) and block-stained with uranyl acetate (1% in distilled water). Sections were then dehydrated in ascending series of ethanol to 100% followed by propylene oxide and flat-embedded on glass slides in Durcupan (Fluka). The CA1 region of the hippocampus was cut at 70-90 nm on an ultramicrotome (Reichert Ultracut E; Leica, Austria) and collected on 200-mesh nickel grids. Staining was performed on drops of 1% aqueous uranyl acetate followed by Reynolds's lead citrate. Ultrastructural analyses were performed in a Jeol-1010 electron microscope.

Microarray analysis

RNA was extracted from dissected hippocampi. Mouse Genome 430 2.0 genechips were hybridized, stained, washed and screened for quality according to the manufacturer's protocol. The Affymetrix GeneChip® data were processed, normalized and statistically analyzed using GCOS (Affymetrix), GeneSpring GX (Agilent Technologies) and dChip softwares (Li and Hung Wong, 2001). After the normalization by the median intensities of the arrays, linearity of the signal intensities between arrays was confirmed and Principal Component Analysis (PCA) was performed to check the similarities of the arrays (Supplemental Figure S5). GeneSpring and dChip softwares were used in parallel and produced highly overlapping lists of significantly changed probe sets after the following filtering: fold change >1.5, signal intensities >20% of the maximal expression (GeneSpring) and PM-MM difference model, >50 (dChip), $P < 0.05$ in Student's t test. For the analysis of 6-weeks old mice, a paired t-Test analysis was conducted (3 wild-type vs. 3 mutant samples, each sample obtained from a group of 4 mice with same sex and age). The list of significantly altered probe sets was analyzed for GO group classification using Pathway Studio 5.0 software (Ariadne Genomic Inc.). We used in the comparison shown in Supplemental Figure S4 the dataset corresponding to the analysis of VP16-CREBhigh mice using U74Av2 genechips (accession no. GSE3965, Barco et al., (2005)).

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D. Discussion

Although the CREB pathway has driven the attention of neuroscientists for many years, there is a number of unanswered questions regarding its regulation, mechanisms of action and physiological roles. The comparison of some previous studies provided contradictory results, probably due to compensation by the other CREB family members. Here, we tried to overcome the limitations of the previous studies and provide a complete characterisation of a new transgenic mouse line with impaired CREB activity, namely A-CREB mice. It is important to note that this is the first analysis of the consequences of A-CREB expression, dominant negative inhibitor of CREB-driven transcription, *in vivo* in the central nervous system. Moreover, A-CREB blocks the function of entire CREB family, including ATF-1 and CREM, providing more potent inhibition of CRE-dependent actions. In addition, we also explored in detail the consequences of the enhancement of the CREB-dependent gene expression. Since many issues were already discussed in previous chapters, I will focus this discussion on the comparison of the observed phenotypes of transgenic mice with enhanced versus reduced CREB activity, especially regarding neuronal survival and excitability.

D1. CREB and neuronal excitability

The epileptic attacks and probable hyperexcitability of the system seem to be main cause of the death in VP16-CREB^{high} animals. Our results showed reduced slow and medium afterhyperpolarization (sAHP, mAHP, respectively) and changed membrane properties of the CA1 neurons in these transgenic animals. It is known that afterhyperpolarization serves to controlling the responsiveness of neurons and that inhibition of afterhyperpolarization can enable a stimulus that trigger E-LTP to induce L-LTP (Sah et al, 1996, Faber et al, 2005) and its regulations was correlated to higher cognitive functions (Wu et al, 2004). Here, we showed that, in addition to this, afterhyperpolarization may also work as a gain control adjuster, by adapting the spike frequency in response to stimuli. Results using field recording also indicated significant increase in the CA1 spontaneous activity. In addition to this, *in vivo* kindling

experiments in VP16-CREB^{high} mice revealed that these mice are also more prone to the epileptic attacks. Recent researches (Dong et al, 2006) also related CREB signaling cascade and excitability in other neuronal types, but exact genes involved in these processes were not revealed. One of the candidates genes that may link broad effects of the CREB pathways and the observed phenotype might be BDNF, the main CREB target that come into view after recent microarray analysis of VP16–CREB^{high} mice (Barco et al, 2005). Moreover, BDNF was already related to epileptogenesis (Gall et al, 1991).

On the contrary, in the 3 weeks old A-CREB mice, intracellular recordings revealed a reduction in the number of action potentials elicited by depolarizing current injections, which was reversed by adding doxycycline for 10 days to mouse diet. The long-term potentiation was also examined and a deficit was found when L-LTP was induced by four tetani. In hippocampal pyramidal neurons of A-CREB mice, we observed the increase in I_M current in A-CREB expressing CA1 neurons. This finding is comparable to the effects of introducing retigabine, an M channel opener that reduces both kindled and epileptic seizures, to wildtype animals (Rostock et al, 1996). Retigabine is currently under evaluation as a new antiseizure drug (Porter et al, 2007). It is thought that M-current underlies medium afterhyperpolarization, as we confirmed in our experiments by detecting increase in mAHP and alteration in I_M. Therefore, modulation of M-current is most likely one of the links between the CREB pathways and reduction of neuronal excitability. In addition, experiments *in vivo* revealed that A-CREB mice show significantly delayed kindling in comparison with their littermate controls.

Here, we described for the first time the bidirectional modulation of the intrinsic excitability on CA1 neurons by CREB-driven gene expression, what may contribute to some of the LTP phenotypes associated to manipulation of CREB function. Thus, it can be hypothesized that the activation of the CREB pathway, in addition to its role in consolidation of synaptic changes, may play role in the sensitization process by facilitating future neuronal responses in a given time range. It is worth of mentioning that in VP16-CREB mice it was only CREB overexpressed, but in the case of A-CREB animals, we cannot exclude that the other members of the CREB family that are downregulated had any effect regarding neuronal excitability. Therefore, we cannot exclude that the reduction in excitability is produced by those because the mechanism is different than in VP16-CREB. In the case of mice with constitutively active CREB, we

found the alteration of after-hyperpolarisation as the underlying mechanism, whereas and in the mice with reduced CREB family activity, M-current was changed. Therefore, different underlying mechanisms found to underlay altered excitability in these transgenics may suggest the involvement of other, most likely, downstream to CREB genes.

D2. Activity driven CREB-dependent gene expression

In order to link the observed phenotypes and the effects of genetical manipulation of the CREB activity level, we compared hippocampal total RNA samples of our mutants and their littermates using Affymetrix microarrays. We have to note that the transgenes are strongly express in CA1 neurons, but not expressed in glial cells, interneurons or CA3 neurons (also, the percentage of DG neurons expressing the transgene is higher in VP16-CREB than in A-CREB mice). In our experiments, we used mRNA extracted from the whole hippocampus, so there is a possibility that we are missing the possible changes restricted to expressing neurons in the DG and diluting the changes in CA1 neurons, which should be counted as important limitation of the technique. However, the analyses of VP16-CREB mice demonstrated that CREB can be sufficient for the expression of many IEGs (Barco, 2005, and my own analyses). In contrast, we looked for CREB downstream targets in basal state and after external stimulation by kainic acid injection in A-CREB mice; we found that the transcriptional changes triggered by A-CREB expression were more restricted than expected. The induction of proenkephalin and prodynorphin were slightly impaired in response to kainate in A-CREB mice. Our analysis also detected some alterations in the expression of IEGs at the basal state in A-CREB mice. Arc (activity-regulated, cytoskeleton-associated protein), Homer1, Egr1 and Egr2 (early growth factors 1 and 2) were reduced in the basal condition. Thus, although these mutants have the potential of inducting most of the IEGs, the expression of some relevant IEGs seems to be impaired by A-CREB at basal states.

Overall, it seems that CREB is sufficient, but not always necessary for activity-driven gene expression. Other transcriptional factors may be equipped to compensate insufficient CREB activity and be recruited to CRE motif or alternative sites in their promoter. Recent studies in mice lacking CREB in the forebrain, accompanied by

deletion of the cAMP responsive element modulator gene (CREM), confirmed that the role of CREB binding proteins in activity-induced gene expression is surprisingly selective and highly context dependent (Lemberger et al, 2008).

Not only IEGs were monitored in our gene profiling analyses. It was found that the sodium channel subunit β4 (Scn4b), related to Huntington's disease, was significantly downregulated in A-CREB mice. Furthermore, gene Trat1 was almost ten times upregulated in A-CREB expressing neurons. Trat1 stands for the T cell receptor associated transmembrane adaptor 1 and is exclusively expressed in lymphocytes and in the brain, although its role in neurons remains unexplored. The highly significant increase in the expression of this gene in the hippocampi of A-CREB mice may help to deciphering its role in the nervous system.

D3. CREB and neuronal survival

D3.1. Time-course and anatomical pattern of neurodegeneration differs in mice with enhanced versus reduced CREB activity.

CREB is an essential protein in different cellular processes and, as such, its activity needs to be precisely regulated. Therefore, inhibition or enhancement of CREB-dependent gene expression may be beneficial for some cellular processes, but deleterious for others. Whereas A-CREB mice despite the significant neurodegeneration have unaffected life span, VP16-CREB^{high} animals suffered spontaneous epileptic attacks frequently followed by premature death. It seems that only prolonged and sustained CREB activity can have such a dramatic effect for the organism. The reason for this may be that enhanced CREB function can also promote the activation of unwanted gene cascades. In the nervous system of A-CREB mice, either it is possible to overcome the lethal consequences of the inhibition of the CREB transcriptional activity by the action of other genes and transcription factors; or the transgene itself has no effect on the animal's viability.

Histomorphological analyses of mice expressing a constitutively active variant of CREB revealed a slightly earlier onset and faster progression of degeneration in the neurons of the dentate gyrus in comparison to CA1. Glial cells are more rapid to spread

in this hippocampal subfield and the apoptotic markers are more abundant than in the CA1 area. On the other hand, continuous inhibition of CREB activity also results in the significant loss of hippocampal cell, but exclusively in the CA1 subfield. Taking in account the lower level of transgene expression in the DG and comparing it to similar of the VP16-CREB^{high} animals, it is difficult to differentiate whether A-CREB-expressing granular layer neurons of the dentate gyrus are more resistant to the deleterious effects of the transgene, or the level of transgene expression was insufficient to produce observable neurodegeneration.

D3.2. The nature of cell loss in the hippocampal subfield CA1 varies between the transgenic mice expressing enhanced versus reduced CREB-driven gene expression

The CA1 hippocampal subfield of A-CREB mice show the presence of so-called dark neurons, which have intracellular inclusions, condensation of nuclei and cytoplasm and ruffling of the plasma membrane. No significant inflammatory signals were observed. These features resemble the ones seen in some neurodegenerative diseases. The mechanisms mediating cell death in neurological diseases are not always well defined. It is thought that the greater stimulus causing cell death in acute diseases results in both necrotic and apoptotic cell death, whereas the milder insults in chronic diseases initiate apoptotic cell death (Friedlander, 2003). Therefore, it seems that there is no always a clear difference between the features of apoptotic and non-apoptotic (necrotic) death. Apoptotic cells may undergo secondary necrosis, during which they swell and lyse, so at this point it is not possible to distinguish it from necrotic cell. This implied that necrosis and apoptosis shared biochemical network through both caspase-dependent mechanisms as well as non-caspase-dependent effectors (Zeiss et al, 2003). Present opinion is that instead of two distinct types of cell death, there is a continuum of responses (Syntichaki et al, 2003). In our study, both gain-of and loss-of function in the CREB pathway trigger the expression of apoptotic markers, such as active caspase 3 and positive TUNEL staining, although other aspect of their phenotypes showed interesting differences.

Electron microscopy analyses on VP16-CREB^{high} brains, together with the physiological alterations observed in VP16-CREB expressing neurons, revealed a

dramatic gliosis and strong inflammatory response. These data made us suggest that excitotoxic death is the mechanism taking place in neurons with enhanced CREB-driven gene expression. It was believed that seizure-induced neuronal death is primarily morphologically necrotic and does not depend on the activation of apoptotic cascade (Olney et al, 2003, Ishimaru et al, 1999, Fujikawa et al, 2002). However, recent investigators (Leist et al, 2001) also suggest mixed apoptotic-necrotic mechanisms. One of the typical consequences of deleterious effect of excitotoxicity is damaging neighboring cells by liberation of the cellular contents into the intercellular space and significant induction of the inflammatory response. This was observed only in mice with CREB gain-of function. Microarray experiments confirmed that, in general, the genes involved in inflammation and immune response are markedly induced in these mice.

Taking in account the kindling experiments, which also revealed that these animals are more susceptible to the onset of epileptic seizures, it was interesting to investigate if the change in excitability precedes the neurodegeneration, or extensive cell loss is independently developed. Excitotoxicity appears when high concentrations of glutamate or other substrates acting at the same receptors, cause cell death through the excessive activation of these receptors. Electrophysiological experiments were performed in order to check whether 2 hours of incubation VP16-CREB^{high} slices with glutamate agonist (5μM NBQX) change slow after-hyperpolarisation current and no differences with the adequate controls were found (López de Armentia et al, 2007). This suggests that slow AHP is not dependent on external glutamate and, most likely, higher excitability precedes ongoing neurodegeneration.

In contrast, A-CREB mice have increased resistance to epileptic attacks, but develop dramatic cell loss in most of the areas of transgene expression. The underlying reasons for this feature were searched in inadequate neurotrophine release. Earlier experiments on VP16-CREB^{high} mice confirmed the upregulation of BDNF by enhanced CREB activity (Barco et al, 2005). However, studies in double CREB/CREM knockout in dopaminergic neurons did not associate reduced level of CREB with repression of the common prosurvival factors, such as BDNF (Parlato et al, 2006). Our data on A-CREB mice provided similar conclusions. The absence of change in protein levels of BDNF or other related factors was explained by their ability to diffuse among cells, to enter to affected neurons from healthy cells and promote their survival (Alemida et al, 2005, Parlato et al, 2006). Therefore, altered expression of other, yet unidentified genes might

underlay the severe neurodegeneration in cells with reduced CREB activity. In addition, A-CREB transgene is expressed in murine forebrain during embryonic and postnatal development, so chronic presence of the stimulus that cause neuronal death might not be sufficient to provoke strong antiapototic reaction of the organism. However, it should be noted that, unlike CREB/CREM double knockouts, in A-CREB the activity of entire CREB family is reduced. This implies that the blocking of CREB, CREM and ATF-1 activities contribute to the observed phenotype. Since the previous attempts to knockdown both CREB and ATF-1 resulted in the death at the embryonic state (Bleckmann et al, 2002), and there were no transgenic animals generated to study the role of ATF-1 in the CNS, we cannot exclude the possibility that ATF-1 also contributes to the neuronal survival in the murine hippocampus.

D3.3. Is it possible to dissect the role of CREB in synaptic plasticity and memory of that promoting neuronal survival?

CREB protein is a convergence point for cascades of gene expression that, among others, regulates survival and synaptic plasticity. The ubiquitous CREB protein targets a wide range of downstream genes, which are also involved in many other cellular processes. The genes affected by induction of VP16-CREB transgene for one week were primarily related to synaptic plasticity. At this post-induction time, increment of neuronal activity and facilitated L-LTP were found. Most of the genes changed in the arrays obtained from the brains of animals expressing VP16-CREB from six weeks were associated to pathology, such as immune response, gliosis or inflammation. These animals also suffered severe cell loss in the hippocampus. However, it cannot be deduced if these processes are independent or not. Similar results were observed in A-CREB mice, in which it was not possible to conclude whether the reduced excitability in three weeks old animals is related to severe cell loss in the CA1 area occurring several weeks later.

We also performed behavioral analyses of A-CREB mice. These analyses are time consuming and cannot be performed in animals before weaning age, so all experiments were done at an age in which the significant neurodegeneration already took place. We observed severe deficits in Morris Watermaze task. Moreover, general activity and anxiety were also increased in these animals. Indeed, a significant freezing

behavior was observed already at training in the fear-conditioning chamber that affected the analyses of 24 hours-later task. Time needed to learn rotarod task was also significantly prolonged in the bitransgenic mice. However, when we performed analyses of the transgene expression in the murine forebrain, the remaining A-CREB positive cells were predominantly located in the cortical layers and only a few cells in the hippocampus. Although A-CREB positive neurons in the cerebral cortex may play a role in some of the observed phenotypes, we cannot relate these behavioral abnormalities with A-CREB transgene expression, but rather with the wide spread degeneration detected at this stage. For these reasons, our next studies will be performed in mice in which the expression of the transgene can be induced at adult age or stopped and compared to mice with chronic reduction of CREB activity.

D3.4. Dangers of prolonged alteration of the CREB activity

Our results reveal the dangers of prolonged and uncontrolled manipulation of the CREB signaling cascade. Although neuroprotective role of CREB has been shown in models of cerebral ischemia (Kitagawa et al, 2007), it is essential to be careful with use and strength of CREB-based therapeutics. In wild type animals, the phosphorylation of CREB occurs in response to neuronal activity is transient and promotes the expression of specific downstream genes in a narrow time-window. In our transgenics the activity of CREB is continuously elevated. By its constitutively activation, CREB may lose its selectivity to promote only a subset of downstream targets.

We showed that only well balanced CREB-driven transcription promotes neuronal survival. The possibility to initiate, pause and reactivate neurodegeneration using doxycycline makes the transgenic strains described here powerful models to assay therapies aimed to recover after these two different types of neurodegeneration.

The putative clinical application of manipulation of the CREB pathway stresses the relevance of the study of CREB as convergence point of molecular events, of enormous importance in basic science. Phenomena such as long-term depression and potentiation and switch from short to long-term memory formation and its consolidation are intimately associated to CREB activation. Future studies should be directed toward understanding the downstream genes activated by CREB according to the type of stimulation, cell or tissue type, context and time. Establishing a connection between

specific “stimulation–pathway–target gene–physiological outcome” cascade will provide breakthrough insight into function and dysfunction of the mammalian brain.

E. Conclusions

In order to investigate how CREB activity contributes to the survival and excitability of hippocampal neurons, I investigated the phenotype of transgenic mice expressing a constitutively active CREB variant (VP16-CREB mice) and mice with reduced CREB-dependent transcription (A-CREB mice). Some of the major findings of these studies and their comparison are:

1) We demonstrated bidirectional regulation of neuronal excitability by CREB activity in hippocampal CA1 neurons *in vivo*. Enhanced CREB-dependent gene expression increases neuronal excitability of CA1, most likely by significant reduction of both mAHP and sAHP in CA1 pyramidal neurons**.** The M potassium current underlies the observed decrement in neuronal excitability in the CA1 hippocampal subfield of A-CREB. (This project was coordinated together with Dr. Mikel López de Armentia.)

2) Kindling experiments in bitransgenic mice with chronic inhibition or enhancement of CREB function showed the important relevance of CREB for the accurate regulation of neuronal excitability in epilepsy. When daily treated with subconvulsive doses of proepileptic drugs, A-CREB mice showed a higher resistance to attacks. VP16-CREB mice presented, on the other hand, a higher incidence of seizures. These findings suggest that the activation of CREB-dependent gene expression cannot only contribute to the stabilization of ongoing reinforcements of synaptic connections (consolidation), but also facilitate future neuronal responses in a given time range (sensitization).

3) The experiments in mice with constitutively active CREB mice in which the transgene was expressed at different level (VP16-CREB^{high} and VP16-CREB^{low} mice) showed a clear dose-dependent effect in physiological properties, such as LTP and neuronal excitability.

4) CREB function is sufficient, but not always necessary for activity-driven gene expression. We dissected the sets of downstream genes that are transcribed in response to kainate, and revealed modest effects in activity driven gene expression in the hippocampi of A-CREB mice. However, several synaptic plasticity related genes (*Arc*,

Egr1, *Egr2*, *Trat1*, *Scn4b*, *Homer*1) are found to be altered in A-CREB mice in the basal condition.

5) The chronic inhibition of CREB function causes severe loss of neurons in the CA1 subfield as well as in other brain regions. Interestingly, the chronic enhancement of CREB activity also leads to degeneration of CA1 neurons.

6) The mechanisms of cell death in A-CREB and VP16-CREB mice differ. Whereas neuronal loss in mice with overexpression of CREB seems to be associated to strong inflammation and excitotoxic insult, apoptosis and atrophy may predominantly underlay cell loss in A-CREB animals. Therefore, the survival of hippocampal pyramidal neurons relied on precisely controlled activity of the CREB mediated gene expression cascade.

7) We found severe defects in the behavior of adult mice with reduced CREB-driven transcription, likely caused by concurrent effect by severe cellular loss and CREB inhibition.

Conclusiones

Con el objetivo de investigar el papel de la actividad dependiente de CREB en la supervivencia y excitabilidad de neuronas de hipocampo, se investigaron los fenotipos de ratones transgénicos que expresan una variante de CREB constitutivamente activa (ratones VP16-CREB) y de ratones en los que la trascripción dependiente de CREB está reducida (ratones A-CREB). Las conclusiones que aportan estés analices y su comparación pueden resumirse de la siguiente forma:

1) Se demostró una regulación bidireccional de la excitabilidad neuronal mediante la actividad de CREB en neuronas de la región CA1 del hipocampo. Mientras que en ratones VP16-CREB el incremento en la expresión de genes dependientes de CREB produjo un aumento en la excitabilidad neuronal de CA1, causado por una significante reducción de tanto mAHP como sAHP en neuronas piramidales de dicha región, en ratones A-CREB se observó un decremento en la excitabilidad neuronal, la cual fue atribuida a la corriente M de potasio (proyecto coordinado con Mikel López de Armentia).

2) Experimentos de kindling en ratones bitransgénicos en los que la función de CREB está o bien inhibida o bien facilitada mostraron la importante relevancia de CREB en la precisa regulación de la excitabilidad neuronal en epilepsia. Tras un tratamiento diario con dosis subconvulsivas de drogas pro-epilépticas, los ratones A-CREB mostraron una mayor resistencia a los ataques. Los ratones VP16-CREB presentaron, en cambio, una mayor incidencia en el número de ataques. Estos hallazgos sugieren que la activación de la expresión génica dependiente de CREB no sólo puede contribuir a la estabilización de los refuerzos de las conexiones sinápticas (consolidación) sino también facilitar las futuras respuestas neuronales en un periodo de tiempo dado (sensibilización).

3) Los experimentos en ratones en los que CREB estaba constitutivamente expresado a diferentes niveles (VP16-CREB^{high} y VP16-CREB^{low}) mostraron un claro efecto dosis-dependiente en propiedades fisiológicas como LTP y excitabilidad neuronal.

4) La función de CREB es suficiente aunque no siempre necesaria para la

expresión génica inducida por actividad. El análisis del conjunto de genes que son transcritos en respuesta a kainato reveló un modesto efecto en la expresión génica inducida por actividad en el hipocampos de ratones A-CREB. En estos ratones encontramos una reducción en la expresión basal de genes relacionados con plasticidad (*Arc, Egr1, Egr2, Scn4b, Homer1*).

5) La inhibición crónica de la función de CREB causa una pérdida severa de neuronas en el subcampo CA1 así como en otras áreas cerebrales. Interesantemente, la facilitación crónica de la actividad de CREB también lleva a la degeneración de neuronal de CA1

6) Tanto el incremento crónico como la inhibición de la actividad de CREB produjo pérdida neuronal en CA1. Sin embargo, los mecanismos causantes de muerte cellular en ratones A-CREB y VP16-CREB difieren. Mientras que la muerte celular causada por la sobreexpresión de CREB parece asociada a una prominente inflamación y consecuente excitoxicidad, la pérdida celular presente en ratones A-CREB podría ser debida a apoptosis y atrofia. De este modo, la supervivencia de neuronas piramidales de hipocampo es controlada de un modo preciso por la cascada de expresión génica mediada por CREB.

7) Se hallaron severos trastornos en el comportamiento de ratones adultos con reducida transcripción mediada por CREB, los cuales pudieron ser un efecto conjunto de la grave pérdida celular y la inhibición de CREB.

F. References (Introduction and Discussion sections)

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G. Publications and Attendance to Congresses

In addition to the three chapters of Results presented above I have participated in the preparation of two additional publications during my thesis:

1. An opinion article published on *The Journal of Neuroscience* "Hunting for synaptic tagging and capture in memory formation" by Jose Viosca, Dragana Janci, Jose P. Lopez-Atalaya and Eva Benito (*J. Neurosci.* 27(47):12761-1276).

2. The chapter "CREB-dependent transcription and synaptic plasticity" in the book "Regulation of transcription by neuronal activity: To the nucleus and back" by Angel Barco, Dragana Jancic and Eric R. Kandel. (Springer Science publishers, 2007).

Both publications are annexed to this Thesis as **Annex 1** and **Annex 2** respectively.

Full list of publications:

1. Barco A, **Jancic D**. and Kandel. E.R. (2007) CREB-Dependent Transcription and Synaptic Plasticity, Transcriptional Regulation by Neuronal Activity To the Nucleus and Back,10.1007/978-0-387-73609-9_7, editor Serena M. Dudek, Springer Science, US

2. Viosca J, **Jancic D**, López-Atalaya JP, Benito E. Hunting for synaptic tagging and capture in memory formation. J Neuroscience, 2007 Nov 21;27(47):12761-3.

3. Lopez de Armentia M, **Jancic D**, Alarcon JM; Olivares R, Kandel ER, Barco A, (2007), Physiological and pathological increase of neuronal excitability by CREB-mediated gene expression, Journal of Neuroscience, Dec 12;27(50):13909-18.

4. **Jancic D**, Lopez de Armentia**,** M, Lujan R, Valor LM, Olivares R, Barco A, (2008), Inhibition of cAMP-response element binding protein reduces neuronal excitability and plasticity and triggers degeneration of CA1 pyramidal neurons, submitted

5. **Jancic D**, Valor LM, M, Lujan R, Olivares R, Barco A, (2008) Bidirectional control

of CA1 pyramidal neuron survival by cAMP response element binding protein dependent gene expression, manuscript in preparation

Attendance to congresses:

1. CA1 neuron survival depends on normal levels of CREB-dependent gene expression. **Jancic D**. Lujan R. Kandel E. R & Barco, *poster*, FENS Vienna, July 2006.

2. CA1 neuron survival depends on normal levels of CREB-dependent gene expression. **Jancic D**. Lujan R. Kandel E. R & Barco, *poster,* Satellite Symposia at Molecular Cognition Society, Vienna, Austria, July 2006.

3. The survival of CA1 hippocampal neurons depend on normal levels of activity in the CREB pathway, **Jancic D**. Lopez de Armentia, M, Lujan R. Kandel E. R & Barco A, poster, Neurizons, PhD students´congress, Goettingen, Germany, May 2007.

4. The survival of CA1 hippocampal neurons depend on normal levels of activity in the CREB pathway, **Jancic D**. Lopez de Armentia, M, Lujan R. Kandel E. R & Barco A ENI-NET Minisimposia, The sparkinling genome: epigenetic mechanisms in brain function, April, 2008, Alicante, Spain, poster

5. Changes in CREB-driven transcription modulate level of neuronal excitability, **Jancic D**, Lopez De Armentia M, Lujan R, Olivares R, Kandel E. R. & Barco A, *poster,* FENS, Geneve, July 2008

6. Changes in CREB-driven transcription modulate level of neuronal excitability, **Jancic D**, Lopez De Armentia M, Lujan R, Olivares R, Kandel E. R. & Barco A, *poster*, Satellite Symposia at Molecular and Cellular Cognition Society, Geneve, July 2008

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Annex 1

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Journal Club

Editor's Note: These short reviews of a recent paper in the *Journal*, written exclusively by graduate students or postdoctoral fellows, are intended to mimic the journal clubs that exist in your own departments or institutions. For more information on the format and purpose of the Journal Club, please see http://www.jneurosci.org/misc/ifa_features.shtml.

Hunting for Synaptic Tagging and Capture in Memory Formation

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Review of Moncada and Viola (http://www.jneurosci.org/cgi/content/full/27/28/7476)

Long-term potentiation (LTP) and some forms of long-term memory share a number of properties, such as associativity, durability, and protein synthesis dependence. These similarities suggest that LTP could be the cellular substrate for memory (Bliss and Collingridge, 1993). The recently reported changes in synaptic transmission caused by learning in behaving animals (Gruart et al., 2006; Whitlock et al., 2006) have provided significant support to this view and attracted attention to additional properties of LTP potentially relevant in memory formation. Ten years ago, Frey and Morris (1997, 1998) performed a series of two-pathway stimulation experiments that led them to propose the synaptic tagging and capture hypothesis. In their seminal work, Frey and Morris (1997, 1998) showed that early-LTP, a transient form of LTP, which is induced by a weak stimulus, could be converted into late-LTP, a more persistent form of LTP, if the weak and the strong stimuli were applied temporally close to each other on different synapses of the same neuron. These results suggested an interaction between the molecular events connecting early- and late-LTP in different pathways. Thus, the authors speculated that such a mechanism might explain why inconsequential events are remembered for much longer if they occur around the same time as relevant well remembered events. A decade later, a recent study by Moncada and Viola (2007) in *The Journal of Neuroscience* tested the validity of this prediction *in vivo*.

By combining two behavioral tasks, Moncada and Viola (2007) investigated whether a behavioral experience could extend the duration of the memory for an independent task and whether this interaction displayed properties consistent with synaptic capture. In the inhibitory avoidance paradigm (IA), a hippocampal-dependent task (Whitlock et al., 2006), rodents receive an electrical foot shock immediately after they step down from a platform, so they suppress the step-down behavior the next time they are placed on the platform. Thus, the step-down latency can be used as a parameter to assess memory retention. To answer the question of whether this memory could be promoted by its coincidence with an independent task, the authors combined this paradigm with the exploration of a novel spatial context (an open field), which also activates neurons in area CA1 (Vianna et al., 2000). They showed that weak training in the IA task produced short-term memory, lasting no longer than 15 min [Moncada and Viola (2007), their Fig. 1*a* (http://www.jneurosci.org/cgi/content/full/27/28/7476/F1)]. However, when a 5 min session of spatial novelty was given just before the IA task, the short-term memory was converted into a persistent memory lasting 24 h [Moncada and Viola (2007), their Fig. 1*b* (http://www.jneurosci.org/cgi/content/full/27/28/7476/F1)]. Similarly, when the spatial novelty was given after IA training, memory was also extended [Moncada and Viola (2007), their Fig. 4*a* (http://www.jneurosci.org/cgi/content/full/27/28/7476/F4)]. These results resembled the symmetry of the seminal electrophysiological experiments, where a strong tetanus applied either before or after weak tetanization in a different pathway resulted in late-LTP in the weakly stimulated pathway (Frey and Morris, 1997, 1998). Like the previous data, Moncada and Viola defined a restricted temporal window within which coincidence of the two behavioral experiences leads to long-term memory for the weak IA training.

Originally, Frey and Morris (1997, 1998) proposed that weak stimulation established a "tag" in certain synapses. This tag would then capture newly synthesized plasticity proteins that were required for a more persistent potentiation and that were provided by the strong stimulation. Extending the model to the behavioral

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data in Moncada and Viola (2007), certain synapses, tagged by the weak IA training, would capture the plasticity proteins provided by spatial novelty. To explore this possibility, the authors blocked new protein synthesis after the open field session, but before IA training. Stereotaxic infusions of the protein synthesis inhibitor anisomycin into CA1 immediately after the open field session disrupted long-term memory for the weak IA training performed 1 h after the first task [Moncada and Viola (2007), their Fig. 3*a* (left) (http://www.jneurosci.org/cgi/content/full/27/28/7476/F3)]. However, anisomycin had no effect on strong IA training [Moncada and Viola (2007), their Fig. 3*a* (right) (http://www.jneurosci.org/cgi/content/full/27/28/7476/F3)], indicating that it did not affect IA memory consolidation. Furthermore, IA long-term memory was impaired by anisomycin infusions immediately before training, and it was rescued when preceded by an open field session [Moncada and Viola (2007), their Fig. 3*b* (http://www.jneurosci.org/cgi/content/full/27/28/7476/F3)]. Together, these results suggest that new protein synthesis triggered by spatial novelty was necessary and sufficient to drive memory consolidation for IA training. Additionally, because anisomycin acts intracellularly, the observed effects indicate that both behavioral experiences converge onto common cellular substrates. As reported for CA1 responding neurons in animals exposed to two spatial contexts (Vazdarjanova and Guzowski, 2004), the extent of overlapping between open field and IA within CA1 seemed not to be complete, because the memory rescue was only partial [compare open field rescued with vehicle injected in Moncada and Viola (2007), their Fig. 3*b* (http://www.jneurosci.org/cgi/content/full/27/28/7476/F3)]. However, both behavioral tasks very unlikely activated the very same synapses within the neurons onto which they converge. This scenario is reminiscent of the two-pathway stimulation experiments, in which two electrodes stimulated different synaptic inputs to the same neuron. Moreover, from a temporal point of view, the stimuli presented here were applied too far in time to be integrated by means of the coincidence detection mechanism of the NMDA receptor. Additional mechanisms are required to extend the temporal frame for late associativity (Reymann and Frey, 2007). Given that the behavioral interaction required new protein synthesis and seemed to involve a heterosynaptic phenomenon, the synaptic tagging and capture model may be a suitable explanation (Fig. 1).

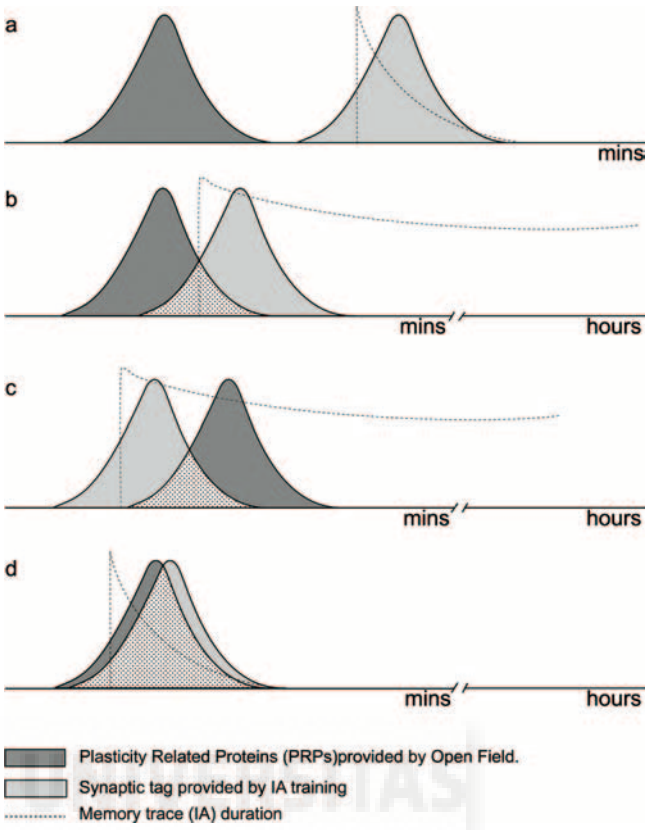

Figure 1. Schematic summary of the behavioral tagging results provided by Moncada and Viola (2007). The magnitude and duration of the variety of phenomena involved are schematically depicted: the IA memory trace and the putative molecular requirements occurring within the same CA1 neurons activated by both spatial novelty and IA training. These neurons would drive the consolidation of IA memory induced by a weak training through a heterosynaptic plasticity process. A set of synapses specifically tagged by the weak IA training-induced neuronal activity would capture the newly synthesized PRPs provided by the spatial novelty stimulation. ***a***, As expected, weak IA training (light gray) led to short-term memory (dotted line), and previous exposure to open field (dark gray) did not affect the IA memory duration when both tasks were spaced by at least 120 min. A similar result was observed when the open field was performed after the weak IA training (data not shown). ***b***, ***c***, Conversely, previous (***b***) or posttraining (***c***) open-field exposure promoted IA long-term memory when both situations were sufficiently close to each other. ***d***, However, novel environment exploration either just before or immediately after training did not result in a switch from short- to long-term IA memory. Adapted from Morris (2006).

A number of parallels emerge from the comparison with previous studies. In addition to the symmetry and the existence of a restricted temporal window, the dopamine neurotransmitter system has been involved in both kind of results (Sajikumar and Frey, 2004). Moreover, the IA LTM rescue by spatial novelty mimics the paradoxical induction of LTP in presence of anisomycin described in the seminal experiments of Frey and Morris (1997, 1998).

It is noticeable, however, that a number of issues need to be addressed. First, the original hypothesis itself remains undemonstrated, because no direct observation of a tag-mediated capture of plasticity factors has been reported. Imaging of subcellular targeting of activity synthesized molecules or biochemical evidences of a molecular interaction required for synaptic capture would provide significant insight. Second, there are some important discrepancies between behavioral and electrophysiological tagging results. For instance, the switch from short- to long-term memory was not achieved when the spatial novelty was applied 30 min before [Moncada and Viola (2007), their Fig. 1*b* (http://www.jneurosci.org/cgi/content/full/27/28/7476/F1)] or immediately after [Moncada and Viola (2007), their Fig. 4*a* (http://www.jneurosci.org/cgi/content/full/27/28/7476/F4)] the weak IA training. Such exceptions within the permissive time window were not observed in hippocampal slices during early-to-late LTP conversion.

In summary, the results provided by Moncada and Viola (2007) share several phenomenological properties with those

in two-pathway stimulation experiments. It is possible that the observed discrepancies reflect minor differences that can be fit within the original model, although they might also reflect unrelated phenomena. All these questions remain to be solved. In the meantime, the results by Moncada and Viola (2007) suggest that synaptic tagging and synaptic capture could underlie long-term memory formation and might provide an explanatory mechanism for the recall of irrelevant events that happened around outstanding events.

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Annex 2

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CREB-Dependent Transcription and Synaptic Plasticity

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Abstract. The CREB family of transcription factors are involved in controlling the transcriptional responses to a wide range of extracellular stimuli in neurons. In this chapter we discuss the role of the CREB pathway in synaptic plasticity. We first describe how learning-related stimuli, of different nature and intensity, can activate signaling pathways that converge on the induction of CRE-driven gene expression and how the nuclear response orchestrated by CREB can alter future synaptic activity. Second, we will discuss how CREB's control of synaptic plasticity contributes to learning, memory and other complex brain function. Finally, we will briefly outline how dysfunction of this activation pathway may lead to disorders of the nervous system.

1 The CREB Family of Transcription Factors

The CREB family of transcription factors refers to a group of highly homologous proteins encoded by the genes *creb*, *crem* and a*tf-1* encode for a group of highly homologous proteins that is frequently referred as. This family is characterized by a highly conserved basic region/leucine zipper (bZIP) domain that bind to a specific DNA sequence called cAMP-responsive-element (CRE) found in one or several copies in the promoters of many genes (Figure 1). Although both, the CRE sequence and CREB, the prototypic member of this family of regulatory molecules, were first identified through studies investigating the regulation of the expression of the hormone somatostatin (Montminy and Bilezikjian, 1987), it was later found that CRE sites were present in the promoter of many other genes and that CREB contributed to the regulation of a variety of cellular responses (Habener et al., 1995; Johannessen et al., 2004; Mayr and Montminy, 2001). In particular, CREB has been involved in many aspects of nervous system function (Carlezon et al., 2005; Lonze and Ginty, 2002), from activity-dependent synaptic plasticity during development (Pham et al.,

1999) and in the adult brain (Martin et al., 2000; Pittenger and Kandel, 1998), to neuronal survival (Dawson and Ginty, 2002; Walton and Dragunow, 2000).

ATF1, CREB and CREM are not the only transcription factors that bind to CRE sites. The ATF/CREB family of transcription factors represents A broader group of bZIP transcription factors, including ATF2, ATF3 and ATF4, share structural features with the CREB family and can also bind to CRE sites. Different ATF/CREB proteins can form selective heterodimers with each other and with other transcription factors such as AP-1 and C/EBP that do not belong to this family but share a bZIP DNA-binding domain.

Although homology in the bZIP domain is the main structural feature used to classify transcription factors belonging to the ATF/CREB family of transcription factors, other structural features are common to the several family members (Mayr and Montminy, 2001). Transcription activation in the CREB family is mediated by two types of transactivation domains: (a) The central kinase-inducible domain (KID), that contains several sites recognized by protein kinases and whose phosphorylation state determines the binding of the transcriptional co-activator CBP and that triggers the inducible transcriptional activity of CREB; and (b) the glutamine-rich domains that contribute to basal transactivation activity by interacting with the transcription machinery and stabilizing the interaction with CRE sites. In the case of CREB two glutamine-rich domains, designated Q1 and Q2, flank the KID domain.

Whereas the *atf-1* gene encodes only one major protein product, the *creb* and *crem* genes have a complex structure, with multiple exons and introns (Habener et al., 1995; Hoeffler et al., 1990). The alternative splicing of CREB and CREM RNAs generates transcripts encoding both repressors and activators (Bartsch et al., 1998; Habener et al., 1995; Mayr and Montminy, 2001). These shorter variants lack transactivation domains and form dimers with reduced or null transactivation capability, but still bind to DNA competing for CRE sites. In the case of CREM, the existence of different translation initiation codons, two alternative spliced bZIP domains and an alternative intronic CRE-driven promoter, which drive the expression of the inducible cAMP early repressor (ICER), provide additional diversity to the capability of dimerization with other bZIP proteins and binding to CRE sites (Habener et al., 1995).

CREB and ATF1 are ubiquitously expressed, whereas CREM is expressed primarily in testis and the neuroendocrine system. Since CREB is the most abundant CRE-binding protein expressed in the nervous system (Hummler et al., 1994) and a large number of studies support a critical role for CREB in controlling transcriptional responses in neurons, we will focus primarily in this transcription factor.

2 Regulation of CREB Activation by Synaptic Activity

CREB participates in numerous cell processes and stands at the crossroad of different signaling pathways. Synaptic activity, hormones, growth factors released during development, hypoxia and stress, among other stimuli, can trigger the phosphorylation of CREB, causing its activation and subsequent induction of CREB-dependent

gene expression. Overall, more than 300 different stimuli have been reported to act through the CREB pathway (Johannessen et al., 2004).

2.1 Brief Overview of Signalling in the CREB Pathway

CREB binds constitutively to CRE sites present in the promoter of cAMP-responsive genes, but it is inactive. The activity of CREB-regulated promoters increases several orders of magnitude when CREB is activated by phosphorylation. The activation of CREB is typically depicted as the consequence of an increase in the levels of the second messengers cAMP and Ca^{2+} (West et al., 2002). Both Ca^{2+} influx or cAMP production trigger the activation of protein kinase cascades that phosphorylates CREB (Figure 2). The phosphorylated form of CREB (pCREB) then recruits the coactivator CREB-binding protein (CBP), which triggers transcription initiation by both acetylating histones in chromatin and bringing the RNA polymerase II complex to the promoter. This description provides a simplified view of a complex and highly regulated process. As we will discuss in the following sections, several kinase cascades can converge on CREB and a large number of proteins contribute to the activation of CREB-dependent gene expression.

2.2 Enzymatic Cascades Leading to CREB Phosphorylation at Ser133

The point of convergence of numerous signalling pathways is the phosphorylation of serine 133 (Ser133) on the KID domain of CREB. Similarly, Ser117 in CREM and Ser63 in ATF1 are the main phospho-acceptor sites in these proteins. Although dozens of kinases have been reported to phosphorylate these serine residues *in vitro*, only a few have been shown to contribute to the activation of CREB-dependent transcription *in vivo*. *In vivo* the phosphorylation of CREB is triggered by a wide variety of stimuli, such as an increase in cAMP after activation of G-coupled receptors, an increase of Ca^{2+} through activation of voltage- or ligand-gated channels, or the activation of receptor tyrosine kinases by growth factors (Lonze and Ginty, 2002). Protein kinase A (PKA), Ca^{2+}/calmodulin-dependent (CaM) kinases and Ras/mitogen-activated protein kinase (MAPK) appear to be the most common kinase activities or cascades responsible for phosphorylating CREB at Ser133 after neuronal stimulation (Deisseroth and Tsien, 2002; Mayr and Montminy, 2001).

PKA was the first protein kinase found to target Ser133 of CREB (Gonzalez et al., 1989). PKA is a tetrameric enzyme that is regulated by intracellular levels of cAMP. This second messenger binds to the regulatory subunits of PKA causing its dissociation of the catalytic subunits, which can then catalyze the transfer of phosphate groups to serine or threonine residues of various proteins, including the major isoforms of CREB, CREM and ATF1. In neurons, the intracellular level of cAMP increases as a consequence of the activation G protein-coupled receptors (GPCRs), such as the receptors for serotonin, dopamine and other important neurotransmitters. The activation of these receptors stimulates the activity of adenylyl cyclases (AC) that converts ATP into cAMP. Some ACs also respond to increases in intracellular Ca^{2+}. Therfore, both Ca^{2+} influx and exposure to dopamine

or other neurotransmitters can lead to CREB phosphorylation through PKA (Johannessen et al., 2004).

Membrane depolarization, for example during NMDA recepor mediated glutamatergic synaptic transmission, can increase the local concentration of Ca^{2+} in the postsynaptic cell by several orders of magnitude and trigger the phosphorylation of CREB (Deisseroth et al., 1996). Ca^{2+} influx occurs not only via ligand-gated cation channels, such as NMDA, but also through voltage-sensitive calcium channels (VSCC). Calmodulin (CaM), a small Ca^{2+} binding protein, transduces these changes in Ca^{2+} level to changes in CREB phosphorylation. In fact, CaM plays a critical role on both the activation and inactivation of the CREB pathway, first through its modulation of CaMKs, and second through interaction with the phosphatase calcineurin (Bito et al., 1996). The binding of Ca^{2+}/CaM causes the rapid activation of CaMKIV, a kinase highly expressed in neurons and located in the cell nucleus where it can directly and efficiently phosphorylate CREB (Deisseroth et al., 1998). Genetic deletion of CaMKIV or its inhibition by antisense oligonucleotides or specific drugs causes a significant reduction in Ser133 phosphorylation (Bito et al., 1996). Although CaMKIV seems to be the most important CaMK regulating the phosphorylation of CREB by synaptic stimulation *in vivo*, CaMKI and CaMKII can also phosphorylate CREB (Dash et al., 1991; Deisseroth et al., 1996; Sheng et al., 1991).

The Ras/mitogen-activated protein kinase (MAPK) and the extracellular signal-regulated protein kinase (ERK) are part of a complex signalling cascade involving the activation of many other kinases, including different members of the ribosomal S6 kinase (RSK) and mitogen and stress-activated kinase (MSK) families and have been also implicated in phosphorylation of CREB (Thomas and Huganir, 2004). In the case of MAPK, activation is typically triggered in response to diverse stressful stimuli or by the binding to receptor tyrosine kinases (RTK) of growth factors, such as the brain derived neurotrophic factor (BDNF) or the nerve growth factor (NGF). RTKs are also connected to the PI3-kinase/Akt pathway, still another signalling cascade that controls CREB phosphorylation under certain circumstances (Lin et al., 2001; Perkinton et al., 2002).

The number of protein kinases found to phosphorylate CREB *in vitro* is even larger, but the relevance of these activities in neuronal function is not clear (Johannessen et al., 2004; Lonze and Ginty, 2002). The convergence of all these signaling cascades, which respond to different stimuli and have different kinetics, allows a fine discrimination and integration of stimuli of different nature and enables the production of highly specific transcriptional responses. For example, the Ras/MAPK pathway is thought to be specifically effective in promoting a slow phase of CREB phosphorylation, whereas CaMKIV signalling is more rapid and transient (Wu et al., 2001). Whereas stimuli that produce a modest Ca^{2+} influx trigger only the fast CaMKIV pathway, stronger stimuli can recruit, in addition, the MAPK pathway and cause a sustained increase in pCREB (Deisseroth et al., 1998; Deisseroth and Tsien, 2002). Furthermore, neurons are highly polarized and compartmentalized cells and not only the nature and intensity of the stimulus, but also the timing and cellular context of the stimulus are important. Thus, whereas synaptic activation of NMDA receptors induces CREB phosphorylation and triggers gene expression, its extrasyn-

aptic activation, acting through divergent signaling, dephosphorylates CREB and promotes neuronal death (Hardingham et al., 2002).

2.3 CREB Can be Phosphorylated at Different Sites

The regulation of CREB activation is more complex than a single phosphorylation switch at Ser133. Several other serine residues are now known to be targets of kinase activities *in vitro*, including Ser89, Ser98, Ser108, Ser111, Ser114, Ser117, Ser121, Ser129, Ser142, Ser143 and Ser156 (Johannessen et al., 2004). The biological relevance of most of these variants is still unknown. The serine residues located in the KID domain contribute to the interaction with CBP and are especially relevant. Although transfection studies in cell culture suggested that phosphorylation at Ser142 has a negative effect on CREB-dependent expression (Sun et al., 1994; Wu and McMurray, 2001) because this modification physically disrupts the interaction between CREB and CBP (Radhakrishnan et al., 1997), more recent studies have demonstrated positive regulation by phosphorylation at this site and suggest that some forms of CREB-dependent gene expression may require an alternative, CBP-independent, mechanism (Gau et al., 2002; Kornhauser et al., 2002). For effective stimulation of CREB-dependent transcription by Ca^{2+} influx is required the triple phosphorylation of CREB at Ser133, Ser142 and Ser143. The elevation of nuclear Ca^{2+} causes the phosphorylation by CaM kinases of both Ser 133 and Ser142, which, in turn, favors a the phosphorylation by casein kinase II (CKII) of Ser143 (Kornhauser et al., 2002). On the other hand, both glutamate and exposure to light induce the phosphorylation of CREB at Ser142 in the suprachiasmatic nucleus, a brain region known to control the circadian rhythm. Knock-in mice bearing a mutation that specifically blocks phosphorylation at Ser142 showed light-induced phase shifts of locomotor activity and an attenuated expression of c-Fos and mPer1, two genes known to participate in the control of circadian rhythms, suggesting that there is a positive role for phosphorylation of Ser142 in the circadian process (Gau et al., 2002).

2.4 CREB Dephophorylation

To keep CREB activity precisely balanced is essential coordinated regulation of both kinases and phosphatases. The most important phosphatases known to directly dephosphorylate CREB are PP-1 and PP-2A, although PP-1 seems the most likely candidate to constraint CREB activation in neurons (Genoux et al., 2002; Jouvenceau et al., 2006). Calcineurin, also called PP-2B, is regulated by CaM and Ca^{2+} influx and functions as negative regulator of the CREB pathway by potentiating PP-1 activity through the inhibition of inhibitor-1 and DARPP-32, two negative regulators of PP-1 (Cohen, 1989). The balance of kinase and phosphatase activities modulates, among other parameters, the duration of CREB phosphorylation (Bito et al., 1996; Wu et al., 2001). Whereas weak synaptic stimulation leads to rapid dephosphorylation of pCREB via calcineurin-mediated PP-1 activation, strong stimuli provoke the inactivation of calcineurin thereby enabling an extended phosphorylation of CREB and, as a consequence, robust CREB-dependent transcription (Bito et al., 1996).

Interestingly, histone deacetylase 1 (HDAC1) interacts directly with both CREB and PP1 and it is thought that the formation of HDAC1/PP1 complex may control the duration of CREB activation, as well as CREB-mediated gene expression (Canettieri et al., 2003). It has not been determined, however, whether this interaction, originally discovered in an immortalized cell line, takes also place in neurons. Furthermore, as we described for activation of CREB, there is cross-talk between different signalling cascades and, at least in non neuronal tissues, other phosphatases, such as the phosphatase and tensin homologue deleted on chromosome 10 phosphatase (PTEN) and the tyrosine phosphatase 1B (PTP1B), can play indirect roles in regulating the activation of the CREB pathway (Gum et al., 2003; Huang et al., 2001).

2.5 Modulation of the CREB Pathway by Acetylation and Other Posttranslational Modifications

The phosphorylation of the KID domain, although necessary, is not sufficient to trigger CREB-dependent transcription. As mentioned above, the recruitment of the co-activators, CREB-binding protein (CBP) and p300, is critical for successful CREB activation (Chrivia et al., 1993). CBP and p300 are histone acetyltransferases (HATs), but, in addition, they also acetylate other proteins including CREB itself (Chan and La Thangue, 2001). Thus, CBP acetylates CREB at 3 lysine residues (at Lys-91, Lys-96, and Lys-136) within the activation domain (Lu et al., 2003). Interestingly, the location of Lys-136, proximal to Ser133, is conserved in other transcription factors of the CREB family. Although it has been postulated that acetylation may diminish phosphatase-dependent attenuation of CREB activity, the role of this posttranslational modification remains unclear (Lu et al., 2003).

CREB is also substrate of other enzymatic activities: ubiquitination and SUMOylation of CREB have been observed after hypoxia (Comerford et al., 2003) and *O*-GlcNAc glycosylation, at specific residues, has been found to be associated with reduced CREB-mediated transcription in cell culture (Lamarre-Vincent and Hsieh-Wilson, 2003). The relevance of these modifications in brain function is not known.

2.6 Regulation of the CREB Pathway Downstream of CREB

The induction of CRE-driven gene expression requires the interaction of the phosphorylated KID domain of CREB with the KIX domain of its co-activators CBP and p300, which are themselves targets of several posttranscriptional modifications. The ability of CBP to recruit specific transcription factors increases selectively after phosphorylation by growth factor–dependent signaling pathways at Ser436 (Zanger et al., 2001). CBP is also phosphorylated at Ser301 by CaM kinases (Impey et al., 2002) and is methylated at Arg residues by the methylase CARM1 (Xu et al., 2001). Whereas phosphorylation contributes to CBP-mediated transcription, methylation interferes with CREB interaction and prevents CREB-dependent gene expression. The activation of the PKA, CaMKIV and p42/44 MAPK pathways also increase the transactivation capability of CBP (Hu et al., 1999; Liu et al., 1999).

CBP and p300 are not exclusive coactivators of CREB. They interact with many other transcription factors and nuclear proteins (Chan and La Thangue, 2001). As a

consequence, competition for occupancy of the KIX domain represents an additional mechanism of control in the CREB pathway. Our understanding of the significance of these regulatory steps in CREB-dependent gene expression *in vivo* is still limited, but regulation of CREB co-activators undoubtedly provides additional mechanisms of control for stimulus-induced gene expression.

2.7 CRE Availability and Competition for Binding Sites

In response to activation of CREB different cell types express different sets of target genes. The availability of different CRE sites for binding is likely regulated by epigenetic changes in the chromatin, such as DNA methylation. Even with a open chromatin configuration, the availability of CRE sites also depends on the particular balance between inhibitor and activator forms of CRE-binding proteins expressed in that particular cell under those condition. Whereas some CRE sites can be occupied by CREB under basal conditions, as traditionally proposed, others may require the elimination of repressor constrains and would be occupied only after cAMP stimulation (Cha-Molstad et al., 2004; Guan et al., 2002).

The complex transcriptional regulation and alternative splicing of the *creb* and *crem* genes provide additional mechanisms for tissue or cell type-specific regulation of CREB-dependent gene expression. The expression of different repressor isoforms of CREM, such as ICER and the CREMα, β and γ variants, can be restricted to specific brain areas and play a pivotal role in the regulation of CREB-mediated gene expression (Mellstrom et al., 1993; Mioduszewska et al., 2003). Even non-phosphorylated CREB can contribute to the regulation of transcriptional responses by competing with other transcription factors for DNA binding sites. Similar balances of repressor and activators forms of CRE-binding proteins have been described in invertebrates. In *Aplysia*, the competition between the activator ApCREB1a and the repressors ApCREB2 and ApCREB1b controls the expression of CRE-driven genes and neuronal responses (Bartsch et al., 1998; Bartsch et al., 1995; Guan et al., 2002). In *Drosophila*, the nomenclature is different and the activator dCREB2 competes with the repressors dCREB1 to drive CRE-mediated gene expression (Yin et al., 1995; Yin et al., 1994)

There are, therefore, many mechanisms by which CRE-driven transcription is modulated. We have tried to present here a comprehensible although non-exhaustive overview of this highly regulated process. The complexity of the network of enzymatic activities and transcription factors that regulates the activation and shut-off of the CREB pathway emphasizes the physiological relevance of the transcriptional responses orchestrated by this family of transcription factors.

3 Regulation of Synaptic Function by CREB

Learning and memory storage are thought to depend on long-lasting changes in the strength of synaptic connections (Kandel, 2001). These stable changes may last hours, days or even years, and are known to depend on *de novo* gene expression. This is the case of the late phase of long-term potentiation (LTP) in the mammalian

hippocampus or long-term facilitation (LTF) in *Aplysia* sensory-neurons, two processes that are blocked by inhibitors of transcription and that have provided so far the most compelling view of how the induction of gene expression can modify synaptic function (Barco et al., 2006). The CREB pathway has been identified as a major regulator of both processes. Although we will focus in hippocampal synaptic plasticity, CREB function has been related to the regulation of synaptic plasticity not only in the hippocampus, but also in the amygdala, basal ganglia and various regions of the neocortex and cerebellum.

3.1 Brief Overview of the Control of Synaptic Function by CREB

CREB is located in the cell nucleus where it regulates transcription. CREB is, therefore, not directly involved in synaptic function, although it can play a critical role in synapse formation and in synaptic transmission by driving the expression of synaptic proteins (Figure 3). The electrical or chemical stimulation of a neuron can induce or enhance the expression of a number of genes, which based on their time-course of induction can be classified either as immediate early genes (IEGs), intermediate or delayed response genes. Immediate early genes represent the initial nuclear response to the activation of intracellular signaling cascades (Tischmeyer and Grimm, 1999) and have been extensively used for mapping neuronal activity (Guzowski et al., 2005; Kaczmarek and Chaudhuri, 1997). Many IEGs have one or more CRE sites in their promoters and are induced after activation of the CREB pathway. The expression of the mRNAS for IEGs is fast, transient, and does not depend on de novo protein synthesis indicating that the transcription factors controlling their induction, such as CREB, are already present in the basal state. Some of the IEGs regulated by CREB, such as c-fos, egr1 or C/EBPβ, are themselves transcription factors, whose induction trigger a second wave of gene expression and may lead to the expression of intermediate and delayed response genes. Overall, the gene expression cascade initiated by CREB activation seems to provide the building blocks required for the stabilization of the otherwise transient strengthening of synaptic connections. Both the formation of new synapses and the remodeling of pre-existing synapses are thought to play a critical role in this stabilization.

3.2 Evidence for a Role of CREB Controlling Synaptic Plasticity

Studies in the sea snail *Aplysia* first established the critical role of the cAMP signaling pathway in long-term facilitation (LTF, a term equivalent to LTP as described in mammals) (Brunelli et al., 1976) and have provided a refined view of the role of CREB in synaptic plasticity (Kandel, 2001). ApCREB-1 controls the transcription of several immediate-early response genes that contribute to the stabilization of the short-term process and its conversion to the long-term process. This is the case of the ubiquitin hydrolase, which regulates the degradation of the regulatory subunit of PKA that constrains long-term facilitation (Hegde et al., 1997), and the transcription factor C/EBP, which initiates a second wave of gene expression (Alberini et al., 1994). In agreement with this model, the injection of phosphorylated ApCREB-1 into cultured sensory neurons can by itself induce LTF (Bartsch et al., 1998; Casadio

et al., 1999). Conversely, the inhibition of the repressor ApCREB-2 enhances long-term facilitation (Bartsch et al., 1995). This burst of gene expression stabilizes the strengthening of synaptic connections and leads to the growth of new synapses.

Many aspects of the role of CREB regulating synaptic plasticity seem to be conserved through evolution from mollusk to mammals. Thus, the enhanced expression of CRE-driven genes also favors the formation and stability of LTP in the mouse hippocampus (Barco et al., 2002; Marie et al., 2005). Conversely, inhibition of CRE-driven expression using a dominant negative form of CREB, which prevents the binding to DNA of different members of the CREB family, caused clear deficits in different forms of LTP (Huang et al., 2004; Pittenger et al., 2002). The results obtained with CREB hypomorphic mutants (mice homozygous for a deletion of the α and δ isoforms) and brain-restricted knockouts were, however, more controversial. The deficit in the late phase of LTP first detected in CREB hypomorphic mutant (Bourtchuladze et al., 1994) was later found to be sensitive to genetic background and gene dosage (Balschun et al., 2003; Gass et al., 1998) and no deficit was found in mice in which CREB was specifically depleted in forebrain neurons (Balschun et al., 2003). The overexpression of other CRE-binding proteins, such as CREM and the CREBβ isoform, observed in CREB hypomorphic and knockout mice suggests that the up regulation of these genes can compensate for the loss of CREB and attenuate the deficits (Balschun et al., 2003; Blendy et al., 1996; Hummler et al., 1994).

3.3 Identifying the CREB Transcriptome

CREB binds with high affinity to the palindromic consensus sequence for CRE, TGACGTCA, which is present on average once every 65 Kbp of random DNA sequence. This ratio represents 45,000 CRE sites in the human genome, the actual number is however much lower. A recent study identified only about 10,000 CRE palindromes in our genomic sequence (Zhang et al., 2005). The number of putative CREB binding sites is actually much higher because the promoters of many cAMP responsive genes often contain only a half-site TGACG sequence. The same study identified almost 750,000 half CREs in the human genome. However, the methylation of CRE sequences restricts CREB occupancy to functionally relevant sites and most of these sites do not bind CREB (Zhang et al., 2005). While classical biochemical and molecular studies, both *in vitro* and *in vivo*, demonstrated the participation of CREB in the regulation of the expression of more than one hundred genes, the availability of the complete sequence of the mouse and human genome, the constant refinement of bioinformatics tools for their analysis and the widespread application of genome-wide transcriptional profiling tools has allowed the recent identification of many more potential targets.

The widespread application of high-density cDNA and oligonucleotide microarrays, to both, studies on synaptic plasticity and behavior (Lee et al., 2005; Park et al., 2006) and the characterization of CREB mutant mice (Barco et al., 2005; McClung and Nestler, 2003), has confirmed many previously identified targets, and revealed hundred of candidate genes to be regulated directly or indirectly by CREB (Barco et al., 2005; Lee et al., 2005; McClung and Nestler, 2003; Park et al., 2006). Interest-

ingly, a recent time-course microarray analysis of LTP-induced gene expression in the dentate gyrus revealed that activity-induced genes are frequently clustered on chromosomes. Many of the genes identified in this study located in chromosomal domains enriched with CREB-binding sites and displayed CREB-mediated transcription (Park et al., 2006).

An important recent development in the analysis of global transcription has been the application of chromatin immunoprecipitation (ChIP) techniques to genome-wide studies. In these techniques, chromatin is immunoprecipitated using a CREB antibody, and DNA bound sequences are amplified and identified using different approaches. In contrast to expression arrays, this methodology can distinguish between direct and indirect targets of CREB. Using serial analysis of chromatin occupancy (SACO), a technique that combines ChIP assays with the sequencing of short tags similar to those used in serial analysis of gene expression (SAGE), Impey and colleagues identified a large number of genes in rat PC12 cells regulated by CREB (Impey et al., 2004). More than 60% of the CRE sites occupied by CREB in the basal condition were located in or near transcriptionally active regions, frequently within previously identified CREB-regulated genes. Strikingly, some of these CRE sites were located in bidirectional promoters and in promoters driving the expression of miRNAs and antisense transcripts (Impey et al., 2004).

An alternative approach for the analysis genome-wide transcription is the production of arrays of promoters suitable for what has been called "ChIP on chip" analysis. Here, immunoprecipitated DNA is amplified by PCR, labeled and hybridized to a microarray displaying promoter sequences. This technique was recently applied to screen for CREB target genes located in the chromosome 22, which represents about 1% of the human genome. This study revealed 215 binding sites corresponding to 192 loci. Most of the sites did not correspond to consensus CREs, but to shorter variants, and were located in regions outside known promoters. Only a subset of these candidate genes was affected by forskolin in cultured cells (Euskirchen et al., 2004). Another recent screen for CREB target genes, also based on ChIP-on-chip technology but with a genome-wide approach, demonstrated that CREB occupies at least 4000 promoter sites *in vivo*. Although the profiles of CREB occupancy were very similar in different human tissues, in a given cell type only a small proportion of CREB target genes were induced by forskolin (Zhang et al., 2005). Similar results were obtained using a different approach based on a hidden Markov model (HMM) trained on known CREB binding sites. This model, a bioinformatics tool, identified more than one 1,600 putative functional CRE sites in the human genome (Conkright et al., 2003), some of which were tested in cell culture. Only those promoters with a TATA box proximal to the CRE site exhibited a strong up regulation in response to forskolin. When the CRE site was moved farer from the TATA box the capability to drive cAMP responsive transcription was reduced. For technical reasons, most of these studies focused on culture cell lines, but it is only a matter of time that these unbiased, genome-wide screening approaches will be applied to the intact nervous system.

The current list of CREB target genes is heterogeneous and includes several hundred genes with very different functions, from transcription and metabolism regulation to cell structure or signaling. Some of the major classes are: transcrip-

tional/nuclear factors (c-Fos, nurr77, zif268); growth factors (BDNF), signalling molecules (IGF-1, MKP-1, TGF-2, VGF), neuronal genes (synaptotagmin IV, presenilin-2), metabolic factors (cytochrome-c, SOD-2), molecules involved in the cell cycle, DNA repair and proliferation (DNA polymerase, cyclin 2), opioid receptors, neurotransmitter receptor subunits (GluR1), molecules important for transport, structural proteins and factors of immune response (Barco and Kandel, 2005; Impey et al., 2004; Lonze and Ginty, 2002; Zhang et al., 2005). Despise the recent advances in the field, the complete set of CRE sites bound by CREB in a specific cell type or under an specific stimuli is still not known and it is still not clear how many of these downstream genes are really regulated in the brain under physiological conditions.

3.4 Transport of mRNAs, Local Protein Synthesis and Synaptic Capture

Upon transcription activation following high frequency stimulation, as occurs with LTP, or heterosynaptic stimulation, as described for LTF, the mRNAs produced *de novo* are translocated specifically to the synapses that received the stimulation (Steward and Worley, 2001). This specifity may reach the level of single synapses and is one of the most relevant properties of LTP and LTF as the cellular correlate of memory formation (Govindarajan et al., 2006; Martin et al., 2000). The participation of the cell nucleus and the requirement of *de novo* gene expression in long-lasting forms of synaptic plasticity impose a critical requirement for any model trying to explain learning-related plasticity: there must be mechanisms that restrict the action of the newly expressed gene products to active synapses but not to others. To address this problem, Frey and Morris suggested that the persistence of LTP is mediated by the generation of a transient local synaptic tag at recently activated synapses and by the production of plasticity-related proteins that can be used only at those synapses marked by a tag (Frey and Morris, 1997), an idea referred to us as the synaptic capture or synaptic tagging hypothesis. They found that once transcription-dependent LTP has been induced at one pathway, the long-term process can be "captured" at a second pathway receiving a stimulation that would normally produce only E–LTP, but can elicit the formation of the tag. Further studies revealed the precise time course of the tag and showed that synapses can be tagged shortly before, at the same time or after the stimulus that elicited the long-term process (Frey and Morris, 1998; Sajikumar and Frey, 2004).

In parallel, Martin and colleagues working in *Aplysia* cultured neurons independently described the phenomenon of synaptic capture in the nervous system of this organism (Martin et al., 1997). Indeed, studies on the gill-withdrawal reflex of *Aplysia* first revealed a direct role of CREB-dependent gene expression in synaptic capture. Kandel and co-workers demonstrated that repeated application of the neurotransmitter serotonin to one synapse branch caused CREB activation at the cell nucleus, and branch-specific LTF and the growth of new synaptic connections in the stimulated branch (Martin et al., 1997). Moreover, they found that the mere injection of phospho-CREB when paired with a single pulse of 5-HT in one of the branches increased varicosities formation. This single pulse of 5-HT marked the branch enabling the capture of the gene products produced by CREB activity and provided the building blocks necessary for the formation of new synaptic connections (Casadio et

al., 1999). Similarly, in CA1 hippocampal neurons of transgenic mice, the expression of a constitutively active variant of CREB, VP16-CREB enhanced CRE-driven gene expression and reduced the threshold for eliciting a persistent late phase of LTP in the Schaffer collateral pathway (Barco et al., 2002). The pharmacological characterization of this form of facilitated L-LTP suggested that VP16-CREB activity can lead to a cell-wide priming for LTP by seeding the synaptic terminals with proteins and mRNAs required for the stabilization of L-LTP. As described in *Aplysia*, these gene products can then be used productively for L-LTP in synapses that have been tagged by stimulation of the sort normally needed for eliciting E-LTP (Barco et al., 2002). Transcription profiling analysis identified the neurotrophin BDNF as the most relevant effector molecule contributing to this enhanced LTP phenotype. Indeed, experiments in BDNF deficient mice suggested that presynapticaly released BDNF contributes to tagging the synapse in normal mice (Barco et al., 2005).

The presynaptic release of BDNF is not the only molecular process involved in synaptic tagging. Since the proposal that previously activated synapses are marked was introduced, the molecular nature of this mark has been intensively investigated both in mammals and *Aplysia* (Martin and Kosik, 2002; Morris, 2006). Several lines of evidence now point to a PKA-mediated phosphorylation event as being critical for the mark (Barco et al., 2002; Navakkode et al., 2004; Young et al., 2006) and that local protein synthesis is a required step that enable the functional capture of the transcripts produced at the nucleus (Casadio et al., 1999; Martin and Kosik, 2002; Si et al., 2003).

The presence of functional machinery for protein synthesis in neuronal processes suggests that local protein synthesis may play a major role in the control of synaptic strength (Schuman et al., 2006; Sutton and Schuman, 2005). Indeed, the induction of LTP in the Schaffer collateral pathway is accompanied by the transport of polysomes from dendritic shafts to active spines of CA1 neurons (Ostroff et al., 2002). In this way, the mRNAs synthesized as a consequence of nuclear activation can be translated exactly where the mRNAs are needed and lead to rapid changes in synaptic strength (Steward and Schuman, 2003).

3.4 Synaptic Growth and Remodeling

Long-lasting forms of LTF and LTP require *de novo* gene expression. The newly synthesized gene products are thought to participate in the formation of new synaptic connections (see recent reviews by (Hayashi and Majewska, 2005; Lamprecht and LeDoux, 2004; Segal, 2005)). Synaptic growth has been found to accompany various forms of learning-related plasticity, a phenomenon particularly well documented in *Aplysia* (Bailey and Kandel, 1993). In this organism, the injection of phosphorylated CREB-1 into cultured sensory neurons can causes long-lasting synaptic changes and leads to the formation of stable new synaptic connections when paired to weak synaptic stimulation (Casadio et al., 1999). However, in the mammalian brain, these structural changes are subtler and more difficult to study. The production of LTP has been associated with the generation and enlargement of dendritic spines in organotypic hippocampal slices (Matsuzaki et al., 2004; Nagerl et al., 2004) and acute slices of neonatal animals (Zhou et al., 2004), but the structural changes are much more

discreet in the adult brain (Lang et al., 2004). In the adult brain, there is only a modest production of new spines (Zuo et al., 2005) and learning-related plasticity seems to rely more on subtle functional changes than in frank anatomical changes. However, as found in *Aplysia*, CREB-dependent gene expression when coupled to subthreshold synaptic activation seems to be sufficient to drive long lasting changes in synaptic function, including the conversion of silent synapses into active ones (Marie et al., 2005).

4 CREB and Brain Function

Given the important role of CREB regulating synaptic plasticity it is not surprising that this transcription factor had been involved in a variety of critical brain functions. Here will focus on the role of the CREB pathway in regulating different forms of learning and memory as an example of CREB's more general role controlling the persistence of changes in synaptic strength. However, it should be noted that the stabilization of the potentiation of synaptic connections is critical not only for learning but for many other aspects of brain function. Long-lasting changes in circuits controlling reward or emotional responses can also be regulated by activation of the CREB pathway, as we will discuss in the next section in reference to drug addiction and depression.

4.1 Learning and Memory

As described above for synaptic plasticity, studies in the sea snail *Aplysia* first identified the cAMP/CREB pathway as a core component of the molecular switch that converts short- to long-term memory for a simple form of learning called sensitization (Brunelli et al., 1976). This role was soon confirmed by the pioneer investigation by Benzer and coworkers on the genetic basis of memory in *Drosophila*. *Dunce* and *rutabaga*, two of the first memory mutants identified by genetic screenings in *Drosophila* (Dudai et al., 1976), were found to affect respectively a cAMP-dependent phosphodiesterase and a Ca^{2+}/CaM-regulated adenylyl cyclase, two important proteins gating the activation of CREB (Byers et al., 1981; Dudai et al., 1983; Waddell and Quinn, 2001). The demonstration of a direct role of CREB in memory formation in flies was provided few years later by the behavioral analysis of transgenic flies overexpressing opposing forms of CREB. Whereas the activator dCREB2 enhanced the formation of long-term memory, the repressor dCREB1suppressed it (Yin et al., 1995; Yin et al., 1994). Parallel studies on CREB hypomorphic mutant mice demonstrated that the reduced expression of CREB caused specific deficit in long-term memory that correlated with deficits in the late phase of LTP also in mammals (Bourtchuladze et al., 1994). These results were soon confirmed by experiments in rats, in which the intra-hippocampal infusion of CREB antisense oligos caused deficits in spatial learning (Guzowski and McGaugh, 1997). However, some of these earlier findings in flies and mammals are now appreciated to be controversial. The memory enhancing effect of overexpressing dCREB2 in flies could not be replicated, although there is agreement that over-expression of the

dCREB1 repressor blocks long-term memory (Perazzona et al., 2004). Similarly, further analyses in mice of CREB hypomorphic and other CREB deficient mutants demonstrated that some of the memory deficits associated to CREB were sensitive to gene dosage, genetic background or molecular mechanism of blocking CREB function (Balschun et al., 2003; Gass et al., 1998; Graves et al., 2002; Rammes et al., 2000). Further work with regulatable CREB deficient mutants has provided a more precise examination of the role of CREB on learning and memory. Thus, the inducible and transient repression of CREB function specifically blocked the consolidation of long-term fear memories (Kida et al., 2002) and caused reversible deficits in spatial navigation at the Morris water maze (Pittenger et al., 2002). In agreement with these results, the expression of a dominant negative CREB mutant in amygdala using a recombinant herpes virus inhibited the consolidation of fear conditioning memories (Josselyn et al., 2004), whereas the acute overexpression of CREB facilitated its formation (Josselyn et al., 2001).

The picture that emerges from these studies suggests that CREB itself may be dispensable for certain forms of explicit memory, likely because the lack of CREB can be compensated by the action of other CRE-binding factors. However, the induction of CRE-driven gene expression seems to be a general requirement for different types of long-lasting memory, both implicit and explicit. Genetic manipulation studies using recombinant viruses or mutant mice have shown that blocking the CREB pathway leads to deficits in a large number of memory processes, including spatial navigation, object recognition, social transmission of food preferences, conditional taste or odor aversion, contextual and cued fear conditioning (Balschun et al., 2003; Brightwell et al., 2005; Kogan et al., 1997; Pittenger et al., 2002; Zhang et al., 2003). Furthermore, the expression of diverse CREB downstream genes (Bito et al., 1996; Deisseroth et al., 1996; Impey et al., 1996; Lu et al., 1999) and the induction of a CRE-driven *lacZ* reporter construct (Impey et al., 1998) in specific brain regions correlate with the acquisition of long-term forms of memory dependent of activity in that region. By extension, these studies suggest that the CREB pathway is an attractive target for drugs aimed at improving or disrupting memory consolidation (Barco et al., 2003; Tully et al., 2003).

5 CREB and Brain Malfunction

The CREB pathway regulates cellular responses critical for the proper functioning of neurons and circuits. It is, therefore, not surprising that malfunction of this pathway has severe consequences for brain function and underlies several important brain disorders.

5.1 Pathologies of Synaptic Plasticity

Dysfunction of synaptic plasticity can have devastating consequences for the organism and can trigger pathological symptoms that rank from memory impairments and long-term alterations in behavior to neuronal cell loss. Interestingly, at least two human disorders characterized by cognitive impairments have been directly related

to the CREB activation pathway (Trivier et al., 1996). Mutations in the gene encoding RSK-2, one of the kinases regulating CREB phosphorylation, cause the Coffin-Lowry syndrome a rare X-linked mental retardation disorder (Trivier et al., 1996). Notably, the cognitive performance of human patients with this syndrome correlates with the cellular capacity to activate RSK2 (Harum et al., 2001). On the other hand, mutations in the gene encoding for CREB co-activator CBP are associated with a second mental retardation syndrome, the Rubinstein-Taybi syndrome (RTS), a complex autosomal-dominant disorder. The recent characterization of several mouse models for RTS has revealed a direct role of both the histone acetyltransferase activity of CBP and its capability to activate the CREB pathway in the etiology of this condition (Alarcon et al., 2004; Bourtchouladze et al., 2003; Korzus et al., 2004; Wood et al., 2005). A new point of view emerging from these studies on mouse models is the thought that a component of the cognitive impairments associated to these syndromes could have a non-developmental origin and be primarily caused by the chronic reduction of CBP enzymatic activity in the postnatal or adult brain.

The dysregulation of the CREB pathway has also been associated with age-associated memory impairment (AAMI) (Brightwell et al., 2004; Chung et al., 2002). Drugs that enhance the activity of this pathway, such as inhibitor of phosphodiesterases, can prevent memory decline in old mice (Bach et al., 1999) and are currently evaluated in clinical trials in humans (Barco et al., 2003; Tully et al., 2003).

Alterations in synaptic plasticity may also underlie other pathological aspects of behavior. For example, much as is the case with long-term memory, addiction to drugs are life-long conditions responsible for permanent behavioral abnormalities and are considered nowadays a pathological manifestation of abnormal synaptic plasticity. Recent studies have highlighted the similarities between memory and addiction at the cellular and molecular level. Both processes depend on stimulus-induced long-lasting changes in neuronal function that correlate at the cellular level with changes in synaptic strenght and at the molecular level with the activation of CREB-dependent gene expression. After exposure to various drugs of abuse, such as ethanol or cocaine, the regions of the CNS known to be involved in addiction, such as the locus coeruleus and nucleus accumbens, show significant increases in CREB phosphorylation and CRE-mediated gene expression. Recent studies in mutant mice with an altered signaling to CREB or reduced CREB activity suggest that CREB-dependent gene expression may be universally involved in addiction, although its precise role may differ depending of the drug studied and its protocol of administration (see reviews by (Carlezon et al., 2005; Nestler, 2001; Pandey et al., 2005)).

Also, recent studies have revealed a critical role of the cAMP cascade in depression (Conti and Blendy, 2004). Our understanding of the molecular bases of depression and other mood disorders is still incomplete, but it is likely that long-term changes in synaptic plasticity underlie this persistent alteration in brain function. Both the expression and the activity of CREB are increased by chronic antidepressant treatment (Nibuya et al., 1996; Thome et al., 2000), suggesting that CRE-driven expression may be one of the targets for these treatments and, therefore, contribute to the etiology of this condition.

5.2 Neurodegenerative Diseases

There is evidence that dysfunction in the CREB pathway may be involved in early manifestations in Alzheimer's disease and that it may also play a central role in Huntington disease (HD) and other forms of polyglutamine pathogenesis. Although, we focused this review on the role of CREB in the signaling cascades that regulate synaptic plasticity, the CREB pathway has been also involved on the control of neuronal survival and response to neuronal stress (Dawson and Ginty, 2002; Lonze and Ginty, 2002; Walton and Dragunow, 2000). Whereas the pathological conditions discussed above are likely caused primarily by failures of synaptic plasticity, the participation of CREB signaling in the etiology of neurodegenerative disorders could be related to a different role for CREB, its role in controlling neuronal survival. However, given that abnormal synaptic plasticity can lead to atrophy or excitotoxicity and triggers neurodegenerative processes, and that neuronal loss by itself may have significant effects in synaptic plasticity, it is difficult to dissect what aspects of CREB malfunction are primarily associated to these pathologies.

CREB regulates the expression of a number of pro-survival factors, such as the neurotrophin BDNF and the anti-apoptotic protein bcl-2. Indeed, CREB-mediated gene expression is necessary and sufficient for the survival of several neuronal subtypes *in vitro* (Bonni et al., 1999; Riccio et al., 1999; Walton and Dragunow, 2000). Experiments with CREB-deficient mice supported this view and showed that dorsal root ganglia (DRG) sensory neurons indeed require the expression of CREB for survival *in vivo* (Lonze et al., 2002). Further studies in knockout mice have demonstrated that different neuronal types differ in their requirement for transcription factors of the CREB family. Sensory neurons of the peripheral nervous system are much more dependent of CREB activity than neurons in the CNS. Experiments in $CREB^{-/-}$ /$CREM^{-/-}$ double mutants demonstrated that when both factors are eliminated in the same cell, survival is compromised also for CNS neurons. (Mantamadiotis et al., 2002). These studies also revealed that different members of the CREB family can compensate for each other for the control of neuronal survival *in vivo*. The reduction on CREB expression causes an upregulation of CREM that likely ameliorates some of the deleterious consequences of the lack of CREB.

Based on this evidence, it is not surprising that transcriptional dysregulation in the CREB pathway had been proposed to play a central role in the pathogenesis of neurodegenerative disorders. For example, polyglutamine repeat disorders, a family of related neurological diseases whose more relevant member is Huntington's disease (HD), have been associated to deficient CREB-mediated gene expression. These diseases are caused by expansions of polyglutamine-encoding sequences that make the mutant protein toxic to neurons, possibly through abnormal interactions with polyglutamine tracts present in proteins important for neuronal survival (McMurray, 2001). CREB co-activator CBP exhibits a C-terminus very rich in glutamine residues that may interact with polyglutamine expansions (McCampbell et al., 2000; Steffan et al., 2000). As a consequence, CBP can be depleted from the cell nucleus by being sequestrated in the cytoplasmic aggregates, resulting in abnormal CREB-dependent transcriptional activity and cellular toxicity (Bates et al., 2006; Higgins et al., 1999; Obrietan and Hoyt, 2004; Sugars et al., 2004; Yu et al., 2002). Consistent with this

view, the overexpression of CBP partially rescues the cell death accompanying expression of mutant huntingtin in neurons (Nucifora et al., 2001), whereas the expression of mutant huntingtin leads to a reduction on HAT activity *in vivo* (Igarashi et al., 2003). The administration of histone deacetylase (HADC) inhibitors ameliorates poly-Q dependent neurodegeneration in Drosophila and HD mouse models (Ferrante et al., 2003; Hockly et al., 2003; Steffan et al., 2001).

Also, the PKA/CREB pathway has been involved in early manifestations of Alzheimer's disease (AD), the most prevalent neurodegenerative disease in humans. The treatment of cultured hippocampal neurons with A□ peptide leads to the inactivation of PKA and reduces the activation of CREB in response to glutamate (Vitolo et al., 2002). Compounds that enhance the cAMP-signaling pathway, such as rolipram, could reverse these effects (Vitolo et al., 2002) and ameliorated synaptic plasticity and memory deficits in a mouse model for AD (Gong et al., 2006; Gong et al., 2004).

6 Conclusions

CREB plays a critical role translating specific patterns of synaptic stimulation, such as the temporal convergence of two different stimuli or the repetition of a stimulus until surpass a given threshold, into long-term changes in synaptic plasticity. This is achieved through the induction of a cascade of gene expression that leads to the synthesis of proteins involved in synaptic function that we are starting now to identify. The stabilization of, otherwise, transient alterations in synaptic plasticity has critical consequences for diverse aspects of brain function, from different forms of learning and memory in the hippocampus to experience-dependent plasticity in different areas of the cortex and addiction in the nucleus accumbens or the locus coeruleus. Although we do not have yet a complete picture of the complex network of protein interactions involved on the activation of CREB-dependent gene expression, the work during the last decade in this field has provided reasonable insight. Probably, the most important challenge ahead is the resolution of the CREB *transcriptome*, or better said, *transcriptomes*: the different sets of genes regulated by CREB in a given cell type in response to a given stimulus. Important efforts in this direction have been made, but the long lists of candidate genes provided by these studies needs to be refined and the targets need to be validated. The same techniques used to investigate CREB-dependent gene expression *in vitro* have to be applied *in vivo* and to different brain areas. The promised reward for these studies is likely to be well worth the effort. We will not only understand better what happens in a healthy brain during learning, but also the identification of specific CREB downstream genes may provide therapeutics for relevant neurodegenerative diseases and disorders of synaptic plasticity of difficult treatment.

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