Inhibition of cAMP Response Element-Binding Protein Reduces Neuronal Excitability and Plasticity, and Triggers Neurodegeneration

The cAMP-responsive element-binding protein (CREB) pathway has been involved in 2 major cascades of gene expression regulating neuronal function. The first one presents CREB as a critical component of the molecular switch that controls long-lasting forms of neuronal plasticity and learning. The second one relates CREB to neuronal survival and protection. To investigate the role of CREB-dependent gene expression in neuronal plasticity and survival in vivo, we generated bitransgenic mice expressing A-CREB, an artificial peptide with strong and broad inhibitory effect on the CREB family, in forebrain neurons in a regulatable manner. 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. Our experiments confirmed previous findings in CREB-deficient mutants and revealed new aspects of CREB-dependent gene expression in the hippocampus supporting a dual role for CREB-dependent gene expression regulating intrinsic and synaptic plasticity and promoting neuronal survival.

Keywords: activity-driven gene expression, CREB, neurodegeneration, neuronal excitability, synaptic plasticity

Introduction

The activation of the cAMP-responsive element-binding protein (CREB) pathway has been involved in 2 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 long-term potentiation (LTP) and memory deficits originally reported for CREB hypomorphic mutants (mice homozygous for a deletion of the α and δ isoforms; Bourchuladze 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; Viosca et al. 2009).

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; Papadía 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 (CNS) were not affected by CREB depletion (Lonze et al. 2002; Parlatò et al. 2006). In contrast, double mutants for CREB and CREM (cAMP response element modulator) exhibited a marked and progressive cell loss in specific CNS structures, such as cortex, hippocampus and striatum (Mantamadiotis et al. 2002). This loss of neurons has not been observed in transgenic lines expressing CREB dominant-negative mutants (Rammes et al. 2000; Kida et al. 2002; Pittenger et al. 2002), likely because the time window and/or the level of CREB inhibition achieved in loss-of-function studies using gene targeting or transgenesis-based strategies were different.

The comparison of different CREB-deficient mouse strains has left important open questions concerning the role of CREB in neuronal plasticity and survival because these roles have never been investigated in parallel in the same mutant strain. We describe here a novel bitransgenic 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-terminal of the CREB leucine zipper region, binds with very high affinity and specificity to the members of the CREB family (CREB, CREM, and ATF1) blocking their binding to CRE sites (Olive et al. 1997; Ahn et al. 1998). We 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.

Materials 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 Not I fragment containing A-CREB downstream of the tetO promoter was injected into mouse oocytes. We selected line tetO-A-CREB-95 (AC95) mice for further studies. AC95 mice were backcrossed to C57Bl6 F1/J mice more than 8 times. We referred as A-CREB mice those bitransgenic animals that result of the crossing of pCaMKII+TA mice (line B, (Mayford et al. 1996) and line AC95. A-CREB mice were usually raised without doxycycline (dox). Transgene repression was achieved by dox administration (40 mg/kg of food) for at least 1 week. VP16-CREBhigh mice have been described before (Barco et al. 2002). Mice were
genotyped by PCR using the oligonucleotides pMM400-3404: AGCTCG- TTAGTGGAACGGCTGACAT; pMM400-3548: CTCGAGACGGGCTTGCTA; and CamKII3` end2: TTGTTGACAACTTGTGGCAGTC. The PCR reaction starts at 94 °C for 2 min and has 35 cycles: 94 °C for 45 s, 60.5 °C for 25 s and 72 °C for 3 min. This reaction allows the simultaneous identification of both the pcAMKIIz-xTA 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 xTA 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 μM was bath applied for 5 min to ensure a complete block of IA current. LTP experiments were performed as previously described (Barco et al. 2002).

Kindling

Five-weeks-old A-CREB and control littermates were ip injected with subconvulsive dosage of pentylentetrazole (50 mg/kg, dissolved in saline) for several consecutive days. In the case of VP16-CREB mice, dox was removed from mouse diet one week prior to the start of the kindling experiment. The behavioral 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 raking; 4: clonic convulsions with loss of posture; and 5: repeated, forceful, clonic-tonic or lethal convulsions. All subjects were monitored for at least 20 min 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 cell layer thickness were counted from 50-μm coronal brain sections from 6 months old A-CREB and their wild-type 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. α-M2-flag, α-synaptophysin, α-MAP-2, α-calbindin, α-GAP-43, and secondary antibodies were obtained from Sigma (Barcelona, Spain); α-CREB antibodies were purchased to Cell Signaling; and α-CREM antiserum was a gift from Günther Schütz’s lab. In situ hybridizations were performed as previously described using appropriate cRNA probes labeled with digoxigenin (Shumyatsky et al. 2002).

Quantitative RT-PCR

qPCR was carried out in an Applied Biosystems 7300 real-time PCR unit using SYBR mix (Invitrogen, Carlsbad, CA) and primers specific for Arc, brain derivative neurotrophic factor (BDNF), c-Fos, CREM, the N-terminus of CREB, and Glyceraldehyde 3-Phosphate Dehydrogenase. Each independent sample was assayed in duplicate and normalized using GAPDH levels.

Microarray Analysis

RNA was extracted from dissected hippocampus. Mouse Genome 430 2.0 genephips 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 GCGS (Affymetrix, Santa Clara, CA), GeneSpring GX (Agilent Technologies, Santa Clara, CA) and dChip softwares (Li and Hung Wong 2001). This dataset is accessible at the GEO database (GSE14320). See additional details in Supplementary Methods.

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 months old and finished when they were 4 months old. The experimenter was blind to genotypes. Further detail on procedures can be found in Viosca et al. 2008.

SHIRPA Primary Screen

Mice were evaluated using a modification of Irwin procedure (Irwin 1968).

Open Field

Mice were placed in 50 × 50-cm2 open-field chambers and monitored throughout the test session (30 min) by a video-tracking system (SMART, Panlab S.L., Barcelona, Spain), which records the position of each animal every 0.5 s.

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- to 100-min intertrial interval were given daily. Probe trials (60 s) were performed to assess retention of the previously acquired information.

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 CamKIIz-xTA 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 CamKIIz-xTA/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 dox to regulate transgene expression. As expected, we found that addition of dox to the mouse diet turned off transgene expression in less than 2 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 et al. (2007) showing that tetO constructs, when turned off during embryonic development, are some times irreversibly silenced.

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, D and Supplementary Fig. S2). 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 (Fig. 2D). 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 function than CA1 neurons (Fig. 2D and Supplementary Fig. S1C). Interestingly, it was possible to stop and reinitiate the degenerative process by turning off and on transgene expression in adult animals (Fig. 2E). 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 has been also observed in CREB/CREM double knockout mice (Mantamadiotis et al. 2002). This might suggest that the disruption of CREB signaling in A-CREB mice was comparable to that in CREB/CREM double knockouts and likely more dramatic 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

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 littersates (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 4 weeks (OFF/ON). Scale bar: 140 \(\mu m\).
CREB 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 and susceptibility to seizure. Although we primarily focused on the early effects of CREB inhibition in hippocampal physiology and gene expression prior to neuronal damage (Figs 3–6), we also

Figure 2. Neuronal loss in the hippocampus of A-CREB mice. (A) A-CREB mice (closed diamonds, n = 7) did not gain weight in the same progression that control littersmates (open square, 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 Fig. 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 littersmates are indistinguishable. However, 7 weeks later (A-CREB 10w), massive loss of neurons was observed in the CA1 subfield. At least 2 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. Scale bar: 140 μm. (D) We quantified the loss of neurons in different brain regions in adult A-CREB mice (=6 months old; WT: n = 6; A-CREB: n = 7). CA1: The thickness of the CA1 cellular layer was significantly reduced in A-CREB mice (P < 0.001). DG: The thickness of the dentate gyrus upper and lower blades was slightly reduced in A-CREB mice (P < 0.001). Cortex: The thickness of the cortex was significantly reduced in A-CREB mice (P < 0.001), but no change in cell density was observed (P = 0.14). Layers 2 and 3, in which transgene expression is stronger (DIG in situ at the right panel and Fig. 1B), were particularly affected (layer 2/3: P < 0.001; layer 5: P = 0.04), but still showed transgene expression. (E) Neuronal loss was prevented by feeding the mice with dox before the onset of cellular death (A-CREB 3w On/8w Off) and was triggered in adulthood by removing dox from the mouse diet (A-CREB 3w On/8w Off/3w On).
Figure 3. Impaired plasticity in the hippocampus of A-CREB mice. (A) Input-output curve of field Excitatory Postsynaptic Potential (fEPSP) slope (V/s) versus stimulus (V) at the Schaffer collateral pathway of hippocampal slices from 3-week-old A-CREB mice (closed circle, n = 24) and control littermates (open square, 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 s) 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.

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 (closed circle) and control littermates (open square, upper panel P < 0.001 ANOVA). This effect was reversed 10 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 μM) (D), and after transgene repression by dox for 10 days (E).
explored the late consequences of the sustained inhibition of CREB function and the severe cell loss (Fig. 7).

Inhibition of CREB Activity Impairs L-LTP
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-week-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,B) or alterations in spontaneous activity at the CA1 subfield (Fig. 3C). E-LTP in response to one standard 100-Hz tetanus train of 1 s 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 4 tetani was impaired after 2 h (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 3-week-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,B; 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). Because we observed differences in the amplitude 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.

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 pentylentetrazol (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-CREBhigh 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.

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 3-weeks-old transgenic and control mice using microarrays MouseArray 430 2.0 (Affymetrix). Because we were interested in activity-driven gene expression, we compared both genotypes in the basal condition and 2 h after kainate (KA)-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 5 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 (Clusters 1 and 2, respectively, in Fig. 6B, see also Supplementary Table 2). The largest fold changes (FCs) in the short list of genes consistently downregulated in A-CREB mutants, both in mice injected with saline or with KA, corresponded to Scn1b and Penk1 probe sets. Scn1b encodes the sodium channel subunit β4, 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 2 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 2 probes complementary to A-CREB sequence (Fig. 6C).

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, Ergr-1, Ergr-2, Ergr-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 KA. 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 (C330006G03Rik) and arc (Supplementary Table 4), that were not initially detected, probably due to the stringency of the 2-way ANOVA analysis (see Supplementary Methods for further details). However, these IEGs where still strongly upregulated by KA in the hippocampus of A-CREB mice. The list of genes significantly altered in A-CREB mice and differentially upregulated in response to KA in A-CREB mice was surprisingly short (Supplementary Table 2) and included penk1 and pdyn, which encode for 2 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 4 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 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 4 IEGs correlated well with seizure intensity in the lower range of the Racine scales (1–5). 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 KA (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). 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 (Table 2), immunostaining using an antiserum against CREM (Fig. 6J), nor qRT-PCR (Fig. 6J) revealed significant changes of CREM expression in the hippocampus of A-CREB mice at the basal stage. Because A-CREB can bind and block the activity of both CREB and CREM (Ahn et al. 1998), these results could suggest that the upregulation of CREM observed in CREB knockout mice could be mediated by CREM itself. We did not observe either upregulation of ATF1 mRNA in the hippocampus of A-CREB mice (Table 2).

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 (Fig. 3A), 1-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 (Fig. 7A). We also tested adult A-CREB mice in an extensive battery of behavioral tasks and detected alterations in some basal behaviors, such as touch escape or geotaxis (Supplementary Table 5), hyperactivity in an open field (Fig. 7B), and strong impairments in spatial navigation (Fig. 7C,D). These behavioral abnormalities are consistent with the wide spread degeneration detected at this stage.

Discussion

The multilevel analysis of A-CREB mice has allowed to address a number of important open questions concerning the role of CREB in plasticity and survival, contributing to clarify some of controversies concerning CREB loss-of-function studies.
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 littersmates \( (n = 10) \) in response to 14 mg/kg of kainic acid. (B) Two-dimensional hierarchical clustering of 21 probe sets significantly affected by genotype in the 2-way ANOVA analysis and showing at least 2-fold change in the comparison between genotypes in animals injected either with saline or KA. The right panels show the clusters obtained by K-means clustering: 1: WTSAL, wild-type saline; 2: WTKA14, wild-type injected with 14 mg/kg of KA; 3: ACSAL, A-CREB saline; 4: ACKA14, A-CREB with 14 mg/kg; and 5: ACKA18, A-CREB with 18 mg/kg KA (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 2 oligonucleotides complementary to A-CREB sequence. (D) Hierarchical clustering of the 209 probe sets significantly affected by drug treatment in the 2-way ANOVA analysis showing a FC equal or bigger than 2 in the comparison between drug treatments for at least one of the 2 genotypes. A number of genes in this list, specially those presenting largest changes (bright red traces in KA 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 209 probe sets showed in panel 6D. (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 littersmates 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 littersmates 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 2 h after injection with 14 mg/kg of 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 h. 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 \( (n = 6) \): dark gray bars.
Thus, we described here the consequences in neuronal gene expression, plasticity and survival of blocking CREB-dependent gene expression and 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.

**A Novel Mouse Model to Investigate CREB Function In Vivo**

The bitransgenic mouse strain described here has a number of advantages for investigating the role of CREB in the adult brain. First, A-CREB mice express a stronger repressor than those used in previous studies on transgenic strains. CREB-M1, the point mutant (S133A) used in 2 previous studies (Rammes et al. 2000; Kida et al. 2002), cannot be phosphorylated at Ser 133 and inhibits CREB-dependent gene expression by competing with CREB for CRE sites, although it has been shown that CREB/CREB-M1 heterodimers can still exhibit significant transactivation capability (Loriaux et al. 1993). K-CREB, the point mutant (R287L) used by Pittenger et al. (2002), blocks gene activation by binding to CREB and other CREB family members and preventing their interaction with CRE sites. In contrast, A-CREB is a short polypeptide specifically designed to form highly stable heterodimers with CREB family members. A-CREB/CREB heterodimers are formed with an affinity 3.3 orders of magnitude greater than CREB homodimers (Ahn et al. 1998) and, likely, than CREB heterodimers with K-CREB or CREB-M1 because neither one of these point mutations affects dimerization. Because of this, A-CREB may have a stronger dominant-negative effect than K-CREB or CREB-M1. Studies in cell culture supports this view (Ching et al. 2004). Second, A-CREB mice are likely more adequate than CREB knockout mice to investigate the consequences of disrupting the CREB pathway. Because the leucine zipper domain of CREB has a high degree of homology with those of CREM and ATF1 and because these 3 proteins can form heterodimers with each other, A-CREB should have, in principle, also the capability of blocking the binding of CREM and ATF1 to DNA. Experiments in cultured cells have demonstrated this capability in the case of ATF1 and CREB (Ahn et al. 1998). The reduction of endogenous CREB detected by immunohistochemistry indicates that A-CREB can

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Figure 7. Long-term consequences of chronic inhibition of CREB function by A-CREB. (A) Input/output curve of IEPSP slope (V/s) versus stimulus at the Schaffer collateral pathway of hippocampal slices from 1-year-old A-CREB mice (closed circle, $n = 23$) and control littersmates (open square, $n = 26$, $P = 0.01$). (B) Ten-week-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 associated with chronic A-CREB expression in both the visible platform and the hidden platform tasks (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 thigmotaxis were not significantly affected. (D) Spatial memory was assessed in 2 probe trials. Values represent percentage of time in the target quadrant (white and black bars) compared with the average in other quadrants (gray bars). Control mice spent more time in the platform quadrant in the 2 probe trials, whereas A-CREB mice did not show a memory for the platform location (chance value: 25%). Asterisks indicate $P < 0.05$. 

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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 littersmates (white bar) (3 mice per group, $P = 0.69$).
also dimerize with CREB in the brain of A-CREB mice, whereas the dramatic loss of neurons observed after sustained transgene expression suggests that both CREB and CREM may be target of A-CREB inhibition. Therefore, our transgenic approach most likely does not distinguish between the role of CREB and that of highly related ATF1 and CREM as positive mediators of CRE-driven transcription. Third, we did not detect in our model the compensatory upregulation of other ATF/CREB family members highly related ATF1 and CREM as positive mediators of CRE-driven transcription. Third, we did not detect in our model the compensatory upregulation of other ATF/CREB family members.

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 (Bourchuladze 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 adaptation 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 (this study).
and ICER-overexpressing transgenics (Kojima et al. 2008). The increase in 14,3- 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 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; Impney 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 (McGlung 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).

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 KA 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 KA (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 and ATF1 expression in A-CREB mice (Table 2) suggested that this is not likely the case. However, we cannot completely discard that alternate CRE-binding factors may escape A-CREB inhibition. A third explanation would be that the induction of IEGs by KA is not mediated solely by CREB and CREs. The promoter region of many IEGs contain binding sites 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 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. In contrast, constitutively active CREB was not sufficient to trigger arc expression (Barco et al. 2005), but we found now that it is necessary to achieve normal levels of basal expression. Future chromatin occupancy experiments on prototypical IEG promoters should clarify the role of CREB in constitutive and activity-driven neuronal gene expression in vivo.

The microarray analysis revealed transcriptional changes that can be highly relevant to explain the phenotype of A-CREB mice. Thus, genes that are known to play critical roles in epileptogenesis, excitability and plasticity were reduced in the basal condition (arc, egfl, egr2), or in response to KA (penk, dyn). 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 analysis of the hippocampus of A-CREB mice revealed massive loss of neurons in the CA1 subfield, likely as a consequence of

### Table 2

Probesets targeted to members of the CREB family of transcription factors

<table>
<thead>
<tr>
<th>Probe set ID</th>
<th>FC</th>
<th>P-value</th>
<th>Unigene ID</th>
<th>Gene symbol</th>
<th>Description</th>
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<td>0.80</td>
<td>Mm.676</td>
<td>Atf1</td>
<td>Activating transcription factor 1</td>
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<tr>
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<td>0.99</td>
<td>Mm.66818</td>
<td>CREB</td>
<td>cAMP-responsive element-binding protein 1</td>
</tr>
<tr>
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<td>0.27</td>
<td>Mm.466618</td>
<td>CREB</td>
<td>cAMP-responsive element-binding protein 1</td>
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<tr>
<td>1452529_a_at</td>
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<td>0.60</td>
<td>Mm.676</td>
<td>CREB</td>
<td>cAMP-responsive element-binding protein 1</td>
</tr>
<tr>
<td>1426755_at</td>
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<td>0.42</td>
<td>Mm.5244</td>
<td>Crem</td>
<td>cAMP-responsive element modulator</td>
</tr>
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<td>0.48</td>
<td>Mm.5244</td>
<td>Crem</td>
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<td>1418322_at</td>
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<td>1.00</td>
<td>Mm.5244</td>
<td>Crem</td>
<td>cAMP-responsive element modulator</td>
</tr>
<tr>
<td>1449037_at</td>
<td>1.19</td>
<td>0.65</td>
<td>Mm.5244</td>
<td>Crem</td>
<td>cAMP-responsive element modulator</td>
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<tr>
<td>1430647_a_at</td>
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<td>0.38</td>
<td>Mm.5244</td>
<td>Crem</td>
<td>cAMP-responsive element modulator</td>
</tr>
</tbody>
</table>

Note: FC = fold change wild-type saline versus A-CREB saline. P values correspond to unpaired t-test analysis.

Probe set 1421582_a_at contains 2 probes that recognize a sequence common to A-CREB and wild-type CREB (Fig. 6C). This abnormal pattern of upregulation, affecting only 2 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 outlier probes was considered.

Probe set 1418322_at and 1449037_at are targeted to the 3' untranslated region of CREM and can recognize the transcripts encoding the inducible repressor isoform ICER. The signals for both probe sets were not affected by transgene expression, but significantly increased in KA-treated samples (see Table 1). Probe set 1430847_a_at, which is targeted to the second Q-rich domain specific of CREM, showed changes neither by KA nor by transgene expression.

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the dramatic and sustained inhibition of CREB function in pyramidal neurons. The neurodegenerative process observed in A-CREB mice presented some similarities with that described for Creb(CREb/Crem)−/− double mutants (Mantamadiotis et al. 2002), suggesting that both CREB and CREM function were effectively blocked by A-CREB expression.

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, Scr-fb, the cell death-related genes Csd7 and Ccld10 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 dox 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).

Supplementary Material
Supplementary material can be found at: http://www.ercor.oxfordjournals.org/

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