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ORIGINAL ARTICLE

Wide distribution of CREM immunoreactivity in adult and fetal human brain, with an increased expression in dentate gyrus neurons of Alzheimer's as compared to normal aging brains

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Abstract Human cyclic AMP response modulator proteins (CREMs) are encoded by the *CREM* gene, which generates 30 or more different CREM protein isoforms. They are members of the leucine zipper protein superfamily of nuclear transcription factors. CREM proteins are known to be implicated in a plethora of important cellular processes within the CNS. Amazingly, little is known about their cellular and regional distribution in the brain, however. Therefore, we studied by means of immunohistochemistry and Western blotting the expression patterns of CREM in developing and adult human brain, as well as in brains of Alzheimer's disease patients. CREM immunoreactivity was found to be widely but unevenly distributed in the adult human brain. Its localization was confined to neurons. In immature human brains, CREM multiple neuroblasts and radial glia cells expressed CREM. In Alzheimer's brain, we found an increased cellular expression of CREM in dentate gyrus neurons as compared to controls. We discuss our results with regard to the putative roles of CREM in brain development and in cognition.

Keywords CREM · Adult human brain · Developing human brain · Alzheimer's disease · Immunohistochemistry · Western blot

Abbreviations

Aβ	Beta amyloid
AD	Alzheimer's disease
Aka	Also known as
APP	Amyloid precursor protein
ATF	CREM/activating transcription factor
BA	Brodmann area
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CRE	cAMP response element
CREB	cAMP response element-binding protein
CREM	cAMP response element modulator
Da	Dalton
DG	Dentate gyrus
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ICER	Inducible cAMP early repressor
IgG	Immunoglobulin G
LCD	Leucine-charged residue-rich domains
SDS	Sodium dodecyl sulfate
bZIP	Leucine zipper (protein superfamily)

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Introduction

Human cyclic AMP response modulator proteins (CREMs) are encoded by the *CREM* gene (mapped to chromosome10p12.1-p11.1). This gene is transcribed, partially by alternative promoter usage and alternative splicing, to multiple mRNAs, from which 30 or more CREM protein isoforms are synthesized (AceView; http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly). They all are members of the leucine zipper (bZIP; Wu et al. 2012) superfamily of nuclear transcription factors, which is represented by various CREM isoforms (homodimeric and heterodimeric variants of CREMs α, β, γ, τ, τ1, τ2, θ1 and θ2) and some closely-related CREM homologues, such as inducible cAMP early repressor (ICER), cAMP responsive element-binding proteins (CREBs)-1 and -2, and the CREM/activating transcription factors (ATF)-1, -2, and -3 (reviewed in Rauen et al. 2013). Common properties of all superfamily members are (1) high sequence homology within their DNA binding domains, (2) binding to the so-called cAMP responsive element (CRE), the palindromic octapeptide 5′-TGACGTCA-3′ (Barcellos et al. 2009), and (3) the ability to become phosphorylated (and thereby activated) by certain protein kinases upon an increase of intracellular cAMP levels in response to various adenylate cyclase stimulating, extracellular signals (such as hormones and growth factors; Rauen et al. 2013). The functional consequences of this phosphorylation-dependent activation of CREBs and CREMs may be quite different, however. While CREBs, which regulate about 4,000 target genes in the human genome (Zhang et al. 2005), and CREMτ, strongly activate gene transcription in most tissues, CREMs α, β, and γ typically repress them (Ahlmann et al. 2009). Individual CREB and CREM members also differ with regard to their expression patterns throughout the body: whereas CREBs appear to be nearly ubiquitously expressed in organs and tissues, CREMs are more restricted in their occurrence, with sometimes tissue-specific expression of single CREM variants (Uyttersprot and Miot 1997; Rauen et al. 2013). Target genes of CREMs were identified in T lymphocytes, spleen, bone, muscle, adrenal gland, heart, ovary, testis, brain, and other organs (Hummler et al. 1994; Herdegen and Leah 1998; Borsook et al. 1999; Behr and Weinbauer 2000; Rauen et al. 2013 and others). CREM proteins are known to be implicated in a plethora of important cellular processes within the CNS [regulation of cell proliferation and migration in the developing brain (Della Fazia et al. 1997; Díaz-Ruiz et al. 2008), early synaptogenesis (Aguado et al. 2009); participation in the control of circadian rhythms (Stehle et al. 1993; Foulkes et al. 1996; Link et al. 2004); transcriptional regulation of proenkephalin and thyrotropin releasing hormone and, possibly, gonadotropin releasing hormone gene expression (Borsook et al. 1999; Chiappini et al. 2013; Kwakowsky et al. 2012), involvement in seizures (Fitzgerald et al. 1996; Zhu et al. 2012), regulation of adult neurogenesis (Luzzati et al. 2011), shaping of synaptic plasticity (Cortés-Mendoza et al. 2013); regulation of behavioral responses to psychostimulants (Madsen et al. 2012), role in neurodegeneration (Konopka et al. 1998), learning, and memory (Kadar et al. 2011), pain (Naranjo et al. 1997) and others]. In humans, polymorphisms in the *CREM* have been linked to gene expression regulation in neuroblastoma cells (Ledo et al. 2000), agoraphobia (Domschke et al. 2003), and panic disorder (Hamilton et al. 2004). In addition, *CREM* gene and its products might play a role in the pathophysiology of Alzheimer's disease (AD; Grupe et al. 2006; Avramopoulos et al. 2007; Giedraitis et al. 2009), Parkinson's disease (Simón-Sánchez et al. 2009) recurrent, early-onset major depression (Zubenko et al. 2003), and schizophrenia (Crisafulli et al. 2012). Although *CREM* gene transcripts are prominently implicated for normal and disturbed brain functions, amazingly little is known about regional distribution and cellular localization of cerebral CREM proteins. While a few anatomical studies show a wide but weak neuronal expression of CREM in rat brain neurons, and an up-regulation of its cellular expression after injury (Mellström et al. 1993; Gomez-Villafuertes et al. 2005; Wu et al. 2012), nearly no information is yet available about CREM expression patterns in human brain. Since revealing the precise neuroanatomical location of CREM may potentially contribute to a better understanding of certain aspects of normal human brain function and its pathology, and their modulation might even be a possible therapeutic option for the future, we decided to investigate by immunohistochemical means the differential distribution of CREM in adult and developing human brain, and to look at possible alterations in CREM expression patterns in late-onset AD.

Materials and methods

Post-mortem human brain tissue

Twenty-one human brains were investigated. Sampling of the human brain material and asservation was done in accordance with the Declaration of Helsinki (1984), German law and approval by the local Ethics commission.

For immunohistochemical analysis, we used adult brain tissue from six neuropathologically confirmed AD cases (mean age 76 years) and eight non-AD controls (mean age 70 years; Table 1). After formalin fixation for 2 weeks, brains were dissected into smaller specimens. These tissue samples were embedded in paraffin and then cut into 20-µm thick sections. Control cases had no history of

Table 1 Clinicopathological data of Alzheimer's disease and control cases submitted to immunohistochemical analysis

No.	Diagnosis	Age/sex	PM interval (h)	Brain weight (g)	Braak stage	CERAD
1	Control	75/F	28	1.380	–	–
2	Control	79/F	30	1.360	–	–
3	Control	64/F	36	1.200	–	–
4	Control	70/F	20	1.150	–	–
5	Control	84/F	48	1.080	–	–
6	Control	62/F	48	1.160	IV	B
7	Control	64/M	32	1.250	IV	B
8	Control	61/F	26	1.090	V	C
9	AD	83/F	40	1.100	V	C
10	AD	84/F	36	1.315	V	C
11	AD	65/M	30	1.115	V	C
12	AD	69/F	40	1.170		
13	AD	77/M	28	1.220		
14	AD	80/F	50	1.240		

PM post-mortem

neurological disorder and showed no relevant neuropathological changes in routine histological and immunohistochemical stainings. Every tenth section was Nissl-stained for morphological orientation as described (Bernstein et al. 2012a).

Frozen brain tissue from three AD cases and four controls stored at −80 °C was used for Western blotting analysis. The following brain regions were investigated: superior frontal lobe (Brodmann area [BA] 8), superior temporal lobe [BA 21] angular gyrus [BA 40]), hippocampus, thalamus, habenula, hypothalamus, cerebellar cortex, and brain stem.

Pre- and perinatal brains

Human pre-and perinatal brains were obtained from six preterm stillborn children and spontaneously aborted fetuses. The ages of these fetuses were 20 ($N = 2$), 25 ($N = 1$), 30 ($N = 1$) and 33 ($N = 2$) weeks of gestation. Brains were removed as quickly as possible (post-mortem delay less than 3 h after death) and dissected into tissue blocks of about 1 cm^3 or smaller volumes as described earlier (Bernstein et al. 1987). Further tissue processing procedures were carried out as for adult brains. The comprehensive "The Embryonic Human Brain: An Atlas of Developmental Stages" (O'Rahilly and Müller 2005) was used for morphological orientation and help with the identification of developmental stages.

Human testicular tissue

CREM proteins are highly expressed in post-meiotic male germ cells of different mammals including man, where CREM is nearly exclusively localized in cell nuclei of spermatids (Hummler et al. 1994; Blöcher et al. 2005 and others). Hence, human testicular tissue from a fertile man who died at the age of 41 years from heart failure, was used here as a positive control tissue.

Rat brains

By using the same CREM antiserum as we have here, Wu et al. (2012) studied the cellular expression of CREM in rat brain and found it up-regulated after injury. Hence, as an additional positive control, the cellular expression of CREM protein(s) was investigated in select brain regions of three normal male Sprague–Dawley rats as well as of three rats postnatally lesioned in the ventral hippocampus with ibotenic acid (2 weeks after lesion, Bernstein et al. 1999a). For all procedures with rats ethical approval was sought according to the National Act on the Use of Experimental Animals (Germany).

Immunohistochemistry

Immunohistochemistry was carried by employing a CREM polyclonal antiserum (CREM-1, X-12): sc-440, from Santa Cruz Biotechnology, Santa Cruz, CA; working dilution 1:200). According to supplier's information, this antiserum recognizes the isoform CREM-1, but might also cross-react with other CREM isoforms. Because of the reportedly weak cellular expression of CREM in normal human brain (Human Protein Atlas; http://www.proteinatlas.org) and rat brain outside hypothalamus (Ginsberg et al. 2004; Wu et al. 2012; Chiappini et al. 2013), we employed a slightly different staining protocol for CREM, including antigen demasking by boiling sections for 4 min in 10 mM citrate buffer (pH 6.0) and enhancement of the reaction product by nickel ammonium sulfate as described earlier (Bernstein et al. 1999b). Staining controls included omission of the primary antibody, its substitution with an irrelevant IgG antibody or normal rabbit serum, and preabsorption of CREM antiserum with blocking peptide sc-440P (Santa Cruz Biotechnology, CA).

Cell counting

To determine the percentage of immunostained dentate gyrus neurons in AD and controls, we counterstained these sections with hematoxylin after having taken microphotographs (Fig. 6a, b). CREM immunoreactive neurons located in the left hippocampal dentate gyrus (granule cells and

molecular cells) were counted at 200-fold magnification (Olympus BH-2 light microscope), using a counting frame and the optical dissector as recently described (Bernstein et al. 2012b). The counting boxes were placed next to each other in the areas investigated (per slice 7 boxes). Neuronal perikarya lying on the left and the lower border of the counting frame were not taken into account. Neuronal cell bodies lying on the right and the upper border on the frame were counted, however. Neurons were counted by one of the authors (H.-G.B.) who was blind to the diagnosis. To establish inter-rater reliability between investigators, repeated measurements were carried out by a different investigator (C.M.), who counted immunostained cells on sections of four hippocampi. Intra-class correlation coefficient was 0.85.

Western blot analysis

Frozen brain samples or cells were homogenized in lysis buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 % Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, and 10 µg/mL aprotinin) using an UltraTurrax device, and incubated on ice for 30 min. After centrifugation at 13,000 rpm for 15 min, the supernatant was collected to measure the total protein content. The amount of protein was determined using the BCA assay (Pierce, Rockford, IL). Thirty micrograms of protein were loaded on 10 % SDS-polyacrylamide gels for electrophoresis. After separation, proteins were transferred to a nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech, Freiburg, Germany) at 150 mA for 2 h. Western blot analysis was performed after blocking of the membrane with 5 % skim milk in TBST buffer for 1 h. The membrane was incubated with the specific antibody at 4 °C overnight. The membrane was then washed 4 times in TBST buffer. Secondary detection was performed using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2000) (Amersham Pharmacia Biotech, Freiburg, Germany). After four times washing with TBST, HRP activity was visualized by applying enhanced chemiluminescent substrate (ECL; Amersham Pharmacia Biotech, Freiburg, Germany) followed by exposure of the membrane to X-ray film. Equal protein loading was confirmed by reprobing the membranes with anti-actin antibody (Sigma) following antibody stripping using the Restore Western Blot Stripping Buffer (Pierce). Densitometric analysis of band densities implied normalization to the actin signal.

Statistical analysis

The frequencies of CREM immunopositive neurons were analyzed with the Mann–Whitney *U* test. Changes in the expression levels of cultured cells were compared using the Student *T* test. Significance level was $p < 0.05$; all tests were carried out with SPSS 11.0 statistic software.

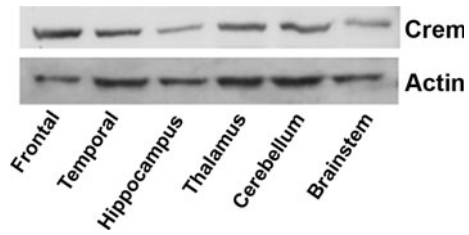


Fig. 1 Detection of CREM protein by Western blotting in normal human brain tissue. Membrane was re-probed for different antibodies. Actin served as loading control for all blots shown

Results

Expression of CREM within adult human brain

Western blot analysis revealed that CREM immunoreactive material is present in many human brain regions, being abundantly expressed in the frontal lobe, thalamus, and cerebellum (Fig. 1). Immunohistochemically, CREM immunoreactivity was found to be widely but unevenly distributed throughout human brain. With the exception of the strong immunostaining of a subset of plexus choroideus epithelia cells, CREM immunoreactive material was exclusively confined to neurons (Fig. 2a–h). Multiple, moderately immunopositive pyramidal and non-pyramidal cells were observed in most cortical areas (Fig. 2a, b) as well as in the hippocampus (Fig. 2c). The habenula stood out by intense immunostaining of neurons and a dense network of fibers (Fig. 2d). Hypothalamic neurons belonging to the paraventricular (PVN) and supraoptic (SON) nuclei were found to highly express CREM protein (Fig. 2e), whereas, suprachiasmatic neurons were only very weakly immunostained. Numerous CREM immunoreactive neurons were scattered throughout thalamic nuclei. Remarkably, nearly all corpus geniculatum laterale neurons were positive for CREM. Moderately immunostained neurons were located in the substantia nigra (Fig. 4f), the locus coeruleus (Fig. 2g), the cerebellum (immunolabeling of most Purkinje cells and a subset of granule cells, Fig. 2h), and brain stem. Remarkably, analysis of intraneuronal CREM immunoreactivity revealed that a vast majority of neurons show an extranuclear immunostaining. CREM immunoreaction was most frequently observed in the cytoplasm and sometimes associated with plasma membranes. Some neurons, however, demonstrated both nuclear and extranuclear immunolocalization of CREM (virtually all Purkinje cells), and a small subset of

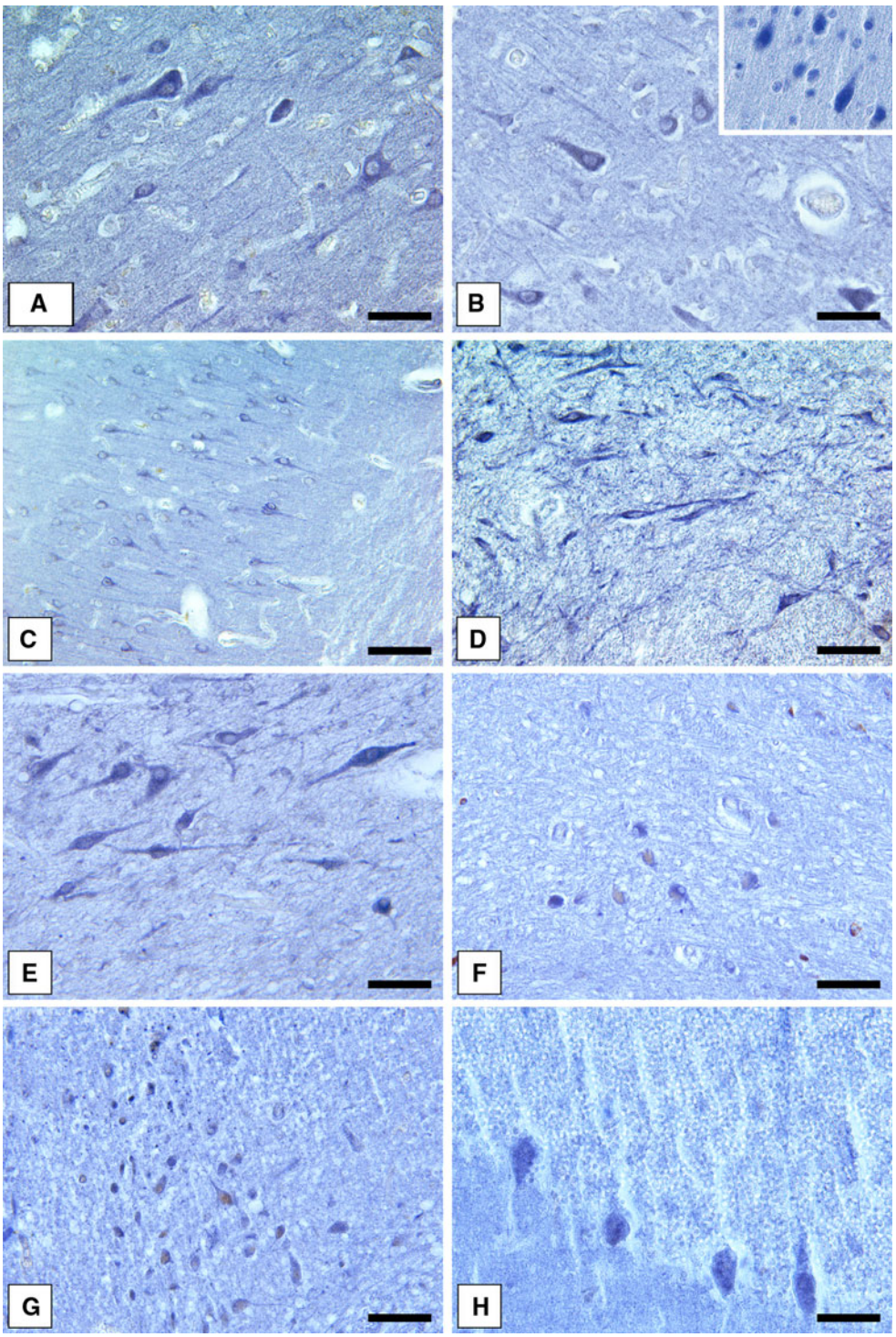


Fig. 2 Regional and cellular distribution of CREM in aging human brain. **a** CREM immunolocalization in pyramidal and non-pyramidal neurons of the angular gyrus. *Bar* 25 μm. **b** CREM immunolocalization in temporal cortex neurons. *Bar* 25 μm. *Insert* A very small percentage of cortical neurons showed a nuclear localization of CREM. Neurons with nuclear CREM immunoreactivity appeared organized in small clusters (photomicrograph from the temporal cortex). *Bar* 35 μm. **c** CREM immunopositive pyramidal cells in hippocampal CA1 region. *Bar* 75 μm. **d** CREM immunoreactive neurons and fibers in the habenula. *Bar* 25 μm. **e** Strongly immunostained neurons belonging to the hypothalamic paraventricular nucleus. *Bar* 25 μm. **f** A subset of nigral neurons is CREM immunopositive. *Bar* 40 μm. **g** CREM immunoreactivity in multiple locus coeruleus neurons. *Bar* 60 μm. **h** CREM in the cerebellum. CREM is expressed in Purkinje cells and granule cells. Please note the appearance of nuclear and cytoplasmic CREM in Purkinje cells. *Bar* 25 μm

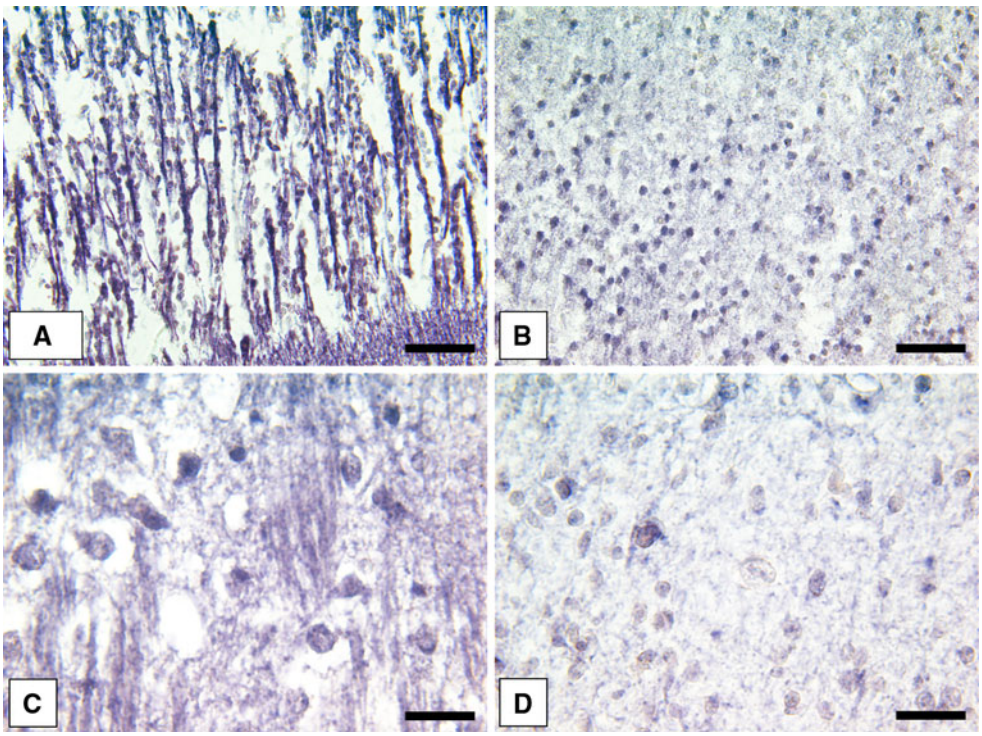


Fig. 3 Aspects of CREM immunolocalization in developing human brain. **a** Fetal brain (20th gestational week). CREM immunoreactivity can be found in many migrating neuroblasts and radial glia. *Bar* 50 µm. **b** Fetal brain (22nd gestational week). CREM immunopositivity can be observed in almost all neuroblasts. Please note the nuclear localization of CREM. *Bar* 50 µm. **c**, **d** Perinatal brain (preterm stillborn child, 32nd gestational week). Many thalamic (**c**) and striatal (**d**) neurons are shown to express CREM. *Bars* (**c**) 25 µm and (**d**) 35 µm

neocortical neurons had a nuclear CREM immunolabeling only (Fig. 2b, insert).

Cellular expression of CREM in developing human brain

By the end of the embryonic period (20th gestational week) CREM immunoreactivity is ubiquitously distributed throughout cells. Interestingly, besides, in multiple migrating neuroblasts (van Strien et al. 2011) a very strong immunostaining appears in a vast majority of cortical radial glia cells (Fig. 3a). At a gestational age of 22 weeks, almost all neuroblasts seem to express CREM. Remarkably, its intracellular localization is exclusively confined to cell nuclei (Fig. 3b). In preterm stillborn children (32th gestational weeks) many neurons are immunopositive for CREM. Besides, in neuronal nuclei, an extranuclear, cytoplasmic CREM immunostaining, which is typically found in adult brain nerve cells (see above), can also be observed (Fig. 3c, d).

Expression of CREM in human testicular tissue

To test the specificity of CREM immunostaining protocol, we used human testicular tissue and rat brain. As predicted, in human testicular tissue, CREM immunoreactivity was merely confined to the nuclei of multiple spermatids (Fig. 4a).

Expression of CREM in normal and injured rat brain

In normal rat brains, a moderate CREM immunoreaction was found in multiple neurons as described by others (Wu et al. 2012). The cell nuclei of most immunostained neurons were devoid of reaction product. However, in ibotenic acid lesioned animals, we saw a strong increase in intracellular CREM immunostaining in certain cortical areas close to the lesion (Fig. 4c), with a clear increase of the portion of those neurons, where both nuclear and extracellular compartments were immunolabeled, or even only cell nuclei were immunopositive (Fig. 4b).

Protein expression and cellular localization of CREM in hippocampi of normal and AD brains

Next, we analyzed CREM in hippocampi of AD patients and controls. Compared to controls we found only insignificantly increased CREM protein levels (considering the main protein band representing the 43,000 Da protein) after densitometrical analysis of Western blots of

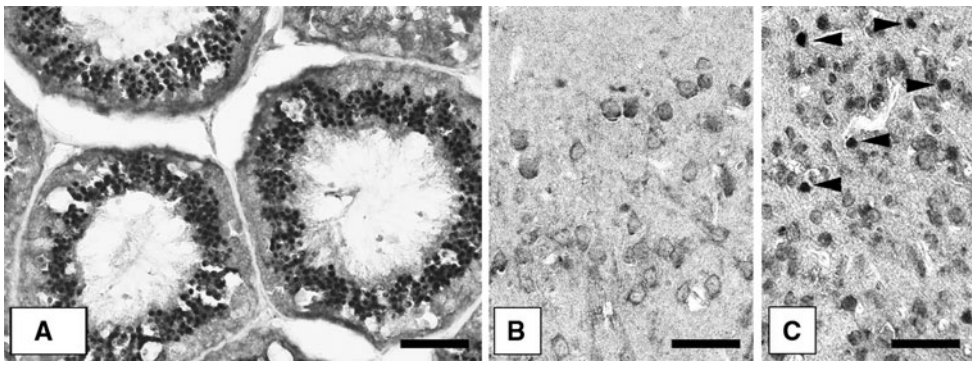
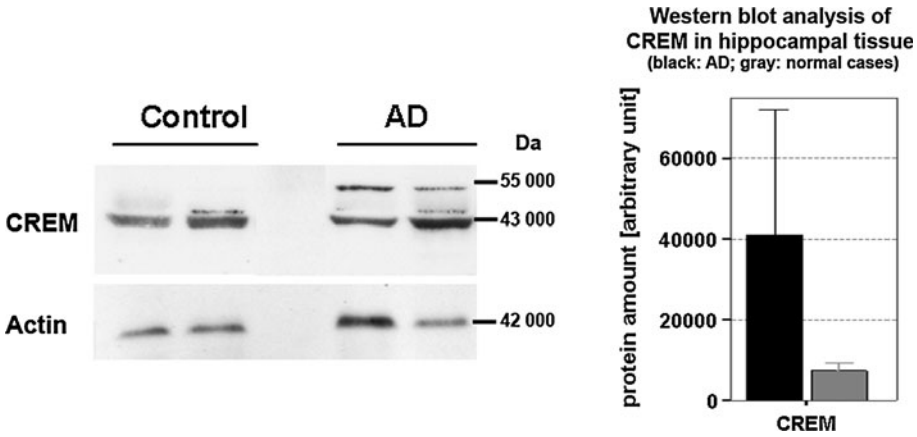


Fig. 4 Positive control tissues for CREM. **a** Immunolocalization of CREM in cells nuclei of spermatids. *Bar* 60 µm. **b** CREM immunoreactive neurons in the rat brain perirhinal cortex. Note the absence of nuclear immunostaining. *Bar* 35 µm. **c** CREM immunoreactive neurons in the rat brain perirhinal cortex 2 weeks after ibotenic acid induced lesion of the ventral hippocampus (located in close neighborhood to this cortical area). Please notice the increased density of immunoreactive neurons as well as the appearance of immunostained neuronal nuclei (indicated by *arrows*). *Bar* 35 µm

Fig. 5 *Left side* Examples of Western blot runs of hippocampal tissue from AD patients and controls to detect CREM. Note the appearance of an additional CREM protein band in AD specimens but not controls. *Right side* Densitometrical analysis of Western blots revealed only an insignificantly increase of main CREM protein band in AD patients compared to normal cases



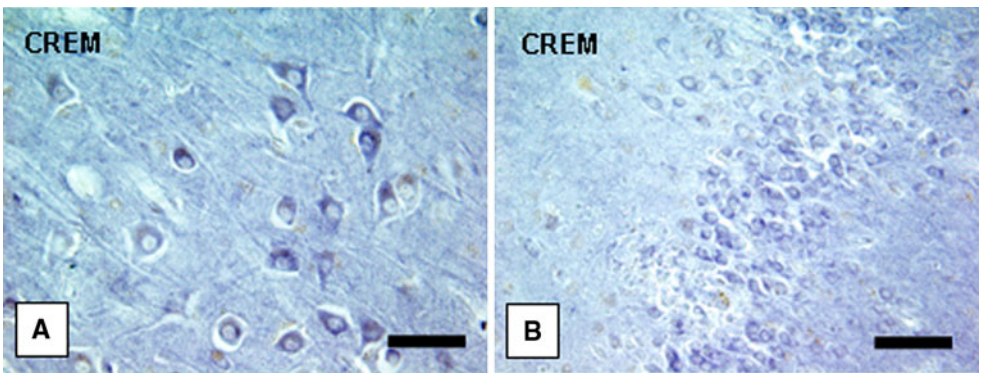


Fig. 6 Immunolocalization of CREM in DG in normal brain neurons. **a** CREM immunoreactive neurons in the DG molecular layer. *Bar* 25 µm. **b** CREM immunoreactive neurons in the DG granule cell layer. *Bar* 25 µm

hippocampal tissue from AD patients (Fig. 5), which was mainly due to the variable expression of CREM proteins in individuals with AD. Interestingly, an additional CREM protein of higher molecular weight (about 54,000 Da) was identified in AD patients but not in controls (Fig. 6a).

Morphometric analysis of CREM-expressing granule cells and molecular layer neurons (shown in Fig. 6a, b) revealed a statistically significant increase in AD (DG granule cells, $p = 0.020$; molecular cells, $p = 0.004$; Fig. 7). A very small number of CREM immunoreactive

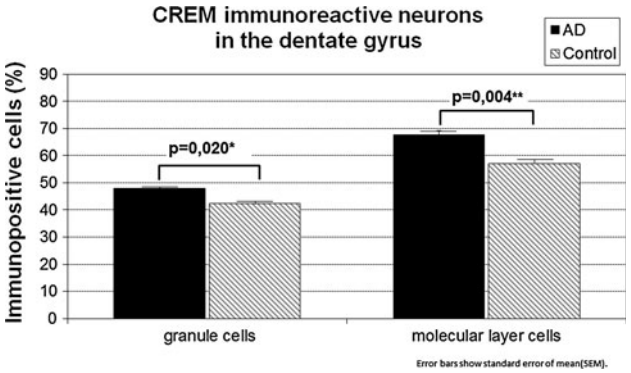


Fig. 7 Morphometric analysis of DG cells. Cell countings revealed that compared with controls in AD cases significantly more DG granule and molecular cells express CREM

diffuse and neuritic plaques were found in cortical areas of two AD patients.

Discussion

To the best of our knowledge this is the first detailed study on the expression patterns of CREM in developing and adult human brain. We, herein, show that CREM immunoreactivity is well detectable in normal human autopsy brain tissue, and that in hippocampal tissue from AD cases the cellular expression of CREM is increased. The CREB homologue CREM, which exists in various, alternatively spliced transcript variants, is best known for its role as a component of cAMP-mediated signal transduction during spermatogenesis, but plays also significant roles in other tissues and organs including the brain. Though originating from the same gene, CREM isoforms have different intracellular functions, with CREMα, β, and γ acting as antagonists and CREMτ acting as an activator of nuclear transcription (Foulkes et al. 1993). As aforementioned, is cerebral CREM prominently involved in the regulation of the synthesis of certain hypothalamic neuropeptides (Kwakowsky et al. 2012 and others). Besides, as an important part of the CRE/CREB signaling system, CREM might well have a function in normal cognition and AD-related cognitive decline (De Felice et al. 2007; Wang and Bibb 2011; Scott Bitner 2012 and others). CREM-deficient mice show various behavioral abnormalities, including altered locomotor activity and lower anxiety (Maldonado et al. 1999). Moreover, mice with postnatal disruption of both *CREB* and *CREM* genes display progressive neurodegeneration in the hippocampus and the striatum (Mantamadiotis et al. 2002). However, lack of information about the regional and cellular distribution of CREM in the human brain (especially regarding its expression in cognition-and AD-relevant brain regions) makes it difficult to say, how relevant the established or proposed roles of CREM might be for human brain functioning. Using Western blotting and immunohistochemistry we have mapped human brains for the distribution of CREM immunoreactivity. CREM was found to be widely distributed within aged human brain. In particular, CREM was found in multiple neurons located in those brain regions which are known to be involved in learning and memory (i.e., hippocampus, prefrontal and temporal cortex and others). With regard to its cellular localization CREM was confined almost exclusively to neurons. Confusingly enough, in a majority of adult human brain neurons, we found the nuclear transcription factor CREM not in cell nuclei, but in the cytoplasm. After having dissipated possible doubts with regard to the specificity of our CREM immunodetection system by doing additional control experiments (localization of CREM in testicular tissue, rat brains etc.), we performed an in-depth search of the existing literature and learned that a localization of CREM outside the cell nucleus had been observed before in human neocortical neurons and several non-neural types of cells (Human Protein Atlas; http://www.proteinatlas.org). Interestingly, some CREM isoforms are subjected to export from cell nucleus into cytoplasm and back (Fenaroli et al. 2004). This nucleo-cytoplasmic shuttling of CREM gene products is dependent on the presence of a "nuclear export activity" sequence in the CREM molecule, which is obviously recognized by anti-CREM polyclonal antibodies (Fenaroli et al. 2004). The functional relevance of this intracellular transit is poorly understood, since CREM has only a very few putative protein interaction partners in the cytoplasmic compartment of the cell (Fenaroli et al. 2004; BioGrid database, http://thebiogrid.org/107780/summary/homo-sapiens/crem.html). Remarkably, our rat experiments demonstrate that extranuclear immunolocalization of CREM is not a peculiarity of aged human brain neurons, but occurs in many nerve cells of non-injured postnatal rat brains, too. In midgestational human brains, however, a vast majority of immature neurons shows a clear nuclear localization of CREM immunoreactivity. Interestingly, in embryonic human brains, radial glial cells were found to abundantly express CREM.

In AD dentate gyrus (and in the angular gyrus, not shown), cell counts revealed an increased number of CREM-expressing neurons, which was accompanied by a strong, though insignificant, increase of hippocampal CREM protein in AD as well as the appearance of an additional CREM protein band. Although the CRE/CREB/cAMP-dependent protein kinase system is believed to play a major role in AD (Yamamoto-Sasaki et al. 1999; Kim et al. 2001; Matsuzaki et al. 2006; Wang and Bibb 2011; and others), there is very little information on CREM expression related to this

disorder. With severe hippocampal neurodegeneration in CREB/CREM double knockout mice in mind (Mantamadiotis et al. 2002), we had expected a decrease, rather than an increase, in neuronal CREM expression in AD. In search for supportive information we could identify only three publications on this topic. Two of them demonstrated that the CREM gene on chromosome 10q11.23 is not a major susceptibility gene in late-onset AD (Grupe et al. 2006; Giedraitis et al. 2009), the third one reported about normal CREM mRNA levels in the temporal cortex of AD patients (Avramopoulos et al. 2007). The observed increased protein expression in hippocampal tissue of AD patients may either point to regional differences in CREM expression, or come from the influence of other factors (for examples, micro RNAs, Smalmeiser and Lugli 2009). It should be taken into account that the increased percentage of CREM immunoreactive neurons in AD was registered in the hippocampal DG, which is known to significantly contribute to disturbances in memory and learning observed in AD, but largely withstands the formation of plaques, tangles, and neuronal death until late stages of AD (Thal et al. 2000; Ohm 2007). Instead, DG neurons show subtle changes related to a disconnecting process and intracellular alterations (Ohm 2007). Interestingly, CREM is known to control the expression of cyclins A, B, D1, and E (Servillo et al. 1998). When analyzing the expression of various cyclins (which are reliable markers of cell cycle disturbances) in the hippocampus of AD brains, Nagy et al. (1997) revealed that one of them, cyclin B was not detectable in control subjects but was expressed in the dentate gyrus, subiculum, dentate gyrus, and CA1 region in AD patients. Hence, it is conceivable that CREM, through regulation of cyclin B and other cyclins, is part of the molecular machinery that triggers the aberrant re-entering of a subset of neurons in the cell cycle, well-known to occur in AD (Nagy et al. 1997; Arendt 2005 and others). Collectively, our data on CREM in AD are preliminary and need to be substantiated by additional studies.

Limitations of the study

A clear limitation is the small sample size of AD patients and controls. Another problem may result from the properties of the antibodies used. Multiple CREM transcripts are present in the brain and other tissues. Although prepared against CREM-1 and tested in sufficient detail by Western blotting and immunocytochemistry, it cannot be said with ultimate certainty, which CREM isoforms besides CREM-1 are recognized by the antibody (and which not). Acknowledging this putative source of errors we prefer to speak about CREM instead of CREM-1.

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References

Aguado F, Díaz-Ruiz C, Parlato R, Martínez A, Carmona MA, Bleckmann S, Ureña JM, Burgaya F, del Río JA, Schütz G, Soriano E (2009) The CREB/CREM transcription factors negatively regulate early synaptogenesis and spontaneous network activity. J Neurosci 29:328–333

Ahlmann M, Varga G, Sturm K, Lippe R, Benedyk K, Viemann D, Scholzen T, Ehrchen J, Müller FU, Seidl M, Matus M, Tsokos GC, Roth J, Tenbrock K (2009) The cyclic AMP response element modulator alpha suppresses CD86 expression and APC function. J Immunol 182:4167–4174

Arendt T (2005) Alzheimer's disease as a disorder of dynamic brain self-organization. Prog Brain Res 147:355–378

Avramopoulos D, Wang R, Valle D, Fallin MD, Bassett SS (2007) A novel gene derived from a segmental duplication shows perturbed expression in Alzheimer's disease. Neurogenetics 8:111–120

Barcellos LF, May SL, Ramsay PP, Quach HL, Lane JA, Nititham J, Noble JA, Taylor KE, Quach DL, Chung SA, Kelly JA, Moser KL, Behrens TW, Seldin MF, Thomson G, Harley JB, Gaffney PM, Criswell LA (2009) High-density SNP screening of the major histocompatibility complex in systemic lupus erythematosus demonstrates strong evidence for independent susceptibility regions. PLoS Genet 5:e1000696

Behr R, Weinbauer GF (2000) CREM activator and repressor isoforms in human testis: sequence variations and inaccurate splicing during impaired spermatogenesis. Mol Hum Reprod 6:967–972

Bernstein HG, Schön E, Ansorge S, Röse I, Dorn A (1987) Immunolocalization of dipeptidyl aminopeptidase (DAP IV) in the developing human brain. Int J Dev Neurosci 5:237–242

Bernstein HG, Grecksch G, Becker A, Höllt V, Bogerts B (1999a) Cellular changes in rat brain areas associated with neonatal hippocampal damage. NeuroReport 10:2307–2311

Bernstein HG, Baumann B, Danos P, Diekmann S, Bogerts B, Gundelfinger ED, Braunewell KH (1999b) Regional and cellular distribution of neural visinin-like protein immunoreactivities (VILIP-1 and VILIP-3) in human brain. J Neurocytol 28:655–662

Bernstein HG, Smalla KH, Dürrschmidt D, Keilhoff G, Dobrowolny H, Steiner J, Schmitt A, Kreutz MR, Bogerts B (2012a) Increased density of prohibitin-immunoreactive oligodendrocytes in the dorsolateral prefrontal white matter of subjects with schizophrenia suggests extraneuronal roles for the protein in the disease. Neuromolecular Med 14:270–280

Bernstein HG, Stich C, Jäger K, Dobrowolny H, Wick M, Steiner J, Veh R, Bogerts B, Laube G (2012b) Agmatinase, an inactivator of the putative endogenous antidepressant agmatine, is strongly upregulated in hippocampal interneurons of subjects with mood disorders. Neuropharmacology 62:237–246

Blöcher S, Fink L, Bohle RM, Bergmann M, Steger K (2005) CREM activator and repressor isoform expression in human male germ cells. Int J Androl 28:215–223

Borsook D, Smirnova O, Behar O, Lewis S, Kobierski LA (1999) PhosphoCREB and CREM/ICER: positive and negative

regulation of proenkephalin gene expression in the paraventricular nucleus of the hypothalamus. J Mol Neurosci 12:35–51
Chiappini F, Ramadoss P, Vella KR, Cunha LL, Ye FD, Stuart RC, Nillni EA, Hollenberg AN (2013) Family members CREB and CREM control thyrotropin-releasing hormone (TRH) expression in the hypothalamus. Mol Cell Endocrinol 365:84–94
Cortés-Mendoza J, Díaz de León-Guerrero S, Pedraza-Alva G, Pérez-Martínez L (2013) Shaping synaptic plasticity: the role of activity-mediated epigenetic regulation on gene transcription. Int J Dev Neurosci 31:359–369
Crisafulli C, Chiesa A, Han C, Lee SJ, Shim DS, Balzarro B, Andrisano C, Sidoti A, Patkar AA, Pae CU, Serretti A (2012) Possible influence of CREB1, CREBBP and CREM variants on diagnosis and treatment outcome in patients with schizophrenia. Neurosci Lett 508:37–41
De Felice FG, Wasilewska-Sampaio AP, Barbosa AC, Gomes FC, Klein WL, Ferreira ST (2007) Cyclic AMP enhancers and Abeta oligomerization blockers as potential therapeutic agents in Alzheimer's disease. Curr Alzheimer Res 4:263–271
Della Fazia MA, Servillo G, Sassone-Corsi P (1997) Cyclic AMP signalling and cellular proliferation: regulation of CREB and CREM. FEBS Lett 410:22–24
Díaz-Ruiz C, Parlato R, Aguado F, Ureña JM, Burgaya F, Martínez A, Carmona MA, Kreiner G, Bleckmann S, Del Río JA, Schütz G, Soriano E (2008) Regulation of neural migration by the CREB/CREM transcription factors and altered Dab1 levels in CREB/CREM mutants. Mol Cell Neurosci 39:519–528
Domschke K, Kuhlenbäumer G, Schirmacher A, Lorenzi C, Armengol L, DiBella D, Gratacos M, Garritsen HS, Nöthen MM, Franke P, Sand P, Fritze J, Perez G, Maier W, Sibrowski W, Estivill X, Bellodi L, Ringelstein EB, Arolt V, Martin-Santos R, Catalano M, Stögbauer F, Deckert J (2003) Human nuclear transcription factor gene CREM: genomic organization, mutation screening, and association analysis in panic disorder. Am J Med Genet B Neuropsychiatr Genet 117B:70–78
Fenaroli A, Vujanac M, De Cesare D, Zimarino VA (2004) Small-scale survey identifies selective and quantitative nucleo-cytoplasmic shuttling of a subset of CREM transcription factors. Exp Cell Res 299:209–226
Fitzgerald LR, Vaidya VA, Terwilliger RZ, Duman RS (1996) Electroconvulsive seizure increases the expression of CREM (cyclic AMP response element modulator) and ICER (inducible cyclic AMP early repressor) in rat brain. J Neurosci 66:429–432
Foulkes NS, Schlotter F, Pévet P, Sassone-Corsi P (1993) Pituitary hormone FSH directs the CREM functional switch during spermatogenesis. Nature 362(6417):264–267
Foulkes NS, Borjigin J, Snyder SH, Sassone-Corsi P (1996) Transcriptional control of circadian hormone synthesis via the CREM feedback loop. Proc Natl Acad Sci USA 93:14140–14145
Giedraitis V, Kilander L, Degerman-Gunnarsson M, Sundelöf J, Axelsson T, Syvänen AC, Lannfelt L, Glaser A (2009) Genetic analysis of Alzheimer's disease in the Uppsala Longitudinal Study of Adult Men. Dement Geriatr Cogn Disord 27:59–68
Ginsberg SD, Elarova I, Ruben M, Tan F, Counts SE, Eberwine JH, Trojanowski JQ, Hemby SE, Mufson EJ, Che S (2004) Single-cell gene expression analysis: implications for neurodegenerative and neuropsychiatric disorders. Neurochem Res 29:1053–1064
Gomez-Villafuertes R, Torres B, Barrio J, Savignac M, Gabellini N, Rizzato F, Pintado B, Gutierrez-Adan A, Mellström B, Carafoli E, Naranjo JR (2005) Downstream regulatory element antagonist modulator regulates Ca2+ homeostasis and viability in cerebellar neurons. J Neurosci 25:10822–10830
Grupe A, Li Y, Rowland C, Nowotny P, Hinrichs AL, Smemo S, Kauwe JS, Maxwell TJ, Cherny S, Doil L, Tacey K, van Luchene R, Myers A, Wavrant-De Vrièze F, Kaleem M, Hollingworth P, Jehu L, Foy C, Archer N, Hamilton G, Holmans P, Morris CM, Catanese J, Sninsky J, White TJ, Powell J, Hardy J, O'Donovan M, Lovestone S, Jones L, Morris JC, Thal L, Owen M, Williams J, Goate A (2006) A scan of chromosome 10 identifies a novel locus showing strong association with late-onset Alzheimer disease. Am J Hum Genet 78:78–88
Hamilton SP, Slager SL, Mayo D, Heiman GA, Klein DF, Hodge SE, Fyer AJ, Weissman MM, Knowles JA (2004) Investigation of polymorphisms in the CREM gene in panic disorder. Am J Med Genet B Neuropsychiatr Genet 126B:111–115
Herdegen T, Leah JD (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Brain Res Rev 28:370–490
Hummler E, Cole TJ, Blendy JA, Ganss R, Aguzzi A, Schmid W, Beermann F, Schütz G (1994) Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. Proc Natl Acad Sci USA 91:5647–56451
Kadar E, Aldavert-Vera L, Huguet G, Costa-Miserachs D, Morgado-Bernal I, Segura-Torres P (2011) Intracranial self-stimulation induces expression of learning and memory-related genes in rat amygdala. Genes Brain Behav 10:69–77
Kim SH, Nairn AC, Cairns N, Lubec G (2001) Decreased levels of ARPP-19 and PKA in brains of Down syndrome and Alzheimer's disease. J Neural Transm Suppl 61:263–272
Konopka D, Szklarczyk AW, Filipkowski RK, Trauzold A, Nowicka D, Hetman M, Kaczmarek L (1998) Plasticity- and neurodegeneration-linked cyclic-AMP responsive element modulator/inducible cyclic-AMP early repressor messenger RNA expression in the rat brain. Neuroscience 86:499–510
Kwakowsky A, Herbison AE, Ábrahám IM (2012) The role of cAMP response element-binding protein in estrogen negative feedback control of gonadotropin-releasing hormone neurons. J Neurosci 32:11309–11337
Ledo F, Carrión AM, Link WA, Mellström B, Naranjo JR (2000) DREAM-alphaCREM interaction via leucine-charged domains derepresses downstream regulatory element-dependent transcription. Mol Cell Biol 20:9120–9126
Link WA, Ledo F, Torres B, Palczewska M, Madsen TM, Savignac M, Albar JP, Mellström B, Naranjo JR (2004) Day-night changes in downstream regulatory element antagonist modulator/potassium channel interacting protein activity contribute to circadian gene expression in pineal gland. J Neurosci 24:5346–5355
Luzzati F, De Marchis S, Parlato R, Gribaudo S, Schütz G, Fasolo A, Peretto P (2011) New striatal neurons in a mouse model of progressive striatal degeneration are generated in both the subventricular zone and the striatal parenchyma. PLoS One 6:e25088
Madsen HB, Navaratnarajah S, Farrugia J, Djouma E, Ehrlich M, Mantamadiotis T, Van Deursen J, Lawrence AJ (2012) CREB1 and CREB-binding protein in striatal medium spiny neurons regulate behavioural responses to psychostimulants. Psychopharmacology 219:699–713
Maldonado R, Smadja C, Mazzucchelli C, Sassone-Corsi P (1999) Altered emotional and locomotor responses in mice deficient in the transcription factor CREM. Proc Natl Acad Sci USA 96:14094–14099
Mantamadiotis T, Lemberger T, Bleckmann SC, Kern H, Kretz O, Martin VA, Tronche F, Kellendonk C, Gau D, Kapfhammer J (2002) Disruption of CREB function in brain leads to neurodegeneration. Nat Genet 31:47–54
Matsuzaki IK, Yamakuni T, Hashimoto M, Haque AM, Shido O, Mimaki Y, Sashida Y, Ohizumi Y (2006) Nobiletin restoring beta-amyloid-impaired CREB phosphorylation rescues memory deterioration in Alzheimer's disease model. Neurosci Lett 400:230–234
Mellström B, Naranjo JR, Foulkes NS, Lafarga M, Sassone-Corsi P (1993) Transcriptional response to cAMP in brain: specific

distribution and induction of CREM antagonists. Neuron 10:655–665
Nagy Z, Esiri MM, Cato AM, Smith AD (1997) Cell cycle markers in the hippocampus in Alzheimer's disease. Acta Neuropathol 94:6–15
Naranjo JR, Mellström B, Carrion AM, Lucas JJ, Foulkes NS, Sassore-Corsi P (1997) Peripheral noxious stimulation induces CREM expression in dorsal horn: involvement of glutamate. Eur J Neurosci 9:2778–2793
O'Rahilly R, Müller F (2005) The embryonic human brain: an atlas of developmental stages, 3rd edn. Wiley, New York
Ohm TG (2007) The dentate gyrus in Alzheimer's disease. Progr Brain Res 163:723–740
Rauen T, Hedrich CM, Tenbrock K, Tsokos GC (2013) cAMP responsive element modulator: a critical regulator of cytokine production. Trends Mol Med. doi:10.1016/j.molmed.2013.02.001
Scott Bitner R (2012) Cyclic AMP response element-binding protein (CREB) phosphorylation: a mechanistic marker in the development of memory enhancing Alzheimer's disease therapeutics. Biochem Pharmacol 83:705–714
Servillo G, Della Fazia MA, Sassone-Corsi P (1998) Transcription factor CREM coordinates the timing of hepatocyte proliferation in the regenerating liver. Genes Dev 12:3639–6943
Simón-Sánchez J, Schulte C, Bras JM, Sharma M, Gibbs JR, Berg D, Paisan-Ruiz C, Lichtner P, Scholz SW, Hernandez DG, Krüger R, Federoff M, Klein C, Goate A, Perlmutter J, Bonin M, Nalls MA, Illig T, Gieger C, Houlden H, Steffens M, Okun MS, Racette BA, Cookson MR, Foote KD, Fernandez HH, Traynor BJ, Schreiber S, Arepalli S, Zonozi R, Gwinn K, van der Brug M, Lopez G, Chanock SJ, Schatzkin A, Park Y, Hollenbeck A, Gao J, Huang X, Wood NW, Lorenz D, Deuschl G, Chen H, Riess O, Hardy JA, Singleton AB, Gasser T (2009) Genome-wide association study reveals genetic risk underlying Parkinson's disease. Nat Genet 41:1308–1312
Smalmeiser NR, Lugli G (2009) microRNA regulation of synaptic plasticity. Neuromolecular Med 11:133–140
Stehle JH, Foulkes NS, Molina CA, Simonneaux V, Pévet P, Sassone-Corsi P (1993) Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland. Nature 365(6444):314–320
Thal DR, Holzer M, Rüb U, Waldmann G, Günzel S, Zedlick D, Schober R (2000) Alzheimer-related tau pathology in the perforant path target zone and in the hippocampal stratum oriens and radiatum correlates with onset and degree of dementia. Exp Neurol 163:98–110
Uyttersprot N, Miot F (1997) Dog CREM transcription factors: cloning, tissue distribution, and identification of new isoforms. Biochem Biophys Res Commun 237:74–78
van Strien ME, van den Berge SA, Hol EM (2011) Migrating neuroblasts in the adult human brain: a stream reduced to a trickle. Cell Res 21:1523–1525
Wang A, Bibb JA (2011) Is CREB the angry bird that releases memory in Alzheimer's? Neuropsychopharmacology 36:2153–2154
Wu X, Jin W, Liu X, Fu H, Gong P, Xu J, Cui G, Ng Y, Ke K, Gao Z, Gao Y (2012) Cyclic AMP response element modulator-1 (CREM-1) involves in neuronal apoptosis after traumatic brain injury. J Mol Neurosci 47:357–367
The Human Protein Atlas (Uppsala Universiteit). http://www.proteinatlas.org/
Yamamoto-Sasaki M, Ozawa H, Saito T, Rösler M, Riederer P (1999) Impaired phosphorylation of cyclic AMP response element binding protein in the hippocampus of dementia of the Alzheimer type. Brain Res 824:300–303
Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E, Kadam S, Ecker JR, Emerson B, Hogenesch JB, Unterman T, Young RA, Montminy M (2005) Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci USA 102:4459–4464
Zhu X, Han X, Blendy JA, Porter BE (2012) Decreased CREB levels suppress epilepsy. Neurobiol Dis 45:253–263
Zubenko GS, Maher B, Hughes HB 3rd, Zubenko WN, Stiffler JS, Kaplan BB, Marazita ML (2003) Genome-wide linkage survey for genetic loci that influence the development of depressive disorders in families with recurrent, early-onset, major depression. Am J Med Genet B Neuropsychiatr Genet 123B(1):1–18