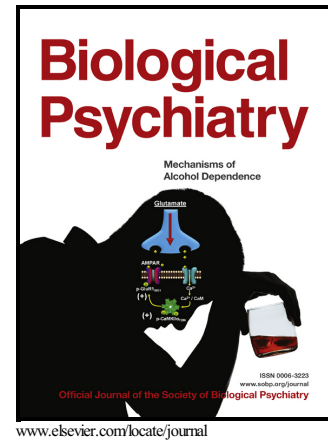# Author's Accepted Manuscript

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CRTC1 function during memory encoding is disrupted in neurodegeneration

Arnaldo Parra-Damas[1,2], Meng Chen[1,2], Lilian Enriquez-Barreto[1,2], Laura Ortega[1], Sara Acosta[1], Judith Camats Perna[1], M. Neus Fullana[1], José Aguilera[1,2] José Rodríguez-Alvarez[1,2] and Carlos A. Saura[1,2*]

[1] Institut de Neurociències, Department de Bioquímica i Biologia Molecular, [2] Centro de Investigación Biomédica en Red Enfermedades Neurodegenerativas (CIBERNED), Universitat Autònoma de Barcelona, Barcelona 08193, Spain.

*Corresponding author: Carlos A. Saura, Institut de Neurociències, Facultat de Medicina M2-113, Universitat Autònoma de Barcelona, Barcelona, Spain 08193. Tel. +34-935868398, FAX: +34-935814152, E-mail: carlos.saura@uab.es

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Short title: CRTC1-dependent gene expression in neurodegeneration

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Abstract

Background: Associative memory impairment is an early clinical feature of dementia patients, but the molecular and cellular mechanisms underlying these deficits are largely unknown. In this study, we investigated the functional regulation of the CREB-regulated transcription coactivator-1 (CRTC1) by associative learning in physiological and neurodegenerative conditions.

Methods: We evaluated the activation of CRTC1 in the hippocampus of control mice and mice lacking the Alzheimer´s disease-linked *presenilin* genes (*PS* cDKO) after one trial contextual fear conditioning by using biochemical, immunohistochemical and gene expression analyses. *PS* cDKO display classical features of neurodegeneration occurring in Alzheimer´s disease including age-dependent cortical atrophy, neuron loss, dendritic degeneration and memory deficits.

Results: Context associative learning, but not single context or unconditioned stimuli, induces rapid dephosphorylation (Ser151) and translocation of CRTC1 from the cytosol/dendrites to the nucleus of hippocampal neurons in the mouse brain. Accordingly, context associative learning induces differential CRTC1-dependent transcription of *c-fos* and the nuclear receptor subfamily 4 (Nr4a) genes *Nr4a1-3* in the hippocampus through a mechanism that involves CRTC1 recruitment to CRE promoters. Deregulation of CRTC1 dephosphorylation, nuclear translocation and transcriptional function are associated with long-term contextual memory deficits in *PS* cDKO mice. Importantly, CRTC1 gene therapy in the hippocampus ameliorates context memory and transcriptional deficits and dendritic degeneration despite ongoing cortical degeneration in this neurodegeneration mouse model.

Conclusions: These findings reveal a critical role of CRTC1 in the hippocampus during associative memory, and provide evidence that CRTC1 deregulation underlies memory deficits during neurodegeneration.

Introduction

Alzheimer´s disease (AD) is a neurodegenerative disorder characterized by neuropsychiatric symptoms and amnesia. Dementia patients develop early deficits in encoding and retrieval of associative episodic memories (1, 2), a clinical feature already present in persons at risk for developing AD (3, 4). Functional magnetic resonance imaging studies show decreased activity and connectivity of the medial temporal lobe, particularly the hippocampus, during associative and emotional memory tasks in AD patients (2, 4-8). Memory decline is accompanied by the presence of

pathological features, including degeneration of synapses, dendrites and neurons in memory encoding brain regions (9). Despite the evidences of associative memory impairments and neurodegeneration in the hippocampus of dementia patients, the cellular and molecular mechanisms linking these features are largely unclear.

Associative memories related to learning new information of people, places or locations is common in daily human activities. Fear conditioning is an associative learning paradigm that allows acquisition and consolidation of emotional-related context memories that depends on a neural circuitry that includes the hippocampus, amygdala and prefrontal cortex (10). The hippocampus encodes context representations and sends projections to the amygdala, which encodes, stores and retrieves contextual cues associated with aversive stimulus (11, 12). Whereas different hippocampal regions contribute to acquisition of fear contextual memory (13, 14), the CA3 subregion is activated during associative encoding and critical for initial context representations (15, 16). Besides participating in adaptive behavior, fear conditioning is implicated in the mechanisms that mediate psychopathological fear and anxiety (17), whereas dementia patients develop associative memory impairments in fear conditioning (18, 19).

The transcription factor cAMP-response element binding protein (CREB) plays a crucial role in contextual memory encoding, consolidation and reconsolidation (20-22). Contextual learning induces CREB phosphorylation at Ser133 and gene transcription (23). However, CREB phosphorylation is essential but not sufficient for gene transcription (24, 25), a process that requires the specific transcriptional coactivators CREB binding protein (CBP) and CREB-regulated transcription coactivators (CRTCs). CRTCs act as selective regulators of CREB-dependent gene expression by directing CREB occupancy to specific gene promoters (26-28). Consistent with its role in CREB signaling, CRTC1 modulates dendritic growth, long-term synaptic plasticity and memory consolidation through still unclear downstream mechanisms (27, 29-33). Disruption of CREB/CRTC association impairs CREB-dependent transcription, synaptic plasticity and long-term memory (34), whereas CRTC1 dysfunction causes transcriptional changes leading to memory impairments in an AD

mouse model (35, 36). Given this scenario, this study was aimed to investigate the specific role of CRTC1 signaling in the hippocampus during associative memory encoding in physiological and pathological conditions.

Methods and Materials

Mice

2-6 month-old male mice (C57BL/6 background) and WT or *PS* cDKO mice (C57BL/6/129 hybrid background) lacking expression of both *PS* genes (*PS1* and *PS2*) in forebrain glutamatergic neurons were used (37). Littermate control (WT; f*PS1*/f*PS1*; *PS2*+/+ or f*PS1*/f*PS1*; *PS2*+/-) and *PS* cDKO mice (f*PS1*/f*PS1*; *PS2*-/-; CaMKIIαCre) were obtained by crossing floxed *PS1*/*PS2*-/- (f*PS1*/f*PS1*; *PS2*-/-) or *PS2*+/- (f*PS1*/f*PS1*; *PS2*+/-) males to heterozygous *PS1* cKO; *PS2*+/- females (f*PS1*/f*PS1*; *PS2*+/-; CaMKIIαCre). Experimental procedures were conducted according to the Animal and Human Ethical Committee of the Universitat Autònoma de Barcelona (CEEAH 1783 and 2896) following the European Union guidelines (2010/63/EU).

Behavioral studies

For contextual fear conditioning, mice handled for three days (3 min/day) were placed in a conditioning chamber (15.9 x 14 x 12.7 cm; Med Associates, St. Albans, Vermont) for 3 min, foot-shocked (1s/1mA) and retained in the chamber for 2 min (*immediate freezing*) (38). Fear memory was tested as freezing behavior, which was defined as a complete cessation of all movement except for respiration, in the same conditioning chamber for 4 min 2 h or 24 h after trainingusing *Video Freeze Software* (Med Associates) (**Fig. 1A**). Naïve mice were handled but neither exposed to the conditioning chamber nor shocked, context groups were placed in the chamber without receiving footshock and shocked groups were shocked and immediately returned to their home cages. For biochemical and immunohistochemical analyses mice were sacrificed 15 min after context training or memory retention by dislocation or a lethal dose of pentobarbital, respectively.

Adeno-associated virus injections

Adeno-associated virus (AAV2/10) from rhesus macaque (AAVrh.10) containing Crtc1-myc under the β-actin promoter was generated by subcloning pcDNA3-Crtc1-myc (27) into pVAX1 (Thermo Fisher Scientific, USA) and pGV-IRES2-GFP vectors as described (35). For viral injections, 4-4.5 month-old mice (n= 6-8 mice/group) were anesthetized with isofluorane and injected bilaterally into the dorsal hippocampus with AAV-GFP or AAV-Crtc1-myc (3 µl; 5.1×10^{11} gc/ml; 0.5 µl/min). The sterotaxic injection coordinates were (in mm) as follows: anteroposterior: -2.0 from Bregma; mediolateral: ±1.8 from Bregma; ventral: -1.8 from dural surface, according to (Paxinos and Franklin, 2004). Mice were tested six weeks after injection in contextual fear conditioning before processed for histological and biochemical analyses.

Gene expression analysis

Primary neurons (4 DIV) were infected with scramble or *Crtc1* ShRNAs lentiviral vectors (1–2 transducing units/cell) and treated (12 DIV) with vehicle or KCl (30 mM) plus forskolin (20 µM; Sigma) for 0-12 h. CRE luciferase assays were performed by triplicate in at least three independent experiments (36). RNA was purified using the PureLink RNA Mini Kit (Thermo Fisher Scientific, USA). RNA integrity number (RIN) was measured using the Agilent 2100 bioanalyser (Agilent Technologies). RNA (1 µg; RIN > 8.0) was reverse-transcribed in 50 µl of a reaction mix containing 1 µM of Oligo (dT) primers, 1 µM random hexamers, 0.5 mM dNTP, 0.45 mM DTT, RNAseOut (10 units) and SuperScript™ II reverse transcriptase (Thermo Fisher Scientific) at 25ºC for 10 min, 42ºC for 60 min and 72ºC for 10 min. Quantitative real time RT-PCR (qRT-PCR) was performed in duplicate in at least 3-5 samples using an Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific). Data analysis was performed by the comparative ΔCt method using the Ct values and the average value of PCR efficiencies obtained from LinRegPCR software (39). Gene expression was normalized to *Gapdh* for cultured neurons or the geometric mean of *Gapdh*, hypoxanthine guanine phosphoribosyl transferase (*Hprt*) and peptidylprolyl isomerase A (*Ppia*) for brain samples (40).

Biochemical analysis

Tissue was lysed in cold-lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1% NP-40, 0.1% SDS, 1mM Na_3VO_4, 50 mM NaF, 1 mM PMSF) containing protease and phosphatase inhibitors (Roche España, Barcelona, Spain). Proteins were quantified with the BCA protein assay kit (Thermo Fisher Scientific), resolved on SDS-polyacrylamide gel electrophoresis (PAGE) and blotted with the following antibodies: rabbit anti-CRTC1 (1:5,000), CREB (1:250), phosphorylated CREB (Ser133; 1:1,000) from Cell Signaling (Danvers, Massachusetts); phosphorylated Ser151 CRTC1 (1:1,000) (36) and ABE560 (Merck-Millipore, Darmstadt, Germany) and mouse anti-GAPDH (1:5,000; Abcam, Cambridge,UK). Protein bands were quantified with ImageJ software.

ChiP-qPCR analysis

Cortical neurons (12 DIV) were crosslinked with 1% formaldehyde before lysis and sonication in ChIP buffer (50 mM Tris-HCl pH 8.1, 100 mM NaCl, 5mM EDTA, 1% SDS, 0,1% Na deoxycholate and protease/phosphatase inhibitors). Fragmented chromatin (200-500 bp) was analyzed using the High Sensitivity DNA Kit (Agilent Technologies). Chromatin immunoprecipitation (2.5 μg) was performed overnight in diluted ChIP buffer (0.1% SDS, 1,1% Triton X-100) with or without rabbit anti-CRTC1 and CREB antibodies (Cell Signaling) (35). Input and immunoprecipitated DNA were decrosslinked and amplified by real-time qPCR using specific primers, and the fold enrichment was calculated over an irrelevant region.

Histological, immunohistochemical and immunofluorescence staining

For CRTC1 translocation analysis, mice in home conditions or exposed to shock, context or context plus shock were anesthesized with a lethal dose of pentobarbital (200 mg/kg, i.p.) 15 min after CFC training. Mice were perfused intracardially with 0.9% NaCl followed by 4% buffered formalin for 2 h. Coronal or saggital brain sections (5 μm) were deparaffinized in xylene, rehydrated and microwave

heated for 10 min in citrate buffer (10 mM, pH=6.0). Sections were incubated with rabbit anti-CRTC1 antibody (1:300; Cell Signaling), rabbit anti-CBP (1:200; Santa Cruz Biotechnology, Santa Cruz, California) and mouse NeuN (1:2,000; Merck-Millipore) or MAP2 (1:200; Sigma, St. Louis, Missouri) antibodies and AlexaFluor-488/594-conjugated goat IgGs (1:400) and Hoechst (1:10,000; Thermo Fisher Scientific). Nissl staining was performed in floating sections (40 μm) after incubation with cresil violet solution (5 g/l) for 5 min, and cortical thickness of somatosensory cortex was measured using ImageJ (n=4-5 mice/group; n=3 sections/mouse).

Confocal image acquisition and analysis

Images (20x; zoom 0.5) obtained with a Zeiss Axio Examiner D1 LSM700 laser scanning microscope (Carl Zeiss Microcopy, Jena, Germany) were analyzed with ImageJ software (v.1.6x). CRTC1 staining intensity in the selected regions was measured using a sum projection of six Z-sections (1 μm/section). Hoescht staining was used to determine the nuclear area, whereas the area comprising 2 μm around the nucleus was considered cytoplasmic CRTC1. Nuclear/cytosol CRTC1 staining intensity ratio in caudal, medial and rostral hippocampal regions was used as measure of CRTC1 nuclear translocation (n=3-4 sections/mouse; n=3-5 mice/group). Dendritic CRTC1 was analyzed by quantifying CRTC1/MAP2 colocalization in the rostral CA3 hippocampus (n=3 sections/mouse; n= 8/group). Dendrite analysis was measured using MAP2 staining intensity in a sum projection of five Z-sections (1 μm/section) and dendritic fiber thickness was measured automatically by generating a Plot Profile of the pixels and peak thickness intensity along a grid line using ImageJ (n=3 sections/mouse; n=4-6 mice/group).

Statistical analysis

Statistical analysis was performed using one- or two-way analysis of variance (ANOVA) and Bonferroni or Tukey´s *post hoc* tests for multiple comparisons using GraphPad. Behavioral results were analyzed by using two-way ANOVA with repeated measures and Bonferroni or Scheffé's S *post hoc* with SuperANOVA v1.11. Differences with *P* < 0.05 were considered significant.

Results

Contextual fear conditioning induces CRTC1 dephosphorylation, nuclear translocation and transcriptional activity in the hippocampus

Previous studies have shown that CRTC1 activation is mediated by activity-dependent CRTC1 dephosphorylation and nuclear translocation (31, 35, 41). We first investigated the regulation of CRTC1 by associative learning in the hippocampus, a region essential for early context representations (15, 42). Contextual fear conditioning, but not context alone, induces a time-dependent increase of freezing responses in mice after training, indicating efficient contextual memory association ($P < 0.0001$, one-way ANOVA; **Figures 1A,B**). Consistent with previous reports (23, 43), CREB phosphorylation at Ser133 was increased in the mouse hippocampus after context or context plus shock compared with naïve conditions ($P < 0.05$), whereas CRTC1 phosphorylation at Ser151, a residue involved in CRTC1 inactivation (33, 36), was significantly decreased 15 min-2 h after contextual training ($P < 0.05$, one-way ANOVA; **Figures 1C, D**).

To explore the possibility that CRTC1 dephosphorylation could mediate CREB-dependent transcription in the hippocampus during associative learning, we analyzed mRNA levels of CREB target genes implicated in contextual learning, including *Arc*, *c-fos* and *Nr4a 1*, *2* and *3* (44). In agreement with previous reports *Arc* is similarly induced by a novel context and contextual conditioning (45, 46). By contrast, levels of *c-fos*, *Nr4a1* and *Nr4a2* transcripts, but not *Nr4a3,* are significantly increased after fear conditioning ($P< 0.05$-0.01, one-way ANOVA) but not by context or shock alone (**Figure 2A**). Quantitative real-time RT-PCR analysis revealed that neuronal activity rapidly increases ($t_{1/2} < 1$ h) transcript levels of *Arc* (10 fold), *c-fos* (40 fold), *Nr4a1* (40 fold), *Nr4a2* (50 fold) and *Nr4a3* (4 fold) ($P< 0.001$, one-way ANOVA; **Figure 2B**). Since *Crtc1* inactivation using *Crtc1* ShRNAs significantly decreases activity-induced expression of *Arc, c-fos, Nr4a1 and Nr4a 2* ($P < 0.05$), whereas *Nr4a3* mRNA levels are minimally affected (**Figure 2B**), we explored the possibility that CRTC1 could bind differentially to the promoter regions of *Nr4a* genes. Quantitative

8

chromatin immunoprecipitation (ChiP-qPCR) analyses demonstrated an activity-dependent recruitment of CRTC1 to the proximal CRE-TATA promoter regions of *c-fos*, *Nr4a1* and *Nr4a2* ($P <$ 0.001, one-way ANOVA) but not to the CRE-TATA-deficient region of *Nr4a3* ($P > 0.05$; **Figure 2C**). By contrast, CREB strongly binds to *c-fos*, *Nr4a1* and *Nr4a2* promoters in basal non-stimulated conditions (**Figure 2C**). This result suggests that activation of CREB/CRTC1-dependent transcription is mediated by binding of CRTC1 to proximal CRE-TATA rich gene promoters after contextual learning in the dorsal hippocampus.

CRTC1 is mostly expressed in cell bodies and fibers of neurons in the mouse hippocampus (CA1, CA3 and dentate gyrus), cortex, striatum, thalamus and amygdala (**Figure 3A**; data not shown). The pattern of CRTC1 staining is similar in naïve, context or shock conditions ($P > 0.05$; **Figures 3A and 3C**). Interestingly, CRTC1 is abundantly localized in the nucleus of CA3 pyramidal neurons and moderately in CA1 neurons 15 min after contextual fear conditioning compared with in naïve, context or shock conditions ($P < 0.01$, one-way ANOVA; **Figures 3A,C** and data not shown). Indeed, CRTC1 colocalizes with MAP2 in dendrites of CA3 hippocampal neurons in naïve conditions, whereas this colocalization is significantly reduced 15 min after CFC ($P < 0.02$; **Figures 3B,C**). These results suggest that contextual learning induces a rapid translocation of CRTC1 from the cytosol and dendrites to the nucleus of neurons in the mouse hippocampus.

Altered CRTC1-dependent transcription and nuclear translocation are associated with contextual memory deficits during neurodegeneration

Since associative memory deficits and reduced hippocampal activity occur in AD patients (2, 4-7), we next investigated the role of CRTC1-dependent transcription in contextual fear memory deficits in *presenilin* (*PS*) cDKO mice lacking both *PS1* and *PS2* genes in neurons of the postnatal forebrain (37). *PS* cDKO mice display classical features of neurodegeneration occurring in AD including age-dependent cortical atrophy, enlargement of lateral ventricles, neuron loss associated with increased apoptosis and activation of caspases 3 and 9, neuroinflammation, dendritic

9

degeneration and synapse loss (37, 47, 48). Indeed, control (WT) and *PS* cDKO mice at 2 months of age display similar freezing responses 2 h and 24 h after contextual fear conditioning ($P > 0.05$, two-way ANOVA; **Figure 4A**). By contrast, 6 month-old *PS* cDKO mice show reduced freezing responses 2 h ($P < 0.05$) and 24 h ($P < 0.001$) after contextual training compared with control mice (two-way ANOVA; **Figure 4A**), which indicates short- and long-term contextual memory deficits in *PS* cDKO mice.

Gene expression analysis shows that *Arc* ($P < 0.05$) and *c-fos* ($P < 0.0001$) transcripts are significantly increased after contextual training but without significant differences between genotypes ($P > 0.05$, two-way ANOVA; **Figure 4B**). *Nr4a1* and *Nr4a2* transcripts, but not *Nr4a3*, are significantly increased 24 h after contextual learning in control and *PS* cDKO mice ($P < 0.001$, two-way ANOVA) but with significant differences between genotypes. *Post hoc* analysis revealed a significant reduction of *Nr4a1* ($P < 0.001$) and *Nr4a2* ($P < 0.05$) transcripts, but not those of *Nr4a3*, in the hippocampus of 6 month-old *PS* cDKO mice at 24 h (**Figure 4B**). Levels of CRTC1 are not significantly different ($P = 0.13$) whereas CREB is slightly decreased ($P < 0.02$) in the hippocampus of *PS* cDKO mice. After contextual training, phosphorylated Ser151 CRTC1/CRTC1 levels are significantly decreased in control mice ($P < 0.01$) but not in *PS* cDKO mice ($P = 0.47$), whereas phosphorylated CREB was increased in both WT and *PS* cDKO mice but with significant changes between genotypes ($P < 0.005$, two-way ANOVA; **Figure 4C**). These results demonstrate age-related memory impairments associated with differential downregulation of CRTC1/CREB target genes in the hippocampus of *PS* cDKO mice.

We next investigated the relationship between CRTC1 nuclear translocation and contextual memory deficits in *PS* cDKO mice. Contextual fear learning induces after 15 min a significant translocation of CRTC1 to the nucleus of CA3 pyramidal neurons in control (WT) mice ($P < 0.05$), whereas CRTC1 staining is found mainly in the cytosol and sporadically in the nucleus in *PS* cDKO mice ($P < 0.05$, two-way ANOVA; **Figures 5A, C**). Moreover, CRTC1 is significantly decreased in dendrites in control but not *PS* cDKO mice 15 min after contextual learning ($P < 0.05$; two-way

ANOVA; **Figures 5B, C**). Together, these results suggest deficient CRTC1 nuclear translocation and transcriptional function associated with contextual memory deficits in *PS* cDKO mice.

CRTC1 gene therapy ameliorates transcriptional and contextual memory deficits in PS cDKO mice

To evaluate whether CRTC1 dysfunction contributes to associative memory deficits in *PS* cDKO mice, we overexpressed CRTC1 *in vivo* by using adeno-associated virus (AAV) 2/10, a serotype that allows stable long-term (> 2 months) neuronal gene expression (49)and enhances nuclear translocation of CRTC1-myc and CRE-dependent transcription in cultured neurons (**Supplementary Figure S1**). 4-4.5 month-old mice were injected with AAV-GFP (control) and AAV-Crtc1-myc in the CA3 hippocampus and evaluated six weeks later. AAV-Crtc1-myc injection allowed high expression of CRTC1-myc mRNA and protein mainly in neurons of CA1, CA3 and dentate gyrus (**Figures 6A, B** and **Supplementary Figure S2**). CRTC1 overexpression does not affect basal freezing responses of WT and *PS* cDKO mice exposed in a novel context ($P = 0.78$, two-way ANOVA; **Figure 6C**). By contrast, AAV-Crtc1 increases significantly freezing responses 24 h after contextual training both in WT ($P < 0.05$) and *PS* cDKO mice ($P < 0.03$) (two-way ANOVA; **Figure 6D**). These results suggest a memory-enhancing rather than an anxiety effect of CRTC1 overexpression in the hippocampus. Contextual fear conditioning significantly induces *Nr4a1* and *Nr4a2* mRNAs in the hippocampus of all groups ($P < 0.001$). Importantly, CRTC1 overexpression increases significantly *Nr4a1* and *Nr4a2* mRNAs in *PS* cDKO mice compared to GFP-injected *PS* cDKO mice ($P < 0.05$, two-way ANOVA; **Figure 6E**). This result indicates that CRTC1 gene therapy in the hippocampus ameliorates transcriptional and long-term contextual memory deficits in *PS* cDKO mice.

CRTC1 ameliorates dendritic degeneration in the hippocampus

PS cDKO mice develop cortical neuron loss and dendritic degeneration in the neocortex and hippocampus during aging (37, 48). In agreement, 6 month-old *PS* cDKO mice injected with AAV-GFP or -Crtc1 in the hippocampus show similar enlargement of lateral ventricles and reduced cortical

thickness and dendritic MAP2-stained fibers in the neocortex (**Figures 7A,B**). Quantitative confocal imaging analysis reveals significant reduction of total MAP2 staining intensity and dendritic fibers in CA3 hippocampus of *PS* cDKO-GFP mice compared with WT-GFP mice ($P < 0.05$, two-way ANOVA; **Figure 7B).** Interestingly, AAV-Crtc1 increases total MAP2 intensity staining and also moderately dendrite thickness in CA3 hippocampus of *PS* cDKO mice (two-way ANOVA; **Figure 7B**). Notably, MAP2 staining intensity and dendrite thickness in CA3 hippocampus of *PS* cDKO-Crtc1 mice are not significant different from WT-GFP mice ($P > 0.05$, two-way ANOVA). These results indicate that CRTC1 gene therapy ameliorates dendritic degeneration in the hippocampus without affecting cortical neurodegeneration.

Discussion

The transcription factor CREB facilitates contextual memory by regulating neuronal excitability and recruitment of neurons into active memory networks (50-53). However, the CREB-dependent transcriptional programs and their regulatory mechanisms that mediate associative memory encoding are largely unclear. In this study, we found that contextual learning induces time-dependent dephosphorylation (Ser151), nuclear translocation and transcriptional activation of CRTC1 in the hippocampus. Importantly, deregulation of CRTC1 nuclear translocation and function in the hippocampus is associated with contextual memory impairments and dendrite degeneration in a mouse model of neurodegeneration, whereas CRTC1 gene therapy reverses these deficits. These results strongly suggest that CRTC1-dependent transcription in the hippocampus is critical for long-term associative memory encoding in normal and pathological conditions.

A relevant finding of our study is that associative learning activates CRTC1 in the hippocampus by a mechanism that involves CRTC1 dephosphorylation and translocation from the cytosol and dendrites to the nucleus. Contextual learning, but not context or shock alone, induces CRTC1 nuclear translocation in CA3 hippocampus, and to a minor extent in CA1 region (not shown), suggesting that CRTC1 activation in the hippocampus can mediate rapid spatial context acquisition

during memory encoding. Indeed, CRTC1 inactivation in the hippocampus by using AAV-Crtc1 ShRNA negatively affects long-term associative memory in control mice (unpublished). A role of CRTC1 in associative memory is further supported by previous findings indicating that spatial memory training induces CRTC1 nuclear translocation in the hippocampus (35), and that CRTC1 overexpression in the dorsal hippocampus enhances contextual fear memory (30, 31) (**Figure 6C**). In agreement, contextual learning induces CREB-mediated transcription in CA1/CA3 hippocampus, whereas cued fear-conditioning activates CREB in the amygdala (23). Alternatively, CRTC1 is activated in the amygdala one day after contextual learning, i.e. during memory consolidation (31), which is consistent with a role of this circuit in associating contextual cues with aversive events (11, 12). Based on these results, we suggest that CRTC1 participates in transcriptional events mediating contextual memory in the dorsal hippocampus, a region required for contextual memory encoding (15, 54).

Consistent with a role of CRTC1 in associative memory encoding, contextual fear learning induces expression of memory-related CRTC1/CREB target genes in the hippocampus. We found a time-dependent differential induction of CREB target *Nr4a* family genes *Nr4a1-3* in the hippocampus, a result that agrees with the requirement of *Nr4a* genes for contextual memory (44, 55). CRTC1-mediated transcription may involve CRTC1 dephosphorylation at Ser151, a critical event for activity-induced CREB-mediated transcription (33, 36). Since the histone deacetylase CBP mediates CREB-dependent transcription through cooperative interactions with CRTC2 and CREB (26), it is possible that CRTC1 dephosphorylation and nuclear translocation could mediate its recruitment to CREB target promoters through cooperative interactions with CBP and CREB. This idea is reinforced by recent results indicating that a constitutive CRTC1 S151A/S245A mutant enhances contextual memory by increasing CREB-dependent transcription in the hippocampus (31). However, it is still unclear whether CBP-mediated histone acetylation plays a role on CRTC1/2/CREB complex formation. Other alternative regulatory transcriptional mechanisms may include kinase/phosphatase activities, synapse-nuclear translocation, acetylation or CREB glycosylation (34, 36, 56, 57).

Genetic and biochemical evidences suggest a role of CREB signaling in cognitive and neurodegenerative disorders (58). The age-related CRTC1-dependent transcription and nuclear translocation deficits in *PS* cDKO mice is the first evidence linking CRTC1 dysfunction and associative memory impairments during neurodegeneration. Although the molecular mechanisms linking PS and CRTC1 are still largely unclear, reduced calcium influx caused by loss of PS function (59) could potentially lead to reduced calcineurin/PP2B activity resulting in the observed reduced CRTC1phosphorylation in *PS* cDKO mice. Indeed, memory deficits in *PS* cDKO mice were previously associated with CBP dysfunction (37), which is consistent with fear memory deficits observed in CBP-deficient mice (60-63). Since selective expression of CREB target genes requires cooperative interaction of CRTC/CBP with CREB (26), a correct balance of this complex may be crucial for activity-dependent gene transcription during memory processing. Indeed, CBP/CRTC1/CREB-dependent transcriptional deregulation is associated with cognitive deficits and neurodegeneration in Huntington´s disease (57, 64). Interestingly, *PS* cDKO mice show contextual memory impairments associated with hippocampal deficits of the CRTC1 target genes *Nr4a1* and *Nr4a2*. Particularly, *Nr4a2* (*Nurr1*) is required for CREB-dependent neuronal survival induced by a number of neural signals (65, 66). Since *Nr4a* genes (i.e. *Nr4a2*) are downregulated in sporadic AD and Parkinson´s disease brains and mouse models (67), our result may have important pathological and therapeutic implications in neurodegenerative diseases.

Do CRTC1-dependent transcription changes contribute to associative memory deficits in neurodegeneration? CRTC1-dependent transcriptional deficits were recently associated with early pathological and memory changes in APP mice and a rat AD model that do not develop neurodegeneration (35, 68). Pharmacological activation of CREB signaling has been useful to reverse memory and synaptic deficits in AD mice (69-71). Our current gene therapy strategy indicates that enhancing especifically CRTC1 in the hippocampus ameliorates long-term contextual memory deficits and CRTC1-dependent transcriptional deficits in *PS* cDKO mice during neurodegeneration. As shown previously (31), CRTC1 significantly increased associative memory although had minor effects on *Nr4a1/2* levels in control mice. It is possible that CRTC1 overexpression *in vivo*: (1) may

14

affect differentially the timing of gene induction, (2) cause a differential expression of particular set of genes as observed after spatial memory training (35), and/or (3) does not affect *Nr4a1/2* levels in conditions where induction is maximal as happens after memory training. Interestingly, CRTC1 overexpression in the hippocampus ameliorated dendrite degeneration in *PS* cDKO mice suggesting a direct link between CRTC1 dysfunction and dendrite degeneration. Although the exact mechanism by which CRTC1 ameliorates dendrite degeneration needs further investigation, one possibility is that CRTC1 improves dendrite morphology through BDNF signaling (72).

In conclusion, CRTC1 gene transfer ameliorates dendrite degeneration, transcriptional deficits and associative memory symptoms during neurodegeneration. These results are highly relevant for AD therapy since dementia patients develop early deficits in associative memory encoding and retrieval caused by decreased activity of the hippocampus. Targeting CRTC1 to increase selectively expression of genes mediating neuronal excitability and associative memory may represent a promising avenue for future therapeutics in AD and other cognitive-related disorders.

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Figure Legends

Figure 1. **Contextual fear learning induces CRTC1 dephosphorylation in the hippocampus**

A, Design of the contextual fear conditioning (CFC) test used in this study. *B*, Freezing responses of 2-3 month-old mice exposed to context (n=20) or context plus shock and measured immediately (n=16), 2 h (n=5) or 24 h (n=5) after training. Statistical analysis shows a significant increase of freezing after training ($F_{(3,\ 42)} = 9.26$, $P = 0.0001$). *C*, *D,* Western blot and quantitative analyses of CRTC1, pCRTC1 (Ser151), CREB and pCREB (Ser133) in the hippocampus of home cage (naïve), context, shocked and CFC (15 min, 2 h and 24 h) groups. pCRTC1 levels are significantly decreased 15 min and 2 h after training ($F_{(4,\ 16)} = 4.34$, $P = 0.01$). Values represent fold changes ± s.e.m (n=4-5 mice/group). Statistical analysis was determined by one-way ANOVA followed by Scheffé's S (A) or Bonferroni (B) *post hoc* tests. *$P < 0.05$, ** $P < 0.01$ and ** *$P < 0.0001$ compared to naïve mice.

Figure 2. Contextual learning induces expression of CRTC1 target genes in the hippocampus

A, Hippocampal mRNA levels of CREB target genes in 2-3 month-old mice in naïve, context, shock and CFC groups analyzed by qRT-PCR. Contextual fear conditioning induces a significant overall effect on hippocampal levels of *Arc* ($F_{(5,30)} = 2.4$, $P = 0.05$), *c-fos* ($F_{(5,30)} = 6.7$, $P = 0.0003$), *Nr4a1* ($F_{(5,30)} = 3.5$, $P = 0.01$) and *Nr4a2* ($F_{(5,30)} = 2.8$, $P = 0.03$), but not *Nr4a3* ($F_{(5,30)} = 0.8$ $P = 0.55$). Values are normalized to the geometric mean of *Gapdh*, *Hprt1* and *Ppia*. Data represent mean ± s.e.m (n=4-6 mice/group). *B*, Western blot analysis of CRTC1 (top) and qRT-PCR analysis of CREB target genes normalized to *Gapdh* (bottom) in non-infected (NI)-, scramble (Scr)- and *Crtc1* ShRNA-treated hippocampal neurons. Data are the mean ± SD of three independent experiments. *C*, Chromatin immunoprecipitation (IP) analysis of *c-fos*, *Nr4a1*, *2* and *3* gene promoters using anti-CRTC1 (left) and anti-CREB (right) antibodies in vehicle- and FSK/KCl-treated primary neurons. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to naïve (A) or vehicle control (B,C), and $^{\#}P < 0.05$, compared to FSK/KCl scramble ShRNA (B) or IP CRTC1 vehicle (C) as determined by one-way ANOVA followed by Bonferroni (A,C) or Dunnett's (B) *post hoc* tests.

Figure 3. **Contextual fear learning induces CRTC1 dendritic delocalization and nuclear translocation in the hippocampus**

A, Confocal microscopy images showing CRTC1 (green), MAP2 (red) and nuclear (Hoescht, blue) staining in CA3 hippocampal neurons of mice in naïve or context, shocked and CFC conditions 15 min after training. Scale bar: 50 μm. *B*, Confocal microscopy images showing CRTC1 (green), MAP2-stained dendrites (red) and nuclei (Hoescht, blue) in CA3 hippocampal neurons. MAP2 staining is detected as punctuate staining pattern due to its transversal position in coronal sections. Insets: magnified images of the dashed regions showing localization (yellow) of CRTC1 in MAP2 fibers in naïve conditions and its nuclear redistribution (arrowheads) 15 min after CFC. Scale bar: 50 μm. **C**, Quantitative analysis of CRTC1 in the nucleus (top) and dendrites (bottom). Values represent mean ± s.e.m (nucleus: n=4-5 mice/group, n=6-12 sections/mouse; dendrites: n=8 mice/group, 4-6 sections/mouse). *$P < 0.05$, ** $P < 0.01$ and n.s. (non-significant) compared to naïve mice. Statistical analysis was determined by one-way ANOVA followed by Bonferroni *post hoc* test (nucleus) and t-test (dendritic).

Figure 4. **Age-dependent contextual memory and CRTC1-mediated transcription deficits in *PS cDKO* mice**

A, Freezing responses of control (WT) and *PS* cDKO mice at 2 or 6 months of age tested in contextual fear conditioning. Mice were tested immediately (context, n=14-24), 2 h (n=5-10) or 24 h (n=5-10) after contextual training. Two-way ANOVA reveals a training effect ($F_{(3,72)} = 22.6$, $P = 0.0001$) but not a genotype effect ($F_{(1,72)} = 0.005$, $P = 0.94$) at 2 months of age, whereas there are training ($F_{(3,121)} = 25$, $P = 0.0001$) and genotype ($F_{(1,121)} = 21$, $P = 0.0001$) effects at 6 months. *B,* Hippocampal levels of mRNAs in naïve and CFC trained WT and *PS* cDKO mice at 6 months of age. Two-way ANOVA indicates a significant time-dependent effect for *Arc* ($F_{(2, 29)} = 6.9$, $P < 0.003$), *c-fos* ($F_{(2, 29)} = 20.0$, $P < 0.0001$), *Nr4a1* ($F_{(2, 29)} = 33.0$, $P < 0.0001$) and *Nr4a2* ($F_{(2, 29)} = 27.9$, $P < 0.0001$) but not *Nr4a3* (*$F_{(2, 29)}$*

=2.5 , $P = 0.1$), and a genotype effect for *Nr4a1* ($F_{(1, 29)}$ = 18.1, $P < 0.0002$), *Nr4a2* ($F_{(1, 29)}$ = 14.8, $P <$ 0.001) and *Nr4a3* ($F_{(1, 29)}$ = 14.6 , $P < 0.001$) but not for *Arc* ($F_{(1,29)}$ = 0.85, $P = 0.36$) and *c-fos* ($F_{(1, 29)}$ = 0.3, $P = 0.6$). mRNA levels were quantified by real-time qRT-PCR and normalized to the geometric mean of standard genes *Gapdh*, *Hprt1* and *Ppia*. ***C***, Western blot and quantitative analyses of CRTC1, pCRTC1 (Ser151), CREB and pCREB (Ser133) in the hippocampus of naïve and CFC (2 h) WT and *PS* cDKO mice at 6 months. Statistical analyses shows a group effect in pCREB/CREB ($F_{(1, 26)}$ = 10.1, $P < 0.004$), CREB ($F_{(1, 26)}$ = 5.4, $P < 0.03$) and pCRTC1/CRTC1 treatment effect ($F_{(1, 26)}$ = 6.1, $P < 0.02$). Values represent mean of fold changes ± s.e.m (A, B: n=4-6 mice/group; C: n=6-9 mice/group). *$P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$, compared to WT mice or the indicated group. Statistical analyses were performed by two-way ANOVA followed by Scheffé´s S (A,C) or Bonferroni (B) *post hoc* tests.

Figure 5. Reduced translocation of CRTC1 to the nucleus of hippocampal neurons in *PS* cDKO mice

A, Confocal microscopy images showing CRTC1 (green, left images) and merged CRTC1/NeuN (red) (right image) staining in CA3 pyramidal neurons of 6 month-old WT and *PS* cDKO mice. Arrowheads indicate some neurons showing nuclear CRTC1. Scale bar: 80 μm. ***B***, Confocal images showing CRTC1 (green) and MAP2 (red) staining in CA3 hippocampal neurons of 6 month-old WT and *PS* cDKO mice in naïve and CFC (15 min) conditions. Arrowheads indicate nuclear CRTC1. Scale bar: 60 μm, 15 μm (inset). ***C***, Quantitative analysis of nuclear (left) and dendritic (right) CRTC1 in CA3 hippocampal neurons in WT and *PS* cDKO mice. Statistical analysis shows a genotype effect on CRTC1 nuclear localization ($F_{(1, 24)}$ = 4.03, $P = 0.05$). Values represent mean ± s.e.m of multiple mice (n=4-8 mice/group), each analyzed in multiple brain sections (n= 4-6 per mouse). *$P < 0.05$, compared to naïve control. Statistical analysis was determined by two-way ANOVA followed by Bonferroni multiple comparison *post hoc* test.

Figure 6. CRTC1 gene therapy ameliorates hippocampal CRTC1-dependent transcription and associative memory deficits in *PS* cDKO mice

A, AAV2/10-CRTC1-myc injection increases CRTC1-myc levels in the adult mouse dorsal hippocampus. Confocal images showing expression of exogenous GFP (green, left) or CRTC1-myc (green, right) in CA3 pyramidal neurons of 6 month-old WT and *PS* cDKO mice six weeks after AAV injection. Mice were sacrificed 24 h after CFC training. Insets are magnified images of the dashed regions showing expression of GFP and CRTC1-myc in NeuN-positive neurons. Hoescht (blue): nucleus. Scale bar: 100 μm. *B*, CRTC1-myc protein (top) and mRNA (bottom) levels in the hippocampus of WT and *PS* cDKO mice six weeks after AAV injection. *C,* Effect of CRTC1 overexpression in freezing responses in WT and *PS* cDKO mice in a novel context (n=6-7 mice/group). No significant differences were found among groups (group effect: $F_{(1,21)}$ = 1.7, P = 0.2, treatment effect: $F_{(1,21)}$ = 0.07, P = 0.8). *D*, Contextual fear conditioning in control and *PS* cDKO mice (n=6-7 mice/group) six weeks after AAV-GFP and -Crtc1 injection. Two-way ANOVA reveals significant effects of groups ($F_{(3,42)}$ = 4.3, P < 0.01), time ($F_{(1,42)}$ = 36.8, P < 0.0001) and group x time interaction ($F_{(3,42)}$ = 3.5, P < 0.02). *E*, Levels of *Nr4a1* and *Nr4a2* transcripts in the hippocampus of AVV-GFP or-Crtc1 injected mice. Values normalized to the geometric mean of *Gapdh*, *Hprt1* and *Ppia* represent mean ± s.e.m (n=4-6 mice/group). Data. *P < 0.05, ** P < 0.01, *** P < 0.0001, compared to WT GFP or the indicated group. Statistical analyses were determined by two-way ANOVA and Scheffé´s S (behavior) or Bonferroni (gene expression) *post hoc* tests.

Figure 7. CRTC1 overexpression ameliorates dendritic degeneration in the hippocampus of *PS* cDKO mice
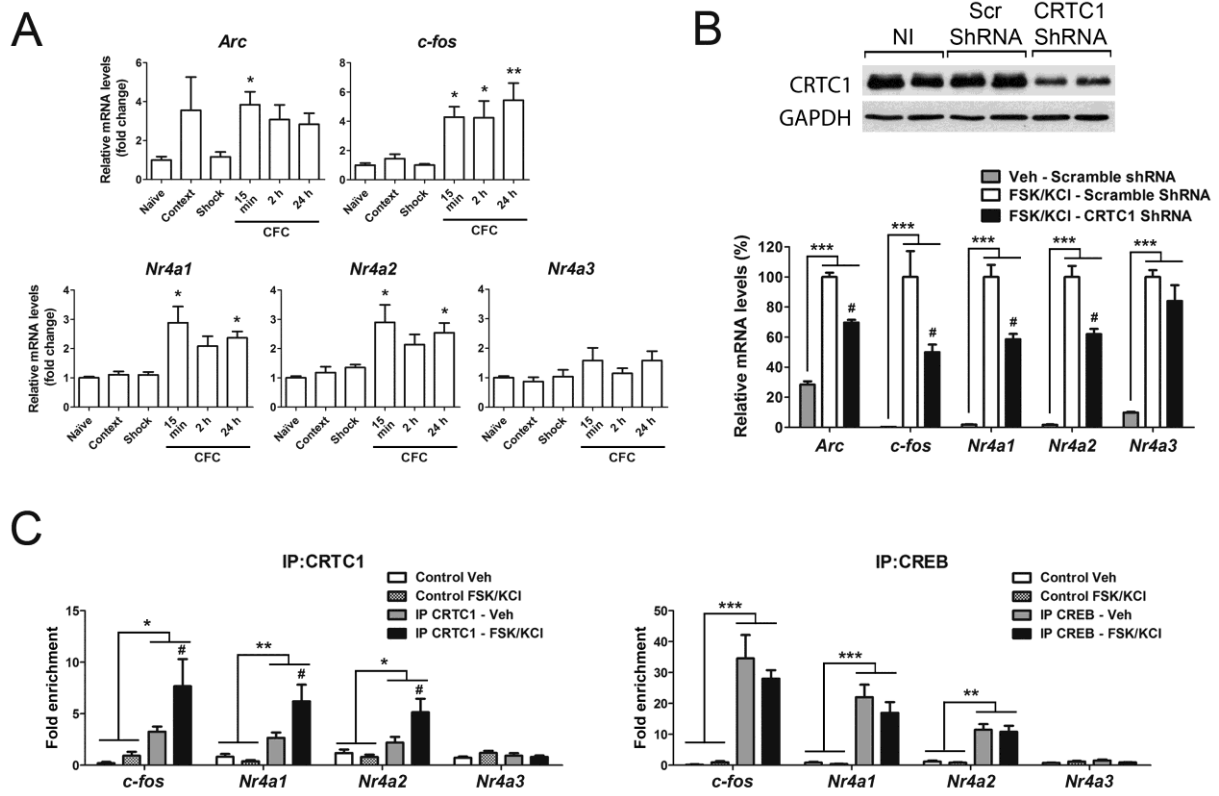
A, Reduced cortical thickness in *PS* cDKO mice at 6 months. Left: Nissl staining of neocortex (top) and hippocampus (bottom) showing reduced cortical thickness (dashed lines) but normal hippocampal morphology in AAV-GFP- and -Crtc1 injected *PS* cDKO mice. Scale bar: 200 μm. Right: Quantification of cortical thickness indicates a significant group effect ($F_{(1,14)}$ = 8.5, P < 0.01) but not treatment effect ($F_{(1,14)}$ = 0.003, P = 0.96). *B*, Dendritic degeneration in the hippocampus is reduced

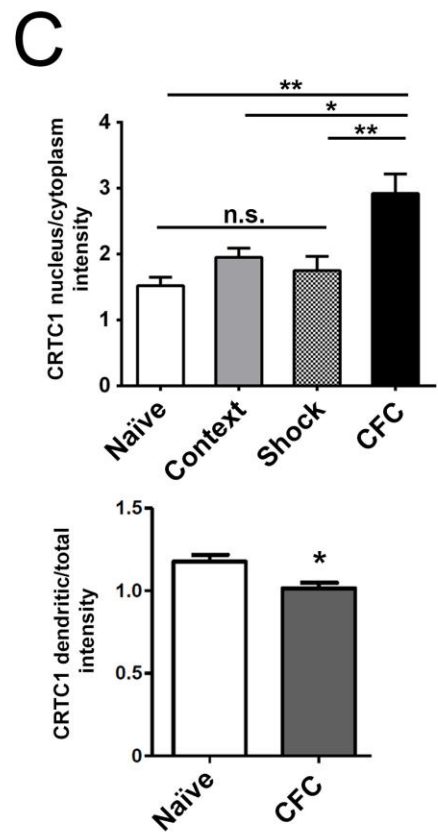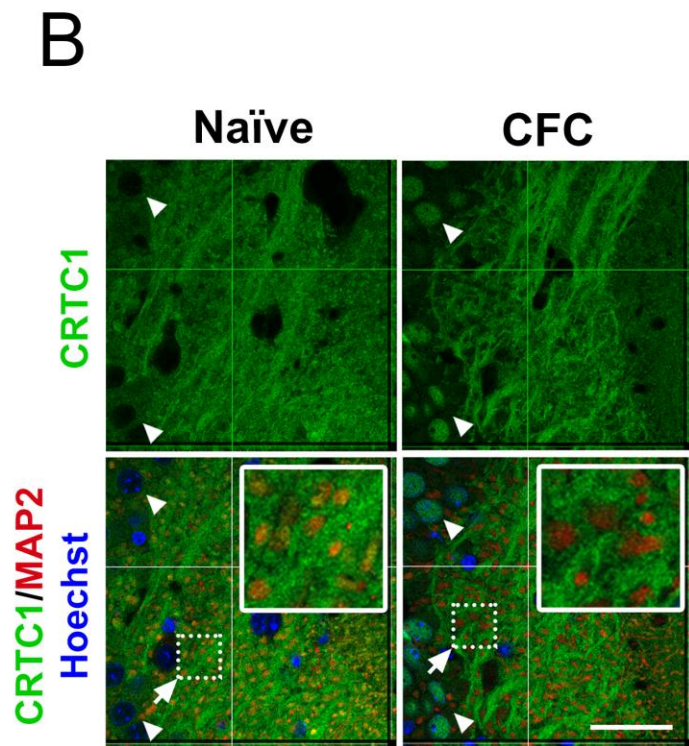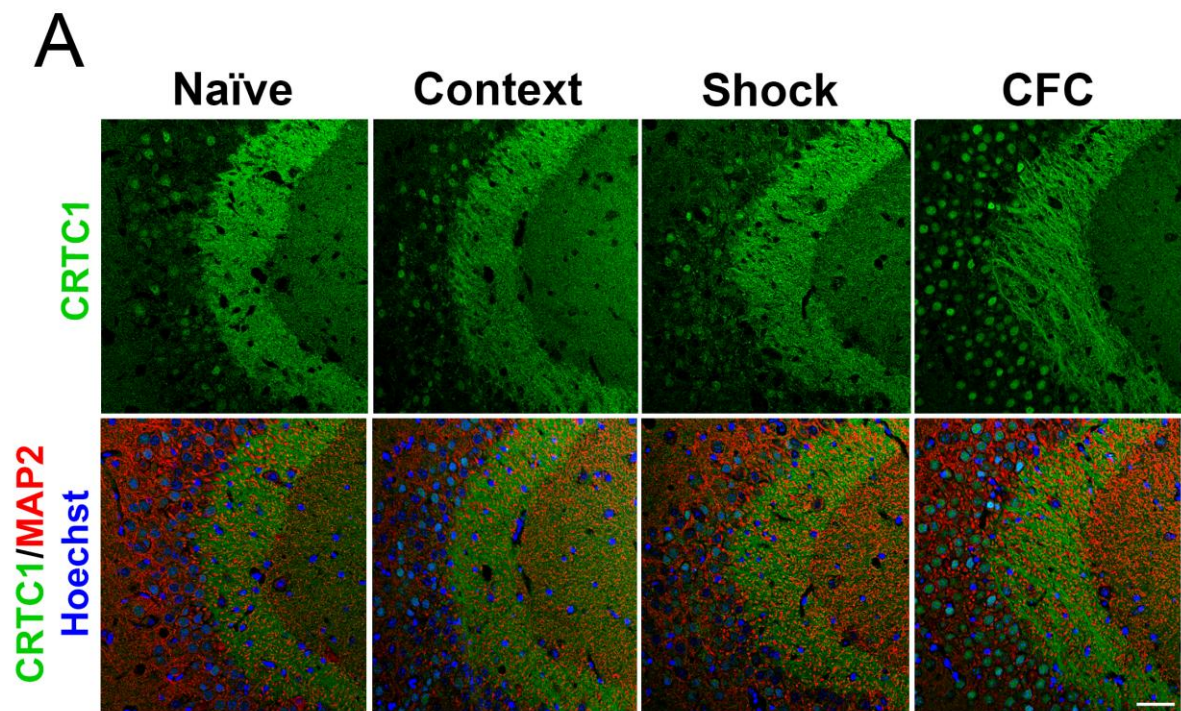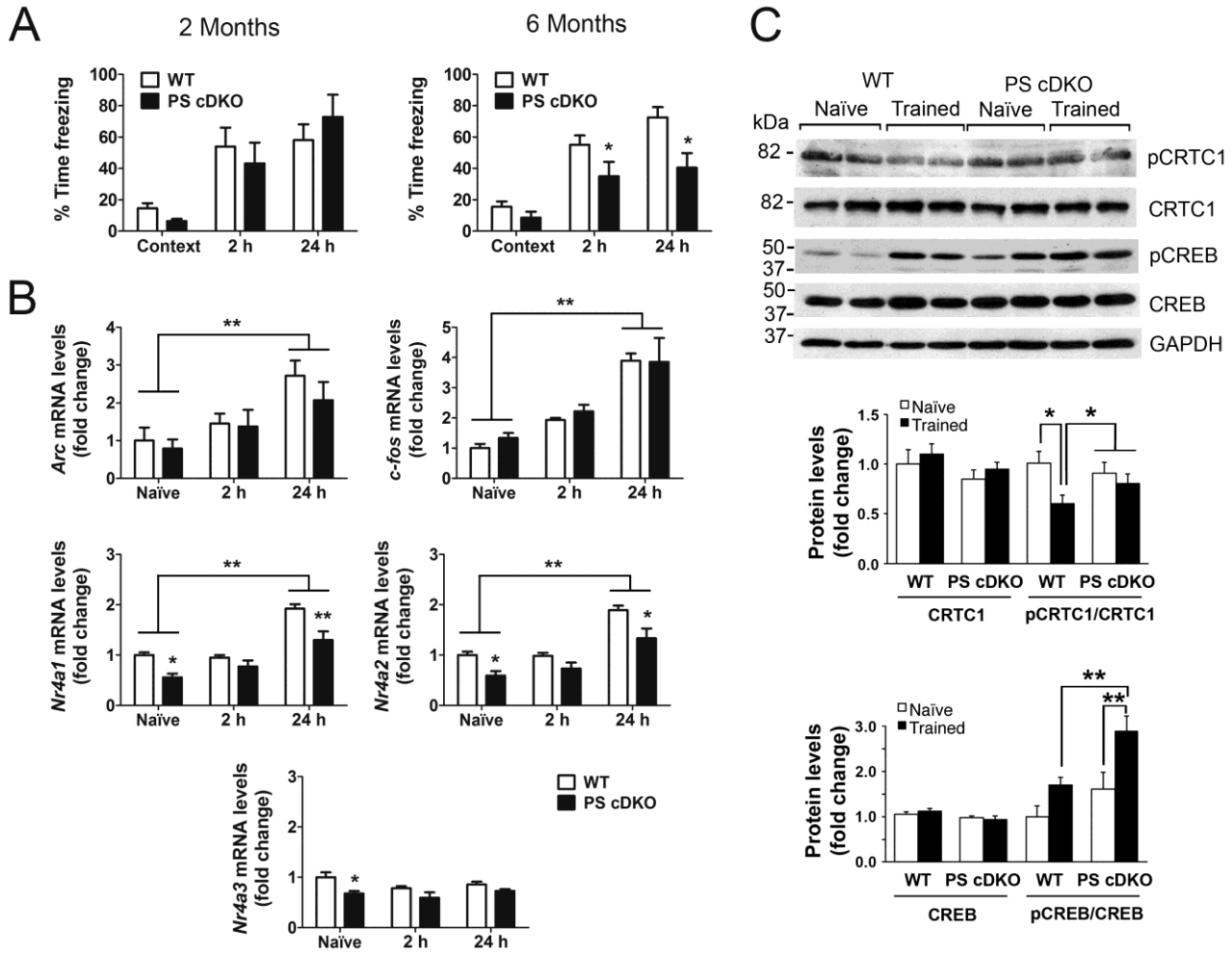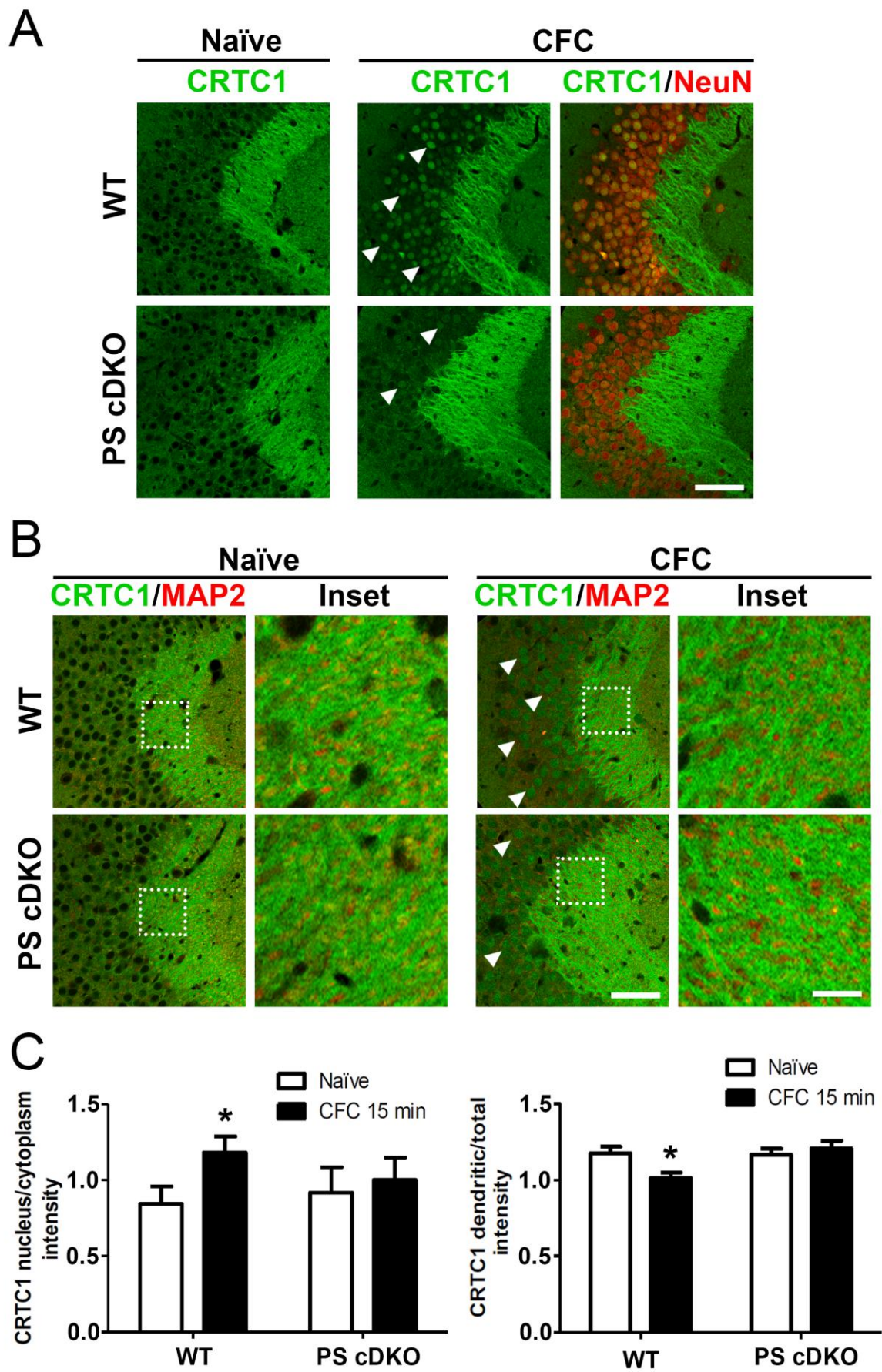after AAV-Crtc1 injection in *PS* cDKO mice. Left: Confocal microscope images showing MAP2-stained fibers (red) in the neocortex (top) and CA3 hippocampus (middle) in brain sections of AAV-GFP- and -Crtc1-injected mice. Magnified dendrites in CA3 region are shown at the bottom. Scale bars: 20 μm (Cortex) or 10 μm (CA3). Right: Quantification of MAP2 staining intensity and dendrite thickness in CA3 hippocampus. Data represent percentage of control ± s.e.m of cortical thickness and MAP2 staining intensity or average of dendrite thickness (μm) in multiple mouse brains (n=4-5 mice/group; n=3 sections/mouse). $*P < 0.05$, $** P < 0.01$, $*** P < 0.0001$, compared to WT GFP mice or the indicated group. $^{\#}P = 0.05$. Statistical analyses were determined by two-way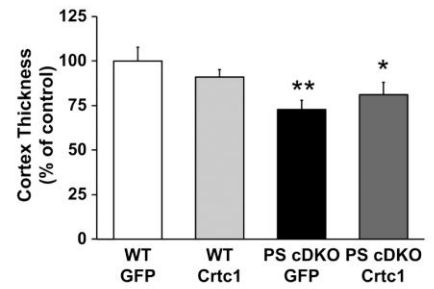 ANOVA and Scheffé´s S *post hoc* test.