Universitat Autònoma de Barcelona

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Arnaldo J. Parra Damas

Institut de Neurociències
Universitat Autònoma de Barcelona
Departament de Bioquímica i Biologia Molecular
Unitat de Bioquímica, Facultat de Medicina

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Papel de la transcripción génica regulada por CRTC1/CREB durante memoria dependiente de hipocampo en modelos murinos de la enfermedad de Alzheimer

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A mi madre

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II. List of abbreviations

αCaMKII	Ca^{2+}/calmodulin-dependent protein kinase II, isoform α
AAV	Adeno-associated virus
AD	Alzheimer's disease
Aβ	β-amyloid peptide
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APOE	Apolipoprotein E
APP	β-amyloid precursor protein
Aph-1	Anterior pharynx-defective 1
Arc	Activity-regulated cytoskeleton-associated protein
ATF-1	Activating transcription factor 1
BDNF	Brain-derived neurotrophic factor
BACE	beta-site APP cleaving enzyme
bZIP	Basic Leucine Zipper
CA	cornu ammonis
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
cKO	Conditional knockout
CREB	cAMP response element-binding protein
CREM	cAMP response element modulator
CRTC	CREB regulated transcriptional coactivator
CS	Conditioned stimulus
DG	Dentate gyrus
DNA	Deoxyribonucleic acid

Egr1	early growth response protein 1
EC	Entorhinal cortex
ER	Endoplasmic reticulum
FAD	Familial AD
FSK	Forskolin
FTD	Frontotemporal dementia
IEG	Immediate-early gene
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
E-LTP	Early-phase LTP
L-LTP	Late-phase LTP
MAPK	Mitogen-activated protein kinases
MSK1	Mitogen and stress-activated kinase 1
Nct	Nicastrin
NFTs	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NR	Nuclear receptors
PBS	Phosphate-buffered saline
Pen2	Presenilin enhancer 2
PDE4	Phosphodiesterase IV
PKA	Protein kinase A
PKC	Protein kinase C
PS	Presenilin

Pol II	RNA polymerase II
RE	Response element
RIN	RNA integrity number
RIPA (buffer)	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
SD	Splicing domain
SRF	Serum response factor
STM	Short-term memory
STP	Short-term potentiation
TAD	Transactivation domain
TAF	TBP-associated factor
TFII(A/B/D-F)	General transcription factors
TBP	TATA box-binding protein
TBS	Tris-buffered saline
US	Unconditioned stimulus
VGCCs	Voltage-gated calcium channels

III. Abstract

Altered gene transcription in the brain is associated with impaired synaptic function and cognitive decline during normal aging and age-related cognitive disorders. However, the molecular mechanisms underlying deregulation of gene expression during Alzheimer's disease (AD) remain largely unknown. Gene expression regulated by the transcription factor cAMP response element binding protein (CREB) is essential for synaptic plasticity and consolidation of long-term memory. CREB-dependent transcription is regulated by transcriptional co-regulators such as CBP/p300 and the CREB regulated transcription coactivator (CRTC) family. CRTC1 is the most abundant isoform in neurons, and has been implicated in neuronal plasticity mechanisms. However, little is known about the role of CRTC1-dependent transcription during AD-related pathology. Recent evidence indicates that CRTC1 activity is reduced in neurons from transgenic mice overexpressing the human amyloid precursor protein (APP) harboring the Swedish and Indiana mutations ($APP_{Sw,Ind}$) linked to familial Alzheimer's disease, suggesting that altered CRTC1 activity may be related to synaptic and memory deficits during AD. In this doctoral thesis we studied the role of CREB/CRTC1-regulated transcription during hippocampal-dependent memory tasks in $APP_{Sw,Ind}$ mice and in conditional knockout mice lacking the Presenilins (PS) 1/2 genes (PScDKO mice). We show that CRTC1 activity is affected in both models, suggesting that CRTC1 function may be compromised by different pathogenic pathways. Importantly, we provide evidence that CRTC1-regulated transcription is also affected in the human AD hippocampus. Taken together, our results support a model in which different pathogenic events may lead to a common AD-like phenotype (synaptic and memory deficits) due to the integration of distinct signaling pathways on CRTC1/CREB-dependent transcription required for synaptic plasticity and memory. Furthermore, we show that increasing CRTC1 function in the hippocampus reverses transcriptional and memory deficits in both mouse models during early pathological stages, suggesting that targeting CRTC1 signaling may be a valuable therapeutic strategy for AD and related neurodegenerative dementias.

IV. Introduction

Memory allows us to store and retrieve the knowledge that we acquire during our life, and the ideas that we generate about the world and ourselves. Thus, *"we are who we are in large part because of what we have learned and what we remember and forget"* (Kandel et al. 2014). Neurodegenerative dementias, including Alzheimer's disease (AD), progressively impair memory and other cognitive abilities of patients, with devastating consequences for their lives and their families. To date, there are no effective treatments that can revert or stop the progression of the disease, and the emergence of new therapeutic strategies requires understanding the mechanisms that subserve memory and how these are affected by the pathological processes that take place during the course of the disease. One of the most significant discoveries in the field of memory research during the last century was that stabilization of short-term memories into long-term memories, a process known as memory consolidation, requires new gene expression that is largely dependent on the transcription factor cAMP response element binding protein (CREB) (Kandel 2001). Gene expression mediated by CREB is regulated by transcriptional co-regulators such as CBP/p300 and the CREB regulated transcription coactivator (CRTC) family, comprising CRTC1, 2 and 3. CRTC1 is the most abundant isoform in neurons, and has been shown to participate in neuronal plasticity mechanisms, including long term potentiation (LTP), which has been postulated as a cellular correlate of memory. However, little is known about the role of CRTC1-dependent transcription during AD-related pathology. Recent evidence indicates that CRTC1 activity is reduced in neurons from transgenic mice overexpressing the human amyloid precursor protein (APP) harboring familial Alzheimer's disease (FAD)-linked mutations (España et al. 2010), suggesting that alteration of this signaling pathway may be relevant for the memory deficits associated to AD.

In order to provide a comprehensible conceptual framework, I will start this introduction by presenting relevant concepts concerning hippocampal-dependent memory, which is the main type of memory affected during AD. Then I will focus on the role of gene transcription induced by neuronal activity as a fundamental process underlying memory consolidation, with particular emphasis on the CREB/CRTC1 signaling pathway. Finally, I will describe the main clinical and neuropathological features of AD, as well as the molecular mechanisms contributing to synaptic/neuronal dysfunction and neurodegeneration during the disease, and how they affect the mentioned transcriptional mechanisms required for synaptic plasticity and memory.

1. Hippocampal-dependent learning and memory

Learning is the process of acquiring new information about the world and memory is the process of storing and retrieving that information over time (Sweatt 2010c; Kandel et al. 2014). From an experimental perspective, learning can be defined as the *"acquisition of an altered behavioral response"* due to a specific stimulus (Sweatt 2010b; Barron et al. 2015), while memory refers to the process of storage and recall of the learned items. These are functional definitions that prove to be useful in order to experimentally test the occurrence of learning and memory. However, it becomes obvious that the experimental usefulness of these definitions depend on the ability to design and perform behavioral experiments that allows to unequivocally identify and quantify the expression of the "altered behavioral response", which should be specific for the provided stimulus. This is often not an easy task, since the complexity of even the apparently simpler forms of learning in mammals.

During the last several decades of modern memory research, it has become clear that memory function is a dynamic process that results from several interactive sub-processes that comprise encoding or acquisition of information, initial storing in the form of short-term and intermediate-term memory, consolidation and maintenance of long-term memory, as well as destabilization and re-stabilization while memories are recalled, updated, and associated or integrated with further memories (Alberini & Ledoux 2013; Kandel et al. 2014; Nader 2015). Thus, memory storage is a time-dependent process in which short-term memories are gradually stabilized into long-term memories, a process known as memory consolidation (Squire et al. 2015). While short-term working memory is supported by sustained neuronal activity mainly in the prefrontal and parietal cortices, consolidation and initial persistence of long-term memories are thought to depend on long-lasting structural changes in the hippocampus and related cortical regions (Sweatt 2010c; Jeneson & Squire 2011; Kandel et al. 2014).

According to the nature of the information to be stored, human memory can be subdivided into declarative (also known as explicit memory), for events, people, places, facts and objects; and non-declarative (implicit memory), for perceptual and motor skills. Declarative memory is encoded during conscious learning and requires the medial temporal lobe system, including the hippocampus, dentate gyrus, entorhinal and perirhinal cortices (Sweatt 2010b). On the contrary, non-declarative implicit memory does not require conscious awareness and relies on other brain areas like the cerebellum and the striatum. Declarative memory can be further classified into episodic

memory, which refers to the ability to recall personally experienced events, and semantic memory, regarding the meaning of words and concepts. Declarative learning is considered to be associative, which means that items are usually learned in the context of other related facts or objects (Sweatt 2010c). Spatial learning also depends on the hippocampus, since it has been well established that hippocampal lesions lead to spatial learning deficits in both human patients and animal models. Importantly, declarative episodic memory is specially affected during neurodegenerative dementias such as Alzheimer's disease, dementia with Lewy bodies and frontotemporal dementia (Burgess et al. 2002; Budson & Price 2005).

1.1. Hippocampal processing of learning and memory

The requirement of the hippocampal formation for consolidation of long-term declarative memories was first established during studies of human patients undergoing bilateral medial temporal lobe (MTL) resections that were performed in order to treat their neurological and psychiatric conditions, which included epilepsy and schizophrenia (Scoville & Milner 2000). These studies demonstrated that bilateral removal of the hippocampi, along with the adjacent cortical tissue and the amygdala, impaired the patient's ability to form new long-lasting declarative memories, but did not affect short-term working memory or long-term declarative memory for events that occurred several years before the surgery, which remained mostly intact (Scoville & Milner 2000; Jeneson & Squire 2011). Therefore, it was evident that 1) short-term memory and long-term memory rely on different brain systems; 2) short-term memories must be gradually stabilized into long-term memory by a process of memory consolidation; and 3) the MTL system is required for explicit memory consolidation but it seemed to be no longer required for storage or recall of long-term consolidated memory. Further studies of amnestic patients suffering from selective damage to the hippocampal formation confirmed that this region is involved in declarative memory consolidation, although in these cases the memory impairment was not as severe as after complete MTL resection, suggesting that associated cortical areas also contribute to memory formation (Squire & Zola-Morgan 1991). This notion has been confirmed in animal models using monkeys and rodents, where selective lesions to the hippocampus as well as to the associated entorhinal, perirhinal and parahippocampal cortices lead to specific deficits on memory function, while damage to the amygdala disrupts the normal processing of relevant emotional information (Squire & Zola-Morgan 1991). Current evidence suggests that learned items are initially stored by the MTL system which gradually drives the establishment of persistent connections

between the different cortical regions that were active during learning and thus comprise the neural representations of the memory (Squire et al. 2015).

The essential role of the hippocampus and adjacent cortical areas during episodic memory acquisition may be explained in terms of their anatomy and connectivity with other cortical regions. The hippocampus receives inputs from every cortical association area in the brain, taking part of a multimodal integration system (the limbic association area) that allows conversion of short-term stored percepts into long-term memories by relating representations of an event from the different sensory systems (Squire & Zola-Morgan 1991). Highly processed sensory information is conveyed from unimodal and polymodal association areas to the hippocampal formation via the entorhinal, perirhinal and parahippocampal cortices. The hippocampal formation comprises the dentate gyrus (DG), the hippocampus proper and the subiculum. The hippocampus proper, also known as *Cornu Ammonis* (CA), is divided into subfields CA1, CA2, and CA3. The main input to the hippocampus originates from layer II of the entorhinal cortex projecting to the DG and CA3 through the perforant pathway. The glutamatergic granule cells of the DG project to CA3 via the mossy fibers (Fig 1), which are unmyelinated axons that extend through the polymorphic layer (known as hilus or hilar region) until reaching the CA3 field where they form distinctive large synaptic boutons onto the proximal apical dendrites of excitatory CA3 pyramidal cells and interneurons. Importantly, the mossy fibers comprise a potent excitatory input that is able to induce strong depolarization of CA3 neurons (Yassa & Stark 2011).

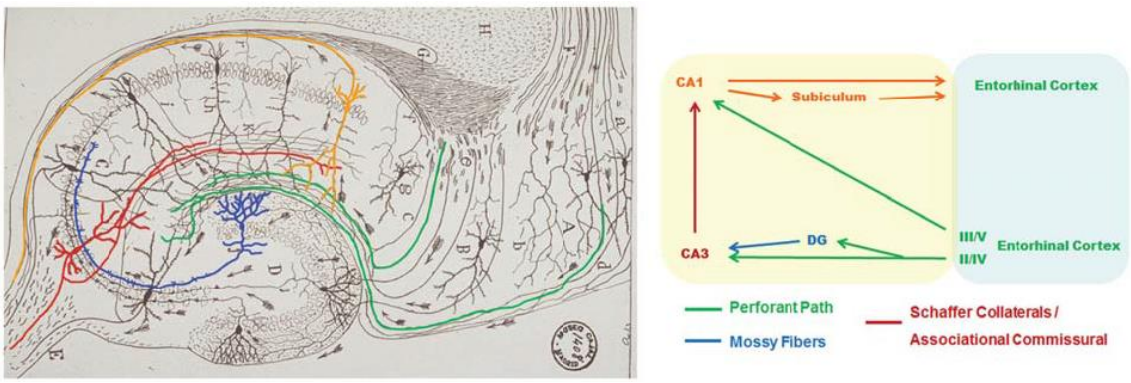

Figure 1. Modified drawing from Santiago Ramón y Cajal (1911, left) and schematic diagram (right) of the rodent hippocampal circuitry. The picture shows direct excitatory projections (arrows) from entorhinal cortex (EC) neurons (green) onto CA1 (orange) or CA3 (red) pyramidal neurons, and indirect projections to the dentate gyrus (DG, blue) through the perforant pathway. DG granule neurons project along the mossy fibers to CA3 pyramidal neurons. CA3 axons then project through the Schaffer collaterals to CA1 pyramidal neurons, which finally project to the subiculum and deep EC IV-VI layers.

A relevant feature of CA3 cells in terms of hippocampal connectivity and function is that they form an extensive recurrent collateral network, due to the considerable amount of recursive projections that originate and terminate within the CA3 field. Thus, CA3 neurons receive inputs from layer II of the EC through the direct perforant pathway, from the DG through the mossy fibers, and finally from the recurrent collateral projections originating within CA3. CA3 then sends the main projection to CA1 via the Schaffer collateral pathway, although CA1 and the subiculum also receive a direct projection from layer III of the entorhinal cortex through a performant path to CA1, also known as temporoammonic pathway (van Strien et al. 2009; Clark & Squire 2013). The main outputs of the hippocampus arise from CA1 and the subiculum projecting to the deep layers of entorhinal cortex, where CA1 projects to the subiculum and to layer V of the entorhinal cortex, while the subiculum projects mainly to layers IV and V of the entorhinal cortex (Allen & Fortin 2013).

Importantly, the hippocampus and the prefrontal cortex (PFC) are functionally interconnected. On the one hand, there is a direct projection from CA1 to the PFC, as well as indirect connections relaying on the parahippocampal region, while PFC outputs are sent to the hippocampus through projections to the entorhinal, perirhinal, and parahippocampal cortices. In the light of these connections it is thought that the hippocampus, the parahippocampal region, and PFC comprise interacting modules of a neural system that subserve different aspects of declarative/episodic memory processing (Allen & Fortin 2013; Preston & Eichenbaum 2013).

It is worth to mention that the basic plot of hippocampal structure, connectivity and function, as well as the associated memory mechanisms described above, has been conserved through evolution across mammal species, ranging from rodents to non-human primates and humans, which validates the study of hippocampal dependent memory in nonhuman mammals, including mice, as models of human declarative memory function and dysfunction (Manns & Eichenbaum 2006; Allen & Fortin 2013; Clark & Squire 2013; Nielson et al. 2015).

1.2. Spatial memory

Spatial information is a key component of episodic memories. Spatial memory refers to our ability to learn, remember and navigate through places in the environment, which are usually associated to events and facts (Bannerman et al. 2014). It is thought that spatial memory can allocate paired associated and map-like representations of the

environment, as well as motivational representations regarding the value of the places (Peters et al. 2015). Electrophysiological recording studies on freely moving rats led to the discovery of “place cells”, principal hippocampal neurons that fire at specific spatial locations or “place fields” (O’Keefe & Dostrovsky 1971; O’Keefe 1976), as well as “grid cells” in the entorhinal cortex, which fire at multiple regularly spaced locations (Fyhn et al. 2004). Further studies have demonstrated the existence of other spatially-regulated cell types, including “border cells” and “head direction cells”, shedding light on the neural mechanisms supporting positioning and spatial navigation and suggesting that the hippocampal formation encoded an internal cognitive map of the spatial environment (Moser et al. 2015). Furthermore, as previously mentioned, several lesion studies in animals and in patients suffering from hippocampal damage, as well as functional MRI (fMRI) studies in healthy humans, have supported the idea that the hippocampus plays a critical role in spatial learning and memory.

One of the most extensively used experimental paradigms for assessing hippocampal dependent spatial learning and memory in rodents is the water maze developed by Richard Morris, often referred to as the Morris Water Maze (MWM) (Morris 1984). In this task, a hidden scape platform is placed in a fixed location just below the level of the water contained in a circular tank or pool. The water is usually stained with tempera paint in order to camouflage the platform which must not be visible to the animals, and several distal visual cues are placed at fixed locations surrounding the swimming pool. During several consecutive days the animals are trained to find the hidden platform, several trials per day, using only the distal visual cues as space reference. In each trial the animals are placed in a different starting position of the pool, to avoid the use of egocentric (self-centered) strategies, and the recorded trajectory and latencies (time spent to locate the platform) can be used as a measure of learning over the training sessions. Finally, the spatial reference memory may be assessed during a probe test, frequently referred to as the removal test, in which the scape platform is removed from the pool and the animals are allowed to swim during one minute. If the animal remembers the location of the platform it should spend more time swimming around the area where the platform was located (target quadrant) (Morris 1984; Vorhees & Williams 2006).

1.3. Contextual fear conditioning as a model of hippocampal-dependent associative memory in mice.

The ability to associate aversive stimuli with the context in which they occur is an essential adaptive behavior that has been conserved through evolution. This type of

learning may be exploited experimentally in a behavioral paradigm known as contextual fear conditioning (CFC). CFC is characterized by a rapid memory encoding, allowing the expression of strong fear responses when the animals are presented with the same context at a later time. In the typical configuration of CFC experiments using rodents, the aversive unconditioned stimulus (US) is an electric foot-shock delivered after exposure to the context of a conditioning chamber, which is equivalent to the neutral conditioned stimulus (CS) defined for classical Pavlovian conditioning. In rodents, the fear response elicited by this conditioning is expressed as an immobilization or "freezing" behavior. The rapid acquisition of fear memory induced by this paradigm, which usually requires only one training session, allows to evaluate the long-term memory by quantifying the amount of time that the animals show freezing behavior when they are re-exposed to the context 24 hours later.

Given the essential role of the MTL during episodic and spatial memory it is not surprising that contextual fear memory also involves this brain region. Due to the high complexity of spatial contexts (Maren et al. 2013), the association between the context and the US requires processing of multimodal sensory information by the hippocampus but also by the amygdala and the prefrontal cortex (Wiltgen et al. 2010; Maren et al. 2013; Saez et al. 2015).

1.4. Molecular mechanisms underlying encoding and storage of long-term associative memory

At the end of the 19th century, Santiago Ramón y Cajal hypothesized that changes in the growth and strength of connections between neurons (a phenomenon now known as synaptic plasticity) supported cognitive processes in the brain (Ramón Y Cajal 1894). Later on, Donald Hebb advanced on this idea by postulating that the synaptic efficiency between the presynaptic and the postsynaptic neurons should be selectively strengthened upon activity, suggesting a cellular basis for associative learning:

"When an axon of cell A … excites cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's efficiency as one of the cells firing B is increased" (Hebb 1949).

An experimental model of such a plasticity mechanism in the mammalian nervous system was reported by Tim Bliss and Terje Lømo in 1973, at the synapses formed by the perforant pathway onto the dentate gyrus of the rabbit hippocampus (Bliss & Lømo 1973). Bliss and Lømo observed that high frequency stimulation of the performant pathway induced a long-lasting increase in the strength of the postsynaptic response

recorded at the dentate gyrus, a phenomenon that they called Long-Term Potentiation (LTP) and that along with other forms of synaptic plasticity, such as Long-Term Depression (LTD), has been postulated as cellular correlates of learning and memory.

The specific cellular and molecular mechanisms mediating LTP induction depend on the circuit that is being activated and on the pattern of synaptic stimulation. For instance, induction of LTP at the Schaffer collateral pathway to CA1, using 100 Hz stimulation, critically depends on NMDA glutamate receptors, since the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (AP5 or APV) completely blocks LTP. However, increasing the stimulation frequency to 200 Hz in the same circuit induces a type of LTP that is NMDA receptor-independent (Grover & Teyler 1990). On the other hand, induction of LTP at the direct entorhinal projection to CA1 depends only partially on the activation of NMDA receptors, while induction of LTP at the mossy fibers to CA3 synapses does not depend on NMDA receptors.

Accordingly, different signaling mechanisms may be recruited either in the presynaptic (leading to increased neurotransmitter release) or postsynaptic neuron (inducing enhanced excitability to glutamate release). For instance, the 100 Hz stimulation used for inducing NMDA receptor-dependent LTP at the Schaffer collateral synapses elicits a burst of potent synaptic activity that opens a large amount of AMPA receptors, producing enough depolarization to trigger an action potential in the postsynaptic neuron. Postsynaptic depolarization also triggers back-propagating action potentials to the dendrites, allowing a strong Ca^{2+} influx through activated NMDA glutamate receptors, which otherwise remain blocked by extracellular Mg^{2+} ions at subthreshold potential. This Ca^{2+} influx activates several signaling cascades mediated by protein kinases, including CaMKII, Fyn, and PKC, resulting in addition of new AMPA glutamate receptors into the postsynaptic membrane. On the contrary, induction of LTP at the Mossy Fiber Pathway involves mainly presynaptic mechanisms mediated by Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCC) (Castillo 2012), leading to increased glutamate release from the presynaptic terminals. Thus, the increase of Ca^{2+} concentration, which may also be mediated by release from intracellular stores, is a common essential step shared by both pre- and post-synaptic mechanisms during LTP induction.

Current models hold that LTP develops through at least three phases, including initial short-term potentiation (STP), early LTP (E-LTP), and late LTP (L-LTP) (Sweatt 2010a; Park et al. 2013; Kandel et al. 2014). While STP and E-LTP are transient and depend on rapid signaling mechanisms such as those described before (increased glutamate

release from the presynaptic terminal and anchoring of additional AMPA glutamate receptors at the postsynaptic membrane), the long-lasting L-LTP phase involves additional structural changes leading to formation of new synapses and remodeling and growth of preexisting synapses (Bailey et al. 2015), which requires new gene expression and protein synthesis. The transcriptional programs required for L-LTP are tightly regulated by a complex signaling network that involves activation of different protein kinases including PKA, CaMKII, CaMKIV and MAPK, finally converging on the activation of gene expression mediated by the transcription factor CREB, as will be discussed in the following section.

LTP constitutes an experimental model of synaptic plasticity induced by high frequency stimulation patterns that are not likely to resemble the endogenous neuronal activity occurring in the brain during physiological learning and memory. However, recent evidence suggest that potentiation-like synaptic plasticity mechanisms may actually be induced by physiological neuronal activity during learning and memory tasks (Takeuchi et al. 2014).

The idea that mechanisms of synaptic plasticity such as LTP underlie learning and memory processes was formalized by Richard Morris and colleagues as the synaptic plasticity and memory (SPM) hypothesis:

"activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed" (Martin et al. 2000).

Even though the synaptic plasticity and memory hypothesis is the most accepted and tested hypothesis explaining the physical substrate of long-term memory storage, it is possible that other mechanisms may support the persistence of at least specific forms of long-lasting memories (Gallistel & Balsam 2014; S. Chen et al. 2014; Trettenbrein 2015; Blackiston et al. 2015).

2. Regulation of gene expression during neuronal activity and plasticity induced by memory

As previously mentioned, both long-term memory and long-term synaptic plasticity require de novo gene expression and protein synthesis, since inhibitors of gene transcription and mRNA translation, such as actinomycin-D and anisomycin respectively, impairs both LTP and LTM (Nguyen et al. 1994; Frey et al. 1996; Kandel 2001). This section will cover the molecular mechanisms underlying neuronal activity-regulated gene transcription required for memory, focusing in the CREB/CRTC1 pathway.

2.1. Neuronal activity-induced gene expression

Extracellular stimuli can induce rapid and robust transcription of specific genes, known as “immediate early genes” (IEGs), which mediate the cellular response to changes in the environment. One of the first IEGs identified was the *c-fos* proto-oncogene, described by Michael Greenberg and Edward Ziff after stimulation of 3T3 fibroblasts with purified growth factors (Greenberg & Ziff 1984). Shortly after, Greenberg and colleagues reported the first evidence of activity-induced transcription in PC12 cells, a rat pheochromocytoma cell line that can be differentiated to an excitable neuronal phenotype upon treatment with nerve growth factor (NGF) (Greenberg et al. 1986). This study showed that stimulation of differentiated PC12 cells using cholinergic agonists or depolarizing concentrations of potassium chloride (KCl) triggered a rapid transcription of *c-fos*, which was mediated by calcium influx through L-type voltage-gated calcium channels (L-VGCCs) (Greenberg et al. 1986).

Further studies have shown neuronal expression of *c-fos* and other IEGs in specific brain regions following a wide range of physiological stimuli (Flavell & Greenberg 2008). Due to the rapid and specific induction of IEG during physiological neuronal activity, the detection of IEGs is often used to identify recent activated neurons after specific behavioral tasks. More recently, sophisticated genetic manipulations based on induction of IEGs combined with optogenetics technology have been used to manipulate the neuronal ensembles recruited during encoding of memories, allowing the blocking, expression and alteration of acquired memories and demonstrating that activation of this neuronal ensembles or engrams is sufficient to express the memories encoded by them.

The transcription of IEGs is regulated by activity-dependent transcription factors (TFs), whose activity is tightly controlled, both spatially and temporally, depending on the

specific stimulus, tissue, cell type, and developmental stage. Upon synaptic activity, Ca^{2+} and cAMP signals allow the recruitment of specific signaling pathways that modulate the expression, stability, protein interactions and DNA-binding properties of neuronal activity-regulated TFs such as CREB, SRF, NPAS4, DREAM and MeCP2 (Flavell & Greenberg 2008; Benito & Barco 2014; Nonaka, Kim, Sharry, et al. 2014).

The Ca^{2+}-dependent transcriptional induction of the *c-fos* gene is regulated by a *cis*-acting regulatory element in the proximal promoter, near the transcriptional start site (TSS). The consensus sequence of this response element was originally described by Marc Montminy and colleagues as an 8-base palindrome 5'-TGACGTCA-3' in the somatostatin gene promoter, which was responsible for its transcriptional induction upon increase of intracellular cAMP, and was therefore named cAMP-responsive element (CRE) (Montminy et al. 1986). A further study identified the CRE binding protein (CREB) as the transcription factor that binds to the CRE consensus sequence (Montminy & Bilezikjian 1987). Although CREB binds with higher affinity to the full 8-base consensus CRE site, it also binds to several variations of this sequence, including the so-called “half-CRE” sites 5'-CGTCA-3' and 5'-TGACG-3' which may be found in many promoters of CREB target genes (Zhang et al. 2005).

Although several other TFs are now known to contribute to synaptic plasticity and memory mechanisms (Benito & Barco 2014; Alberini & Kandel 2015), CREB is by far the most well characterized activity-regulated TF in the nervous system, in large part because of the early evidence of its role during memory consolidation. In fact, several studies have demonstrated that genetic disruption of CREB activity affects both LTP and memory consolidation, while increasing CREB transcriptional activity often leads to enhanced synaptic potentiation and memory (Benito & Barco 2014).

2.2. Molecular mechanisms regulating CREB transcriptional activity in neurons

From the aforementioned studies it was clear that the presence of CRE sequences in gene promoter regions confers transcriptional responsiveness to both Ca^{2+} and cAMP signals, which is mediated by CREB. Several CREB isoforms may be encoded by an evolutionary conserved *CREB1* gene. CREB-1 belongs to the CREB subfamily of transcription factors, which includes transcriptional activators, like CREB itself, the activation transcription factor-1 (ATF-1) and the cAMP-responsive element modulator (CREM), but also comprises transcriptional inhibitors such as CREB-2 and ATF-4. The products of the *CREB1* gene are usually referred to simply as CREB. The CREB subfamily belongs to the basic leucine zipper domain (bZIP) family of transcription

factors. Like all members of the bZIP family, CREB contains an amino-terminal transactivation domain (TAD) and a carboxy-terminal bZIP domain. While the bZIP domain mediates CREB dimerization and DNA binding, the TAD contains a regulatory kinase-inducible domain (KID) and a glutamine-rich constitutive active domain (Q2/CAD).

Multiple phosphorylation sites within the KID domain modulate CREB binding to the KIX domain of the histone acetyl-transferase (HAT) paralogues CREB-binding protein (CBP) and p300, which act as CREB transcriptional coactivators. In particular, CREB phosphorylation at Ser-133 has been shown to promote binding to CBP/p300 and transcriptional activation. Increase of both Ca^{2+} and cAMP induce CREB phosphorylation at Ser-133, but the kinases involved are different for each signal: Protein kinase A (PKA) is thought to mediate the cAMP-dependent pathway (Gonzalez & Montminy 1989), while the Ca^{2+}-dependent kinase calmodulin (CaM) and members of the Ca^{2+}/CaM-dependent protein kinase family (including CaMKII and CaMKIV) contribute to Ca^{2+}-dependent phosphorylation (Sheng et al. 1991; Dash et al. 1991; Ma et al. 2014). Thus, in the canonical model of CREB activation in neurons, synaptic activity triggers Ca^{2+} and cAMP signals which activate downstream kinases leading to CREB phosphorylation at Ser-133. This phosphorylation induces the binding of CREB to CBP/p300, which promotes gene transcription by means of histone acetylation

Due to the very high affinity of CREB binding to the CRE sites compared to its dimerization affinity, it is though that DNA binding may precede CREB dimerization (Mayr & Montminy 2001). Accordingly, many studies suggest that CREB binds constitutively to the CRE sites, independently of its activation state, although others report activity-dependent recruitment to specific gene promoters upon stimulation [Reviewed by (Altarejos & Montminy 2011)].

It has been shown that efficient induction of CREB-mediated transcription requires the presence of TATA boxes near the CRE sites (Conkright, Guzmán, et al. 2003). An important genome wide study of CREB occupancy, revealed that about 4000 genes in the human genome contain full- or half- CRE sites in the proximal promoter, within 250 bases of the TSS (Zhang et al. 2005). However, only about 100 genes were induced by cAMP, despite robust induction of CREB phosphorylation by cAMP. Furthermore, the specific induced genes varied among different tissues depending on the DNA methylation state and the recruitment of CBP, although CREB occupancy profiles were similar across the different cell types (Zhang et al. 2005).

Besides Ser-133, other phosphorylation sites within the KID are though to modulate CREB activity, including Ser-129, Ser-142 and Ser-143 among others, although the specific contribution of each phosphorylation site is still unclear, with some studies showing opposite effects on CREB-dependent transcription (Sakamoto et al. 2011). Furthermore, other posttranslational modifications of CREB, including acetylation, O-glycosylation and SUMOylation has been shown to modulate CREB-dependent transcription (Lu et al. 2003; Rexach et al. 2012; Chen et al. 2014). Therefore, CREB-regulated transcriptional activity depends not only on the type (full- or half- CRE) and number of CRE sites within the promoter region but also on their distance to the TSS, the presence of proximal TATA boxes as well as the overall promoter context, the tissue and cellular type and state determining the activity of modulatory signals, transcriptional coactivators and repressors, and finally on the specific signals upstream of CREB activation. In summary, CREB transcriptional activity integrates a very wide range of signaling pathways which likely serves the coordination of specific responses according to the synaptic activity patterns and the current cellular context.

2.3. CRTC signaling in the nervous system

The fact that CREB phosphorylation is not sufficient to promote gene expression suggested that other mechanisms could regulate CREB-dependent transcription. In 2003, two independent studies lead by Marc Montminy and Mark Labow reported a new family of activity-regulated transcriptional coactivators known as CREB-regulated transcriptional coactivators (CRTCs, previously known as TORCs), which induce robust CREB-mediated transcription in response to Ca^{2+} and cAMP signals (Conkright, Canettieri, et al. 2003; Iourgenko et al. 2003). The three members of the mammalian CRTC family (CRTC1, CRTC2 and CRTC3) share the same basic functional domains, consisting on a CREB-binding domain (CDB) at the N-terminal, a central regulatory domain, an RNA binding/splicing domain and a C-terminal transactivation domain (Altarejos & Montminy 2011). Interestingly, while CRTC2 and CRTC3 are ubiquitously expressed in different tissues, CRTC1 is primarily expressed in the brain (Altarejos et al. 2008), been the most abundant isoform in the hippocampus and hypothalamus (Watts et al. 2011) as well as in cultured primary forebrain neurons.

For each CRTC isoform several regulatory phosphorylation sites have been identified, which may be mediated by different kinases including members of the AMP-activated protein kinase (AMPK) family, like salt-inducible kinases (SIKs) and AMPK, but also the microtubule affinity-regulating kinase 2 (MARK2) and the dual leucine zipper kinase (DLK) (Screaton et al. 2004; Mair et al. 2011; Phu et al. 2011; Nonaka, Kim,

Fukushima, et al. 2014). During basal neuronal activity CRTC1 is phosphorylated by SIK1/2 which promotes CRTC1 interaction with 14-3-3 proteins in the cytoplasm (Screaton et al. 2004; Sasaki et al. 2011; Ch'Ng et al. 2012; Jagannath et al. 2013). Following glutamatergic activity-induced Ca^{2+} influx through L-VGCCs CRTC1 is dephosphorylated by the Ca^{2+}-dependent phosphatase Calcineurin (CaN), which disrupts the interaction with 14-3-3 proteins and induces CRTC1 transport to the nucleus (Fig. 2) (España et al. 2010; Ch'Ng et al. 2012). On the other hand, increase of cAMP activates PKA which phosphorylates and inhibits SIK1/2, thereby preventing CRTC1 phosphorylation (Altarejos & Montminy 2011; Ch'Ng et al. 2012).

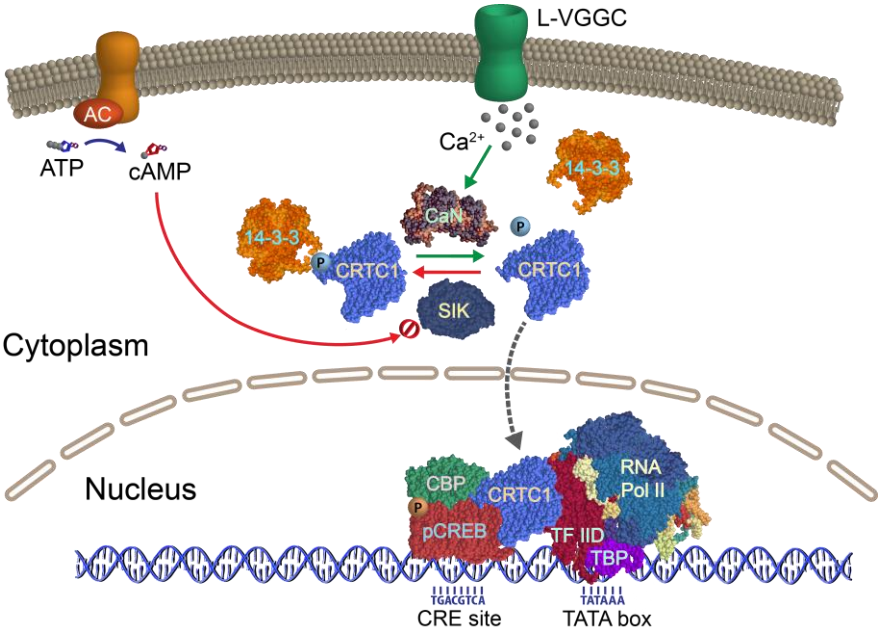

Figure 2. Regulation of CRTC1 activity by Ca^{2+} and cAMP signals. Neuronal activity-dependent increase of intracellular Ca^{2+} and cAMP levels regulate phosphorylation and nuclear translocation of CRTC1 by modulating its interaction with 14-3-3 proteins in the cytoplasm. Upon dephosphorylation by CaN, CRTC1 translocates to the nucleus and binds to CREB, inducing the expression of specific target genes.

Consistent with this model, inhibition of CaN activity using Cyclosporine A (CsA) or FK-506 has been shown to block CREB transcriptional activity by preventing nuclear translocation of CRTC1, while expression of a constitutively active CaN mutant is sufficient to induce nuclear accumulation of CRTC1 (Bittinger et al. 2004) and CREB-regulated transcription in neurons (España et al. 2010). CRTC1 dephosphorylation at Ser64, Ser151 and Ser245 has been shown to regulate activity-induced nuclear translocation and transcription in neurons (España et al. 2010; Ch'Ng et al. 2012; Nonaka, Kim, Fukushima, et al. 2014; Ch'ng et al. 2015).

Nuclear export of CRTCs to the cytoplasm is mediated by CRM1, since treatment with leptomycin B (LMB), an inhibitor of CRM1-mediated nuclear export, induces nuclear CRTC accumulation (Bittinger et al. 2004). Interestingly, a recent study has shown that the gene encoding the 14-3-3 isoform ζ contains a proximal CRE binding site that can be regulated by ATF-1 and CREB (Kasinski et al. 2014), suggesting a potential additional regulatory loop in CREB/CRTC1-regulated transcription.

Once in the nucleus, CRTC1 binds to bZIP transcription factors, including CREB, through their bZIP domains, enhancing the interaction with the TATA box-binding protein (TBP)- associated factor $TAF_{II}130$ (Conkright, Canettieri, et al. 2003), a subunit of the general transcription factor TFIID which also includes TBP (Carey et al. 2009). This TATA box-dependent interaction between the CREB/CRTC1 complex and the transcriptional machinery may explain why TATA boxes are required for robust induction of CREB-mediated gene expression. The interaction between the CREB-binding domain (CBD) of CRTCs and the bZIP domain of CREB has micromolar affinity on both full- and half-site CRE sequences, with a 2:2:1 stoichiometry, indicating that there is one CRTC binding site in each CREB monomer (Luo et al. 2012).

Interestingly, it has been shown that CRTCs can regulate pre-mRNA splicing of CREB target genes in a transcription independent manner by a splice-site selection mechanism that is mediated by their splicing domain and at the same time depends on interaction with CREB (Amelio et al. 2009). Thus, although transcription of CRE-containing genes with TATA-less promoters may not be induced by CREB/CRTC1, their expression could be regulated through CRTC1-dependent alternative splicing (Altarejos & Montminy 2011).

A growing body of recent evidence suggests that CRTC1 regulates a wide range of physiological processes in the nervous system, including neuronal differentiation, survival, circadian rhythms, emotional responses, synaptic plasticity, and learning and memory (Zhou et al. 2006; Kovács et al. 2007; Li et al. 2009; Finsterwald et al. 2010; Sasaki et al. 2011; Sekeres et al. 2012; Jagannath et al. 2013; Sakamoto et al. 2013; Nonaka, Kim, Fukushima, et al. 2014).

Besides its role in the regulation of activity-dependent gene expression in neurons, CREB/CRTC1-mediated transcription also plays important roles in astrocytes, although the signaling mechanisms mediating CRE-transcription seems to be different than in neurons. In astrocytes, adenosine 5'-triphosphate (ATP) and noradrenaline (NE), but not glutamate, induce CREB-dependent transcription mediated by ERK1/2 and CRTC1/2 (Carriba et al. 2012). Interestingly, induction of CREB-regulated transcription

was not dependent on PKA or Ca^{2+}/CaN since treatment of astrocyte cultures with PKA inhibitors, CsA or blocking NE/ATP-induced Ca^{2+} transients with BAPTA, had no effect on CREB-mediated gene expression induced by NE and ATP (Carriba et al. 2012).

Taken together, these studies provide strong evidence for a role of CRTC1 on the regulation of CREB-dependent transcription during a wide range of brain processes, in both neurons and glia.

2.4 Role of CREB/CRTC1-regulated transcription in plasticity, learning and memory processes

Shortly after the identification of CRE and CREB, Eric Kandel and colleagues demonstrated that intranuclear injection of oligonucleotide CRE sequences in sensory neurons of the mollusk *Aplysia californica* impaired a long lasting form of synaptic plasticity known as long-term facilitation, without affecting the earlier transient short-term facilitation (Dash et al. 1990), suggesting for the first time that CRE-regulated transcription mediates synaptic plasticity mechanisms related to memory. Further studies in *Aplysia*, *Drosophila* and rodents supported the idea that CRE-mediated transcription downstream of cAMP/PKA signaling was required for long-term synaptic plasticity and memory in invertebrates (Kaang et al. 1993; Yin et al. 1994) and mammals (Bourtchuladze et al. 1994; Impey et al. 1996; Pittenger et al. 2002).

Mice with homozygous conventional deletion of the CREB α and δ isoforms ($CREB^{\alpha\delta-/-}$) displayed impaired LTP as well as long-term but not short-term memory deficits in both spatial and contextual fear memory induced by training in the MWM and contextual fear conditioning, respectively (Bourtchuladze et al. 1994), suggesting that CREB was necessary for the consolidation of hippocampal-dependent memory in mammals. A later study using conditional expression of a dominant negative CREB mutant (KCREB) restricted to postnatal CA1 neurons of the dorsal hippocampus, reported impaired spatial learning in the MWM but normal contextual fear conditioning (Pittenger et al. 2002). Furthermore, recent studies have shown that conditional expression of a Ser-133 phosphorylation-defective CREB mutant (mCREB) in forebrain neurons impaired contextual fear conditioning, which was associated with learning-induced collapse of CA1 hippocampal spines and reduced levels of the glutamate AMPA receptor subunit GluA1 within the post-synaptic densities (Middei et al. 2012; Middei et al. 2013).

Although the aforementioned studies pointed towards an essential role for CREB-mediated transcription during hippocampal-dependent memory and synaptic plasticity,

some studies using loss-of-function approaches have not found significant alterations in either synaptic plasticity or memory. For instance, a study by Gass and colleagues using F1 hybrids of backcrossed $CREB^{\alpha\delta-/-}$ mice and a mixed strain with one $\alpha\delta$ allele and one CREB-null allele in which all CREB isoforms are disrupted (named $CREB^{comp}$), reported normal LTP and a gene dosage-dependent effect on thigmotaxis (circular swimming close to the border of the pool) but not on the probe trial scores (Gass et al. 1998). A further study analyzed two different lines of neuron-specific conditional CREB knockout mice, in addition to $CREB^{\alpha\delta-/-}$ and $CREB^{comp}$ mice, and found unaltered LTP and LTD, normal contextual fear memory and only slightly deficits in spatial learning in the four different mutant mice analyzed (Balschun et al. 2003). Likewise, a recent study reports normal CREB binding to CRE sites, unchanged CREB-mediated transcription and unaffected hippocampal-dependent memory in a conventional (not conditional) mCREB (S133A) mutant mouse (Briand et al. 2015). However, these results may be explained by compensatory mechanisms involving other members of the CREB family (i.e. ATF-1 and CREM), since upregulation of the CREB β isoform and CREM occurs in $CREB^{\alpha\delta-/-}$ mice (Hummler et al. 1994; Blendy et al. 1996). In this regard, the use of dominant negative strategies for assessing the effects of loss of CREB function seems more appropriate. For example, the dominant negative KCREB used by Pittenger and colleagues has been shown to heterodimerize with wild-type CREB, CREM, and ATF1, thus affecting the whole CREB family by preventing binding of the dimers to the DNA.

Gain-of-function approaches have also been useful for studying the role of CREB on hippocampal-dependent memory. A study by Angel Barco and colleagues reported that enhancement of CREB activity by conditional expression of a constitutively active CREB construct (VP16-CREB) in forebrain neurons interferes with retrieval but not encoding of spatial memory (Viosca et al. 2009), suggesting that optimal hippocampus-dependent memory function requires accurate regulation of CREB activity. On the other hand, viral-mediated overexpression of wild-type CREB but not the S133A-phosphorylation-defective mCREB in the dorsal hippocampus increased spatial memory in both weakly trained wild-type mice and strongly trained heterozygous $CREB^{\alpha\delta+/-}$ mice, compared to control-injected groups (Sekeres et al. 2010). Likewise, expression of the VP16-CREB construct in forebrain neurons has been shown to increase neuronal excitability, hippocampal LTP, and classical eyeblink conditioning memory (Gruart et al. 2012). In summary, despite some apparently contradictory results there is compelling evidence for a role of CREB activity on the regulation of hippocampal-dependent memory processes.

An important role for CREB in the regulation of memory encoding has been successfully established in the lateral amygdala during auditory fear conditioning. Neurons with increased CREB expression in the lateral amygdala were preferentially activated and recruited into an auditory fear conditioning memory trace (Han et al. 2007). It was further demonstrated that the recruitment of individual neurons to the memory trace depended on the neuronal excitability prior to training, which was determined by the levels of CREB activity (Yiu et al. 2014), suggesting a competitive model in which CREB activity increases neuronal excitability to favor recruitment of neurons during memory encoding (Han et al. 2007; Yiu et al. 2014). A mechanism involving CREB regulation of spine density in the lateral amygdala has been proposed to mediate the preferential recruitment of neurons with higher CREB activity (Sargin et al. 2013). Interestingly, CREB levels in neurons of the insular cortex also determine preferential recruitment during memory encoding in a conditioned taste aversion paradigm (Sano et al. 2014).

Due to the pivotal role of CREB transcriptional coactivators during induction of CREB-dependent gene expression, it is expected that in consequence these coactivators should be required for proper regulation of transcriptional programs mediating long-term synaptic plasticity and memory. In fact, partial loss of CBP function in heterozygous $CBP^{+/-}$ and dominant negative CBP transgenic mice leads to long-term memory deficits in passive avoidance, motor skill learning, novel object recognition and fear conditioning, whereas short-term memory is not affected (Oike et al. 1999; Bourtchouladze et al. 2003; Alarcón et al. 2004). Accordingly, learning and short-term memory are normal in CBP transgenic mice expressing a dominant-negative CBP lacking the HAT domain (dnCBP), whereas memory consolidation is selectively impaired (Korzus et al. 2004). Interestingly, hippocampal-dependent spatial learning and memory in the MWM was not affected in in $CBP^{+/-}$ mice, although long-term fear memory deficits were associated with reduced L-LTP in the Schaffer collateral pathway (Alarcón et al. 2004).

Memory deficits caused by reduced CBP activity are at least partially due to decreased histone acetylation and CREB-mediated gene expression, since treatment with histone deacetylase (HDAC) inhibitors improved memory consolidation in both $CBP^{+/-}$ and dnCBP mice (Alarcón et al. 2004; Korzus et al. 2004). On the other hand, complete loss of CBP function at excitatory forebrain neurons in conditional CBP knockout mice (CBP cKO) impaired both short- and long-term memory during MWM, object-recognition and contextual fear conditioning (Chen et al. 2010), suggesting a role for CBP during the initial stages of memory formation in addition to memory consolidation. Similar to

dnCBP mice, conditional transgenic mice expressing a dominant negative p300 lacking the HAT domain, show impaired long-term object recognition and contextual fear memory (Oliveira et al. 2007). However, in contrast to $CBP^{+/-}$ mice, $p300^{+/-}$ mice show normal motor skill learning and mild impairments in hippocampal-dependent spatial memory, although as seen in CREB deficient mice, variations in the genetic backgrounds may also account for these differences (Oliveira et al. 2006; Viosca et al. 2010). Together, these results provide convincing evidence that CBP and p300 are required for proper synaptic plasticity and memory by regulating CREB-dependent transcription.

Only recently, the role of CRTC1 during synaptic plasticity and memory processes has begun to be elucidated. Similar to CBP, a role for CRTC1 on the establishment of L-LTP has been demonstrated (Zhou et al. 2006; Kovács et al. 2007). Furthermore, recent studies have shown that overexpression of CRTC1 in the dorsal hippocampus enhances consolidation of long-term contextual fear memory (Sekeres et al. 2012; Nonaka, Kim, Fukushima, et al. 2014), although nuclear translocation of endogenous CRTC1 during CFC was detected only in the basolateral amygdala, but not in the hippocampus (Nonaka, Kim, Fukushima, et al. 2014). Despite these studies highlight the importance of CRTC1 during contextual associative learning and memory, the potential implication of CRTC1-regulated transcription during age-related memory disorders such as Alzheimer's disease and related neurodegenerative dementias has not been assessed.

3. Role of CREB-regulated transcription in Alzheimer's disease and neurodegeneration

Memory is a fascinating phenomenon itself, but its significance goes beyond merely storing and retrieving information when we consider it as a fundamental process supporting many higher cognitive functions such as abstract thought, planning, reasoning, even creativity and of course consciousness: our ability of being aware of ourselves and our environment. Every one of those processes requires at least a short-term buffer of information available in order to successfully accomplish a specific task, while others require high demands of both working memory and consolidated long-term memories. It is then not surprising that clinical conditions affecting memory performance can be severely detrimental for a person's life, especially when memory deficits are persistent and worsen over time, as occurs in patients suffering from neurodegenerative dementias.

3.1. Clinical features and genetics of AD

Dementia is a broad term referred to several syndromes characterized by persistent deterioration of intellectual or cognitive function, including alterations in behavior and personality, although the term it is more often used to denote the advanced stages of syndromes comprising increasing impairment of memory and other intellectual abilities caused by chronic progressive neurodegenerative diseases (Allan et al. 2014).

According to the 2015 World Alzheimer Report recently issued by *Alzheimer's Disease International*, it is estimated that 46.8 million people worldwide are currently living with dementia, and this number is expected to increase up to 74.7 million in 2030 and 131.5 million in 2050 (Prince et al. 2015). Although recent epidemiological studies suggest that the prevalence of dementia is stablishing at least in North America and Western Europe (Andrieu et al. 2015; Hofman et al. 2015; Wu et al. 2015), the actual number of people affected with dementia is expected to rise as life expectancy and the aging population increases especially in middle- and high-income countries (Andrieu et al. 2015; Hofman et al. 2015; Prince et al. 2015; Wu et al. 2015). At this time, the total worldwide cost, derived from medical and social care associated to dementia, is estimated in US $818 billion, and it will reach the trillion US dollars by 2018, whereas less than 1% of the current costs is destined to research (Prince et al. 2015).

Alzheimer's disease (AD) is the most common cause of dementia, accounting for 50-70% of all cases, followed by vascular dementia, representing about 20% of cases

(Savonenko et al. 2015). AD is clinically manifested by a gradual increase of memory deficits progressing to pronounced impairment of cognitive abilities ultimately leading to dementia. At the neuropathological level AD is characterized by loss of synapses and neurons, and by the accumulation of two main protein aggregates: extracellular amyloid plaques (also known as senile plaques) formed by progressive deposition of β-amyloid peptides (Aβ), and intracellular neurofibrillary tangles (NFT) containing aberrantly hyperphosphorylated species of the microtubule-associated protein tau (Savonenko et al. 2015).

The clinical and neuropathological features of AD were first described in 1906 by the German neuropathologist Aloysius “Alois” Alzheimer, after the histopathological analysis of the brain of patient Auguste D, a 51 year-old woman suffering from severe memory impairment, aphasia, delusions, and auditory hallucinations (Goedert & Spillantini 2006; Hodges 2006). While senile plaques were already known by that time, Alzheimer was the first to describe the neurofibrillary tangle pathology (Goedert & Spillantini 2006). A distinct type of senile plaques, known as neuritic plaques, can be distinguished in close proximity to neurons having “dystrophic neurites” and high levels of pathological tau hyperphosphorylation.

The type of AD initially identified by Alzheimer affects middle-aged adult patients (40 to 50 year-old) who inherit the disease in an autosomal dominant fashion. This type of early-onset AD is also known as “presenile AD”, in order to differentiate it from non-inherited forms of AD affecting older people (usually older than 65). Both the autosomal dominant early-onset AD and the non-inherited late-onset AD share the same histopathological features described by Alzheimer, and they are most often referred to as early-onset or familial AD (FAD) and late-onset or sporadic AD, respectively. Despite its name, sporadic late-onset AD is the most frequent form, accounting for more than 99% of all AD cases. The main genetic risk associated to late-onset AD is the genotype of the apolipoprotein E (APOE) alleles: APOE2, APOE3, or APOE4. Homozygosis for the APOE2 allele is associated with preserved cognitive performance and longevity, whereas the APOE4 allele is associated with higher risk of developing dementia. A single E4 allele increases the risk by a factor of 3, while two E4 alleles increase the risk by a factor of 8-10.

On the contrary, early onset familial AD (FAD) is produced by dominantly inherited mutations in 3 genes: the amyloid-β precursor protein (APP) gene on chromosome 21, presenilin 1 (PS1) on chromosome 14 and presenilin 2 (PS2) on chromosome 1 (Querfurth & LaFerla 2010). To date, more than 200 mutations have been reported to

cause AD, and mutations in PS1 account for most cases of early-onset FAD (http://www.molgen.ua.ac.be/ADMutations - http://www.alzforum.org/mutations).

3.2. Presenilins, γ-secretase and APP processing

Presenilins (PS) are the catalytic subunit of γ−secretase, an aspartyl protease complex that cleavages different type I transmembrane proteins including Notch, APP and cadherins. The proteolytic activity of the γ−secretase complex requires association of PS with three additional subunits: Nicastrin, Anterior pharynx-defective 1 (Aph-1), and Presenilin enhancer 2 (Pen-2) (De Strooper 2010). It has been proposed that nicastrin controls the access of substrates to the catalytic site, whereas Aph1, which has been shown to associate with nicastrin before incorporation of PS and Pen-2, act as an initial scaffold for assembly of the complex. Incorporation of Pen2 is considered the final step in the assembly of the complex, leading to PS cleavage and activation of γ-secretase proteolytic activity (De Strooper 2010).

Aβ peptides are generated through the amyloidogenic processing pathway by the sequential proteolytic cleavage of APP, first by β–secretase and then by γ-secretase. During this amyloidogenic processing APP is first cleaved on the N-terminal side of the Aβ sequence by the β-site APP cleaving enzyme-1 (BACE1), a transmembrane aspartyl protease. The resulting C-terminal fragment is then cleaved by γ-secretase, releasing extracellular Aβ and a C-terminal APP intracellular domain (AICD) fragment. The most abundant form of Aβ has 40 amino-acid residues (Aβ40). However, the longer 42-residue species (Aβ42) is considered more pathogenic because it aggregates more readily than Aβ40. Importantly, many FAD mutations in both APP and PS increase the production of Aβ42 (Shen & Kelleher 2007). On the contrary, cleavage of APP by α-secretase promotes a nonamyloidogenic processing. Through this pathway a large amyloid precursor protein ectodomain (sAPPα) is released and the remaining carboxy-terminal fragment (CTF) is further digested by γ-secretase, generating an extracellular p3 fragment and the AICD (De Strooper 2010). Thus, γ-secretase processing participates in both amyloidogenic and nonamyloidogenic pathways.

3.3. Role of CREB/CRTC1 signalling during AD

Recent evidence suggest that generation and accumulation of Aβ oligomers induce synaptic plasticity and long-term memory deficits (Chapman et al. 1999; Walsh et al. 2002), and these effects are mediated by disruption of the CREB signaling pathway (Vitolo et al. 2002). It has been shown that Aβ42 disrupts PKA activity leading to LTP deficits in hippocampal slices, which was associated with reduced levels of glutamate-induced CREB phosphorylation (Vitolo et al. 2002). It has been suggested that Aβ-induced decrease of CREB phosphorylation is mediated by reduction of NMDA receptors (Ma et al. 2007). Importantly, reduced cAMP levels and altered regulation of PKA induced by Aβ accumulation, are associated with decreased levels of CREB phosphorylation in cultured neurons and AD brains (Yamamoto-Sasaki et al. 1999; Vitolo et al. 2002; Liang et al. 2007).

Previous studies from our group have shown that CRTC1 activity is reduced in neurons from APP transgenic mice harboring the Swedish and Indiana mutations ($APP_{Sw,Ind}$) linked to familial Alzheimer's disease (España et al. 2010). Increase of intracellular cAMP and Ca^{2+} by treatment with forskolin (FSK, an adenylate cyclase activator) and depolarizing concentrations of potassium chloride (KCl) induced similar CREB phosphorylation levels in control and $APP_{Sw,Ind}$ neurons. Interestingly, although FSK-induced CRE-dependent transcription was unchanged, there was a significant reduction in CRE-transcriptional activity induced by KCl or FSK+KCl in cortical and hippocampal $APP_{Sw,Ind}$ neurons (España et al. 2010).

Suppression of CRTC1-dependent gene transcription by Aβ in response to cAMP and Ca^{2+} signals is mediated by reduced calcium influx through L-VGCC and disruption of CaN-dependent CRTC1 dephosphorylation at Ser151 (España et al. 2010). Importantly, reduced mRNA levels of CRTC1/CREB-regulated genes related to memory (Bdnf, c-fos, and Nr4a2) coincided with hippocampal-dependent spatial memory deficits in $APP_{Sw,Ind}$ mice (España et al. 2010), suggesting that CRTC1 may mediate activity-induced gene transcription required for hippocampal-dependent memory, and that Aβ may disrupt memory processes by affecting CRTC1 function.

V. Working hypothesis and objectives

The working hypothesis of this doctoral thesis is that CREB-dependent gene expression essential for hippocampal memory processing is regulated by CRTC1 activity, which is affected by pathogenic mechanisms during Alzheimer´s disease (AD) and neurodegeneration. If this hypothesis is correct, altered CRTC1/CREB-regulated transcription may contribute to early synaptic dysfunction and memory deficits during AD and neurodegeneration, and conversely, experimental therapeutic strategies aimed to restore CRTC1 function in the hippocampus may alleviate synaptic and cognitive deficits in these conditions.

To test this hypothesis we have proposed the following objectives:

1. To analyze the CREB/CRTC1-regulated transcriptome in $APP_{Sw,Ind}$ mice during hippocampal-dependent spatial learning and memory
2. To analyze the expression of CRTC1/CREB-target genes in the human AD hippocampus at different pathological stages
3. To study the activation and nuclear translocation of CRTC1 during hippocampal-dependent associative memory in PS cDKO mice
4. To test the feasibility of using CRTC1-based gene therapy during initial pathological stages in $APP_{Sw,Ind}$ and PS cDKO mice

VI. Materials and methos

1. Mouse models

Mouse colonies used in this work were maintained in standard conditions at the Animal Core facility of the Universitat Autònoma de Barcelona, on a 12 h light/dark cycle with food and water available *ad libitum*. All behavioral experiments were performed during the light phase. Unless otherwise stated, littermates were housed together (maximum 5 mice per cage) keeping males and females separated after weaning. Experimental procedures were conducted according to the animal experimentation protocols approved and supervised by the Animal Care facility and Bioethics Committee of the Universitat Autònoma de Barcelona (protocol CEEAH 1783, Generalitat de Catalunya 6381) following the European Union guidelines.

1.1 $APP_{Sw,Ind}$ transgenic mice:

$APP_{Sw,Ind}$ transgenic mice (line J9) expressing the human APP 695 isoform harboring the FAD-linked Swedish (K670N/M671L) and Indiana (V717F) mutations under the neuronal PDGFβ promoter were generated in the laboratory of Dr. Lennart Mucke at UCSF (Hsia et al. 1999). Mice used in this study were age-matched male littermates obtained by crossing heterozygous $APP_{Sw,Ind}$ to nontransgenic (WT) mice (C57BL/6 background). C57BL/6 $APP_{Sw,Ind}$ mice develop age dependent β-amyloid pathology, hippocampal-dependent memory deficits, reduced density of synaptophysin-positive terminals, altered synaptic transmission, and reduced CREB-dependent gene expression (Hsia et al. 1999; Mucke et al. 2000; España et al. 2010).

1.2 PS cDKO mice:

PS cDKO mice were generated in the laboratory of Dr. Jie Shen at Harvard Medical School as previously described (Saura et al. 2004). Briefly, females PS1 cKO; PS2+/- (fPS1/fPS1; CaMKIIα-Cre; PS2+/-) (Yu et al. 2001) were crossed with floxed PS1;PS2-/- (fPS1/fPS1; PS2-/-) males to generate controls (fPS1/fPS1; PS2+/-), PS1 cDKO (fPS1/fPS1; CaMKIIα-Cre; PS2+/-), PS2-/- (fPS1/fPS1; PS2-/-) and PS cDKO mice (fPS1/fPS1; CaMKIIα-Cre; PS2-/-). The genetic background of all mice was C57BL6/129 hybrid. PS cDKO mice develop progressive impairment of synaptic plasticity, learning and memory deficits, age-dependent neurodegeneration, and reduced CREB-dependent transcription (Saura et al. 2004). In PS cDKO mice Cre-recombinase is mainly expressed postnatally at glutamatergic forebrain neurons, starting at P18.

2. Human brain tissue

Human brain samples were obtained from brain banks of Hospital de Bellvitge (Universitat de Barcelona, Spain) and Fundación CIEN (Instituto de Salud Carlos III, Spain). Neuropathology was classified according to Braak staging for neurofibrillary tangles as described (Braak et al. 2006). We analyzed hippocampal tissue samples from control persons and individuals that displayed neurofibrillary tangle pathology corresponding to early presymptomatic (Braak I-II), mild cognitive impairment (Braak III-IV) and final AD (Braak V-VI) stages (n=14-22/group for gene expression analysis; n=5-12/group for biochemical analysis). Information of sex, age and postmortem delay of the samples used for gene expression analysis and western blotting is provided in Article 1 (Table 3) and Table I, respectively. Western blot analysis of human samples was performed by Meng Chen.

Table I. Summary of human brain samples used for western blotting

Braak stage	*n*	Sex	Age	PMD (h)
Ctrl	12	4F/8M	50,42 ± 2,34	6,33 ± 0,69
I-II	12	2F/10M	68,58 ± 2,74	6,75 ± 1,25
III	5	2F/3M	76,80 ± 3,31	8,55 ± 2,35
IV	5	2F/3M	85,40 ± 3,87	7,12 ± 2,74
V-VI	8	4F/4M	78,63 ± 2,37	9,71 ± 2,09

F, Female; M, male; PMD, postmortem delay (hours). Age and PMD data represent the mean ± SD.

3. Genotyping

For genomic DNA extraction, a small portion of tail (1-2 mm) was incubated overnight at 56ºC in 0.5 ml of digestion buffer containing 0.1 mg/ml proteinase K (Roche). Samples were centrifuged at 12000 rpm for 5 minutes; supernatants were transferred to a new tube and 0.5 ml isopropanol was added to each tube. Samples were centrifuged at 12000 rpm for 10 minutes and 0.5 ml of 70% ethanol was added to the DNA pellet. After centrifugation, the DNA was resuspended in 50-100 μl TE buffer and incubated in a thermomixer (1000 rpm) at 65ºC for 2 hours.

For DNA amplification by Polymerase Chain Reaction (PCR) 2 μl of purified genomic DNA was added to 2.5 μl of 10X PCR buffer (Biotools), 0.5 μl dNTP (10 mM; Biotools), 0.5 μl $MgCl_2$ (50 mM; Biotools), 0.2 μl Taq DNA polymerase (5 U/ml; Biotools), forward and reverse primers (0.5 μM; Life Technologies) (Table 3) in a final volume of 25 μl. The amplification was performed in a PXE 0.2 thermal cycler (Thermo Electron

Corporation) using the appropriate PCR program (Table 4). 15 µl of PCR product was resolved on a 2.5% agarose gel containing 1X SYBR-Safe (Life Technologies).

4. Cell culture

4.1 Primary neuronal culture

Cortical and hippocampal neurons were obtained from E15 mouse embryos. Embryos were extracted and placed in a 100 mm diameter dish containing cold phosphate buffer saline (PBS) containing 30 mM glucose (1 mg/ml) and penicillin/streptomycin (50 U/mL penicillin and 50 µg/mL streptomycin, Life Technologies). After extracting the brains, the hemispheres were separated and meninges were removed. Cortices and hippocampi were dissected and transferred to a sterile centrifuge tube containing 10 ml of Krebs buffer (Solution 1), centrifuged at 300 g for 1 minute and the supernatant was discarded. Tissue was incubated in a trypsin solution (solution 2) at 37ºC during 8 minutes, agitating the tubes gently every 2 minutes. The digestion reaction was stopped by adding trypsin inhibitor (Life Technologies) (Solution 3). The digested tissue was centrifuged (300 g for 1 minute), the supernatant was discarded and the pellet was resuspended in the solution 4. The cell suspension was mechanically dissociated using a Pasteur pipette and filtered through a nylon mesh (40 µm pore size) to eliminate cell clumps. The filtered cell suspension was then transferred to a tube containing solution 5 and centrifuged at 250 g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in B27/glutamine-supplemented Neurobasal medium. Live cells were counted in a hemocytometer using trypan blue. Neurons were seeded in Poly-D-lysine-coated 24 well-dishes (50,000 cells/well for immunocytochemistry and 100,000-150,000 cells/well for luciferase assay), 12-well dishes (200.000 cells/well for molecular assays) 6-well dishes (350.000 cells/well for biochemical and molecular assays) or 60 mm diameter dishes (1.5-2x10^6 cells/dish for ChIP assays). Neurons were maintained in a humidified incubator at 37ºC with 5% CO2, and the culture medium was changed every 4 days by replacing half of the conditioned medium with fresh medium.

Solutions and reagents:
Solution 1: 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 14.3 mM Glucose, 0.3% bovine serum albumin and 0.03% Mg_2SO_4
Solution 2: Solution 1, 0.025% trypsin
Solution 3: Solution 1 plus 0.052% trypsin inhibitor, 0.008% DNAse and 0.03% $MgSO_4$
Solution 4: Solution 1 plus 16% solution 3
Solution 5: Solution 1 plus 0.03% $MgSO_4$ and 0.0014% $CaCl_2$

Complete Neurobasal medium: 500 mL Neurobasal medium supplemented with 2 mM glutamine, 10 ml B27 supplement (Life Technologies), 25 U/mL penicillin and 25 µg/mL streptomycin.
Phosphate-buffered saline (PBS): 136.87 mM NaCl, 2.5 mM KCl, 0.8 mM NaH_4PO_4, 1.47 mM K_2HPO_4, pH 7.4. Supplemented with 30 mM glucose (1 mg/mL) and penicillin/streptomycin (50 U/mL and 50 µg/mL, respectively).

Neurobasal (Life Technologies, 21103-049)
Bovine serum albumin (Sigma T4665)
B27 (50X, Life Technologies 17504-044)
L-Glutamine (200 mM, Life Technologies 25030-081)
Penicillin-Streptomycin (5000 U/mL, Life Technologies 15070-063)
Poly-D-lysine (Sigma P7658)
Trypsin (Sigma T4665)
Trypsin inhibitor (Life Technologies 17075-029)

4.2 Cell lines

Human embryonic kidney (HEK293) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 25 U/mL penicillin, and 25 µg/mL streptomycin. Cells were maintained in a humidified incubator at 37ºC with 5% CO_2. Culture medium was changed every 2-3 days and cell passages were performed once a week.

Reagents:

Dulbecco's Modified Eagle Medium, DMEM (Sigma Aldrich D5796)
Fetal Bovine Serum, FBS (Invitrogen-Gibco 10106-169)
Penicillin-Streptomycin (5000 U/mL, Life Technologies 15070-063)
0.25% Trypsin-EDTA (1X), Phenol Red (Life Technologies 5200-056)

4.3 Transfection and viral infection

CRE-luciferase and TK Renila plasmids were obtained from Stratagene and Promega, respectively. Lipofectamine 2000 reagent was used for tansfection of hippocampal and cortical neurons (7 days in vitro, DIV). 1 µl of Lipofectamine 2000 was diluted in 50 µl OptiMEM containing 1mM glutamine, and kept at room temperature for 5 min. DNA (0.25-0.5 µg) was mixed with 50 µl OptiMEM containing 1mM glutamine, and kept at

room temperature for 20 min. 100 µl of the DNA/Lipofectamine medium were added to each well, containing approximately 200 µl of medium. The cells were incubated for 50 min at 37 ºC before replacing the culture medium.

Annealed oligonucleotides containing the shRNA sequence were cloned into BglII/HindIII sites of the pSUPER.retro.puro plasmid (OligoEngine). The H1 promoter-shRNA sequence was then subcloned into a pLVTHM vector. Lentiviral particles were generated by co transfecting HEK293T cells with pLVTHM-Sh, pSPAX2, and pM2G vectors. Primary cultured neurons (3-4 DIV) were infected overnight using 1 infective particle/cell.

Reagents:

Lipofectamine 2000 reagent: Invitrogen 11668-019

OptiMEM: Invitrogen 31985-062

5. Biochemical methods

5.1 Cell and brain lysis and protein quantification

Mice were scarified by cervical dislocation, and the brain was dissected on ice. Cortices or hippocampi were homogenized using a dounce homogenizer in lysis buffer containing protease and phosphatase inhibitors. 1200 µl lysis buffer was used for 1/2 cortices and 400 µl lysis buffer was used for 1/2 hippocampi. Cell cultures were washed twice with ice-cold PBS (1x) and then lysed in cold RIPA lysis buffer containing protease and phosphatase inhibitors (75 µl/well for 6-well dishes). The lysate was sonicated using 35% of power (relative output 5.5) for 10 sec (Dynatech Sonic Dismembrator model 300), and samples were kept on the ice before storage at -80ºC. Protein concentration was determined using the Pierce BCA kit according to manufacturer's instructions (Pierce #23225).

Solutions:

Lysis buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1% NP-40, .1% SDS, 1 mM Na3VO4, 50 mM NaF, 1 mmol/L PMSF.

RIPA lysis buffer: 50 mM Tris base pH 7.4, 150 mM NaCl, 2.5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4, 1 mM PMSF.

Protease inhibitor cocktail tablets: Roche #11836145001

Phosphatase inhibitors: Roche #04906837001

BCA protein assay kit: Pierce 23225

5.2 SDS-PAGE and Western blotting

Equal amount of protein were diluted with sample loading buffer (3x) and heated at 95ºC for 5 min before loading the polyacrylamide gels (PAGE) (7% -12.5% acrylamide). Proteins were transferred to methanol-activated PVDF membranes which were stained with Ponceau S solution to verify proper transference.

PVDF membranes were incubated with blocking solution for 1 hour and washed with Tris-buffered saline-Tween (TBS-T) (3x 10 min). Membranes were incubated with primary antibody diluted in antibody buffer for 2 h at room temperature or overnight at 4 4ºC. Membranes were washed with TBS-T (10 min x5) followed by incubating with secondary antibody coupled to HRP at room temperature for 45 min. Finally, membranes were washed with TBS-T (10 min x5) before revealing by chemoluminescence reaction with Western Light plus-ECL. If necessary, membrane was stripped in stripping buffer at room temperature for 1h and washed in TBS-T (10 min x3) before starting new blotting.

Buffers and reagents:

Sample loading buffer (1x): 62.5 mM Tris HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol (βME) and 0.01% bromophenol blue

SDS-PAGE electrophoresis buffer (10x): 250 mM Tris buffer, 2 M Glycine, 1% SDS, pH 8.3

Transfer buffer (20x): 200 mM Tris base and 2 M Glycine, pH 8.3
TBS-T: Tris 30.3g, NaCl 80.1g, Tween-20 10 ml, add ddH2O to 1L, pH 7.6
Blocking solution: 5% skimmed milk powder and 0.05% Tween in TBS, pH7.4
Primary antibody buffer: 0.1% BSA and 0.02% thimerosal in TBST, pH7.4
Stripping buffer: 0.1 M Glycine pH2.3
Ponceau S solution: Sigma 81462
Molecular weight marker: Invitrogen 10748-010
Immun-Blot PVDF membrane for protein blotting: Bio-rad 162-0177
Western Light plus-ECL: Peroxide solution (Promega, #W100B), Luminol enhancer solution (Promage, #W101B)

6. Molecular biology methods

6.1 RNA extraction and reverse transcription

Total RNA from human hippocampal tissue was isolated using a combination of Trizol extraction (Life Technologies) and the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, frozen hippocampal tissue was homogenized in Trizol reagent…

Total RNA from cultured neurons or mouse brain tissue was isolated using the PureLink RNA Mini Kit according to the manufacturer's instructions (Ambion, Life Technologies, USA). RNA quality was assessed by measuring the RNA Integrity Number (RIN) using the Agilent RNA 6000 Nano Kit in a 2100 Bioanalyzer instrument (Agilent Technologies, USA). Reverse transcription of total mouse RNA (1 µg; RIN > 8.0) or human RNA (2 µg; RIN > 6.0) was performed in 50 µl of a mix containing Oligo(dT) primers (1 µM; Life technologies), random hexamers (1 µM; Life technologies), dNTPs (0.5 mM; Life technologies), DTT (0.45 mM; Life technologies), RNAseOut (10 units; Life technologies) and SuperScript™ II reverse transcriptase (200 units; Life technologies), using the following program: 25ºC for 10 min → 42ºC for 60 min → 72ºC for 10 min.

6.2 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) experiments for gene expression analysis were performed in compliance with The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009). qPCR reactions were performed in duplicate using 2.5 µl of cDNA (1:100 dilution) and 7.5 µl of a mix containing custom designed primers (400 nM) and SYBR Select Master Mix (Life Technologies), using the Applied Biosystems 7500 Fast instrument. The specificity of the amplified products was verified by detection of a single melting point during melt curve analysis. Amplification data was acquired using the 7500 Software v2.0.6 (Applied Biosystems) and the raw fluorescence data was exported and analyzed using the LinRegPCR software (Ruijter et al. 2009). Gene expression data analysis was performed by the comparative ΔCt method (Pfaffl 2001), using the Ct values and average efficiencies obtained from LinRegPCR. In each experiment, the stability of at least five reference genes was evaluated using the NormFinder algorithm (Andersen et al. 2004). Expression data from cultured neurons or mouse brain tissue was normalized using the geometric mean of the three most stable genes between Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), hypoxanthine guanine

phosphoribosyl transferase-1 (*Hprt1*), peptidylprolyl isomerase A (*Ppia*), β-actin (*Actb*) and TATA box binding protein (*Tbp*). Gene expression data from human samples was normalized using the geometric mean of GAPDH, ACTB, and PPIA, which were the three more stable genes from the Human Reference Gene Panel (TATAA Biocenter AB).

6.3 Quantitative chromatin immunoprecipitation (ChIP-qPCR)

11-12 DIV cortical neurons were treated with vehicle or FSK (20 μM) and KCl (30 mM) for 15 min. Cells were crosslinked with 1% formaldehyde, lysed 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) and chromatin was sheared between 200 and 500 bp by sonication using a BioruptorPlus (Diagenode). Fragmented chromatin was analyzed using the High Sensitivity DNA Kit (Agilent technologies). Chromatin immunoprecipitations (2.5 μg) was performed overnight in diluted ChIP buffer (0.1% SDS, 1,1% Triton X-100) with monoclonal rabbit anti-CRTC1 and CREB antibodies (Cell Signaling) including negative controls without antibodies. Input and immunoprecipitated DNA were decrosslinked and amplified by real-time qPCR using specific primers for CRE-containing promoter sequences of specific genes.

6.4 Microarray analysis

Microarray sample preparation and analysis were performed by Dr. Jorge Valero and Dr. Judit España. Genome-wide microarray analysis was performed using hippocampal total RNA from control (WT) and $APP_{Sw,Ind}$ mice in both naïve and MWM-trained conditions (n=3-4 per group) as described above. 6 Month-old littermates male mice were obtained from WT x heterozygous $APP_{Sw,Ind}$ crossings. Mice were handled and kept in the home cage (naive) or trained in the MWM for five consecutive days and tested in a probe trial 2.5 h later. Trained mice were killed 30 min after the probe trial by cervical dislocation. Hippocampi were dissected on ice, immersed in RNAlater® (Life Technologies), and stored at -80ºC. RNA was purified using the RNAeasy mini kit (Qiagen) and quality tested by using the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was amplified, reverse-transcribed, and fluorescently labeled with either Cy3-CTP or Cy5-CTP using the Agilent Fluorescent Linear Amplification kit (Agilent Technologies). Two individual samples labeled with Cy3 or Cy5 were hybridized (64°C, 20 h) to the mouse Genome 32K Oligo Array containing 33,696 transcripts (Capital-Bio) by the Biotools Custom Microarray Service (Biotools B&M Labs). After hybridization, slides were washed, dried and scanned on a LuxScan 10K Microarray Scanner (Capitalio) and analyzed with LuxScan 3.0 Imaging and Analysis

software (LuxScan Technologies). Microarray normalization was performed by the Global loess method.

Microarray data were analyzed using the Linear Model for Microarray Data (Limma) package in Bioconductor (http://www.bioconductor.org/; R software v2.9.2; http://www.r-project.org) (Durinck et al. 2009). Statistical gene expression differences between the groups, calculated from measures of log2- fold-change (M values), were analyzed with a linear model and empirical Bayes using the Limma package (Smyth, 2005). The *p* values correction for multiple testing was performed using Benjamini and Hochberg algorithm (Benjamini and Hochberg, 1995). Statistical gene changes >1 or < -1 and corrected *p* values <0.05 were considered significant. Mouse microarray data are available at the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2067. Gene ontology (GO) analysis of mouse microarray data were performed with ClueGO v1.4 (Bindea et al. 2009) using the following parameters: enrichment/depletion two-sided hypergeometric statistical test; correction method: Bonferroni; GO term range levels: 3–8; minimal number of genes for term selection: 5; minimal percentage of genes for term selection: 10%; κ-score threshold: 0.5; general term selection method: smallest p value; group method: κ; minimal number of subgroups included in a group: 3; minimal percentage of shared genes between subgroups: 50%.

The CREB-regulated transcriptome consisted of 287 genes, which contained CRE sequences and at least a TATA box in their promoter randomly selected from the CREB Target gene database (http://natural.salk.edu/CREB), plus 63 confirmed CREB-target genes (Zhang et al. 2005). The CREB gene list was filtered in the whole microarray data according to the above statistical criteria. Heat maps were computed with Mayday software 2.10 (Battke et al.. 2010). Differentially expressed CREB genes were submitted to an ontology term enrichment analysis using DAVID (Huang da et al. 2009). Filters used in the functional annotation clusters were established as follows: Similarity Term Overlap 3, Similarity Threshold 0.50, Initial Group size 5, final Group Membership 5, and Multiple Linkage Threshold 0.70.

7. Histological and immunostaining procedures

7.1 Intracardial perfusion and tissue processing

Mice were deeply anesthetized using a lethal dose of phentobarbital (120 mg/kg) and transcardial perfusion was performed using 0.9% NaCl solution during 1 min followed by 4% buffered formaldehyde solution (pH 6.9 Histology grade, Merck) during 10 min.

Brains were removed and submerged in the same fixative for 2 hours. For paraffin embedding, brains were washed in PBS and then transferred to 70% ethanol before inclusion. Brains were dehydrated in a graded series of ethanol followed by xylene and then embedded in paraffin. 5 µm sections were cut using a microtome (Leica RM 2255) and mounted on microscope glass slides (Fisher Superfrost). At least one section of each experimental group was mounted on the same paraffin block to account for staining variability across groups. For cryopreserved free-floating sections, brains were washed in 0.1 M phosphate buffer (PB; pH 7.4) and transferred to a solution containing 30% sucrose in PB. After overnight incubation, brains were frozen in dry ice and mounted in a cutting chuck using Tissue-Tek® O.C.T. Compound. Serial sections (20-40 µm) were obtained using a Leica CM 3050s cryostat and kept in antifreezing solution at -20 ºC.

Antifreezing solution: PB 0.1 M pH 7.4 30ml, Ethylene glycol 40 ml, Glycerol 30 ml.

7.2 Immunohistochemical and immunofluorescence staining

For Aβ staining, paraffin-embedded brain sections (5 µm) were deparaffinized in xylene, rehydrated and incubated with 3% hydrogen peroxide. Sections were incubated in 60% formic acid for 6 min to allow antigen retrieval, washed in 0.1 M Tris-HCl and incubated with anti-Aβ (6E10; 1:1,000; Signet) before immunoperoxidase staining and analysis with a Nikon Eclipse 90i microscope. This protocol was previously shown to label specifically Aβ rather than full-length APP (España et al., 2010a). For CRTC1-myc and Arc staining, floating sections (40 µm) were blocked in 5% normal goat serum in PBS containing 0.2% Triton X-100 and incubated with rabbit myc (1:1,000) and NeuN (1:2,000; Chemicon) antibodies or rabbit anti-CRTC1 (1:300) and mouse anti-myc (1:500; 9E10) or Arc (1:100; Ab62142, Abcam) and the AlexaFluor-488/594-conjugated goat secondary antibodies (1:400; Invitrogen) and Hoechst (1:10,000; Invitrogen). For CRTC1 nuclear staining, mice were deeply anesthetized with pentobarbital (200 mg/kg, i.p.) and intracardially perfused with saline and 4% buffered formaldehyde (Merck) during 10 min after 5 day MWM. Paraffin-embedded coronal brain sections (5 µm) were deparaffinized and microwave heated (10 min) in antigen retrieval citrate buffer. Sections were incubated with CRTC1 (1:300; Cell signaling) and MAP2 (1:300; Sigma) antibodies and Hoechst (1:10000; Invitrogen) followed by AlexaFluor-488/594-conjugated goat IgGs (1:400; Invitrogen). Images (20x; zoom 0.5) of hippocampal CA3 layer (4 sections; n=5-6/group) were obtained using a Zeiss LSM700 laser scanning microscope and nuclear/cytoplasmic intensity ratio was calculated using ImageJ software (v.1.47n).

8. Behavioral experiments

8.1 Morris water maze (MWM)

Morris water maze (MWM) experiments were performed by Judit España as described (Giménez-Llort et al. 2007; España et al. 2010; Parra-Damas et al. 2014). Mice were handled individually during 3 min for 3 consecutive days before starting the MWM experiments. Mice were trained in a circular pool (90 cm diameter; 6.5 cm hidden platform) for three or five consecutive days (4 trials daily; 60 s per trial). Mice were tested for memory retention (probe trial) 2.5 h after training on day five and were euthanized by cervical dislocation 30 min after the probe trial. We selected this time to obtain a measure of memory retention while achieving maximum induction of gene expression, which occurs between 0.5–2 h after spatial training (Guzowski et al., 2001). A control swimming group moved freely in the maze without platform for 5 d and mice were euthanized 30 min after a simulated probe trial. AAV-injected mice were trained in the water maze three weeks after AAV injection, using in a bigger circular pool (120 cm diameter; 11 cm platform) during 5 days (6 trials daily; 60 s per trial). Mice were tested in a probe trial 2.5 h after training, and euthanized 30 min later. Importantly, both maze setups lead to similar escape latencies during spatial training and memory retention (probe trial test) in WT and $APP_{Sw,Ind}$ mice. In all cases, spatial learning and memory parameters were analyzed with SMART software (PanLab) (España et al. 2010).

8.2 Contextual fear conditioning (CFC)

Mice were handled individually during 3 min for 3 consecutive days before starting the CFC experiments. Mice were placed individually in a conditioning chamber (15.9 cm x 14 cm x 12.7 cm; Med Associates Inc.) and were allowed to explore it during 3 min to allow the development of a contextual representation before the onset of the unconditioned stimulus (US; footshock, 1s/1mA). After the shock, mice remained in the chamber during 2 additional minutes, to allow association of the context and the US. Contextual fear memory was assessed by measuring the freezing response when mice were exposed to the same chamber 2 hours (for assessing short-term memory), or 24 hours (for assessing long-term memory) after training. Control groups included: 1) a handled context group, in which mice were placed in the chamber during 5 minutes without receiving footshock, and 2) a handled shock group, in which mice were immediately returned to their home cage after delivery of the footshock, in order to prevent an association between the US and the context. Freezing, defined as absence of movement except for breathing, was automatically measured using the Video Freeze Software (Med Associates Inc.). The conditioning chamber was cleaned every time

using 70% ethanol before placing each mouse. CFC experiments were performed by Meng Chen. For molecular and biochemical analysis, mice were sacrificed by cervical dislocation at the specified time after training. For immunohistochemical analysis, mice were deeply anesthetized at the specified time before transcardial perfusion as described in the following section. For AAV-injected mice, CFC experiments were performed six weeks after AAV injection.

9. Stereotaxic surgery and injection of recombinant AVVs

Adeno-associated viral vectors were generated by the Vector Production Unit of the UAB Center of Animal Biotechnology and Gene Therapy (CBATEG). We used adeno-associated virus (AAV2/10) from rhesus macaque (AAVrh.10) containing AAV2 genome into AAV10 packing vectors, which has been reported to produce specific transduction of neurons (Klein et al. 2008). An AAV2 CMV-Crtc1-myc-IRES2-GFP expression cassette was generated by subcloning a Crtc1-myc construct (Kovács et al. 2007) into pVAX1 (Invitrigen) and pGV-IRES2-GFP vectors. AAV particles were generated by transfecting HEK293T cells with the AAV2 expression cassette along with the pRepAAV2/CapAAV10 and pXX6 vectors. For viral injections, 6-month old $APP_{Sw,Ind}$ mice (8 mice/group) or 4-4.5 month old PS cDKO mice, and the respective age-matched control mice, were anesthetized with isofluorane and placed in a stereotaxic instrument (Model 900, David Kopf instruments). The injection coordinates used for 6-months old mice were as follows: 0.2 cm caudal to bregma; 0.18 cm lateral to bregma; 0.2 cm ventral to dural surface. For 4-4.5 month old mice, 0.18 cm ventral to dural surface was used instead. AAV2/10-GFP or -Crtc1 viral stocks (3 µl per hemisfere; 5.1 x 10^{11} gc/ml; 0.5 µl/min) were injected bilaterally into the hippocampus, using 30 gauge Small Hub RN needles (Hamilton) and 10 µl Hamilton Gas-Tight syringes (Series 1700, Hamilton) coupled to an automated injector (KD Scientific) attached to the stereotaxic instrument.

10. Statistical analysis

Statistical analysis was performed using GraphPad Prim 5 software. A test of normality and homoscedasticity was applied to each data set before further analysis by one-way or two-way analysis of variance (ANOVA). When preliminary data was available, a power analysis was used in order to determine the required sample size using an *a priori analysis* on G*Power 3.1 software (Faul et al. 2009). For analysis of biochemical and molecular data collected from experiments performed during different days, a block design was applied to account for the effect of the different experimental conditions (cells, reagents, solutions, etc). Unless otherwise stated, data is shown as the mean ±

standard error of the mean (SEM) or standard deviation (SD). Differences with *p*-value < 0.05 were considered significant.

VII. Results

Article 1

CRTC1 Activates a Transcriptional Program Deregulated at Early Alzheimer's Disease-Related Stages

Article 2

CRTC1 function in the hippocampus during memory encoding is disrupted in neurodegeneration

Article 3

Gene expression parallels synaptic excitability and plasticity changes in Alzheimer's disease

Neurobiology of Disease

Crtc1 Activates a Transcriptional Program Deregulated at Early Alzheimer's Disease-Related Stages

Arnaldo Parra-Damas,[1,2]* Jorge Valero,[1,2]* Meng Chen,[1,2] Judit España,[1,2] Elsa Martín,[1,2] Isidro Ferrer,[2,3] José Rodríguez-Alvarez,[1,2] and Carlos A. Saura[1,2]

[1]Institut de Neurociències, Department de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Barcelona 08193, Spain, and [2]Centro de Investigación Biomédica en Red Enfermedades Neurodegenerativas (CIBERNED) and [3]Institut de Neuropatologia, IDIBELL-Hospital Universitari de Bellvitge, Universitat de Barcelona, Barcelona 08907, Spain

Cognitive decline is associated with gene expression changes in the brain, but the transcriptional mechanisms underlying memory impairments in cognitive disorders, such as Alzheimer's disease (AD), are largely unknown. Here, we aimed to elucidate relevant mechanisms responsible for transcriptional changes underlying early memory loss in AD by examining pathological, behavioral, and transcriptomic changes in control and mutant β-amyloid precursor protein ($APP_{Sw,Ind}$) transgenic mice during aging. Genome-wide transcriptome analysis using mouse microarrays revealed deregulation of a gene network related with neurotransmission, synaptic plasticity, and learning/memory in the hippocampus of $APP_{Sw,Ind}$ mice after spatial memory training. Specifically, $APP_{Sw,Ind}$ mice show changes on a cAMP-responsive element binding protein (CREB)-regulated transcriptional program dependent on the CREB-regulated transcription coactivator-1 (Crtc1). Interestingly, synaptic activity and spatial memory induces Crtc1 dephosphorylation (Ser151), nuclear translocation, and Crtc1-dependent transcription in the hippocampus, and these events are impaired in $APP_{Sw,Ind}$ mice at early pathological and cognitive decline stages. CRTC1-dependent genes and CRTC1 levels are reduced in human hippocampus at intermediate Braak III/IV pathological stages. Importantly, adeno-associated viral-mediated Crtc1 overexpression in the hippocampus efficiently reverses Aβ-induced spatial learning and memory deficits by restoring a specific subset of Crtc1 target genes. Our results reveal a critical role of Crtc1-dependent transcription on spatial memory formation and provide the first evidence that targeting brain transcriptome reverses memory loss in AD.

Key words: **β-amyloid; CREB; gene expression; memory; neurodegeneration; TORC**

Introduction

Alzheimer's disease (AD), the most common cause of dementia, is characterized pathologically by abnormal accumulation of β-amyloid (Aβ) peptides, hyperphosphorylated tau and synapse dysfunction in the brain. The earliest cognitive symptoms of the disease are temporally associated with progression of tau and amyloid pathologies from the entorhinal cortex and hippocampus to associative and temporal cortical areas (Braak et al., 2006). Memory impairments in AD transgenic mouse models are evident before accumulation of amyloid plaques (Oddo et al., 2003; Saura et al., 2005) suggesting that events downstream of Aβ contribute to synaptic changes early in the disease process. Among these events, transcriptome changes affecting cell signaling, metabolic, inflammation and neurotransmission pathways precede neuropathology in AD brains (Blalock et al., 2004; Bossers et al., 2010; Twine et al., 2011). This raises the possibility that deregulation of mechanisms controlling brain transcriptome may underlie memory loss at early AD stages.

Activity-dependent gene transcription is essential for long-lasting plastic changes in neuronal circuits encoding memory. cAMP-responsive element binding protein (CREB)-dependent transcription, which mediates neuronal excitability, synaptic plasticity, and long-lasting memory in the hippocampus (Lee and Silva, 2009), depends on the transcriptional coactivator CRTC1 (or mouse Crtc1) (Conkright et al., 2003b). In response to synaptic activity, Crtc1 translocates from the cytosol to the nucleus to increase CREB binding to specific gene promoters (Altarejos et al., 2008; España et al., 2010b; Ch'ng et al., 2012). Recent evidences suggest that Aβ negatively affect hippocampal synaptic plasticity, memory and synapse loss by deregulating $cAMP/Ca^{2+}$-mediated CREB signaling (Vitolo et al., 2002; Smith et al., 2009; España et al., 2010b). Consistently, CREB-signaling activation ameliorates learn-

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*A.P.-D. and J.V. contributed equally to this work.

Correspondence should be addressed to Dr Carlos A. Saura, Institut de Neurociències, Facultat de Medicina M2-113, Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain 08193. E-mail: carlos.saura@uab.es.

J. Valero's present address: Center for Neuroscience and Cell Biology, Universidade de Coimbra, 3004-504 Coimbra, Portugal.

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ing and/or memory deficits in transgenic AD mouse models (Gong et al., 2004; Caccamo et al., 2010; Yiu et al., 2011). These results suggest that disruption of CREB signaling may contribute to memory deficits in AD (Saura and Valero, 2011), but the specific CREB-dependent gene programs that mediate early synaptic dysfunction and memory loss in AD are unknown. A better understanding of these mechanisms is crucial for elucidating new signaling pathways for drug discovery in cognitive disorders.

To investigate the molecular mechanisms responsible for transcriptome changes during the progression of AD, we performed extensive pathological, behavioral, transcriptional, and biochemical analyses in WT and APP transgenic mice at 2–18 months of age (Mucke et al., 2000). Genome-wide transcriptome analyses were performed in naive and memory trained $APP_{Sw,Ind}$ transgenic mice at initial pathological and cognitive decline stages. Microarray and bioinformatic enrichment analyses revealed a set of CREB-dependent genes involved in synaptic function and plasticity deregulated in the hippocampus of $APP_{Sw,Ind}$ transgenic mice specifically after memory training. These transcriptional changes were associated with Crtc1 dysfunction but not CREB changes. Crtc1 overexpression in the hippocampus efficiently reversed transcriptome and spatial learning and memory deficits in $APP_{Sw,Ind}$ mice, suggesting that enhancing Crtc1 function may provide therapeutic benefits for transcriptome and memory deficits at early AD stages.

Materials and Methods

Transgenic mice and human samples. $APP_{Sw,Ind}$ transgenic mice (line J9) expressing human APP_{695} harboring the FAD-linked Swedish (K670N/M671L) and Indiana (V717F) mutations under the neuronal PDGFβ promoter were obtained by crossing $APP_{Sw,Ind}$ to nontransgenic (WT) mice. Mice used in this study were age-matched male littermate control and $APP_{Sw,Ind}$ mice (C57BL/6 background). Human brain samples were obtained from brain banks of Hospital de Bellvitge (Universitat de Barcelona, Spain) and Fundación CIEN (Instituto de Salud Carlos III, Spain). Brains samples were matched as closely as possible for sex, age and postmortem interval. Neuropathology was classified according to Braak staging for neurofibrillary tangles and neuritic plaques (Braak et al., 2006). Experimental procedures were conducted according to the Animal and Human Ethical Committee of the Universitat Autònoma de Barcelona (protocol CEEAH 1783, Generalitat Catalunya 6381) following the European Union guidelines.

Viral constructs and transcriptional assays. Lentiviral *Crtc1* shRNAs were generated by transfecting pLVTHM containing mouse *Crtc1* or scramble ShRNA, pSPAX2 and pM2G vectors in HEK293T cells as described previously (España et al., 2010b). Adeno-associated virus (AAV) AAV2/10-Crtc1-myc containing the AAV2 genome into AAV10 packing vectors and under the chicken β-actin promoter was generated by subcloning pcDNA3-Crtc1-myc (Kovács et al., 2007) into pVAX1 (Invitrogene) and pGV-IRES2-GFP vectors. AAV were generated by transfecting HEK293T cells with AAV2 recombinant, pRepAAV2/CapAAV10, and pXX6 vectors. For transcriptional assays, hippocampal neurons were infected at 3 DIV with scramble or *Crtc1* shRNA lentivirus (2 transducing units/cell) or AAV2/10-Crtc1 or AAV2/10-GFP virus (1×10^5 gc/cell). Neurons (10 DIV) were transfected with pCRE-luc (0.5 μg; Stratagene) and TK renilla (0.25 μg; Promega) plasmids using LipofectAMINE 2000 for 24 h before stimulation (FSK/KCl) for 4 h and analyzed with the dual-luciferase activity assay (Promega) in a Synergy HT luminometer (Bio-Tek; España et al., 2010b).

ChIP analysis. Chromatin immunoprecipitation (ChIP) was performed as described previously (Dahl and Collas, 2008; España et al., 2010b). Neurons (12–14 DIV) were treated with vehicle or FSK (20 μM) and KCl (30 mM) for 30 min. Cells were crosslinked with 1% formaldehyde, lysed in ChIP buffer (50 mM Tris-HCl, pH 8.1, 100 mM NaCl, 5 mM EDTA, 1% SDS, 0,1% Na deoxycholate, and protease/phosphatase inhibitors) and sonicated. DNA (2.5 μg) immunoprecipitations were performed overnight in diluted ChIP buffer (0.1% SDS, 1.1% Triton X-100) with rabbit CRTC1 and CREB antibodies or irrelevant IgGs (Cell Signaling Technology). Immunoprecipitated DNA was decrosslinked and amplified by real-time PCR using specific primers for CRE-containing promoter sequences of specific genes.

Behavioral studies and viral injections. The Morris water maze (MWM) was performed in 3 d handled mice in a circular pool (90 cm diameter; 6.5 cm hidden platform) for three or five consecutive days (4 trials daily; 60 s per trial; España et al., 2010a). Mice were tested for memory retention (probe trial) 2.5 h after training on day five, and they were killed 30 min after training. We selected this time to get a measure of memory retention while achieving a maximum peak of gene expression, which occurs ~0.5–2 h after spatial training (Guzowski et al., 2001). The swimming group moved freely in the maze without platform for 5 d and mice were killed 30 min after a simulated probe trial. For viral injections, 6-month-old mice ($n = 8$ mice/group) were anesthetized with isofluorane and placed in a stereotaxic platform (Kopf). The injection coordinates for the hippocampus were as follows: anterior 0.2 caudal to bregma; 0.18 lateral to bregma; depth 0.2 ventral to dural surface. AAV2/10-GFP or -Crtc1 viral stocks (3 μl; 5.1×10^{11} gc/ml; 0.5 μl/min) were injected bilaterally into the hippocampus. Three weeks after AAV injection mice were tested in the water maze (120 cm circular pool; 11 cm platform) for 5 d (6 trials daily; 60 s per trial), tested in a probe trial 2.5 h after training, killed, and dissected. Importantly, both maze setups lead to similar values of escape latencies during spatial training and memory retention in the probe trial test in WT and $APP_{Sw,Ind}$ mice (compare Figs. 1*C*, 4*D*). In all cases, spatial learning and memory parameters were analyzed with *SMART* software (PanLab; España et al., 2010a).

Biochemical analysis. For biochemical analysis, naive and trained mice were killed 30 min after training by cervical dislocation and whole hippocampus dissected out and immediately frozen. 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, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF) containing protease and phosphatase inhibitors (Roche). For nuclear fractionation mouse forebrains were freshly dissected, gently homogenized in ice-cold sucrose buffer A (10 mM HEPES, pH 7.5, 200 mM sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, protease/phosphatase inhibitors) and centrifuged ($1500 \times g$, 15 min). The pellet was homogenized in 1.6 M sucrose buffer, fractionated in 2.2–1.4 M sucrose gradients ($100{,}000 \times g$, 35 min, 4°C) and the pellet (nuclei) was lysed in buffer B (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, and 10% glycerol as described previously (Thormodsson et al., 1995; Saura et al., 2004). Proteins were quantified with the BCA protein assay kit (Pierce) and resolved by SDS-PAGE and Western blotting. Protein bands were quantified with the ImageJ software within a linear range of detection. The following antibodies were used: rabbit anti-Crtc1, CREB, phosphorylated CREB (Ser133), and Nur77 (Cell Signaling Technology); phosphorylated CRTC1 (Ser151; España et al., 2010b); CBP (A-22), BDNF, c-fos (4), rabbit c-myc (A-10), and c-myc (9E10) (Santa Cruz Biotechnology); rabbit APP C-terminal antibody (Saeko; aa 665–695); rabbit αAPPs antibody (1736; aa 595–611) recognizing specifically cleaved αAPPs C-terminus; Nurr1, GAPDH, β-tubulin and α-actin) (AC15) from Abcam, and lamin B1 (Zymed).

Immunohistochemical and immunofluorescence staining. For Aβ staining, sagittal brain paraffin sections (5 μm) were deparaffinized in xylene, rehydrated, and incubated with 3% hydrogen peroxide as described previously (España et al., 2010a). Sections were incubated in 60% formic acid for 6 min to allow antigen retrieval, washed in 0.1 M Tris-HCl, and incubated with anti-Aβ (6E10; 1:1000; Signet) before immunoperoxidase staining and analysis with a Nikon Eclipse 90i microscope. This staining protocol was previously shown to label specifically Aβ in APP transgenic mice (España et al., 2010a). For Crtc1-myc and Arc staining, floating sections (40 μm) were blocked in PBS containing 5% normal goat serum and 0.2% Triton X-100 and incubated with rabbit anti-myc (1:1000) and mouse anti-NeuN (1:2000; Millipore) antibodies or rabbit anti-Crtc1 (1:300) and mouse anti-myc (1:500; 9E10) or anti-Arc (1:100; Ab62142, Abcam) and the Alexa Fluor 488/594-conjugated goat secondary antibodies (1:400; Invitrogen) and Hoechst (1:10,000; Invitrogen). For Crtc1 nuclear translocation analysis, mice were trained in the MWM for 5 d and killed 30 min after training. Mice were deeply anesthetized

with pentobarbital (200 mg/kg, i.p.) and intracardially perfused with saline and 4% buffered formaldehyde. Paraffin sections (5 μm) were deparaffinized, microwave heated (10 min) in antigen retrieval citrate buffer and incubated with CRTC1 (1:300; Cell Signaling Technology) and MAP2 (1:300; Sigma-Aldrich) antibodies and Hoechst followed by AlexaFluor 488/594-conjugated goat IgGs. Images (20×; zoom 0.5) of hippocampal subregions (4 sections; n = 5–6/group) were obtained with a Zeiss LSM700 laser scanning microscope. Crtc1 nuclear staining intensity in the selected regions was measured using a sum projection of six Z-sections (1 μm)/section). Hoechst labeling was used to assign the region of interest for nuclear Crtc1 staining, whereas 2 μm area around the nucleus was considered cytoplasmic. Crtc1 nuclear/cytoplasm intensity ratio was calculated using ImageJ software (v1.47n).

Microarray and bioinformatic analyses. For microarray analyses, nontransgenic control (WT) and $APP_{Sw,Ind}$ mice (n = 3–4/group) were handled and kept in the home cage (naive) or trained in the MWM for five consecutive days and tested in a probe trial 2.5 h later. Trained mice were killed 30 min after the probe trial by cervical dislocation. Hippocampi were dissected on ice, immersed in RNAlater (R), and stored at −80°C. RNA was purified using the RNAeasy mini kit (Qiagen) and quality tested by using the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was amplified, reverse-transcribed, and fluorescently labeled with either Cy3-CTP or Cy5-CTP using the Agilent Fluorescent Linear Amplification kit (Agilent Technologies). Two individual samples labeled with Cy3 or Cy5 were hybridized (64°C, 20 h) to the mouse Genome 32K Oligo Array containing 33,696 transcripts (CapitalBio) by the Biotools Custom Microarray Service (Biotools B&M Labs). After hybridization, slides were washed, dried and scanned on a LuxScan 10K Microarray Scanner (Capitalio) and analyzed with LuxScan 3.0 Imaging and Analysis software (LuxScan Technologies). Microarray normalization was performed by the Global loess method.

Microarray data were statistically analyzed with the open source R statistical software program v2.9.2 (http://www.r-project.org) using the Linear Model for Microarray Data (Limma) package in Bioconductor (http://www.bioconductor.org/; Durinck et al., 2009). Statistical gene expression differences between the groups, calculated from measures of log2-fold-change (M values), were analyzed with a linear model and empirical Bayes using the Limma package (Smyth, 2005). The p values correction for multiple testing was performed using Benjamini and Hochberg algorithm (Benjamini and Hochberg, 1995). Statistical gene changes >1 or <-1 and corrected p values <0.05 were considered significant. Mouse microarray data are available at the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2067. Gene ontology (GO) analysis of mouse microarray data were performed with ClueGO v1.4 (Bindea et al., 2009) using the following parameters: enrichment/depletion two-sided hypergeometric statistical test; correction method: Bonferroni; GO term range levels: 3–8; minimal number of genes for term selection: 5; minimal percentage of genes for term selection: 10%; κ-score threshold: 0.5; general term selection method: smallest p value; group method: κ; minimal number of subgroups included in a group: 3; minimal percentage of shared genes between subgroups: 50%.

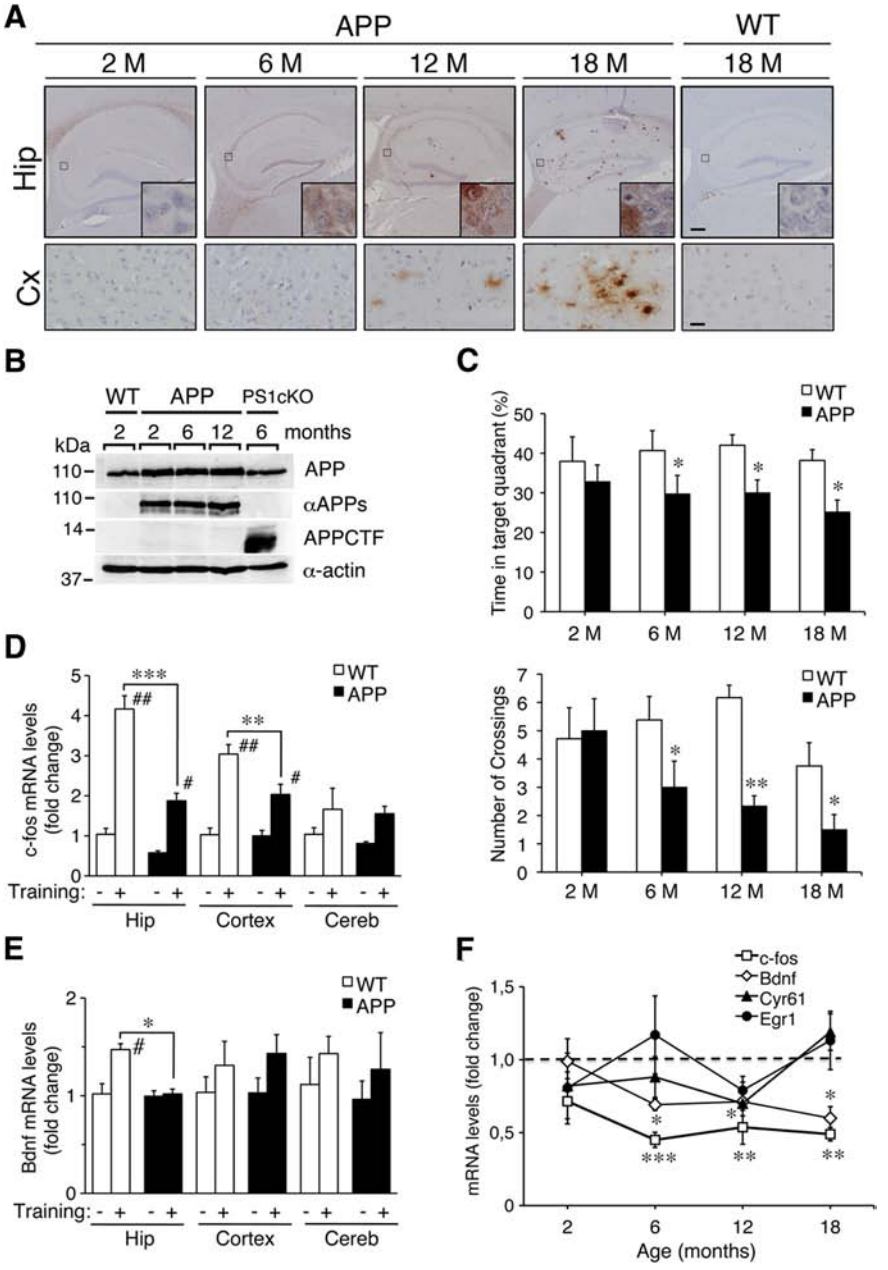

Figure 1. Age-dependent pathological, memory and gene expression changes in $APP_{Sw,Ind}$ mice. ***A***, Age-dependent amyloid pathology in the hippocampus of $APP_{Sw,Ind}$ (APP) mice. Brain sections were stained with an anti-Aβ 6E10 antibody. M, months; Hip, hippocampus; Cx, cortex. Scale bars: Hp, 250 μm; Cx, 20 μm. ***B***, Biochemical analysis of APP and APP C-terminal fragment (CTF; "Saeko" antibody) and α-secretase-derived αAPPs fragment (1736 antibody) in hippocampus of WT, $APP_{Sw,Ind}$ and presenilin-1 (PS1) conditional knock-out mouse (PS1cKO). ***C***, Age-dependent spatial memory deficits in $APP_{Sw,Ind}$ mice analyzed as number of target platform crossings and percentage time in the target quadrant in the probe test in the MWM. Data are mean ± SEM (n = 7–8 mice/group); $*p<0.05$, $**p<0.001$. ***D***, ***E***, Mice were trained for 5 d in the water maze (+) or treated identically without training (−) before analysis of *c-fos* and *Bdnf IV* mRNAs by qRT-PCR in different brain regions. Levels of mRNA were normalized to *Gapdh*. Values represent mean ± SEM (n = 4–5 mice/group); $*p<0.01$, $**p<0.001$, $***p<0.0001$ compared with controls. $\#p<0.01$, $\#\#p<0.001$ compared with nontrained. ***F***, Expression of activity-dependent genes in trained $APP_{Sw,Ind}$ hippocampus at 2–18 months. Values represent gene changes relative to trained nontransgenic controls. Data represent mean ± SEM (n = 4–6 mice/group); $*p<0.05$, $**p<0.001$, $***p<0.0001$ compared with trained controls. Statistical analyses were determined by two-way ANOVA followed by Scheffé's S *post hoc* test.

The CREB-regulated transcriptome consisted of 287 genes, which contained CRE sequences and at least a TATA box in their promoter randomly selected from the CREB Target gene database (http://natural.salk.edu/CREB), and 63 confirmed CREB target genes (Zhang et al., 2005). The CREB gene list was filtered in the whole microarray data according to the above statistical criteria. Heat maps were computed with Mayday software 2.10 (Battke et al., 2010). Differentially expressed CREB genes were submitted to an ontology term enrichment analysis using DAVID (Huang da et al. 2009). Filters used in the functional annotation clusters were established as follows: Similarity Term Overlap 3, Similarity Threshold 0.50, Initial Group size 5, final Group Membership 5, and Multiple Linkage Threshold 0.70.

Quantitative real-time RT-PCR. Human hippocampal RNA was isolated using a combination of Trizol method (Life Technologies) and the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Purified mouse RNA (1 μg; RIN $>$ 8.0) and human RNA (2 μg; RIN $>$ 6.0) were reverse-transcribed and amplified using Power SYBR Green PCR Master Mix (15 μl; Invitrogen) in an Applied Biosystems 7500 Fast system. Data analysis was performed by the comparative ΔCt method using the Ct values and the average value of PCR efficiencies obtained from LinRegPCR software. Gene expression in mouse samples was normalized to *Gapdh* or the geometric mean of three of the most stable following genes determined in each experiment: *Gapdh*, *hypoxanthine guanine phosphoribosyl transferase* (*Hprt*), *peptidylprolyl isomerase A* (*Ppia*), and β-*actin* or *TATA box binding protein* (*Tbp*; Vandesompele et al., 2002). Human genes were normalized to the geometric mean of *GAPDH, ACTB*, and *PPIA*, which were the three more stable genes found from the Human Reference Gene Panel (TATAA Biocenter AB).

Statistical analysis. Statistical analysis was performed using one-way ANOVA and Bonferroni or Student-Newman–Keuls *post hoc* tests. The behavioral data were analyzed using two-way ANOVA with repeated measures and Scheffé's S for *post hoc* comparisons by using the SuperANOVA program v1.11. Data represent the mean $\pm$ SEM. Differences with $p < 0.05$ were considered significant.

Results

Altered activity-dependent genes are associated with early memory loss in an AD mouse model

To elucidate transcriptional mechanisms underlying early memory loss in AD, we first analyzed pathological and cognitive changes in a β-amyloid precursor protein ($APP_{Sw,Ind}$) transgenic mouse that develops AD-like pathological changes (Mucke et al., 2000; España et al., 2010a). $APP_{Sw,Ind}$ transgenic mice show absence of cerebral Aβ staining at 2 months, intracellular Aβ accumulation in the hippocampus at 6 months and amyloid plaques in hippocampus and cortex at 12–18 months (Fig. 1*A*). Levels of human APP were similarly increased ($\approx$2-fold) in $APP_{Sw,Ind}$ at 2 months (1 $\pm$ 0.15-fold), 6 months (0.96 $\pm$ 0.1-fold), and 12 months (1 $\pm$ 0.1-fold; Fig. 1*B*). To examine α-, β-, and γ-secretase-mediated APP processing we performed biochemical analyses of α/β-secretase-derived soluble(s) αAPPs and APP C-terminal fragments (CTFs). Levels of αAPPs were similar, whereas APP CTFs were absent, in the hippocampus of $APP_{Sw,Ind}$ mice at 2–12 months of age (Fig. 1*B*), indicating increased Aβ but unchanged α-, β-, and γ-secretase-mediated APP processing in $APP_{Sw,Ind}$ mice during aging. We next used the MWM test to evaluate hippocampal-dependent spatial memory, a type of memory altered in AD patients at early disease stages (deIpolyi et al., 2007; Laczó et al., 2011). Two-month-old $APP_{Sw,Ind}$ and control mice showed similar escape latencies during training, as revealed by a statistically significant day effect (two-way ANOVA: $F_{(4,60)} = 15.01$; $p < 0.0001$) but no genotype effect ($F_{(1,60)} = 0.31$, $p > 0.05$), and a significant preference for the target quadrant in the probe trial (quadrant effect, $F_{(3,48)} = 9.1$, $p < 0.0001$) without significant effect of genotype ($F_{(1,48)} = 3.45$, $p > 0.05$; Fig. 1*C*; data not shown). By contrast, $APP_{Sw,Ind}$ mice starting at 6 months spent significantly longer latencies during training (two-way ANOVA, genotype effect, 6 months: $F_{(1,70)} = 21.2$, $p < 0.0001$; 12 months: $F_{(1,50)} = 59.6$, $p < 0.0001$, and 18 months: $F_{(1,70)} = 41.5$, $p < 0.0001$; Fig. 1*C*; data not shown). $APP_{Sw,Ind}$ mice developed age-dependent long-term spatial memory deficits starting at 6 months as confirmed by significant reduced target quadrant permanencies (genotype effect, $F_{(1,50)} = 13.3$; $p < 0.001$; age effect: $F_{(3,50)} = 0.46$; $p = 0.71$) and number of target crossings (genotype effect, $F_{(1,50)} = 13.5$; $p < 0.001$; age effect: $F_{(3,50)} = 3.14$; $p < 0.03$) in the probe test (Fig. 1*C*). Groups did not differ in latencies to find the visible platform or swimming speeds ruling out the possibility of visual/motor disturbances in transgenic mice.

To evaluate whether our spatial training protocol was efficient to induce expression of memory-dependent genes, we analyzed by quantitative real-time RT-PCR (qRT-PCR) the levels of activity-dependent CREB target genes, such as *c-fos* and *Bdnf*, in basal and trained conditions as previously reported (Guzowski et al., 2001). Spatial training for 3 or 5 d, but not swimming without spatial cues, induced expression of *c-fos* and *Bdnf* (*exon IV*) transcripts in the hippocampus and/or neocortex but not the cerebellum (Fig. 1*D*,*E*; data not shown). Interestingly, *c-fos* and *Bdnf* levels were significantly reduced in $APP_{Sw,Ind}$ mice after spatial training but not in basal conditions starting at 6 months (Fig. 1*D–F*). By contrast, levels of CREB target genes *Egr-1* and *Cyr61* were unchanged in naive and trained $APP_{Sw,Ind}$ mice at 2–18 months (Fig. 1*F*). These results suggested altered expression of CREB target genes regulated by spatial training coinciding with early pathological and memory changes in $APP_{Sw,Ind}$ mice.

Altered CREB-dependent transcriptome in $APP_{Sw,Ind}$ mice

To identify gene expression changes associated with early memory deficits in AD, we performed genome-wide transcriptome profile analyses by using mouse cDNA microarrays in the hippocampus of 6-month-old nontransgenic (WT) and $APP_{Sw,Ind}$ mice in two distinct experimental situations: nontrained (naive) and spatial trained conditions (Fig. 2*A*). Using a linear regression model and empirical Bayes analysis (using $-1 \geq$ log2-fold ≥ 1 and $p < 0.05$ as statistical criteria), we identified 28 genes (17 upregulated and 11 downregulated) of 33,696 transcripts represented on the mouse genome microarray differentially expressed in $APP_{Sw,Ind}$ mice in basal conditions. By contrast, 932 genes (88% downregulated and 12% upregulated) were differentially expressed in $APP_{Sw,Ind}$ mice compared with WT mice after spatial training (Fig. 2*A*). The microarray data are available in the functional genomic database ArrayExpress (www.ebi.ac.uk/arrayexpress; E-MTAB-2067). Gene-annotation enrichment analysis based on ClueGO, a computational tool that integrates GO terms as well as Kyoto encyclopedia of genes and genomes (KEGG)/ BioCarta pathways (Bindea et al., 2009), revealed a number of functional biological pathways associated with these differentially transcribed genes in trained $APP_{Sw,Ind}$ mice. The biological network with the most significant k score (> 0.5) contains 164 differentially expressed genes grouped in several functional GO terms. This network is depicted in Figure 2*B* as functional biological terms represented as nodes of different colors and sizes, which reflect the enrichment significance of the term, as well as the interrelations (indicated by connecting lines according to k score) of functionally related biological groups deregulated in spatial trained $APP_{Sw,Ind}$ mice. Interestingly, 70 genes of this network are included in five principal biological groups: learning, regulation of neurological system, long-term depression, long-term potentiation, and oxidative phosphorylation (Fig. 2*B*; Table 1). Specifically, the "learning" group is a significant term within the network because it contains five interconnected subgroups (n = number of genes): learning ($n = 10$), memory ($n = 5$), learning or memory ($n = 12$), visual learning ($n = 5$),

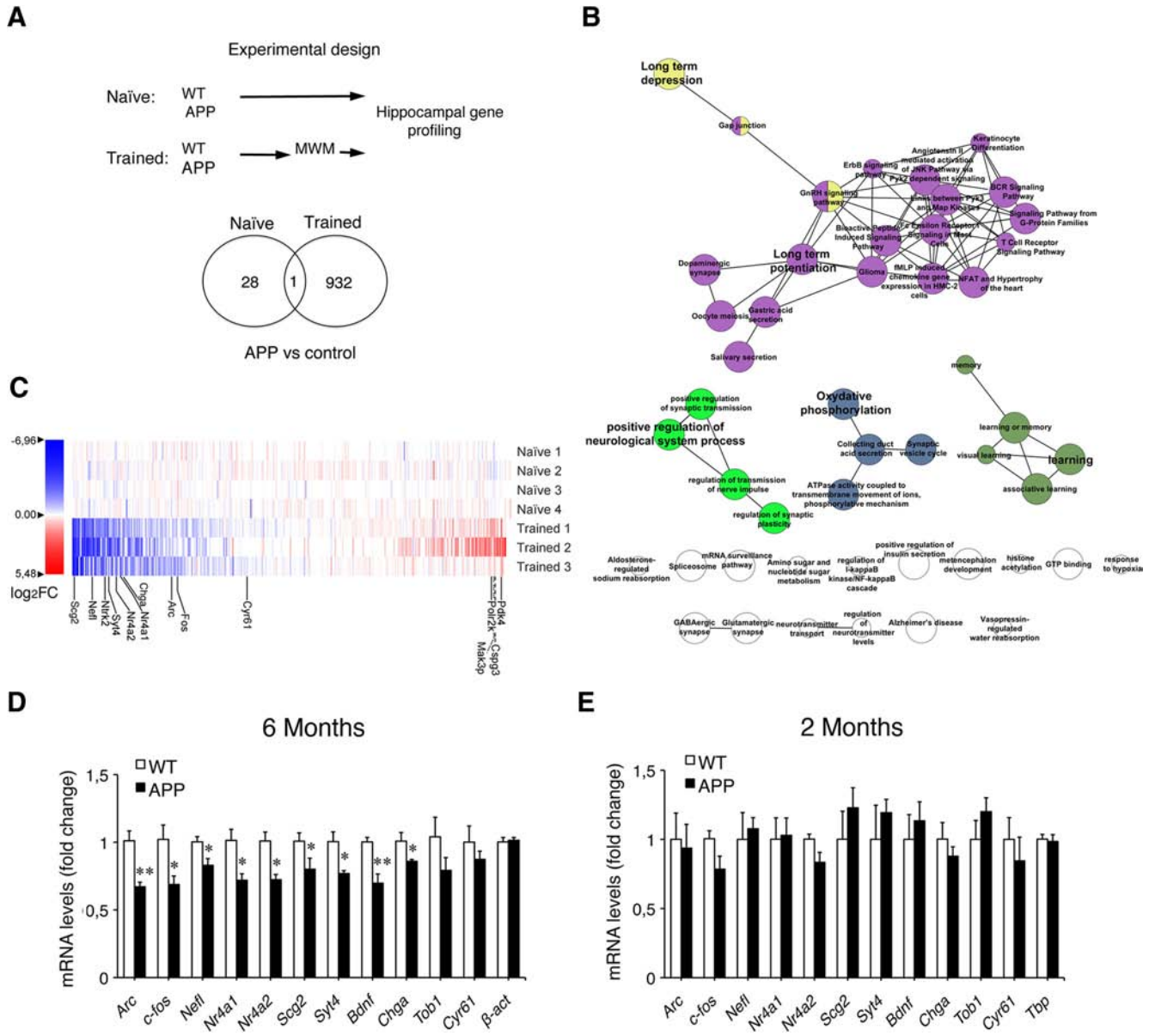

Figure 2. Hippocampal transcriptome changes in spatial trained $APP_{Sw,Ind}$ mice. ***A***, Experimental design of the groups used for gene profiling analyses (top) and Venn diagram (bottom) showing the number of genes differentially expressed in the hippocampus of $APP_{Sw,Ind}$ mice versus control mice in the microarray analysis. ***B***, ClueGO analysis of the whole gene microarray results showing the most significant functional gene network (k score > 0.5) altered in the hippocampus of spatial memory trained $APP_{Sw,Ind}$ mice compared with trained WT mice. Biological pathways are visualized as colored nodes linked with related groups based on their κ score level (≥ 0.3). The node size reflects the enrichment significance of the term and functionally related groups are linked. Not grouped terms are shown in white. ***C***, Heat map of the normalized gene data showing differential expression of CREB target genes in the hippocampus of naive (four top lines) and spatial trained (three bottom lines) $APP_{Sw,Ind}$ mice versus WT mice. Blue and red indicate genes downregulated or upregulated in $APP_{Sw,Ind}$ mice compared with WT mice. (***D***, ***E***) Expression of genes associated with neurotransmission and synaptic plasticity quantified by qRT-PCR in the hippocampus of spatial trained WT and $APP_{Sw,Ind}$ mice at 6 months (***D***) and 2 months (***E***). Values represent fold gene changes ± SEM ($n = 4$–5 mice/group). Values were normalized to the geometric mean of *Ppia, Hprt, and β-actin*. *Bdnf* refers to *Bdnf IV*; $^*p < 0.05$, $^{**}p < 0.001$ (***D***, ***E***), compared with WT control or naive. Statistical analyses were determined by one-way ANOVA followed by Bonferroni *post hoc* test.

and associative learning ($n = 7$). Notably, AD (KEGG:05010) was the functional term with the largest number of differentially expressed genes of the total of the term (10.53%; $n = 20$ genes of 190 genes of the term). However, the AD term was not linked to other groups as it was not sharing enough percentage of genes with any other term.

To identify potential CREB target genes differentially expressed after memory training, we filtered in the raw microarray data 350 genes obtained from the CREB target gene database (http://natural.salk.edu/CREB). Selected genes contained CRE promoter sequences localized within 250 base pairs from the TATA box, a distance that is required for robust transcriptional induction (Conkright et al., 2003a; Zhang et al., 2005). Whereas gene expression profile was similar between naive control and $APP_{Sw,Ind}$ mice, 49 CREB target genes (45 downregulated and 4 upregulated) were differentially expressed in spatial trained $APP_{Sw,Ind}$ mice (Fig. 2*C*; Table 2). Functional enrichment analysis using DAVID identified several biological groups associated with these genes including metabolism (15%), cell signaling (14%), cell adhesion (13%), neuronal transmission/plasticity/neuritogenesis (30%), transcriptional regulation (10%), vesicular trafficking (7%), translation (4%), cell survival (4%), and protein degradation (3%). Expression of genes related to synaptic trans-

Table 1. Genes and functional groups of the relevant gene network deregulated in the hippocampus of trained $APP_{Sw,Ind}$ mice

Group	GO term	Associated Genes (%)	Corrected *p* value	Group/term genes
1	Learning	15.38	0.0004	*Apbb1*, *Atp1a2*, *Chst10*, *Gabra5*, *Gria1*, *Hif1a*, *Mecp2*, *Neto1*, *Neurod2*, *Ntan1*, *Ptn*, *Vdac1*
2	Regulation of neurological system	25.00	0.0002	*Camk2a*, *Hras1*, *Mecp2*, *Mgll*, *Neto1*, *Neurod2*, *Ppp3ca*, *Prkce*, *Prkcz*, *Rnf10*, *Serpine2*, *Slc1a3*, *Snca*, *Ywhag*
3	Long-term depression	15.28	0.00001	*Adcy9*, *Calm1*, *Calm2*, *Camk2a*, *Camk2g*, *Gna11*, *Gnao1*, *Gria1*, *Gucy1b3*, *Hras1*, *Itpr1*, *Jun*, *Map3k4*, *Mapk1*, *Ppp2ca*, *Ppp2cb*, *Prkcb*, *Prkcc*, *Tubb3*
4	Oxidative phosphorylation	10.74	0.00001	*1110020P15Rik*, *Atp1a1*, *Atp1a2*, *Atp5o*, *Atp6v0a1*, *Atp6v0d1*, *Atp6v0e2*, *Atp6v1c1*, *Atp6v1d*, *Atp6v1g2*, *Cox15*, *Cox4i1*, *Cox7a2l*, *Cox7c*, *Cplx1*, *Ndufb2*, *Ndufb8*, *Ndufc2*, *Sdhb*, *Syt1*, *Vamp2*
5	Long-term potentiation	18.84	0.001	*Abl1*, *Adcy9*, *Atp1a1*, *Atp1a2*, *Calm1*, *Calm2*, *Camk2a*, *Camk2g*, *Crk*, *Gna11*, *Gnao1*, *Gng10*, *Gria1*, *Gucy1b3*, *Hras1*, *Itpr1*, *Jun*, *Map3k4*, *Mapk1*, *Ppp1ca*, *Ppp2ca*, *Ppp2cb*, *Ppp2r2c*, *Ppp2r5a*, *Ppp3ca*, *Ppp3cb*, *Prkcb*, *Prkcc*, *Rpl3*, *Skp1a*, *Slc12a2*, *Tubb3*, *Vamp2*, *Ywhag*
Significant nongrouped terms				
None	Alzheimer's disease	10.53	0.0002	*1110020P15Rik*, *Apbb1*, *Apoe*, *Atp5o*, *Calm1*, *Calm2*, *Capn2*, *Cox4i1*, *Cox7a2l*, *Cox7c*, *Itpr1*, *Lpl*, *Mapk1*, *Ndufb2*, *Ndufb8*, *Ndufc2*, *Ppp3ca*, *Ppp3cb*, *Sdhb*, *Snca*
None	Spliceosome	11.43	0.0007	*Cwc15*, *Dhx15*, *Hnrnpk*, *Hspa8*, *Lsm4*, *Lsm5*, *Ncbp2*, *Nhp2l1*, *Prpf19*, *Sf3a3*, *Sf3b5*, *Sfrs7*, *Snrpb*, *Snrpb2*, *Snrpd3*, *Syf2*
None	Glutamatergic synapse	10.85	0.004	*Adcy9*, *Dlgap1*, *Glul*, *Gnao1*, *Gng10*, *Gria1*, *Itpr1*, *Mapk1*, *Ppp3ca*, *Ppp3cb*, *Prkcb*, *Prkcc*, *Slc1a3*, *Slc38a1*
None	Regulation of insulin secretion	26.32	0.0143	*Hif1a*, *Nnat*, *Pfkm*, *Ppp3cb*, *Prkce*
None	GTP binding	14.81	0.017	*Arf1*, *Arf3*, *Arf5*, *Arl3*, *Gnao1*, *Hras1*, *Rraga*, *Rragb*
None	Metencephalon development	13.12	0.039	*Hspa5*, *Kat2a*, *Ldb1*, *Mecp2*, *Neurod2*, *Pfdn1*, *Sdf4*, *Serpine2*
None	GABAergic synapse	10.90	0.041	*Adcy9*, *Gabarap*, *Gabarapl1*, *Gabra5*, *Glul*, *Gnao1*, *Gng10*, *Prkcb*, *Prkcc*, *Slc38a1*

Genes are grouped according to their biological function as determined by GO analysis of the mouse microarray data using ClueGO v1.4. Associated genes indicates the percentage of changed genes of the total of genes of the term.

mission and plasticity was validated by qRT-PCR. Thus, *Arc*, *c-fos*, *neurofilament* (*Nefl*), *nuclear receptor sub 4*, *1*, and *2* (*Nr4a1*, *Nr4a2*), *secretogranin II* (*Scg2*), *synaptotagmin IV* (*syt4*), *chromogranin A* (*Chga*), *transducer of ErB-2* (*Tob1; p = 0.18*), *Rab2a* (10% decrease), and *Ptp4a1* (14% decrease) were downregulated in the hippocampus of trained $APP_{Sw,Ind}$ mice at 6 months ($p < 0.05$) but not at 2 months (Fig. 2*D*,*E*). These results suggested deregulation of a specific CREB-dependent gene program associated with early memory loss in trained $APP_{Sw,Ind}$ mice.

Activity-dependent Crtc1 transcription is deregulated in $APP_{Sw,ind}$ mice

We next investigated the molecular mechanisms underlying differential deregulation of CREB target genes in $APP_{Sw,Ind}$ mice. Biochemical analysis revealed similar levels of phosphorylated (pSer133) and total CREB (WT: 1.0 ± 0.1 vs $APP_{Sw,Ind}$: 1.2 ± 0.1-fold change) in the hippocampus of 6-month-old naive control and $APP_{Sw,Ind}$ mice ($p > 0.05$; Fig. 3*A*). Spatial training similarly enhanced CREB phosphorylation in control (1.8 ± 0.2-fold) and $APP_{Sw,Ind}$ (2.2 ± 0.4-fold) mice (one-way ANOVA, $p > 0.05$). Total levels of Crtc1 were similar in naive or trained WT and $APP_{Sw,Ind}$ mice (naive mice, WT: 1.0 ± 0.1 vs $APP_{Sw,Ind}$: 1.0 ± 0.07-fold change). By contrast, phosphorylated Crtc1 at Ser151, a site that regulates Crtc1 nuclear translocation and transcription (Altarejos et al., 2008; España et al., 2010b), was significantly increased in naive $APP_{Sw,Ind}$ mice ($p < 0.02$). Interestingly, spatial training significantly decreased Crtc1 phosphorylation in both genotypes, although levels of phosphorylated Crtc1 were significantly higher in $APP_{Sw,Ind}$ mice ($p < 0.04$; Fig. 3*A*). Reduced levels of Crtc1, but not CREB, phosphorylated CREB or CBP, were found in nuclear fractions of $APP_{Sw,Ind}$ forebrains (Fig. 3*B*). Confocal microscopy analysis revealed a clear increased of Crtc1 in the nucleus of CA3 pyramidal neurons of WT mice after spatial training (nucleus/cytoplasm ratio, trained: 1.25 vs naive: 1.0), whereas nuclear Crtc1 was reduced in trained $APP_{Sw,Ind}$ mice (~1.05; Fig. 3*C*). By contrast, Crtc1 nuclear translocation was more diffuse and sparser in dentate gyrus (DG) granular neurons and occurs only in specific CA1 pyramidal neurons in WT but not $APP_{Sw,Ind}$ mice after spatial training (Fig. 3*C*).

These results raised the possibility that Crtc1 dysfunction could cause transcriptional changes in $APP_{Sw,Ind}$ mice. To analyze this possibility, we examined whether the above CREB target genes were dependent on Crtc1. In 10 DIV primary neurons, synaptic activity significantly enhanced (~5- to 100-fold) the expression of *Arc*, *c-fos*, *Nefl*, *Nr4a1*, *Nr4a2*, *Scg2*, *Syt*, and *Bdnf*, whereas only slightly increased *Chga*, *Tob1*, and *Cyr61* levels (1.5-fold; Fig. 3*D*). A *Crtc1* shRNA (España et al., 2010b), which efficiently decreases *Crtc1* transcripts (scramble: 100 ± 10% vs Crtc1 shRNA: 23.3 ± 1.7%, $p < 0.0001$ by one-way ANOVA) and CREB transcriptional activity (scramble shRNA: 12.5 ± 0.9 *vs* Crtc1 shRNA: 5.8 ± 0.8-fold, $p < 0.0001$ by one-way ANOVA), significantly reduced expression of those genes, whereas barely affected *Tob1* and *Cyr61* (Fig. 3*D*). Interestingly, *Crtc1* transcripts were decreased by synaptic activity, suggesting that sustained neuronal activity downregulates *Crtc1* expression. Western blotting analysis confirmed increased Nr4a1 (NUR77), Nr4a2 (NURR1), c-Fos, and BDNF proteins in response to neuronal activity and their Crtc1 dependency (Fig. 3*E*). ChiP analyses using antibodies against Crtc1 and CREB (positive control) and an irrelevant IgG (negative control) demonstrated that Crtc1 is specifically recruited to the promoter regions of *c-fos*, *Nr4a1*, and *Nefl* but not *Cyr61* in an activity-dependent manner, which contrasts with binding of CREB to *c-fos*, *Nr4a1*, and *Cyr61* promoters independently of stimulus (Fig. 3*F*). Finally, CA3 pyramidal neurons expressing high Crtc1 levels show elevated Arc expression compared with neurons with low or very low Crtc1 (Fig. 3*G*).

Crtc1 overexpression rescues amyloid-induced transcriptional and cognitive deficits

The above results suggested that disruption of Crtc1 could mediate early Aβ-induced transcriptional and memory deficits. As a proof of concept, we expressed Crtc1-myc *in vivo* by using AAV

Table 2. Potential CREB target genes differentially expressed in the hippocampus of trained $APP_{Sw,Ind}$

Biological pathway/gene	Gene	GenBank	Log fold-change	*p*	Biological function
Metabolism					
Lactate dehydrogenase A	*Ldha*	NM_010699	−5.08	0.0001	Conversion of L-lactate to pyruvate
ATP synthase subunit c1	*Atp5g1*	NM_007506	−4.00	0.001	ATP synthesis
Glu oxaloacet transaminase1	*Got1*	NM_010324	−3.71	0.001	Aspartate aminotransferase activity
Enolase 2/γ-enolase	*Eno2*	NM_013509	−3.03	0.028	Glycolysis
Na^+/K^+ ATPase α1	*Atp1a1*	NM_144900	−2.37	0.01	Na^+/K^+ transport, ATP synthesis
Isopentenyl-diphosphate δ-isomer1	*Idi1*	NM_145360	−1.86	0.008	Isoprenoid biosynthetic pathway
Solute carrier family 38	*Slc38a1*	NM_134086	−1.38	0.011	Glutamine transporter
Uncoupling protein 2	*Ucp2*	NM_011671	−1.12	0.041	Mitochondria proton uncoupling
Pyruvate dehydrogenase kinase 4	*Pdk4*	NM_013743	1.78	0.019	Pyruvate metabolism
N(α)-acetyltransferase 50	*Naa50*	NM_028108	1.12	0.025	Acetyltransferase activity
Neurotransmission, plasticity, ves traff					
Neuritin/cpg 15	*Nrn1*	NM_153529	−4.34	0.001	Neuritogenesis, synaptic plasticity
Secretogranin II	*Scg2*	NM_009129	−3.76	0.007	Vesicle release, neuromodulation
Glutamate receptor GluA1	*Gria1*	NM_008165	−3.00	0.005	Neurotransmission, memory
Syntaxin 4A	*Stx4a*	NM_009294	−2.28	0.004	Docking of synaptic vesicles
Synaptotagmin IV	*Syt4*	NM_009308	−1.99	0.004	Exocitosis of synaptic vesicles
Chromogranin A	*Chga*	NM_007693	−1.50	0.033	Vesicle release, neuromodulation
RAB2A	*Rab2a*	NM_021518	−1.42	0.002	Protein transport from ER to Golgi
Syntaxin 18	*Stx18*	NM_026959	−1.40	0.042	SNAP receptor
Leucin rich repeat TM Neuronal 1	*Lrtm1*	NM_028880	−1.36	0.049	Synapse formation, axon traficking
Cell adhesion/cytoskeleton					
Claudin 5	*Cldn5*	NM_013805	−2.92	0.02	Component of tigh junctions
Neurofilament, light peptide	*Nefl*	NM_010910	−2.60	0.0001	Neurofilament member
Fibronectin 1	*Fn1*	NM_010233	−2.38	0.042	Cell adhesion and migration
Brain angiogenesis inh 1-ass.prot 2	*Baiap2*	NM_130862	−2.25	0.01	Actin cytoskeleton, neuritogenesis
Angio-associated migratory protein	*Aamp*	NM_146110	−1.69	0.003	Cell migration
Chondroitin sulfate proteoglycan 5	*Cspg5*	NM_013884	−1.64	0.013	Dendritic branching and synapses
Calsyntenin 3	*Clstn3*	NM_153508	−1.46	0.005	Cell adhesion, associative learning
Myelin oligodendrocyte glycoprotein	*Mog*	NM_010814	−1.18	0.002	Maintenance myelin sheath
Neurocan	*Ncan*	NM_007789	1.14	0.03	Neuronal adhesion, neurite growth
Cell signaling					
Protein phosph 1, reg sub 11	*Ppp1r11*	NM_029632	−2.29	0.02	Inhibitor of PP1
TrkB	*Ntrk2*	NM_008745	−2.16	0.02	BDNF, NT-3/4/5 receptor
P tyrosine phosphatase 4a1	*Ptp4a1*	NM_011200	−2.15	0.03	Protein tyrosine phosphatase
Transducer of ErbB-2.1	*Tob1*	NM_009427	−1.93	0.02	Antiproliferation, learning/memory
Regulator of G-protein signaling 4	*Rgs4*	NM_009062	−1.83	0.02	Regulates GTPase activity
Cyclin-dependent kinase 5	*Cdk5*	NM_007668	−1.32	0.002	Neurodegen., associative memory
Disabled homolog1	*Dab1*	NM_010014	−1.13	0.03	Adapter molecule, neural develop.
Cyclin-dependent kinase 16	*Cdk16*	NM_011049	−1.09	0.03	Neurite outgrowth, neuron migration
Transcriptional regulation					
Histone cluster 1, H2bj	*Hist1h2bj*	NM_178198	−3.58	0.001	Compaction of chromatin
Activating Transcriptional factor 4	*Atf4*	NM_009716	−3.29	0.004	Transcription activator, binds to CRE
Jun oncogene	*Jun*	NM_010591	−2.64	0.002	Transcription factor
Inhibitor of DNA binding 2	Id2	NM_010496	−2.3	0.032	Inhibitor of transcription factors
Nuclear receptor sub 4, 2	*Nr4a2*	NM_0113613	−1.80	0.007	Transcription factor
Nuclear receptor sub 4, 1	*Nr4a1*	NM_010444	−1.74	0.028	Transcription factor
Polymerase (RNA) II polypeptide K	*Polr2k*	NM_023127	1.013	0.042	RNA polymerase
Translation/cell survival					
Poly(A)binding protein, cytoplas. 1	*Pabpc1*	NM_008774	−3.75	0.003	Poly(A) translation, initiation
Translation initiation factor 3, sub D	*Eif3d*	NM_018749	−1.92	0.009	Component of the eIF-3 complex
Programmed cell death 7	*Pdcd7*	NM_016688	−1.43	0.025	Promotes apoptosis
Protein Degradation					
Myeloid leukemia factor 2	*Mlf2*	NM_145385	−3.03	0.0001	Protein degradation tagging activity
Ubiquitin-conjugated enzyme E2G 1	*Ube2g1*	NM_025985	−1.31	0.041	Attachment of ubiquitin to proteins
Unclassified					
Abhydrolase domain containing 11	*Abhd11*	NM_145215	−2.38	0.001	Deleted Williams-Bernes syndrome

Relative mRNA levels in the hippocampus of $APP_{Sw,Ind}$ mice compared to nontransgenic control mice after spatial training in the MWM. Relevant genes from microarray hybridizations are listed in each column, with log2-fold changes indicating relative decrease (<1) or increase (>1) of mRNA levels in $APP_{Sw,Ind}$ mice compared with controls. Genes are grouped according to their biological pathway and relative gene expression changes. A gene could be assigned to more than one biological function term. Only genes with $p < 0.05$ are given.

vector AAV2/10, a serotype characterized by high and specific gene transduction in neurons of the brain (Klein et al., 2008). AAV-Crct1 efficiently expressed functional Crtc1-myc as shown by enhancement of synaptic activity-induced Crtc1-myc nuclear translocation (data not shown) and CREB-dependent transcription by a CRE-luciferase assay in hippocampal neurons (AAV-GFP: vehicle, 0.10 ± 0.01 and FSK/KCl, 9.3 ± 2.0; AAV-Crtc1: vehicle, 0.17 ± 0.04 and FSK/KCl, 16.8 ± 4.2-fold change; one-way

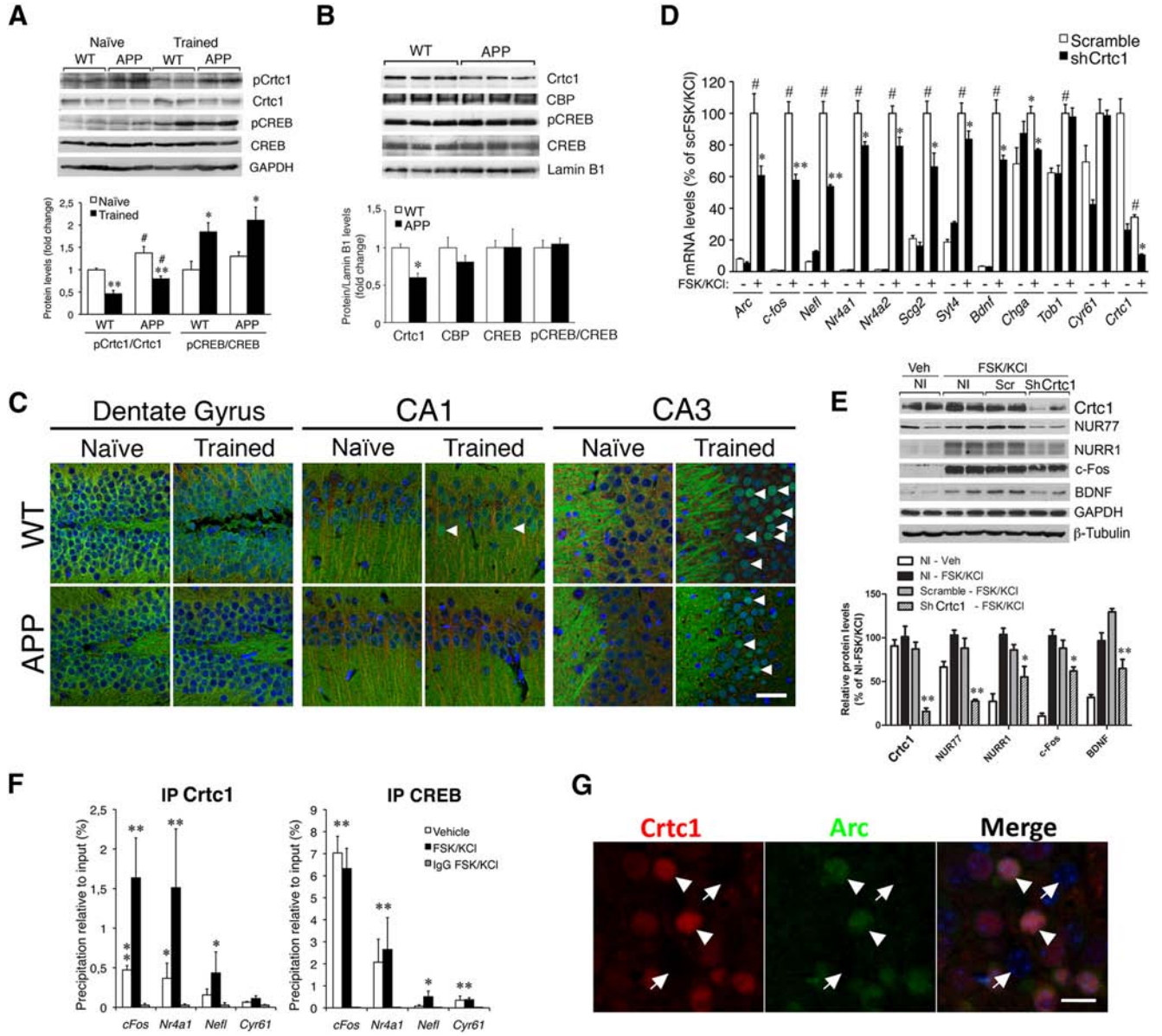

Figure 3. Reduced Crtc1 dephosphorylation, nuclear translocation and activation in $APP_{Sw,Ind}$ mice. ***A***, Biochemical analyses of Crtc1, pCrtc1 (Ser151), CREB, and pCREB (Ser133) in hippocampus of naive and memory trained control (WT) and $APP_{Sw,Ind}$ mice. Values represent fold changes ± s.e.m ($n = 4$ mice/group); $^*p < 0.05$, $^{**}p < 0.002$, and $\#p < 0.05$ compared with naive and WT mice, respectively. ***B***, Reduced Crtc1 and unchanged CBP, CREB, and pCREB in purified nuclear brain extracts of trained $APP_{Sw,Ind}$ mice. Data are the mean ± SEM ($n = 3$–4 mice/group); $^*p < 0.05$ compared with controls. ***C***, Confocal images showing localization of Crtc1 (green) and MAP2 (red) in DG, CA1, and CA3 hippocampus in naive and spatial trained mice. Nuclear translocation of Crtc1, as revealed by colocalization with Hoechst (blue; arrowheads) is more evident in CA3 hippocampal neurons of WT mice after spatial training, and reduced in trained $APP_{Sw,Ind}$ mice. CA3: Green Crtc1 staining in the left side of the images represents terminal axons from DG granular cells (mossy fibers), whereas dendritic MAP2 staining (red) is detected as punctuate staining due to its transversal position in the coronal sections. Images (20×, zoom 0.5) are representative of $n = 5$–6 mice/group. Scale bar, 40 μm. ***D***, Expression of CREB target genes in 10 DIV cultured neurons expressing scramble or *Crtc1* shRNAs treated with vehicle (−) or FSK/KCl (+). Data are normalized to *Gapdh* and represent the mean ± SEM ($n = 3$); $\#p < 0.0001$, $^*p < 0.05$, $^{**}p < 0.01$ compared with vehicle-treated or FSK/KCl-treated control neurons. ***E***, Protein levels of Crtc1-dependent genes in noninfected (NI) or scramble (Scr)- or Crtc1 shRNA-infected neurons (10 DIV; $n = 4$–5 cultures per group); $^*p < 0.05$, $^{**}p < 0.01$ compared with scramble-FSK/KCl. Values are normalized to β-tubulin. ***F***, ChiP analysis shows activity-dependent recruitment of Crtc1 to specific gene promoters. IgG, Irrelevant antibody. Data represent the mean ± SEM of three independent experiments; $^*p < 0.05$, $^{**}p < 0.005$, compared with IgG FSK/KCl IP. ***G***, Expression of Arc (green) is evident in neurons expressing high Crtc1 levels (red; arrowheads) compared with neurons with very low Crtc1 levels (arrows) in CA3 hippocampus of WT trained mice. Scale bar, 20 μm. Statistical analysis was determined by one- or two-way ANOVA followed by Student-Newman–Keuls *post hoc* test.

ANOVA, $p < 0.03$). Viral injections were localized in the septal (dorsal) hippocampus since this region is critical for spatial water-maze acquisition and memory (Moser et al., 1995). AVV-Crtc1 injection resulted in stable and long-term (>1 month) expression of *Crtc1*-myc mRNA and protein mainly in neurons of CA1 and CA3 pyramidal layers, stratum oriens, and hilus of the DG (Figs. 4*A*–*C*, and data not shown). Crtc1-myc overexpression lead to an increase of Crtc1-myc nuclear translocation in AVV-Crtc1-myc injected mice (data not shown). The performance of all groups improved significantly during spatial training in the water maze (day 1 vs day 5, $p < 0.001$), although the latencies of AAV-GFP-injected $APP_{Sw,Ind}$ mice were significantly higher than the rest of groups (two-way ANOVA, genotype effect: $F_{(3,135)} = 10.2$; day effect: $F_{(4,135)} = 45.6$; $p < 0.0001$; Fig. 4*D*). In the probe test, nontransgenic mice injected with AAV-GFP and AAV-Crtc1, and $APP_{Sw,Ind}$ mice injected with AAV-Crtc1 displayed

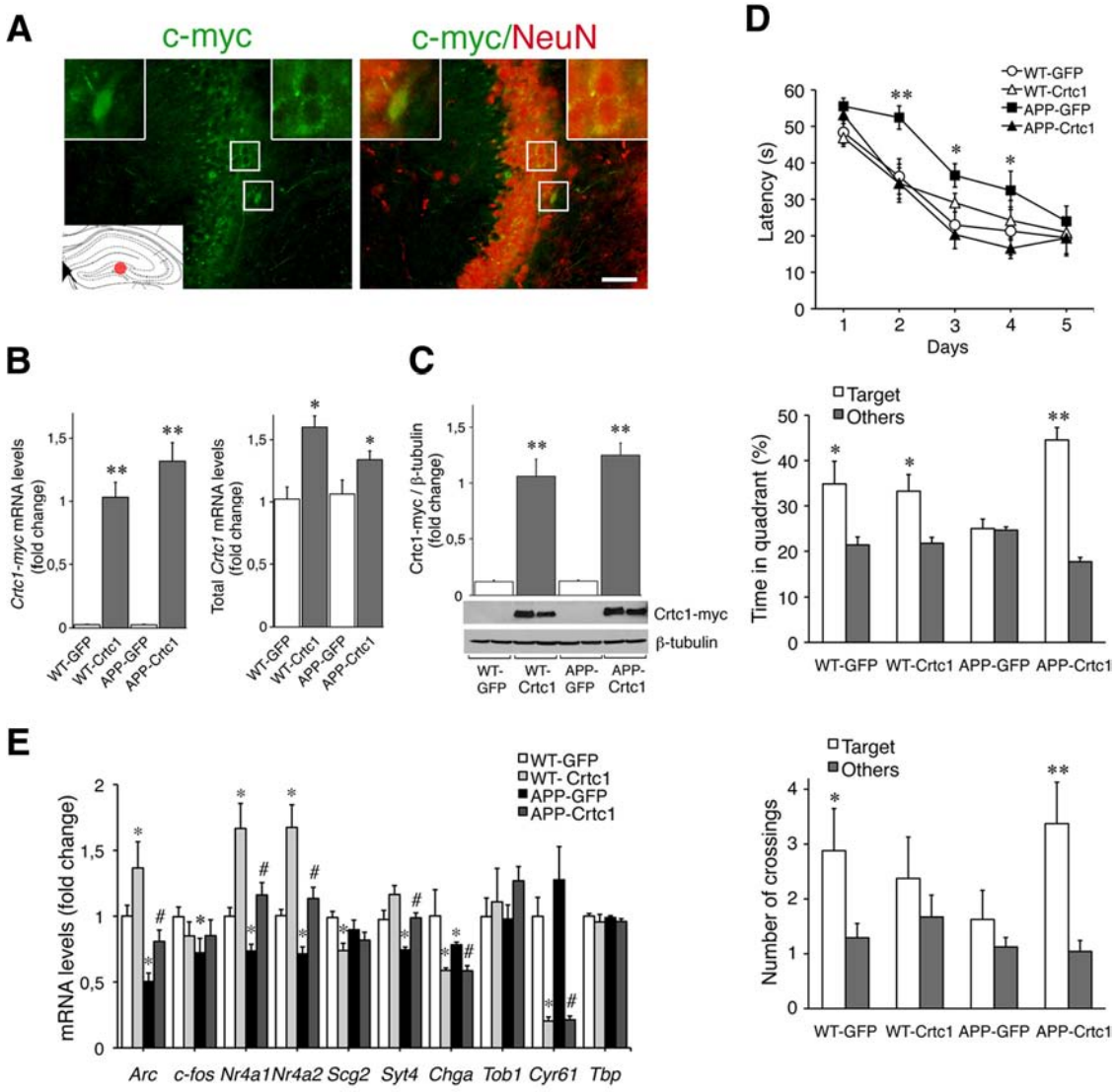

Figure 4. Adeno-associated viral-mediated Crtc1 overexpression prevents early Aβ-induced transcriptional and memory deficits. ***A***, Long-term Crtc1-myc expression in the mouse dorsal hippocampus. Overexpression of Crtc1-myc (green) in CA3 pyramidal neurons (NeuN, red) three weeks after stereotaxic intrahippocampal AAV-Crtc1-myc injection. Injection point is indicated in red in the brain diagram. Insets, Magnified images of the selected regions (square) showing Crtc1-myc localization in the neuronal nucleus (left inset) or cytoplasm (right inset). Scale bar, 50 μm. ***B***, Increased *Crtc1*-myc and total *Crtc1* mRNAs normalized to *Gapdh* in AAV-Crtc1-myc-injected mice. Data are the mean ± SEM ($n = 4$–5 mice/group); $^*p < 0.05$, $^{**}p < 0.001$, compared with AAV-GFP-injected control mice. ***C***, Crtc1-myc protein levels in injected mice. Data are the mean ± SEM ($n = 4$ mice/group); $^{**}p < 0.001$ compared with AAV-GFP-injected control mice. ***D***, Overexpression of AAV-Crtc1 rescues spatial learning (top panel) and long-term memory (middle and bottom panels) deficits in 6-month-old $APP_{Sw,Ind}$ mice. Data indicate percentage of time in the target quadrant or number of target platform crossings compared with the average of time or number of crossings in the three other quadrants, respectively. Data are the mean ± SEM ($n = 8$ mice/group); $^*p < 0.002$, $^{**}p < 0.0001$, compared with controls or the average of other quadrants as determined by two-way ANOVA. ***E***, Crtc1-dependent gene expression normalized to the geometric mean of *Gapdh*, *Hprt1*, and *Tbp* in hippocampus of AAV-injected mice. Data represents the mean ± SEM ($n = 4$–5 mice/group); $^*p < 0.05$ compared with WT-GFP; $\#p < 0.05$ compared with APP-GFP mice. Statistical analyses were determined by one- or two-way ANOVA followed by Student-Newman–Keuls *post hoc* test.

significantly higher occupancies and number of crossings in the target quadrant/platform relative to other quadrants ($p < 0.001$), whereas $APP_{Sw,Ind}$ mice injected with AAV-GFP failed to show such a preference ($p = 0.9$; two-way ANOVA, Scheffé's S *post hoc* test; Fig. 4*D*). Notably, Crtc1 gene delivery significantly increased *Arc*, *Nr4a1*, *Nr4a2*, and *Syt4* levels in control and $APP_{Sw,Ind}$ mice, which were significantly different from those of AAV-GFP-injected $APP_{Sw,Ind}$ mice, but decreased *Chga*, *Scg2* and *Cyr61* or unaffected *c-fos*, *Tob1*, and *Tbp* (Fig. 4*E*). These results demonstrated that Crtc1 efficiently ameliorates hippocampal-dependent learning and memory deficits in $APP_{Sw,Ind}$ mice by enhancing the expression of a specific subset of Crtc1 target genes.

CRTC1-dependent transcriptional changes at early AD stages

To investigate changes in CRTC1-dependent genes during the progression of AD pathology, we analyzed gene expression in the hippocampus of 68 individuals pathologically classified as controls (no pathology, $n = 16$), early (Braak I–II, $n = 22$), intermediate (Braak III–IV, $n = 14$), and advanced (Braak V–VI, $n = 16$) AD pathological cases (Braak et al., 2006). Brain samples were closely matched for age, neurofibrillary pathology, postmortem delay and RIN values (Table 3). To faithfully compare gene expression across different pathological stages, transcripts were normalized to the geometric mean of multiple reference genes (Vandesompele et al., 2002). We observed a differential pattern of

Table 3. Summary of human brain samples used in the gene expression assays

Braak stage	*n*	Sex	Age	PMD (h)	RIN
Control	16	6F/10M	49.9 ± 7..8	7.1 ± 3.6	6.22 ± 1.2
I–II	22	4F/18M	69.6 ± 10.2	6.0 ± 3.6	6.26 ± 0.9
III–IV	14	8F/6M	78.6 ± 6.3	5.0 ± 3.9	6.49 ± 1.0
V–VI	16	8F/8M	79.8 ± 7.2	7.4 ± 5.0	6.27 ± 0.9

Data are represented as mean ± SD. F, Female; M, male; PMD, postmortem delay; h, hours; RIN, RNA integrity number.

Arc mRNA expression across AD stages ($F_{(3,64)} = 4.7$, $p < 0.005$) with significant reduced levels at early (Braak I–II) and intermediate (Braak III–IV) pathological stages compared with controls (one-way ANOVA, $p < 0.02$; Fig. 5*A*). Similarly, *Nr4a2* levels were downregulated at Braak III–IV and V–VI stages compared with controls ($p < 0.04$; Fig. 5A). By contrast, *Cyr61* and *CRTC1* transcripts were not significantly changed during AD pathological progression (Fig. 5*A*). Biochemical analysis revealed a reduction of both total and phosphorylated CRTC1 in human hippocampus at Braak IV and V–VI pathological stages (Fig. 5*B*). These results indicated dysregulation of CRTC1-dependent transcription associated with decreased CRTC1 levels in human brain at intermediate Braak III–VI pathological stages.

Discussion

Gene expression changes in the brain occur at early AD stages (Blalock et al., 2004; Bossers et al., 2010; Twine et al., 2011), but whether deregulation of brain transcriptome causes memory deficits in this disease is still unclear. Genome-wide transcriptome analyses revealed significant differences in genes related to neurotransmission, synaptic plasticity, learning/memory, and oxidative phosphorylation in the hippocampus of memory trained $APP_{Sw,Ind}$ mice, whereas AD was the pathway with the highest number of changed genes relative to those of the term. Specifically, a Crtc1-dependent gene program related to synaptic function and plasticity was deregulated at early AD-related pathological and cognitive stages. Age-dependent Crtc1 transcriptional changes occurred in brain regions affected by amyloid pathology and essential for memory encoding (i.e., hippocampus), a result consistent with previous reports showing region-, neuropathology-, and age-dependent gene changes in AD (Liang et al., 2010; Twine et al., 2011). Notably, synaptic genes identified in this study, including secretogranin II, GluA1, neurofilament, synaptotagmin IV, Nr4a1, and Nr4a2 were previously reported to be reduced in AD brains or CSF (Wakabayashi et al., 1999; Ginsberg et al., 2000; Marksteiner et al., 2002), whereas others, including BDNF and chromogranin A, are altered and may be novel biomarkers at early AD cognitive stages (Li et al., 2009; Perrin et al., 2011). Similarly, *Arc* and *Nr4a2* were significantly reduced in human hippocampus at intermediate AD pathological stages. These changes are consistent with a decline of neurotransmission and plasticity genes coinciding with intraneuronal Aβ at intermediate AD stages (Bossers et al., 2010). Similarly, Crtc1-dependent gene changes coincided temporally with initial amyloid accumulation and memory deficits in $APP_{Sw,Ind}$ transgenic mice, suggesting a casual link between these events. These transcriptome alterations are likely caused by age-dependent Aβ accumulation since APP, αAPPs, or APP CTF are unchanged between 2 and 12 months in $APP_{Sw,Ind}$ transgenic mice. Therefore, we propose that changes on Crtc1-dependent genes related to synaptic function and plasticity are associated with early pathological progression and memory deficits in AD.

Our results indicate that neuronal activity and spatial memory training activate a Crtc1-dependent transcriptional program that includes, among others, genes essential for neurotransmission (*Scg2*, *Syt4*, *Rab2a*, *Chga*), synaptic plasticity and memory (*Arc*, *c-fos*, *Nr4a1*, *Nr4a2*, *Bdnf*), and neuritogenesis (*Nefl*). This result agrees with previous reports showing preferential activation of CREB transcriptional programs by neuronal activity and memory training (Guzowski et al., 2001; Benito et al., 2011). It is interesting that *Crtc1* expression is decreased by sustained neu-

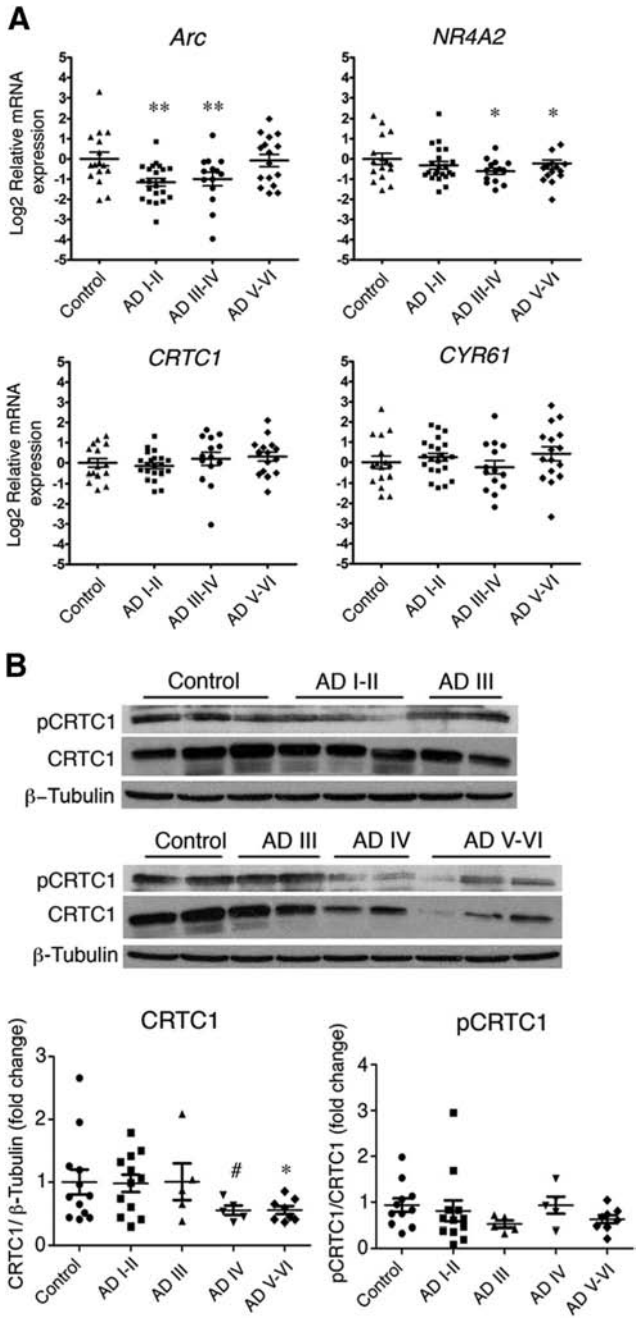

Figure 5. CRTC1 levels and transcriptional changes in human brain at intermediate AD pathological stages. ***A***, Levels of *Arc, NR4A2*, *CRTC1*, and *CYR61* transcripts in the human hippocampus at Braak 0 (Control; $n = 16$), I–II ($n = 22$), III–IV ($n = 14$), and V–VI ($n = 16$) stages. *Arc* is significantly reduced at early (I–II) and intermediate (III–IV) Braak stages compared with controls ($F_{(3,64)} = 4.7$, $p < 0.005$), whereas *NR4A2* is reduced at intermediate stages. Gene changes in log2 scale relative to controls are normalized to the geometric mean of *PPIA, GAPDH, and β-actin*. Values represent mean ± SEM; $^*p < 0.05$, $^{**}p < 0.02$, compared with controls. ***B***, Western blotting and quantification of total and phosphorylated (Ser151) CRTC1 (pCRTC1) in human hippocampus at different AD stages. Values represent mean fold change ± SEM (n = 5–12 per group); $^*p < 0.05$ compared with control as determined by one-way ANOVA followed by Scheffé's S *post hoc* test.

ronal activity suggesting that a still unknown feedback regulatory mechanism tightly controls Crtc-dependent transcription. The mechanism underlying Crtc1 activation involves Crtc1 dephosphorylation at Ser151, a motif that regulates Crtc1 nuclear translocation and function (España et al., 2010b; Ch'ng et al., 2012). Crtc1 dephosphorylation seems to regulate the induction or maintenance rather than basal CREB-dependent gene expression since efficient recruitment of Crtc1 to specific CREB gene promoters depends on synaptic activity (Fig. 3*F*). In addition, Crtc1-dependent transcription depends on the specific cellular stimulus and system, which results for instance in modest (0.5- to 4-fold) or high (5- to 100-fold) gene activation by spatial memory or *in vitro* synaptic stimulation, respectively. Similar to the effect of Crtc1 ShRNA, *Nr4a1-2* are reduced by 30–50% in the hippocampus of *Crtc1*$^{-/-}$ mice, an experimental model characterized by emotional changes (Breuillaud et al., 2012). Whether sustained Crtc-dependent transcription in our experimental conditions is due to the remaining 10–20% *Crtc1* expression, the functional redundancy of *Crtc2*, and/or alternative transcriptional mechanisms need further investigation.

Our findings also provide strong evidence that Crtc1 dysfunction is associated with hippocampal-dependent transcriptome and memory impairments. First, Crtc1-mediated transcriptional changes are evident in the hippocampus of memory trained but not naive $APP_{Sw,Ind}$ mice, which agrees with the genome-wide transcriptome results showing major gene changes after cognitive stimulation. Second, age-related CREB gene changes are specific for genes dependent on Crtc1, whereas genes activated independently of Crtc1 (*Cyr61*, *Egr1*, or *Tob1*; Ravnskjaer et al., 2007; España et al., 2010b), are unaffected in $APP_{Sw,Ind}$ mice. Although changes in *Arc*, *c-fos*, and *Nr4a1* were previously observed in APP transgenic mice (Palop et al., 2005; España et al., 2010b), its contribution to memory loss was unclear. Our results showing that Crtc1 gene transfer efficiently rescued spatial memory impairments by enhancing the expression of specific subset of Crtc1-dependent genes strongly indicate a role of Crtc1 dysfunction on memory deficits at early AD-related stages. Several mechanisms have been proposed to underlie Crtc1 dysfunction in neurons including changes on kinases or phosphatases (i.e., calcineurin), synapse-nuclear translocation, Crtc1 acetylation, or CREB glycosylation (España et al., 2010b; Jeong et al., 2012; Rexach et al., 2012; Ch'ng et al., 2012). Our results suggest that impaired Crtc1 dephosphorylation at Ser151 and nuclear translocation in the CA1/CA3 hippocampus may result in Crtc1-dependent transcriptional changes. However, we cannot rule out the possibility that altered Crtc1 levels in advanced AD stages or disrupted activation of CREB might also contribute to CREB transcriptional changes in AD (Pugazhenthi et al., 2011).

Previous studies showed that pharmacological and genetic activation of CREB signaling ameliorate synaptic and memory deficits in AD transgenic mice (Vitolo et al., 2002; Gong et al., 2004; Smith et al., 2009; Caccamo et al., 2010; Yiu et al., 2011). Similarly, Crtc1 gene transfer reversed early Aβ-induced Crtc1 transcriptome changes and spatial memory deficits. Notably, enhancing expression of a specific subset of CREB target genes was sufficient to reverse learning and memory deficits in APP mice. By contrast, Crtc1 overexpression *in vivo* did not affect *c-fos* or decreased CREB target genes activated independently of Crtc1, such as *Cyr61* and *Chga*. This differential effect on gene transcription could be due to the preferential binding of Crtc1/CREB to specific Crtc1 target promoters (i.e., *Arc*, *Nr4a1*, *Nr4a2*…) in detrimental of Crtc1-independent CREB promoters (Zhang et al., 2005), epigenetic changes caused by binding of Crtc1 to the CBP/CREB complex (Ravnskjaer et al., 2007), and/or a differential timing of RNA polymerase II occupancy over specific promoters and enhancers (Saha et al., 2011). However, spatial learning and memory were similar in WT mice after Crtc1 delivery, a result that contrasts with enhancement of contextual memory by Crct1 overexpression (Sekeres et al., 2012). This apparent discrepancy could be due to distinct neural circuits involved in these memory tasks (associative vs spatial) and different gene-delivered vectors (Herpes simplex virus vs adeno-associated virus) targeting different neuronal populations. Finally, synapse loss and dysfunction tightly correlates with cognitive decline at initial AD stages (Terry et al., 1991; Masliah et al., 2001). Because Crtc1 regulates expression of multiple proteins involved in synaptic morphology, function, and plasticity, our results raise the possibility that Crtc1 dysfunction underlies synapse dysfunction in AD. In conclusion, targeting specifically Crtc1, instead of affecting globally CREB signaling, can represent a novel therapeutic avenue to ameliorate transcriptome, synaptic, and cognitive changes at early AD stages.

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Corresponding Author: Dr. Carlos A. Saura, Ph.D.

Corresponding Author's Institution: Universitat Autónoma de Barcelona

First Author: Arnaldo Parra-Damas, BS

Order of Authors: Arnaldo Parra-Damas, BS; Meng Chen, Ph.D.; Lilian Enríquez-Barreto, Ph.D.; Laura Ortega, Ph.D.; Sara Acosta, BS; Neus Fullana, BS; José Aguilera, Ph.D.; José Rodríguez-Alvarez, Ph.D.; Carlos A. Saura, Ph.D.

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

Methods: We evaluated the activation of CRTC1 in the hippocampus after one trial contextual fear conditioning in control mice and in a knockout mouse model of neurodegeneration lacking the Alzheimer´s disease-linked presenilin genes (PS cDKO) by using biochemical, immunohistochemical and gene expression analyses.

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 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 nuclear translocation and transcriptional function are associated with long-term contextual memory deficits in PS cDKO mice. Importantly, CRTC1 gene therapy in the hippocampus of this model ameliorates context memory and transcriptional deficits and dendritic degeneration despite ongoing cortical degeneration.

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

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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], 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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Abstract

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Introduction

Alzheimer´s disease (AD) is a neurodegenerative disorder characterized by cognitive disturbances, progressive amnesia and neuropsychiatric symptoms. 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 (fMRI) 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 synapse, dendritic and neuron degeneration, in these brain regions essential for memory encoding (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 (10). Dementia patients develop associative memory impairments in fear conditioning (11, 12). Fear conditioning tasks depend on a neural circuitry that includes the hippocampus, amygdala and prefrontal cortex. The hippocampus encodes context representations and sends projections to the amygdala, which encodes, stores and retrieves contextual cues associated with aversive stimulus (13, 14). Whereas different hippocampal regions (CA3, CA1 and dentate gyrus) contribute to acquisition of fear contextual memory (15, 16), the CA3 subregion is activated during associative encoding and critical for initial context representations (17, 18). Besides participating in adaptive behavior, fear conditioning is implicated in the mechanisms that mediate psychopathological fear and anxiety (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 glucose metabolism, long-term synaptic plasticity and dendritic growth (27, 29, 30). Disruption of CREB/CRTC association impairs CREB-dependent transcription, synaptic plasticity and long-term memory (31), whereas CRTC1 dysfunction causes transcriptional changes leading to memory impairments in an AD mouse model (32, 33). CRTC1 regulates fasting-mediated appetitive long-term memory in *Drosophila* and fear memory consolidation through still unclear downstream mechanisms (34-36). 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

Male C57BL/6 mice at the age of 2 or 6 months were used. *PS* cDKO mice (C57BL/6/129 hybrid background) lack expression of both *PS* genes (*PS1* and *PS2*) in forebrain glutamatergic neurons (37). Littermate control (WT; f*PS1*/f*PS1*; *PS2*+/+ or f*PS1*/f*PS1*; *PS2*+/-) and *PS* cDKO mice (f*PS1*/f*PS1*; *PS2*-/-; CaMKIIα-Cre) 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*+/-; CaMKIIα-Cre). Experimental procedures were conducted according to the Animal and Human Ethical Committee of the Universitat Autònoma de Barcelona (protocol CEEAH 1783, Generalitat de Catalunya 6381) following the European Union guidelines (2010/63/EU).

Behavioral studies

For contextual fear conditioning, mice handled for three days (3min/day) were placed in a conditioning chamber (15.9 x 14 x 12.7 cm; Med Associates Inc., 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 in the same conditioning chamber for 4 min 2 h or 24 h after training using *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 group were shocked and immediately returned to their home cages. For biochemical or immunohistochemical analysis 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 AAV2 genome into AAV10 packing vectors transduce mainly neurons (39). AAV2/10-Crtc1-myc was generated by subcloning pcDNA3-Crtc1-myc (27) into pVAX1 (Thermo Fisher Scientific, USA) and pGV-IRES2-

GFP vectors as described (33). For viral injections, control mice were injected with AAV-GFP while the treated group was administered with AAV-Crtc1-myc. Briefly, 4-4.5 month-old mice (n= 6-8 mice/group) were anesthetized with isofluorane and injected bilaterally into the dorsal hippocampus (3 µl; $5.1x10^{11}$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 in 24 h-contextual fear conditioning, sacrificed and brains processed for histological and biochemical analyses six weeks after injection.

Gene expression analysis

For *Crtc1* silencing, ShRNA lentiviral particles were generated by transfecting HEK293T cells with pSPAX2, pM2G and pLVTHM vectors containing *Crtc1* or scramble ShRNAs as described (32). Primary neurons (4 DIV) were infected with scramble or *Crtc1* ShRNAs lentiviral vectors (1–2 transducing units/cell) and treated at 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 in pCRE-luc/TK Renilla transfected neurons by using the dual-luciferase activity assay (Promega, San Luis Obispo, CA) as described (32).

RNA from cultured neurons or hippocampal tissue 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^{TM} 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 (40). 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 using the NormFinder, BestKeeper and geNorm algorithms (41)

Biochemical analysis

Tissue was sonicated 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 8-14% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes before blotting with the following antibodies: rabbit anti-CRTC1 (1:5,000), CREB (1:250), phosphorylated CREB (Ser133) (1:1,000; Cell Signaling, Danvers, Massachusetts), phosphorylated CRTC1 (Ser151; 1:1,000) (32) and mouse anti-GAPDH (1:5,000; Abcam, Cambridge,UK). Protein bands were quantified with ImageJ software.

ChiP-qPCR analysis

Chromatin immunoprecipitation (ChIP) was performed essentially as described (33). 12 DIV cortical neurons were treated with vehicle or FSK (20 μM) and KCl (30 mM) for 15 min. Cells were crosslinked with 1% formaldehyde, lysed 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) and chromatin was sheared between 200 and 500 bp by sonication using a BioruptorPlus (Diagenode, Seraing, Belgium). Fragmented chromatin 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 monoclonal rabbit anti-CRTC1 and CREB antibodies (Cell Signaling). 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; EMD Millipore, Billerica, Massachusetts) 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.

Confocal image acquisition and analysis

Images (20x; zoom 0.5) were obtained with a Zeiss Axio Examiner D1 LSM700 laser scanning microscope (Carl Zeiss Microcopy, Jena, Germany) and analyzed with ImageJ software (v.1.6x). Briefly, 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. The nuclear/cytosol CRTC1 staining intensity ratio was used as a measure of CRTC1 nuclear translocation. CRTC1 translocation was analyzed in caudal, medial and rostral regions using three/four sections per region of each animal (n=3-5 mice/group). Dendritic CRTC1 was analyzed by quantifying colocalization of CRTC1 staining in MAP2-stained dendrites in two brain hemispheres of the rostral CA3 hippocampus of multiple mice (n= 8/group; n=3 sections/mouse).

For dendritic fiber thickness, MAP2 staining intensity in a sum projection of five Z-sections (1 μm/section) was measured by using ImageJ (n=3 sections/mouse; n=4-6 mice/group). Briefly, maximum projection was transformed to 8 bits in black and white and the resulting image was divided

into a grid to establish the region of interest (CA3 region). Three grid lines intersecting perpendicularly the MAP2 fibers within the image were used to measure automatically the thickness of the fibers by generating a Plot Profile of the intensity of the pixels and peak thickness (fiber thickness) along the lines (ImageJ). Cortical thickness of somatosensory cortex was measured (ImageJ) in multiple images (n=3/section) of Nissl stained brain sections (n=4-5 mice/group) captured with a Nikon Eclipse 90i microscope (Nikon Instruments, Badhoevedorp, The Netherlands).

Statistical analysis

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

Results

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

To investigate the regulation of CRTC1 by associative learning, we first analyzed CREB and CRTC1 activation in the hippocampus, a region essential for early context representation during associative memory encoding (17, 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 (training effect: $F_{(3, 42)}= 9.26$, $P = 0.0001$) (**Figure 1B**). Consistent with previous reports (23, 43), CREB phosphorylation at Ser133 was increased in the hippocampus after context or context plus shock (**Figure 1C**). Interestingly, CRTC1 phosphorylation at Ser151, which leads to CRTC1 inactivation (30, 32), was significantly decreased 15 min and 2 h after contextual training ($F_{(4, 16)} = 4.34$, $P = 0.01$) (**Figure 1C**).

To explore the possibility that CRTC1 dephosphorylation could mediate CREB-dependent transcription in the hippocampus during associative learning, we examined the levels of several CREB target genes, including *c-fos* and *Nr4a 1*, *2* and *3*, implicated in contextual learning (44). Contextual fear conditioning induces a significant overall effect on hippocampal levels of *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* ($F_{(5,30)} = 0.8$ $P = 0.55$) (**Figure 2A**). *c-Fos*, *Nr4a1* and *Nr4a2* transcripts, but not *Nr4a3,* are increased after fear conditioning but not by context or shock alone (**Figure 2A**). Since *Crtc1* ShRNA significantly decreases transcripts levels of *c-fos*, *Nr4a1* and *Nr4a2* but not *Nr4a3* (**Figure 2B**), we explored the possibility that CRTC1 could bind differentially to the promoter regions of *Nr4a* genes. Quantitative 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* but not to the CRE-TATA-deficient region of *Nr4a3* (**Figure 2C**; data not shown). 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.

Previous studies have shown that CRTC1 activation is mediated by activity-dependent CRTC1 dephosphorylation and nuclear translocation (33, 36, 45). 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 (**Figures 3A and 3C;** $P > 0.05$). Interestingly, CRTC1 is abundantly localized in the nucleus of CA3 pyramidal neurons and moderately in CA1 neurons 15 min after contextual fear conditioning (**Figures 3A and 3C**). Indeed, CRTC1 colocalizes with MAP2 in dendrites of CA3 hippocampal neurons in naïve conditions, whereas CRTC1/MAP2 colocalization is significantly reduced 15 min after CFC ($P < 0.02$; **Figures 3B and 3C**). 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 during contextual fear memory deficits in neurodegeneration. *PS* cDKO mice lacking both *presenilin* (*PS*) genes (*PS1* and *PS2*) in neurons of the postnatal forebrain develop age-dependent memory and synaptic plasticity deficits prior to cortical degeneration (37). At 2 months, control (WT) and *PS* cDKO mice display similar freezing responses 2 h and 24 h after contextual fear conditioning (training effect: $F_{(3,72)} = 22.6$, $P = 0.0001$; genotype effect: $F_{(1,72)} = 0.005$, $P = 0.94$) (**Figure 4A**).

At 6 months of age, *PS* cDKO mice show reduced freezing responses 2 h and 24 h after training (training effect: $F_{(3,121)} = 25$, $P = 0.0001$; genotype effect: $F_{(1,121)} = 21$, $P = 0.0001$) (**Figure 4A**), which indicates short- and long-term contextual memory deficits. *Nr4a1* and *Nr4a2* transcripts, but not *Nr4a3,* are significantly increased 24 h after contextual learning in control and *PS* cDKO mice (time effect: *Nr4a1*: $F_{(2, 29)} = 33.0$, $P < 0.0001$; *Nr4a2*: $F_{(2, 29)} = 27.9$, $P < 0.0001$; *Nr4a3:* $F_{(2, 29)} = 2.5$, $P = 0.1$), but with differences between genotypes (genotype effect, *Nr4a1*: $F_{(1, 29)} = 18.1$, $P = 0.0002$; *Nr4a2*: $F_{(1, 29)} = 14.8$, $P = 0.0006$; *Nr4a3:* $F_{(1, 29)} = 14.6$, $P < 0.001$) (**Figure 4B**). *Post hoc* analysis revealed a significant reduction of *Nr4a1* and *Nr4a2* transcripts, but not those of *Nr4a3*, in the hippocampus of 6 month-old *PS* cDKO mice at 24 h. These results demonstrated age-related contextual memory impairments associated with CRTC1-dependent genes changes 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 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 at 15 min (genotype effect: $F_{(1, 24)} = 4.03$, $P = 0.05$; **Figures 5A and 5C**). Moreover, CRTC1 is significantly decreased in dendrites in control but not *PS* cDKO mice 15 min after contextual learning (**Figures 5B and 5C**). 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 stably expressed CRTC1 *in vivo* by using adeno-associated virus (AAV) 2/10, a serotype that allows stable long-term (>2 months) neuronal gene expression (33, 39) and enhances nuclear translocation of CRTC1-myc and CRE-dependent transcription in cultured neurons

(**Supplementary Figure S1**). AAV-GFP (control) and AAV-Crtc1-myc were injected in the CA3 hippocampus of 4-4.5 month-old WT (control) and *PS* cDKO mice, and six weeks later mice were evaluated in CFC. AAV-Crtc1-myc injection allowed high expression of CRTC1-myc mRNA and protein mainly in neurons of CA1, CA3 and dentate gyrus (**Figures 6A, 6B** and **Supplementary Figure S2**). We found 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 ($F_{(3,42)} = 3.5$, $P < 0.02$, 2 way ANOVA). Compared to AAV-GFP injected mice, AAV-Crtc1 increases significantly freezing responses both in WT ($P < 0.05$) and *PS* cDKO mice ($P < 0.03$) 24 h after training (**Figure 6C**). 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 ($P < 0.05$; **Figure 6D**). 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 hippocampus and neocortex, which result in reduced cortical thickness and enlargement of lateral ventricles during aging (37, 46). AAV-Crtc1 injection in the hippocampus does not affect cortical thickness and enlargement of lateral ventricles in *PS* cDKO mice (**Figures 7A and 7C**). Confocal imaging analysis reveals reduction of dendritic MAP2-stained fibers in the neocortex and CA3 hippocampus of *PS* cDKO-GFP mice (**Figures 7B and 7C**). AAV-mediated CRTC1 overexpression in the hippocampus does not apparently affect dendrite morphology in the neocortex but significantly increases intensity and thickness of MAP2-stained dendrites in CA3 hippocampus of *PS* cDKO mice (**Figures 7B and 7C**). 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 (47-50). However, the CREB-dependent transcriptional programs and their regulatory mechanisms that mediate associative memory encoding have not been identified. 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. CRTC1 activation during contextual memory encoding involves dephosphorylation and translocation of CRTC1 from the cytosol and dendrites to the nucleus of hippocampal neurons. Contextual learning, but not context or shock alone, induces preferentially CRTC1 nuclear translocation in CA3 hippocampus, and to a minor extent in CA1 region (not shown). This result suggests that CRTC1 activation in the hippocampus can mediate rapid spatial context acquisition during memory encoding. This idea is supported by previous findings indicating that spatial memory induces CRTC1 nuclear translocation in the hippocampus (33), and that CRTC1 expression in the dorsal hippocampus enhances contextual fear memory (35, 36) (**Figure 6C**). These results also agree with previous findings showing that 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 (36), which is consistent with a role of this circuit in associating contextual cues with aversive events (13, 14). 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 (17, 51).

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. Specific CREB target genes (i.e. *c-fos, Nr4a1* and *Nr4a2*) are significantly induced in the hippocampus by context conditioning, that is in conditions that activate CRTC1, but not by shock or context alone. Previous findings showed induction of *Nr4a* genes by contextual learning in the hippocampus, whereas blocking NR4A function impairs contextual memory (44, 52). Indeed, we found a time-dependent differential induction of the CREB target *Nr4a* family genes *Nr4a1-3* in response to synaptic activity and memory training. Activity-dependent recruitment of CRTC1 to CRE-TATA sequences proximal to the transcription starting sites may explain their preferential transcription by CRTC1. Our results suggest that a mechanism by which contextual conditioning induces CRTC1-mediated transcription may involve CRTC1 dephosphorylation at Ser151, a critical event for activity-induced CREB-mediated transcription (30, 32). 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 (36). Other alternative mechanisms may include kinase/phosphatase activities, synapse-nuclear translocation, acetylation or CREB glycosylation (31, 32, 45, 53).

Genetic and biochemical evidences suggest a role of CREB signaling in cognitive and neurodegenerative disorders (54). Of relevance, 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. Memory deficits in *PS* cDKO mice were previously associated with changes on CREB-dependent genes caused by CBP dysfunction (37), which is consistent with fear memory deficits observed in CBP-deficient mice (55-58). 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/CREB-dependent transcriptional deregulation and CREB/CRTC1 dysfunction are associated with cognitive deficits and neurodegeneration in Huntington´s disease (53, 59). 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 (60, 61). Since *Nr4a* genes (i.e. *Nr4a2*) are downregulated in sporadic AD and Parkinson´s disease brains and mouse models (62), our result may have important pathological and therapeutic implications in neurodegenerative diseases.

Do CRTC1-dependent transcription changes contribute to associative memory deficits in neurodegeneration? Our previous studies indicated that CRTC1-dependent transcriptional deficits were associated with early pathological and memory changes in APP mice (33), an AD mouse model that does not develop neurodegeneration (63-65). Our gene therapy strategy indicates that enhancing CRTC1 function in the hippocampus ameliorates long-term contextual memory deficits in *PS* cDKO mice during neurodegeneration. 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 (66).

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 (2, 3, 5-7), 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 and experimental mouse groups used in this study. ***B***, Freezing responses of 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. ***C***, 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. GAPDH was used as loading control. 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 month-old mice in naïve, context, shock and CFC groups were analyzed by qRT-PCR. Values are normalized to the geometric mean of *Gapdh*, *Hprt1* and *Ppia*. Data represent mean ± s.e.m (n=4-6 mice/group). ***B***, CRTC1 regulates expression of CREB target genes in an activity-dependent manner. Western blot analysis of CRTC1 (left) and qRT-PCR analysis of CREB target genes normalized to *Gapdh* (right) in cultured hippocampal neurons treated with scramble or *Crtc1* ShRNA in the presence of vehicle or FSK/KCl. Data are the mean ± s.e.m of three independent experiments. ***C***, ChIP analysis with anti-CRTC1 (left) and anti-CREB (right) antibodies of *c-fos*, *Nr4a1*, *2* and *3* 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) 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) 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 expression of CRTC1 (green) in CA3 hippocampus. Dendritic MAP2 staining (red) is detected as punctuate staining pattern due to its transversal position in coronal sections. Insets: magnified images of the selected square regions showing colocalization (yellow) of CRTC1 in MAP2 fibers in naïve conditions and its redistribution to the nucleus (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 of intensity values of multiple mice (nucleus: n=4-5 mice/group, n=6-12 sections/mouse; dendrites: n=8 mice/group, 4-6 sections/mouse). *$P < 0.05$, ** $P < 0.01$ 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, n=5-24) and *PS* cDKO (n=5-20) mice at 2 or 6 months of age tested in contextual fear conditioning. ***B*,** Hippocampal levels of mRNAs in naïve and CFC trained WT and *PS* cDKO mice at 6 months of age. mRNA levels were quantified by real-time qRT-PCR and normalized to the geometric mean of standard genes *Gapdh*, *Hprt1* and *Ppia*. Values represent mean of fold changes ± s.e.m (n=4-6 mice/group). *$P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, compared to naïve mice. Statistical analyses were performed by two-way ANOVA followed by Scheffé´s S (A) 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. 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 one- or 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-mediated CRTC1 expression in 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. 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*,** Contextual fear conditioning in control and *PS* cDKO mice (n=6-7 mice/group) six weeks after AAV-GFP and -Crtc1 injection. ***D***, Levels of *Nr4a1* and *Nr4a2* transcripts in the hippocampus of AVV-GFP or-Crtc1 injected mice. Values are normalized to the geometric mean of *Gapdh*, *Hprt1* and *Ppia*. Data represents mean ± s.e.m (n=4-6 mice/group). **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*,** Nissl staining of neocortex (Cortex; top) and hippocampus (bottom) showing reduced cortical thickness (dashed lines) in AAV-GFP- and -Crtc1 injected *PS* cDKO mice. Scale bar: 200 μm. ***B, Dendritic degeneration is reduced after AAV-mediated CRTC1 expression in *PS* cDKO mice.

Confocal images showing MAP2-stained fibers (red) in the neocortex (top) and CA3 hippocampus (middle and bottom) in brain sections of GFP- and Crtc1-injected mice. Magnified dendrites in CA3 region are shown at the bottom images. Scale bars: 20 μm (Cortex) or 10 μm (CA3). ***C***, Quantification of cortical thickness (left), total MAP2 staining intensity (middle) and dendrite thickness (right) in WT and *PS* cDKO groups. 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. #*P* = 0.063. Statistical analyses were determined by one-way ANOVA and Scheffé´s S post hoc test.

Figure 1

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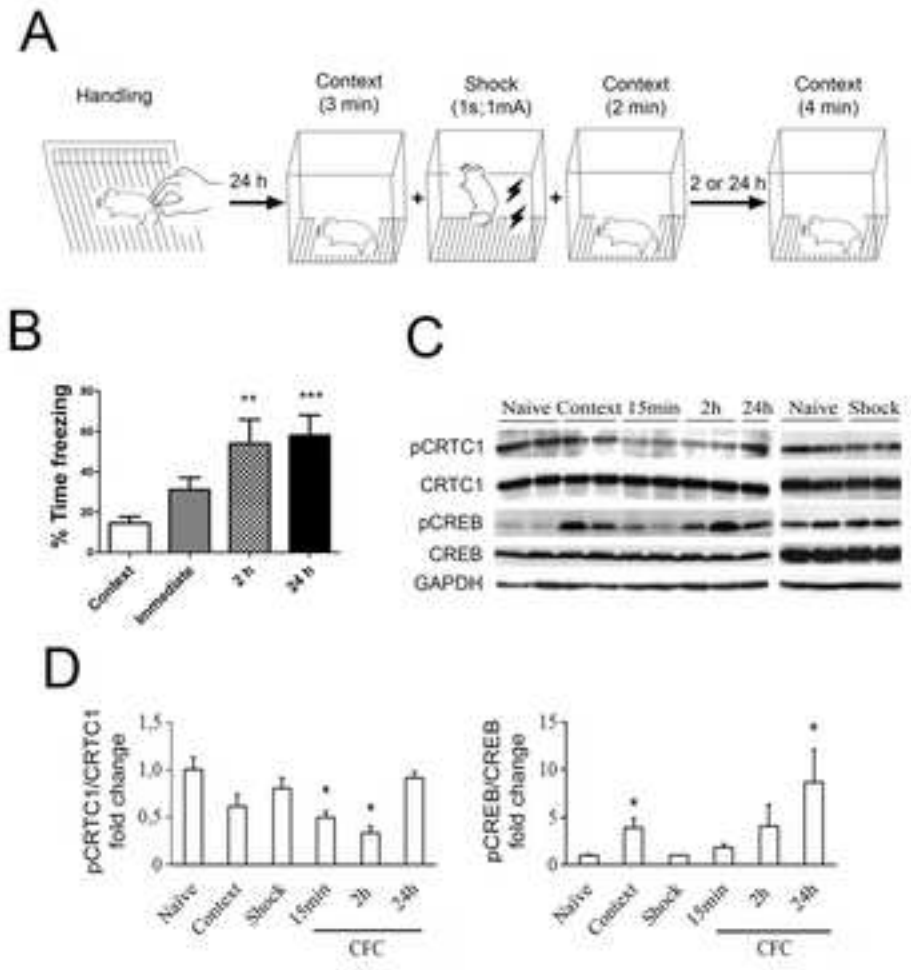

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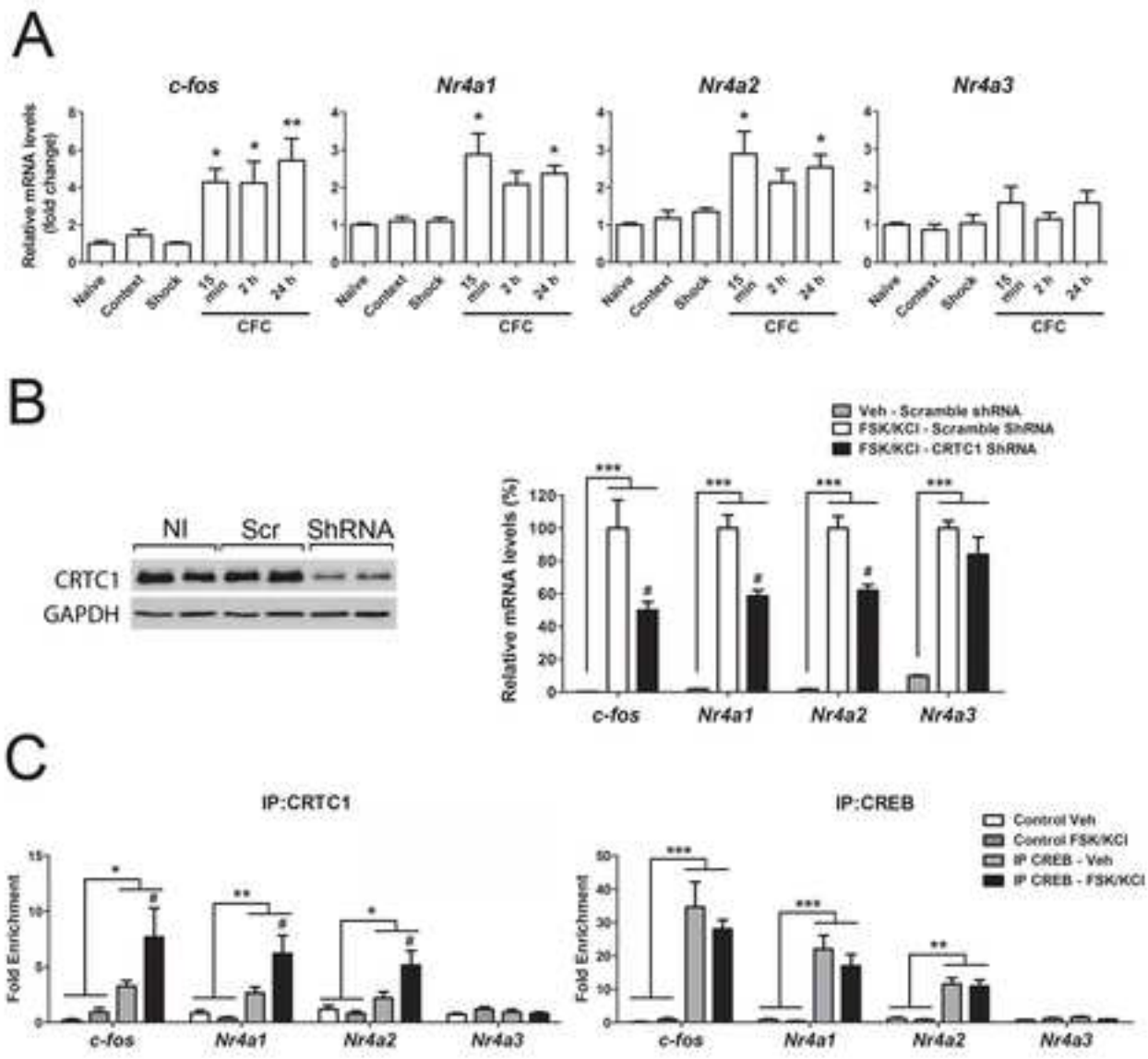

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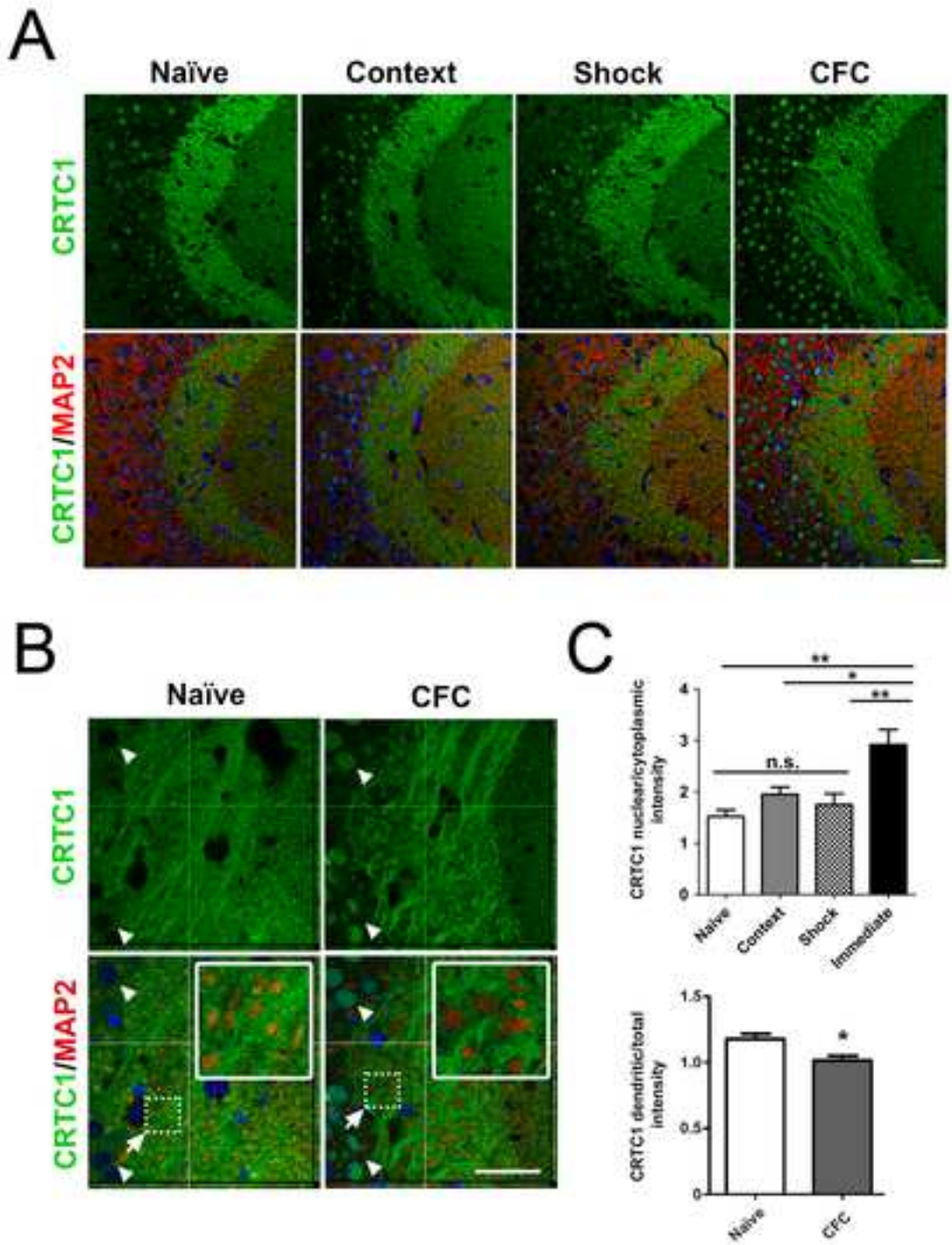

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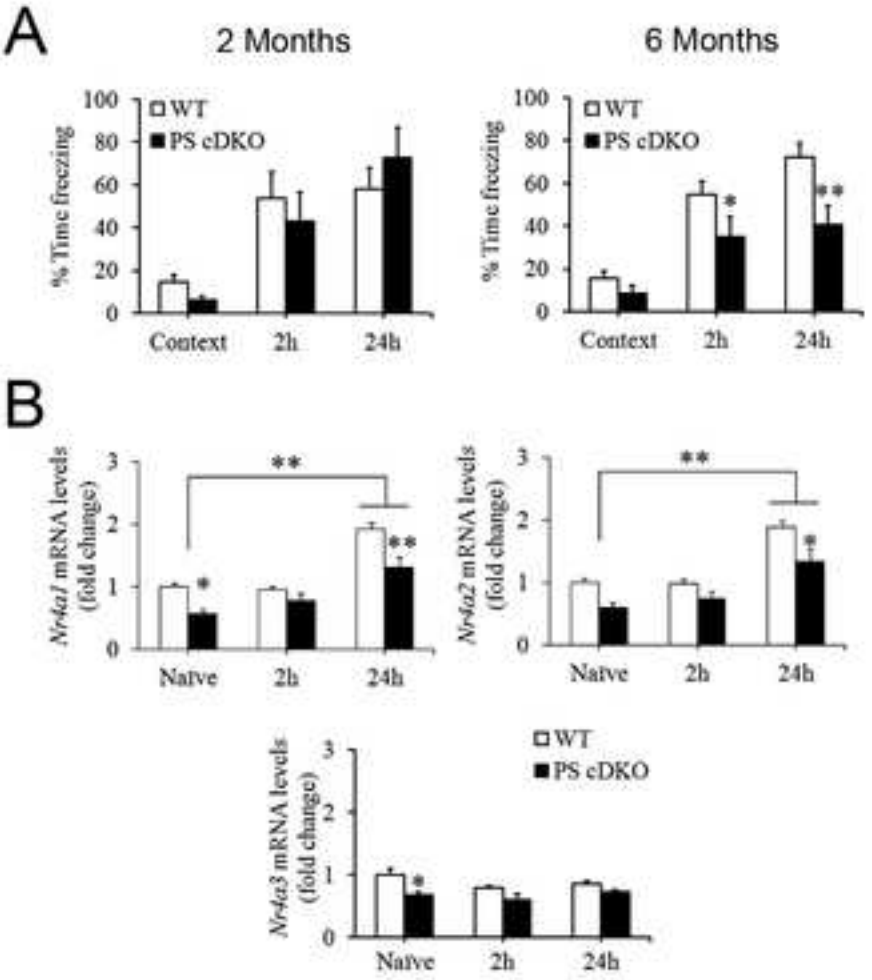

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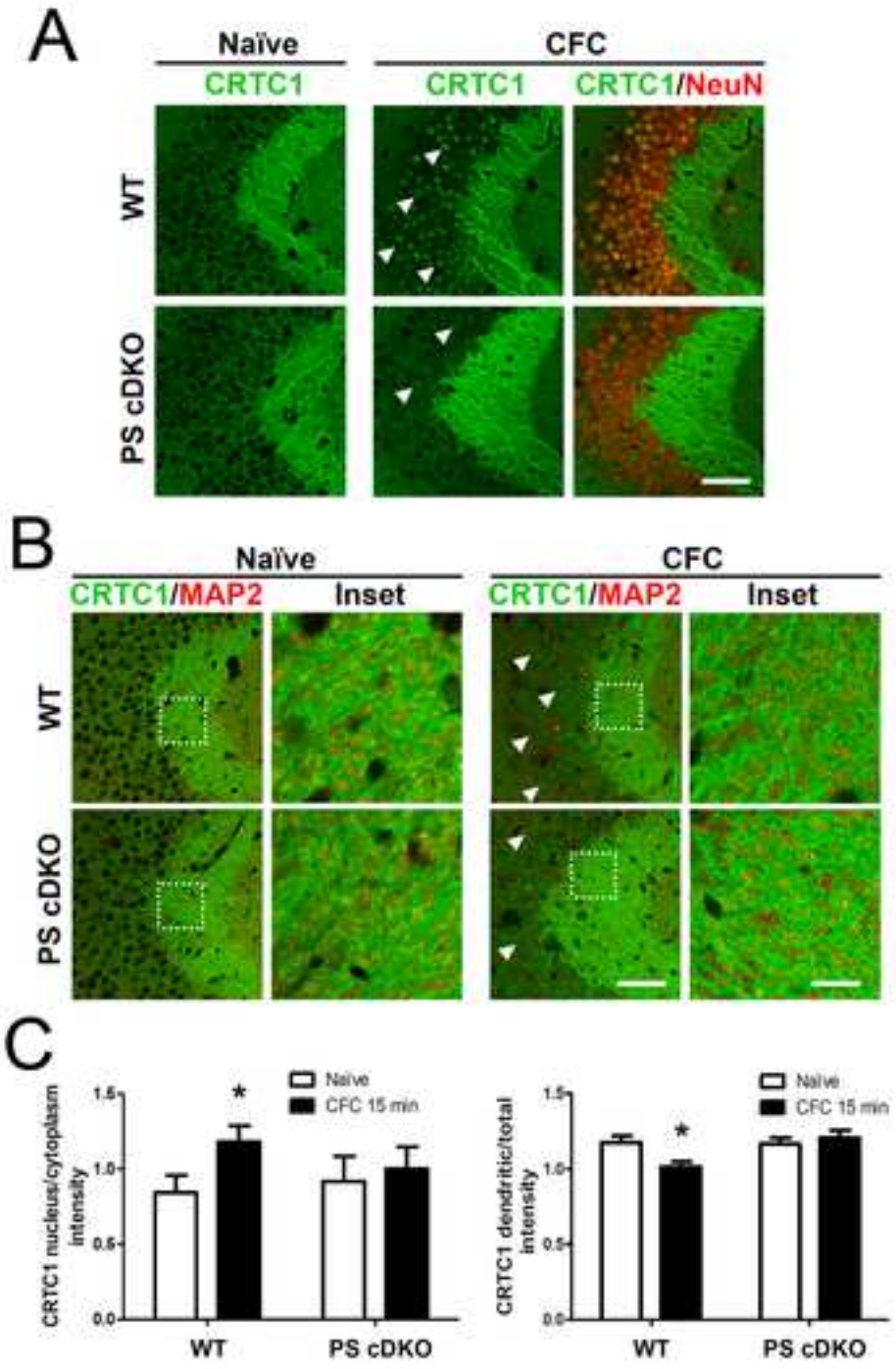

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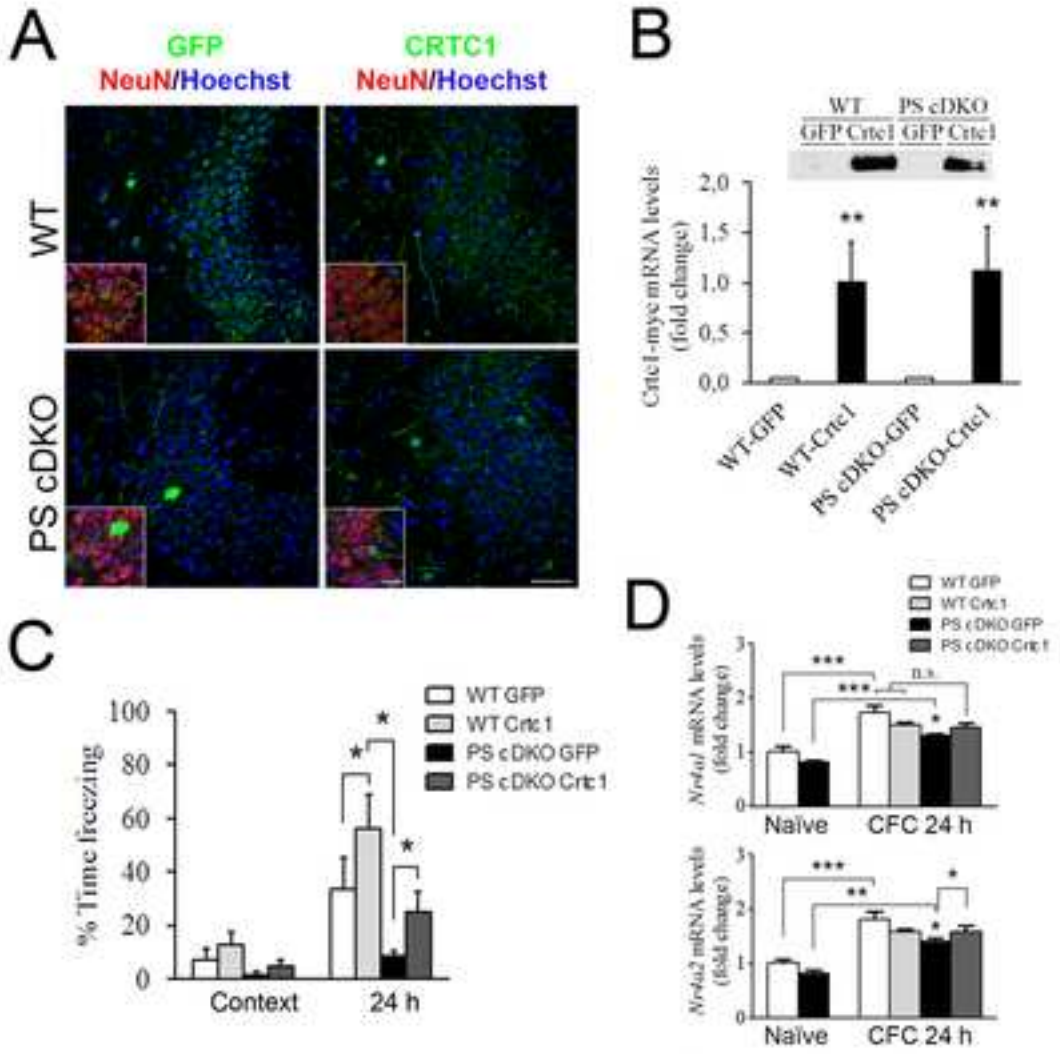

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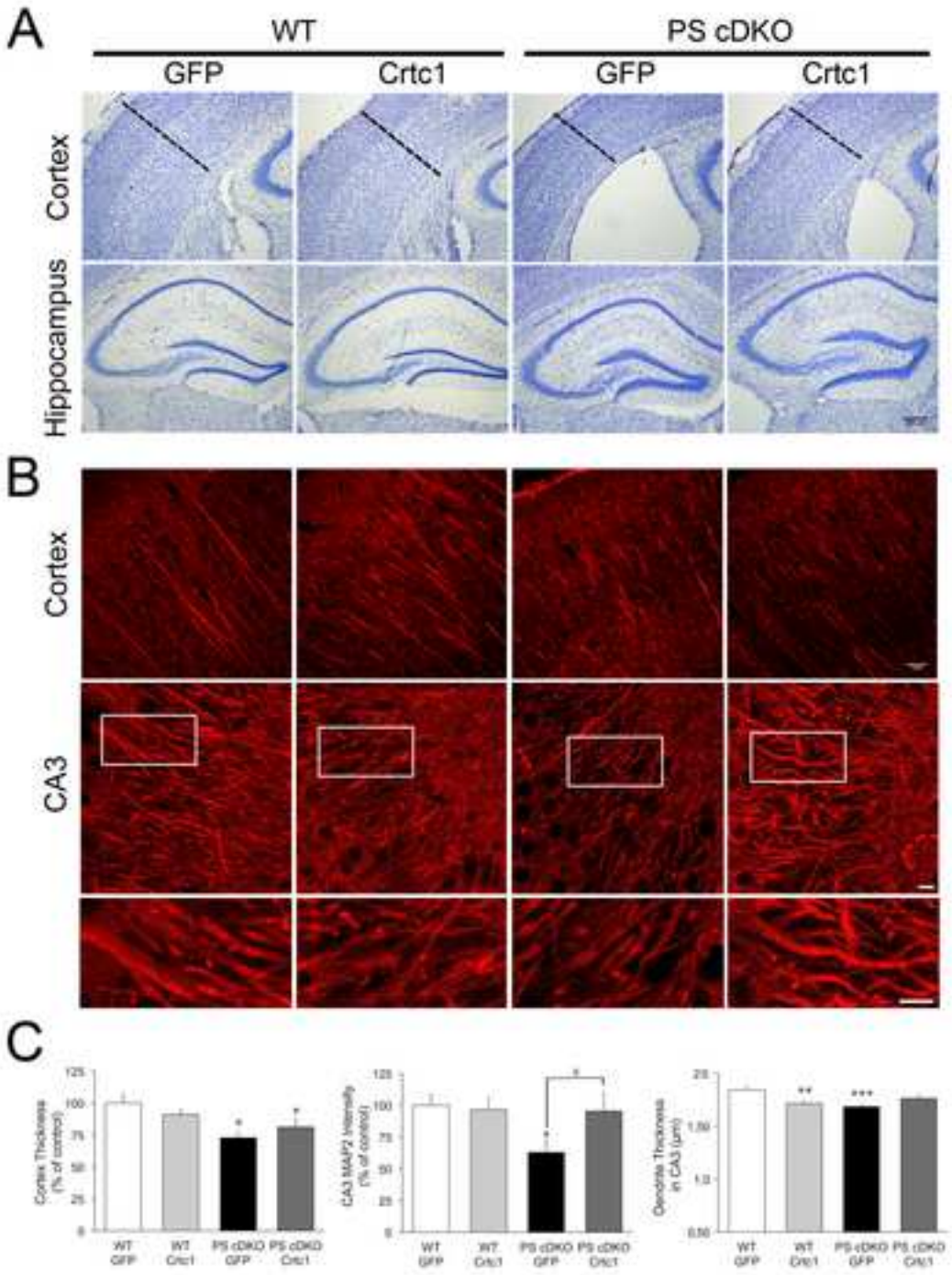

Figure 7

Supplemental Figures

Supplementary Figure S1

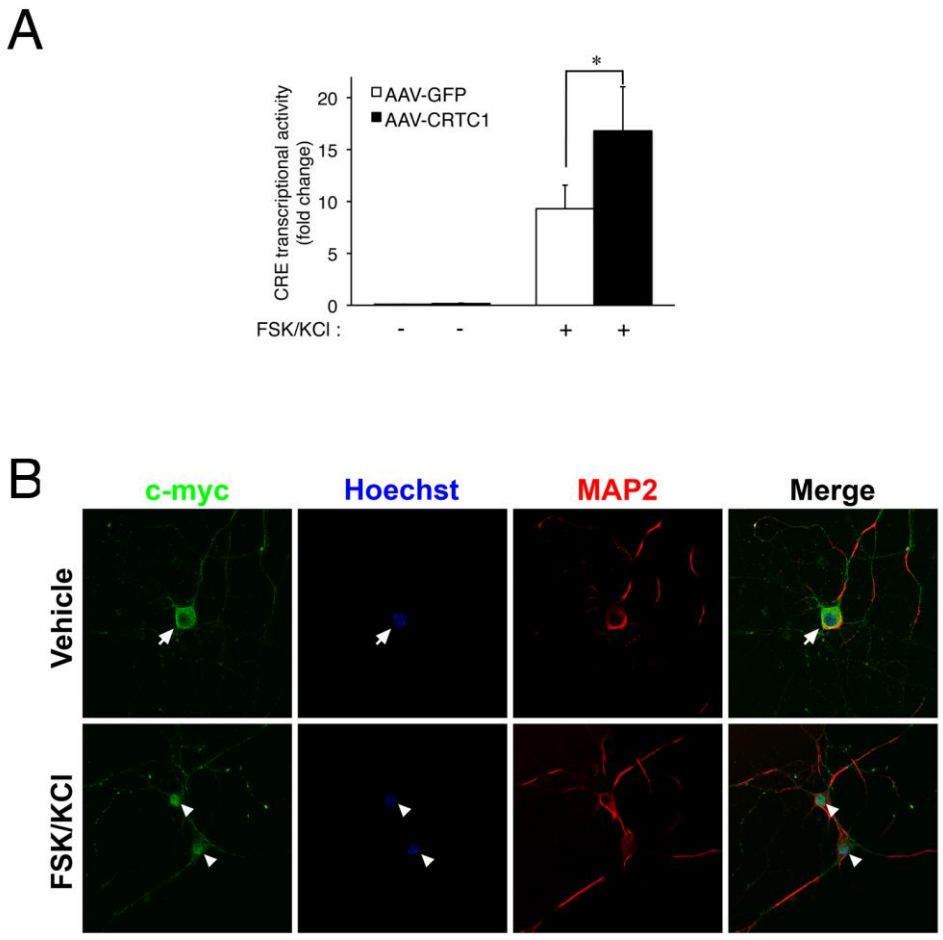

Figure S1. AAV-mediated CRTC1-myc expression increases CREB transcriptional activity.

A, CREB transcriptional activity in primary hippocampal neurons (10 DIV) infected with AAV-GFP or -Crtc1-myc and treated with vehicle (-) or forskolin (FSK)/KCl for 4 h. Data represent mean ± s.d of three independent experiments $*P < 0.05$, as determined by two-way ANOVA. **B**, Confocal images showing localization of CRTC1-myc (myc staining, green) in the cytosol (arrows) and nucleus, as colocalized with Hoechst (arrowheads) in control (vehicle) and activated (FSK/KCl) conditions, respectively. Primary hippocampal neurons were infected with AAV-Crtc1-myc and treated with vehicle or forskolin (FSK)/KCl for 10 min.

Supplementary Figure S2

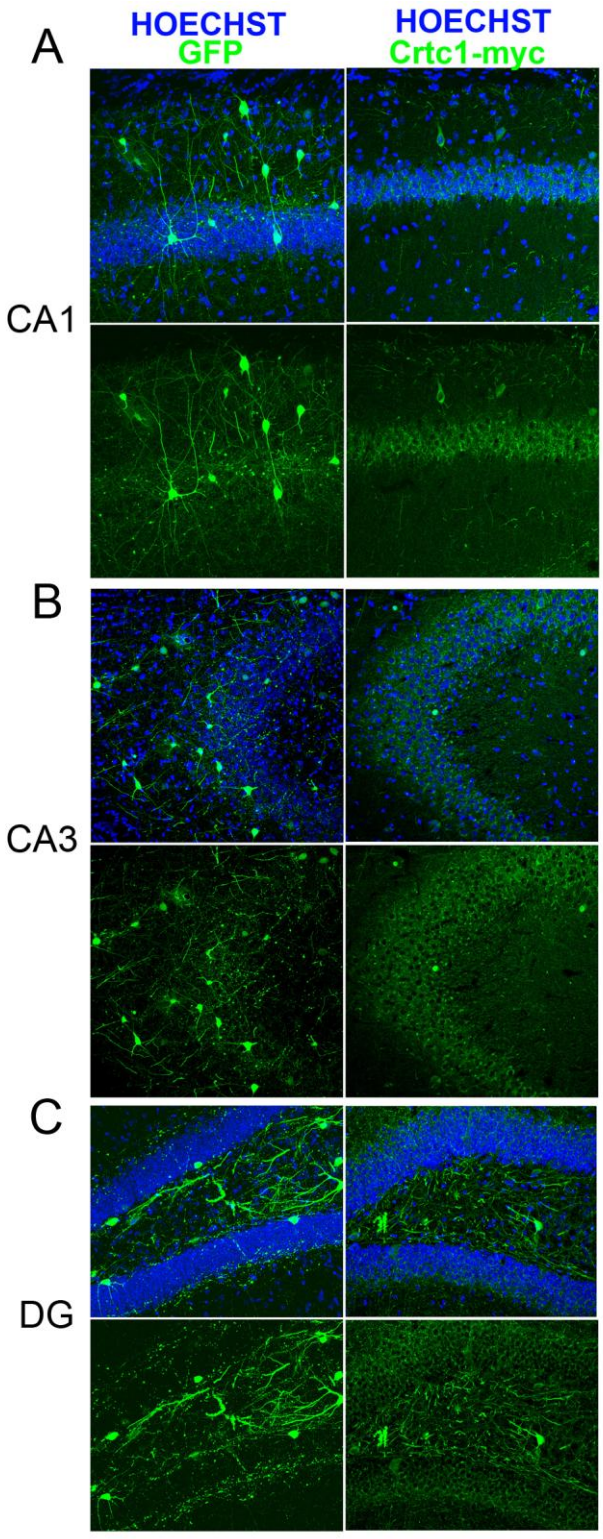

Figure S2. Efficient AAV2/10-mediated CRTC1-myc expression in neurons of the mouse hippocampus

Immunofluorescence images showing efficient expression of GFP (green, left) or CRTC1-myc (green c-myc staining, right) and nucleus (Hoechst, blue) in neurons of CA1 **(A)**, CA3 **(B)** and dentate gyrus (DG, **C)** regions of the hippocampus of adult mice three weeks after AAV-GFP and AAV-Crtc1-myc injection, respectively. Notice high GFP and Crtc1 gene expression in CA1 and CA3 pyramidal neurons although distinct subcellular expression pattern of GFP (cytosolic, neurites and nucleus) and CRTC1-myc (cytosolic and neurites) are observed.

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REVIEW
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Gene expression parallels synaptic excitability and plasticity changes in Alzheimer's disease

Carlos A. Saura, Arnaldo Parra-Damas and Lilian Enriquez-Barreto*

Institut de Neurociències, Departament de Bioquímica i Biologia Molecular, Centro de Investigación Biomédica en Red Enfermedades Neurodegenerativas (CIBERNED), Universitat Autònoma de Barcelona, Barcelona, Spain

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by abnormal accumulation of β-amyloid and tau and synapse dysfunction in memory-related neural circuits. Pathological and functional changes in the medial temporal lobe, a region essential for explicit memory encoding, contribute to cognitive decline in AD. Surprisingly, functional imaging studies show increased activity of the hippocampus and associated cortical regions during memory tasks in presymptomatic and early AD stages, whereas brain activity declines as the disease progresses. These findings suggest an emerging scenario where early pathogenic events might increase neuronal excitability leading to enhanced brain activity before clinical manifestations of the disease, a stage that is followed by decreased brain activity as neurodegeneration progresses. The mechanisms linking pathology with synaptic excitability and plasticity changes leading to memory loss in AD remain largely unclear. Recent studies suggest that increased brain activity parallels enhanced expression of genes involved in synaptic transmission and plasticity in preclinical stages, whereas expression of synaptic and activity-dependent genes are reduced by the onset of pathological and cognitive symptoms. Here, we review recent evidences indicating a relationship between transcriptional deregulation of synaptic genes and neuronal activity and memory loss in AD and mouse models. These findings provide the basis for potential clinical applications of memory-related transcriptional programs and their regulatory mechanisms as novel biomarkers and therapeutic targets to restore brain function in AD and other cognitive disorders.

Keywords: Alzheimer's disease, memory, transcriptome, neurodegeneration, Aβ, gene expression

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Reviewed by:
Hermona Soreq, The Hebrew University of Jerusalem, Israel
Agnes Gruart, Universidad Pablo de Olavide, Spain
Valentina Gatta, University of Chieti-Pescara, Italy

****Correspondence:***
Carlos A. Saura, Institut de Neurociències, Departament de Bioquímica i Biologia Molecular, Centro de Investigación Biomédica en Red Enfermedades Neurodegenerativas (CIBERNED), Universitat Autònoma de Barcelona, Barcelona 08193, Spain
carlos.saura@uab.es

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Introduction

The rise of life expectancy has profoundly increased the aging population, and hence the prevalence of age-related cognitive disorders, including Alzheimer's disease (AD). AD is a devastating neurological disorder characterized by early episodic memory deficits that progresses with cognitive impairments and neuropsychiatric symptoms and finally ends with general disabling dementia. The disease is preceded by a presymptomatic or preclinical stage that can last for years during which the clinical symptoms do not manifest but the pathological process starts (Sperling et al., 2014). In a subsequent prodromal stage named mild cognitive impairment (MCI), the disease

is characterized by impairment of memory (amnesia) and other cognitive functions. The majority of subjects with MCI, which represent 10–20% of population older than 65 years, suffer from the disease in the following years (Petersen, 2011).

Memory decline is accompanied by pathological features in the brain of AD patients, including accumulation of extracellular amyloid plaques composed of β-amyloid (Aβ) peptides and intracellular neurofibrillary tangles (NFTs) formed by aggregated hyperphosphorylated microtubule-associated protein tau. These pathological lesions accumulate in brain regions essential for memory encoding and storage, such as the medial temporal lobe (MTL) and related cortical areas (Spires-Jones and Hyman, 2014). Tau pathology starts in the entorhinal cortex (EC) and then spreads to the hippocampal formation and limbic and association cortices (Braak and Braak, 1991). Progression of NFTs correlates well with cognitive decline and neuron loss, whereas amyloid plaques are abundant in neocortical regions but they do not correlate with the degree of memory loss (Arriagada et al., 1992; Gomez-Isla et al., 1997).

It is becoming clear that specific memory circuits are affected by changes in synaptic function and plasticity during the course of the disease. Indeed, synapse dysfunction and loss is an early pathological feature that correlates closely with cognitive impairment (Terry, 2000; Scheff et al., 2007). Recent functional imaging studies reveal decreased activity of the MTL in AD patients, whereas function of cortical and temporal lobe regions, particularly the hippocampus, are increased during memory tasks in preclinical and early stages of the disease (i.e., MCI; for review see Sperling et al., 2010). This enhancement of brain activity may represent a compensatory mechanism resulting from reduced neuronal connectivity that can maintain memory encoding at the beginning of the disease process. Notably, enhanced neuronal activity parallels increased expression of genes involved in synaptic transmission and plasticity at presymptomatic or very early AD stages, whereas deregulation of synaptic gene programs occurs at early and late pathological stages. Here, we summarize pathological as well as functional features occurring in the brain of human and AD mouse models during aging, and discuss recent evidences suggesting a relationship between gene expression changes and neuronal activity and memory disturbances during the progression of AD.

Hippocampal Pathology and Activity in AD

Declarative episodic memories of live facts and events depend on the MTL and connected cortical regions. The MTL includes the hippocampal formation (CA fields, dentate gyrus and subiculum), amygdala and adjacent cortical regions (entorhinal, perirhinal, and parahippocampal cortices; Squirre and Zola-Morgan, 1991). The EC receives cortical sensory information and projects excitatory inputs directly to CA1 pyramidal neurons or to the dentate gyrus and CA3 hippocampus through the perforant pathway (Van Hoesen and Pandya, 1975). CA3 neurons project Schaffer collaterals to CA1 pyramidal neurons, which finally project to the subiculum and deep EC layers IV, V and VI (EC-IV-VI; **Figure 1**). The MTL undergoes atrophy and hypometabolism not only in AD but also in MCI stages (Press et al., 1989; Mosconi et al., 2005; La Joie et al., 2013), an effect observed at least 4 years in advance to cognitive symptoms (Tondelli et al., 2012). Indeed, disruption of the hippocampus, a critical component of this memory circuit, is sufficient to produce anterograde amnesia (Zola-Morgan et al., 1986).

The EC is severely affected by pathological events and neurodegeneration early in AD, likely contributing to memory impairment. AD brains are characterized by a specific pattern of degenerating neurons in EC-II/IV layers and subiculum (Hyman et al., 1984). Cholinergic neurons are particularly

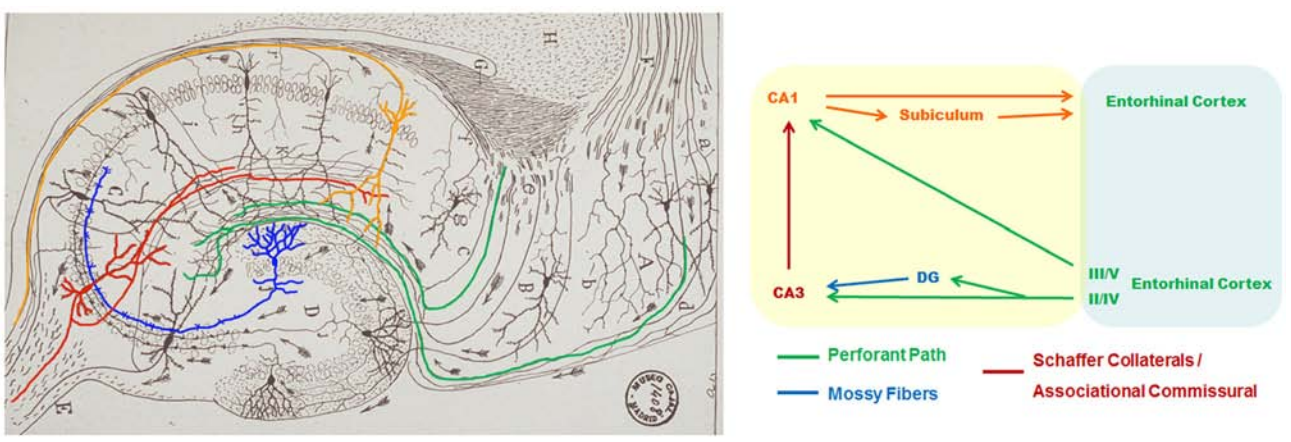

FIGURE 1 | Hippocampal circuitry in the mouse brain. Modified original drawing of Santiago Ramón y Cajal's (1911, left) and schematic diagram (right) of the rodent hippocampal circuitry. The picture shows the flux of excitatory projections (arrows) from entorhinal cortex (EC) neurons (green) directly to CA1 (orange) or CA3 (red) hippocampal pyramidal neurons or indirectly through projections to the dentate gyrus (DG, blue) through the perforant pathway. DG granule neurons project along the mossy fibers to CA3 pyramidal neurons. CA3 axons project through the Schaffer collaterals to CA1 pyramidal neurons, which finally project to the subiculum and deep EC IV- VI layers.

vulnerable in AD, and therefore classical therapeutic treatments are based on acetylcholinesterase inhibition. One of the earliest pathological features linked to AD progression is accumulation of NFTs, which occurrs in at least 70% of brains of healthy individuals at sixties (Nelson et al., 2012). Tau pathology starts in the EC and continues to CA1/subiculum field and amygdala prior to clinical symptoms (Braak stages I–II; Hyman et al., 1986; Arriagada et al., 1992). As AD progresses, tau pathology propagates in a sequential regional fashion to limbic and association cortices (Braak stages III–VI) apparently through an aggregation spreading mechanism (Braak and Braak, 1991; Clavaguera et al., 2013). Indeed, NFTs and amyloid plaques are abundant in the terminal sites of the EC projections such as the dentate gyrus (Hyman et al., 1990). Collectively, progressive accumulation and spreading of pathological hallmarks in the MTL suggests that disruption of this neural circuit may contribute to memory decline during the progression of the disease.

Functional magnetic resonance imaging (fMRI) studies show decreased activity and connectivity of the hippocampus, and temporal and prefrontal cortices during episodic memory tasks in AD patients [(Press et al., 1989; Small et al., 1999; Sperling et al., 2003; Pariente et al., 2005; Bai et al., 2009), for review see (Dickerson and Sperling, 2009)]. By contrast, MCI subjects show abnormal activation of the hippocampus and EC during face-name, visual object and verbal associative memory tasks (Dickerson et al., 2005; Hamalainen et al., 2007; Kircher et al., 2007). Compared with healthy aged controls, asymptomatic subjects at risk for AD, including *presenilin-1* (*PSEN1*) C410Y and E280A carriers, show higher activation of the hippocampus and frontal and temporal cortices during associative memory encoding years before clinical symptoms (Bassett et al., 2006; Mondadori et al., 2006; Yassa et al., 2008; Reiman et al., 2012). This increase of brain activity seems to reflect a compensatory mechanism to overcome neural dysfunction in preclinical stages, a process that may be necessary for appropriate memory encoding and retrieval (Kircher et al., 2007; O'Brien et al., 2010; Sperling et al., 2010). Taken together, these studies demonstrate increased activity of the MTL early during presymptomatic AD stages followed by decreased activity as the disease progresses.

Cortical Default Network in AD

Memory encoding and retrieval are affected by interconnected neocortical regions known as the default mode network, which is active at wakeful rest and deactivates during memory encoding (Buckner et al., 2008). The default mode network is connected to the MTL and includes the medial prefrontal cortex, the posteromedial cingulate cortex, the adjacent ventral precuneus, and the medial, lateral and inferior parietal cortices (Kobayashi and Amaral, 2007).

Specific regions of the cortical default network are functionally disrupted in AD and subjects at risk for AD (i.e., MCI; for a review, see Sperling et al., 2010). Hyperactivation of parietal and prefrontal cortices during memory encoding is accompanied by hippocampal hypoactivation in AD patients (Grady et al., 2003; Pariente et al., 2005). Reduced default network connectivity at rest, but increased activity during performance of attentional and associative memory tasks is observed in MCI and mild AD stages (Koch et al., 2014). Similar to AD patients, older cognitively normal subjects with brain amyloid deposition show significant reduced connectivity of the default network to the hippocampus in resting state (Hedden et al., 2009; Sheline et al., 2010). More striking, asymptomatic subjects with AD-linked autosomal dominant *PSEN1* mutations show decreased activity of the precuneus/posterior cingulate and parietal cortex in resting state (Chhatwal et al., 2013), but increased activity of the frontal, parietal and prefrontal cortex during memory encoding (Wishart et al., 2006; Reiman et al., 2012). These results indicate disruption of the default mode network years before cognitive or behavioral symptoms, which suggests that early AD-associated pathology exerts deleterious functional effects on distinct memory circuits prior to memory impairment.

Effect of Aβ on Hippocampal Activity and Memory in AD Mouse Models

Mice expressing human β-*amyloid precursor protein* (*APP), TAU* and/or *PSEN1* genes harboring familial AD-linked mutations develop AD pathological hallmarks, neuroinflamation and memory impairments (McGowan et al., 2006). APP and APP/PS1 transgenic mice develop age-dependent amyloid deposits and memory impairments in the absence of tau inclusions (Hsiao et al., 1996; Chapman et al., 1999; Koistinaho et al., 2001). APP transgenic mice show spatial and contextual memory impairments tightly associated with changes in long-term potentiation (LTP), a form of synaptic plasticity thought to be the cellular basis of learning and memory. Tg2576 (Swedish: APP KM670/671NL), $APP_{Sw,Ind}$ (J20) and APP V717I transgenic mice that develop amyloid plaques display impaired hippocampal synaptic plasticity and memory deficits (Chapman et al., 1999; Dewachter et al., 2002; Saura et al., 2005). In APP transgenic mice, including PDAPP (Indiana: APP V717F), Tg2576, APPLd2 (London: V642I), APP23 (Swedish), $APP_{Sw,Ind}$ (J20), APP/PS1 and 3xTg-AD (APP Swedish, Tau P301L, PS1 M146V), altered hippocampal synaptic plasticity and memory deficits precede amyloid plaque pathology suggesting that disruption of memory neural circuits is independent of plaque deposition (Dodart et al., 1999; Hsia et al., 1999; Koistinaho et al., 2001; Kelly et al., 2003; Van Dam et al., 2003; Dominguez-del-Toro et al., 2004; Billings et al., 2005; Saura et al., 2005; Jacobsen et al., 2006; Gruart et al., 2008). Notably, hippocampal-dependent synaptic plasticity and memory deficits in 3xTg-AD, $APP_{Sw,Ind}$ and ArcAβ transgenic mice are associated with the presence of intraneuronal Aβ accumulation, which precedes amyloid plaques (Billings et al., 2005; España et al., 2010a).

It has been recently postulated that synaptic excitability changes may alter memory networks leading to cognitive disturbances in AD (Santos et al., 2010, for review). $APP_{Sw,Ind}$ (J20) mice show prior to amyloid plaque deposition enhanced

synaptic plasticity in the Schaffer collateral pathway coinciding with early hippocampal-dependent memory deficits (Saura et al., 2005). During aging, $APP_{Sw,Ind}$ mice develop associative memory deficits accompanied by amyloid plaque accumulation and LTP impairments in the hippocampus (**Figure 2**). Similarly, young free-plaque TgCRND8 and 3xTg-AD mice show increased synaptic plasticity caused by enhancement of synaptic excitability in the hippocampus, a phenotype associated with episodic memory impairments (Jolas et al., 2002; Davis et al., 2014). Several studies have also shown increased neuronal hyperactivity and excitability in the cortex of young APP transgenic mice before or when the first amyloid plaques appear (Palop et al., 2007; Busche et al., 2008; Minkeviciene et al., 2009; Gurevicius et al., 2013). This increased excitability is likely responsible for spontaneous epileptic seizures and premature death of APP mice (Palop et al., 2007; Minkeviciene et al., 2009). Enhancement of neuronal activity associated with early pathological and memory changes in AD mouse

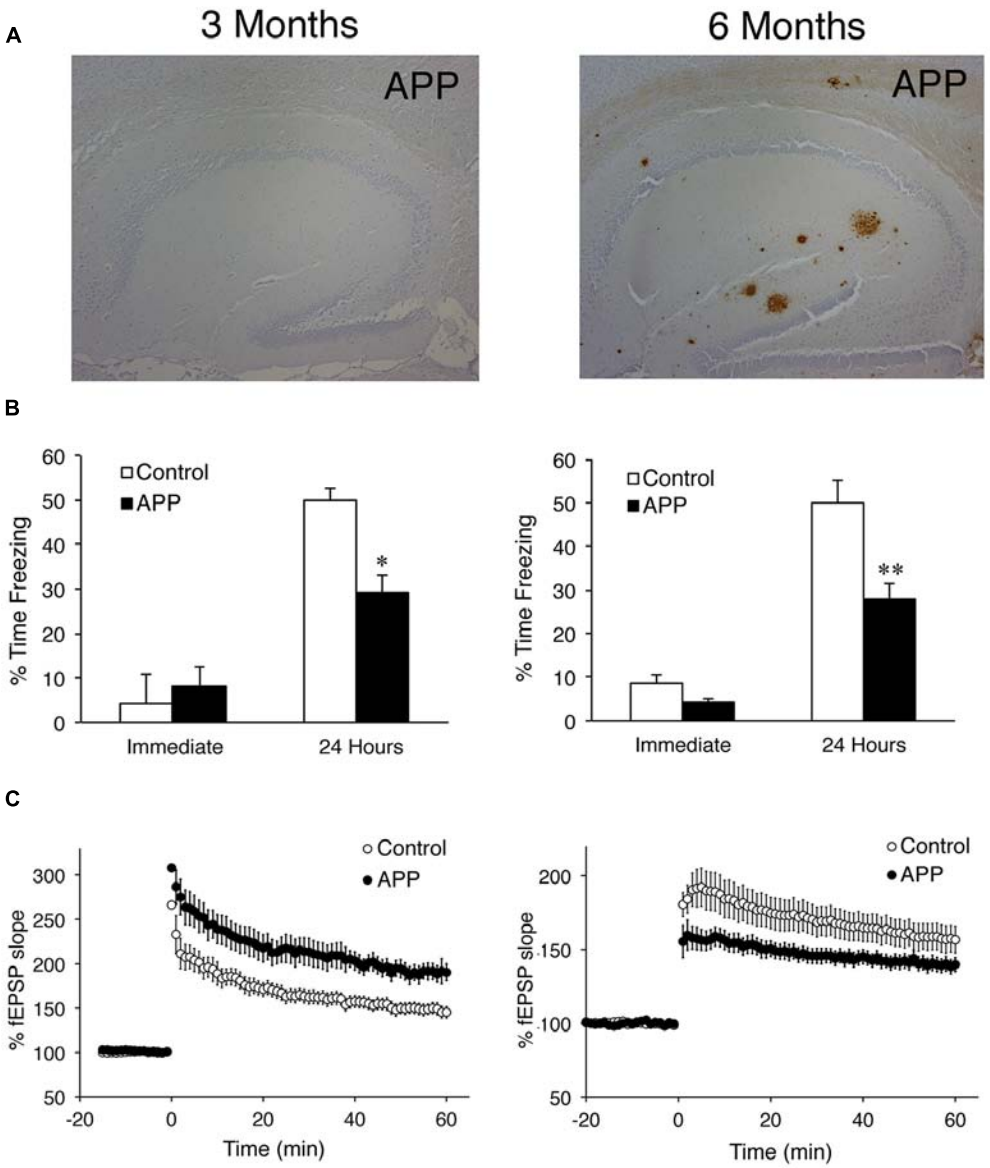

FIGURE 2 | Age-dependent pathological, synaptic plasticity and associative memory changes in APP transgenic mice. (A) Brain sections of 3–6 months-old $APP_{Sw,Ind}$ (J20) transgenic mice (APP) stained with an anti-Aβ antibody revealing the presence of amyloid plaques in the hippocampus at 6 months. **(B)** Contextual associative memory in one-shock contextual fear conditioning task. Three and six month-old APP mice exhibit significantly reduced levels of freezing at 24 h indicating disruption of long-term associative memory. Data represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.0001$. **(C)**, Time course of LTP induction at the CA1 Schaffer collaterals after theta burst stimulation (TBS) in 3- and 6-months old non-transgenic (control) and APP mice ($n = 5$–7). Notice the differential LTP in APP mice at 3 months (up) and 6 months (down) compared to the respective non-transgenic (control) mice. fEPSP, field excitatory postsynaptic potentials. Images are adapted from Saura et al. (2005).

models resembles the clinical symptoms of MCI subjects (see above).

The mechanism by which Aβ induces neuronal hyperexcitability is mediated by an increase of spontaneous action potential firing likely due to an impairment of inhibitory interneuron activity and/or increase of presynaptic vesicle release (Palop et al., 2007; Minkeviciene et al., 2009; Fogel et al., 2014). In this regard, hyperactivity of CA1 hippocampal neurons caused by loss of somatostatin inhibitory interneurons results in memory disturbances in APP transgenic mice (Perez-Cruz et al., 2011). By contrast, increasing the inhibitory activity of parvalbumin interneurons by restoring the voltage-gated sodium channel subunit Nav1.1 improves memory in $APP_{Sw,Ind}$ mice (Verret et al., 2012). Finally, Aβ contributes to emotional psychiatric disturbances by disrupting glutamatergic excitatory/GABAergic inhibitory neurotransmission in the basolateral amygdala (España et al., 2010a). Based on these results, it is plausible that early Aβ accumulation affects the inhibitory/excitatory neuronal balance of specific memory-related neural circuits. This will result in increased neuronal excitability leading to excitotoxicity and synapse and neuronal loss at later pathological stages, when plaque load, synaptic plasticity deficits and memory loss are prominent (**Figure 3**).

Synapse Dysfunction in AD

Synapse dysfunction is a common pathological feature of several dementing disorders being the major correlate of cognitive impairment in AD (Terry et al., 1991). Synapse loss affects different neuronal populations and neurotransmitter systems in brains of AD subjects (Masliah et al., 1990; Scheff et al., 1990, 2007). Individuals with amnestic MCI and AD have significantly fewer synapses and synaptic proteins in CA1 hippocampus and inferior temporal and posterior cingulate gyrus (Scheff et al., 2007). Accumulation of soluble toxic forms of tau and Aβ at synapses may be a crucial event leading to synapse loss and neurodegeneration (Spires-Jones and Hyman, 2014). Thus, loss of dendritic spines in cortical pyramidal neurons parallels tau phosphorylation during aging (Merino-Serrais et al., 2013), whereas soluble Aβ peptides and oligomers induce synapse loss in mice, rats and non-human primates (Forny-Germano et al., 2014). In APP transgenic, synapse loss and morphology changes are common features that precede amyloid deposition (Lanz et al., 2003; Wu et al., 2004; Rutten et al., 2005; Jacobsen et al., 2006; Wilke et al., 2014). Interestingly, reduced spine density in hippocampal neurons is associated with synaptic plasticity and memory deficits in Tg2576 mice (Jacobsen et al., 2006; Rocher et al., 2008; D'Amelio et al., 2011; Perez-Cruz et al., 2011; Ricobaraza et al., 2012).

The molecular mechanisms leading to synapse dysfunction and loss in AD are largely unclear. Aβ oligomers impair glutamatergic neurotransmission in an activity-dependent manner (Lacor et al., 2004; Deshpande et al., 2009) and cause synapse loss by postsynaptic mechanisms involving deregulation, removal and/or mistargeting of extrasynaptic NMDA and synaptic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) glutamate receptors (Shankar et al., 2007; D'Amelio et al., 2011; Miñano-Molina et al., 2011). For instance, reduced phosphorylated and surface expression of GluA1 is associated with early spatial memory deficits in APP transgenic mice (Miñano-Molina et al., 2011). Pharmacological treatments that inhibit aberrant extrasynaptic NMDA receptors or activate cAMP/PKA/CREB signaling reverse Aβ-induced dendritic spine loss and memory deficits (Smith et al., 2009; Talantova et al., 2013).

On the other hand, both Aβ and APP modulate excitatory presynaptic vesicle release in an activity-dependent manner (Abramov et al., 2009; Fogel et al., 2014), whereas neuronal activity modulates generation and deposition of Aβ *in vivo* (Bero et al., 2011), suggesting that neuronal hyperactivity can contribute to Aβ generation and accumulation. Taken together, these results point toward a bidirectional regulation between Aβ and neuronal activity through presynaptic and postsynaptic mechanisms.

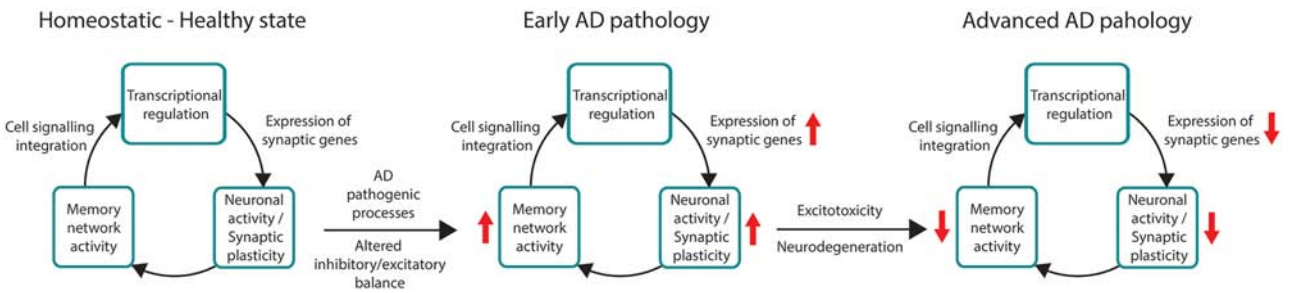

FIGURE 3 | Hypothetical model linking expression of synaptic genes and neuronal and memory network activities during AD progression. In healthy state, gene transcription controls expression of synaptic genes to maintain neuronal activity and synaptic plasticity in active memory circuits. In prodromal and very early AD stages, pathological changes increase expression of synaptic genes contributing to inhibitory/excitatory imbalance resulting in enhancement of synaptic excitability and plasticity in memory circuits. At intermediate and severe AD stages, sustained neuronal dysfunction causes transcriptional deregulation of synaptic genes resulting in synapse dysfunction and plasticity impairments, which contributes to memory network disruption and neurodegeneration.

Differential Brain Gene Expression in Presymptomatic and Pathological AD Stages

Cognitive decline is associated with changes of gene expression in the brain during aging and AD. Transcriptome profile studies indicate that genes related to synaptic function, energy metabolism and protein synthesis are downregulated in the brain during aging, while expression of inflammatory genes increases especially in the sixth to seventh decades of life (Berchtold et al., 2008; Cribbs et al., 2012; Kumar et al., 2013). Transcription of genes associated with neuron loss, glial activation and lipid metabolism increases with aging while inflammatory cytokines and microglial genes are activated early in AD (Podtelezhnikov et al., 2011), which corroborates an early inflammatory response in AD (Parachikova et al., 2007). Systems biology analysis identified two relevant pathways related to mitochondrial/energy metabolism and synaptic plasticity conserved between AD and aging (Miller et al., 2008). Interestingly, *APP* and *tau* transcripts are upregulated and regulators of APP metabolism (*BACE1*, *PSEN1*, *PSEN2*) and tau phosphorylation (*MARK1/3/4*, *CDK5*, *PINK1*) are downregulated in memory-related brain regions in individuals with moderate and clinical diagnosis of AD (Liang et al., 2010).

Altered expression of genes related to synapse, energy metabolism and transcriptional regulation processes exacerbate in the brain during the progression of AD pathology contributing likely to cognitive dysfunction (Yao et al., 2003; Blalock et al., 2004; Liang et al., 2008; Miller et al., 2008; Tan et al., 2010; Silva et al., 2012; Berchtold et al., 2013). Remarkably, downregulation of synaptic gene transcripts in CA1 hippocampal neurons of MCI/AD brains correlates with pathological cognitive status (Ginsberg et al., 2012; Counts et al., 2014). By contrast, genes changes related to metabolic/mitochondrial function occur in neurons and astrocytes in AD brain (Liang et al., 2008, 2010; Sekar et al., 2015). In astrocytes, deregulation of genes associated with cytoskeleton, proliferation, apoptosis, and ubiquitin-mediated degradation occur at early Braak stages, while deregulation of intracellular signaling pathways (PI3K/Akt, MAP, insulin) are associated with late pathological stages (Simpson et al., 2011).

Microarrays comparison analysis of synaptic genes in control and AD brains at different ages (20–99 years) revealed significant expression changes in genes regulating vesicle trafficking/release, neurotransmitter receptors, postsynaptic density, cell adhesion and neuromodulation in normal aging and AD, suggesting that similar synaptic genes are vulnerable to aging and AD (Berchtold et al., 2013). Indeed, expression of genes associated with synaptic signaling and structure, protein biosynthesis and mitochondrial/energy metabolism is predominantly increased in the hippocampus, EC and/or temporal gyrus in MCI and decline in AD (Berchtold et al., 2014). Genes that regulate vesicle and synapse function, including those encoding different isoforms of synaptophysin (*SYP*), SNAP25, synapsin (*SYN*), synaptogyrin (*SYNGR1*), synaptobrevin (*VAMP*), synaptotagmin (SYT), syntaxin-1 (*STX1*), synaptopodin (*SYNPO*) and PSD-95 are downregulated in the hippocampus and EC at moderate and severe AD stages (Liang et al., 2010; Ginsberg et al., 2012; Counts et al., 2014). Indeed, altered expression of genes related to synapse function and plasticity correlates better with AD pathology and clinical severity (Gomez Ravetti et al., 2010; Berchtold et al., 2014). **Table 1** summarizes changes of expression of some synaptic genes in AD brain and mouse models. It should be noticed that transcript changes in AD brain could reflect the loss of neurons and synapses in advanced disease stages, a possibility not generally considered in the majority of these studies.

In the prefrontal cortex, transcriptome changes affecting cell signaling, metabolic, inflammation and neurotransmission pathways occur at early pathological stages coinciding with the presence of intraneuronal Aβ (Bossers et al., 2010). Two patterns of gene regulation can be detected: (1) genes related to synaptic function, ATP synthesis and RNA increase in early pathological stages (Braak 0–III) and decline later (Braak IV–VI), and (2) genes related to cell differentiation/proliferation, metal ion binding, antigen processing and transcriptional regulation decrease early and then increase in late Braak stages (Bossers et al., 2010). Synaptic genes upregulated at early pathological stages include potassium voltage-gated channels (*KCNS3*, *KCNB1*, *KCNA1,* and *KCNAB1*), GABA receptors (*GABRA1*, *GABRD*, *GABRG2*), vesicle exocitosis (*SNAP25*, *CPLX1*, *VAMP7*, *SYT1*, *SYT3*, *SYT4*, *NAPB,* and *SV2C*) and vesicle endocytosis (*clathrin*, *CLTC*, *PACSIN1*) proteins.

These above findings indicate a close relationship between transcriptional deregulation and AD-associated neuropathology in memory-related neural circuits. We therefore hypothesize that increased expression of synaptic genes resulting from excitatory/inhibitory imbalance can enhance neural excitability and circuit activity during pre-symptomatic and very early disease stages of AD. In turn, this leads to global gene deregulation, synaptic dysfunction and degeneration and memory loss during the progression of the disease (**Figure 3**).

Synaptic Gene Expression Changes in AD Mouse Models

Transcriptome profile studies in AD mouse models have revealed altered expression of genes related to mitochondrial function, metabolism, insulin signaling, calcium homeostasis, inflammation, and synaptic plasticity during AD-like pathological progression (see **Table 1**, for synaptic genes). 3xTg-AD mice shows early hippocampal deregulation of genes linked to mitochondrial morphology and function, neuroinflammation, calcium homeostasis, neurotransmission, neuronal loss, and cell cycle (Gatta et al., 2014). 3xTg-AD mice show age-dependent expression changes on AMPA receptor subunits, with marked reduction of *Gria2 and Gria3* in the hippocampus at 12 months. Interestingly, levels of *Gria2, Gria3, and Gria4* transcripts are increased in the hippocampus of young 3xTg-AD animals suggesting a compensatory mechanism against AD-related synaptic dysfunction (Cantanelli et al., 2014). Several synaptic plasticity

TABLE 1 | Summary of expression of synaptic genes in AD and mouse models.

Gene	Gene name	Function	Model	Region	Levels	References
ARC	Activity-regulated cytoskeleton-associated protein	Synapse structure	AD $APP_{Sw,Ind}$ APP/PS1 Tg2576/APP_L	Hip Hip Hip/Cx Hip	Down Down Down Up	Ginsberg et al. (2012), Palop et al. (2005), España et al. (2010a), Parra-Damas et al. (2014), Dickey et al. (2003), Perez-Cruz et al. (2011)
CHGA	ChromograninA	Vesicle trafficking/release	AD AD $APP_{Sw,Ind}$	Hip CSF Hip	Down Down Down	Marksteiner et al. (2002), Simonsen et al. (2008), Perrin et al. (2011) Parra-Damas et al. (2014)
GRIA	GluA1 GluA2,3,4	Synaptic transmission	BraakII-IV/ AD $APP_{Sw,Ind}$,APPTg AD/3xTg-AD	Hip, EC, MTG Hip/Cx Hip/Cx Hip	Down Down Down Up/Down	Liang et al. (2010), Wakabayashi et al. (1999), Ginsberg et al. (2012), Parra-Damas et al. (2014), Dickey et al. (2003), Cantanelli et al. (2014)
NEFL	Neurofilament	Neuron structure	FTD, AD $APP_{Sw,Ind}$ APP/PS1	CSF Hip Hip/Cx	Up Down Down	Sjögren et al. (2000) Parra-Damas et al. (2014) Dickey et al. (2003)
NRN1	Neuritin	Neurite	$APP_{Sw,Ind}$	Hip	Down	Parra-Damas et al. (2014)
NRX1	Neurexin 1	Synapse structure	MCI AD	EC, SFG Hip, EC	Up Down	Berchtold et al. (2014)
NR4A1/2	Nuclear receptor sub 4, 1/2	Synaptic plasticity	AD $APP_{Sw,Ind}$,APPTg	Hip Hip/Cx	Down Down	Dickey et al. (2003), Chu et al. (2006) España et al. (2010b), Parra-Damas et al. (2014)
RAB	RAB2,5,7	Vesicle trafficking	MCI/AD $APP_{Sw,Ind}$	Hip Hip	Up Down	Ginsberg et al. (2010) Parra-Damas et al. (2014)
SCG2	Secretogranin II	Neurotransmission	AD $APP_{Sw,Ind}$	Hip Hip	Down Down	Marksteiner et al. (2002) Parra-Damas et al. (2014)
SNAP25	Synaptosomal-associated protein 25kDa	Vesicle trafficking	MCI AD 3xTg-AD	EC, Hip, PC EC, Hip, MTG Hip	Up Down Up/Down	Berchtold et al. (2014), Bossers et al. (2010), Liang et al. (2010) Gatta et al. (2014)
STX	Syntaxin1A Syntaxin4A Syntaxin 6 Syntaxin 18	Vesicle trafficking/release	MCI/AD AD/$APP_{Sw,Ind}$ MCI AD AD $APP_{Sw,Ind}$	Hip, MTG, EC Hip Hip, PCG Hip Hip, MTG, PC Hip	Down Down Up Down Down Down	Counts et al. (2014), Liang et al. (2010) Ginsberg et al. (2012), Parra-Damas et al. (2014), Berchtold et al. (2014) Liang et al. (2010) Parra-Damas et al. (2014)
SYT	SYT1,3,4 SYT6 SYT1,3,5,6,11,12 SYT4	Vesicle trafficking/ release	BraakII/III MCI AD BraakII/IV $APP_{Sw,Ind}$	PC Hip, PCG Hip, EC, PC Hip, EC, MTG Hip	Up Up Down Down Down	Bossers et al. (2010) Berchtold et al. (2014) Ginsberg et al. (2012), Liu et al. (2006) Liang et al. (2010) Parra-Damas et al. (2014)
VAMP	VAMP1,2,4 VAMP1,2,3,4	Vesicle trafficking	MCI AD	Hip, PCG Hip, EC, PC	Up/Down Down	Berchtold et al. (2013, 2014), Counts et al. (2014), Liang et al. (2010)

AD, Alzheimer's disease; FTD, frontotemporal dementia; MCI, mild cognitive impairment; EC, entorhinal cortex; Hip, hippocampus; MTG, middle temporal gyrus; PC, prefrontal cortex; PCG, post temporal gyrus; SFG, superior frontal gyrus.

genes, including *Arc*, early growth response 1 (*Egr1*), *NR2B*, *Gria1*, *Homer-1* and *Nr4a1*/*Nur77*, are significantly reduced in the hippocampus of 18 months-old APP/PS1 transgenic mice coinciding with amyloid plaques and memory deficits (Dickey et al., 2003). Interestingly, expression of genes directly implicated in learning/memory and plasticity is increased in the hippocampus of environmental enriched APP/PS1 (Lazarov et al., 2005).

Recently, comparison of different lines of APP and tau transgenic mice revealed that elevation of immune system genes is associated with appearance of amyloid plaques, whereas reduced expression of synaptic genes and increased cell death genes correlate with cortical and hippocampal tau pathology (Matarin et al., 2015). This result agrees with previous reports showing reduced expression of genes related to glutamatergic (*Arc*, *Gria1*, *Gria2*, *Grik4*, *Psd95*), or GABAergic (*Gad67*, *Gabarap-11*) neurotransmission and vesicle trafficking (*Syn3*, *Syb*, *Synj*, *Snap29*, *Syp*, *Stx4a*, *Stx7*) in hTau mice (Alldred et al., 2012), and elevation of inflammatory genes in brain regions containing amyloid deposits in APP mice (Dickey et al., 2003; Landel et al., 2014). In summary, transcriptome analysis demonstrates deregulation of common cellular pathways in several AD transgenic mouse models during AD-associated pathology.

Activity-Dependent Gene Expression and Memory Deficits in AD Mouse Models

Activity-dependent gene expression is a fundamental mechanism mediating structural changes at synapses during memory formation. Cognitive deficits in human and mice are associated with dysregulation of activity-dependent genes and transcription factors (West and Greenberg, 2011). Downregulation of activity-dependent genes involved in synaptic plasticity and memory, including the activity-regulated cytoskeleton-associated protein (*Arc*), *c-fos* and *Bdnf*, are associated with learning and memory deficits in AD and APP transgenic mice (Phillips et al., 1991; Dewachter et al., 2009; España et al., 2010b). Notably, *ARC* transcripts are significantly reduced at early and advanced AD pathological stages (Ginsberg et al., 2012; Parra-Damas et al., 2014). Similarly, *Arc* expression is markedly decreased in the hippocampus and visual cortex of APP transgenic mice after experience and memory training (Palop et al., 2005; Rudinskiy et al., 2012; Parra-Damas et al., 2014). Paradoxicaly, *Arc* is increased in individual cortical neurons close to amyloid plaques and CA1 pyramidal neurons in APP mice, an effect attributed to neuronal hyperactivity caused by decreased synaptic inhibition (Perez-Cruz et al., 2011; Rudinskiy et al., 2012). Despite the established disruption of activity-dependent gene expression in AD, the regulatory transcriptional mechanisms underlying gene changes causing memory loss in this disease are largely unknown. Understanding these mechanisms may offer new opportunities for therapeutic intervention in cognitive disorders.

To discern transcriptional mechanisms related to memory impairment in AD, we recently performed genome-wide transcriptome analyses in naïve and memory trained non-transgenic and $APP_{Sw,Ind}$ (J9) mice. Gene-annotation analysis revealed a gene cluster of 164 transcripts deregulated in the hippocampus of 6 months-old $APP_{Sw,Ind}$ mice compared to non-transgenic mice after memory training. The biological pathways associated with these genes are learning/memory, neurotransmission, synaptic plasticity, glutamatergic and GABAergic neurotransmission, oxidative phosphorylation and AD (Parra-Damas et al., 2014). Coinciding with initial intraneuronal Aβ accumulation and memory deficits, $APP_{Sw,Ind}$ mice show deregulation of a transcriptional program dependent on the cAMP-response element binding protein (CREB)-regulated transcription coactivator-1 (CRTC1), which includes genes involved in neurotransmission (*Scg2*, *Syt4*, *Stx4*, *Stx18*, *Rab2a*, *Gria1, Chga*), synaptic plasticity/memory (*Arc*, *c-fos*, *Nr4a1*, *Nr4a2*, *Bdnf*) and neuritogenesis (*Nefl*, *Nrn1*) (Parra-Damas et al., 2014) (**Table 1**). This result is consistent with a decline of synaptic gene transcripts coinciding with the presence of intraneuronal Aβ and preceding synapse loss in human prefrontal cortex at intermediate pathological stages (Bossers et al., 2010).

Genetic and pharmacological studies have shown that disruption of CREB signaling mediates synaptic plasticity and memory impairments in AD (Saura and Valero, 2011). Accordingly, CREB activation ameliorates synaptic and memory deficits in APP transgenic mice (Smith et al., 2009; Yiu et al., 2011), whereas CRTC1 gene therapy reverses early transcriptional changes and memory impairments in AD mice (Parra-Damas et al., 2014). In summary, disruption of CREB/CRTC1-dependent transcription underlies early memory deficits whereas its activation ameliorates AD-related synaptic and memory impairments, which provides evidence that targeting this pathway may be therapeutically beneficial in AD.

Pathogenic and Therapeutic Implications of Gene Deregulation in AD

AD is the most common form of dementia in the aging population but, unfortunately, current therapies are not effective to ameliorate or reverse the clinical symptoms. Classical pharmacological treatments based on inhibition of acetylcholinesterase (e.g., donepezil, rivastigmine) or excitotoxicity (memantine) slow the disease progression but do not prevent or stop the neurodegeneration process. Alternatively, anti-amyloid immunotherapy treatments that efficiently reduce amyloid plaque burden fail to improve cognitive performance in mild-to moderate AD patients (Doody et al., 2014; Salloway et al., 2014). The discouraging failures of anti-amyloid clinical trials have raised doubts about the contribution of Aβ as the initiating factor in AD pathophysiology (Herrup, 2015). Alternatively, several molecular, genetic and cellular events affected by aging, the main risk factor of the disease, may contribute to neuronal dysfunction and degeneration leading to dementia in AD.

In this context, deregulation of genes involved in pathological pathways, including oxidative stress, mitochondrial/energy metabolism, synapse dysfunction and inflammation, may be crucial in the etiology of AD. Thus, despite massive gene expression changes in the brain, few vulnerable biological pathways, including energy metabolism, synaptic function/plasticity and inflammation are generally altered in normal aging and AD (Berchtold et al., 2008, 2013; Cribbs et al., 2012). Gene expression deregulation occurs already in presymptomatic or early diseases phases (Bossers et al., 2010; Liang et al., 2010; Berchtold et al., 2014). Thus, synaptic and energy metabolism gene clusters are upregulated early during the disease process declining later at intermediate/severe pathological stages. Based on these results, we hypothesize that upregulation of synaptic genes contributes to increased neural excitability and memory circuit activity at presymptomatic or very early disease stages, which could then trigger gene deregulation, synaptic dysfunction, degeneration and memory loss during the progression of the disease (**Figure 3**). Indeed, changes in expression of synaptic genes parallel altered activity of memory circuits indicating a close relationship between neuropathology, transcriptional deregulation and activity of susceptible memory circuits in AD (**Figure 4**).

How changes of synaptic gene programs contribute to neuronal activity and connectivity alterations leading to memory loss in cognitive disorders is starting to be elucidated. One suggested possibility is that Aβ enhances intrinsic neuronal excitability leading to memory network changes and clinical manifestations of AD (Palop et al., 2007). Therapeutic approaches aimed at reducing neuronal hyperactivity may be beneficial to

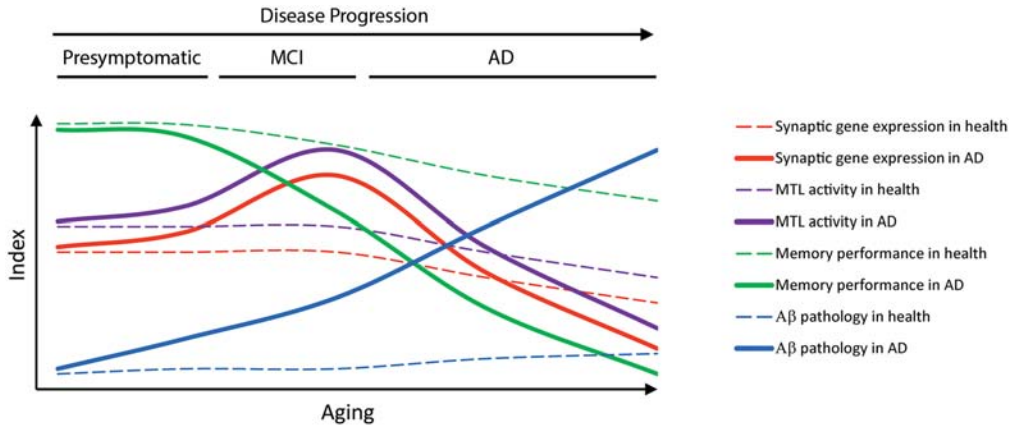

FIGURE 4 | Temporal progression of pathological and transcriptional changes in aging and AD. The diagram represents the hypothetical temporal progression of memory deficits, expression of synaptic genes, medial temporal lobe (MTL) activity and Aβ pathology during aging and AD. Enhanced brain activity at prodromal AD stages is associated with increased expression of synaptic genes, whereas decreased brain activity parallels reduction of synaptic genes as the disease progresses.

stabilize synaptic function and improve cognitive abilities at early disease stages. In support of this idea, a recent clinical trial indicates that levetiracetam, an antiepileptic drug that reduces hippocampal hyperactivity, improves cognition in amnestic MCI subjects (Bakker et al., 2012). Levetiracetam treatment also reverses synaptic gene changes as well as functional abnormalities and cognitive deficits in APP transgenic mice (Sanchez et al., 2012), whereas decreasing oxidative stress, excitotoxicity and hyperexcitability without interfering with amyloid or tau pathologies prevents AD-related memory deficits (Isopi et al., 2014). Likewise, the antiepileptic drug valproate ameliorates memory deficits and neuropsychiatric symptoms in APP transgenic mice (España et al., 2010a; Yao et al., 2014), but fails to slow cognitive decline and behavioral symptoms at late diseases stages (Fleisher et al., 2011). The efficacy of anti-hyperactivity drugs for treating AD will ultimately depend on the drug type, dosage and disease stage.

Gene profiling and transcriptional regulatory mechanisms involved in memory loss could offer clinical applications as diagnostic tools, novel biomarkers and therapeutic targets in AD. First, it is conceivable that meta-analysis of transcriptomic data from large population-based cohorts of sporadic AD patients may reveal differentially altered pathways related to specific pathogenic mechanisms, opening new venues to design personalized therapeutic strategies. Second, individual or particular set of genes altered in AD brain could be applied as novel early biomarkers to predict the progression of the disease and to monitor therapeutic effects in personalized medicine. Of relevance, synaptic genes identified by wide-genome profile analysis in mouse models are deregulated in AD brain or biological fluids (**Table 1**). Several of these synaptic proteins, such as BDNF, secretogranin II, synaptotagmin, chromogranin A and SNAP25, have been proposed as novel biomarkers for AD (Simonsen et al., 2008; Li et al., 2009; Perrin et al., 2011; Brinkmalm et al., 2014). Since testing brain tissue could be of limited application in population-based screenings, the use of CSF and blood samples may represent a valuable non-invasive tool for biomarker analysis. Indeed, deregulation of genes and microRNAs (miRNAs) in CSF and/or blood (plasma and blood cells) occurs in AD patients (Chen et al., 2011; Bekris et al., 2013; Roed et al., 2013), and a gene signature in blood related to inflammation, transcription and cell death was recently used for diagnosis and prediction of MCI to AD conversion (Roed et al., 2013). Interestingly, the abundant changes of transcripts of genes related to synaptic plasticity/transmission, neuritogenesis and neurological diseases (AD, Parkinsons'disease, and mental retardation) in blood cells of AD patients suggest a strong link between blood and brain transcriptional profiles in AD (Naughton et al., 2015). Future identification of relevant biomarkers in biological fluids may be useful for early and accurate diagnosis of AD.

An important point is that gene expression is regulated by multiple mechanisms including transcription, translation and posttranscriptional or posttranslational mechanisms. Among these, epigenetic regulation has been the intense focus of research in neurodegenerative diseases in recent years. Epigenetic chromatin remodeling and DNA modifications regulate gene expression during memory formation, whereas epigenetic dysregulation is associated with aging and cognitive disorders (Graff and Mansuy, 2009). Thus, global reduction of DNA methylation and hydroxymethylation occur in the hippocampus of AD patients at early pathological stages (Chouliaras et al., 2013; Sanchez-Mut et al., 2013; De Jager et al., 2014). Other epigenetic factors such as non-coding RNA, in particular miRNAs and long non-coding RNAs, have also received increasing attention in neurodegenerative diseases due to their role in modulating gene expression. A set of miRNAs and long non-coding RNAs are deregulated in brain, blood, and CSF of AD patients (Dorval et al., 2013; Lau et al., 2013), which raises the possibility that non-coding RNAs may play a key role in gene expression deregulation during the course of the disease. Nonetheless, specific miRNAs and long non-coding RNAs affect

expression of genes involved in AD pathology including gene regulating APP processing, tau, inflammation and apoptosis (Goodall et al., 2013). The diversity of miRNAs and their potential to target gene expression of multiple pathways offer alternative applications of these molecules as novel biomarkers and therapeutic targets for AD and other neurodegenerative diseases.

Based on the above studies, epigenetic therapeutic approaches have been applied in AD mouse models. As an example, histone deacetylase inhibition increases expression of plasticity genes and ameliorates synaptic pathology and cognitive deficits in APP transgenic mice (Ricobaraza et al., 2012). Long-term systemic treatment with epigenetic drugs may, however, cause broad and deleterious effects on brain function. Alternatively, targeting molecules or pathways regulating specific gene expression programs in vulnerable memory circuits may represent potential therapeutic targets for AD. Interestingly, a recent report demonstrates that a gene therapy approach targeting CRTC1 to enhance expression of specific synaptic genes prevents memory impairments in an AD mouse model (Parra-Damas et al., 2014). A future scientific challenge will be the identification of transcriptome signatures in the brain or biological fluids for early diagnosis and prediction of the disease. In parallel, a better understanding of the expression regulatory mechanisms of genes involved in synaptic dysfunction and neurodegeneration will be crucial to develop efficient therapeutic treatments for AD and other cognitive disorders.

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VIII. Discussion

Recent evidence suggests that dysregulation of gene expression may mediate the synaptic and cognitive deficits that occur during normal physiological aging and age-related cognitive disorders such as AD (Coleman & Yao 2003; Blalock et al. 2004; Berchtold et al. 2008; Twine et al. 2011; Pavlopoulos et al. 2013). However, the underlying activity-regulated transcriptional mechanisms that are disrupted during early AD stages remain largely unknown. Abnormal Aβ processing by PS/γ-secretase and loss of PS function are thought to contribute to synaptic dysfunction during initial stages of AD, leading to subsequent neuropathology, cognitive deficits and neurodegeneration (Selkoe 2002; Saura et al. 2004; Shen & Kelleher 2007; Jack et al. 2010; Xia et al. 2015). Previous studies from our group indicate that CRTC1 function is impaired in neurons from $APP_{Sw,Ind}$ mice, suggesting that alteration of this signaling pathway may contribute to the synaptic and memory deficits associated to AD (España et al. 2010). Since the hippocampal formation is particularly vulnerable to AD neuropathology and given the essential role of the hippocampus for declarative memory processing we aimed to analyze the transcriptional deficits mediated by CRTC1 during hippocampal–dependent learning and memory processes in two different mouse models of AD and neurodegeneration: $APP_{Sw,Ind}$ mice and PS cDKO mice; as well as in hippocampal samples from human AD.

At six month of age $APP_{Sw,Ind}$ mice exhibit early intraneuronal Aβ pathology coinciding with initial deficits in CREB-regulated transcription and hippocampal-dependent spatial learning and memory. Our genome-wide transcriptome analyses revealed significant changes in transcriptional pathways related to neurotransmission, synaptic plasticity, learning/memory, and oxidative phosphorylation in the hippocampus of $APP_{Sw,Ind}$ mice specifically after spatial memory training, suggesting that neuronal activity-induced transcription but not basal gene expression is affected by Aβ in the hippocampus of $APP_{Sw,Ind}$ mice. Importantly, we identified several synaptic function-related genes such as secretogranin II, GluA1, Nefl, synaptotagmin IV, Nr4a1, and Nr4a2, which are reduced in AD brains or CSF (Wakabayashi et al. 1999; Ginsberg et al. 2000; Marksteiner et al. 2002). Thus, our results suggest that Aβ-induced CRTC1 dysregulation is associated with altered hippocampal-dependent transcription and memory impairments, since altered gene expression is observed in the hippocampus of memory trained but not naïve $APP_{Sw,Ind}$ mice and these changes are specific for genes dependent on CRTC1, whereas genes activated independently of CRTC1 (Cyr61) (Ravnskjaer et al. 2007; España et al. 2010), are not affected.

In agreement with previous studies showing induction of CREB-regulated transcription upon induction of neuronal activity and memory training (Guzowski et al. 2001; Benito et al. 2011), our gene expression analyses indicate that both neuronal activity and spatial learning induce the expression of CREB-target genes related to neurotransmission (*Scg2, Syt4, Rab2a, Chga*), synaptic plasticity and memory (*Arc, c-fos, Nr4a1, Nr4a2, Bdnf, Nefl*), whose expression seems to be regulated, at least partially, by CRTC1. Interestingly, *Crtc1* mRNA levels were decreased by sustained neuronal activity, suggesting that a possible regulatory feedback mechanism to control CRTC1-dependent transcription. Similar to the effect of *Crtc1* ShRNA in our *in vitro* assays, Nr4a1-2 mRNA levels are reduced in the hippocampus of *Crtc1*$^{-/-}$ mice, which exhibit complex behavioral phenotypes associated to mood dysregulation (Breuillaud et al. 2012). Although residual CREB/CRTC-dependent transcription in our experimental conditions may be due to the remaining expression of CRTC1 (~30%), we cannot exclude a contribution of CRTC2/3 and/or alternative transcriptional regulatory processes including compensatory or cooperative/competition mechanisms involving other transcription factors (including other members of the CREB family) or coactivators.

We next analyzed the expression of CRTC1-dependent CREB target genes related to memory (*ARC, NR4A2*) and genes regulated by CREB independently of CRTC1 or memory (*CYR61*) in the AD hippocampus at early (Braak I-II), intermediate (Braak III-IV) and late (Braak V-VI) AD pathological stages, detecting a reduction in the mRNA levels of *ARC* and *NR4A2* at early and intermediate AD stages. These results are consistent with previous studies showing reduced levels of neurotransmission- and plasticity-related transcripts during intermediate AD stages (Bossers et al. 2010). Contrary to the reduction of target transcripts during early-intermediate AD stages, total CRTC1 protein levels were reduced at intermediate-late AD stages (Braak IV-VI) whereas no changes in CRTC1 phosphorylation were detected. However, it is difficult to draw definite conclusions from these results because the stability of the proteins and the phosphorylation epitopes may be affected by the post-mortem delay and this could not be quality-checked for proteins, unlike RNA. Taken together, these results suggest that CRTC1/CREB-regulated transcription is affected in the human AD hippocampus. Although the above analyses of human samples may provide valuable information about the underlying transcriptional mechanisms affected by CRTC1 deregulation, there are important technical limitations concerning the integrity of the samples and the fact that the analysis was performed on a whole tissue. Whereas the sample integrity could be controlled by including specimens with short post-mortem delay and low RNA

degradation (Weis et al. 2007; Vennemann & Koppelkamm 2010), the second issue is more difficult to control because of the many different cell types that contribute to the overall mRNA profile, and how these cell populations (both neurons and glia) are affected during the progression of AD by neurodegeneration and inflammation processes.

APP transgenic mice are useful for studying the effects of altered Aβ accumulation in the brain. However, most APP transgenic mice, including $APP_{Sw,Ind}$ mice, fail to reproduce the neurodegeneration that take place during AD. In order to study the potential role of CRTC1 during neurodegeneration we used PS cDKO mice which develop early synaptic plasticity and memory deficits followed by age dependent neurodegeneration (Saura et al. 2004). In PS cDKO mice PS1 inactivation is restricted to excitatory neurons of the postnatal forebrain starting at P18. After one month of presenilin inactivation, these mice exhibit deficits in hippocampal-dependent memory as well as specific presynaptic and postsynaptic alterations without significant neurodegeneration. However, by 6 and 9 months of age, there is a reduction in the number of cortical neurons of 18% and 24%, and this neuronal loss is associated with poorer performance on hippocampal-dependent learning and memory tasks (Saura et al. 2004).

We found that deregulation of CRTC1 activation and nuclear translocation in the hippocampus of PS cDKO mice is associated with reduced expression of target genes and contextual memory impairments, suggesting that CRTC1-dependent transcription in the hippocampus is critical for long-term associative memory encoding, and this process is affected during loss of PS function. Interestingly, PS cDKO mice show contextual memory impairments associated with hippocampal deficits of the CRTC1 target genes Nr4a1 and Nr4a2. Interestingly, Nr4a genes have been shown to play important roles during memory (Hawk et al. 2012). and particularly Nr4a2 (Nurr1) is required for CREB-dependent neuronal survival (Volakakis et al. 2010). Since Nr4a genes (i.e. Nr4a2) are downregulated in sporadic AD and Parkinson's disease brains and mouse models (Skerrett et al. 2014), our result may have important pathological and therapeutic implications in neurodegenerative diseases. Furthermore, recent evidence suggest that CRTC1 plays important roles in the regulation of neuronal survival and neuroprotection during brain insults triggered by ischemia and mutant huntingtin (Sasaki et al. 2011; Jeong et al. 2012), supporting the view that it could also mediate neuroprotection in other neuronal insults such as AD and other neurodegenerative diseases.

It has been shown that CRTC1 translocate from dendrites to nucleus upon neuronal activity in hippocampal slices (Ch'ng et al. 2015). Our results suggest that this phenomenon also occur in vivo during physiological learning. Mobilization of CRTC1 from dendrites to the nucleus may be a mechanism used by the neuron to compare different states of synaptic activity, at the transcriptional level. Thus, local depolarization at dendrites could activate a different CRTC1 transcriptional program than action potential-mediated CRTC1 translocation from the cytoplasm, based for example on competition or cooperativity between transcription factors. Recent evidence has unveiled a mechanism by which dendritic Ca^{2+} concentration may be temporarily sustained at dendritic spines (Johenning et al. 2015), which could be relevant for postsynaptic CaN-dependent CRTC1 activation. Interestingly, we have also observed abundant CRTC1 localization in the axonal terminals of mossy fibers, which clearly colocalizes with the presynaptic marker synapsin and the light neurofilament marker SMI-312 (data not shown). Although retrograde transport of CRTC1 from axon to nucleus has not been demonstrated yet, it could be possible that such a mechanism could contribute to presynaptic forms of synapse tagging. Furthermore, CRTC1 may form different complexes in dendrites vs. nucleus which may have functional implications besides transcriptional regulation. For example, it was been shown that γ-CaMKII acts as a carrier shuttling CaM from membrane to nucleus (Ma et al. Neuron 2014).

Based on our studies on CRTC1 translocation, we think that analysis of cellular CRTC1 localization (nuclear and dendritic) at specific brain regions may be used as an analytical tool for identifying active circuits during specific cognitive tasks. An interesting approach would be to combine immunohistochemical CRTC1 labeling with in situ hybridization (ISH) analysis of target genes over a time course during the behavioral tasks.

Besides the visual-spatial and other sensorial elements of the context, there is also an important temporal component that may be learned during the CFC protocol that we use, since the US (Shock) is delivered at a fixed time during exposure to the chamber. It has been shown that these temporal cues are rapidly learned (often after a single trial) and may influence the ways in which the behavioral responses are expressed after learning, leading to misleading interpretations about the resulting memory that has been measured (Gallistel & Balsam 2014). For example, an animal with a more accurate memory about the temporal occurrence of the US may show the expected behavioral response (i.e. freezing) only when the time of the US delivery is approaching, which could be assumed as a memory deficit if the freezing response is

expected to occur during a broader temporal window (i.e. from the initial re-exposure to the context). Therefore, it would be interesting to assess the effect of CRTC1 overexpression on the timing expression of the freezing responses in particular, as well as the possible role of CRTC1 activity during time learning and memory in general.

The role of CRTC1 on CREB-dependent transcription and memory deficits observed in $APP_{Sw,Ind}$ and PS cDKO mice suggests that enhancement of CRTC1 function may be therapeutically beneficial for AD. To test this hypothesis, we overexpressed CRTC1 in the hippocampus of $APP_{Sw,Ind}$ and PS cDKO mice using adeno-associated virus (AAV) expressing myc-tagged CRTC1 (CRTC1-myc). CRTC1 overexpression in the hippocampus was able to rescue spatial and associative memory deficits in these models, even when upstream Ca^{2+} signals and CaN activity is reduced. This may be explained because, although CaN activity seems to be the limiting step leading to CRTC1 activation, there must be competition between the different CaN substrates. Therefore, increasing the amount of CRTC1 protein will favor its overall interaction with CaN, compared to other targets. Our results are consistent with recent studies showing that overexpression of CRTC1 in the dorsal hippocampus enhances consolidation of long-term hippocampal-dependent memory (Sekeres et al. 2012; Nonaka, Kim, Fukushima, et al. 2014).

Taken together, our results in APP transgenic mice and PS cDKO mice indicate that CRTC1 activity is affected in both models, suggesting that CRTC1 function may be compromised by different pathogenic pathways. Importantly, we provide evidence that CRTC1-regulated transcription is also affected in the human AD hippocampus. Taken together, our results support a model in which different pathogenic events may lead to a common AD-like phenotype (synaptic and memory deficits) due to the integration of distinct signaling pathways on CRTC1/CREB-dependent transcription required for synaptic plasticity and memory. Furthermore, altered neuronal excitability during initial stages of AD may further contribute to dysregulation of activity-dependent gene programs regulated by CRTC1. Finally, we have shown that increasing CRTC1 function in the hippocampus reverses transcriptional and memory deficits in both mouse models during early pathological stages, suggesting that targeting CRTC1 signaling may be a valuable therapeutic strategy for AD and related neurodegenerative dementias.

IX. Conclusions

- Aβ accumulation is associated with spatial memory deficits and altered hippocampal expression of CRTC1/CREB-target genes in $APP_{Sw,Ind}$ transgenic mice
- Specific CRTC1/CREB-regulated plasticity genes are decreased in the human hippocampus during early and intermediate Alzheimer's disease pathological stages
- Reduced activation and CRTC1 nuclear translocation in the hippocampus is associated with decreased mRNA levels of CRTC1/CREB-target genes and associative memory deficits during neurodegeneration in PS cDKO mice
- CRTC1 overexpression in the hippocampus ameliorates spatial and contextual fear memory deficits induced by Aβ and PS inactivation, via transcriptional induction of specific plasticity-related CREB-target genes

X. Reference List

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