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Commentary

Cyclic AMP response element-binding protein (CREB) phosphorylation: A mechanistic marker in the development of memory enhancing Alzheimer's disease therapeutics

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ABSTRACT

CREB-mediated transcription can be initiated by membrane receptor stimulation and subsequent activation of intracellular pathways to the cell nucleus, and has been described as a molecular switch required for learning and memory. While CREB dimers are thought to be constitutively bound to response elements on DNA under basal conditions, it is CREB phosphorylation that is believed to be responsible for transcriptional activation leading to gene products such as BDNF that play a key role in synaptic plasticity and cognitive function. Conversely, preclinical and clinical findings now suggest that impaired CREB phosphorylation may be a pathological component in neurodegenerative disorders, in particular Alzheimer's disease (AD). In this regard, pharmacological-induced CREB phosphorylation in brain regions associated with cognition, i.e. cortex and hippocampus may represent a mechanistic basis for the development of novel AD therapeutics. The purpose of this commentary is to describe an experimental strategy to biochemically characterize the pharmacological induction of CREB phosphorylation as a mechanistic marker across different pharmacological classes of compounds for the potential treatment of AD that include: α7 nicotinic agonists, H3 antagonists and 11β HSD1 inhibitors.

1. Introduction

The neurophysiological definition of memory dates as far back as 1650 when the French Philosopher René Descartes described the ability of the mind to remember as: "*nerve impulses inclined toward different parts of the brain until they come upon the part where the traces are left of the thing it wishes to remember*" [1]. This early concept of specific anatomical brain substrates being involved in the formation, storage and retrieval of a neuronal "engram" remains a well-accepted theory in learning and memory. In contrast, the biochemistry of memory does not share a similar long history, and has only made an appearance in the last 40 years with advances in cellular and molecular neurobiology occurring in the second half of the 20th century. Initial studies in the 1960s demonstrated that transcriptional changes in RNA and subsequent protein synthesis were often necessary for memory formation [2–6]. Such studies along with molecular gene cloning paved the way for the purification and isolation of key molecules thought to be involved in biochemical signaling necessary for memory formation and cognitive function.

In particular, the isolation and purification of cAMP response element binding protein (CREB) in vitro from undifferentiated neuronal-like PC12 cells [7] and in vivo from mouse brain [8] led to a series of studies in the 1990s during which CREB was subsequently described as the molecular memory gene serving as a switch of long-term memory [9,10]. As illustrated in Fig. 1A, CREB functions as a transcription factor under physiological conditions through the convergence of multiple cellular signaling cascades that include: increased intracellular cAMP following stimulation of G protein-coupled receptors (GPCRs); increased intracellular calcium through activation of voltage- or ligand-gated ion channels, or the activation of neurotrophic receptor tyrosine kinases. Constitutively expressed throughout the brain, CREB is localized within the nucleus where it dimerizes and binds to the DNA cAMP response element (CRE). Transcriptional activation occurs through phosphorylation of CREB that serves as a substrate to the numerous upstream cascade pathways, many of which are thought to be involved in cognitive processing. Several lines of evidence now indicate that CREB activation represents a key stimulus-transcription coupling resulting in the translation-expression of gene products involved in neuronal, i.e. synaptic plasticity required for long-term memory in response to environmental learning cues.

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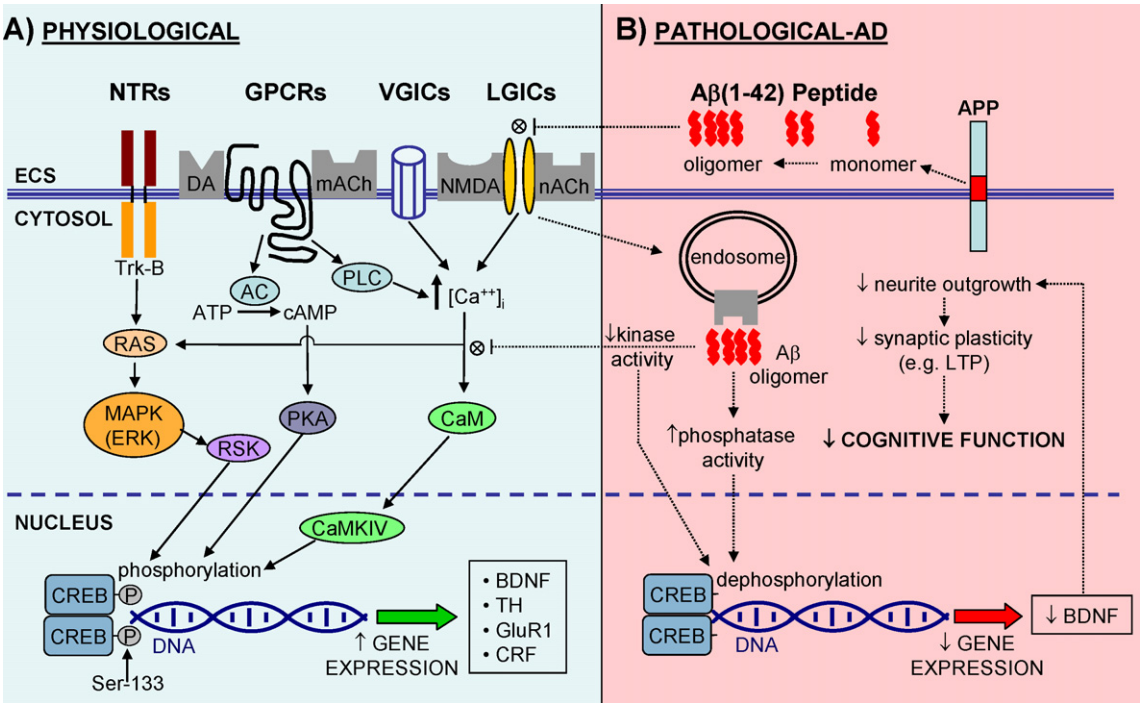

Fig. 1. (A) Under physiological conditions (left side of diagram; solid black lines), nuclear CREB phosphorylation and subsequent transcription-gene expression in the CNS can occur through the stimulation of multiple neuronal cell surface receptors and ion channels that includes neurotrophin receptors (NTR), e.g. TrkB; G-protein coupled receptors (GPCRs), e.g. dopamine (DA) and muscarinic acetylcholine (mACh) receptors; ligand-gated ion channels (LGICs), e.g. N-methyl-D-aspartic acid (NMDA) and nicotinic acetylcholine (nACh) receptors; and voltage gated ion channels (VGICs). Subsequently, CREB phosphorylation at Ser-133 is regulated through second messenger cascades that include activation of: RAS-dependent MAPK/ERK-mediated ribosomal s6 kinase (RSK); Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV); and cAMP-mediated protein kinase A (PKA). The phosphorylated CREB dimer is known to initiate transcription of numerous gene products, some of which include: brain derived neurotrophic factor (BDNF); tyrosine hydroxylase (TH); and corticotrophin releasing factor (CRF). (B) Under pathological conditions involved in AD (right side of diagram; broken black lines), there is growing evidence that soluble Aβ(1–42) peptide is the toxic species associated with AD pathology. Specifically, Aβ monomer peptides are believed to assemble into progressively larger soluble oligomers that play a neurotoxic role in early neuronal and synaptic dysfunction by targeting cellular effectors involved in cognitive function, such as the NMDA and α7 nACh receptors. Endocytotic receptor internalization of oligomeric Aβ may lead to decreased kinase and increased phosphatase activity thereby reducing CREB phosphorylation and the subsequent reduction in gene products such as BDNF, thereby resulting in impaired synaptic plasticity and cognitive function.

While the majority of CREB research over the last 30 years has largely focused on the cellular and molecular aspects of memory formation in the developing and adult brain under normal physiological conditions, more recently there has been an emergence to examine CREB's involvement under pathological conditions of memory impairment, in particular Alzheimer's disease (AD), as illustrated in Fig. 1B. As cognitive dysfunction and profound memory loss represents a major manifestation of AD, it is reasonable to speculate that aberrant CREB signaling may comprise elements of AD pathogenesis. AD pathophysiology is classically defined by the two focal proteins β-amyloid (Aβ), a product of aberrant amyloid precursor protein (APP) leading to production of extracellular Aβ plaques; and tau, a microtubule-associated protein that when hyperphosphorylated results in the formation of intracellular neurofibrillary tangles (NFTs) [11]. Originally identified as insoluble fibril filaments, it is now widely accepted that pathological Aβ and tau can also exist as soluble species that may be even more capable of generating neuropathology through increased fluidity leading to synaptic dysfunction via pathogenic signaling prior to aggregate accumulation. In particular, soluble Aβ has been proposed to interact and interfere with signaling pathways involved in cognitive function that includes CREB, where studies in AD transgenic (Tg) mice and patients have demonstrated reduced CREB phosphorylation.

As a memory switch, nuclear phosphorylation of dimerized CREB is downstream from numerous intracellular pathways that may indeed be vulnerable to neurotoxic insult associated with neurodegenerative pathology, such as soluble Aβ production in AD. In this regard, assessing CREB signaling may provide utility as a preclinical strategy in the identification and development of therapeutic agents for treating AD and other cognitive disorders. Specifically, pharmacological induction of phosphorylated CREB (pCREB) under physiological conditions or restoration of reduced pCREB expression associated with neurodegenerative pathology may represent a mechanistic signal of procognitive efficacy in AD drug development. As highlighted in this review, studies in our laboratories have employed such an approach in the experimental development of target platforms that have included α7 nicotinic acetylcholine receptor (nAChR) agonists, histamine H3 receptor antagonists, and inhibitors of 11β-hydroxysteroid dehydrogenase type 1 (HSD1).

2. CREB biology

CREB was first identified and described as a cAMP-responsive transcription factor regulating the somatostatin gene in PC12 cells where adenyl cyclase (AC) activation by forskolin produced a 3–4-fold increase in the phosphorylation of the 43 kD CREB protein leading to activation of somatostatin gene transcription [7]. In addition to the purification and secondary sequencing of CREB, this pioneering study marked one of the first examples of how hormones act on neuroendocrine cells through receptor stimulation and the subsequent activation of phosphoinositol and cAMP-dependent second messenger pathways, causing changes in cellular activity through specific protein kinases phosphorylating cytoplasmic and nuclear proteins resulting in the biosynthesis and release of a neuropeptide. Studies followed demonstrating that neurotransmitter stimulation of Gs-coupled CPCRs, such as

dopamine D1-like receptors, resulted in AC-mediated accumulation of cAMP, liberating the catalytic subunits of cAMP-dependent protein kinase (PKA) to enter the cell nucleus to phosphorylate CREB [12,13]. CREB phosphorylation, a required event for transcriptional initiation, activates recruitment of associated proteins such as CREB-binding protein (CBP) and assembly of a transcriptional complex that promotes nuclear processes such as histone acetylation, which alter conformational changes in surrounding chromatin leading to RNA polymerase II-mediated RNA synthesis. Like other transcription factors, CREB binds as a dimer to the specialized CRE stretch of DNA that contains the consensus sequence TGACGTCA found within the regulatory promoter region of many genes. The mouse and human CREB gene is comprised of 11 exons in which alternative splicing generates the three major CREB isoforms: α, δ, and β [14–16]. The molecular biology and biochemistry of CREB and its transcriptional role have been reviewed in detail elsewhere [17–20] and therefore a more succinct discussion of CREB biology will be described here in context of CREB phosphorylation as mechanistic marker in the development of cognitive memory enhancing agents for treating AD.

2.1. Phospho-regulation of CREB transcription in the CNS

Neuronal CREB is found throughout the brain where expression is particularly high as might be expected in anatomical regions associated with cognitive processing, including the hippocampus and cortex [21,22]. However, the abundance of total CREB alone does not necessarily translate to transcriptional sensitivity as phosphorylation of CREB is required for it to function as an activator of gene expression. Rather activity is differentially regulated through various phosphorylation sites on the CREB protein, in particular Ser-133. While CREB was originally shown to be phosphorylated at Ser-133 by activated PKA, several other kinases, such as Ca^{2+}/calmodulin-dependent kinases (CaMKs), mitogen-activated protein kinases (MAPK), protein kinase C (PKC), and MAPK-activated ribosomal S6 kinases (RSKs), are known to phosphorylate Ser-133 to stimulate CBP recruitment and subsequent gene transcriptional activation. The biological diversity of CREB-mediated transcription is exemplified by literature reports of over 300 different cellular and environmental stimuli capable of inducing phosphorylation of CREB on Ser-133 [18]. This includes stimulation of dozens of neurotransmitter receptors and neuronal ion channels involved in neuronal signaling pathways linked to complex behaviors. Other phosphoacceptor residues on the protein may represent alternative mechanisms influencing the transcriptional state of CREB. However, while additional phosphorylation events besides Ser-133 have been described, the biological consequences are not fully understood, although post-translational modifications such as ubiquitination and proteosome-mediated degradation have been proposed [23].

Although best known for its roles in learning and memory, described in detail below, CREB is recognized in general as a crucial transcriptional mediator of neuroadaptive changes in response to external and internal stimuli-stressors that influences a variety of neuropsychiatric behaviors. In this regard, CREB-mediated transcription in the brain represents a complex interaction between environmental stimuli and the subsequent activation of distinct signaling cascades beginning with stimulation of a plethora of cell surface receptor or ion channel proteins, and terminating with recruitment of phosphorylation-directed enzyme kinases and phosphatases that act on CREB to regulate gene transcription in mediating experience-based behavioral responding.

However, while CREB phosphorylation is required, it is not always sufficient to induce gene transcription. Studies examining CREB-mediated transcription in the CNS suggest that CREB-mediated transcription may require a cooperative interaction with other transcription factors that permit CREB to induce specific sets of genes in response to a specific signal [24]. In addition, the kinetics of CREB phosphorylation may also be a determining factor for transcriptional activation, as transient increases in CREB phosphorylation may be insufficient to stimulate CREB-dependent gene expression. Interestingly, cellular signaling through the CaMKIV pathway generates a rapid, but transient phosphorylation, whereas a late onset, but prolonged phosphorylation of CREB is seen with signaling through the ERK-MAPK pathway [25,26]. It has been proposed that such a biological scenario may explain functional specificity where CREB-mediated transcription is permitted through stimuli that engage both pathways, but not through activation of either pathway alone, in this case CaMK or ERK alone. In the CNS, such conditional activation involving the timing and context of incoming biochemical information may be critical in determining CREB's ability to regulate gene expression in response to environmental stimuli during learning and subsequent memory formation.

2.2. CREB-mediated gene expression in the CNS

Whether at a cellular, systems and organism level, the effect of CREB on neuronal function is determined primarily through its ability to mediate expression of target genes. In the last two decades, CREB activation has been reported to result in transcriptional activation of over a hundred different genes. Studies to determine the CREB "transcriptome", i.e. genes regulated by CREB have revealed that CREB can bind to over a thousand different gene promotors under in vitro cell culture conditions. However, CREB-mediated gene expression in vivo, particularly in the brain, likely differs dramatically whereby CREB regulates distinct subsets of genes within different anatomical regions [27]. These differences may result from the biological requirements of distinct intracellular signaling pathways for initiation of CREB-activated gene expression, such as the dependency of other transcriptional co-factors that may also be differentially expressed across cell types and anatomical brain regions. Although it becomes more difficult and complicated to elucidate CREB-mediated gene products in vivo, there are examples of CREB regulation of several genes within specific brain regions linked to physiological and pathological CNS function [28] that includes: neurotransmitter synthetic enzymes, such as tyrosine hydroxylase; neuropeptide transmitters, such as corticotrophin releasing factor; neurotransmitter receptor subunits, such as GluR1; and growth factors, such as brain-derived neurotrophic factor (BDNF). Given this wide and diverse range of gene products, the biological importance of CREB becomes apparent. In particular, BDNF is very likely a key gene product that contributes to CREB's involvement in cognitive processing through changes in synaptic plasticity, as described below.

3. Role of CREB in cognition

The cellular consolidation model of learning and memory asserts that memory storage is dependent on long lasting changes in the strength of synaptic connections that results in the stabilization and formation of a memory trace in critical brain regions, such as the hippocampus and neocortex [2]. This process of "synaptic plasticity" is known to be dependent on *de nova* gene expression and can last hours, days or even years after initial acquisition. Consolidation has been described as a biochemical "fixing" that represents the conversion of short-term memory, independent of protein synthesis, to long-term memory, dependent on protein synthesis. The activation of CREB-mediated gene expression by convergent signaling pathways in response to environmental stimuli has led to numerous studies implicating a crucial role for CREB in memory consolidation and long-term memory formation.

3.1. CREB-mediated memory in invertebrates

Initial studies supporting CREB's role in long-term memory consolidation were conducted in the invertebrate mollusk *Aplysia* in which the gill-withdrawal reflex was used as a model of consolidation-dependent, nonassociative learning. Specifically, CREB inactivation through exogenous injection of DNA containing the CRE sequence into *Aplysia* sensory neurons impaired formation of long-term synaptic facilitation, without affecting short-term facilitation [29], while serotonin-induced long-term facilitation in *Aplysia* was shown to require phosphorylation of CREB on Ser119 by PKA [30]. Cloned *Aplysia* CREB-1a injected in a phosphorylated form into *Aplysia* was also shown to initiate the long-term memory process [31]. Together, these landmark studies provided evidence demonstrating that CREB-phosphorylation induced transcriptional activation was required for long-term facilitation in *Aplysia*. Other invertebrate systems have also been employed in studying the molecular mechanisms of CREB-mediated long-term memory. In *Drosophilia*, expression of a dominant negative CREB transgene inhibits the acquisition on long-term memory, whereas CREB induction shows enhanced memory formation [32,33].

3.2. CREB-mediated memory in mice

Studies conducted in invertebrates paved the way to examine the role of CREB across various learning paradigms in vertebrate species, in particular genetically engineered mice. Studies with fear conditioning and water maze showed that mice with a null mutation to alpha and delta isoforms of CREB exhibited deficits in long-term, but not short-term memory [34], suggesting that similar to invertebrates, CREB-dependent transcription in vertebrates is required for lasting synaptic plasticity associated with long-term memory. Conversely, transgenic mouse studies have shown that CREB over expression results in the augmentation of long-term memory [35]. While numerous studies have demonstrated the requirement of CREB-mediated transcription for long-term memory consolidation, the mechanisms by which CREB facilitates memory are not fully understood, in particular the target genes of CREB that are involved in cognitive processing within various brain regions and species studied. However, the ability of CREB to induce gene expression of BDNF is viewed as a key mechanistic constituent to its memory enhancing effects. Similar to CREB, numerous studies have shown that BDNF produces pro-cognitive effects through its neurotrophic effects leading to neurite outgrowth and enhanced synaptogenesis associated with neuroplasticity [36]. As such, BDNF has been shown to enhance learning in both short-term and long-term memory paradigms [37]. Moreover, enhanced short-term memory produced by BDNF can be linked to upregulation of CREB activity. Studies in CREB null mutant knockout mice suggest that CREB plays a regulatory role in short-term memory through the regulation of BDNF expression [38]. Thus, it can be argued that CREB indirectly enhances short-term memory in addition to its effects on long-term memory formation.

3.3. ERK regulation of CREB-mediated memory

CREB's critical role in memory, both long-term consolidation, as well as indirect regulation of short-term memory, raises the intriguing question whether the connection between CREB and enhanced memory can lead to novel approaches to improve deficits in cognitive function associated with neurodegenerative disorders, such as AD. Whether targeting CREB directly or indirectly can provide utility as a novel AD therapeutic to date is unproven. However, many of the upstream pathways that converge to phosphorylate CREB and activate transcription are recognized to be involved in memory enhancement. In particular, the MAPK ERK1/2 pathway is known to be stimulated through a variety of cell surface protein receptors that may represent viable targets for mediating cognitive function through ERK and subsequent CREB activation. The MAPK-ERK pathway regulates a diverse array of cellular functions, such as cell growth, differentiation and survival that may underlie the synaptic plasticity required for cognitive processing and memory formation [39,40]. ERK1/2 phosphorylation activation is required for several forms of memory that includes LTP [41], fear conditioning [42], spatial memory [43], inhibitory avoidance [44] and object recognition [45]. However, in addition to ERK, CREB is a substrate to other kinase signaling cascades activated through cell surface proteins that lead to Ser-133 phosphorylation [28], as previously noted. In this regard, CREB phosphorylation represents a common downstream link involving multiple signaling pathways initiated by membrane receptor-mediated actions to the cell nucleus, where CREB phosphorylation induces gene transcription required for cognitive processing, both short and long-term memory formation.

4. Role of CREB in AD

As the physiological role of CREB in memory formation has been well established over the last 20 years, it is not surprising that more recently there has been interest in examining impaired CREB signaling as part of the neurodegenerative pathology associated with cognitive disorders, in particular AD. The most prevalent neurodegenerative disease today, AD affects approximately 25 million patients worldwide and is the most common cause of dementia of aging consisting of progressive cognitive failure and neuropsychiatric symptoms [46].

4.1. AD pathology and oligomeric Aβ

The hallmark of AD pathophysiology involves brain, in particular temporal and frontal lobe, accumulation of: (1) extracellular neuritic plaques consisting of fibril Aβ peptide, a product of aberrant amyloid precursor protein (APP) and (2) intracellular neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau, a microtubule-associated protein involved in axonal transport [11]. Most AD experts agree that abnormal proteolytic processing of beta amyloid peptide is the primary pathogenic event behind the manifestation of AD symptomatology, i.e. cognitive dysfunction [47,48]. However, the correlation between plaque formation and cognitive dysfunction does not always hold as memory impairment is often observed in AD patients prior to substantial plaque development. Rather, more recent evidence suggests that Aβ monomer peptide assembles into progressively larger soluble polymers (dimmers, trimers, etc.), and that these diffusible oligomers play a neurotoxic role in early neuronal and synaptic dysfunction associated with AD pathogenesis by targeting cellular effectors involved in cognitive function [49]. Thus, the ability of soluble Aβ oligomers to disrupt cellular signaling associated with learning and memory may represent the underpinnings of AD cognitive neuropathology at its earliest stages.

4.2. Oligomeric Aβ and LTP

As previously discussed, multiple signaling pathways converge on CREB leading to gene expression changes that are thought to regulate synaptic plasticity, which includes glutamate stimulation of the NMDA receptor and the subsequent elevation of intracellular calcium. Functionally, the phenomena of hippocampal LTP, defined as a strengthening of interneuronal communications that enhance synaptic transmission, involves both NMDA receptor and CREB activation. It is believed that the neurotoxic mechanism of

oligomeric Aβ may occur through the dysregulation of calcium signaling and disruption of hippocampal LTP, regarded as a cellular-anatomical correlate of learning and memory [50]. Several studies have shown that hippocampal LTP can be inhibited by both synthetic and naturally secreted human Aβ. Over expression of mutant Aβ in transgenic AD mice exhibit age-dependent attenuated hippocampal LTP, as well as behavioral memory impairment [51,52]. Furthermore, oligomeric Aβ-induced LTP deficits in vivo can be attenuated through passive immunization with anti-Aβ antibodies. Together, results from these studies are consistent with the hypothesis that oligomeric Aβ inhibits NMDA receptor activity and disrupts the subsequent activation of downstream signaling pathways that converge on CREB-mediated signaling and gene expression, and thereby interfere with synaptic plasticity ranging from LTP to long-term memory.

4.3. Effects of Aβ on pCREB expression and signaling in AD

Similar to LTP, deficits in CREB signaling may be implicated in AD pathology through the detrimental effects of Aβ. Reduced phosphorylation of CREB has been observed in postmortem brain of AD patients [53] and in Tg-AD mice overexpressing Aβ [54]. In vitro, the ability of NMDA or high potassium to stimulate CREB phosphorylation in cultured cortical neurons culture can be blocked by concentrations of Aβ(1–42) that do not elicit neurodegeneration or changes in total CREB expression [55]. Also in these studies, Aβ was shown to interfere with events downstream to activated CREB, specifically reduced gene expression of BDNF. In a similar in vitro design, Aβ decreased cAMP and PKA-sensitive glutamate-evoked CREB phosphorylation that was reversed by the phosphatase inhibitor rolipram, which also rescued LTP deficits produced by Aβ [56]. This finding was one of the first demonstrations of how Aβ-induced synaptic dysfunction, as measured by LTP, could be reversed by pharmacological maintenance of CREB phosphorylation, thus suggesting that agents that enhance CREB signaling may have potential for the treatment of AD. Further evidence for the involvement of Aβ-mediated CREB dysregulation in AD pathology have come from transgenic AD-mice. Phospho, but not total, ERK and CREB expression in cortex of Aβ overexpressing Tg2576 AD mice is reduced compared to wild type [57]. Moreover, attenuation of Aβ-induced deficits in CREB signaling have been observed in vitro and in vivo with treatments that include antibody neutralization of soluble Aβ [57], phosphodiesterase-5 inhibition [58] and the *Ginkgo biloba* extract EGb 761 [59]. Together, these studies indicate that reduced CREB phosphorylation may represent a component of AD pathology mediated through neurotoxic Aβ production. However, while these studies support reduced CREB phosphorylation as an experimental marker of Aβ-mediated AD pathology, further studies demonstrating a correlation between changes in pCREB expression and cognitive performance in Aβ overexpressing transgenic mice are warranted.

5. Assessing CREB phosphorylation in development of cognitive enhancing agents for AD

Our behavioral pharmacology group has utilized a number of preclinical models representing distinct domains of cognitive function across experimental species to assess the memory enhancing properties of compounds being developed for the potential treatment of AD. Specifically, these cognition assays have included: monkey delayed matching to sample, a model of visual working memory; rat social recognition, a model of short-term recognition memory; and mouse inhibitory avoidance, a model of long-term memory consolidation. In addition to behavioral assessment, we have adopted a strategy to examine pharmacological-induced changes in vivo suggestive of recruitment of signaling pathways involved in cognitive processing that includes CREB phosphorylation, as well as activation of the upstream MAPK ERK1/2 cascade.

Specifically, studies are routinely conducted in mice or rats where changes in ERK1/2 or CREB phosphorylation are examined immunohistochemically in anatomical regions linked to learning and memory, such as the hippocampus and cortex following administration of compounds at doses known to produce plasma and/or brain concentrations associated with behavioral efficacy in models of cognition. It should be noted that immunohistochemical (IHC) quantification is semi-quantitative at best, and does not provide an absolute measurement of protein phosphorylation. However, by adhering to a number of experimental conditions to reduce extraneous variability, we have demonstrated that IHC assessment of cellular signaling provides a viable means to examine pharmacological and physiological-induced phosphorylation signaling in situ yielding consistent changes across subjects with low within-group variability. Moreover, as an index of "biochemical memory", these data not only provide a mechanistic readout of compound efficacy, but equally important substantiate the behavioral efficacy associated with a given compound. In this section, utilization of such a strategy examining the pharmacological effects on pCREB expression in rodent CNS will be described across target platforms that include α7 nAChR agonists, histamine H3 antagonists and HSD1 inhibitors.

5.1. Nicotinic α7 agonists

Representing one of the two primary native nicotinic acetylcholine receptors (nAChRs) in the mammalian brain, the α7 nAChR subtype is a homopentameric ligand-gated ion channel that is distinct from its α4β2 counterpart with a relatively higher permeability for calcium (Ca^{2+}). For this reason, α7 nAChRs have been described as having metabotropic-like properties resulting from the activation of Ca^{2+}-dependent 2nd messenger cellular signaling [60,61]. Abundantly expressed in the frontal cortex and hippocampus, pharmacological and genetic studies support a role of the α7 nAChR in cognitive processing. We have hypothesized that the cognitive enhancing properties of α7 nAChR agonism may involve in part activation of calcium-sensitive signaling pathways that lead to CREB activation [62] (Fig. 2A). In particular, activation of MAPK ERK1/2 and the subsequent phosphorylation of CREB, as described above, is recognized as a key biochemical event in CREB-mediated cognitive processing [39]. Studies have shown that pharmacological or molecular inhibition of ERK leads to memory impairment, whereas genetic over-expression can produce memory enhancement [39,63].

Early reports of ERK and CREB activation by α7 nAChR stimulation involved in vitro studies with nicotine, which displays higher affinity for α4β2 versus α7, and where thus hampered by issues of non-selectively as well as receptor desensitization [64,65]. With the more recent experimental development of selective and CNS permeable α7 agonists producing broad spectrum efficacy across rodent and primate models of cognition, we have examined the ability of selective α7 nAChR agonists to activate ERK and CREB phosphorylation [62,66]. Specifically, A-582941 activated ERK1/2 and CREB signaling in vitro and in vivo. In PC-12 cells that endogenously express α7 nAChRs, agonist-evoked ERK1/2 phosphorylation was attenuated by the selective α7 antagonist methyllycaconitine. In mice, A-582941 at doses associated with enhanced cognition (e.g. memory consolidation) increases ERK1/2 and CREB phosphorylation in the cingulate cortex (Fig. 2B and C). The MEK inhibitor SL327 completely prevents A-582941-induced ERK1/2 phosphorylation, validating that increased ERK1/2 phosphorylation following A-582941 is mediated

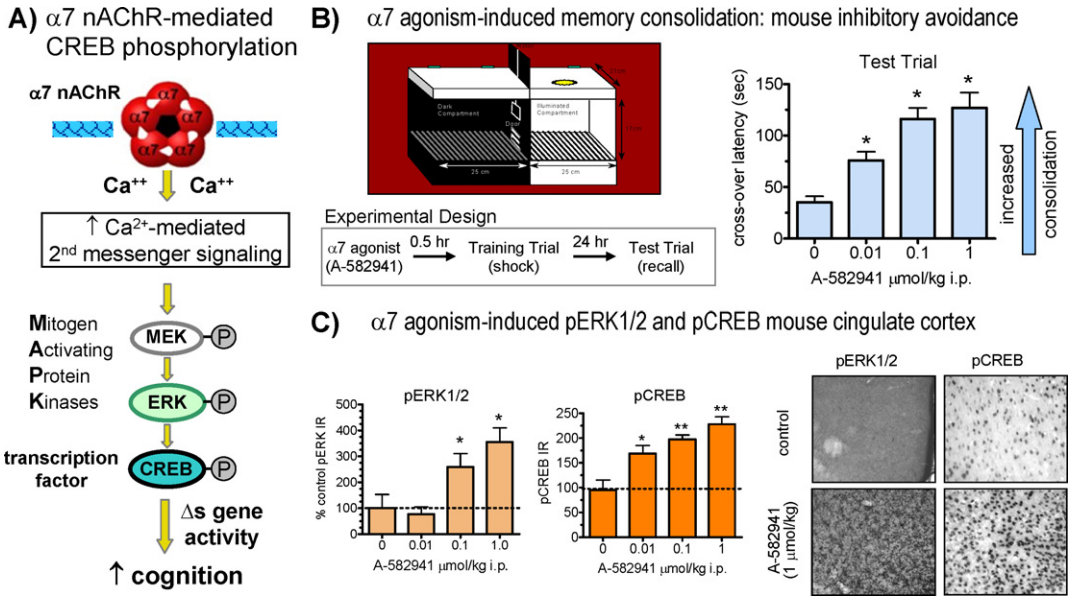

Fig. 2. (A) Stimulation of the α7 nAChR results in increased intracellular Ca^{2+} permeability and the subsequent activation of signaling cascades leading to phosphorylation-activation of the MAPK (MEK and ERK) and the transcription factor CREB, a proposed mechanism of α7 agonism-mediated procognition. (B) α7 nAChR agonism-induced memory consolidation is depicted where the selective α7 agonist A-582941 improved memory in the mouse inhibitory avoidance test, a model of long-term memory consolidation. Administered (i.p.) 30-min prior to the training (shock) trial, A-582941 increased crossover latency during the test trial (no shock) 24-h later, an index of memory enhancement [62]. (C) A-582941 also increased ERK1/2 (left) and CREB (right) phosphorylation in mouse cingulate cortex and hippocampus (data not shown) at behaviorally efficacious doses [62].

through the ERK-MAPK pathway. However, in contrast to the complete block of ERK1/2 phosphorylation, SL327 pretreatment in mice only partially attenuates A-582941-induced CREB Ser-133 phosphorylation [62]. This appears to suggest that independent of the MAPK/ERK pathway, α7 nAChR agonism leads to activation of other calcium-dependent kinases known to phosphorylate CREB, such as CamKIV or PKA, albeit additional studies need to be undertaken to further elucidate these mechanisms. Similar to A-582941, the selective α7 agonist ABT-107 increases cortical CREB phosphorylation in normal mice at doses associated with behavioral efficacy in cognition tests [66]. Consistent with an α7 nAChR-mediated effect, a lack of ABT-107-induced CREB phosphorylation is observed in α7 knock-out mice (unpublished data). Together, these results provide experimental evidence suggesting that selective α7 nAChR agonism may translate to novel therapeutics for treating cognitive disorders, in particular AD, that involve activation of MAPK and CREB-linked pathways.

5.2. Histamine H3 antagonists

Originally described as a presynaptic autoreceptor [67], Gi-coupled H3 receptors are also expressed as heteroreceptors located on axoaxonic postsynaptic terminals of non-histaminergic neurons that when occupied by histamine inhibit the release of neurotransmitters that in addition to histamine include: acetylcholine (ACh), dopamine (DA), norepinephrine (NE), and serotonin (5-HT) [68]. Conversely, H3 antagonists are known to evoke the release of these neurotransmitters in vitro and in vivo. In addition, H3 receptor antagonists have been shown to produce procognitive activity in preclinical animal models that has led to the investigation of these agents in the treatment of cognitive disorders, in particular AD [68]. Increased neurotransmitter release associated with this pharmacology may lead to activation of signaling pathways relevant to cognition through the stimulation of both GPCRs and ligand gated ion channels (LGICs) transmitter receptors. It can be hypothesized that H3 antagonists produce procognitive effects by acting as indirect agonists across a number of neurotransmitter receptors that activate postsynaptic signaling cascades, which could include calcium-dependent kinases culminating in the phosphorylation-activation of CREB (Fig. 3A).

In a series of studies in both normal and Tg-AD mice, we examined the effects of the H3 antagonist ABT-239 on CREB phosphorylation [69]. ABT-239 has been characterized preclinically and shown to produce broad spectrum procognitive efficacy as well as increased neurotransmitter release, specifically ACh, in rodents [70]. When administered at doses consistent with exposure levels associated with its procognitive effects, ABT-239 produced an increase in CREB phosphorylation in the cingulate cortex of normal mice (Fig. 3B). As suggested for α7 agonists, the ability of ABT-239 to elevate pCREB expression was interpreted as a biochemical surrogate of behavioral procognitive efficacy. Moreover, H3 antagonist-induced CREB phosphorylation supports the contention that H3 antagonist-evoked neurotransmitter release may lead to signaling changes germane to improved memory formation via CREB phosphorylation-activation.

We also examined the effects of ABT-239 on CREB phosphorylation in APP/Aβ overexpressing Tg2576 AD mice (Fig. 3C). As earlier described, reduced CREB phosphorylation has been reported in transgenic AD mice and patients. Similarly, a significant reduction of pCREB expression was observed in the frontal cortex of 12- to 13-month-old Tg2576 mice. Continuous 2-wk infusion of ABT-239 in Tg2576 mice normalized the reduced CREB phosphorylation to levels observed in vehicle-treated wild type mice. Consistent with acute administration in normal CD1 mice, ABT-239 infusion in wild type mice resulted in elevated pCREB expression. Although these results represent to our knowledge the first report of a H3 antagonist rescuing a biochemical phenotype associated with a Tg-AD mouse, the H3 antagonist clobenpropit was shown to protect against Aβ42-induced neurotoxicity in PC12 cells, where it was suggested that the H3 antagonist may be acting through regulation of glutamate release and NMDA receptor trafficking [71]. As suggested above, these findings support the hypothesis that H3-antagonist-evoked neurotransmitter release and the subsequent activation of postsynaptic signaling pathways may account for the therapeutic mechanism of this novel class of memory enhancing agents.

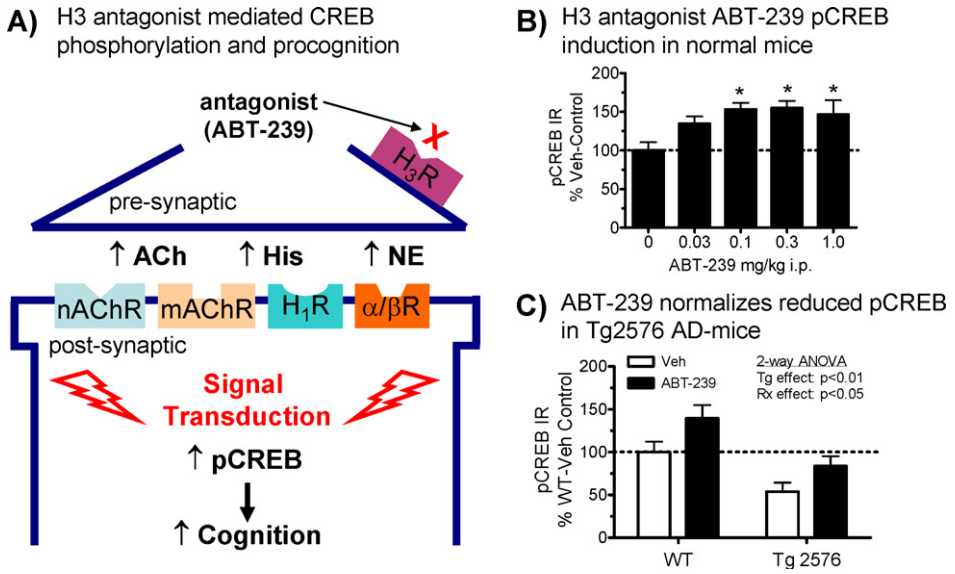

Fig. 3. (A) As a proposed mechanism of pharmacological-mediated procognition, presynaptic H3 receptor antagonism leads to an increase in multiple neurotransmitters such as acetylcholine (ACh), histamine (HA) and norepinephrine (NE). Synaptic elevation of neurotransmitter levels then results in the activation of postsynaptic signaling pathways relevant to cognition, specifically increased CREB phosphorylation. (B) The acute effects of H3 antagonism on CREB phosphorylation in vivo are shown where ABT-239 increased phosphorylation of CREB in the cingulate cortex of normal CD1 mice 15–20 min after injection [69]. (C) Cortical CREB phosphorylation was reduced in Tg2576 AD-mice, as compared to wild-type (WT) controls, that was normalized near WT levels in Tg2576 mice receiving 2-wk infusion (s.c.) of ABT-239 [69].

However, while the ability of ABT-239 to activate CREB provides biochemical support to behavioral cognition and neurochemical results, further investigation involving pharmacological antagonist and/or genetic knock out approaches will be necessary to better understand which upstream receptors and 2nd messengers are involved in H3 antagonist-mediated CREB phosphorylation.

5.3. 11β HSD1 inhibitors

Accumulating evidence suggests that prolonged exposure to elevated glucocorticoids in the brain may be linked to AD and age-associated memory impairment [72]. In particular, the cortex and hippocampus are vulnerable regions due to the high expression of

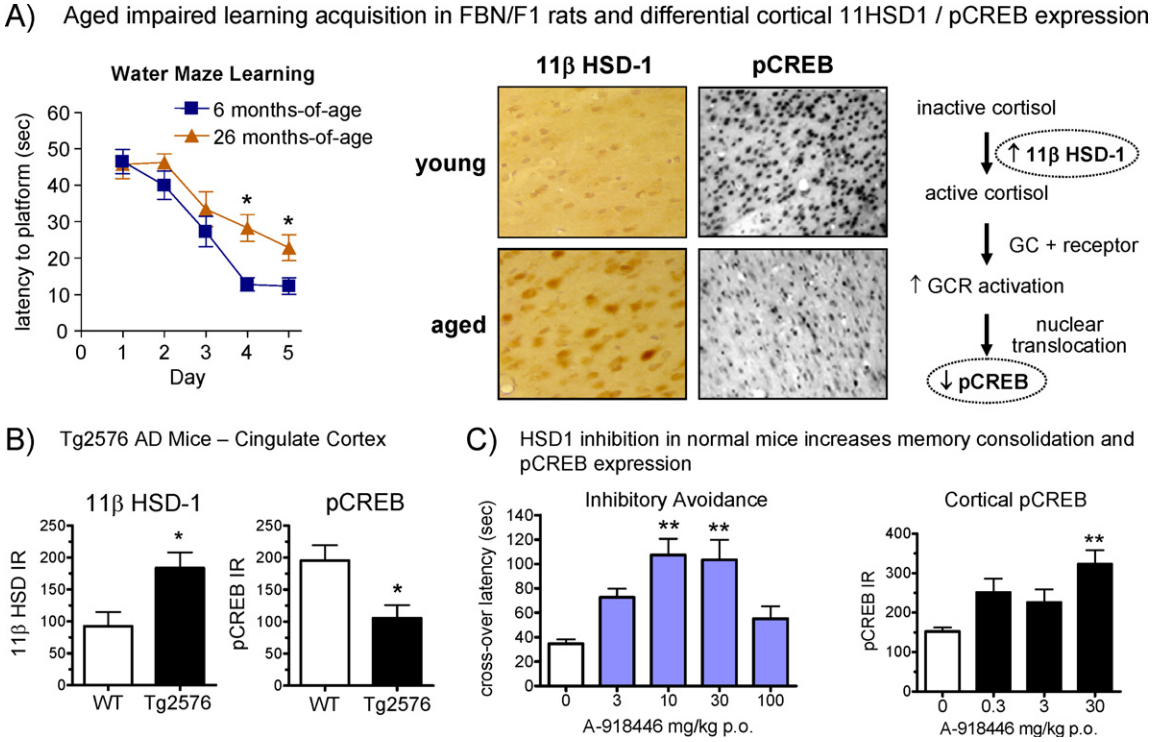

Fig. 4. (A) In aged Fischer 344 Brown Norway F1 Hybrid (FBN/F1) rats (32 months-of-age), impaired learning acquisition (days 4 & 5) in the Morris water maze is observed as compared to young rats (6 months-of-age) (*$p < 0.05$ vs young rats). Elevated 11βHSD1 and reduced pCREB expression observed in aged rats may suggests HSD1-mediated increased glucocorticoid activity results in decreased CREB phosphorylation associated with cognitive impairment (unpublished results). (B) A similar inverse relationship between elevated HSD1 and decreased pCREB expression was observed in the cingulate cortex of 12- to 13-month-old Tg2576 AD-mice (unpublished results). (C) Conversely, acute administration (3–100 mg/kg p.o.) of the selective HSD1 inhibitor A-918446 in normal CD1 mice enhances memory recall in two-trial inhibitory avoidance, an index of memory consolidation. In separate mice, doses of A-918446 that enhanced inhibitory avoidance recall increased pCREB expression in the cingulate cortex 1-h following administration, the pre-injection interval used for inhibitory avoidance training, thus representing a critical period for consolidation formation [77].

glucocorticoid receptors (GRs), in addition to being anatomical substrates of cognition. Conversion of glucocorticoids from inactive to active forms is catalyzed by HSD1, a key enzyme in regulating glucocorticoid activity, and therefore may play an important role in glucocorticoid-mediated cognitive impairment. Aged HSD1 knock-out mice have improved cognition and increased long-term potentiation relative to age-matched controls [73]. In addition, aged C57BL/6 mice display water maze deficits that correlate with increased HSD1 expression in the hippocampus and forebrain, with genetically engineered overexpression accelerating the impaired performance [74]. Taken together, inhibition of HSD1 has recently been proposed as a novel therapeutic approach for cognitive disorders such as AD.

There is experimental support suggesting that the mechanism associated with glucocorticoid-mediated cognitive impairment may in part involve CREB. As a nuclear receptor unbound in the cellular cytoplasm, the GR when stimulated by glucocorticoid translocates to the nucleus where the complex binds to a specific glucocorticoid response element (GRE) in the regulatory regions of various genes, or alternatively enhance or inhibit the transcriptional activity of other transcription factors such as CREB by means of protein–protein interactions [75]. It has been demonstrated that long-term glucocorticoid exposure in clonal neurons inhibits CREB phosphorylation and transcriptional activity, suggesting a link between the glucocorticoid pathway and CREB that provide a molecular basis for glucocorticoid-mediated cognitive impairment [76]. In support of a pathogenic role of HSD1 in this process, we have observed an inverse relationship between HSD1 and pCREB expression in the cortex of aged rats that display impaired water maze performance, as well as in transgenic AD-mice (Fig. 4A and B; unpublished data). Specifically, increased HSD1 correlated with a decrease in pCREB expression in the cingulate cortex of 26-month-old Fischer 344 Brown Norway F1 Hybrid rats exhibiting impaired water maze performance. Elevated cortical HSD1 expression associated with decreased CREB phosphorylation was also observed in 12- to 13-month-old Tg2576 AD-mice. Conversely, the selective HSD1 inhibitor A-918446 enhanced 24-h recall performance in normal mice tested in the inhibitory avoidance model of memory consolidation and increased pCREB expression in the anterior cingulate cortex 1-h after dosing, a time frame that parallels the pre-training injection regimen used in the inhibitory avoidance testing [77] (Fig. 4C). Taken together, these results provide support to the idea that the cognitive enhancing mechanism of HSD1 inhibition involves reduced glucocorticoid receptor activity and subsequent increased CREB phosphorylation and transcriptional activation.

6. Conclusions

CREB's involvement in memory formation has been extensively studied over the last three decades that has led to the accepted view that environmental, physiological and cellular stimuli are biochemically transduced through intracellular signaling cascades culminating in nuclear CREB-dependent gene expression that have critical roles in the neuroplasticity associated with cognitive function. However, the biological role of CREB in the CNS is not limited to learning and memory, but an assortment of complex behavioral systems involved in neuropsychiatric control. Thus, while targeting CREB phosphorylation as a therapeutic approach for developing novel memory enhancing agents may be cause for excitement, the fact that CREB is involved in numerous cellular activities would likely make it difficult to control specific processes. Nonetheless examining CREB phosphorylation and CREB-dependent gene products, in particular BDNF, may have utility as mechanistic markers in the experimental development of memory enhancing agents for treating cognitive deficits associated with AD where there is growing evidence that impaired CREB phosphorylation is involved in AD pathophysiology. This raises an interesting question as to whether pharmacological rescue of CREB phosphorylation in the AD brain represents a biochemical index of not only symptomatic, but also disease modifying efficacy in the treatment of AD. Pharmacologically induced CREB phosphorylation in normal, as well as normalization of reduced CREB phosphorylation in transgenic AD mice has served as a preclinical strategy in conjunction with cognitive behavioral testing within our discovery group to advance novel AD therapeutics for clinical development.

7. Supplemental: Methods (Figs. 4A and B)

7.1. Animals

Adult (17–18 months) and aged (31–33 months) Fischer 344 Brown Norway F1 Hybrid (FBN/F1) rats were used for Morris water maze testing and assessment of cortical HSD1 and pCREB expression. Male Tg2576 mice (Taconic Farms), along with wild type, were used for assessing cortical HSD1 and pCREB expression. All animals were acclimated to the animal facilities for a period of at least one week prior to the beginning of experimental procedures. Animals were grouped housed in an AAALAC approved facility at Abbott Laboratories in a temperature-regulated environment with lights on between 7:00 and 20:00 h. All experimental procedures involving animals were conducted under protocols approved by Abbott's Institutional Animal Care and Use Committee (IACUC) and National Institute of Health Guide for Care and Use of Laboratory Animals guidelines.

7.2. Morris water maze training

Adult (17–18 months) and aged (31–33 months) FBN/F1 rats were trained in the Morris Water maze, an index of spatial memory acquisition. Specifically, rats were given 2 trials a day for 5 days to learn the location of a hidden platform submerged 2 cm below the water surface. Latency to find the platform, distance swum, and swim speeds were recorded for each trial.

7.3. 7.3.Immunohistochemical analysis of HSD1 and pCREB

For immunohistochemical assessment, rats and mice were deeply anesthetized (CO_2) and perfused through the aorta with normal saline (5-min) followed by 10% formalin (10-min). Following perfusion, brains were removed and postfixed in 30% sucrose-phosphate buffered saline (PBS) overnight and subsequently cut frozen on a cryostat (40 μm coronal sections) and collected as free-floating sections in PBS. Sections containing cingulate cortex were then immunostained using a three-step ABC (avidin–biotin complex)-peroxidase technique beginning with a 30-min incubation with blocking serum. Sections were next incubated with anti-HSD1 antibody (rabbit polyclonal IgG, 1:1000, Zymed Laboratories) or anti-phospho-CREB (rabbit polyclonal IgG 1:1000, Cell Signaling) antibody (Ab) overnight at room temperature, washed with PBS and incubated for 1-h with biotinylated secondary anti-rabbit Ab (1:200). After that, sections were washed in PBS, incubated with ABC reagent (Vectastain Elite, Vector) and then developed in a peroxidase substrate solution (diaminobenzidine, 0.625 mg/ml). All incubations were done at room temperature.

Following IHC processing, four to six serial sections from each animal were cover slipped and photographed with a light microscope (Leica, DMRB). Sections were chosen on the basis of optimal immunoreactivity and anatomical similarity for immunoquantification, in which the experimenter was blind to treatment

conditions. Phospho-CREB and HSD1 immuno-reactivity in the cingulate cortex was quantified using an image analysis system (Leica, Quantimet 500) that determined relative intensity of peroxidase substrate-positive stained neurons from digitized photomicrographs according to a pixel gray level empirically determined prior to analysis.

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