CAMP Response Element Binding Protein (CREB) As A Marker Of Cognitive Function In Alzheimer's Disease

BY

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THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Anatomy and Cell Biology
in the Graduate College of the
University of Illinois at Chicago, 2017

Chicago, Illinois

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Dedication

To James

Acknowledgements

I would first like to thank my family for their constant encouragement and support during my education. My parents, Patricia Long and Christopher Long gave me the foundation and opportunities to pursue my chosen academic path, and I am grateful for their support. My husband, James Bartolotti, who is endlessly patient in listening to me talk about my research, and selflessly offers assistance and encouragment whenever I need it. I also appreciate the enthusiasm and support during my education from my siblings, Ginny Long and Phillip Long, as well as from the Bartolotti family, Betsy, Vinny, Eric and Matthew.

Next, I would like to acknowledge my academic mentors who promoted my intellectual growth. First, I would like to thank Dr. Jun Yoshino from Colgate University, Hamilton, NY, who taught me the foundations of research and critical scientific thought. Second, I would like to thank Dr. William Karpus, Northwestern University, Chicago, who employed me as a technician in his lab and taught me about research on a professional level. I would also like to thank Dr. Doug Feinstein and Dr. John Larson for their mentorship during my rotations in their labs. Finally, I am deeply grateful for the time and support that Dr. Orly Lazarov has invested teaching me how to be an independent scientist.

I would also like to acknowledge the members of my thesis committee, Dr. John Larson, Dr. Doug Feinstein, Dr. Amy Lasek, Dr. Orly Lazarov, and Dr. Gopal Thinakaran, for their time and advice during my committee meetings, and their responsiveness whenever I asked for advice outside of my meetings.

I would like to thank the members of the Lazarov lab who helped cultivate a supportive, productive lab environment. I would particularly like to thank Yuan-Shih (Jennifer) Hu whose thesis provided the groundwork for my research, and for teaching me lab techniques when I first joined the lab. I would also like to thank Laura Segura who provided me with valuable assistance on the CRE-reporter mouse behavior experiments during her rotation. In addition, I appreciate the positive influence and friendship of my fellow graduate students, Jackie Bonds, Matthew Tobin, and Caryn Davis. Finally, I would like to thank the other past and present members of the lab who all contributed valuable advice and assistance during my work, including Dr. Carolyn Hollands, Dr. Rachana Mishra, Dr. Yafit Kutner, Dr. Bich Duong, and Dr. Mike Demars.

I would also like to acknowledge those who provided me with materials and technical advice during the course of my work including Dr. Daniel Storm and his lab from the University of Washington, Seattle, for providing the CRE-β-galactosidase mice, and Dr. David Bennett and his lab from Rush University, Chicago, for the human samples. I am also grateful for the help I have received from other labs in the department of Anatomy and Cell Biology at UIC, particularly the labs of Dr. Ernesto Bongarzone, Dr. Scott Brady, Dr. Irene Givogri, Dr. Gerardo Morfini, and Dr. Kamal Sharma. I would also like to thank Dr. Balaji Ganesh for his advice on designing and analyzing the flow cytometry experiments. Finally, I would like to thank the staff in the Anatomy and Cell Biology department office for their assistance with the administrative aspects of my Ph.D.

Finally, I would like to thank the funding sources that supported this work, including NIA AG033570 (OL) and the PECTS Award CCTS Pre-doctoral Education

Clinical and Translational Scientists Program (NB), as well as P30AG10161, RF1AG15819, R01AG17917 (DAB).

Contribution of Authors

The following thesis includes methods, concepts, and figures that I have previously published as first author. The publications represented in this thesis are:

1. Bartolotti N, Segura L, Lazarov O (2016) Diminished CRE-Induced Plasticity is Linked to Memory Deficits in Familial Alzheimer's Disease Mice. *Journal of Alzheimers Disease* **50**, 477-489.

I was the primary researcher and author for this paper. Ms. Laura Segura, a rotating graduate student, performed analysis of behavior from CRE reporter mice and my research mentor, Dr. Orly Lazarov, assisted in writing the manuscript.

2. Bartolotti N, Bennett DA, Lazarov O (2016) Reduced pCREB in Alzheimer's disease prefrontal cortex is reflected in peripheral blood mononuclear cells. *Molecular Psychiatry* **21**, 1158-1166.

I was the primary researcher and author for this paper. Dr. Orly Lazarov, my research mentor, and Dr. David Bennett, a collaborator from Rush University, Chicago, IL, assisted in writing the manuscript.

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LIST OF ABBREVIATIONS

Aβ	Beta Amyloid
AD	Alzheimer's disease
ANOVA	Analysis of Variance
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
APPswe/PS1ΔE9	APP Swedish mutation and PS1 Deletion at Exon 9
A.U.	Arbitrary Units
BACE1	β-secretase 1, or β-site amyloid precursor protein cleaving enzyme
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
CaMKII	Calcium/Calmodulin-Dependent Protein Kinase II
CaMKIV	Calcium/Calmodulin-Dependent Protein Kinase IV

cAMP	Cyclic adenosine monophosphate
CBP	CREB Binding Protein
CRE	cAMP Response Element; may also refer to CRE-LacZ reporter mice
CRE-AD	Transgenic mice positive for CRE-LacZ reporter and APPswe/PS1ΔE9 mutations
CREB	CRE Binding Protein
DAPI	4',6-diamidino-2-phenylindole
DG	Dentate Gyrus
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's PBS
ECL	Enhanced Chemiluminescence
EE	Environmental Enrichment
EGR-1	Early growth response protein 1
ERK	Extracellular signal-regulated kinases
FAD	Familial Alzheimer's Disease
FBS	Fetal Bovine Serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase

HRP	Horse Radish Peroxidase
IEG	Immediate Early Gene
LTP	Long Term Potentiation
mA	milliampere
mM	millimolar
MAP	Memory and Aging Project
MAPK	Mitogen-activated protein kinase
MAR	Minority Aging Research study
MCI	Mild Cognitive Impairment
mg	milligram
mL	milliliter
MSK	Mitogen- and stress-activated protein kinase 1
NOR	Novel Object Recognition
OCT	Optimal cutting temperature compound
p300	Also known as EP300, or E1A (adenovirus early region 1A) binding protein p300
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
pCREB	Phosphorylated CREB (at Ser133)

PDEI	Phosphodiesterase Inhibitor
pERK	Phosphorylated ERK (at Tyrosine 204)
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
PHFtau	Paired Helical Filament tau
PKA	Protein Kinase A
PKC	Protein Kinase C
PS1	Presenillin-1
PVA-DABCO	Polyvinyl alcohol- 1,4-diazabicyclo[2.2.2]octane
RCF	Relative Centrifugal Force
RPM	Revolutions Per Minute
RSK	Ribosomal s6 kinase
SEM	Standard Error of the Mean
Ser133	Serine 133
SF	Superior Frontal gyrus
SH	Standard Housing
TBS	Tris-Buffered Saline
μM	Micromolar

X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Zif268	Zinc finger protein 225

SUMMARY

This study presents evidence that cyclic-AMP Response Element Binding Protein (CREB) signaling is dysfunctional in Alzheimer's disease (AD) and may underlie cognitive dysfunction in that disease. We show that multiple factors in the CREB signaling machinery are impaired in both a mouse model of familial Alzheimer's disease (FAD) and in the blood and postmortem brain of persons with sporadic AD.

Alzheimer's disease (AD) is an aging related neurodegenerative disorder with no known cause for the sporadic form. AD is characterized by severe cognitive impairments and memory dysfunction leading to decreased quality of life. There is no cure for AD, and treatments are limited and ineffective, alleviating initial symptoms at best. Understanding the cause of AD, and in particular the mechanism underlying cognitive dysfunction characterizing the disease, is essential to the discovery of effective and meaningful therapies.

Research on therapeutic interventions for AD has historically targeted the pathological hallmarks of the disease, the β-amyloid (Aβ) plaques and the neurofibrillary tangles. However, the severity of the pathology observed pre- and postmortem is poorly correlated with cognitive functioning during life, suggesting that other molecular mechanisms may underlie or contribute to cognitive impairments in AD.

Here, we investigate the role of CREB in AD. CREB is known to be critical for the formation of memory in the hippocampus. It is thought that CREB contributes to memory formation by regulating genes important for memory, such as the immediate early gene *Egr-1* (*zif268*). CREB drives activation of these genes following

phosphorylation at Ser133 (pCREB) and the recruitment of the transcription cofactors CREB Binding Protein (CBP) and p300, forming a transcription complex in the nucleus which binds the cyclic-AMP Response Element (CRE) sequence in the promoter region of genes essential for synaptic plasticity, learning, and memory, thus driving their transcription.

To determine the contribution of CREB signaling to memory dysfunction in AD we used the FAD-linked mouse model APPswe/PS1ΔE9. Female APPswe/PS1ΔE9 mice typically begin to exhibit plaque pathology between 4 and 6 months of age. In this study, we used female mice between 2 and 3 months of age. This distinction is important because it emphasizes that our observations are not simply the result of secondary alterations taking place as a result of advanced pathology. We examined baseline levels of CREB signaling components in the nuclear fraction of the hippocampus of these mice and show critical impairments in this signaling pathway. Specifically, we observed reduced expression of CREB, pCREB, p300 and CBP in the hippocampi of APPswe/PS1ΔE9 mice. We then had the novel observation that 3 month old female APPswe/PS1ΔE9 mice exhibit hippocampus-dependent memory impairments in the contextual fear conditioning task, and that this behavior deficit corresponded to diminished expression of CREB signaling components.

Next, we show that the reduction in CREB signaling machinery in the APPswe/PS1ΔE9 hippocampus is manifested by reduced CRE-based gene transcription. Crossing the APPswe/PS1ΔE9 mouse with a CRE-β galactosidase reporter mouse allowed us to visualize CRE-based gene transcription. This reduction is associated with an impairment in performance in another hippocampus-based behavior

test, Novel Object Recognition, demonstrating that memory impairments in 3 month old female APPswe/PS1ΔE9 mice are not task-specific. We then further confirm the functional deficit of impaired CRE-based gene transcription by examining protein levels of a CRE-driven immediate early gene, *Egr-1*, which did not increase in the hippocampus of APPswe/PS1ΔE9 mice following learning as it did in nontransgenic mice. These observations demonstrate that CREB signaling is impaired in young-adult FAD-linked mice, and that these impairments correspond to memory deficits.

Our observations in mice led us to investigate whether CREB signaling is compromised in human tissue of Alzheimer's disease patients. Very little work has been done to date to determine the role of CREB in AD, but reduced pCREB and Total CREB in the postmortem AD hippocampus has been previously reported. Here, we show that pCREB and Total CREB are also reduced in the nuclear fraction of AD prefrontal cortex (PFC), indicating that dysfunction of CREB signaling may not be restricted to the hippocampus in the AD brain. In addition, we are the first to show that the transcription cofactors CBP and p300 are also reduced in the postmortem AD brain. Importantly, we also showed lack of correlation between the severity of plaque or tangle pathology in the postmortem brain and the level of pCREB. These results strengthen the observation that CREB signaling may contribute to cognitive dysfunction in AD independent of neuropathological hallmarks.

While these observations suggest that CREB may be impaired at an end stage of AD, we next asked if we could observe CREB signaling impairments in the blood. The relevance of brain-blood communication is increasingly recognized, and AD brain hallmarks, such as β-amyloid (Aβ) and tau species are detected in the blood. Detection

of CREB signals in the blood would not only allow for potential diagnostic parameters, but could also serve as a readout for the efficacy of therapeutic intervention. To this end, we analyzed the nuclear fraction of peripheral blood mononuclear cells (PBMC) of persons clinically diagnosed with AD, mild cognitive impairment (MCI; the prodromal form of AD), and cognitively- intact, age-matched controls. We observed a reduction in pCREB in AD PBMC samples when compared to non-AD PBMC samples (i.e., cognitively normal combined with MCI). Similarly, we observed a decrease in CBP in AD PBMC samples when compared to non-AD PBMC samples. We observed a trending reduction in Total CREB in AD PBMC, albeit statistically not significant, as well as a nonsignificant reduction in the ratio of pCREB/Total CREB, suggesting that the reduction in pCREB may be a combination of reduced total protein and reduced activation. Finally, we observed a significant positive correlation between pCREB expression in the postmortem brain and pCREB expression in PBMC drawn fewer than 3 years prior to death, particularly in AD individuals. This novel observation suggests that CREB signaling in the periphery has the potential to serve as a marker for CREB signaling in the brain and, by extension, cognitive function in AD.

Taken together, this work in both an FAD-linked mouse model and human tissue from sporadic AD offers several novel contributions to the field, including (1) a comprehensive description of several points of impairment along the CREB signaling pathway corresponding to memory deficits in young FAD mice, (2) the novel description of CREB signaling impairments in postmortem AD PFC, and (3) the description of CREB signaling components in the periphery as markers of CREB signaling in the brain. This study offers exciting new evidence that (1) CREB signaling dysfunction could be an

important contributing factor to cognitive function in AD, (2) CREB dysfunction could be an early event in AD etiology, and (3) that monitoring CREB signaling in the periphery may serve as a valuable marker for AD disease progression and for assessing the effectiveness of therapies aimed at enhancing cognitive function in AD.

I. Introduction

A. Overview

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by memory impairments and cognitive decline. AD is a pressing public health concern due to the increase of the aged population and the lack of a cure or effective therapeutics. Historically, much of the research aimed at treating AD has focused on the amelioration of the pathological hallmarks of AD, namely Aβ plaques and neurofibrillary tangles. However, the relationship between cognitive functioning during life and the amount of pathology observed in postmortem tissue is poorly correlated (Mesulam, 1999; Terry et al., 1991), suggesting that pathology may not be the only or even main cause of cognitive decline in AD. Therefore, it is possible that other markers may be better suited for measuring cognitive ability during AD.

Cyclic-AMP Response Element Binding Protein (CREB) has long been known to be important for the formation of memories (Kandel, 2012; Yin et al., 1994). It is thought that activation of CREB by phosphorylation at Ser133 (pCREB), followed by the recruitment of critical transcription factors such as CREB Binding Protein (CBP) and p300, allows the transcription of genes such as *Egr-1* (*zif268*) which are required for memory formation (Jones et al., 2001a; Lakhina et al., 2015) (**Figure 1**). While CREB signaling has been investigated previously in mouse models of FAD, the results are often conflicting and focus on a single aspect of CREB signaling (Caccamo, Maldonado, Bokov, Majumder, & Oddo, 2010a; Y. M. Chen et al., 2012; Dineley et al., 2001; Francis, Diss, Kariti, Stephanou, & Latchman, 2007; Francis, Stephanou, & Latchman, 2006; Marambaud et al., 2003; Muller, Cardenas, Mei, Cheung, & Foskett, 2011; Nishimoto, Okamoto, Matsuura, Takahashi, Okamoto, et al., 1993; Saura et al., 2004; R. S. Wang

et al., 2006). Therefore, the nature of CREB signaling deficits in FAD remains unclear. In addition, the contribution of CREB signaling in human tissue from sporadic AD is a relatively unexplored area. Therefore, we designed a series of experiments to examine the relationship between CREB signaling and cognitive functioning in the APPswe/PS1ΔE9 mouse model of FAD. This mouse model coexpresses the FAD-linked APPswe and PS1ΔE9 mutations under the PrP promoter (Jankowsky et al., 2001). While a mouse model that faithfully replicates AD has yet to be developed, the APPswe/PS1ΔE9 is thought to model several important aspects of AD progression (for review see (Malm, Koistinaho, & Kanninen, 2011)). Importantly, these mice exhibit plaques and tau hyperphosphorylation, progress at a fast enough rate to allow us to examine cognitive dysfunction in a timely manner, while still progressing at a slower rate than some of the more aggressive mouse models of FAD, allowing us to study cognitive performance and CREB signaling following full development, but prior to the onset of amyloid deposition. Using this mouse model, we have shown that levels of pCREB and its cofactors, p300 and CBP are significantly reduced in the hippocampus of APPswe/PS1ΔE9 mice. Moreover, following learning, levels of these proteins are upregulated in the wild type brain, but not in the APPswe/PS1ΔE9 brain, suggesting that impairments in CREB signaling may play a role in learning and memory deficits in FAD.

Next, we attempted to translate these observations and examined the expression of these proteins in human tissue of AD patients. Remarkably, we observed that levels of pCREB, p300 and CBP are significantly reduced in the AD compared to the non-AD brain. Moreover, we observed that levels of pCREB in the blood [peripheral blood mononuclear cells (PBMC)] mirrors its level in the brains of these individuals. Taken

together, these experiments suggest that CREB signaling may play a critical role in learning and memory deficits in AD, and offers information on potential therapeutic targets. Importantly, these results suggest that pCREB may be a potential biomarker for the diagnosis of cognitive deficits and may serve as a diagnostic tool or a measure of the efficacy of therapeutic interventions.

Figure 1

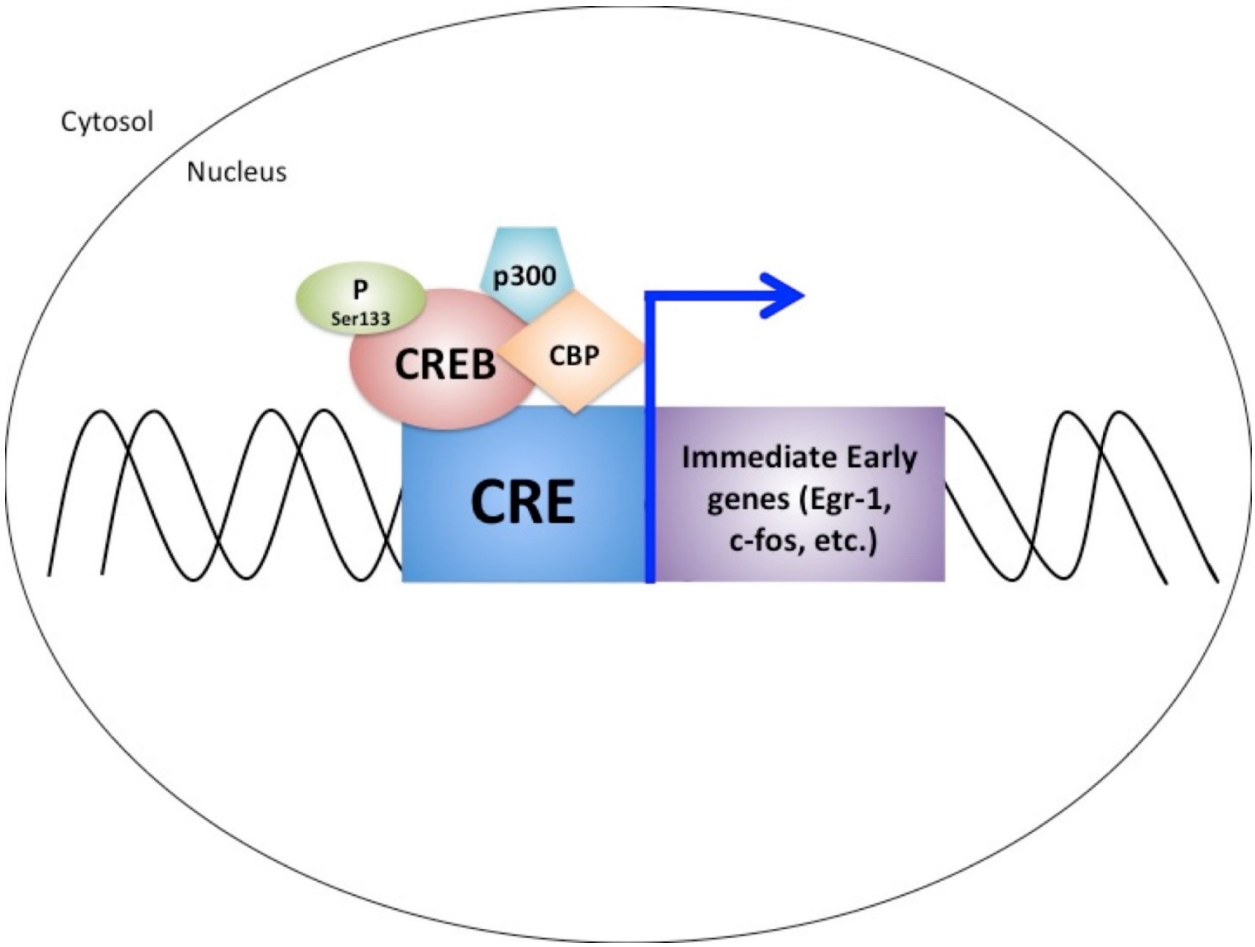

Figure 1: Schematic illustration of the components of CREB-based signaling resulting in expression of CRE-driven genes thought to be important for the formation of memory.

B. Research questions

The purpose of this study was to determine the role of CREB signaling in Alzheimer's disease, both at a steady state and during learning. In addition, we aimed to determine the relationship between CREB signaling in the periphery and CREB signaling in the brain. The primary goal of this study was to identify potential therapeutic targets and biomarkers of cognitive function in AD.

Hypothesis:

Deficits in CREB signaling may underlie learning and memory impairments in Alzheimer's disease.

C. Specific Aims

To address our hypothesis, we generated the following Specific Aims:

1. To determine the mechanisms underlying impaired CREB signaling in FAD

We have previously shown that activation of CREB is impaired in young adult FAD-linked APPswe/PS1ΔE9 mice following environmental enrichment (Y. S. Hu, Long, Pigino, Brady, & Lazarov, 2013a). In addition, abnormal CREB signaling in mouse models of FAD has been previously reported by others (Caccamo et al., 2010a; Y. M. Chen et al., 2012; Dineley et al., 2001; Francis et al., 2007; Francis et al., 2006; Marambaud et al., 2003; Muller et al., 2011; Nishimoto, Okamoto, Matsuura, Takahashi, Okamoto, et al., 1993; Saura et al., 2004; R. S. Wang et al., 2006). However, the results of these studies are conflicting, and so the nature and functional relevance of CREB

signaling in FAD remains unclear. Experiments in this Aim will utilize Western blot analysis of protein expression in the hippocampus of APPswe/PS1ΔE9 mice and their nontransgenic littermates to examine the hypotheses that (1) *CREB signaling is impaired in FAD* and (2) *Kinases that activate CREB are defective in FAD, leading to impaired induction of CREB phosphorylation.*

2. To determine whether impairments in CREB signaling interfere with the formation of long-term memories in FAD.

While CREB signaling impairments are thought to lead to deficits in memory formation, the extent of abnormality necessary to have a functional impact on memory formation remains unclear. Experiments in this Aim will relate performance on hippocampus-dependent learning and memory tasks to protein expression of CREB signaling components and reporter expression of CRE-gene transcription to examine the hypotheses that (1) *Impairments in CREB phosphorylation interfere with learning and memory in FAD*, and (2) *Learning induced CRE-driven gene transcription is impaired in the FAD mouse model APPswe/PS1ΔE9*.

3. To determine if CREB signaling components are reduced in sporadic AD, and whether peripheral CREB can be used as a marker for CREB in the brain.

Very little work has been done to examine the expression of CREB signaling components in sporadic AD, and the need for an accessible biomarker for cognitive functioning in AD is of paramount importance. Experiments in this aim will utilize Western blot analysis to examine protein expression in the brain and blood of individuals with sporadic AD to examine the hypotheses that (1) *CREB signaling is impaired in*

sporadic AD brain, (2) *CREB signaling components are impaired in AD peripheral blood mononuclear cells* and (3) *CREB expression in peripheral blood mononuclear cells is reflective of CREB expression in the brain*.

D. Significance of the study

Dementia, or the progressive loss of cognitive functioning, is an urgent health concern that is thought to affect over 25 million individuals worldwide and is estimated to rise to over 80 million by 2040 (Ferri et al., 2005). Alzheimer's disease is the most prevalent form of dementia (Ballard et al., 2011). Understanding the mechanisms underlying the cognitive impairments of this disease is imperative to developing therapies for AD. Currently there is no cure for AD, treatments are limited, and even diagnosis remains challenging. Many efforts at treatment and diagnosis have focused on ameliorating the pathological hallmarks of AD, the amyloid plaques and neurofibrilary tangles. However, these neuropathological hallmarks may not be indicative of cognitive functioning, and the efficacy of treating these hallmarks on improving cognitive impairments is controversial.

This study focuses on the molecular mechanism underlying cognitive function in AD. Specifically, we examine the role of CREB signaling, both in genetically-linked, familial forms of AD using a mouse model, as well as utilizing human tissue from the more common sporadic form of AD. Our results suggest that CREB signaling is defective in both FAD and sporadic AD, and these defects correlate with learning and memory impairments. Importantly, we report for the first time that CREB signaling components are reduced in the peripheral blood cells of persons with AD, and that this reduction correlates with a reduction in CREB signaling components in the brain. This

study paves the way for the development of new biomarkers and therapeutic targets for the diagnosis and treatment of AD.

II. Background and Relevant Literature

A. Alzheimer's disease (AD)

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by severe, progressive cognitive impairments. These impairments can include difficulty in learning or remembering information, impairments in judgment, deficits in object recognition and language abilities, as well as alterations in mood or psychiatric status to an extent to which normal daily life is severely impacted (McKhann et al., 2011). Postmortem hallmarks of AD include the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles (Braak & Braak, 1991). The initiating cause of these deposits is unknown, but some clues have come from the familial forms of AD (FAD), which arise from mutations in either *PSEN1* or *PSEN2,* or *APP.* Amyloid Precursor Protein (APP) is thought to contribute to the formation of amyloid deposits when it is processed via the amyloidogenic pathway which refers to sequential processing of APP first by the enzyme β-secretase, followed by processing by the enzyme γ-secretase (for review see (Lazarov & Demars, 2012)). Presenilin is a critical component of the catalytic core of γ-secretase and mutations in either *APP or PSEN* are thought to contribute to AD etiology by accelerating the production of β-amyloid and therefore plaque deposition. However, alterations in these proteins can affect additional physiological processes, such as the formation of new neurons in the brain via neurogenesis, which may also contribute to cognitive impairments (for review see (Lazarov & Marr, 2013)). Other neuropathological effects of AD include neuronal loss and brain atrophy, particularly in the hippocampus (Schroder & Pantel, 2016).

Importantly, most forms of AD are of the sporadic kind with no known cause. Risk factors for sporadic AD include age, APOE ε4 carrier status, and lifestyle factors such as

mental activity level, physical activity level, and nutrition, and there is substantial evidence that environmental manipulation can alter cognitive function in AD in spite of the presence of AD neuropathology (for review see (N. Bartolotti, & Lazarov, O., 2016)). This suggests that cognitive impairments in AD may not be exclusively tied to the progression of neuropathology.

B. Cognitive function in AD

The poor correlation between end state tangle and plaque pathology and cognitive performance in AD has long been known (Mesulam, 1999; Terry et al., 1991). In addition, there is substantial evidence that participation in cognitively complex activities during life can reduce risk of AD and slow cognitive decline, even in the presence of extensive pathology (for review see (N. Bartolotti, & Lazarov, O., 2016)). For example, studies from religious order participants, for whom lifestyle factors are relatively similar, show that the extent of participation in cognitively complex tasks during life modified AD risk, as well as superior cognitive functioning and reduced AD-related pathology (D. A. Bennett, Schneider, Wilson, Bienias, & Arnold, 2005; Mortimer, 2012; Riley, Snowdon, Desrosiers, & Markesbery, 2005; Snowdon et al., 1996; Tyas, Snowdon, Desrosiers, Riley, & Markesbery, 2007). Similarly, in twin studies, the twin with greater lifestyle complexity either through leisure activities or through occupation exhibited reduced risk for AD, particularly when the twins were carriers for APOE ε4 (Andel et al., 2005; Carlson et al., 2008; Crowe et al., 2003; Potter, Helms, Burke, Steffens, & Plassman, 2007).

The protective aspects of cognitive complexity have been shown to arise from many experiences, such as education or bilingualism, as well as participation in

cognitively demanding activities such as reading, writing, or playing an instrument or games (Akbaraly et al., 2009; Alladi et al., 2013; Farfel et al., 2013; Freedman et al., 2014; Meng & D'Arcy, 2012; Rovio et al., 2005; R. S. Wilson, Scherr, Schneider, Tang, & Bennett, 2007) (Gold, 2014) (Sattler, Toro, Schönknecht, & Schröder, 2012; R. S. Wilson, Boyle, Yang, James, & Bennett, 2015; R. S. Wilson et al., 2002).

The mechanism underlying the benefits of cognitive activity for AD risk and progression is not well understood. Some studies have examined the effect of cognitive complexity on the pathological hallmarks of AD. For example, some preliminary experiments suggest that greater education level and participation in cognitively complex leisure activities may reduce the presence of amyloid plaques in the brain, although the effect that this reduction has on cognitive performance is less clear (J. Wang et al., 2014) (Lo & Jagust, 2013; Yasuno et al., 2014) (Landau et al., 2012)

However, cognitive activity has been shown to ameliorate the negative effects of AD even without altering amyloid pathology. For example, many studies have described enhanced cognitive functioning in individuals with greater education level when matched with individuals with similar levels of postmortem amyloid pathology (D. A. Bennett, Schneider, Tang, Arnold, & Wilson, 2006; D. A. Bennett et al., 2005; D. A. Bennett et al., 2003; Meng & D'Arcy, 2012; Roe, Xiong, Miller, & Morris, 2007). Similarly, in the bilingual brain, postmortem pathology is often more severe that the cognitive symptoms during life would have suggested (Schweizer, Ware, Fischer, Craik, & Bialystok, 2012). A similar protective effect has been described for participation in cognitively stimulating activities like reading and playing games, which allows for greater cognitive functioning even in the presence of AD pathology (Negash et al., 2013).

The mechanism underlying the phenomemon of preserved cognitive functioning in spite of AD neuropathology is still not understood, but some clues may come from animal experiments. The role of the environment in cognitive performance is perhaps most extensively described in rodents. Famously, Donald Hebb showed that rats raised in a more complex environment as pets were more adept at learning and memory tasks compared to standard housed lab rats, indicating that environmental manipulation can affect brain function (Hebb, 1947, 1949). Subsequent studies expanded on these observations by demonstrating that experience in a complex environment enhanced many readouts of brain functioning such as increased cortex weight, increased expression of neurotrophic factors, enhanced synaptic formation and hippocampal neurogenesis, as well as increased number of dendrites and dendritic branching (E. L. Bennett, Diamond, Krech, & Rosenzweig, 1964; Globus, Rosenzweig, Bennett, & Diamond, 1973; Hamilton, Bennett, & Silver, 1964; Turner & Greenough, 1985; Volkmar & Greenough, 1972).

This experimental paradigm, known as environmental enrichment (EE), was then extended to mice modeling FAD. The benefits of EE in FAD mice includes reduced neuropathology such as oligomeric Aβ and tau hyperphosphrylation as well as enhanced plasticity, as evidenced by increased neurogenesis and long-term potentiation (LTP) (Y. S. Hu et al., 2010).

The mechanism underlying the improvements following EE in the FAD mice may be in part due to reduction of pathology. Amyloid pathology in particular seems to be particularly malleable by EE (Ambree et al., 2006; Berchtold, Chinn, Chou, Kesslak, & Cotman, 2005; Billings, Green, McGaugh, & LaFerla, 2007; Costa et al., 2007; Herring et al., 2011; Lazarov et al., 2005; Min et al., 2010b; Mirochnic, Wolf, Staufenbiel, &

Kempermann, 2009; K. Nichol, Deeny, Seif, Camaclang, & Cotman, 2009; K. E. Nichol et al., 2008; Parachikova, Nichol, & Cotman, 2008; Perreau, Adlard, Anderson, & Cotman, 2005; Russo-Neustadt, Beard, & Cotman, 1999). EE has also been shown to modify tau pathology while improving cognitive performance in multiple mouse models of FAD (Billings et al., 2007; Y. S. Hu et al., 2010).

However, improvements in learning and memory performance following EE have been observed even when pathology is not ameliorated, suggesting that EE may offer cognitive benefits independent of amyloid clearance (Joanna L. Jankowsky et al., 2005; Verret et al., 2013). The mechanism for this improvement may include enhancing neurotrophic factors and synaptic function, such as has been demonstrated for example in the 3xTg-AD mouse model of FAD, in which, following the onset of pathology, use of a running wheel increased proteins important for synaptic function normally reduced in this mouse model (Revilla et al., 2014). We, and others, have made attempts to begin to unravel the molecular mechanisms of the benefits of EE for AD (Berchtold et al., 2005; Billings et al., 2007; Y. S. Hu et al., 2013a; Lazarov et al., 2005; Perreau et al., 2005). One important observation from our work included the evidence that the CREB signaling pathway is enhanced following EE in nontransgenic mice, *but not in the APPswe/PS1ΔE9 mouse model of AD* (Y. S. Hu, Long, Pigino, Brady, & Lazarov, 2013b). This observation led us to ask the question whether EE could completely rescue cognitive functioning in AD, and whether dysfunctional CREB signaling could be, at least in part, responsible for cognitive impairments in AD.

C. CREB signaling

1. CRE

Formation of long term memories is thought to depend on protein synthesis resulting from gene transcription (Squire & Barondes, 1973). Genes containing a cyclic-AMP Response Element on the promoter region of the DNA in particular are thought to be important for this aspect of memory encoding. Long-term potentiation (LTP) experiments indicate that CRE-based gene transcription is driven by neuronal activity and may play a role in regulating synaptic plasticity (Impey et al., 1996). These experiments suggest that during learning, Ca^{2+} influx is sensed by either Ca^{2+} sensitive adenylyl cyclases that in turn activate PKA and cause it to translocate to the nucleus where it phosphorylates CREB, or via other Ca^{2+} sensitive kinases. While other brain cells have been described to express CRE-driven reporters, the majority of the cells that respond to stimulation by enhancing CRE-activity appear to be primarily neurons (Sugiura et al., 2004). It is thought that following neuronal stimulation, CREB activation induces the transcription of genes that play a crucial role in the initiation of long-term memory (Lee & Silva, 2009) and in the consolidation and retrieval of spatial and fear memories (R. Kim, Moki, & Kida, 2011). Many genes, known as immediate early genes, have been described to respond to neuronal activity, and many of these genes possess a CRE region in the promoter region, including *c-fos, c-jun,* and *Egr-1* (Worley et al., 1990). In addition, these genes have been independently shown to be important for memory formation (Fleischmann et al., 2003; Jones et al., 2001b). Therefore, CRE-driven gene transcription is an essential part of neuroplasticity and learning.

CRE-induced plasticity may not be confined exclusively to the hippocampus. For example, stimulating the whisker barrel in CRE-reporter mice results in increased CRE-reporter expression in the part of the cortex on which this area is mapped (Barth et al., 2000). This evidence suggests that appropriate regulation of CRE-driven gene transcription is a critical component of neuroplasticity throughout the brain and may not be exclusively confined to the hippocampal formation.

2. CREB

One of the most critical steps in the initiation of CRE-driven gene transcription is the activation of the transcription factor cyclic-AMP response element binding protein (CREB) by phosphorylation at Ser133 (pCREB) [for review see (Alberini & Chen, 2012)]. Activation of CREB is necessary and sufficient for the recruitment of the transcriptional coactivators CBP and p300 to the promoter and to initiate transcription (Du, Asahara, Jhala, Wagner, & Montminy, 2000). Experiments examining the formation of memory when CREB or the activation of CREB is impaired can offer insight to the importance of CREB signaling in memory.

There are three isoforms of CREB: α, δ, and β, with α and δ being the major isoforms. CREB mutant mice have been generated that lack the major α and δ isoforms, though retaining small amounts of CREB activity due to the β isoform (Lonze & Ginty, 2002). These CREB mutant mice have been described to have impairments in LTP and in memory-based behavior tests such as fear conditioning (Bourtchuladze et al., 1994). Interestingly, impaired CREB function has also been shown to prevent memory formation in Drosopholia, and the importance of CREB in learning for *C. elegans* has also been described, suggesting that this pathway is evolutionarily conserved (Lakhina

et al., 2015; Yin et al., 1994). The results of impaired CREB signaling may not be limited to impaired hippocampal plasticity. For example, CREB mutant mice show impaired cortical plasticity as well (Glazewski et al., 1999). The importance of CREB in memory has been confirmed by the use of additional genetic methods including a CREB repressor (Kida et al., 2002), and expression of a dominant negative form of CREB in mice (Pittenger et al., 2002).

A few attempts have been made to increase CREB signaling in mice. Viral manipulation to enhance CREB signaling in mice generally improves memory performance (Brightwell, Smith, Neve, & Colombo, 2007; Josselyn et al., 2001). However, impartial upregulation of CREB, particularly of basal CREB, may be equally problematic for memory formation and retrieval (Viosca, Malleret, et al., 2009). This is in line with experiments that describe abnormal upregulation of activated CREB in mouse models of AD that may correspond to memory impairments (Muller et al., 2011). In an additional example, expression of a dominant active form of CREB has been reported to impair as well as enhance memory performance (Lopez de Armentia et al., 2007; Suzuki et al., 2011; Viosca, Lopez de Armentia, Jancic, & Barco, 2009; Viosca, Malleret, et al., 2009). The difference in these reports may be in part due to the magnitude of increase in CREB activity, with more moderate CREB activity enhancement providing a more beneficial outcome in memory performance. Alternatively, it may depend on the mechanism of action of the dominant active CREB, with forms that mimic wild-type CREB signaling in terms of downstream effects, such as the recruitment of transcription cofactors, offering more beneficial effects, suggesting that therapeutics aimed at CREB should focus on specificity to mimic normal CREB signaling, as well as sensitivity to stimuli normally activating CREB (Suzuki et al., 2011). These experiments also indicate

that additional components of the CREB signaling pathway, such as the cofactors CBP and p300, are critical for appropriate regulation of gene transcription and thus memory formation.

3. CBP and p300

CREB binding protein (CBP) and p300 are transcriptional coactivators that share great similarity in both function and sequence (Arany, Sellers, Livingston, & Eckner, 1994). They interact with proteins such as activated CREB and are necessary to drive CRE-based gene transcription (Chrivia et al., 1993). Like CREB, CBP is activated as a transcriptional coactivator following neuronal activity (Hardingham, Chawla, Cruzalegui, & Bading, 1999), and mice with deficient CBP are known to have impairments in memory formation. For example, CBP deficient mice show a strong defect in enriched environment (EE)-induced neurogenesis as well as impairments in EE- mediated enhancement of spatial navigation and pattern separation (Lopez-Atalaya et al., 2011b). Similarly, mice with p300 conditionally knocked out in the hippocampus and cortex show impairments in several memory tests, including NOR and contextual fear conditioning (A. M. Oliveira et al., 2011).

In addition to serving as scaffolding for coordinating the transcription complex, CBP and p300 possess intrinsic histone acetyltransferase (HAT) activity and may facilitate gene transcription by loosening chromatin and allowing access of the transcriptional machinery (Bannister & Kouzarides, 1996; Ogryzko, Schiltz, Russanova, Howard, & Nakatani, 1996). Importantly, this HAT activity of CBP is essential for memory consolidation, as evidenced by experiments in which the HAT activity of CBP is

blocked, while the scaffolding function remains intact (Korzus, Rosenfeld, & Mayford, 2004). In addition, preliminary studies using an activator of CBP/p300 HAT activity have shown promising enhancement of memory performance in mice, suggesting that the HAT activity may be a critical component of the function of CBP/p300 in learning and memory (Chatterjee et al., 2013). Similarly, while CBP mutant mice show impairments in Novel Object Recognition, treatment with an HDAC inhibitor to restore histone acetylation can restore recognition memory in this task (Stefanko, Barrett, Ly, Reolon, & Wood, 2009). Indeed, even in a CREB mutant mouse, an HDAC inhibitor was able to enhance memory performance, indicating a strong role of the HAT activity of CBP (Vecsey et al., 2007a). However, in a CBP conditional knockout, HDAC inhibition by the same inhibitor was not sufficient to restore memory impairments in spite of rescuing histone acetylation, so the mechanism by which CBP might be used as a therapeutic target remains an open question (Chen, Zou, Watanabe, van Deursen, & Shen, 2010).

4. PKA, PKC, CAMKs, and ERK

An additional point of regulation for CREB signaling is through the kinases that phosphorylate CREB. The kinases that have been shown to activate CREB *in vivo* include protein kinase A (PKA), protein kinase C (PKC), the calcium/calmodulin-dependent protein kinases CaMKII and CaMKIV, and the extracellular signal-regulated kinase (ERK)-activated kinases mitogen- and stress-activated protein kinase (MSK) and the 90 kDa ribosomal S6 kinase (RSK) (Lonze & Ginty, 2002). Therefore, we will confine the discussion of kinase activation of CREB to these kinases.

PKA, a kinase activated by cAMP, was one of the first kinases recognized to phosphorylate and activate CREB (for review see (Kandel, 2012)). The PKA activator forskolin has been shown to enhance CRE-driven gene expression in hippocampal slices, and PKA inhibitors blocked LTP and the associated increase in CRE-driven gene expression (Impey et al., 1996). EE increases PKA activity while improving performance in a contextual fear conditioning task (Duffy, Craddock, Abel, & Nguyen, 2001). Mice that are genetically modified to have impaired PKA expression or activity have been described to have a wide range of memory impairments, including impairments in NOR and contextual fear conditioning (Abel, Nguyen, Barad, Deuel, & Kandel, 1997; Bourtchouladze et al., 1998; Isiegas, Park, Kandel, Abel, & Lattal, 2006; Sharifzadeh, Sharifzadeh, Naghdi, Ghahremani, & Roghani, 2005). However, overactive PKA has also been described to interfere with memory formation in several behavioral paradigms, including NOR (Giralt et al., 2011). Interestingly, in the Giralt study they did not observe increased pCREB in spite of overactive PKA, which is line with the hypothesis that rather than enhancing levels of upstream CREB signaling factors, it is more important to enhance the functional signaling that results from the components of the CREB signaling pathway (reviewed by (Abel & Nguyen, 2008)).

PKC is also known to activate CREB *in vivo* and is thought to also play a role in synaptic plasticity across species (Choi, Smith, Buratowski, & Quinn, 1991; Manseau, Sossin, & Castellucci, 1998; Paylor, Morrison, Rudy, Waltrip, & Wehner, 1992; Sacktor, Kruger, & Schwartz, 1988; Sheu, Mccabe, Horn, & Routtenberg, 1993). EE, a paradigm known to enhance learning and memory performance, has also been shown to enhance PKC activity in the hippocampus (Paylor et al., 1992). Colombo *et al*. found a

relationship between strength of performance on a memory-based task and concentration of PKC in the hippocampus (Colombo, Wetsel, & Gallagher, 1997). However, a great deal of redundancy exists between the different isoforms of PKC, and the extent of the contribution of PKC to memory may be dependent on the isoform, subcellular localization, and task (Colombo et al., 1997; Sossin, 2007; Volk, Bachman, Johnson, Yu, & Huganir, 2013; Weeber et al., 2000).

The calcium/calmodulin-dependent protein kinases (CaM) are kinases that respond to Ca^{2+} and have also been shown to be important for activation of CREB and for learning and memory. Both CaMKII and CaMKIV activate CRE-based gene expression, and mice genetically modified to express reduced CaMKII or CaMKIV signaling show widespread deficits in many memory tasks (Bachstetter et al., 2014; Takao et al., 2010). The importance of CaM kinases in plasticity may not be confined to the hippocampus, as paradigms of cortical plasticity have elucidated a critical role for CaMKII (Glazewski, Chen, Silva, & Fox, 1996). Mice overexpressing CaMKIV have been described to have enhanced activation of CREB corresponding to improved memory performance in tasks such as contextual fear conditioning (Fukushima et al., 2008; Wu et al., 2008). Interestingly, CaMK mutant mice have also been described to have normal memory in certain tasks, and neuronal cultures from CaMKIV mutant mice have even been described to have normal CREB activation (Song et al., 2015; Takao et al., 2010). This evidence suggests that while the CaM kinases are important for CREB activation and memory formation, other signaling pathways may be able to compensate when the CaMK cascade is dysfunctional.

The MAPK signaling cascade, and the downstream kinase ERK in particular, is important for memory and CREB function through a pathway involving *zif268* (reviewed in (Bozon et al., 2003)). Appropriate regulation of the ERK kinase pathway is known to be crucial for many processes related to cognitive functioning, including neurogenesis and activation of IEG, likely through CREB (Mazzucchelli et al., 2002; Satoh et al., 2007; Satoh et al., 2011). ERK can activate CREB through activation of the kinases MSK and RSK. MSK knockout mice have been reported to have impaired CREB phosphorylation and memory deficits on multiple learning and memory tasks, including contextual fear conditioning (Arthur et al., 2004; Besnard, Laroche, & Caboche, 2014; Chandramohan, Droste, Arthur, & Reul, 2008; Chwang, Arthur, Schumacher, & Sweatt, 2007; Impey et al., 1998a; Sindreu, Scheiner, & Storm, 2007). Similarly, loss of RSK function can cause impairments in synaptic activity and alterations in ERK-based function (Beck et al., 2015). These studies show that ERK, through MSK and RSK, may be another important point of regulation of CREB activation and cognitive function.

The interaction between CREB and its kinases is complex, and the redundancy and cross-talk between these pathways suggests that a single kinase pathway may not be solely responsible for the regulation of CREB (Ahi, Radulovic, & Spiess, 2004; Giese & Mizuno, 2013). However, taken together, the evidence that interfering with upstream activation of CREB by altering its kinases results in memory impairments demonstrates the critical role of proper regulation of CREB in memory formation.

D. CREB signaling in AD

The importance of CREB signaling for neural plasticity and cognitive performance suggests that this pathway may be dysfunctional in neurodegenerative disorders, such as AD, in which memory and cognitive function are impaired (Cowburn, Oneill, et al., 1992; Schnecko, Witte, Bohl, Ohm, & Lemmer, 1994b; Yamamoto-Sasaki, Ozawa, Saito, Rosler, & Riederer, 1999a). Indeed, the evidence that compromised CREB signaling results in impairments in spatial reference memory in mice is particularly interesting for the study of cognitive functioning in AD, as spatial reference memory is known to be impaired in persons with AD (Mizuno et al., 2002).

However, relatively little work has been done to investigate CREB in persons with AD. Two papers have examined CREB in the postmortem AD brain and demonstrated that total and activated CREB are reduced in the post-mortem hippocampus of individuals with AD, which supports the idea that CREB signaling is defective in AD (Pugazhenthi, Wang, Pham, Sze, & Eckman, 2011; Yamamoto-Sasaki, Ozawa, Saito, Rosler, & Riederer, 1999b). However, there were limitations to these studies. First, these studies utilized a small sample size (N=7 per group for Yamamoto-Sasaki, N=12 for Pugazhenthi) of mixed sex samples. Second, the analysis in both papers was restricted to the hippocampus and lacked cellular fractionation, both of which are important considerations for the interpretation of CREB function (Cammarota et al., 2000; Cammarota et al., 1999; Crino et al., 1998; Vianna et al., 2000). Finally, both studies utilized postmortem tissue only, which represents an end state. Therefore, how CREB signaling is altered during early AD and during disease progression remains unknown.

Other components of the CREB signaling pathway have been studied in postmortem brain. For example, the level and activity of Type I adenylyl cyclase, an

enzyme responsible for the generation of cAMP and hence CREB activation, is reduced in AD (Cowburn, O'Neill, et al., 1992; Schnecko, Witte, Bohl, Ohm, & Lemmer, 1994a; Yamamoto et al., 1996; Yamamoto et al., 1997). Similarly, the kinases that phosphorylate CREB, including PKA, PKC, CamK, and ERK have been repeatedly described to exhibit abnormal expression or activity in postmortem AD brain (Battaini, Pascale, Lucchi, Pasinetti, & Govoni, 1999; Bonkale, Cowburn, Ohm, Bogdanovic, & Fastbom, 1999; S. H. Kim, Nairn, Cairns, & Lubec, 2001; Perry et al., 1999; Reese, Laezza, Woltjer, & Taglialatela, 2011; H. Y. Wang, Pisano, & Friedman, 1994). Therefore, the evidence suggests that the CREB signaling pathway may be dysfunctional at multiple points in the postmortem AD brain. Again, how these components change during the course of the disease remains an open question.

E. CREB signaling in mouse models of AD

While data concerning CREB signaling in AD is limited, many groups have examined CREB signaling in mouse models of FAD. However, the results from these studies remain controversial (Francis et al., 2007; Francis et al., 2006; Marambaud et al., 2003; Saura et al., 2004). For example, in studies of baseline expression of activated CREB, both decreased (Caccamo et al., 2010a; Y. M. Chen et al., 2012; Marambaud et al., 2003; Nishimoto, Okamoto, Matsuura, Takahashi, Okamoto, et al., 1993; Saura et al., 2004; R. S. Wang et al., 2006) and increased (Dineley et al., 2001; Marambaud et al., 2003; Muller et al., 2011) levels have been reported. These discrepancies may be due to a number of factors, including the mouse model used, as well as the age and sex of the mice included in the study. Interestingly, even when baseline levels of CREB activity

are higher, such as in the 3XTg-AD mouse, learning-induced CREB activation remains impaired, indicating that higher levels of CREB activity alone are not sufficient for proper learning-induced CREB signaling, and that excessive levels of CREB activity may have harmful consequences on synaptic function and retrieval of spatial information (Muller, Cardenas, Mei, Cheung, & Foskett, 2011, Caccamo, Maldonado, Bokov, Majumder, & Oddo, 2010a). In line with this idea, EE is not sufficient to rescue memory impairments in a mouse model expressing dominant active CREB mouse, though EE has been previously reported to increase CREB signaling and enhance memory performance (Viosca, Malleret, et al., 2009; Williams et al., 2001), and we have previously shown that EE enhances activated CREB in the hippocampus of nontransgenic, but not in the hippocampus of APPswe/PS1ΔE9 mice (Y. S. Hu et al., 2013b).These results suggest that CREB signaling is not only a critical modulator of memory formation, but that abnormal CREB signaling can singly prevent memory enhancement by other means such as EE.

In the mouse models of FAD, dysregulation of CREB signaling is thought to result from the mutations in PS1 and or APP (Y. Chen et al., 2012; Marambaud et al., 2003; R. Wang et al., 2006). For example, mutations in PS1 are thought to interact with CREB signaling components. For example, one recent paper suggests that a cleavage product generated by PS1 may promote the degradation of CBP, and that a mutation in PS1 may alter this process (Marambaud et al., 2003). Interestingly, CBP has also been described to be repressed following mutations in PS1 (Francis et al., 2006).

Similarly, APP mutants have been shown to negatively regulate CREB activity (Nishimoto, Okamoto, Matsuura, Takahashi, Murayama, et al., 1993). Other work

suggests that increased Aβ toxicity resulting from the mutations in APP may negatively impact CREB (Dineley et al., 2010b; Dineley et al., 2001; Espana et al., 2010; Ma et al., 2007). Indeed, mice injected with Aβ oligomers exhibit decreased pCREB in the hippocampus and impaired performance in the fear-conditioning task (Dineley et al., 2010a). In addition, treatment of the Tg2576 mouse model of FAD with anti-Aβ antibodies rescued impaired CREB activation (Ma et al., 2007). However, β-secretase, which is thought to be increased in AD, has been shown to reduce CREB activation independent of its effects through Aβ, suggesting that CREB dysfunction may not be solely dependent on Aβ expression (Y. Chen et al., 2012). Therefore, while the mutations in APP and PS1 in mouse models of AD are undoubtedly the original source of CREB impairments, the proximate cause of these deficits remains unclear.

A few groups have made attempts to enhance memory in FAD mouse models by facilitating CREB signaling. For example, rescuing CREB signaling in the hippocampus of the TgCRND8 mouse model of AD through a viral paradigm ameliorated memory deficits (Yiu, Rashid, & Josselyn, 2011). In a separate study using virus technology, upregulating CBP expression by reduced memory impairments without affecting changes in amyloid or tau pathology in the 3xTg-AD mouse model of AD (Caccamo et al., 2010a). Pharmacological interventions aimed at increasing CREB signaling have also been tested in mouse models of FAD. For example, phosphodiesterase inhibitors (PDEI) have been proposed to increase CREB activity by preventing the reduction of cAMP by phosphodiesterases and thus enhancing CREB signaling through the PKA pathway. The PDEI rolipram has been shown to reverse Aβ induced deficits in LTP, as well as rescue deficits in pCREB, dendritic complexity, and memory deficits in mouse

models of FAD (Cheng et al., 2010; Gong et al., 2004; Smith, Pozueta, Gong, Arancio, & Shelanski, 2009; C. Zhang et al., 2014). In the APPswe/PS1ΔE9 mouse model, treatment with a PDEI was shown to enhance memory function (Jin et al., 2014).

An alternative pharmacological approach to enhancing CREB signaling is via HDAC inhibition. CBP and p300 are HATs, and a reduction in their expression or activity may reduce gene transcription by preventing the opening of the chromatin. HDAC inhibition prevents the deactylation of the chromatin allowing the transcription machinery continued access to the chromatin, and it has been proposed that they act through the CREB/CBP pathway in the brain (Vecsey et al., 2007b). HDAC inhibition has been described to increase pCREB and reverse memory impairments in mouse models of AD (Francis et al., 2009; Ricobaraza et al., 2009; Roy et al., 2015). Recently, therapies combining PDEI and HDAC inhibition have been tested in the APPswe/PS1ΔE9 mouse model, and a synergistic improvement in memory and dendritic complexity was observed (Cuadrado-Tejedor et al., 2015).

However, these drugs have some important limitations, including a lack of specificity to the brain and to the CREB signaling pathway, low blood brain-barrier permeability, and negative side effects (Bischoff, 2004; Hebenstreit et al., 1989; Kazantsev & Thompson, 2008). Even with these limitations in mind, the studies showing rescued memory function following enhancement of the CREB signaling pathway highlight the importance of this pathway in learning and memory and the dysfunctional nature of this pathway in AD. The appropriate therapeutic strategy will require further study and a greater understanding of the CREB signaling deficits in AD (A. Wang & Bibb, 2011).

This study contributes to the current body of literature on CREB signaling in AD by examining both steady state and learning induced CREB signaling in young adult FAD mice, as well as CREB signaling components in postmortem AD prefrontal cortex and PBMC isolated during life. This study adds mechanistic details to the body of literature indicating that CREB signaling is dysfunctional in mouse models of FAD, as well as introducing a novel means of monitoring CREB signaling during life in Alzheimer's disease through the use of PBMC.

III. Materials and Methods

Parts of this chapter were previously published as

1. Bartolotti N, Segura L, Lazarov O (2016) Diminished CRE-Induced Plasticity is Linked to Memory Deficits in Familial Alzheimer's Disease Mice. *Journal of Alzheimers Disease* **50**, 477-489.

or

2. Bartolotti N, Bennett DA, Lazarov O (2016) Reduced pCREB in Alzheimer's disease prefrontal cortex is reflected in peripheral blood mononuclear cells. *Molecular Psychiatry* **21**, 1158-1166.

A. Materials

1. Chemicals and Reagents

Molecular biology grade chemicals and reagents used in this study were purchased from Fisher, Sigma, Life Technologies, and ThermoFisher.

2. Transgenic animals

All animal experiments were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee (IACUC). APPswe/PS1ΔE9 have been previously described (Jankowsky et al., 2001). These mice coexpress human PS1 with the ΔE9 mutation and human APP with the Swedish mutation (K595N, M596L). CRE-AD mice were generated by breeding a CRE-LacZ male transgenic mouse (previously described (Impey et al., 1996) and generously provided by Dr. Daniel Storm, University of Washington) with a female APPswe/PS1ΔE9 mouse. Animals were maintained in standard conditions (14/10 hour light/dark cycle) with full access to food and water ad libitum. Female APPswe/PS1ΔE9 and their nontransgenic littermates were used for fear conditioning experiments. CRE and CRE-AD mice were used for novel object recognition experiments. Each mouse was used for only a single behavioral task and then immediately sacrificed. To control for time-dependent expression of CREB

signaling, the time between the test and sacrifice was recorded and mice were time-matched between genotype for comparison.

3. Human participants

Clinical data and biospecimens came from participants in the, MAR, MAP and Religious Orders Study (D. A. Bennett, Schneider, Arvanitakis, & Wilson, 2012; D. A. Bennett, Schneider, Buchman, et al., 2012). Participants without dementia are recruited from more than 40 Catholic Religious Groups across the USA. Individuals undergo clinical evaluation, blood draw, and brain donation at death. A subset agreed to annual blood draws. The clinical evaluation includes 21 cognitive performance tests which are used to inform on diagnoses of dementia, AD, and MCI (D. A. Bennett, Schneider, Aggarwal, et al., 2006; D. A. Bennett et al., 2002). The follow-up rate exceeds 95% and the autopsy rate exceeds 90%. At the time of death, a neurologist reviews all clinical data and provides a summary diagnosis without access to neuropathologic data. At the time of these analyses more than 1200 participants had enrolled and more than 600 autopsies obtained. The study was approved by the Institutional Review Board of Rush University Medical Center. All participants signed an informed consent and an Anatomic Gift Act for organ donations. Only female persons were used in this study. Demographic data is summarized in Supplementary Table 1. See supplementary methods for description of PBMC and PFC isolation.

B. Methods

1. Environmental enrichment

Two- to three-month old mice were either exposed to an enriched environment for one week or were maintained in standard housing conditions as described previously (Y. S. Hu et al., 2010) with the following modification: the mice remained in the cage for an entire week, and were removed immediately prior to sacrifice.

2. Contextual fear conditioning

Three-month-old APPswe/PS1ΔE9 or nontransgenic female mice were placed into a 17.8 x 17.8 x 30.5 cm chamber with two clear Plexiglass walls, two metal walls, and a stainless steel grid floor (Coulbourn Instruments). The chamber was located inside an opaque isolation cabinet. The mice remained in the cage for 148 seconds before receiving a single 0.75 mA foot shock lasting 2 seconds. The mice remained in the cage for another 30 seconds after the shock before being returned to their home cage. Twenty-four hours later the mice were returned to the chamber for 5 minutes. Freezing was measured using a digital video camera mounted above the chamber and FreezeFrame software (Actimetrics). Percentage freezing time was analyzed using FreezeView software (Actimetrics). For statistical analysis, a two tailed, paired t-test was used (*p<0.05).

3. Novel object recognition

Novel object recognition was performed as previously described in (Singh & Thakur, 2014). Three-month-old female CRE-LacZ, APPswe/PS1ΔE9 and CRE-LacZ/APPswe/PS1ΔE9 mice were habituated to the experimental box (24x17x11 inches) for 5 minutes on two consecutive days. On the third day the mice were familiarized to

two identical objects placed in the center of the box for 5 minutes. On the fourth day the mice were placed in a box with one familiar object and one novel object. The mice were recorded by video on the familiarization and novel object days. The videos were analyzed by counting the number of frames the mice spent exploring each object. "Exploring" was defined as pointing the nose at the object or touching the object with the front paws. The time was determined by the frame rate of the video. Mice that explored for fewer than 7 seconds on either the familiarization or novel object day were excluded from the analysis. Statistical analysis was performed by a two-tailed, paired t-test (*p<0.05).

4. Nuclear protein extraction and Western blot

Nuclear protein from mouse hippocampus was extracted using CelLytic NuCLEAR Extraction Kit (Sigma NXTRACT) per the manufacturer's instructions. The sample preparations were supplemented with protease and phosphatase inhibitors (2 mM sodium orthovanadate, 50 nM RR microcystin, 50 nM okadaic acid, 100 nM K52a, 100 nM staurosporine, 1 µL/100 µL buffer mammalian protease inhibitor cocktail II (Sigma P8430), 1uL/100uL buffer potassium phosphatase cocktail II (Milipore 524625), 1mM PMSF). The concentration of protein was determined by BCA assay (ThermoScientific). The samples were run on a 5% (CBP and p-300) or 7.5% (all other proteins) Tris-glycine gel and transferred to a 0.45 µM nitrocellulose membrane (Biorad) at 100mV for 2 hours. Membranes were blocked for 1 hour in 5% milk in TBST (TBS + 0.1% Tween-20) supplemented with the phosphatase inhibitors 1mM sodium orthovanadate and 10mM sodium fluoride. The primary antibodies (pCREB 1:1000 Cell Signaling, Total CREB 1:1000 Cell Signaling, CBP 1:2000 Santa Cruz, p-300 1:1000

Santa Cruz, PKA 1:1000 Santa Cruz, PKC 1:1000 Santa Cruz, PKA 1:1000 Santa Cruz, CaMKIV 1:500 eBioscience, pERK 1:1000 Santa Cruz, Total ERK 1:1000 Santa Cruz, Lamin B 1:500 Abcam) were incubated at 4 degrees C overnight with shaking in 2% BSA in TBST with phosphatase inhibitors. The membranes were rinsed 3 times for 15 minutes each time in TBS-T with phosphatase inhibitors and then placed in the appropriate secondary (Rabbit HRP 1:10,000 Promega) for 2 hours at room temperature with shaking. The membranes were rinsed again 3 times for 15 minutes each time and then imaged using an ECL chemiluminesence substrate (GE Healthcare). Relative levels of protein were quantified using densiometric measurements from ImageJ software. For statistical analysis, a two-tailed, unpaired t-test was used. The ratio of phosphorylated protein to total protein was determined for each mouse followed by averaging the ratios for each group.

5. PKA activity assay

The PKA kinase activity assay was performed according the manufacturer's instructions (Enzo) using 0.2 µg of total hippocampal protein. Absorbance was measured at 450 nm on a DTX 880 Multimode Detector (Beckman Coulter). For statistical analysis, a two-tailed, unpaired t-test was used.

6. X-gal staining

Mice were perfused with ice cold PBS, and the right hemisphere fixed in 4% PFA for 4 hours. The hemispheres were then transferred to a 30% sucrose solution for 16 hours, after which they were frozen in OCT and stored at -80 degrees C. Sections of 20 µm thickness were prepared using a cryostat. X-gal staining was performed as previously described (Barth et al., 2000). Briefly, slides were washed for 30 minutes

twice at room temperature in a PBS buffer containing 2 mM magnesium chloride, followed by a 30 minute wash at room temperature in a PBS buffer containing 2m M magnesium chloride, 0.02% Nonidet P40, and 0.01% sodium deoxycholate. The slides were then incubated overnight at 37 degrees C in a PBS buffer containing 2 mM $MgCl_2$, 0.02% Nonidet P40, 0.01% sodium deoxycholate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 0.6 mg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (Sigma B4252). Slides were then rinsed in PBS, and mounted in PVA-DABCO.

7. Immunoflourescent staining

For immunofluorescent staining, 50 µm floating sagittal sections were prepared on a microtome (Leica SM2000 R). The slides were blocked in TBS containing 5% Normal Donkey Serum (Jackson) and 0.25% Triton X-100. The sections were then incubated with primary antibodies pCREB (1:400 Cell Signaling 9197) or Egr-1 (1:250 Santa Cruz) overnight at 4 degrees C for 72 hours with gentle shaking. The sections were rinsed 3 times in TBS for 5 minutes each and then incubated with Donkey anti-rabbit Cy3 (1:250 Jackson Immunoresearch) for 2 hours at room temperature in the dark. The sections were rinsed in TBS 3 times for 5 minutes each. Finally, the sections were counterstained for DAPI, then mounted using and aqueous mounting medium (PVA-DABCO) and stored at 4 degrees C. Sections were imaged on a Ziess LSM 510 confocal microscope. For quantification of Egr-1 immunoflourescence, the area of interest (CA1 region) was traced in ImageJ software and the mean intensity was quantified. The mean intensity of the background fluorescence was also measured and

subtracted from the value for the region of interest. For statistical analysis, an unpaired, two-tailed t-test was used (*p<0.05).

8. Mouse blood and PBMC preparation

At time of sacrifice, whole blood was removed from the mouse heart by cardiac puncture. Whole blood was collected into an EDTA treated vacutainer (BD Biosceinces). To isolate PBMC, whole blood was separated using a Ficoll-Paque 1.084 gradient (GE Healthcare Life Sciences) according to the manufacturer's instructions. Briefly, equal volumes of whole blood from each mouse were combined to total 2mL of whole blood. This volume was combined with 2mL of Dulbecco's PBS free of calcium and magnesium (Life Technologies). The combined 4mL was then layered on top of 3mL of Ficoll in a 15mL conical tube and centrifuged at 400g for 30 minutes. The mononuclear cells were removed and rinsed twice in Dulbecco's PBS for 10 minutes at 400g. The cells were then fractionated using the Sigma NXTRACT kit as described above.

9. Flow cytometry

Mouse blood was removed at the time of sacrifice by cardiac puncture. Whole blood was collected into an EDTA treated vacutainer (BD Biosceinces), and 100µL of whole blood was used per staining condition. The red blood cells were lysed using 1x red blood cell lysis buffer (eBioscience). The remaining cells were then fixed in 100µL of 4% PFA for 15 minutes at room temperature. The PFA was rinsed off in excess flow buffer containing 0.5% BSA in 1x PBS. The cells were then resuspended in 50% ice cold methanol at -20 degrees overnight. Prior to staining, the methanol was rinsed twice using excess flow buffer. The Fc receptors were blocked using purified CD16/CD32 (eBioscience) according to the manufacturer's instructions. The cells were then stained

for 1 hour with the primary, unconjugated antibodies pCREB 1:800 (Cell Signaling), Total CREB 1:1000 (Cell Signaling), or the appropriate volume of isotype control calculated based on the concentration of the antibodies (determined on a lot by lot basis by communication with Cell Signaling). The primary antibody was washed off using excess flow buffer, and the cells were then incubated with a rabbit AF647 1:4000 (Cell Signaling) conjugated secondary antibody for 30 minutes at room temperature. The secondary antibody was washed off using excess flow buffer, and the cells were resuspended in 300μL of flow buffer. A single cell suspension was prepared by filtering the cells into a polystyrene tube with a cell strainer cap (BD Falcon). The samples were then run on a Gallios cytometer (Beckman Coulter). The data was analyzed using Kaluza Flow Cytometry Analysis Software (Beckman Coulter).

10. Human PFC preparation

At death, the brain is removed and weighed. The brainstem is removed and the brain hemisected. One hemisphere is cut into 1cm slabs in a Plexiglass jig, the slabs are placed in individual freezer bags on a metal tray and placed in a -80^{o}C freezer. Prefrontal cortex was frozen without fixation following autopsy. Approximately 50mg of PFC tissue was used per sample for fractionation. Individuals were classified as normal or AD based on clinical data. We also quantified β-amyloid load and PHFtau neurofibrillary tangle density by image analysis and stereology (D. A. Bennett, Schneider, Tang, et al., 2006; D. A. Bennett, Schneider, Wilson, Bienias, & Arnold, 2004). For pCREB and CBP analysis, 20 normal and 21 AD female persons were used. The mean ages were 93.49±1.16 years (Normal) and 93.14±1.44 (AD). The mean education was 15.84±0.55 (Normal) and 15.30±0.66 (AD). PFC expression of pCREB and CBP were

compared using a two-tailed, unpaired t test. Associations of PFC pCREB and CREB with β-amyloid load and PHFtau tangle density were analyzed using a two-tailed, unpaired t-test.

Protein levels were normalized using densiometric measurements from ImageJ software by dividing the densiometric value for pCREB or CBP by the densiometric value for Lamin. For the PFC and the associated PBMC, either 5 or 4 samples per AD or Normal group were run per gel. Z-scores were then calculated for the normalized values (Cheadle, Vawter, Freed, & Becker, 2003).

11. Human PBMC preparation

PBMC were prepared from whole blood samples within 2 hours of blood draw. Whole blood was centrifuged at 1500 RCF for 30 minutes at room temperature. The PBMC layer was isolated, and rinsed in 30mL of 1x DPBS at 300 RCF for 15 minutes at room temperature. Cells were counted and approximately 3 million cells were preserved per PBMC sample. The cells were washed again in 10mL 1x DPBS and centrifuged at 300 RCF for 15 minutes at room temperature. The cells were then transferred in 1mL 1x DPBS to a cryogenic tube and centrifuged at 5000 RPM for 5 minutes at room temperature. The pellet was resuspended in 1mL of freezing media containing 10% DMSO and 90% FBS and placed in a Mr. Frosty container overnight at -80 degrees C. The samples were then stored at -160 degrees C cyrotanks (Bradshaw et al., 2013). Before fractionation, samples were thawed on ice and rinsed twice in 1mL 1x DPBS followed by centrifugation at 400 RCF for 5 minutes at 4 degrees C. For pCREB analysis, samples from 38 cognitively normal (mean age 88.55±0.95, mean education 17.00±0.42), 19 MCI (mean age 86.92±1.23, mean education 17.31±0.63), and 31 AD

(mean age 88.33±1.00, mean education 16.52±0.60) female persons were used. For Total CREB analysis, samples from 28 cognitively normal (mean age 87.86± 1.05, mean education 16.27± 0.49), 11 MCI 85.13 ± 1.76 (mean age 85.13± 1.76, mean education 18.00± 0.65), and 23 AD female persons were used (mean age 89.00± 1.05, mean education 17.21± 0.64). The pCREB/Total CREB ratio was limited by the number of Total CREB samples so the statistics for the ratio are the same as for Total CREB. For CBP analysis, samples from 34 cognitively normal (mean age 88.71±1.04, mean education 16.58±0.44), 14 MCI (mean age 85.16±1.30, mean education 18.15±0.54), and 25 AD female persons (mean age 88.13±1.13, mean education 16.46±0.69) were used. The individuals were classified using cognitive scoring at time of sample draw. For statistical analysis, the associations of PFC and PBMC pCREB and CREB with clinical diagnosis were analyzed using a one-way ANOVA. For the correlation between PBMC and PFC, the mean age at time of sample draw was years 90.79±1.15 (Normal, N=20) and years 88.47±1.55 (AD, N=16). The correlation between PBMC and PFC and the correlation between PFC and neuropathologic indices was performed with linear regression analysis.

Protein levels were normalized using densiometric measurements from ImageJ software by dividing the densiometric value for pCREB or CBP by the densiometric value for Lamin. For most of the PBMC samples, 9 samples were run per gel; 3 AD, 3 MCI, and 3 Normal. Z-scores were then calculated for the normalized values (Cheadle et al., 2003).

12. Statistical analysis for human data

To compare the intensity of samples across blots, Z-scores were calculated for each sample by first calculating the density of each band by ImageJ. The mean and standard deviation of the band intensity of each western blot were then determined, and the Z-score for each sample was then calculated using the following calculation: (density value for sample-mean density value for blot)/standard deviation of density for blot. To determine normality of the distribution, a Shapiro-Wilk test was used (Prism Graphpad). To determine sample number, a power analysis was done on a preliminary experiment of PFC samples (G*Power). For the PBMC experiments, the PFC sample size was used as a starting point for sample number, although the effect size may be smaller for the PBMC than for PFC, and thus more samples will be needed for stronger power in future analyses. For PFC comparisons, a two-tail, unpaired t-test was used. For PBMC comparisons, a one-way ANOVA was used, and one-tail, unpaired t-tests were used for secondary analysis. For PBMC and PFC correlations, linear regression was used. For amyloid and tau pathology comparisons, a two-tail, unpaired t-test was used. For PFC pCREB and pathology correlations, a Pearson test was used.

Table 1

Prefrontal cortex	Normal PFC (N=20)	AD PFC (N=21)
Age (years)	93.49±1.16	93.14±1.44 (N=21)
Education (years)	15.84±0.55	15.30±0.66
Interval between PBMC and death (years)	2.30±0.12	1.90±0.15
Postmortem interval (hours)	7.51±0.69	8.03±0.81
Sex	Female N=20	Female N=21

PBMC pCREB	Normal PBMC (N=38)	MCI PBMC (N=19)	AD PBMC (N=31)
Age (years)	88.55±0.95	86.92±1.23	88.33±1.00
Education (years)	17.00±0.42	17.31±0.63	16.52±0.60
Sex	Female N=38	Female N=19	Female N=31
PBMC CBP	**Normal PBMC (N=34)**	**MCI PBMC (N=14)**	**AD PBMC (N=25)**
Age (years)	88.71± 1.04	85.16± 1.30	88.13± 1.13
Education (years)	16.58± 0.44	18.15±.054	16.46± 0.69
Sex	Female N=34	Female N=14	Female N=25
PBMC Total CREB	**Normal PBMC (N=33)**	**MCI PBMC (N=11)**	**AD PBMC (N=23)**
Age (years)	89.26± 1.13	85.13± 1.76	88.42± 1.28
Education (years)	16.30± 0.46	18.00± 0.65	17.21± 0.73
Sex	Female N=33	Female N=11	Female N=23
PBMC pCREB/ Total CREB ratio	**Normal PBMC (N=33)**	**MCI PBMC (N=10)**	**AD PBMC (N=22)**
Age (years)	89.26± 1.13	86.13± 1.42	89.01± 1.18
Education (years)	16.3± 0.46	18.00± 0.71	16.82± 0.64
Sex	Female N=33	Female N=10	Female N=22

Table 1: Demographic information for human tissue.

IV. Results

Parts of this chapter were previously published as

1. Bartolotti N, Segura L, Lazarov O (2016) Diminished CRE-Induced Plasticity is Linked to Memory Deficits in Familial Alzheimer's Disease Mice. *Journal of Alzheimers Disease* **50**, 477-489.

or

2. Bartolotti N, Bennett DA, Lazarov O (2016) Reduced pCREB in Alzheimer's disease prefrontal cortex is reflected in peripheral blood mononuclear cells. *Molecular Psychiatry* **21**, 1158-1166.

A. CREB signaling is impaired in young adult APPswe/PS1ΔE9 mice

1. Basal levels of CREB signaling components are reduced in the nuclear fraction of the hippocampus of APPswe/PS1ΔE9 mice

While others have examined baseline levels of CREB signaling components in mouse models of FAD, the results remain controversial and may depend on many factors that influence the progression of the disease, such as the mouse model used as well as the age and sex of the mice used (Francis et al., 2007; Francis et al., 2006; Marambaud et al., 2003; Saura et al., 2004). We previously reported that APPswe/PS1ΔE9 mice exposed to EE from one month to two months of age did not show an increase in CREB activation as we observed in the nontransgenic littermates (Y. S. Hu et al., 2013b). This data suggests that CREB impairments may be apparent early in the APPswe/PS1ΔE9 mouse model. Therefore, we used young adult mice between two and three months old to examine basal levels of CREB signaling components.

First, we examined whether basal levels of CREB and pCREB were altered in the hippocampus of young adult APPswe/PS1ΔE9 mice. Using Western blot analysis of nuclear fractions prepared from hippocampal protein extract APPswe/PS1ΔE9 mice and their nontransgenic littermates, we observed that expression levels of total CREB were

comparable, in the hippocampus of APPswe/PS1ΔE9 and wild type mice, although we did observe a trending reduction (**Figure 2A-B**). Levels of pCREB were significantly reduced in the hippocampus of APPswe/PS1ΔE9 compared to their nontransgenic counterparts.

We next wanted to ask if additional components of the CREB signaling pathway were altered in this mouse model of FAD at this age. For this purpose we examined CREB's transcriptional coactivator paralogs, CBP and p300. We observed that protein levels of both CBP and p300 were reduced in the nuclear fraction of the hippocampus of APPswe/PS1ΔE9 mice compared to their nontransgenic littermates (**Figure 3A-B**). These results indicate a reduction in the availability of critical CREB signaling components in the hippocampus of young adult APPswe/PS1ΔE9 mice, and that these impairments are not confined to a single aspect of CREB signaling but may be more widespread.

While the deficits in CREB signaling in this mouse model are undoubtedly tied to the mutations in *APP* and/or *PS1*, we wanted to start to understand the more proximal mechanism underlying these deficits. First, examined levels of the β-secretase enzyme, BACE1, which cleaves APP. Elevated BACE1 levels have been shown to reduce CREB phosphorylation, PKA activity and cAMP levels (Y. M. Chen et al., 2012). We observed significantly higher levels of BACE1 in the hippocampus of APPswe/PS1ΔE9 compared to nontransgenic mice (**Figure 4A-B**). This observation is in line with previous reports of abnormal BACE1 in the APPswe/PS1ΔE9 mouse model (Li et al., 2014; Y. Wang, Tang, & Zhang, 2012).

Another potential cause of impairments in CREB activation includes dysfunctional kinase expression or activity. For this reason, we asked whether reduced levels of CREB phosphorylation could result from alterations in the kinases that phosphorylate CREB. The kinases that have been shown to phosphorylate CREB and activate CREB-dependent gene transcription in vivo include protein kinase A (PKA), protein kinase C (PKC), the calcium / calmodulin-dependent protein kinases CaMKII and CaMKIV, and the mitogen- and stress-activated protein kinase (MSK) and the 90 kDa ribosomal S6 kinase (RSK), which are activated by ERK (Lonze & Ginty, 2002). Therefore, we focused our analysis on these kinases. We observed that protein levels of PKA were significantly reduced in the hippocampus of APPswe/PS1ΔE9 compared to their nontransgenic littermates (**Figure 5A-B**). Similarly, expression levels of PKC and CaMKIV were reduced in hippocampal extracts of APPswe/PS1ΔE9 compared to wild type mice, although this difference was not statistically significant (**Figure 5A-B**). While both CaMKII and CaMKIV activate CRE-based gene expression, it is thought that this may occur through different mechanisms, and that CaMKIV may act as a more direct activator of CRE-based gene transcription by entering the nucleus (Matthews et al., 1994).

Finally, we examined levels of ERK and activated ERK (pERK), which activate MSK and RSK. Interestingly, we observed that levels of pERK and Total ERK, as well as the ratio of pERK to Total ERK were significantly increased in the hippocampus of APPswe/PS1ΔE9 compared to nontransgenic mice (**Figure 5A,C**). Taken together, these results suggest that the reduction in activated CREB in the hippocampus of APPswe/PS1ΔE9 mice may be due in part to dysregulation of the kinases that

phosphorylate CREB. Specifically, the significant reduction in PKA and trending reductions in PKC and CaMKIV protein levels suggest that these kinases may not be readily available to activate CREB. The observation that ERK is overactive suggests that ERK is not sufficient to overcome the reduction in the protein level of the other kinases. It is also possible that in spite of increased ERK activity, it may not be appropriately activating its downstream targets RSK and MSK, which would be responsible for the activation of CREB.

This series of experiments is important as it indicates that multiple points of dysfunction exist in the CREB signaling pathway in the hippocampus of young adult APPswe/PS1ΔE9 mice even at a steady state, suggesting a reduction in availability of critical factors for memory formation.

Figure 2

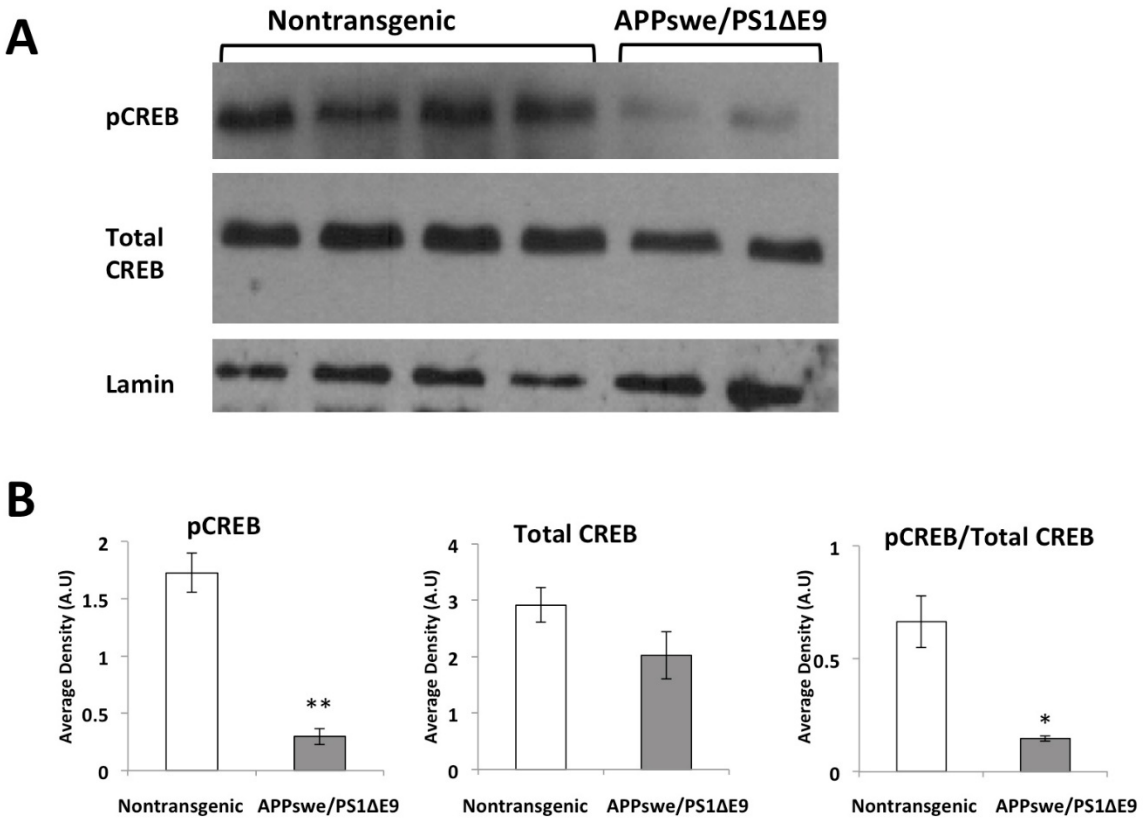

Figure 2: Basal levels of activated CREB are reduced in the nuclear fraction of the hippocampus of young adult female APPswe/PS1ΔE9 mice. **A**. Representative blot of pCREB and Total CREB in 3 month old female nontransgenic (N=14) and APPswe/PS1ΔE9 (N=8) mice. **B**. ImageJ analysis of pCREB normalized to Lamin (p=0.01), Total CREB normalized to Lamin (p=0.22), Ratio of pCREB/Total CREB (p=0.05)

Figure 3

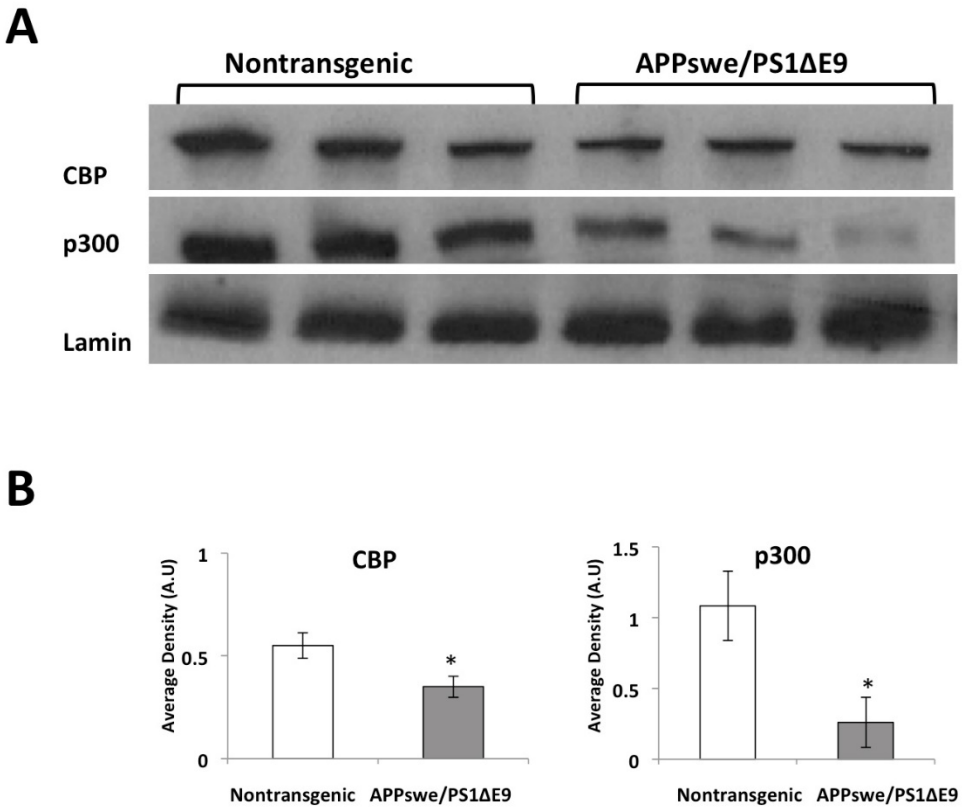

Figure 3: Basal levels of CBP and p300 are reduced in the nuclear fraction of the hippocampus of young adult female APPswe/PS1ΔE9 mice. **A**. Representative blot of CBP and p300 in 3 month old female nontransgenic (N=4) and APPswe/PS1ΔE9 mice (N=3) **B**. ImageJ analysis of CBP normalized to Lamin (p=0.04), p300 normalized to Lamin (p=0.05).

Figure 4

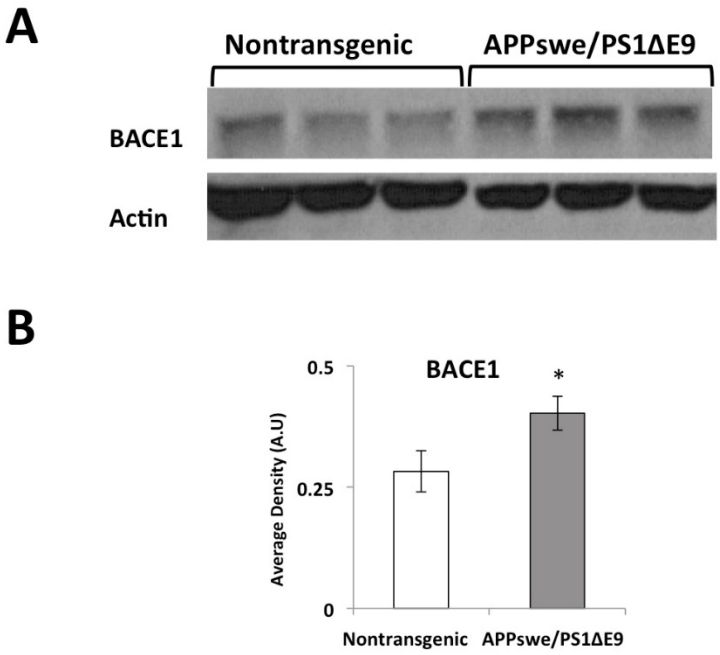

Figure 4: BACE1 is elevated in the hippocampus of young adult female APPswe/PS1ΔE9 mice A. Representative blot of BACE1 expression in total hippocampal homogenate in 3 month old nontransgenic (N=8) and APPswe/PS1ΔE9 (N=6) mice. **B**. ImageJ analysis of BACE1 normalized to actin (p=0.05).

Figure 5

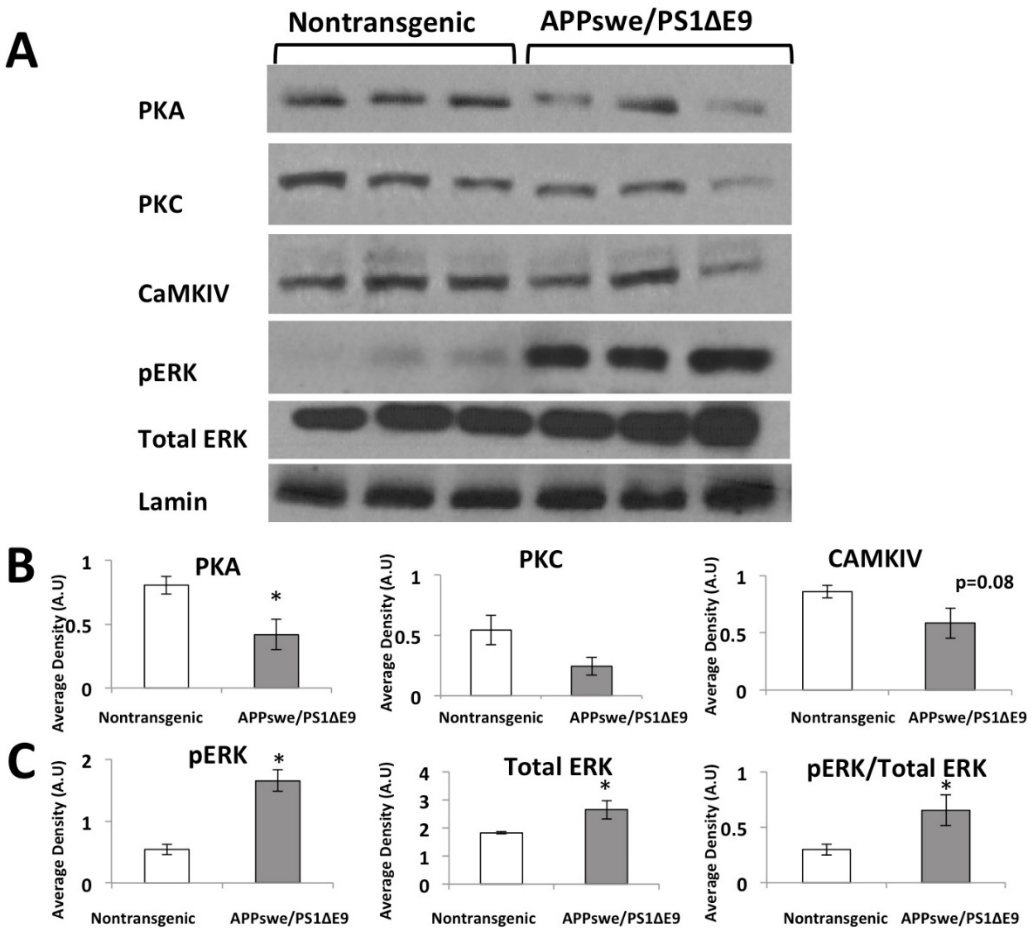

Figure 5: Basal levels of kinases that phosphorylate CREB are aberrantly regulated in the nuclear fraction of the hippocampus of young adult female APPswe/PS1ΔE9 mice A. Representative blots of PKA, PKC, CaMKIV, pERK, and Total ERK in 3 month old female nontransgenic (N=4) and APPswe/PS1ΔE9 (N=3) mice. **B**. ImageJ analysis of PKA normalized to Lamin (p=0.03), PKC normalized to Lamin (p=0.1), CaMKIV normalized to Lamin (p=0.08). **C**. ImageJ analysis of pERK normalized

to Lamin (p=0.001), Total ERK normalized to Lamin (p=0.03) and the ratio of pERK to Total ERK (p=0.04).

2. Impairments in CREB signaling correspond to deficits in associative learning in young adult APPswe/PS1ΔE9 mice, and are apparent in brain regions that process learning and memory

In the previous set of experiments, we observed numerous deficits in CREB signaling components in the hippocampus of young adult APPswe/PS1ΔE9 mice. However, it was not clear if these mice would exhibit memory deficits. Previous data on learning and memory in young APPswe/PS1ΔE9 mice is conflicting, with some groups reporting no memory deficits as late as 6 months of age (Savonenko et al., 2005), and others observing impairments earlier (W. Zhang et al., 2012). However, these studies combined male and female mice, which progress at different rates and may have increased variability in performance, which, at low sample size, may have obfuscated any observable significant difference in memory performance. Therefore, we restricted our analysis to female mice at the age at which we observed impairments in CREB signaling.

In addition, we chose behavioral tests that have been previously reported to discern memory impairments in CREB or CBP deficient mice. While CREB signaling is important for the formation of memory in general, different experimental tests and designs may allow for mice to exhibit normal memory in spite of impairments in CREB (Kogan et al., 1997). Therefore, we were careful to tailor our behavioral experimental tests to resemble those which have been previously shown to be sensitive to impairments in CREB signaling.

One such test is contextual fear conditioning, a test that depends on a functioning hippocampus and amygdala. CREB and CBP deficient mice have been previously shown to exhibit impairments in contextual fear conditioning (Barrett et al., 2011; Bourtchuladze et al., 1994; Kogan et al., 1997). Therefore, to associate learning ability and CREB impairment in FAD, we tested the performance of APPswe/PS1ΔE9 mice in a contextual fear conditioning paradigm and examined CREB signaling in the brain following the task. We modeled our experimental paradigm from previous work on CREB mutant mice (Impey et al., 1998b), which is summarized in **Figure 6A**. Briefly, the mouse is placed in a particular context and allowed 2.5 minutes to learn the context before receiving a mild footshock. After an additional 30 seconds post shock to learn the context, the mouse is removed. The next day when the mouse is re-introduced to the context the amount of freezing is indicative of whether or not a fear memory was encoded.

We observed no differences in mobility prior to the shock, and no differences in the magnitude of the response to the shock between the nontransgenic and APPswe/PS1ΔE9 mice (**Figure 6C**). However, young adult APPswe/PS1ΔE9 mice froze significantly less than nontransgenic mice when re-introduced to the context 24 hours later (**Figure 6B,C**). These results indicate that at 3 months old female APPswe/PS1ΔE9 mice do exhibit memory impairments at the same age that impairments in baseline CREB signaling are apparent.

To confirm that the impairments in memory were also associated with impairments in CREB signaling components following learning, we analyzed the nuclear fraction of the hippocampus of APPswe/PS1ΔE9 mice and their nontransgenic

counterparts following contextual fear conditioning. Again, we observed significantly lower levels of pCREB and the ratio of pCREB/Total CREB, as well as reduced protein levels of CBP (**Figure 7A-B**). While we did observe significantly reduced levels of PKA protein in the nuclear fraction of the hippocampus of APPswe/PS1ΔE9 mice following fear conditioning, we only observed a trending reduction in the activity of PKA as measured by a colorometric PKA activity assay, suggesting that PKA activity may not be reflected by total protein level of the kinase alone (**Figure 7A-B**). These experiments show that CREB signaling is impaired not only at a basal state, but also following a learning task, suggesting a functional connection.

Contextual fear conditioning is known to depend on functioning of both the hippocampus and the amygdala, so it is possible that APPswe/PS1ΔE9 mice are unable to learn the task due to dysfunction in the amygdala, Indeed, CREB signaling is thought to play a role in the amygdala during fear conditioning (Josselyn, Kida, & Silva, 2004). In addition, the two primary areas of the hippocampus, namely the dorsal and ventral hippocampus, are thought to have different roles in memory formation, where the dorsal hippocampus is thought to be especially critical for fear memory formation, and the ventral hippocampus, which receives input from the amygdala, is thought to be critical for the processing of emotional state (Pittenger et al., 2002). The analysis of freezing following the shock showed no difference in response between nontransgenic and APPswe/PS1ΔE9 mice (**Figure 6C**), which suggests that APPswe/PS1ΔE9 were able to sense the shock and experienced a fear response as a result of the shock, which suggests that the deficit in performance on the following day was primarily a result of memory failure. However, to more specifically examine the localization of CREB

activation following fear conditioning, we immunolabeled brain sections with pCREB. In agreement with our western blot analysis, we observed reduced pCREB in the hippocampus of the APPswe/PS1ΔE9 mice at baseline levels compared to their nontransgenic counterparts, particularly in the dorsal hippocampus (**Figure 8A, C**). We observed low levels of pCREB staining in the amygdala at baseline, with no apparent difference in intensity between APPswe/PS1ΔE9 and nontransgenic mice (**Figure 8I, K**). Following fear conditioning in nontransgenic mice, we observed dramatic upregulation of pCREB in the dorsal hippocampus (**Figure 8B,A**) and amygdala (**Figure 8J,I**), and a moderate increase in pCREB in the ventral hippocampus (**Figure 8F,E**) compared to baseline expression. These results indicate CREB activation is increased in both the hippocampus and the amygdala during contextual fear conditioning. However, in the APPswe/PS1ΔE9 we observed very little change in pCREB staining in either the dorsal (**Figure 8C,D)**, or ventral (**Figure 8G,H**) hippocampus following fear conditioning. We did however observe an increase in pCREB staining in the amygdala of the APPswe/PS1ΔE9 mice following fear conditioning (**Figure 8K,L**). In agreement with the behavior results, the immunostaining results suggest that during a fear conditioning task, CREB signaling in the amygdala is intact in APPswe/PS1ΔE9, and that the impairment in performance is more likely due to CREB signaling impairments in the hippocampus, particularly in the dorsal hippocampus which is thought to be involved in learning and memory, rather than mood and emotion*,* and strengthening the evidence that impairments in pCREB following fear conditioning are apparent in neuronal populations implicated in explicit memory.

Figure 6

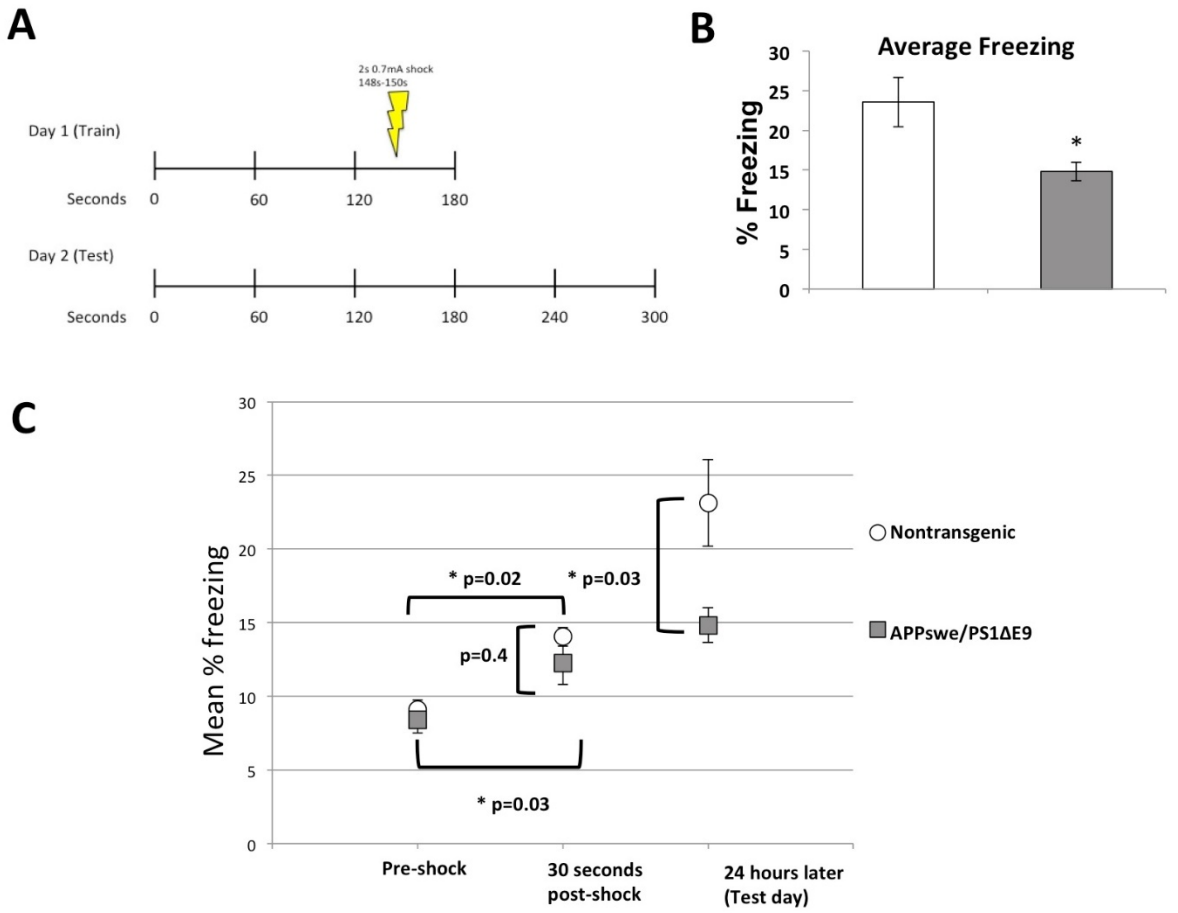

Figure 6: Memory impairments are apparent in contextual fear conditioning of 3 month old female APPswe/PS1ΔE9 mice A. Experimental paradigm. Long-term memory consolidation was tested by a contextual fear conditioning paradigm. Mice were placed in the box for 2 minutes and 28 seconds before receiving a 2 second shock (0.7mA). The mice remained in the cage for another 30 seconds and were then removed. 24 hours later the mice were placed in the cage for 5 minutes and average freezing time was measured. **B**. Average freezing for 3 month old female mice (nontransgenc N=14, APPswe/PS1ΔE9 N=11 p=0.03). **C**. Nontransgenic and APPswe/PS1ΔE9 show similar freezing responses prior to receiving the shock. Following the shock, both nontransgenic

(p=0.02) and APPswe/PS1ΔE9 (p=0.03) mice freeze significantly more than they did prior to the shock, and there is no difference in freezing between groups (p=0.4). Twenty-four hours later, nontransgenic mice freeze significantly more than APPswe/PS1ΔE9 mice (p=0.03).

Figure 7

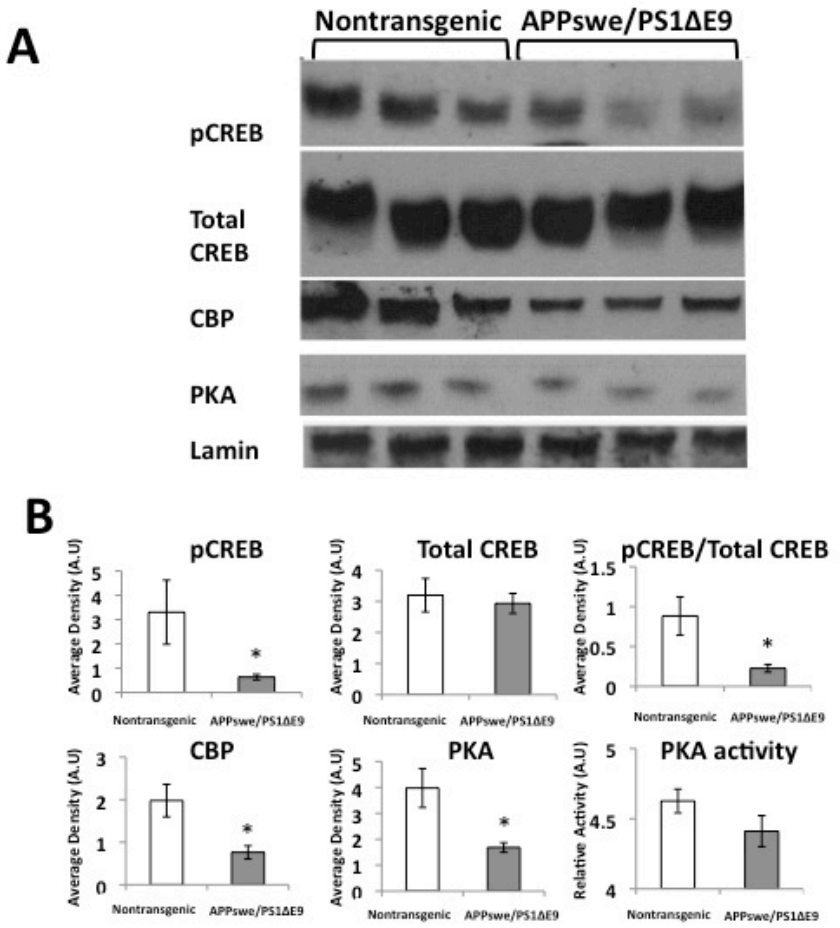

Figure 7: Impairments in contextual fear conditioning of 3 month old female APPswe/PS1ΔE9 mice correspond to decreased CREB signaling components in nuclear fractions of the hippocampus. A. Representative blot of pCREB, Total CREB and CBP expression levels following contextual fear conditioning training. **B**. ImageJ analysis of pCREB expression normalized to Lamin B (nontransgenc N=7, APPswe/PS1ΔE9 N=7, p=0.06), Total CREB expression normalized to Lamin B (p=0.68), the ratio of pCREB expression to Total CREB (p=0.02), CBP expression normalized to Lamin B (p=0.01), PKA expression normalized to Lamin (p=0.04), and PKA activity (p=0.14)

Figure 8

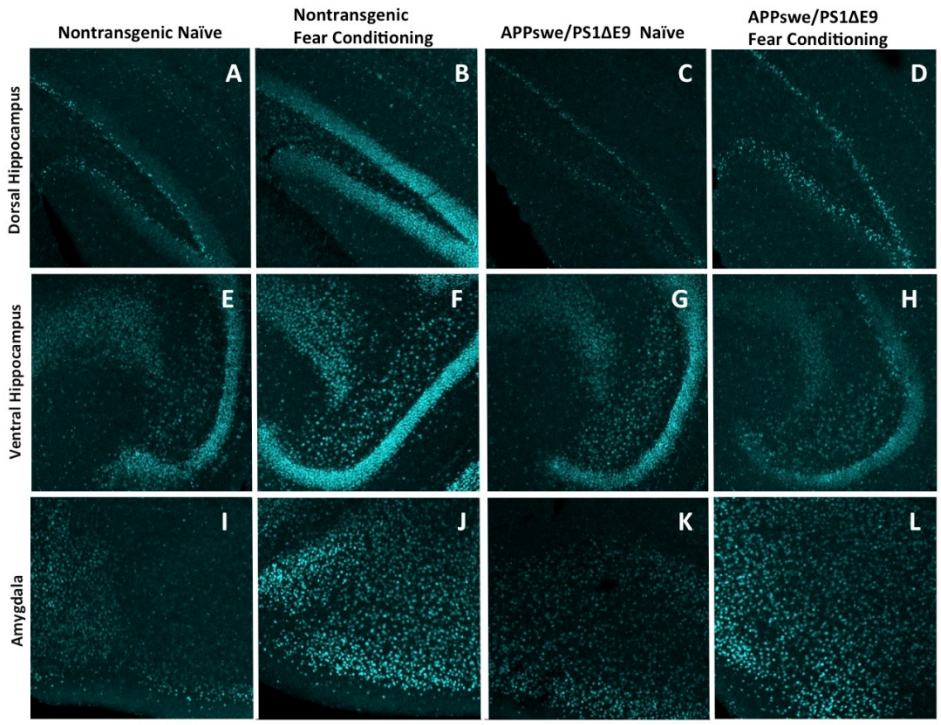

Figure 8: Impairments in pCREB in APPswe/PS1ΔE9 mice are apparent primarily in the hippocampus. A-D. Representative staining of pCREB staining in the dorsal hippocampus **A.** pCREB staining in the dorsal hippocampus in a naive nontransgenic mouse. **B**. pCREB staining the dorsal hippocampus in a nontransgenic mouse following fear conditioning. **C.** pCREB staining in the dorsal hippocampus of a naïve APPswe/PS1ΔE9 mouse. **D**. pCREB staining in the dorsal hippocampus of an APPswe/PS1ΔE9 mouse following fear conditioning. **E-H.** Representative staining of pCREB staining in the ventral hippocampus **E.** pCREB staining in the ventral

hippocampus in a naive nontransgenic mouse. **F**. pCREB staining the ventral hippocampus in a nontransgenic mouse following fear conditioning. **G.** pCREB staining in the ventral hippocampus of a naïve APPswe/PS1ΔE9 mouse. **H**. pCREB staining in the ventral hippocampus of an APPswe/PS1ΔE9 mouse following fear conditioning. **I-L.** Representative staining of pCREB staining in the amygdala **I.** pCREB staining in the amygdala in a naive nontransgenic mouse. **J**. pCREB staining the amygdala in a nontransgenic mouse following fear conditioning. **K.** pCREB staining in the amygdala of a naïve APPswe/PS1ΔE9 mouse. **L**. pCREB staining in the amygdala of an APPswe/PS1ΔE9 mouse following fear conditioning.

3. CRE and CRE-AD reporter mice allow for visualization of CRE-based gene transcription

The CRE-reporter mouse generated in the lab of Dr. Daniel Storm allows for visualization of CRE-based gene transcription by utilizing a LacZ reporter that express β-galactosidase driven by the CRE promoter (**Figure 9A**, generously provided by Dr. Daniel Storm (Barth et al., 2000; Impey et al., 1996). This mouse model has been used to demonstrate CRE-driven gene expression in the hippocampus following learning tasks (Impey et al., 1998b). Our observation that levels of CBP and pCREB do not increase in the hippocampus of APPswe/PS1ΔE9 mice following learning in the fear conditioning task indicates that CREB signaling is nonresponsive to a stimulus, and suggests that the transcription of CRE-driven genes required for synaptic plasticity and long-term memory may be impaired (Alberini, Ghirardi, Huang, Nguyen, & Kandel, 1995; S. C. Hu, Chrivia, & Ghosh, 1999; Schwaninger et al., 1995; Shieh, Hu, Bobb, Timmusk, & Ghosh, 1998; B. E. Wilson, Mochon, & Boxer, 1996). To test the hypothesis that reduced CREB signaling during learning is resulting in reduced CRE-based gene transcription, we utilized the CRE reporter mouse and crossed this mouse with the APPswe/PS1ΔE9 mouse (**Figure 9B**) in order to visualize the effect that these transgenes have on CRE-based gene transcription during learning.

We initially decided to use environmental enrichment (EE) for the enhancement of CRE-driven gene transcription, to get a maximal effect of experience on brain plasticity, compared to the confined effect of a specific learning task. We have previously shown that in contrast to wild type mice, pCREB is not upregulated in the hippocampus

of young adult APPswe/PS1ΔE9 mice following EE (Y. S. Hu et al., 2013a). To directly address whether CRE-driven gene transcription is impaired in FAD in response to a stimulus, we allowed the mice to experience EE or standard housing (SH) conditions (**Figure 9C**). We observed abundant X-gal staining throughout the hippocampus following EE in the CRE reporter mouse, particularly in the CA1 region (**Figure 9D i-iii**). In contrast, X-gal staining in the hippocampus of the CRE-AD mice was limited (**Figure 9D iv-vi**), suggesting that EE-induced CRE-driven gene expression is defective in the APPswe/PS1ΔE9 mouse. In agreement with our previous observation that pCREB is not increased in the APPswe/PS1ΔE9 hippocampus following EE as it is in nontransgenic mice, this observation indicates that, in the APPswe/PS1ΔE9 mouse, the CREB pathway is not responding to a stimulus known to enhance neuroplasticity and memory function, and that this deficit indeed results in reduced CRE-driven gene transcription.

Figure 9

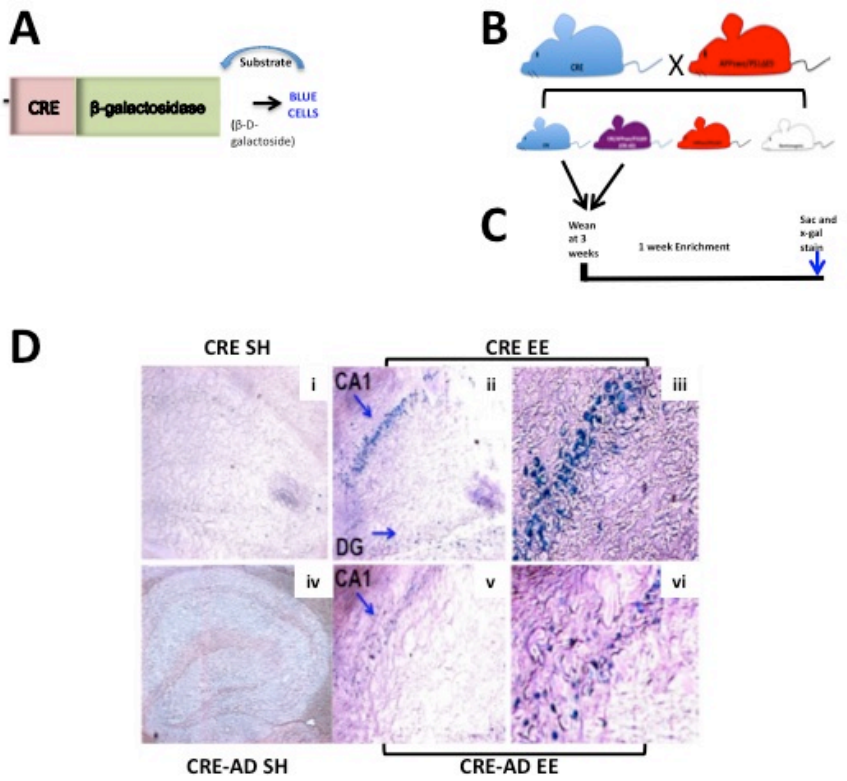

Figure 9: CRE-AD reporter mice allow visualization of CRE-driven gene expression in the hippocampus of APPswe/PS1ΔE9 mice. A. Schematic of CRE-reporter. B. Breeding scheme of CRE-reporter mouse with APPswe/PS1ΔE9 mice to generate the CRE-AD mouse. C. Enrichment timeline scheme. D. CRE-gene transcription is increased following EE in CRE mice (i-iii) but is not increased following EE in CRE-AD mice (iv-vi) relative to standard housing (SH) conditions.

4. CRE- driven gene transcription is increased in the hippocampus following a Novel Object Recognition task in CRE mice, but not CRE-AD mice

While we have shown that young adult APPswe/PS1ΔE9 mice exhibit deficits in the contextual fear conditioning task, and that this impairment appears to be primarily localized to the hippocampus and not the amygdala, we wanted to test an additional learning and memory behavioral paradigm that is primarily dependent on the hippocampus. In addition, this would allow us to determine if early memory impairments in young adult APPswe/PS1ΔE9 mice are task specific. For this purpose we used the Novel Object Recognition (NOR) task.

NOR exploits the instinct of an animal to explore novel objects (Antunes & Biala, 2012). This task is thought to depend primarily on the hippocampal formation and particularly the dorsal hippocampus, but lesions in the cortex can also impact performance (Albasser, Davies, Futter, & Aggleton, 2009; Buckmaster, Eichenbaum, Amaral, Suzuki, & Rapp, 2004; Clark, Zola, & Squire, 2000; Goulart et al., 2010). Importantly, it models object recognition memory, a type of memory impaired during AD. CBP mutants have been shown repeatedly to exhibit impairments in NOR (Alarcon et al., 2004; Bourtchouladze et al., 2003; Korzus et al., 2004; A. M. M. Oliveira, Wood, McDonough, & Abel, 2007; Wood, Attner, Oliveira, Brindle, & Abel, 2006). Interestingly, CBP mutant mice show impairments in this task, and treatment with an HDAC inhibitor to restore histone acetylation can restore recognition memory in this task (Stefanko et al.,

2009). In addition, the importance of CREB signaling in object recognition memory has also been demonstrated through experiments using a mouse generated to have a CREB-repressor (Kida et al., 2002).

For our NOR experiments, we based the paradigm on previous reports in which mice with deficient CBP function were shown to have impairments in performance (Singh & Thakur, 2014). In this setup, we habituated CRE and CRE-AD mice to the experimental arena for 5 minutes on two consecutive days (**Figure 10A**). On the third day, the mice were exposed to two identical objects and allowed to explore the objects for 5 minutes total. We observed no difference in preference for either object in either the CRE or CRE-AD mice (**Figure 10B-C**). On the fourth day, the mice were exposed to one familiar object from the previous day and one novel object and allowed to explore for 5 minutes. While the CRE mice preferred to explore the novel object, the CRE-AD mice did not show a preference for the novel object (**Figure 10D-E**). These experiments demonstrated that memory impairments in in young adult APPswe/PS1ΔE9 are apparent in multiple behavior tests that depend on the hippocampus.

Following the task, we observed a clear expression of CRE-gene transcription in the hippocampus of the CRE mice, but not the CRE-AD mice (**Figure 10F**). These experiments demonstrate that not only are CREB signaling components dysfunctional during learning tasks in young adult APPswe/PS1ΔE9 mice, but that these impairments are indeed resulting in a reduction of CRE-based gene transcription. In addition, these experiments demonstrate that impairments in CRE-based gene transcription correspond to memory impairments in young adult APPswe/PS1ΔE9 mice.

Figure 10

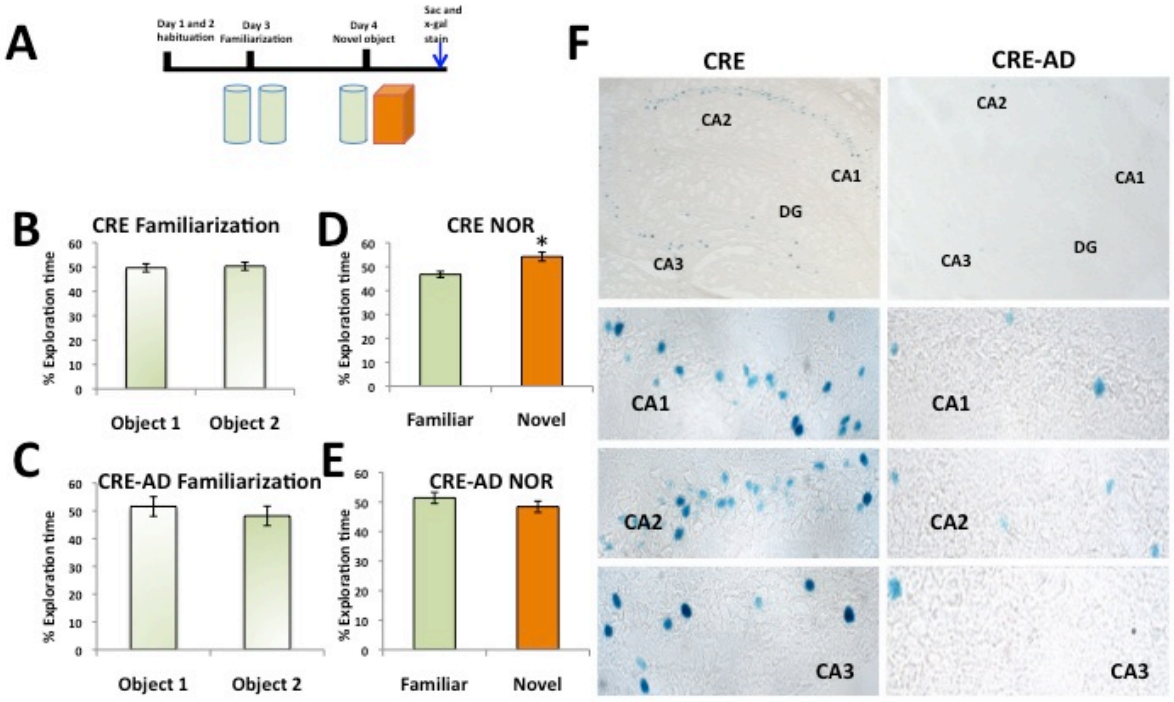

Figure 10: Novel Object Recognition and CRE-driven gene expression are impaired in 3 month old APPswe/PS1ΔE9 mice. A. Schematic of NOR experimental design. **B.** CRE mice show no preference for either object during Familiarization (N=9, p=0.84). **C.** CRE-AD mice show no preference for either object during Familiarization (N=10, p=0.64). **D**. CRE reporter mice significantly prefer a novel object over a familiar object (N=9, p=0.04). **E**. CRE-AD mice do not show a preference for a novel object over a familiar object (N=10, p=0.4). **F**. Representative images showing X-gal staining in the hippocampus of CRE or CRE-AD mice following NOR (30 minutes post-task).

5. Egr-1 expression is not increased in hippocampus of APPswe/PS1ΔE9 mice following learning

After we established that CRE-based gene transcription was impaired in young adult APPswe/PS1ΔE9 mice, we next asked if this impairment results in an observable impairment in the expression of a CRE-driven gene known to be important for learning and memory. We chose to examine protein expression of the CRE-driven gene *Egr-1* (*zif268*) which has been previously shown to activate in response to an animal's exposure to novel environments or learning (Hall, Thomas, & Everitt, 2000; Tischmeyer & Grimm, 1999) (Lakhina et al., 2015), and Egr-1 mutant mice have been described to have impairments in LTP maintenance and in several long term memory tasks including object recognition (Jones et al., 2001b).

Our first experiment was to examine Egr-1 expression following learning in the hippocampus of CRE and CRE-AD mice following completion of the NOR task. We did observe colocalization between X-gal and Egr-1 in the hippocampus (**Figure 11A-B**). Notably, there were more cells that expressed Egr-1 than were positive for X-gal. This may be attributed to differences in activation time of the different genes following learning, as well as to the processing time of the β-galactosidase activity.

Next, we examined Egr-1 expression in the hippocampus of APPswe/PS1ΔE9 or nontransgenic mice following contextual fear conditioning. We observed that Egr-1 expression was increased following learning in the hippocampus of nontransgenic mice (**Figure 12A**), but not in the hippocampus of APPswe/PS1ΔE9 mice (**Figure 12B**). In

order to quantify Egr-1 expression in the CA regions of the hippocampus, we measured the intensity of the positive signal from the immunolabeling and observed that Egr-1 was significantly increased following fear conditioning in nontransgenic mice, but not in the APPswe/PS1ΔE9 mice (**Figure 12C**). We did not observe any differences in Egr-1 expression in the somatosensory regions of the cortex (**Figure 12D**). These results strongly suggest that the upregulation of Egr-1, and possibly other important CRE-driven genes, is compromised following learning in the hippocampus of APPswe/PS1ΔE9 mice, leading to deficient brain plasticity and memory formation.

Taken together, the experiments in young adult APPswe/PS1ΔE9 mice yield three important observations: 1. CREB signaling components are impaired at an early age in APPswe/PS1ΔE9 mice, 2. These impairments result in deficient CRE-based gene transcription, and 3. These deficits correspond to functional impairments in learning and memory-based behavioral experiments.

Figure 11

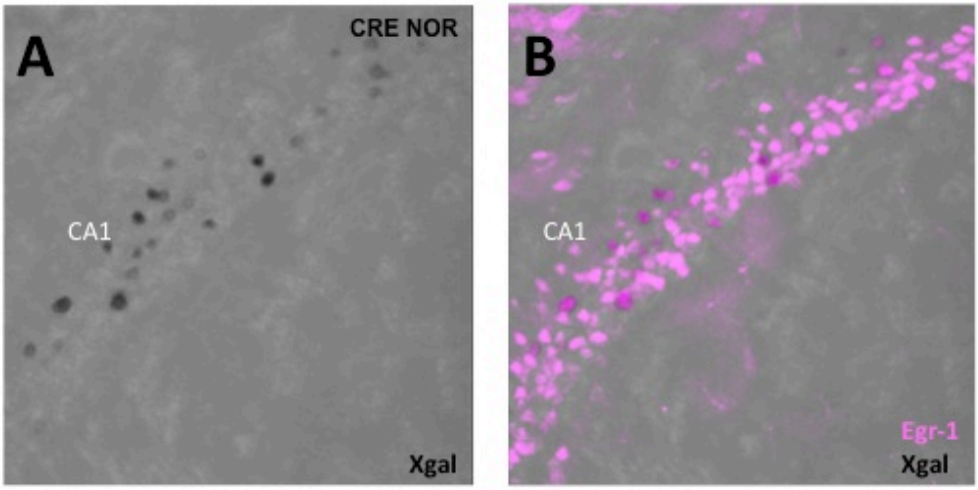

Figure 11. **X-gal staining overlaps with Egr-1 staining in the CA1 region of the CRE mouse following NOR**. **A.** X-gal expression in a CRE mouse following NOR. **B**. Overlap of X-gal and Egr-1 staining in the same section showing colocalization.

Figure 12

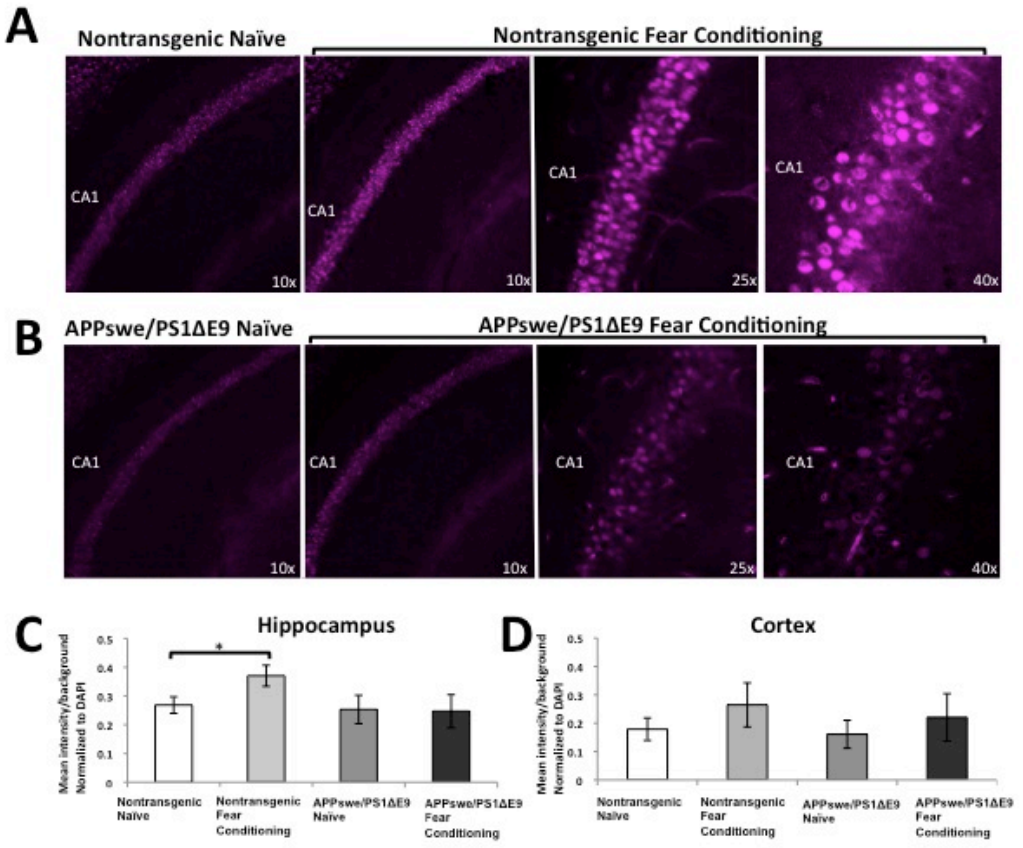

Figure 12. Egr-1 expression is not increased in the hippocampus of APPswe/PS1ΔE9 mice following fear conditioning as it is in nontransgenic mice. **A**. Egr-1 expression in naïve nontransgenic mice and following fear conditioning (N=4 per group). **B**. Egr-1 expression in naïve APPswe/PS1ΔE9 mice and following fear conditioning (N=3 per group). **C**. ImageJ analysis average intensity of Egr-1 staining relative to background in the hippocampus and normalized to intensity of DAPI**. D**. ImageJ analysis average intensity of Egr-1 staining relative to background in the

somatosensory regions of the cortex and normalized to intensity of DAPI.

B. Expression of CREB signaling components is impaired in postmortem AD prefrontal cortex, and are not associated with severity of AD pathology

1. CREB signaling components are reduced in the AD prefrontal cortex

While we and others have shown that CREB signaling is impaired in mouse models of FAD, how this impairment relates to the progression of AD remains an under-explored area. A few studies have examined expression of pCREB and total CREB in the postmortem hippocampus, and demonstrated a reduction of pCREB and Total CREB in the AD hippocampus (Pugazhenthi et al., 2011; Yamamoto-Sasaki et al., 1999b). We asked if these deficits are limited to the hippocampus by analyzing CREB signaling components in the prefrontal cortex. While much of AD research is focused on the hippocampus, the PFC is also vulnerable to neurodegeneration in AD (DeKosky & Scheff, 1990; Grady, Furey, Pietrini, Horwitz, & Rapoport, 2001; Salat, Kaye, & Janowsky, 2001; van Veluw et al., 2012a) indicating that impairments in CREB signaling may not be confined to the hippocampal formation.

For this reason, we first examined the levels of pCREB and Total CREB in the nuclear fraction of postmortem PFC of individuals with a clinical diagnosis of AD and cognitively intact controls matched for age and education. We observed reduced levels of pCREB and Total CREB in the PFC of AD individuals (**Figure 13A-C**). To our knowledge, this is the first report of reduced Total CREB and pCREB in AD PFC, and this observation indicates that CREB signaling dysfunction is not confined to the hippocampal formation in AD.

In light of our observations of multiple impairments in the CREB signaling pathway in the APPswe/PS1ΔE9 mouse model of AD, we next asked if other components of the CREB signaling pathway are decreased in AD PFC. We examined expression of the transcription cofactors CBP and p300, and, as in the hippocampus of the APPswe/PS1ΔE9 mice, we observed for the first time reduced expression of p300 and CBP in the postmortem AD PFC (**Figure 13A,D-E**). These results indicate that multiple impairments may exist in the CREB signaling components in AD brain and also suggest that reduced CREB signaling in the AD brain may play a role in cognitive dysfunction in AD.

In an effort to begin to understand the source of reduced CREB activation in AD PFC we examined levels of total PKA protein. We previously observed reduced levels of total PKA protein in the hippocampus of the APPswe/PS1ΔE9 mouse model of AD both at steady state (**Figure 5A,B)** and following learning (**Figure 7A,B**). Interestingly, we did not observe reductions in nuclear protein levels of PKA in the postmortem AD PFC, suggesting that pCREB impairments may not be a result of decreased protein levels of PKA (**Figure 13A,F**). However, we cannot rule out the possibility that the activity level of PKA is reduced in the AD PFC, in spite of comparable levels of total protein. Therefore, while we have shown that multiple impairments exist in the CREB signaling pathway and that these impairments are not confined to the hippocampus, the question of the source of these impairments remains an open question.

Figure 13

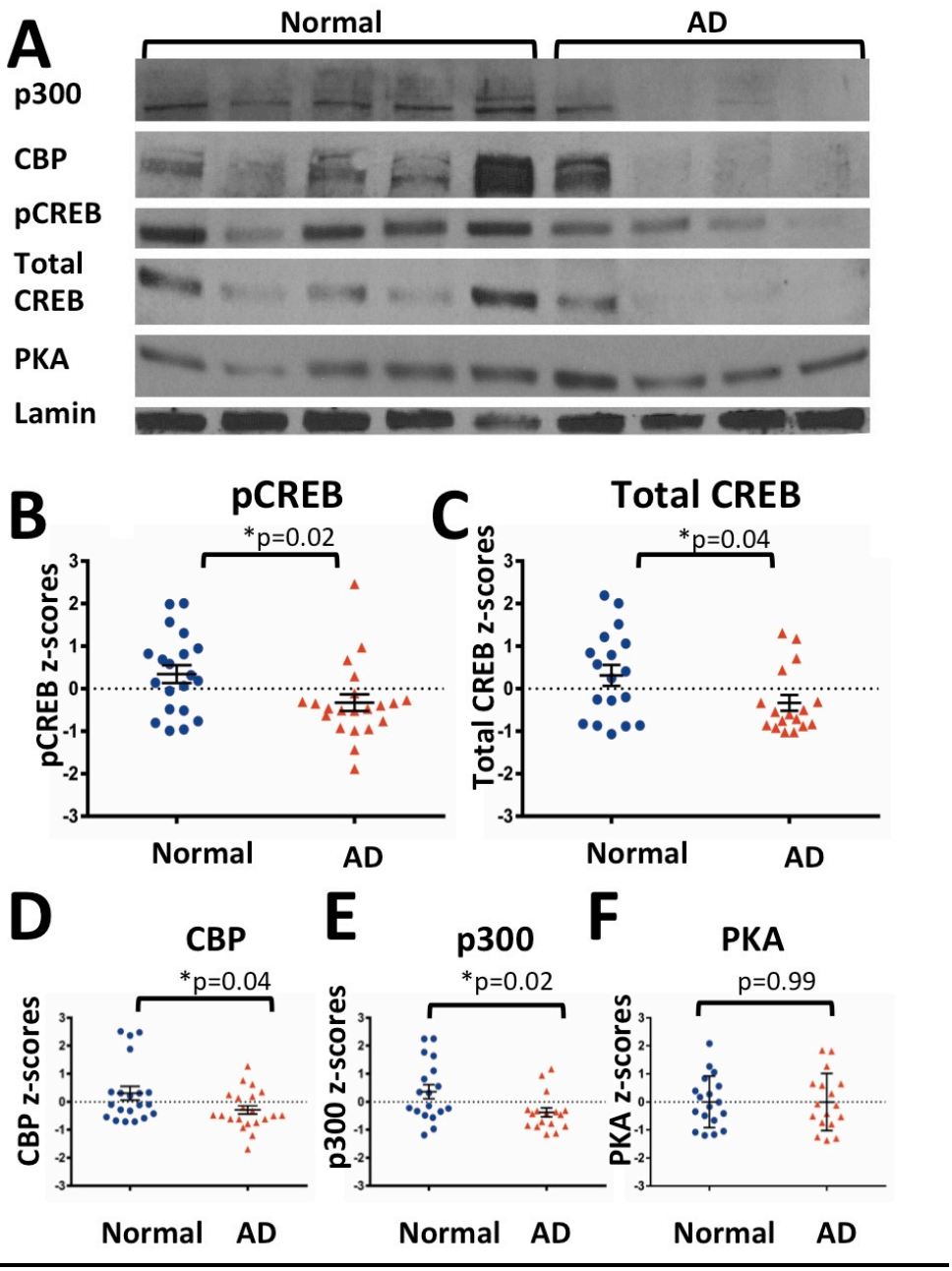

Figure 13. CREB signaling components in AD PFC are lower compared to age- and education-matched, cognitively-intact controls A. Representative Western blot image of p300, CBP, pCREB, Total CREB, and PKA in human PFC. B. pCREB

expression (normalized to Lamin) in nuclear fractions of PFC of AD individuals and cognitively intact controls (p=0.02). C. Total CREB expression (normalized to Lamin) in nuclear fractions of PFC of AD individuals and cognitively intact controls (p=0.04) C. CBP expression (normalized to Lamin) in nuclear fractions of PFC of AD individuals and cognitively intact controls (p=0.04). D. p300 expression (normalized to Lamin) in nuclear fractions of PFC of AD individuals and cognitively intact controls (p=0.02). E. PKA expression (normalized to Lamin) in nuclear fractions of PFC of AD individuals and cognitively intact controls (p=0.99). Error bars represent SEM.

2. pCREB expression in the PFC does not correlate with pathological hallmarks of AD

Our observation that CREB signaling is dysfunctional in young adults in the APPswe/PS1ΔE9 mouse model of AD suggest that CREB dysfunction in AD may not be inherently tied to severity of pathology. Plaque deposition in the hippocampus of the APPswe/PS1ΔE9 mouse is typically observed starting between 4 and 6 months of age (Jankowsky et al., 2004; J. L. Jankowsky et al., 2005), but we observe deficits in CREB signaling as early as 2-3 months of age (Y. S. Hu et al., 2013b). Therefore, to examine the hypothesis that CREB impairments are not simply a function of pathology status in the human disease, we compared pCREB expression in the PFC with amyloid and tau pathology scores for the entire brain and for the superior frontal gyrus.

While we did observe increased pathology severity of both plaques and tangles in the AD group as expected (**Figure 14A,C**), we did not observe a relationship between either local amyloid or local tau pathology and PFC pCREB expression by a Pearson correlation analysis (**Figure 14B, D**). We then conducted an additional analysis in which we compared the PFC pCREB expression with global brain amyloid and tau pathology, and again, we did not observe a relationship between pCREB expression and severity of pathology (**Figure 15A-D**).

These results support the hypothesis that dysfunctional CREB signaling in AD is not simply the result of increased amyloid or tau pathology, in contrast to a previous report (Pugazhenthi et al., 2011), and offers the intriguing possibility that CREB signaling

components may serve as novel, accessible markers of cognitive function and disease progression.

Figure 14

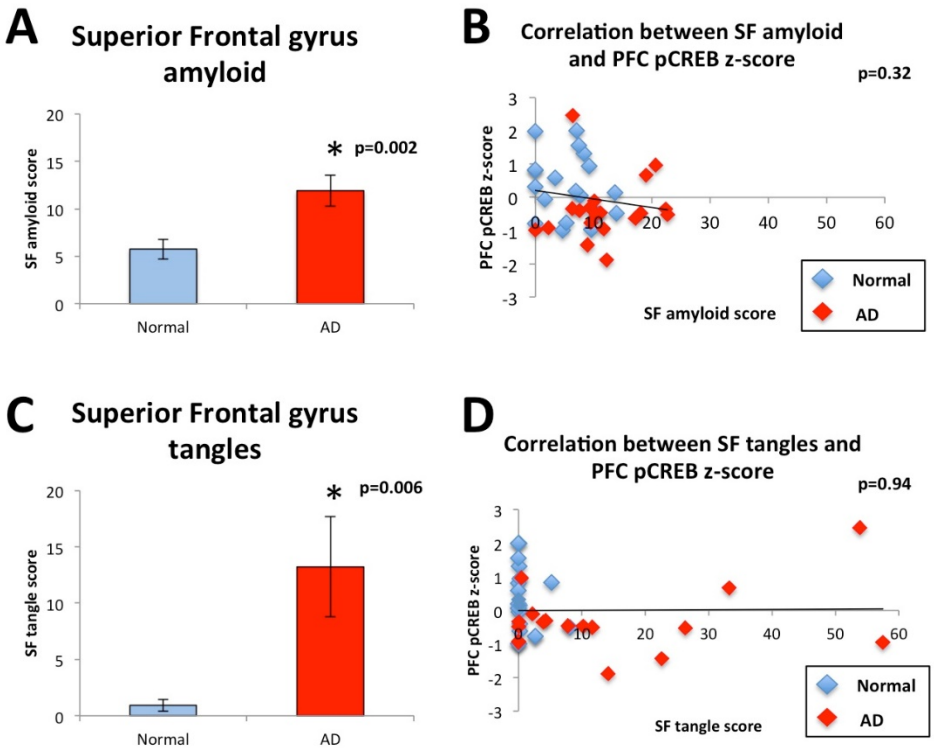

Figure 14. PFC pCREB expression is not associated with severity of local amyloid or tau pathology. A. Amyloid counts are higher in the superior frontal gyrus (SFG) of AD compared to cognitively normal SFG (p=0.002). B. pCREB in the PFC is not correlated with severity of amyloid pathology in SFG by a Pearson correlation (p=0.32). C. Tangle counts are higher in the postmortem SFG of AD compared to cognitively normal (p=0.006). D.pCREB in the PFC is not correlated with severity of tangle pathology in the SFG by a Pearson correlation (p=0.94).

Figure 15

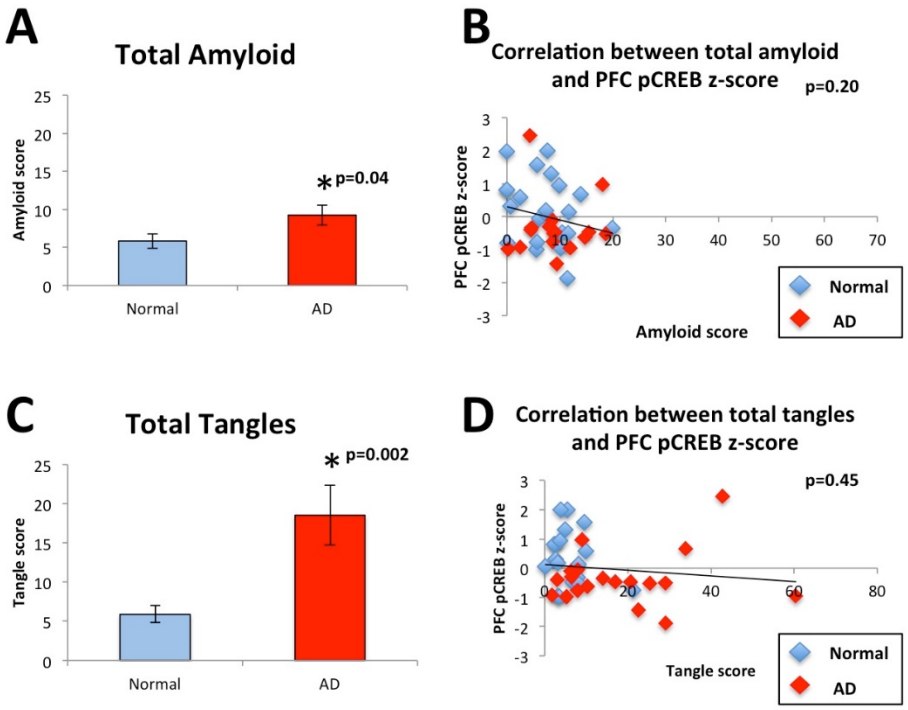

Figure 15. **PFC pCREB expression is not associated with severity of global amyloid or tau pathology.** A. Total amyloid pathology is higher in postmortem AD brain compared to cognitively normal brain (p=0.04). B. pCREB in the PFC is not correlated with severity of amyloid pathology (Pearson R=-0.214, p=0.197). C. Total tangle pathology is higher in postmortem AD brain compared to cognitively normal brain (p=0.002). D. pCREB in the PFC is not correlated with severity of tangle pathology (Pearson R=-0.126, p=0.451).

C. Impairments of CREB signaling components is reflected in peripheral blood mononuclear cells

1. pCREB and CBP are reduced in the PBMC of young adult APPswe/PS1ΔE9 mice

Our observations that CREB signaling components are decreased in the postmortem brain of AD patients indicate that CREB signaling is dysfunctional at death, but does not give information about CREB signaling during disease progression. In an effort to understand how CREB signaling changes during disease progression we decided to investigate CREB signaling components in peripheral blood mononuclear cells (PBMC). First, we examined pCREB and CBP in the pooled PBMC from APPswe/PS1ΔE9 and nontransgenic following contextual fear conditioning. The nuclear fractions of the hippocampus from these mice is shown in **Figure 7**, in which we observed significantly lower levels of CREB signaling components. After pooling the whole blood from these mice and isolating the PBMC, we then performed nuclear fractionation and observed a lower level of pCREB and CBP in the APPswe/PS1ΔE9 PBMC compared to the nontransgenic littermates (**Figure 16A-C**). We confirmed this observation by flow cytometry analysis, which allowed us to analyze mice individually rather than pooling the samples. In this analysis we observed significantly reduced expression of pCREB in blood isolated from 3 month old female APPswe/PS1ΔE9 mice (**Figure 17A-C)**. These results offered the intriguing possibility that the expression of CREB signaling components in the PBMC may serve as an indicator of CREB signaling in the brain.

Next we asked if the reduced expression of pCREB and CBP in the APPswe/PS1ΔE9 PBMC was a direct result of the mutations in APP and PS1. These transgenic mice were generated using the PrP promoter that preferentially targets neuronal cells, and previous reports have shown that under this promoter the mutations in PS1 are primarily present in the brain and kidneys, with low expression in the heart, but are not present in other organs, such as the spleen (Borchelt et al., 1996; Jankowsky et al., 2001). Therefore, we examined the expression of PS1 and APP in the PBMC of APPswe/PS1ΔE9 and nontransgenic mice. As expected, in the brain of the APPswe/PS1ΔE9 mice we observed accumulation of the ~42kDa PS1 fragment that occurs as a result of the ΔE9 mutation, and increased expression of full-length APP that occurs as a result of the Swedish mutation. However, we observed no difference in either PS1 or APP expression in the PBMC of APPswe/PS1ΔE9 mice compared to their nontransgenic littermates. These results suggest that the CREB signaling deficit observed in the APPswe/PS1ΔE9 PBMC may respond to systemic signaling rather than being the result of a cell autonomous effect. This strengthens the argument that CREB signaling in the periphery may serve as an indicator of CREB signaling in the brain.

Figure 16

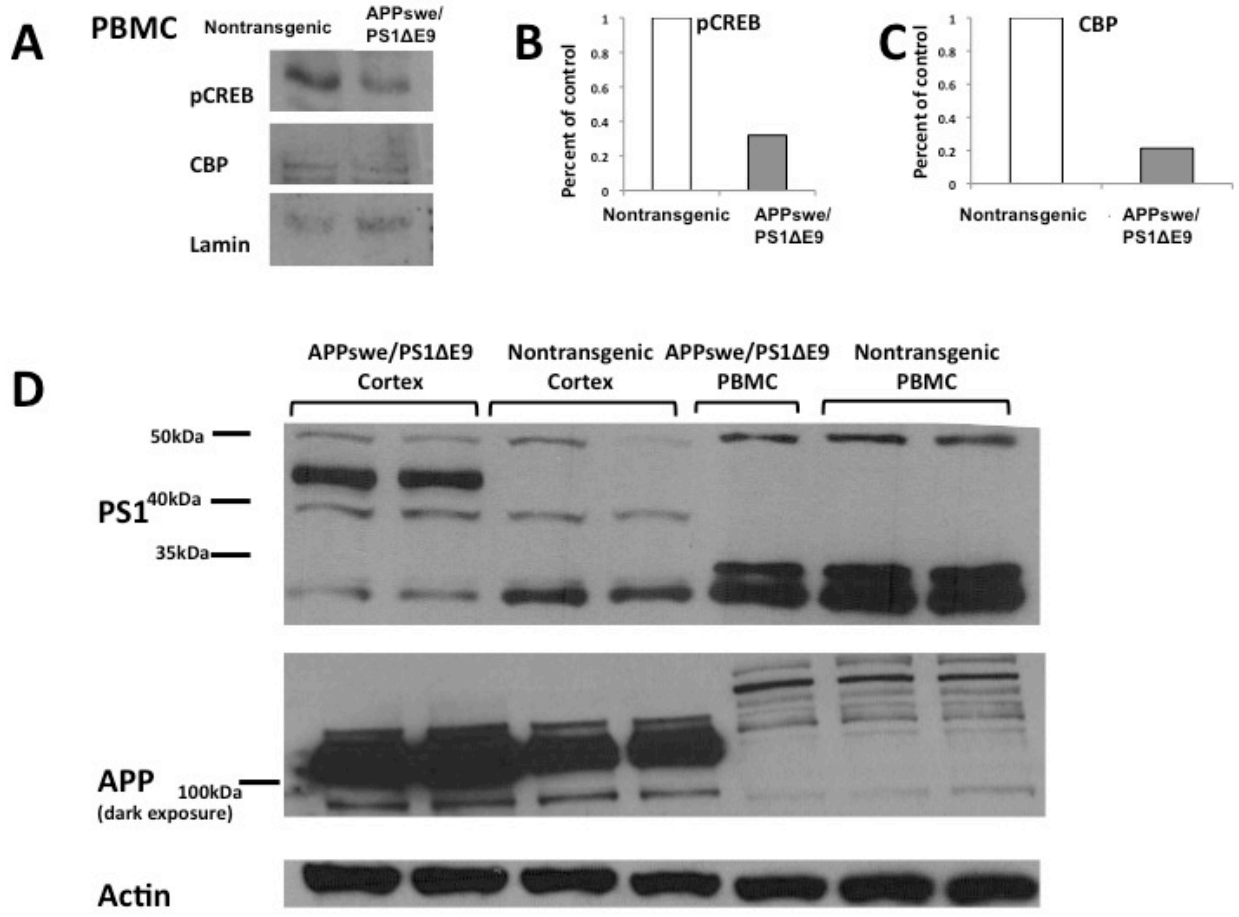

Figure 16. pCREB and CBP are reduced in the PBMC of APPswe/PS1ΔE9 mice. A. Western blot showing pCREB and CBP expression in nuclear fractions of pooled PBMC samples (Nontransgenic N=7, APPswe/PS1ΔE9 N=7). **B.** pCREB expression normalized to lamin. **C**. CBP expression normalized to lamin. **D.** Expression of PS1 and APP in the brain and PBMC of APPswe/PS1ΔE9 and nontransgenic mice. Each cortex band represents one mouse, while each PBMC band is the result of protein from PBMC pooled from 4 mice.

Figure 17

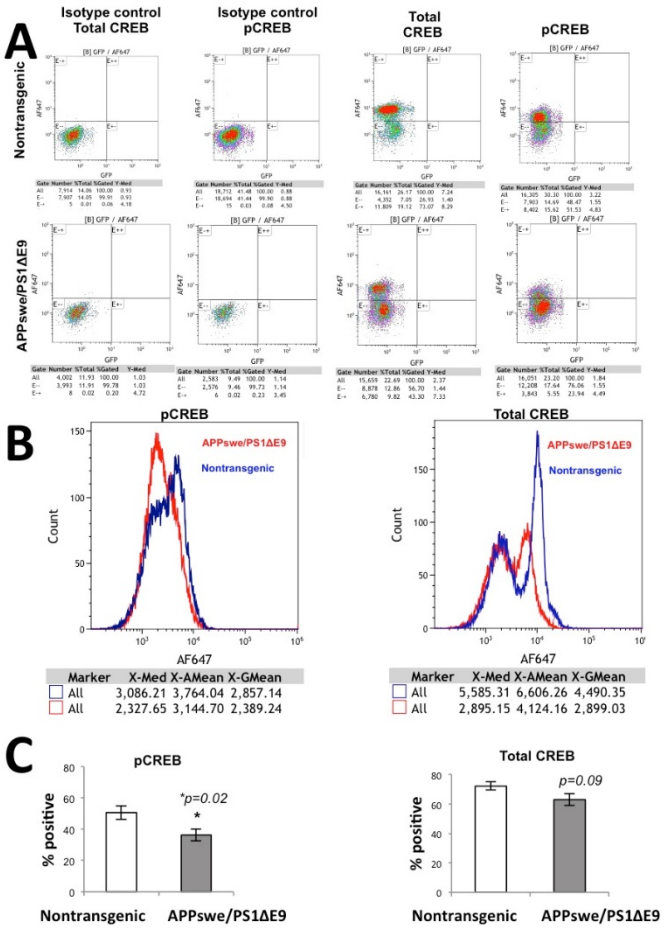

Figure 17. pCREB expression is diminished in blood isolated from APPswe/PS1ΔE9 mice. Whole blood was drawn from 2-3 month old female nontransgenic or APPswe/PS1ΔE9 mice by cardiac puncture. RBC were lysed and remaining WBC were prepared for FACS and stained with pCREB or Total CREB antibodies. Dot plots show intensity of pCREB or Total CREB signal on the Y-axis and intensity of GFP signal on the X-axis. Data shown here was first gated on PBMC using Forward and Side scatter, and on single events. Quadrants were set based on the isotype controls, and the percent of cells staining brighter than this threshold are considered the positive population (value found in “% gated” column for the E-+

quadrant). Histograms were derived from the same gating strategy as the dot blots, and show signal intensity for either pCREB or Total CREB on the X-axis and event count on the Y-axis. **A.** Representative dot plots from FACS analysis of Total CREB and pCREB in PBMC isolated from nontransgenic or APPswe/PS1ΔE9 mice. **B.** Representative histograms of FACS analysis of Total CREB and pCREB pCREB in PBMC isolated from nontransgenic (blue) or APPswe/PS1ΔE9 (red) mice. **C.** Mean of percent Total CREB and pCREB positive PBMC in nontransgenic (N=9) and APPswe/PS1ΔE9 (N=11) mice.

2. pCREB and CBP are reduced in the PBMC of individuals with AD

Based on our observations in the APPswe/PS1ΔE9 PBMC, we decided to examine CREB signaling components in the PBMC of individuals with clinically diagnosed AD, MCI, and age- and education-matched controls. First, we examined pCREB, Total CREB, and CBP in the PBMC to determine if pCREB and CBP are reduced in AD PBMC as they are in AD PFC. We observed a nonsignificant reduction in pCREB expression in the PBMC of individuals with AD when compared by a one-way ANOVA to individuals with MCI or cognitively normal individuals, and observed no difference between PBMC from normal or MCI individuals (**Figure 18A,C**). To examine whether reduced pCREB is indicative of a definitive AD diagnosis, we examined pCREB expression in PBMC of individuals with AD compared to non-AD. pCREB level was significantly lower in AD PBMC compared to non-AD PBMC by a one-tail, unpaired t-test. (**Figure 18D**). These results indicate that the reduction in pCREB expression in PBMC may not be indicative of general cognitive dysfunction, but rather an indicator of the conversion from general cognitive impairments to AD dementia.

Next, we examined Total CREB in PBMC and observed a trending, but non-significant, decrease in Total CREB in AD PBMC when compared to non-AD PBMC (**Figure 18E-F**). We then compared the ratio of pCREB/Total CREB in PBMC and observed a non-significant reduction in the ratio in AD PBMC when compared to non-AD PBMC (**Figure 18G-H**). These results suggest that reduced pCREB in AD PBMC may be a combination of reduced levels of total protein and impaired phosphorylation.

Next, we measured CBP in the PBMC of clinically diagnosed AD patients, MCI, or age-and education-matched controls. We did observe a significant difference in CBP expression by a one-way ANOVA, and follow-up analysis revealed that CBP expression was significantly lower in individuals with AD compared to individuals with MCI (**Figure 18I**). Since we did not observe a difference in CBP expression between normal and MCI, we hypothesized that levels of CBP too are reduced in definitive AD. As with pCREB, we observed that CBP is significantly lower in the PBMC of individuals with AD when compared to individuals without AD (one-tail, unpaired t-test, **Figure 18J**). These results indicate that the impairments in CREB signaling in PBMC are strongest upon conversion to AD rather than a general indicator of cognitive impairment. In addition, these results suggest for the first time that CREB signaling deficits in the brain may also be apparent systemically, and offers the intriguing possibility that the expression of CREB signaling components in PBMC may be used as a readout for CREB signaling in the brain.

Figure 18

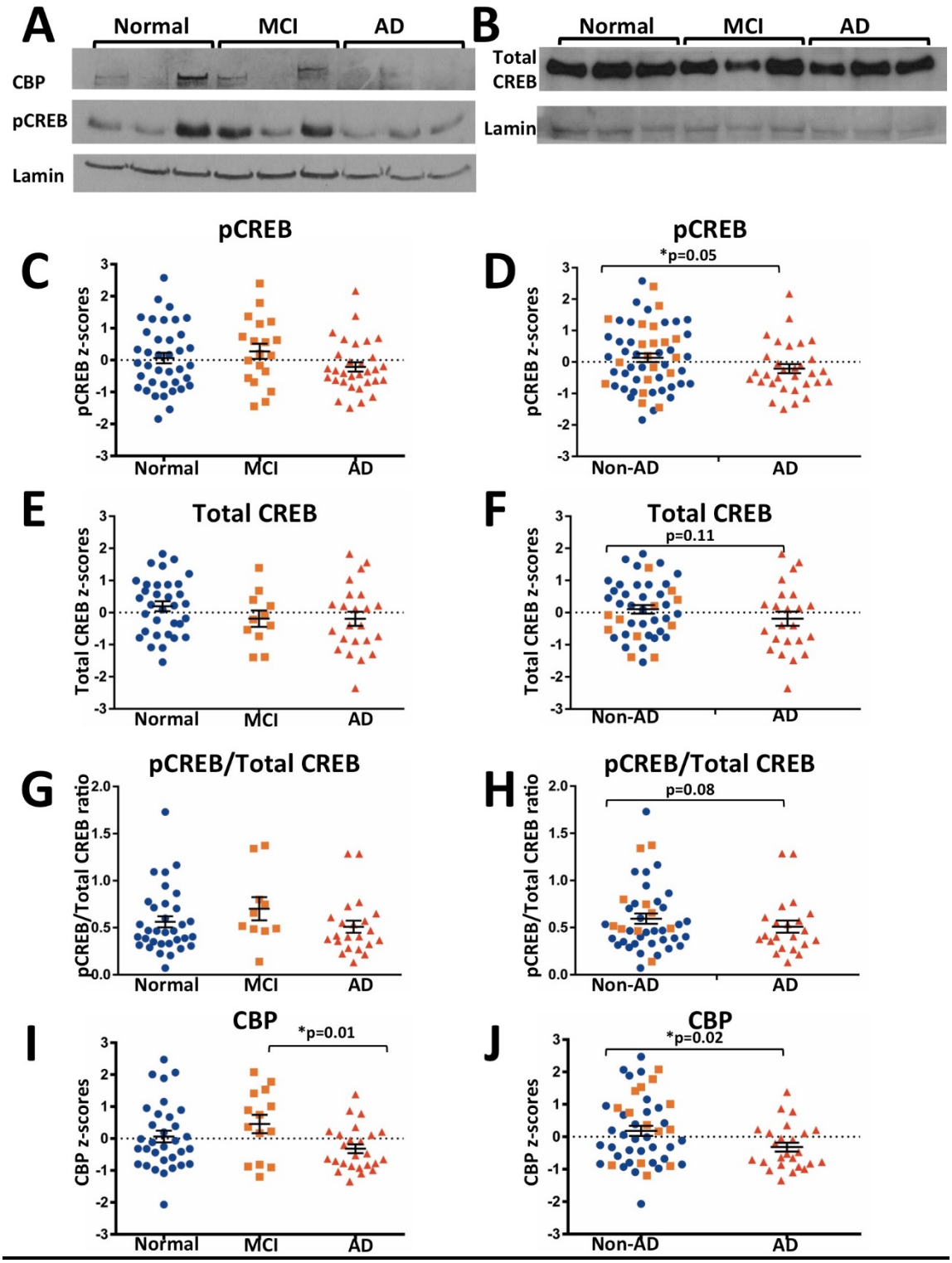
A
Normal
MCI
AD
CBP
pCREB
Lamin
B
Normal
MCI
AD
Total CREB
Lamin
C
pCREB
pCREB z-scores
Normal
MCI
AD
D
pCREB
*p=0.05
pCREB z-scores
Non-AD
AD
E
Total CREB
Total CREB z-scores
Normal
MCI
AD
F
Total CREB
p=0.11
Total CREB z-scores
Non-AD
AD
G
pCREB/Total CREB
pCREB/Total CREB ratio
Normal
MCI
AD
H
pCREB/Total CREB
p=0.08
pCREB/Total CREB ratio
Non-AD
AD
I
CBP
*p=0.01
CBP z-scores
Normal
MCI
AD
J
CBP
*p=0.02
CBP z-scores
Non-AD
AD

Figure 18. **pCREB and CBP are reduced in AD PBMC**. A. Representative Western blot of pCREB and CBP in nuclear fractions of PBMC of AD, MCI and cognitively normal controls. B. Representative Western blot of Total CREB in nuclear fractions of PBMC of AD, MCI and cognitively normal controls. C. pCREB z-scores of PBMC in Normal, MCI and AD individuals were not different by a one-way ANOVA ($p=0.20$). D. pCREB is significantly lower in the PBMC of AD individuals compared to non-AD individuals when compared by a one-tail, unpaired t-test ($p=0.05$). E. Total CREB is not significantly different by a one-way ANOVA ($p=0.25$). F. Total CREB is not significantly different in AD PBMC when compared to non-AD PBMC by a one-tail, unpaired t-test ($p=0.11$). G. The ratio of pCREB to Total CREB was not significantly different by a one-way ANOVA ($p=0.33$). H. The ratio of pCREB to Total CREB was reduced in AD PBMC when compared to non-AD PBMC by a one-tail, unpaired t-test ($p=0.08$). I. CBP z-scores of PBMC in Normal, MCI and AD individuals were significantly different by a one-way ANOVA ($p=0.049$). Follow up analysis using a two-tailed, unpaired t-test revealed that CBP is significantly reduced in AD PBMC compared to MCI PBMC ($p=0.01$). J. CBP is significantly lower in the PBMC of AD individuals compared to non-AD individuals when compared by a one-tail, unpaired t-test ($p=0.02$). Error bars represent SEM.

3. Association of pCREB in PBMC with pCREB in brain PFC

To address the possibility that CREB signaling components in the PBMC might be indicative of CREB signaling in the brain, we compared pCREB and CBP in PBMC of AD or cognitively-intact individuals to pCREB and CBP of the corresponding postmortem PFC. For this purpose, we used PBMC and PFC from 20 normal and 21 AD individuals (**Figure 19A**). We observed a weak positive correlation between pCREB expression in PBMC and expression in the brain by a linear regression analysis (**Figure 19B**). However, the PBMC in this experiment were isolated as many as 4-5 years prior to death, and thus temporally mismatched with brain readout. We hypothesized that by narrowing the interval between blood sample draw and death, we might observe a stronger relationship between expression in the blood and expression in the brain. Therefore, we performed an additional analysis in which PBMC were drawn from patients 3 years or less from the time of death (i.e., time of postmortem brain collection; 13 normal and 11 AD). We observed a significant positive correlation by a linear regression analysis between pCREB expression in the PBMC and pCREB expression in the PFC for all samples, when the time interval between PBMC sample draw and postmortem brain sample collection was less than 3 years (**Figure 19C**). Examining the association between pCREB expression in PBMC and brain of cognitively intact individuals revealed weak correlation (**Figure 19D**). However, a very strong positive correlation was observed in the AD individuals (**Figure 19E**). These results strongly suggest that pCREB in the PBMC reflects pCREB signaling in the brain, and that this correlation is particularly strong in AD. These results suggest that pCREB in PBMC may serve as a noninvasive marker for pCREB expression in the brain.

Figure 19

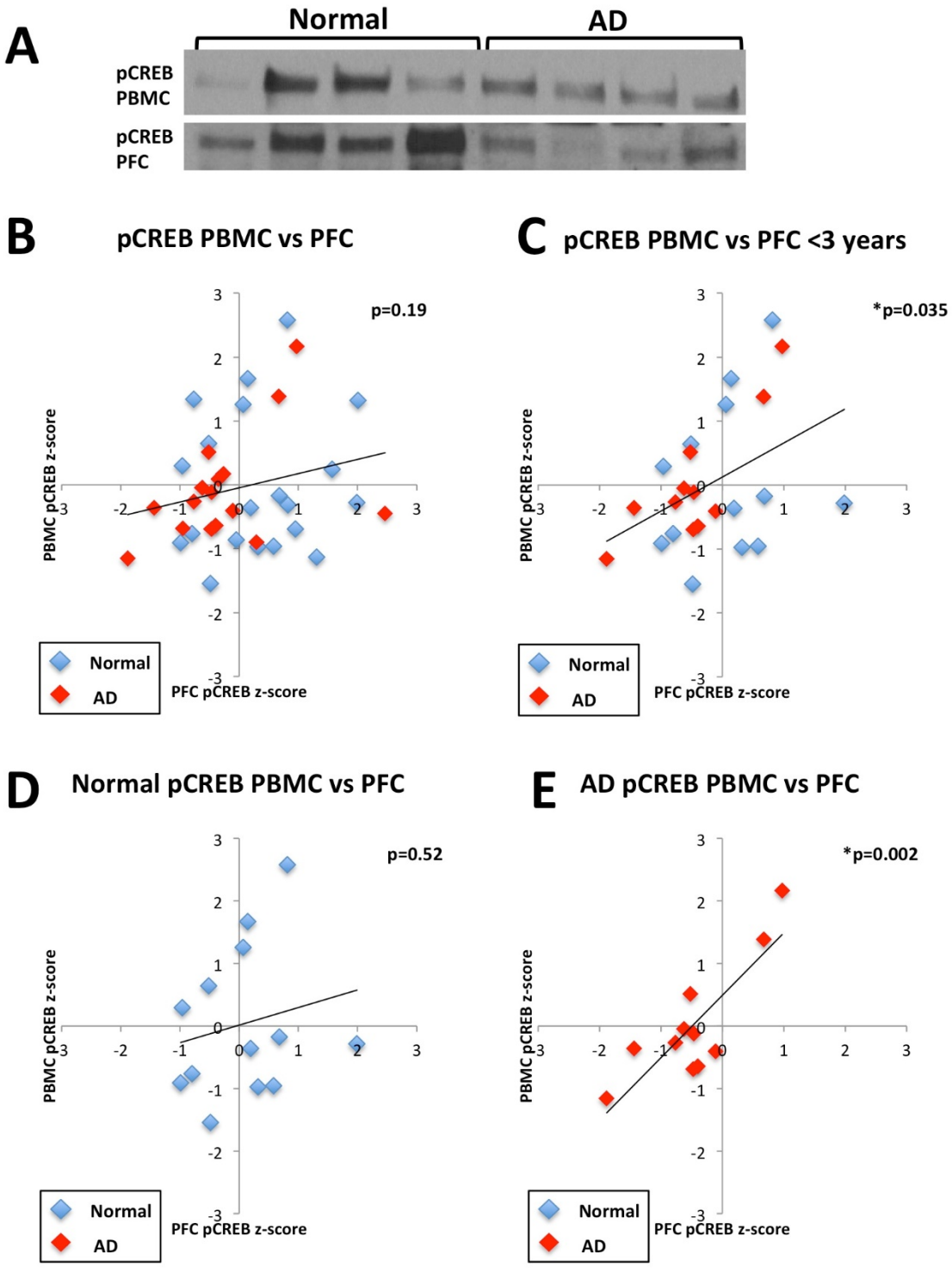
A
Normal
AD
pCREB PBMC
pCREB PFC
B
pCREB PBMC vs PFC
p=0.19
PBMC pCREB z-score
PFC pCREB z-score
Normal
AD
C
pCREB PBMC vs PFC <3 years
*p=0.035
PBMC pCREB z-score
PFC pCREB z-score
Normal
AD
D
Normal pCREB PBMC vs PFC
p=0.52
PBMC pCREB z-score
PFC pCREB z-score
Normal
AD
E
AD pCREB PBMC vs PFC
*p=0.002
PBMC pCREB z-score
PFC pCREB z-score
Normal
AD

Figure 19. **pCREB expression in PBMC correlates with pCREB expression in PFC**. A. Representative blot showing expression of pCREB in PBMC and PFC in the same individuals. B. pCREB in the PBMC is weakly correlated with pCREB expression in the PFC ($p=0.19$). C. Linear regression analysis revealed that pCREB expression in PBMC correlates with pCREB expression in the PFC when the temporal interval between blood and brain sample is < 3 years ($p=0.035$). D-E. No correlation is observed in cognitively normal individuals between PFC pCREB and PBMC pCREB (D; $p=0.52$). However, a strong positive correlation exists between PFC pCREB and PBMC pCREB in the AD individuals (E; $p=0.002$).

4. Association of CBP in PBMC with CBP in brain PFC

We also compared CBP expression in the PBMC to CBP expression in the PFC (**Figure 20A**). We observed a nonsignificant, weakly negative correlation between PBMC CBP and PFC CBP (**Figure 20B**). Analysis of PBMC samples drawn within 3 years prior to death (**Figure 20C**) also showed a weak negative correlation. Analyzing the correlation inside diagnostic group, i.e., in normal individuals or in AD patients revealed weak negative correlations between CBP in PBMC and in PFC (**Figure 20D,E**). None of these weak correlations was statistically significant. This result suggests that while CBP may be independently reduced in both PFC and PBMC in AD, the relationship between PBMC and PFC on an individual level is not correlative at our sample size.

Figure 20

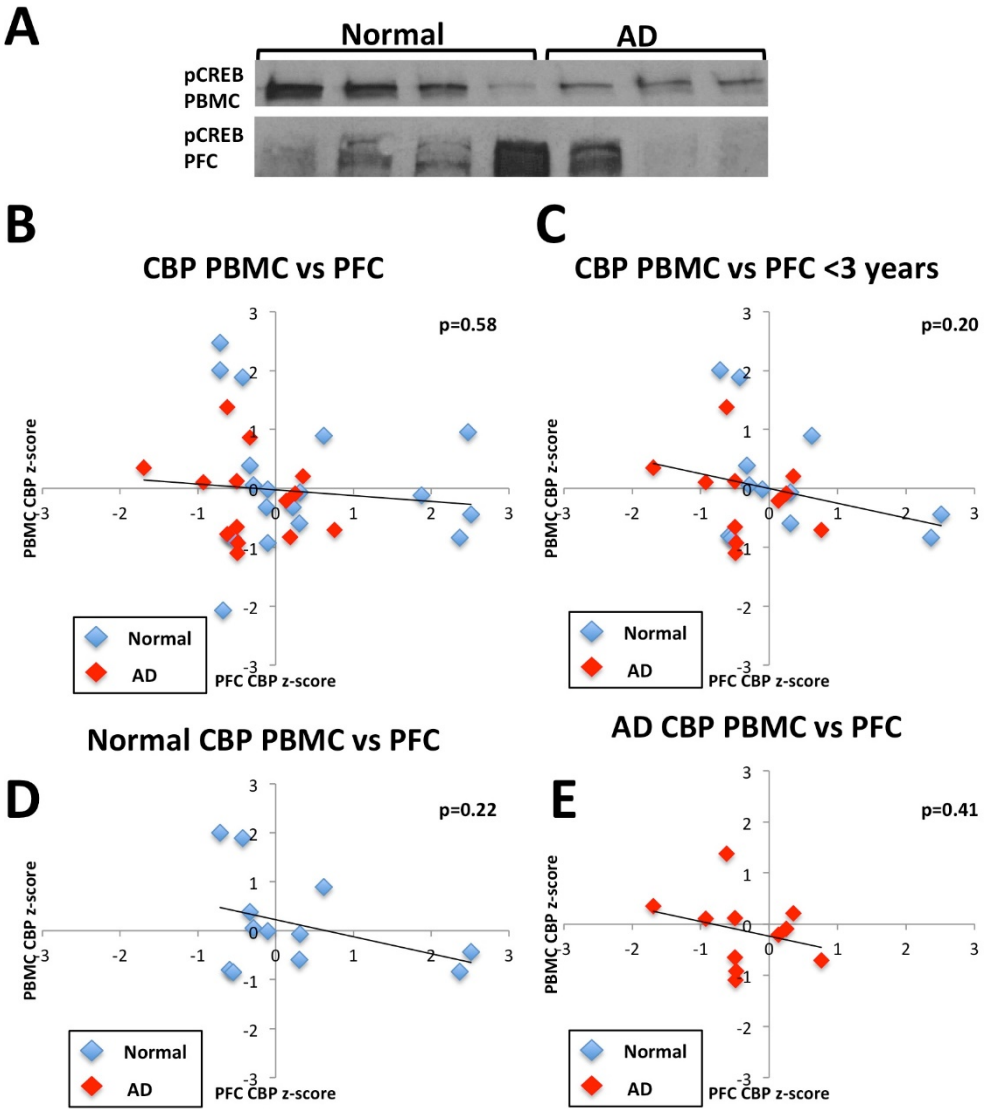

Figure 20. CBP expression in PBMC is not related to CBP expression in the PFC. A. Representative blot showing expression of CBP in PBMC and PFC in the same individuals B. CBP expression in the PBMC is not correlated with expression in the PFC by a Pearson correlation (p=0.58). C. CBP expression in PBMC drawn less than 3 years prior to death is weakly correlated with CBP expression in the PFC (p=0.20). D-E. When

the data is split into cognitively normal and AD individuals, weak negative correlations are observed between PFC CBP and PBMC CBP in cognitively normal individuals (D; p=0.22) and AD individuals (E; p=0.41).

V. Discussion

Parts of this chapter were previously published as

1. Bartolotti N, Segura L, Lazarov O (2016) Diminished CRE-Induced Plasticity is Linked to Memory Deficits in Familial Alzheimer's Disease Mice. *Journal of Alzheimers Disease* **50**, 477-489.

or

2. Bartolotti N, Bennett DA, Lazarov O (2016) Reduced pCREB in Alzheimer's disease prefrontal cortex is reflected in peripheral blood mononuclear cells. *Molecular Psychiatry* **21**, 1158-1166.

A. Characterization of CREB signaling in young adults of the APPswe/PS1ΔE9 mouse model of AD.

1. CREB signaling components are diminished in young adult APPswe/PS1ΔE9 at a basal state.

This study provides several novel aspects concerning the role of CREB signaling in cognitive functioning in sporadic AD and in a mouse model of FAD. First, we show that basal levels of pCREB, CBP and p300 are reduced in the nuclear fraction of the hippocampus of APPswe/PS1ΔE9 mice. Lower levels of basal pCREB and CBP could indicate a reduction in the availability of these critical factors for learning and memory, resulting in impaired CRE-based gene transcription and failure to form a memory. We also show that EE-induced CRE-driven gene transcription is deficient in APPswe/PS1ΔE9 mice. While levels of pCREB, CBP, and CRE-gene transcription are significantly increased following learning or EE in wild type or CRE-reporter mice, this activation is deficient in the APPswe/PS1ΔE9 mice. Clearly, these impairments are not a result of age or disease progression, as they are detected as early as 2-3 months of age in this study. This suggests that enhancement of plasticity by experience in EE may not be sufficient for a complete rescue of cognitive impairments in AD.

The cause of reduced levels of active CREB and the transcription cofactors is unclear. One potential explanation could be impairments in the level or activity of kinases that phosphorylate CREB. Here, we observed significantly reduced levels of PKA, and trending reductions in PKC and CaMKIV, which might support the hypothesis that impaired CREB phosphorylation results from reduced activation by kinases. Interestingly, we observed increases in pERK, total ERK, and the ratio of pERK to total ERK in APPswe/PS1ΔE9 mice. This observation is interesting in light of the fact that ERK signaling has been previously reported to be abnormally increased in AD (Perry et al., 1999). It has been suggested that increased ERK signaling may even result in accelerated neurodegeneration (Subramaniam et al., 2004). One study suggests that ERK is hyperactive in astrocytes in early stages of AD, before being reduced in later stages (Webster et al., 2006). A similar phenomenon has been reported in the Tg2576 mouse model of AD, with overactive ERK observed at early stages and a reduction in later stages (Dineley et al., 2001). Importantly, this data demonstrates the enhanced ERK expression and activation cannot compensate for other deficits in mechanisms that activate CREB. We do not examine the downstream kinases MSK and RSK, so another important consideration is that we do not know if these kinases are dysfunctional in spite of overactive ERK, representing the broken link of activation between ERK and CREB.

There are several potential mechanisms by which the mutations in PS1 and APP might contribute to defects in CREB activation and expression of CREB signaling components. For example, several studies suggest that PS1 regulates CREB expression and function, (Bonds et al., 2015; Marambaud et al., 2003; Muller et al., 2011; Watanabe et al., 2009). It is thought that normal presenilin may promote CBP-induced

transcription, and that FAD mutant PS1 can interfere with this transcription, offering one potential mechanism for the deficits observed in the FAD mice (Francis et al., 2006; Saura et al., 2004). The mutations in APP may also contribute to this dysfunction, possible through the dysregulation of APP processing. For example, in 6 month 3xTg-AD mice which also have mutations in PS1 and APP, pCREB was reduced in the hippocampus concordant with abnormal expression of PKA and pERK, which the authors suggest may be a result of Aβ (Caccamo, Maldonado, Bokov, Majumder, & Oddo, 2010b). Another study showed that increased levels of Aβ result in chronic activation of the ERK/MAPK cascade, leading to decreased phosphorylation of CREB (Dineley et al., 2001), an idea in line with our observation of increased ERK activation but reduced CREB activation, in spite of the documented role of ERK in activating CREB (Lonze & Ginty, 2002). While we and others have described impairments in cognitive functioning in APPswe/PS1ΔE9 mice prior to plaque deposition, these mice do have aberrant APP processing and increased levels of soluble Aβ at this age (Bonardi, de Pulford, Jennings, & Pardon, 2011; Min et al., 2010b; W. Zhang et al., 2012). Female APPswe/PS1ΔE9 mice exhibit increased levels of Aβ42 as well as oligomeric Aβ with onset of deposition around 4-5 months of age (Lazarov, Lee, Peterson, & Sisodia, 2002; Lazarov et al., 2005). We did not observe plaques in the brains of the mice in this study, but cannot exclude the possibility of an effect of soluble Aβ on CREB expression. Indeed, administration of oligomeric forms of Aβ to the Tg2576 mouse model of AD, results in aberrant PKA, ERK and CREB activation, suggesting that even in the absence of plaques, the increased oligomeric forms of Aβ that may be present at this age may contribute to impairments in the CREB signaling pathway (Gong et al., 2006; Ma et al.,

2007). In addition, aberrant APP processing may impact CREB signaling components independent of the effects on Aβ generation. For example, we observed increased levels of BACE1, which can negatively regulate CREB via PKA signaling (Y. M. Chen et al., 2012), provides an Aβ-independent, alternative mechanism for memory impairments in AD. Our observation that BACE1 is increased in these mice is in line with other reports of abnormal BACE1 in the APPswe/PS1ΔE9 mouse model (Li et al., 2014; Y. Wang et al., 2012). In addition, BACE1 activity is known to be increased in AD (Ahmed et al., 2010). Perhaps increased BACE1 activity may partially account for poor cognitive performance even in the presence of relatively mild neuropathology. While PKA expression was significantly reduced, difference in PKA activity was not statistically significant. However, it is not clear what extent of activity loss is required for a significant effect on the level of pCREB.

This series of experiments demonstrates that CREB signaling is impaired at multiple levels at an early stage of this mouse model of FAD, and identifies several potential targets for future therapeutic interventions.

2. Impairments in CREB signaling correspond to memory impairments in young adult APPswe/PS1ΔE9 mice

While the results of the previous set of experiments would suggest that memory impairments would be apparent at the same time as CREB signaling impairments, this phenomenon was not guaranteed, as other studies have failed to observe memory impairments in APPswe/PS1ΔE9 mice even as late as 6 months of age (Savonenko et al., 2005). One reason for this difference may be the use of males in their analysis, as cognitive decline occurs more slowly in males in this mouse model. Indeed, when the

analysis is confined to females memory deficits have been previously reported as early as 3.5 months of age in this mouse model (W. Zhang et al., 2012). The other important consideration is experimental design. It is important to note that we designed our experiments to replicate paradigms previously used to show memory deficits in CREB or CBP deficient mice (Barrett et al., 2011; Bourtchuladze et al., 1994; Valor et al., 2011). It has previously been suggested that the experimental design may have profound effects on behavioral outcomes, particularly in regard to CREB signaling (Kogan et al., 1997), and our targeted selection and design of the behavioral tasks used may also have contributed to detection of early impairments. Indeed, following the contextual fear conditioning experiments, we observed impairments in CREB signaling components that corresponded to the impaired memory performance in the APPswe/PS1ΔE9 mice. Contextual fear conditioning depends on the amygdala as well as the hippocampus. However, we observed that deficits in pCREB were mainly localized to the dorsal hippocampus, and no differences were detected in the amygdala of wild type and APPswe/PS1ΔE9 mice. In part to ensure that the deficits in learning and memory were not task specific, we utilized another experimental paradigm previously described to demonstrate memory impairments in CREB-signaling deficient mice, Novel Object Recognition, as well as EE. Taken together, these results lead us to conclude that the impairments in performance on learning tests result from deficient CREB signaling in memory pathways in the hippocampus.

We also generated a novel mouse model by crossing the CRE-reporter mouse with the FAD-linked APPswe/PS1ΔE9 mice. This allowed us to visualize CRE-driven gene transcription during the formation of long-term memories. While our previous

experiments indicated that CRE-mediated gene transcription might be impaired due to diminished CREB signaling components, this is the first time that impaired CRE-based gene transcription has been visualized in APPswe/PS1ΔE9 mice using this reporter. We performed NOR with these CRE-AD mice, and not only did we observe a lack of preference for the novel object in our CRE-AD mice, we also observed reduced CRE-driven gene transcription throughout the hippocampus immediately following the NOR task. These results profoundly demonstrate that impairments in CRE-driven gene expression are associated with (and may lead to) deficits in long-term memory formation.

Finally, we investigated the expression of the CRE-driven gene Egr-1 following contextual fear conditioning in order to confirm that a reduction in CRE-driven gene expression was having a functional impact on CRE-driven genes known to be important for learning and memory. We showed overlap between X-gal and Egr-1 staining in the CA regions of the hippocampus, although not every Egr-1+ cell was X-gal positive. A few explanations for this observation are possible. First, X-gal staining is dependent on the activity of the galactosidase enzyme, which begins to decay during tissue processing. Time of tissue processing was carefully controlled across groups, but the interval of processing may have resulted in some decay of enzymatic activity. Second, X-gal expression following stimulation is transient, and may be returning to baseline by the time the epitope for Egr-1 is fully expressed. However, the fact that we do see overlap between X-gal and Egr-1 staining further supports that the CRE-driven gene expression observed following learning results in the transcription of factors important for the formation of long-term memories. The observation that Egr-1 levels are not upregulated

in the hippocampus of APPswe/PS1ΔE9 mice following learning further supports our result that CRE-based gene transcription is impaired in these mice.

The results from these behavior experiments indicate that critical components of CREB signaling are reduced and nonresponsive to stimulus in APPswe/PS1ΔE9 mice, resulting in impaired transcription of genes important for long-term memory formation and leading to deficits in long-term memory.

B. Characterization of CREB signaling components in AD postmortem prefrontal cortex

1. CREB signaling components are diminished in the AD PFC.

In this study we have made several novel observations and suggest that they are mechanistically and diagnostically indicative of AD. First, we confirmed an earlier observation that Total CREB and pCREB are decreased in postmortem AD brain (Pugazhenthi et al., 2011; Yamamoto-Sasaki et al., 1999b). While transient activation of CREB is known to be critical for learning and memory formation, we have previously shown that, in a mouse model of familial Alzheimer's disease, CREB signaling is impaired both during learning and at a steady state, suggesting that CREB impairments in AD are not restricted to the active learning (N. Bartolotti, Segura, & Lazarov, 2015). For this reason, we interpret the postmortem data to be relevant to cognitive function in AD. We have also made the novel observation that in addition to the hippocampus, this deficit is also present in the prefrontal cortex. The PFC is also critical for cognitive functioning and learning and memory, and he PFC is known to be vulnerable in AD

(Salat et al., 2001; Shimamura, 1995; van Veluw et al., 2012b). Enhancing CREB phosphorylation in the brain through phosphodiesterase inhibitors has been shown to improve performance in PFC-dependent memory tasks in macaques (Rutten, Basile, Prickaerts, Blokland, & Vivian, 2008). In addition activation of the CRE-driven gene Egr-1 has also been observed in the macaque cortex during memory task (Okuno & Miyashita, 1996). Two papers have described reduced total and activated protein in aged hippocampus suggesting that the impairments are due to both reductions in total protein as well as activation (Pugazhenthi et al., 2011; Yamamoto-Sasaki et al., 1999a). These papers also describe a negative correlation between age and pCREB and Total CREB in the postmortem hippocampus, suggesting that the age-related impairments may be a combined result of reduced total protein as well as impaired phosphorylation

Furthermore, we are the first to report that the CREB transcription cofactors CBP and p300 are reduced in the postmortem AD brain. CBP recruitment following CREB activation is a critical event in facilitating the CRE-based gene transcription thought to underlie the formation of long-term memory, and enhancing CBP has been previously shown to rescue cognitive deficits in a mouse model of AD (Caccamo et al., 2010b; Lopez-Atalaya et al., 2011a). Dysregulation of CBP may contribute to the abnormal epigenetic regulation of immediate early genes in the PFC (Hendrickx et al., 2014). Our observation that CBP is reduced in AD PFC strengthens the evidence that CREB signaling is dysfunctional in AD, and offers another potential therapeutic target for the treatment of AD. Our observation that protein levels of p300 are reduced in AD PFC is intriguing in light of the fact that p300 has been hypothesized to be overactive in its function of acetylating tau in AD (Aubry et al., 2015; Min et al., 2015; Min et al., 2010a).

We cannot exclude the possibility that protein expression of p300 may not be indicative of its activity level, and future experiments will determine the nature of p300 dysfunction in AD. Therefore, our data suggest that one mechanism underlying cognitive impairment in AD may be reduced CREB signaling, and that dysfunctional CREB signaling may not be exclusively localized to the hippocampus in AD.

One open question is why CREB is impaired in AD. In the APPswe/PS1ΔE9 mouse model, deficits in CREB signaling could be linked back to mutations in APP or PS1. However, our observations here are made from patients with sporadic AD, for which there is no known genetic cause. While the cause of these deficits remains unknown, advanced age may contribute to this dysfunction (Kudo, Wati, Qiao, Arita, & Kanba, 2005; Porte, Buhot, & Mons, 2008). Age is the primary risk factor for AD, and some experiments have shown a decrease in total CREB protein and activation as a function of age in the postmortem hippocampus (Yamamoto-Sasaki et al., 1999b). While the mechanism underlying reduced CREB during aging is unclear, reactive oxygen species may partially play a role (Ozgen et al., 2009; Waldron & Rozengurt, 2000). In support of this idea, antioxidants have been shown to increase CREB phosphorylation via mitochondrial PKA, which is also thought to be important for learning and memory (Ryu, Lee, Impey, Ratan, & Ferrante, 2005) (Bevilaqua et al., 1999). However, our cognitively normal individuals were age-matched for the AD individuals, so age alone cannot explain this phenomenon. Therefore, more experiments are needed to determine the cause of CREB dysfunction in AD, particularly sporadic AD.

2. CREB signaling impairments in the AD PFC are not related to severity of pathological hallmarks.

We next asked if deficits in CREB signaling components were simply a function of advanced pathology. However, we did not observe a correlation between amyloid or tau pathology and pCREB expression in the PFC. This observation is important because it suggests that declining pCREB expression is not simply the result of increased AD-pathology. While it has been proposed that amyloid and tau may interact with CREB signaling components (Liu et al., 2015; Min et al., 2010b; Pugazhenthi et al., 2011; Z. H. Zhang et al., 2010), our experiments in a mouse model of AD suggest that CREB signaling components may be dysfunctional prior to the onset of amyloid deposition and neurofibrillary tangles, and therefore the cause of CREB dysfunction may be independent of theses lesions (N. Bartolotti et al., 2015). Similarly, enhancing CBP in a mouse model of AD is able to rescue cognitive function without affecting pathology (Caccamo et al., 2010b). Indeed, it is well known that the severity of AD pathology is poorly associated with cognitive function during life (Mesulam, 1999; Terry et al., 1991). Therefore, the fact that we do not observe a correlation between pathology and pCREB might mean that pCREB could be more indicative of cognitive function than severity of pathology.

These studies are important because they not only indicate that CREB signaling is dysfunctional in AD, and may underlie cognitive impairments, but that these impairments are not simply a function of advanced amyloid or plaque pathology, suggesting that CREB signaling components may be a better indicator of cognitive function than end state pathology scores.

C. Characterization of CREB signaling components in AD peripheral blood mononuclear cells.

1. CREB signaling components are impaired in AD PBMC

While it is useful to know that CREB signaling is impaired in postmortem AD tissue, it still remains unclear how CREB signaling changes with disease progression. In order to examine CREB signaling during disease progression we investigated the expression of CREB signaling components in PBMC. Here, we show that pCREB and CBP expression is significantly reduced in PBMC of AD patients. Examination of CREB expression in PBMC offers a non-invasive route for monitoring its signaling in the brain and thus perhaps cognitive functioning. We therefore asked if Total CREB, pCREB and CBP were also reduced in the PBMC of individuals with AD as they are in the PFC. We observed that levels of pCREB and CBP in the PBMC of individuals with AD were significantly reduced compared to the non-AD samples. This data suggests that the reduction in CREB and CBP expression may be tied to the onset of AD, rather than general cognitive impairments. We did observe high variability in our PBMC analysis. One explanation for this may be that many of the individuals in our PBMC analysis are still alive, and we can not rule out the possibility that the individuals in the normal or MCI groups may yet develop AD or other neurodegenerative disease. Future experiments should examine a time course of samples drawn from the same individual over time to determine if pCREB and CBP decline following the onset of AD. In addition, we observed a trending reduction in Total CREB expression in AD PBMC, as well as a trending reduction in the ratio of pCREB to Total CREB. These results suggest that the reduction in pCREB may be a result of both reduced levels of Total CREB and

impairments in phosphorylation. Additional experiments will be necessary to determine the cause of impaired phosphorylation in AD PBMC, and whether this mechanism is autonomous. One possible mechanism for the impairments in CREB phosphorylation is through kinase function. CREB in PBMC responds to many of the same kinases as neuronal CREB (for review (Kuo & Leiden, 1999)), suggesting the possibility of common regulatory pathways. For example, Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) has been shown to activate CREB in leukocytes via RSK (Mitton, Dutta, Hsu, & Sakamoto, 2014). Interestingly, peripheral administration of GM-CSF to a mouse model of AD has been shown to improve cognitive function (Boyd et al., 2010). Similarly, mice deficient in CaMKIV not only exhibit impairments in memory, but also exhibit impaired CREB phosphorylation in T cells (Anderson & Means, 2002; Takao et al., 2010). MSK has also been shown to activate CREB in T cells, and are thought to have an anti-inflammatory role in the cells of the immune system (Kaiser, Wiggin, Lightfoot, Arthur, & Macdonald, 2007; Reyskens & Arthur, 2016). Therefore, impairments in kinase function may be a possible mechanism underlying dysregulation of CREB phosphorylation in both blood and brain. In addition, we cannot exclude the possibility that brain-derived factors may play a role in CREB metabolism in PBMC.

2. Expression of pCREB in PBMC correlates to expression of pCREB in PFC.

One of the most important observations of this study is the positive correlation between pCREB expression in PFC samples and temporally-matched blood samples. This correlation was particularly strong in AD individuals. This analysis provides the first evidence that pCREB expression in PBMC may be correlated with pCREB expression in

the brain. Thus, it appears that impairments in pCREB in AD may be a long-lasting phenomenon. More experiments are warranted to determine the predictive power of pCREB as a blood biomarker for cognitive dysfunction in AD. We also analyzed the relationship of CBP expression in the PBMC and PFC. In these analyses we did not observe a significant relationship between PBMC and PFC CBP. We observed no correlation when we separated the analysis by diagnosis which suggests that CBP may be inversely regulated in the PBMC. Surprisingly, we also observed a no correlation when the normal and AD samples were combined. However, it is clear from our data that a great deal of variability exists in the CBP samples and therefore this phenomenon may be explained by sample size too low to account for the variability in the samples. Indeed, we did not observe a significant reduction in CBP in our primary analysis of normal PBMC compared to AD PBMC, and only observed a significant difference when we combined the normal and MCI individuals for higher power. Therefore, while it appears that a negative correlation exists between PFC CBP and PBMC CBP in the normal and AD groups when analyzed separately, the overall correlation may be too weakly powered to draw definitive correlations when analyzing across groups.

This series of experiments is a critical first step for the monitoring of CREB signaling in persons with AD. The correlation between blood and brain in particular offers the exciting possibility of a biomarker for cognitive function in AD, which would allow not only for the development of a diagnostic test for cognitive functioning in AD, but could also serve as a potential readout for the testing of new therapies aimed at improving cognitive function in AD.

VI. Conclusion and Future directions

This thesis has described several important experiments that elucidate the role of CREB signaling in AD. While previous studies in the field have investigated the role of CREB signaling in mouse models of FAD, the results from these studies have been contradictory and the nature of CREB signaling in mouse models of FAD remains unclear. This study contributes to the discussion of CREB signaling in FAD by carefully controlling the sex and age of the experimental mice, and designing the behavioral paradigms to specifically examine the contribution of CREB impairments to learning and memory. In addition, this study emphasizes the early nature of CREB impairments in the progression of FAD, indicating that these impairments may not simply be a result of advanced pathology, and may not be corrected by therapies targeting pathology. In addition, we have rigorously demonstrated the impairments at multiple levels of the CREB signaling cascade, which demonstrates that therapies targeting the CREB signaling pathway may need to target multiple aspects of the CREB signaling pathway to fully restore CRE-based gene transcription and rescue cognitive functioning.

This thesis also provides several important observations regarding CREB signaling in sporadic AD. We first expanded on previous observations that CREB is reduced in the postmortem AD hippocampus, by showing that CREB is reduced in the PFC as well. This suggests that CREB signaling deficits may be more widespread than previously thought, and contributes to the idea that the PFC may also be an important consideration for treatment of AD. We also expanded on previous literature by describing for the first time impairments in the transcription cofactors CBP and p300 in postmortem AD brain, indicating that, as in the mouse model, multiple aspects of the CREB signaling cascade may be dysfunctional in AD and require thoughtful therapeutic

interventions to comprehensively address the impairments in CREB signaling. In addition, the observation that CREB dysfunction is not simply a result of advanced pathology suggests that therapies targeting plaques and tangles may not be sufficient to restore CREB signaling and cognitive functioning.

Perhaps the most important contribution of this thesis is the observation that CREB signaling deficits are reflected in the periphery through the PBMC. This offers the possibility of monitoring CREB in the brain via CREB in the more easily accessible PBMC. Not only would this offer the potential to monitor disease progression, but would perhaps allow monitoring the efficacy of therapies aimed at enhancing cognitive functioning in AD through modulating CREB signaling.

Several future experiments could be initiated from these results. First, future experiments will need to determine the strength of the association between pCREB in the blood and brain, as well as the predictive power of pCREB in PBMC as a diagnostic for AD. In addition, methods for absolute quantification, rather than the relative description provided by the Western blot, of the amount of pCREB in the blood, will be necessary for practical clinical use of this data. Finally, while many therapies aimed at targeting CREB impairments have not been successful in mouse models of FAD, the data presented here suggests that sensitive and specific targeting multiple aspects of the CREB signaling may be beneficial for enhancing cognitive functioning. The development of novel molecules that enhance CRE-based gene transcription may offer the most direct path to the generation of a CREB based therapy for cognitive dysfunction in AD.

In summary, the goal of this study was to determine the molecular mechanism underlying cognitive impairments in AD. We presented novel information showing the

association of impairments in multiple aspects of the CREB signaling pathway with cognitive impairments as an early event in the progression of FAD. Similarly, we presented novel information regarding CREB signaling impairments in the postmortem AD brain, and introduced the possibility of CREB as a biomarker for cognitive functioning.

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Reynolds ND, Lukacs NW, **Long N**, & Karpus WJ (2011). Delta-like ligand 4 regulates central nervous system T cell accumulation during experimental autoimmune encephalomyelitis. *J Immunology*, 187, 2803-2813. doi:10.4049/jimmunol.1100160

Dogan RN, **Long N**, Forde E, Dennis K, Kohm AP, Miller SD, & Karpus WJ (2011). CCL22 regulates experimental autoimmune encephalomyelitis by controlling inflammatory macrophage accumulation and effector function. *J Leukoc Biol*, 89, 93-104. doi:10.1189/jlb.0810442

REVIEWS

Hollands C, **Bartolotti N**, Lazarov O (2016). Alzheimer's Disease and Hippocampal Adult Neurogenesis; Exploring Shared Mechanisms. *Frontiers in Neuroscience*, 10. doi: ARTN 178 10.3389/fnins.2016.00178

BOOK CHAPTERS

Bartolotti N, Lazarov O (2016). Lifestyle and Alzheimer's disease: The role of environmental factors in disease development. In *Genes, Environment and Alzheimer's disease*. Elsevier, Inc. 197-237.

Bartolotti N, Lazarov O (in press). Modulation of Hallmarks of Brain Aging by Environmental Enrichment. In *Inflammation, Oxidative Stress and Age-Related Disease*.

PROFESSIONAL PRESENTATIONS

Oral Presentations

Bartolotti N, Bennett DA, Lazarov O (2016, November). CREB signaling components as blood biomarkers for cognitive function in Alzheimer's disease. Talk presented at Society for Neuroscience 46th annual meeting 2016 Nanosymposium. San Diego, CA.

Bartolotti N, Bennett DA, Lazarov O (2016, September). CREB signaling components as blood biomarkers for cognitive function in Alzheimer's disease. Talk presented at UIC Anatomy and Cell Biology Graduate Student Seminar. Chicago, IL.

Bartolotti N, Bennett DA, Lazarov O (2016, April). CREB signaling as a biomarker for cognitive functioning in Alzheimer's disease. Talk presented at Association for Clinical and Translational Science annual meeting. Washington, DC.

Bartolotti N, Bennett DA, Lazarov O (2016, February). CREB as a biomarker for cognitive function in Alzheimer's disease. Talk presented at UIC Neuroscience Graduate Student Symposium, Chicago, IL.

Bartolotti N, Lazarov O (2015, February). Impaired CREB signaling may underlie learning and memory impairments in Alzheimer's disease. Talk presented at UIC Neuroscience Graduate Student Symposium. Chicago, IL.

Bartolotti N, Hu Y-S, Storm D, Lazarov O (2014, November). Cyclic-AMP Response Element Binding Protein (CREB) signaling is impaired in a mouse model of Alzheimer's disease. Talk presented at Society for Neuroscience 44th annual meeting 2014 Nanosymposium. Washington, DC.

Poster Presentations

Bartolotti N, Bennett DA, Lazarov O (2016, November). CREB signaling components as blood biomarkers for cognitive function in Alzheimer's disease. College of Medicine Research Forum, Chicago IL

Bartolotti N, Bennett DA, Lazarov O (2016, April). CREB signaling as a biomarker for cognitive functioning in Alzheimer's disease. Association for Clinical and Translational Science annual meeting. Washington, DC.

Bartolotti N, Segura L, Storm D, Bennett DA, Lazarov O (2016, January). CREB as a biomarker for cognitive function in Alzheimer's disease. Brain Research Foundation annual meeting. Chicago, IL.

Bartolotti N, Segura L, Storm D, Lazarov O (2015, October). Dysfunction of CREB signaling is associated with learning and memory impairments in a mouse model of Alzheimer's disease. Society for Neuroscience 45th annual meeting 2015. Chicago, IL.

Bartolotti N, Storm D, Lazarov O (2015, April). Impairments in CREB signaling in a mouse model of Alzheimer's disease. Association for Clinical and Translational Science annual meeting. Washington, D.C.

Bartolotti N, Storm D, Lazarov O (2015, March). Impairments in CREB signaling in a mouse model of Alzheimer's disease. Chicago chapter of Society for Neuroscience Annual meeting. Chicago, IL.

Bartolotti N, Hu Y-S, Storm D, Lazarov O. (2014 November). Cyclic-AMP Response Element Binding Protein signaling is impaired in a mouse model of Alzheimer's Disease. UIC College of Medicine Research Forum. Chicago, IL. (Winner of honorable mention in graduate student category)

Bartolotti N, Hu Y-S, Storm D, Lazarov O (2014, September). Cyclic-AMP Response Element Binding Protein signaling is impaired in a mouse model of Alzheimer's Disease. UIC Neuroscience Day. Chicago, IL

Bartolotti N, Hu Y-S, Pigino G, Brady ST, Lazarov O (2014, March). Environmental Enrichment enhances Akt/GSK3_ Neurotrophin-3 and CREB signaling pathways in wild-type mice, but not in a mouse model of Alzheimer's disease. Chicago chapter of Society for Neuroscience Annual meeting. Chicago, IL.

Bartolotti N, Hu YS, Pigino G, Brady ST, Lazarov O (2014, January). Environmental Enrichment enhances Akt/GSK3_ Neurotrophin-3 and CREB signaling pathways in wild-type mice, but not in a mouse model of Alzheimer's disease. Brain Research Foundation Neuroscience Day Abstracts. Northwestern University, Chicago, IL.

Bonds JA, Kuttner-Hirshler Y, **Long N**, Pizzi M, Lazarov O (2013, November). Presenilin-1 down-regulation in neural progenitor cells impairs learning and memory. Program No.766.12. Neuroscience 2013 Abstracts. San Diego, CA: Society for Neuroscience, 2013.

Munkacsy E, **Long N**, & Larson JR (2012, October). Dendritic compartmentalization of theta burst-induced associative LTP in hippocampal field CA1. Abstracts of the Society for Neuroscience 42nd Annual Meeting. New Orleans, LA.

Yoshino JE, **Long N**, Hoover B, Brown T, Francis D, Castel J, & Beaudett E (2009, March). The effect of melatonin on nitric oxide production in vivo and in vitro. Abstracts of the American Society for Neurochemistry 40th Annual Meeting. Charleston, SC.

RESEARCH EXPERIENCE

- Graduate student, University of Illinois at Chicago (Orly Lazarov lab), 2012-present. Cyclic AMP Response Element Binding (CREB) Protein as a therapeutic target for treating dementia in a mouse model of Alzheimer's disease
- Research rotation, University of Illinois at Chicago (John Larson lab), Spring 2012 Dendritic compartmentalization of theta burst-induced associative LTP in hippocampal field CA1
- Research rotation, University of Illinois at Chicago (Doug Feinstein lab), Winter 2011 The effect of dimethyl fumarate on GM-CSF production by stimulated T cells derived from patients with Multiple Sclerosis
- Research rotation, University of Illinois at Chicago (Orly Lazarov lab), Fall 2011 Alterations in neural stem cell lineages following treatment by a gamma-secretase inhibitor
- Full-time Research Technician, Northwestern University (William Karpus lab), 2009-2011
- Summer Research Assistant, Colgate University (Jun Yoshino lab), Summer 2007
- Research Assistant, Colgate University (Jun Yoshino lab), 2005-2009

AWARDS

- Sherman Fairchild research fellowship ($4,000), 2007
- Horatio Alger National Scholar Finalist ($1,000), 2005
    - Scholarship awarded for critical financial need and demonstrated integrity and perseverance in overcoming adversity
- Center for Clinical and Translational Science (CCTS) Predoctoral Education in Clinical and Translational Science (PECTS) Fellow (2014-2015)
    - One year full tuition and stipend, two years of $2,000 trainee related expenses
- Paul D. Doolen Graduate Award for the study of aging alternate winner, 2014
- Honorable mention in UIC College of Medicine Research Forum, 2014
- Paul D. Doolen Graduate Award for the study of aging alternate winner, 2015
- First place gold medal winner in UIC College of Medicine Research Forum, 2015
    - $500 award, $750 in research support
- Association for Clinical and Translational Science Burroughs Wellcome Fund Trainee Travel Award
    - $500 travel award

RESEARCH SKILLS

- Primary cell culture
- Cell line maintenance
- Flow Cytometry
- PCR
- Real-time PCR
- Tissue Harvest
- Tissue Sectioning
- ELISA Assays
- Western Blot
- Oral gavage
- Animal Care and Handling
- IP, SC Injections
- Peritoneal macrophage isolation
- Protein Assay
- DNA/RNA isolation
- Confocal microscopy
- EAE immunization, observation
- Histology and Immunohistochemistry
- Recording LTP from hippocampal slices
- Nitrite Assay

TEACHING EXPERIENCE

University of Illinois at Chicago

- Neuroanatomy Teaching Assistant - Spring 2014

Colgate University

- Brain and Behavior Tutor - Fall 2007
- German Tutor - Fall 2006

RELEVANT COURSEWORK

University of Illinois at Chicago - UIC Graduate College

- Human Neuroanatomy
- Biochemistry
- Molecular Biology
- Stem Cells
- Molecular and Cellular Mechanisms of Neurodegenerative Disease
- Receptor Pharmacology
- Cell Biology
- Integrative Biology
- Scientific Integrity and Responsible Research

Northwestern University - School of Continuing Studies

- Biochemistry
- Physics I, II, III

Colgate University

- Neuroanatomy
- Brain and Behavior
- Molecular Analysis
- General Chemistry I, II
- Organic Chemistry I
- Language and Cognition
- Neurochemistry/ Neuropharmacology
- Visual Perception and Cognition
- Molecules, Cells, and Genes
- Quantitative Methods of Behavioral Research
- Neurobiological Foundations of Personality