NORTHWESTERN UNIVERSITY

CREB Overexpression Ameliorates Age-related Behavioral and Biophysical Deficits

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

for the degree

DOCTOR OF PHILOSOPHY

Field of Neuroscience

By

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EVANSTON, ILLINOIS

March 2017

ABSTRACT

Age-related cognitive deficits are observed in both humans and animals. Yet, the molecular mechanisms underlying these deficits are not yet fully elucidated. In aged animals, a decrease in intrinsic excitability of pyramidal neurons from the CA1 sub-region of hippocampus is believed to contribute to age-related cognitive impairments, but the molecular mechanism(s) that modulate both these factors has yet to be identified. Increasing activity of the transcription factor cAMP response element-binding protein (CREB) in young adult rodents has been shown to facilitate cognition, and increase intrinsic excitability of their neurons. However, how CREB changes with age, and how that impacts cognition in aged animals, is not clear. Therefore, we first systematically characterized age- and training-related changes in CREB levels in dorsal hippocampus. At a remote time point after undergoing behavioral training, levels of total CREB and activated CREB (phosphorylated at S133, pCREB) were measured in both young and aged rats. We found that pCREB, but not total CREB was significantly reduced in dorsal CA1 of aged rats. Importantly, levels of pCREB were found to be positively correlated with short-term spatial memory in both young and aged rats i.e. higher pCREB in dorsal CA1 was associated with better spatial memory. These findings indicate that an age-related deficit in CREB activity may contribute to the development of age-related cognitive deficits. However, it was still unclear if increasing CREB activity would be sufficient to ameliorate age-related cognitive, and biophysical deficits. To address this question, we virally overexpressed CREB in CA1, where we found the age-related deficit. Young and aged rats received control or CREB virus, and underwent water maze training. While control aged animals exhibited deficits in long-term

spatial memory, aged animals with CREB overexpression performed at levels comparable to young animals. Concurrently, aged neurons overexpressing CREB had increased excitability. This indicates that overexpression of CREB was sufficient to rescue both the cognitive deficits, and the biophysical dysfunction normally seen in aged animals. Together, the results from this thesis identify CREB as a new mechanism underlying age-related cognitive deficits. This not only furthers our understanding of how cognitive processes change with age, but also suggests that increasing activity of CREB or its downstream transcription targets may be a novel therapeutic for the treatment of age-related cognitive decline.

Acknowledgements

I would like to thank my mentor, John Disterhoft for his guidance, support, and encouragement throughout my graduate career. I also thank Dr. Murali Prakriya, Dr. Jelena Radulovic, Dr. Robert Vassar, and Dr. Jerry Yin for generously serving on my thesis committee. Their advice and guidance was invaluable.

I thank former and current members of the lab, in particular, Dr. Matt Oh, Dr. Craig Weiss, Dr. Dan Curlik, Carmen Lin, Jenny Benson, Mary Kando, Dr. Shoai Hattori, Dr. Eugenie Suter, and Dr. Matthew Schroeder, for creating a supportive lab environment.

My girls - Eileen McIver, Meriel Owen, and Agnes Bao have been amazing pillars of support. What would I have done without our lunches, games, and dinners?

I thank Tom, and the Menagerie for coming on this crazy ride with me, for their unwavering support and love, and for believing in me even when I didn't.

Lastly, I thank my parents, who have always supported me to do whatever I wanted. This never would have been possible without your love and encouragement.

Table of Contents

List of Tables and Figures

Chapter 1 – General Introduction

Cognitive and biophysical deficits in aging

Like humans, laboratory animals display impairments in their ability to learn and remember as they age. However, there are currently no effective pharmacotherapies available to treat these deficits, as the underlying mechanisms are poorly understood. In rats, impairments are observed in tasks that utilize spatial memory, such as the Barnes circular maze and the Morris water maze, as well as tasks that require complex temporal processing, such as trace associative conditioning (Barnes, 1979; Foster, 1999; Knuttinen et al., 2001a; Thompson et al., 1996). Along with cognitive impairments, numerous studies have found that aged animals have neurons which are intrinsically less excitable than those from young animals. Specifically, pyramidal cells from area CA1 of dorsal hippocampus exhibit these changes (Disterhoft and Oh, 2007; 2006; Landfield et al., 1978; Landfield and Pitler, 1984; Oh et al., 2010). One measure of neuronal excitability is the post-burst AHP. This is a hyperpolarization that occurs after the cell has fired a train of action potentials. By bringing the cell further from action potential threshold, a larger AHP means the cell is less excitable. After an animal successfully learns a task, the AHPs of their CA1 pyramidal neurons are reduced – the cells are more excitable (Moyer et al., 2000; 1996; Oh et al., 2010). Thus, the reduced intrinsic excitability seen in aged animals is thought to be linked to their cognitive impairments.

Fortunately, numerous compounds have been found which can increase intrinsic excitability of CA1 pyramidal neurons. These compounds are very promising as when given *in*

vivo, they also improve performance on behavioral tasks. One example is the L-type calcium channel blocker nimodipine. When applied onto cells *in vitro*, both young and aged CA1 pyramidal cells show reduced AHP and spike-frequency accommodation - another measure of intrinsic excitability where more accommodation is fewer action potentials fired in response to a prolonged current step (Moyer et al., 1992). Furthermore, when nimodipine is given systemically, it increases firing in CA1, and facilitates aged animals' ability to learn trace eyeblink conditioning (Deyo et al., 1989; Moyer et al., 1992). Metrifonate, a cholinesterase inhibitor, is another example: it reduces AHP and accommodation in CA1 pyramidal cells *in vitro*, and improves acquisition of trace eyeblink conditioning when delivered *in vivo* for young and aged animals (Kronforst-Collins et al., 1997; Oh et al., 1999). Conversely, compounds which increase the AHP, such as NS309, result in a behavioral impairment when given *in vivo* to young adult animals (McKay et al., 2012). These findings suggest a very tight link between the intrinsic excitability of CA1 pyramidal neurons, and an animal's ability to learn and remember. Therefore, it is important to identify the underlying molecular pathways that are disrupted with age, and underlie these changes on both the biophysical and cognitive level.

CREB is required for the formation of new memories

Biophysical experiments have shown us that monoamine and learning-induced reductions in the AHP depend on the cAMP/PKA pathway (Oh et al., 2009; Pedarzani and Storm, 1995; 1993; Zhang et al., 2013). This same pathway also activates CREB, a transcription factor long known for its role in memory formation (Bernabeu et al., 1997; Dash et al., 1990; Deisseroth et al., 1998; Kaang et al., 1993). CREB is activated when it is phosphorylated at S133, it can then

dimerize and stimulate transcription of downstream target genes such as BDNF and c-fos (Alberini, 2009; Deisseroth et al., 1998; Impey et al., 1998; Kaang et al., 1993). CREB is often referred to as an integrator of various extracellular signals, which it then converts into changes in gene transcription (Fig. 1.1).

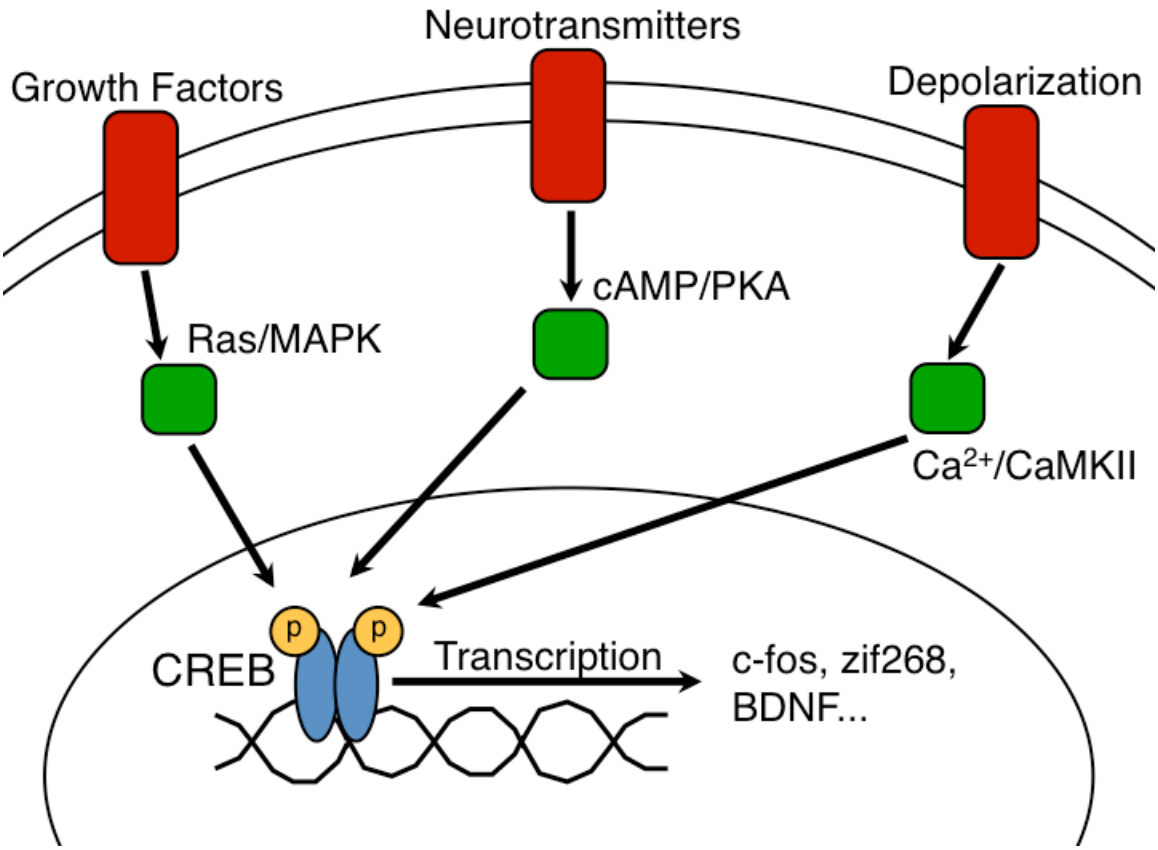


Figure 1.1. Simplified schematic of CREB signaling. CREB is often referred to as an integrator of extracellular signals, as several of them lead to the activation (via phosphorylation) of CREB. Examples used here are signaling by growth factors, neurotransmitters, and simple depolarization. These then activate their respective second messenger pathways and kinases, which lead to the phosphorylation of CREB. When CREB is phosphorylated and dimerized in the nucleus, it can then bind to target genes containing the consensus CRE sequence, and with the help of co-activators, stimulate their transcription. Example target genes include c-fos, zif268, and BDNF.

Studies preventing activity of CREB have shown just how important it is for memory formation. Dash et al. (1990) were the first to show that blocking CREB function impaired memory. When cAMP-responsive element (CRE) was injected into the nucleus of Aplysia sensory neurons, long-term facilitation normally induced by serotonin was blocked, while short-term facilitation was intact. This showed that by injecting exogenous CRE, CREB's normal binding site, CREB could no longer bind to CRE sites on DNA and did not give the downstream transcription needed for expression of long-term facilitation. Guzowski and McGaugh (1997) demonstrated a similar finding by using an antisense oligodeoxynucleotide against CREB in rats. CREB antisense oligodeoxynucleotides were injected into dorsal hippocampus and caused a 36% reduction in CREB protein levels. When rats were injected prior to training on the Morris water maze, acquisition of the task was not impaired, nor was short-term memory in a retention test carried out 2 hours later. However, a long-term memory test given 48 hours later showed an impairment only in the antisense-injected animals. This indicated that reducing CREB levels impaired long-term memory on a spatial task. A CREB knock-out mouse line was also generated (Bourtchuladze et al., 1994). When these mutants were trained in fear conditioning, they showed very little freezing to context or tone, both in tests of short- and long-term memory. When these mutants were trained on Morris water maze, they had slight impairments in acquisition, and showed no preference for the target quadrant during the probe trial. When long-term potentiation (LTP) experiments were conducted on slices made from these mice, it was found to be small in amplitude, and short-lasting. These results suggest that CREB knock-out results in impairments in memory as well as synaptic plasticity/neuronal excitability.

More temporally restricted blocking of CREB activity started with Yin et al. (1994). Here, a dominant negative form of CREB was temporally inducible in drosophila via heat-shock. Dominant negative CREB heterodimerizes with wild-type CREB to prevent it from binding to the DNA. Heat-shocked flies performed poorly on a long-term memory test after learning an olfactory association task while short-term memory was unimpaired. In the same vein, Pittenger et al. (2002) generated a transgenic mouse expressing KCREB, a dominant negative CREB which heterodimerizes with CREB, and related proteins CREM and ATF-1, reducing their affinity for DNA. These mice were impaired in both acquisition and retention of the Morris water maze task. Their object recognition memory was also impaired. Kida et al. (2002) took another approach by utilising a tamoxifen-sensitive estrogen receptor ligand binding domain. Tamoxifen induced expression of a mutated form of CREB which had an alanine at 133, making it impossible to activate (phosphorylate). When tamoxifen was injected prior to fear conditioning, mice were impaired in long-term memory for the context and the cue, while short-term memory was left intact. Injecting another group of mice with anisomycin gave the same results, indicating the CREB-blocking effects were due to prevention of downstream *de novo* protein synthesis which is required for long-term memory formation (Davis and Squire, 1984). These studies show that blocking CREB activity in different ways all result in impairments in memory, typically long-term. However, caution must be used when blocking CREB. When an inducible transgenic mouse expressed ACREB, a potent CREB activity blocker, neurons showed impaired LTP and reduced intrinsic excitability, were susceptible to seizures, and showed severe neurodegeneration (Jancic et al., 2009).

Increasing CREB facilitates cognition in young adult animals

Increasing CREB activity using exogenous compounds, or via transgenic and viral means has also been found to facilitate behavioral performance. When rats received systemic rolipram, a phosphodiesterase inhibitor, for 5 days, hippocampal levels of CREB and CREB phosphorylated at S133 (pCREB) were increased (Monti et al., 2005a). It also resulted in increased long-term memory for fear conditioning. Transgenic drosophila expressing a CREB activator exhibited long-term memory for olfactory memory when trained with a protocol that normally only produces short-term memory (J. C. J. Yin et al., 1995). Suzuki et al. (2011) generated transgenic mice which had increased affinity for either PKA, and would be more likely to be phosphorylated ($CREB^{Y134F}$), or for CREB-binding protein (CREB-DIEMDL), CREB's co-activator. These mice showed facilitation in long-term memory for social recognition, fear conditioning, Morris water maze, and passive avoidance, all the while showing no differences in short-term memory or learning. Another transgenic mouse line expressed constitutively-active VP-16 CREB (caCREB) in the amygdala (Viosca et al., 2009a). This resulted in strong contextual and cued fear conditioning, which even overcame the effects of anisomycin.

While transgenic animals are useful tools, since their gene expression is changed prior to birth, it can be difficult to directly implicate the genetic manipulation to the phenotypes seen. Thus, viral vector expression of genes has the advantage of using animals who develop normally. One example used the Sindbis virus to express $CREB^{Y134F}$ in CA1 hippocampal cells of mice. This resulted in an increased contextual fear memory (Restivo et al., 2009). In an elegant experiment using HSV to express wild-type CREB, Sekeres et al. (2010) demonstrated the

necessity and sufficiency of CREB for long-term memory. HSV-CREB was injected into either wild-type mice then given weak Morris water maze training, or CREB knock-out mice. In both cases, long-term spatial memory was observed. Josselyn et al. (2001) also produced long-term memory with a weak fear conditioning protocol (which normally only produces short-term memory), when HSV-CREB was injected into the amygdala of rats. A similar experiment showed HSV-CREB injections into hippocampus improved memory for water cross maze (Brightwell et al., 2007). Most recently, mice injected with HSV-CREB in the retrosplenial cortex also showed improved memory for water maze (Czajkowski et al., 2014). While these viral vector experiments do a great job of showing CREB's ability to improve long-term memory, the infection gained from these viruses are short-lived and restrict the types and number of behavioral testing that can be carried out. Adeno-associated viruses (AAVs), on the other hand, have been shown to stably express for more than 15 months (Klein et al., 2002; Mouravlev et al., 2006), so the work described in later chapters utilized AAVs. All the studies discussed so far have been conducted in young adult animals, only one has looked at the effect of increasing CREB in aged animals. Mouravlev et al. (2006) did a longitudinal preventative study using AAV-CREB. Injections in 8 week-old rats resulted in a two-fold increase of CREB protein in all regions of the hippocampus. Testing 12 week-old rats on Barnes circular maze and passive avoidance tasks showed no change in behavior between injected and control animals. Waiting until animals were 15 months-old showed better performance in both tasks, only in the injected animals. While this study shows increasing CREB can partly prevent age-related cognitive decline, my project aims to reverse the decline once it has already occurred, a more likely scenario for therapeutic intervention.

Given all the evidence for CREB's role in modulating behavior, it is no surprise that CREB phosphorylation is seen in response to training or learning on many different behavioral tasks. Both mice and rats have exhibited increased pCREB levels following training on inhibitory avoidance, contextual fear conditioning, cross-maze training, radial arm maze, and Morris water maze (Bernabeu et al., 1997; Colombo et al., 2003; Koutaro Kudo et al., 2005; Mizuno et al., 2002; Porte et al., 2008a; 2008b; Stanciu et al., 2001; Taubenfeld et al., 2001). In these studies, pCREB has been found to rise in a biphasic fashion: an initial increase occurs immediately after training/testing and is attributed to stress, anxiety, or simply from exposure to and doing the task. A later wave of pCREB, 15 min to 6 hr later, depending on the task, has been found to be correlated with how well the animal learned or performed on memory tests. Of interest, aged animals who fail to learn or remember a task have been shown to also not exhibit the learning-associated increase in pCREB levels (Koutaro Kudo et al., 2005; Monti et al., 2005b; Porte et al., 2008a). This inability to activate CREB may underlie their cognitive deficits.

How does CREB change with age?

While it has been shown that aged animals are not able to appropriately phosphorylate CREB in response to new stimuli, how CREB levels change with age is still unclear. Bach et al. (1999) were the first to demonstrate that aged mice may have deficits in CREB signaling. Aged mice displayed impaired performance on the Barnes circular maze performance and *in vitro* slices exhibited reduced LTP as compared to young counterparts. Both of these effects were rescued by compounds that activated the cAMP/PKA pathway. Concordantly, CREB was shown

to have lower binding to DNA in extracts from aged rat brains, this was also rescued by increasing cAMP levels (Asanuma et al., 1996). Interestingly, the only study looking at CREB mRNA levels in aged and young rats showed no change in the dorsal hippocampus (Calabrese et al., 2013). In a study correlating performance to basal levels of CREB and pCREB, young rats were trained on fear conditioning, left alone for 30 days, then sacrificed to examine pCREB and CREB levels (Cowansage et al., 2013). Their pCREB/CREB ratio correlated well with performance on the memory test. The story in basal CREB levels in aged animals is far from clear. Foster et al. (2001) used western blotting to show that total CREB levels are not changed, but that pCREB is decreased in hippocampus of naïve aged Fischer 344 rats. However, this result only applies to the cytosolic fraction of their cellular extracts, as the nuclear fraction was excluded from this analysis. Brightwell et al. (2004) found a different result: total CREB levels were found to be decreased in aged Long-Evans rats, specifically those who were impaired in learning Morris water maze. One caveat of this finding is that animals were sacrificed just 1 hour after the last retention test, so these data may be influenced by the animals' performing the task. Monti et al. (2005b) showed a different result again; total CREB was unchanged, but pCREB was increased in aged Wistar rats. These studies used different rat strains and different antibodies for CREB, possibly leading to the different findings. Additionally, these studies used protein extracts from the entire hippocampus. Given that dorsal and ventral hippocampus carry out different functions, as well as do the major sub-regions (CA1, CA3, and DG), it's likely that combining all these regions masked subtle differences that may have been present. One study did examine pCREB levels with age using immunohistochemistry. Levels of pCREB were found to drop by middle age, and further decrease into old age (Hattiangady et al., 2005). All

hippocampal sub-regions showed a decrease in pCREB: CA1 by ~25%, CA3 by ~25%, and DG by ~28%. Unfortunately, the antibody used was not specific only to pCREB, it also recognised pCREM, and pATF-1, meaning the results cannot be attributed purely to changes to pCREB. Additionally, only looking at pCREB levels do not tell us if the ratio of pCREB/CREB changed, or if total levels of CREB were reduced. Chapter 2 of this dissertation characterizes how CREB and pCREB levels change with age using western blotting, and determines how these levels relate to cognitive abilities of the rats.

CREB also modulates excitability in young adult animals

While CREB's ability to modulate performance on behavioral tasks is unsurprising, recent studies have shown that CREB is also able to modulate intrinsic neuronal excitability. Expression of caCREB has resulted in increased excitability in many brain areas: CA1 of hippocampus, locus ceruleus, nucleus accumbens, and amygdala (Dong et al., 2006; M.-H. Han et al., 2006; Lopez de Armentia et al., 2007; Viosca et al., 2009b; Zhou et al., 2009). This indicates there is a general mechanism activated by CREB, which works in a universal, non-cell specific manner. By expressing dominant negative forms of CREB, excitability can also be reduced (Dong et al., 2006; M.-H. Han et al., 2006; Jancic et al., 2009). This indicates that the modulation is bidirectional. Further evidence also suggests that expression of wild-type CREB is sufficient to give rise to these changes (Yiu et al., 2011; Zhou et al., 2009).

CREB's ability to augment both neuronal excitability and behavioral performance makes it an ideal candidate for rescuing age-related cognitive deficits. There is evidence that CREB

levels and/or activity are reduced with age. Therefore, the following chapters describe how CREB levels change with age, how these levels are related to the animals' cognition, and how increasing CREB levels ameliorate age-related cognitive deficits. Together, the results suggest that a reduction in CREB activation may underlie age-related cognitive deficits, and increasing CREB may be a new therapeutic target to treat these deficits.

Chapter 2 – Young and aged pCREB levels in CA1, but not CA3 or dentate gyrus, correlate with spatial memory

ABSTRACT

Age-related cognitive deficits are observed in both humans and animals. Yet, the molecular mechanisms that underlie these deficits, or more importantly, prevent them, remain to be identified. Here we examined age- and training-related changes to cAMP response element binding protein (CREB) levels in the dorsal hippocampus. We found that activated (pCREB), but not total CREB, was significantly reduced in dorsal CA1 of aged rats. Importantly, we observed that CA1 pCREB, measured 2 weeks after behavioral testing, positively correlated with spatial memory in both young and aged animals, i.e. higher pCREB in dorsal CA1 was associated with better spatial memory. Of note, we also observed a long-lasting change in CREB levels, which exceeds the duration of biophysical changes observed after learning. Together these findings suggest that the deficit in CREB signaling is a molecular mechanism that underlies age-related cognitive deficits. Furthermore, they indicate that the CREB pathway is a new therapeutic target for preventing and/or ameliorating aging-related cognitive deficits.

INTRODUCTION

Age-related cognitive impairments are observed across multiple species, including laboratory rodents and humans. Specifically, forms of learning that require an intact hippocampal formation, such as spatial navigation, are severely impacted in aged humans (Etchamendy et al.,

2012), rats (Gallagher and Nicolle, 1993), and mice (Bach et al., 1999). For therapeutic purposes, it is crucial to identify the molecular changes that underlie age-related cognitive deficits. Since many forms of learning that are impaired during aging require an intact hippocampal formation, much research has focused on the hippocampus to identify the molecular mechanisms that mediate these impairments.

Numerous studies have revealed that the hippocampus is sensitive to age-related changes (Blalock et al., 2011; Foster, 2007; Josselyn et al., 2015). For example, pyramidal neurons in the CA1 sub-region of the hippocampus display age-related reductions in cellular excitability, which correlate with age-related cognitive deficits; i.e. aged cognitively-impaired animals have less excitable CA1 pyramidal neurons than aged cognitively-unimpaired animals (Disterhoft and Oh, 2006; Foster, 1999; Landfield et al., 1978; Landfield and Pitler, 1984). Of great interest, recent experiments in young rodents have shown that activating CREB or overexpressing it can lead to increased neuronal excitability, and can also facilitate behavioral performance (Brightwell et al., 2007; Josselyn et al., 2001; Lopez de Armentia et al., 2007; Yiu et al., 2011). This suggests that a reduction in CREB levels and/or activation may underlie the age-related decrease in neuronal excitability, and age-related cognitive deficits. Unfortunately, few studies have examined this hypothesis, and results of these studies are conflicted, reporting that CREB activity increases, decreases, or remains unchanged in aged hippocampus (Brightwell et al., 2004; Foster et al., 2001; Monti et al., 2005b; Ramos et al., 2003). In order to determine if age-related changes to CREB activity in the hippocampus relate to age-related cognitive deficits, we systematically characterized how levels of total and activated CREB within the major hippocampal sub-regions

change with aging, and how this CREB activity relates to behavioral performance in young and aged rats. We hypothesized that total CREB and/or activated CREB would be reduced in the aged hippocampus. Moreover, we hypothesized that total and/or activated CREB levels would positively correlate with performance on hippocampal-dependent tasks.

MATERIALS AND METHODS

Subjects

Thirty young adult (3-6 months old, mo) and 28 aged (29-31 mo) male F1 hybrid Fischer 344XBrown Norway (F344XBN) rats were used for the current study. All animals were obtained from the National Institute on Aging colony at Charles River Laboratories (Raleigh, NC). All rats were housed in a temperature-controlled facility with a 14 hr light/10hr dark cycle and allowed free access to food and water. They were group housed and allowed to acclimate in the Northwestern University vivarium for a minimum of one week prior to the any experimentation. All procedures were conducted according to protocols approved by Northwestern IACUC following NIH guidelines. Fourteen young and 12 aged animals were first trained on a massed Morris water maze protocol. After water maze training, a subset of the animals (5 young and 8 aged) received trace fear conditioning. All other animals remained behaviorally-naïve.

Morris Water Maze

We assessed spatial learning and memory using a massed Morris water maze task (Guidi et al., 2014). Training and testing were conducted in a circular pool (180 cm diameter). The water (25 ± 1 °C) was made opaque by the addition of white non-toxic paint. Rats were first

trained using a visible platform to ensure they were capable of locating, navigating to, and climbing onto the escape platform. The escape platform (20.3 cm x 25.4 cm) was placed in 1 of 4 locations, in the centre of one quadrant of the pool, so that the bottom of the platform was at the top of the water level. The visible platform had several visual cues attached to it, and no distinctive cues surrounded the pool. During each trial, animals were placed approximately 7.5 cm away from the wall of the pool, in the centre of a quadrant. The starting quadrant was randomly assigned to be one of the three quadrants that did not contain the platform. Each animal was given 60 s to locate the visible platform. If the platform was not located after 60 s, the experimenter gently guided the animal to the platform. Each animal received three trials per block of training, after which the platform location changed. Visible platform training consisted of 5 blocks with 3 trials each, for a total of 15 trials. This visible platform testing ensured that rats tested had no sensorimotor deficits that prevented them from swimming, seeing cues, and climbing onto the escape platform. All animals successfully located the escape platform during the last trial of visible platform training, and no animals were excluded from further water maze training.

Three days following visible platform training, all animals were trained with a hidden platform procedure. Distinctive, high-contrast cues were placed on each of the walls surrounding the water maze pool. The escape platform (20 cm diameter) was placed in one quadrant submerged 1 cm below the water level. The location of the hidden platform did not change throughout hidden platform training. Each animal received 3 trials of training per block, over 5 blocks (total 15 trials). Each trial began with the animal being placed in one of the three

quadrants that did not contain the hidden platform, facing the wall of the pool. Each animal was given 60 s to locate the escape platform. If an animal failed to swim to the hidden platform after 60 s, the experimenter gently guided the animal to the platform. Upon reaching the platform, animals remained on the platform for a further 30 s, before being removed by the experimenter. Animals' cumulative proximity to the platform was measured for each training trial, then the average calculated for each block of 3 trials, giving 5 blocks. Cumulative proximity was chosen as it has been shown to be sensitive to age-related cognitive impairments (Gallagher et al., 1993).

To test for memory of the platform location, we performed probe trials 1 hour, 1 day, and 6 days after the end of hidden platform training. The escape platform was removed from the pool for the duration of the probe trial where each rat was given 60 s to swim in the pool. The percent time animals spent in the quadrant of the pool that the hidden platform was during training was measured for each probe trial. Similar to the cumulative proximity measure, percent time in target quadrant has been shown to be sensitive to age-related memory impairments (Foster, 2012). WaterMaze software was used for computerized tracking during trials and for subsequent offline analysis (Actimetrics, Wilmette, IL).

Trace Fear Conditioning

At least 3 weeks after the end of Morris water maze training and testing, a subset of rats were trained with a trace fear conditioning task. Training occurred in context A, which consisted of transparent perspex training boxes with a removable shock grid lined with stainless steel floor

bars. The boxes in context A were cleaned with Clydox. Animals were placed into the box and allowed a 180 s baseline period to explore the environment, which was followed by 5 pairings of the conditioned stimulus (15 s, 85 dB white noise) and the unconditioned stimulus (1 s, 1 mA footshock), which were separated by a 30 s trace interval. These pairings were presented over the course of 30 min, with an average inter-trial interval of 312 ± 30 s. The percent time animals spent immobile during the 30 s trace period on each trial was assessed.

Contextual memory was tested 24 hours after training by returning the rats to context A for 10 min in the absence of the tone or the shock. The percent of time spent immobile during the full 10 min test was used as a measure of contextual memory. Cued memory (i.e. immobility in response to the CS) was tested 48 hours after training, in a novel context B, which was changed from the original context A in the following manner. The transparent walls were covered, the cage was illuminated with only red light, cleaned with ethanol, and the shock grid was removed and replaced with cage bedding, Rats were placed into context B, given 180 s baseline, then presented with the conditioned stimulus 3 times. All rats then underwent extinction training where they received up to 4 more sessions (in the two days following cued testing) in context B with 3 presentations of the conditioned stimulus, as described above. Immobility during what would have been each trace period was measured. All reported immobility data are normalized to the initial baseline period for that session. FreezeFrame software was used for computerized tracking during acquisition, and subsequent offline analysis (Actimetrics).

Western Blotting

Two weeks after the end of behavioral testing, rats were euthanized to collect hippocampal tissue. After anaesthetizing the animals with isoflurane, they were rapidly decapitated, their brains were removed, and placed into ice-cold artificial cerebrospinal fluid (in mM): 124 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 25 glucose, 2.4 $CaCl_2$, pH 7.4, oxygenated with 95%:5% O_2:CO_2. Hippocampi were then extracted and immediately frozen on dry ice. While frozen and on ice, transverse sections were manually made from dorsal hippocampus, and the three hippocampal sub-regions (CA1, CA3, and DG) were manually dissected under a Stemi DV4 dissecting microscope (Zeiss, CA). Tissue from each sub-region was lysed in RIPA buffer (150 mM NaCl, 25 mM Tris, 1 mM EDTA, 0.1% SDS, 1 % Triton X-100, and 0.5 % sodium deoxycholate) containing protease and phosphatase inhibitors (Pierce, Rockford, IL). Each sample was manually dissociated with a syringe, then shaken at 4 °C for 30 min. All samples were then centrifuged at 14,000 g for 15 min. The supernatants were retained and protein concentration measured by BCA assay (Pierce). Fifteen micrograms of each sample was boiled in 2x Laemmli buffer containing 5 % β-mercaptoethanol (Bio-Rad Hercules, CA). Samples were separated using 4-20 % Mini Protean TGX gels (Bio-Rad), and transferred to Immobilin-P PVDF membranes (Millipore, Temecula, CA). Blots were first probed for pS133CREB (#9189, 1:1,000, Cell Signaling, Beverly, MA), and detected using an anti-rabbit secondary (Jackson Immunoresearch, West Grove, PA). The blots were then stripped using Restore Western Blot Stripping Buffer (Pierce), and re-probed with primary antibodies against CREB (#04-767, 1:5000, Millipore) and GAPDH (#MA1-16757, 1:40000, Thermo Scientific, Waltman, CA). Anti-rabbit secondary was first used to react with the anti-CREB antibody. After imaging, HRP signal was quenched using 15 % H_2O_2. An anti-mouse secondary (Jackson) was

then used to react with the anti-GAPDH primary. Immunoreactive bands were visualized using a ChemiDoc XRS+ Molecular Imager System with ImageLab Software (Bio-Rad). Offline quantification of reactive bands also used ImageLab software. All gels were loaded with the same reference sample to allow for comparison across gels. All samples were normalized for loading error using GAPDH signal.

Statistics

Water maze acquisition data were analyzed using a two-way, repeated measures analysis of variance. Two-way analysis of variance was used to compare protein levels between trained and untrained, young and aged animals. All other comparisons for behavioral performance and protein levels were performed using unpaired student t-tests when data was normally distributed, and Mann Whitney test when they were not. Pearson's R values were determined to test for strength of correlations between protein levels and behavioral performance. All data are reported as mean ± SEM. All statistical tests were run using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

RESULTS

Aged animals are impaired in acquisition of water maze

As previously reported, the spatial water maze is a well-characterized, hippocampal-dependent task that is sensitive to age-related cognitive deficits. (Gallagher et al., 1993). Therefore, to asses age-related, hippocampal-dependent, learning and memory impairments, we

trained young adult and aged rats with the massed Morris water maze task (Guidi et al., 2014). Additionally, a subset of these animals was then trained with trace fear conditioning.

Prior to hidden platform water maze training, all rats were tested on the visible platform version of this task. Both young and aged animals decreased their cumulative proximity to the visible platform across the 5 blocks of visible platform training ($F_{4, 96} = 49.62$, $p < 0.001$, inset, Fig. 2.1A). However, young rats acquired this task more quickly than aged rats ($F_{1, 24} = 106.60$, $p < 0.001$). With that said, young and aged rats performed comparably on the third, fourth, and fifth blocks of visible platform training. Together, these results indicate that both the young and aged groups were able to reliably locate, and navigate to the visible platform by the end of visible platform training. Therefore, no rats were excluded from further testing. Hidden platform training commenced three days after visible platform testing.

During water maze training, all animals were trained with five blocks of hidden platform training over the course of one day, with three trials per block. Performance during hidden platform training was assessed by measuring cumulative proximity from the platform during each trial (Gallagher et al., 1993). Repeated measures two-way analysis of variance, with block as the repeated measure, and age as the between groups measure revealed significant main effects of block ($F_{4, 92} = 34.02$, $p < 0.001$), age ($F_{1, 23} = 27.91$, $p < 0.001$), and interaction between block and age ($F_{4, 92} = 2.547$, $p = 0.045$), indicating that aged animals were impaired during acquisition of the hidden platform water maze task. Post-hoc tests using Sidak's correction for multiple comparisons indicated that aged animals had significantly larger cumulative proximities

during the first two blocks of training (Blocks 1 and 2 $p < 0.001$, Fig. 2.1A). However, there was no difference in cumulative proximity between young and aged rats on the last three blocks of training (Blocks 3, 4, and 5, $p > 0.05$, Fig. 2.1A). These results are consistent with those from previous studies revealing that while aged animals tend to be slower to learn than their young counterparts, given enough trials, they can perform at the same level (Beatty et al., 1985; Gallagher et al., 1993; Gallagher and Pelleymounter, 1988; Matzel et al., 2009).

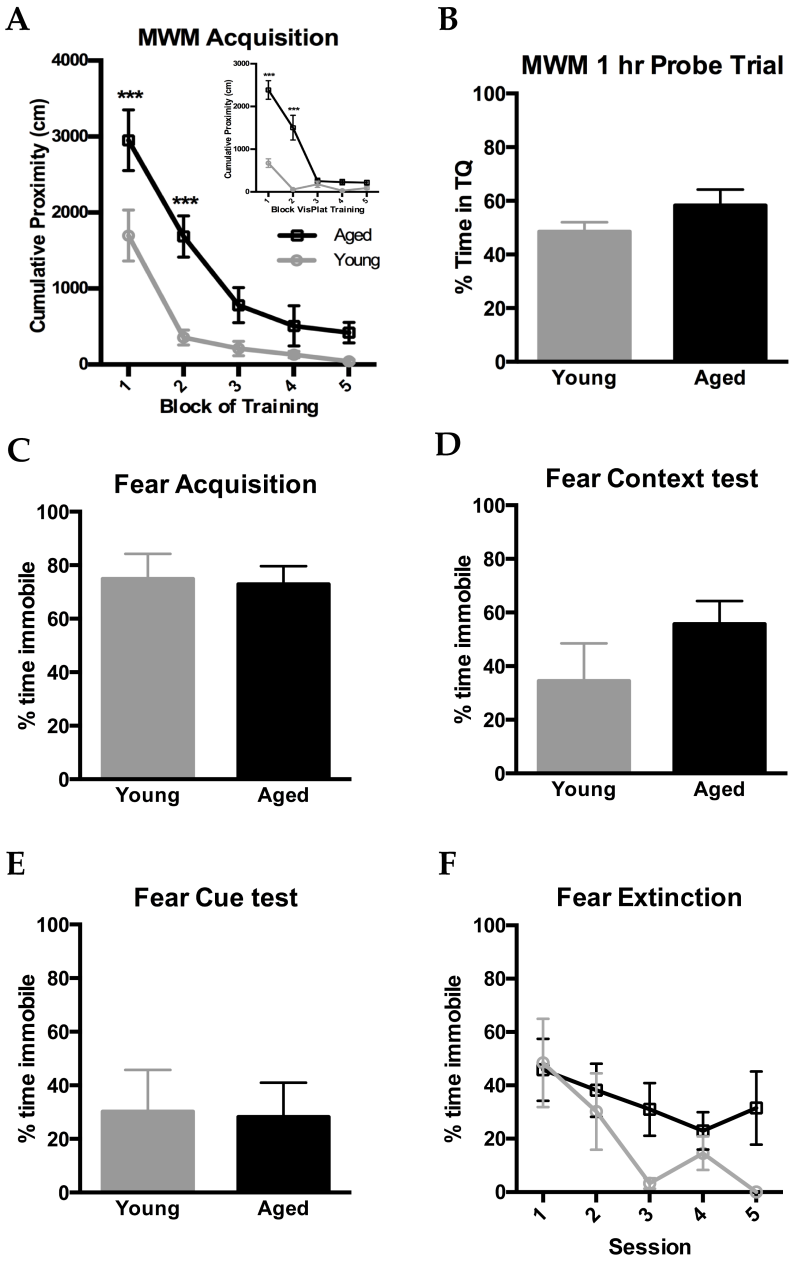


Figure 2.1. Aged animals were impaired during acquisition of Morris water maze and extinction of Fear memory. A. Learning curves of young (grey, n = 14), and aged (black, n = 11) rats. Aged rats were impaired during blocks 1 and 2. Inset: learning curves for visible platform training. Young and aged rats performed comparably during the end of visible platform training. B. Young and aged rats performed comparably during a probe trial conducted 1 hr after the last block of hidden platform training. C. Young (n = 5) and aged (n = 8) rats acquired the task comparably, and spent similar amounts of time immobile during the last trial of acquisition, D. contextual testing, and E. cued testing. F. Aged animals were found to be impaired on extinction of their cued memory.

We assessed spatial memory by conducting probe trials 1 hr, 1 day, and 6 days after the last session of hidden platform training. The percent time spent in the target quadrant was used as a measure of memory for the platform location. There was no difference in percent time spent in the target quadrant between young and aged rats during the 1 hr probe test (young = 48.5 ± 3.5 % time, aged = 58.3 ± 5.9 % time, $t_{23} = 1.50$, $p = 0.15$, Fig. 2.1B). We also found no age-related difference during the 1 day probe test (young = 39.5 ± 5.27 % time, aged = 34.3 ± 6.80 % time, $t_{23} = 0.062$, $p = 0.54$) or 6 days probe test (young = 32.4 ± 7.0%, aged = 31.8 ± 6.80%, $t_{23} = 0.056$, $p = 0.96$). While it was surprising to not observe an age-related spatial memory deficit, these results are consistent with previous studies reporting that aged and young animals perform comparably on probe trials if they are trained to the same level (Matzel et al., 2009).

We also tested for an age-related cognitive deficit in trace fear conditioning. Surprisingly, contrary to previously published results (Moyer and Brown, 2006), we observed no age-related impairments during trace fear conditioning. Young and aged rats spent a comparable amount of time immobile during the last trial of acquisition (young = 74.9 ± 9.3 %, aged = 72.9 ± 6.7 %, $t_{11} = 0.18$, $p = 0.86$, Fig. 2.1C). There was also no difference in the percent of time spent immobile between young and aged animals during a contextual memory test 24 hours after training (young = 34.5 ± 14.0 %, aged = 55.7 ± 8.6 %, $t_{11} = 1.37$, $p = 0.20$, Fig. 2.1D), and during a cued retention test 24 hours after contextual testing (young = 30.2 ± 15.6 %, aged = 28.2 ± 12.8 %, $t_{11} = 0.098$, $p = 0.92$, Fig. 2.1E). While it was surprising to not observe age-related deficits in either acquisition of trace fear conditioning, or retention of memory for context or cue, we did find that the aged animals were impaired on extinction for their cued memory. A two-way, repeated

measures ANOVA revealed a significant main effect of extinction session (Fig. 2.1F, $F_{4, 51}$ = 3.15, p = 0.022), and a main effect of age ($F_{1, 51}$ = 4.37, p = 0.042), indicating that while all animals improved with more sessions, aged animals did not improve as much as young did. In fact, by the last extinction session, while all young animals showed 0% immobility, aged animals were immobile 31.52 ± 13.70 % of the time.

While relationships between behavior and CREB levels immediately after behavioral training and testing have previously been studied with aging (Brightwell et al., 2004), it's unclear whether CREB or pCREB levels at a more distant time point would still have any predictive value on an animal's behavioral performance. Therefore, two weeks after the end of behavioral testing, a time point at which learning-induced changes on the electrophysiological level have returned to baseline (Moyer et al., 1996; Thompson et al., 1996; Tombaugh et al., 2005), we euthanized the rats to collect tissue for western blots.

Hippocampal CREB and pCREB levels change with age

We used western blotting on the trained animals to identify how their CREB and pCREB levels change with age in the three major hippocampal sub-regions. We found no age-related difference in CREB levels in CA1 (young = 100.0 ± 8.8 %, aged = 108.8 ± 19.5 %, t_{24} = 0.43, p = 0.67, Fig. 2.2A). However, aged animals had substantially less pCREB in CA1 (young = 100.0 ± 21.7 %, age = 25.8 ± 4.7 %, t_{24} = 3.11, p = 0.005). This indicates that there was a drastic reduction in the pCREB/CREB ratio in CA1 of aged animals. CA3 showed a significant age-related decrease in CREB levels (young = 100.0 ± 12.9 %, aged = 47.0 ± 6.4 %, U_{24} = 22, p <

0.001, Fig. 2.2B), and a concurrent trend towards less pCREB (young = 100.0 ± 21.7 %, aged 50.23 ± 9.3 %, U_{24} = 47, p = 0.059). Finally, we found an age-related decrease in CREB (young = 100.0 ± 10.8 %, aged = 71.8 ± 9.3 %, U_{24} = 45, p = 0.046, Fig. 2.2C) and pCREB (young = 100.0 ± 20.0 %, aged = 60.9 ± 12.4 %, U_{24} = 44, p =0.040) in the dentate gyrus. This demonstrates that the decrease in pCREB in CA3 and DG can be attributed to the decrease in total CREB i.e. no change in pCREB/CREB ratio. Moreover, these results indicate that CA1 undergoes a mechanistically different change with aging that specifically results in a decreased pCREB/CREB ratio. These results point to the changes in CA1 as more functionally significant, however, the decreases in CA3 and DG could also lead to functional deficits due to a drop in absolute amount of pCREB.

In order to determine whether the amount of CREB or pCREB levels in the three major hippocampal sub-regions were related to cognitive performance in young or aged animals, we ran correlations between their behavioral performance and CREB or pCREB levels.

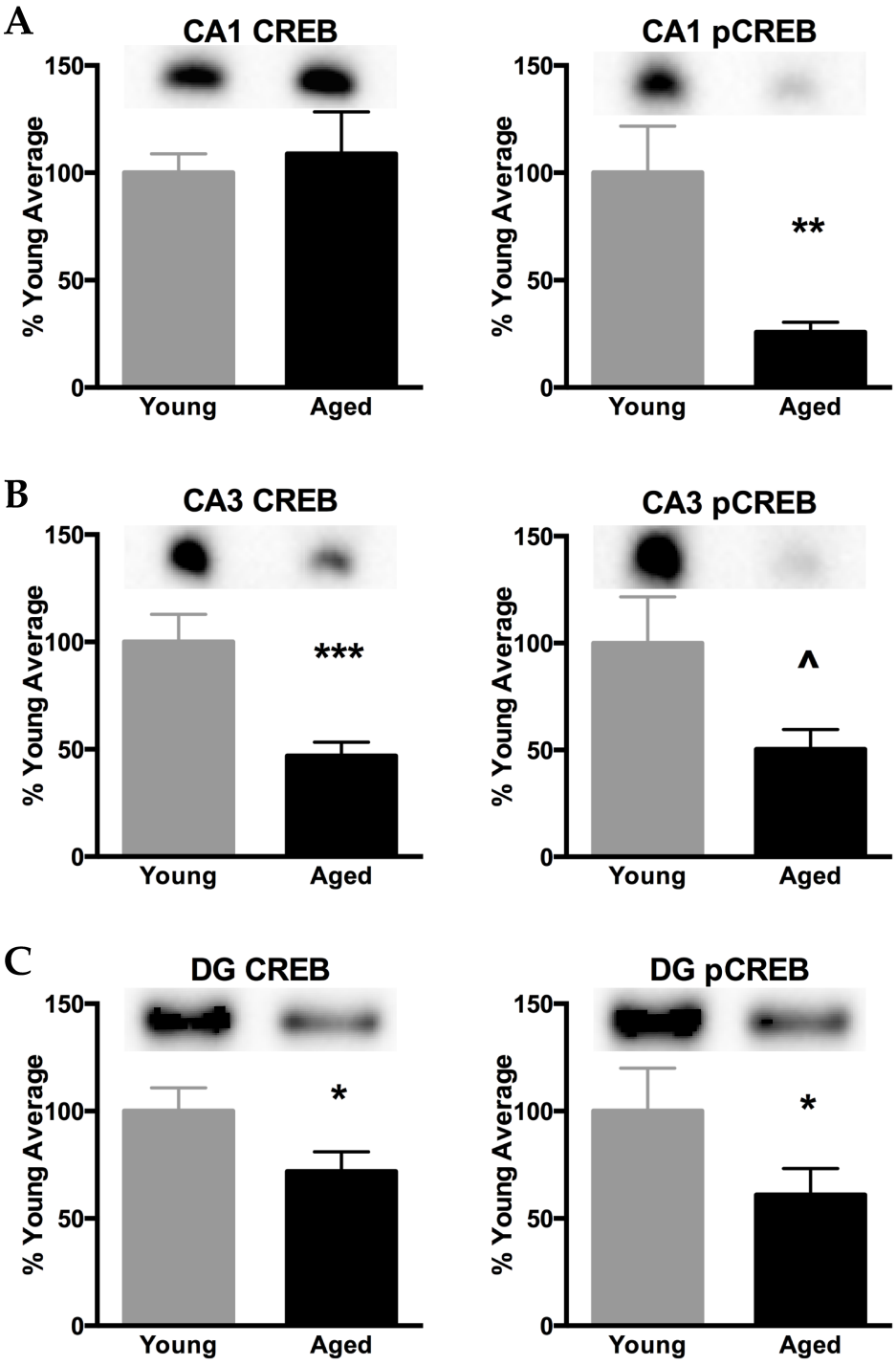


Figure 2.2. Aged rats have less pCREB in all three major hippocampal sub-regions. Aged animals also have less total CREB in CA3 and DG. Total and phosphorylated CREB levels in regions A. CA1, B. CA3, and C. DG. Bars represent mean ± SEM, normalized to the young average, and representative of 3 independent experiments. Representative bands from each group are shown, ^ $p = 0.06$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

pCREB levels correlate with spatial memory in young and aged rats

Based on previous studies in young animals reporting that increasing CREB signaling can facilitate memory for a spatial task (Brightwell et al., 2007), while knocking CREB out can impair both learning and memory (Bourtchuladze et al., 1994), we hypothesized that animals with higher CREB or pCREB would perform better on the spatial water maze task. Furthermore, while many studies have already explored levels of CREB and its activation immediately following training or learning, we wanted to characterize CREB levels two weeks after the end of behavioral testing to determine whether these levels were related to an animal's cognitive ability.

We found that pCREB levels in CA1 were significantly correlated with performance on the 1 hr water maze probe trial, in both young ($r = 0.55$, $p = 0.040$) and aged ($r = 0.66$, $p = 0.028$) animals (Fig. 2.3A); i.e. animals with the greatest amount of pCREB in dorsal CA1 spent the most time in the target quadrant during testing. Interestingly, this relationship between pCREB and spatial memory was only observed in area CA1. No such correlation was found in CA3 (young $r = 0.27$, $p = 0.35$, aged $r = 0.07$, $p = 0.84$, Fig. 2.3B) or the dentate gyrus (young $r = 0.29$, $p = 0.32$, aged $r = 0.21$, $p = 0.53$, Fig 2.3C). Moreover, no correlations were observed between CREB or pCREB levels and performance on later probe trials.

Additionally, no relationships between pCREB levels and performance during fear training, testing, or extinction were observed across any of the three hippocampal sub-regions. Together, these results indicate that pCREB levels in area CA1, but not area CA3 or the dentate

gyrus, is important for short-term memory of a spatial task. This suggests, that similar to age-related changes in neuronal excitability (Disterhoft and Oh, 2007), deficits in CA1 have crucial implication on behavioral performance. This could in part be due to CA1 being the major output of the hippocampus, or perhaps because CA1 is crucial for the formation or retrieval of spatial memory.

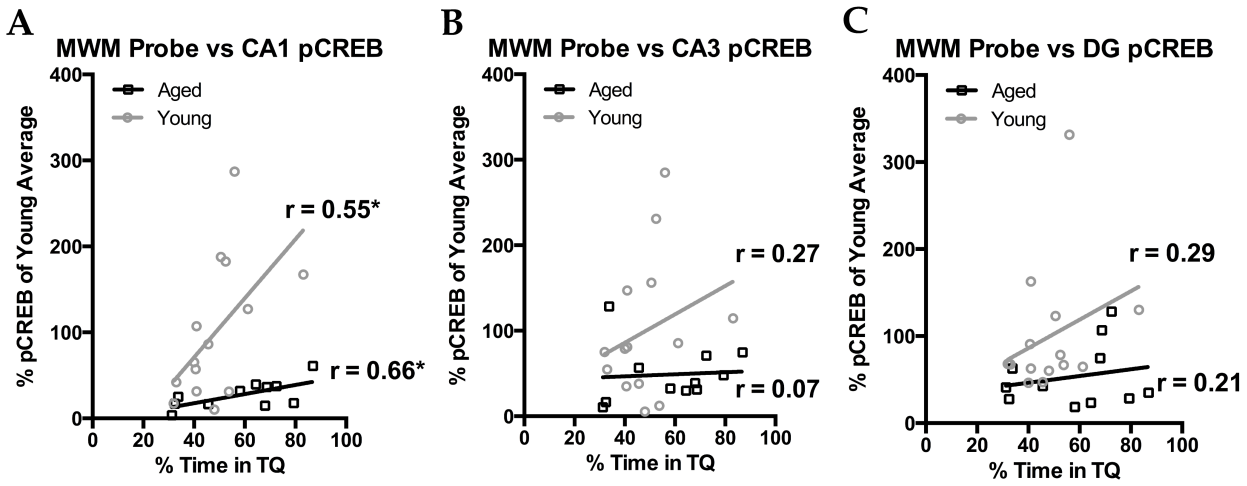


Figure 2.3. pCREB levels correlate with short term spatial memory. A. There was a positive correlation between pCREB levels in dorsal CA1 and spatial memory, as assessed by a probe test conducted 1 hr after the last block of water maze training. B. Level of pCREB in area CA3, C. and in the dentate gyrus did not correlate with spatial memory. * $p < 0.05$.

Training induces long-lasting changes at the protein level

Previous studies have shown that learning or training-induced changes at the electrophysiological level returned to baseline two weeks after the end of training (Moyer et al., 1996; Thompson et al., 1996; Tombaugh et al., 2005). Based on these results, we hypothesized that levels of total and activated CREB would return to steady-state or basal levels two weeks after the end of training. To test this hypothesis, we compared CREB and pCREB levels from the trained rats to those of behaviorally-naïve rats. Two-way ANOVAs were run to determine the effect of age and or training on CREB and pCREB levels. Of interest, we also note that while

these changes did not reach statistical significance, when comparing young to aged naïve animals, similar trends in decreased pCREB were found, as compared to the trained animals as described above (Table 2.1).

In CA1, a main effect of training was found to be significant for CREB ($F_{1, 53} = 4.92$, $p = 0.031$). However, no significance was found for the main effect of age ($F_{1, 53} = 0.058$, $p = 0.81$). Together, these results indicate that CA1 CREB levels were reduced with training in both age groups. No significant effects of age or training were found for CA1 pCREB levels.

Table 2.1. Training induces a long lasting change to CREB levels and activation

	Young Naïve (n = 16)	Aged Naïve (n = 16)	Young Trained (n = 14)	Aged Trained (n = 12)
CA1 CREB	100.0 ± 39.41	83.44 ± 37.68	24.21 ± 2.13	26.34 ± 4.73
CA1 pCREB	100.0 ± 37.36	45.15 ± 20.55	55.83 ± 12.11	14.39 ± 2.60
CA3 CREB	100.0 ± 34.23	18.39 ± 2.37	30.93 ± 3.99	14.52 ± 1.97
CA3 pCREB	100.0 ± 26.61	32.12 ± 12.75	102.1 ± 22.13	51.35 ± 9.47
DG CREB	100.0 ± 29.90	55.14 ± 20.79	26.10 ± 2.82*	18.74 ± 2.42
DG pCREB	100.0 ± 21.10	51.27 ± 13.08	210.2 ± 41.97**	128.1 ± 26.04

Numbers represent mean ± SEM, normalized to young naïve average, and representative of 3 independent experiments, * $p < 0.05$, ** $p < 0.01$ relative to young naïve average.

A significant effect of training was also found for CA3 CREB ($F_{1, 53} = 4.85$, $p = 0.032$). Again, no significance was found for the main effect of age ($F_{1, 53} = 0.002$, $p = 0.96$), indicating that CA3 CREB levels were reduced with training in both age groups. Unlike in CA1, a significant effect of age was found for pCREB levels in CA3 ($F_{1, 53} = 7.91$, $p = 0.007$). No significance was found for the main effect of training ($F_{1, 53} = 0.441$, $p = 0.51$), indicating that CA3 levels of pCREB are reduced with age in both naïve and trained groups.

A significant effect of training was again found for DG CREB ($F_{1, 52} = 6.43$, $p = 0.014$). Post-hoc analysis using Sidak's multiple comparisons test revealed that young trained animals had significantly less CREB in DG than young naïve ($p = 0.045$). Significant effects were found for DG pCREB levels on both main effects of training ($F_{1, 53} = 12.97$, $p < 0.001$), and age ($F_{1, 53} = 5.22$, $p = 0.026$). Post-hoc analysis using Sidak's multiple comparisons test revealed that young trained animals had significantly more pCREB in DG than young naïve ($p = 0.006$).

Together, these results reveal that training-induced molecular changes (such as for CREB and pCREB) take more than two weeks to return to basal levels, unlike changes on the biophysical level.

DISCUSSION

This is the first study to show a differential change in CREB levels and activation in the three major sub-regions of the hippocampus. The decrease in pCREB in aged animals observed in CA1 was independent of any change to total CREB levels i.e., a decrease in pCREB/CREB ratio,

indicating that CA1 specifically has a functional loss of CREB signaling. CA1 is again unique in the hippocampal sub-regions studied, in that it is the only sub-region that displayed a correlation to short-term spatial memory. Of interest, we also identified a long-lasting down-regulation of CREB signaling across the entire hippocampus, as a result of undergoing training.

It has previously been shown pCREB levels rise almost immediately following learning, and that aged animals who fail to learn also lack the learning-associated increase in pCREB (Koutarou Kudo et al., 2004; Monti et al., 2005b; Porte et al., 2008a). However, while CREB activation had been shown to be related to cognitive performance soon after the behavioral test (Porte et al., 2008b), it was not known if CREB activation was correlated to task performance when observed at a later time point. Here, we show that individual animals with more pCREB in CA1 (two weeks after the end of training) perform better on a one-hour probe trial of the Morris water maze. This suggests that pCREB levels could be an indicator or predictor of an animals' short-term memory on a spatial task. Interestingly, we did not observe any relationships between CREB levels or activation in hippocampus, with animals' performance on trace fear conditioning, another hippocampal-dependent task. It is possible that despite the requirement for the hippocampus to acquire fear conditioning, the molecular changes in the amygdala are more important (LeDoux, 2000). Thus, it may be that pCREB levels in the amygdala, and not the hippocampus, correlate with performance during fear conditioning. This is supported by previous studies highlighting the importance of amygdala CREB levels for fear conditioning (Josselyn et al., 2001; 2004; Kida et al., 2002; Zhou et al., 2009).

Regulation of the binding step between CREB and DNA could provide another possible reason why the whole cell pCREB levels examined in this study did not correlate with water maze acquisition, or performance on fear conditioning. Studies have now shown that CREB may not, as previously suggested, be constitutively bound to cAMP-response element-containing DNA stretches (Cha-Molstad et al., 2004). Instead, numerous factors including nitric oxide pathway activity (Riccio et al., 2006), phosphorylation of serines upstream of s133 (Horiuchi et al., 2004), and reduction of cysteines in CREB's bZIP region (Goren et al., 2001) regulate CREB's ability to bind to target DNA. Thus, it is possible that a closer relationship between behavioral performance and CREB may be revealed with careful analysis of nuclear levels of DNA-bound CREB.

Previous studies examining age-induced changes to CREB levels have reported disparate results (Brightwell et al., 2004; Foster et al., 2001; Monti et al., 2005b). The inconsistent results may be due to the use of different strains of rat, and/or possibly due to the use of different antibodies. However, most importantly, all of the previous studies carried out western blots on tissue homogenates from the entire hippocampus. We probed the issue further, by carrying out dissections of the hippocampal sub-regions, and only focusing on tissue from dorsal hippocampus, the area most relevant to the behavioral tasks used. Our findings of decreased pCREB across all sub-regions (but especially CA1) seem to concur with the findings of Foster *et al*. (2001), who also found no change in total CREB, but a drop in pCREB in aged animals. Our findings also confirm those of Hattiangady *et al*. (2005) who carried out immunohistochemistry for pCREB on aged and young tissue, and reported a decrease in pCREB in aged hippocampus.

However, this study could not attribute the entirety of their antibody signal to pCREB, and therefore they could not conclusively attribute the age-related decrease to pCREB alone, a problem we have overcome by only examining the signal from the expected 43kDa band for pCREB on our western blots.

One rather surprising finding is a long-lasting change in CREB levels following behavioral training. While many studies have established the timeline of how CREB activation changes immediately after training, this is the first report of a molecular change in response to behavioral training, which persists for two weeks after the final training/testing session. This result suggests that following a large activation of CREB signaling in response to the stimuli of behavioral training, the system may undergo a homeostatic reduction of both CREB levels and activation, in order to prepare for further incoming signals.

Our present findings suggest that increasing pCREB levels in dorsal CA1 may ameliorate and/or prevent age-related cognitive deficits. Viral overexpression of CREB has been shown to facilitate the behavioral performance of young adult rodents (Brightwell et al., 2007; Czajkowski et al., 2014; Josselyn et al., 2001), indicating that CREB overexpression may also have therapeutic benefits for age-related cognitive impairments. Virally increasing total CREB levels and subsequently levels of pCREB, could rescue cognitive impairments seen with normal aging. Furthermore, dysfunctional CREB signaling has been reported in age-related diseases such as Alzheimer's Disease (Chen et al., 2012; Pugazhenthi et al., 2011; Satoh et al., 2009). Therefore,

CREB signaling may also be a viable therapeutic target to reverse cognitive changes in neurodegeneration (Yiu et al., 2011).

In an attempt to tease out the contributions of the combined water maze and fear conditioning, a new cohort was subjected to the original training schedule, with fear conditioning removed. Upon examination of western blot data, these animals did not exhibit an age-related decrease in pCREB in CA1, or the corresponding correlation with probe trial performance. Several factors may contribute to the varying results we observed; one vs. two behavioral tasks, cohort effects, antibody lots. Each of these factors may differ in the weight of their contribution to our observations, and more work will be needed to further tease them apart.

ACKNOWLEDGMENTS

This work was supported by NIH grants R37 AG008796 (JFD), RF1 AG017139 (JFD), R01 NS063245 (JCPY), T32 AG020506 (DMC and XY) and the Glenn/AFAR Scholarship for Research in the Biology of Aging (XY). These funding sources had no involvement in the design of this study; the collection, analysis, or interpretation of the data; or in the writing of this report and where to submit for publication. Morris water maze and fear conditioning experiments were carried out with the help of the Northwestern University Behavioral Phenotyping Core.

Chapter 3 – CREB overexpression in dorsal CA1 ameliorates long-term memory deficits in aged rats

ABSTRACT

The molecular mechanisms underlying age-related cognitive deficits are not yet fully elucidated. In aged animals, a decrease in the intrinsic excitability of CA1 pyramidal neurons is believed to contribute to age-related cognitive impairments.

Increasing activity of the transcription factor cAMP response element-binding protein (CREB) in young adult rodents facilitates cognition, and increases intrinsic excitability. However, it has yet to be tested if increasing CREB expression also ameliorates age-related behavioral and biophysical deficits. To test this hypothesis, we virally overexpressed CREB in CA1 of dorsal hippocampus. Rats received CREB or control virus, before undergoing water maze training. CREB overexpression in aged animals ameliorated the long-term memory deficits observed in control animals. Concurrently, cells overexpressing CREB in aged animals had reduced post-burst afterhyperpolarizations, indicative of increased intrinsic excitability. These results identify CREB modulation as a potential therapy to treat age-related cognitive decline.

INTRODUCTION

Age-related cognitive impairments are observed across multiple species, including laboratory rodents and humans. Forms of learning that require an intact hippocampal formation, such as spatial navigation, are severely impacted in aged humans (Etchamendy et al., 2012), rats

(Gallagher and Pelleymounter, 1988) , and mice (Bach et al., 1999). Although age-related cognitive deficits have been observed across numerous tasks and species, not all aged subjects display these impairments (Gallagher and Pelleymounter, 1988; Knuttinen et al., 2001a; 2001b). Therefore, the aging population can be split into aged individuals who are cognitively-impaired, and others who are cognitively-unimpaired. These cognitively-unimpaired "super agers" are capable of learning and remembering at young-like levels (Curlik et al., 2014; Gallagher and Pelleymounter, 1988; Knuttinen et al., 2001a; Rogalski et al., 2013).

Identifying the molecular mechanisms that differentiate successful from unsuccessful cognitive-agers is highly desirable, as knowledge of the underlying mechanisms will greatly facilitate treatment of these impairments. One likely mechanism contributing to age-related cognitive deficits is a decrease in the intrinsic excitability of CA1 pyramidal neurons. Numerous studies have revealed that CA1 pyramidal neurons from aged animals have reduced intrinsic excitability when compared to those from young animals. Specifically, pyramidal neurons from area CA1 of the dorsal hippocampus of aged animals exhibit a larger post-burst afterhyperpolarization (AHP) than those from young animals (Disterhoft and Oh, 2007; 2006; Gant et al., 2006; Landfield and Pitler, 1984; Oh et al., 2013). The magnitude of this age-related decrease in neuronal excitability correlates with age-related cognitive deficits (Tombaugh et al., 2005). Aged impaired (AI) animals have larger AHPs than both young animals, and aged unimpaired (AU) animals. Interestingly, the AHP amplitude from AU animals is no different than that of young animals (Matthews et al., 2009; Moyer et al., 2000; Tombaugh et al., 2005). Moreover, pharmacological compounds that reduce the amplitude of the AHP *in vitro*,

ameliorate age-related cognitive impairments *in vivo* (Kronforst-Collins et al., 1997; Moyer et al., 1992; Oh et al., 1999). Based on these findings, we are searching for molecular pathway(s) that modulate both cognition and intrinsic cellular excitability.

One such pathway is activated by the transcription factor, cAMP response element-binding protein (CREB; (Alberini, 2009). Numerous studies have manipulated CREB activation in young animals to demonstrate its essential role in memory formation (Bernabeu et al., 1997; Bourtchuladze et al., 1994; Dash et al., 1990; Deisseroth et al., 1998; Kaang et al., 1993). Memories for spatial and cued information were impaired in transgenic mice expressing a dominant negative form of CREB (Pittenger et al., 2002). Likewise, mutations which prevent CREB from being activated by inhibiting its phosphorylation also impaired memory (Kida et al., 2002). Conversely, increases in CREB activity via transgenic or viral means facilitated memory. For instance, expressing a partially-active form of CREB (VP16-CREB) in the amygdala resulted in stronger memories for contextual and cued fear conditioning (Viosca et al., 2009b). Likewise, infusion of HSV-CREB into hippocampus or amygdala, results in CREB overexpression and facilitation of memory for the Morris water maze (Sekeres et al., 2010), water cross maze (Brightwell et al., 2007), or fear conditioning (Josselyn et al., 2001) in young animals.

Notably, manipulations that increase CREB activity in young animals have also been found to increase intrinsic excitability of their neurons. For example, expression of VP16-CREB resulted in increased neuronal excitability of numerous brain regions, including CA1 of

hippocampus, the locus coeruleus, the nucleus accumbens, and the amygdala (Dong et al., 2006; M.-H. Han et al., 2006; Lopez de Armentia et al., 2007; Viosca et al., 2009b). Moreover, overexpression of wild-type CREB has also been shown to be sufficient to enhance excitability (Yiu et al., 2011; Zhou et al., 2009). Taken together, these previous studies in young adult animals indicate that increasing CREB activity facilitates memory and increases intrinsic neuronal excitability.

Contrary to young adults, very little is known about age-related changes to hippocampal CREB levels and/or activity. Furthermore, the few published studies which have examined CREB levels in aging appear to present conflicting results. One study reported that levels of total CREB protein were unchanged with aging, however, levels of CREB phosphorylated at S133 (pCREB) decreased with age (Foster et al., 2001). Another study reported a decrease in total CREB with aging, which was specifically observed in aged animals that had impairments in spatial memory (Brightwell et al., 2004). A third study found that total CREB was unchanged, while pCREB increased with age (Monti et al., 2005b). While the exact nature of age-related CREB levels is unclear, these findings do suggest that CREB levels in the hippocampal formation change with age. Additionally, upstream activators of CREB, such as cAMP, have been found to be decreased with age (Bach et al., 1999). Activation of protein kinase A, the next step in this activation cascade, is required for the learning-induced reduction in post-burst AHPs (Oh et al., 2009; Zhang et al., 2013), and reduced in aged rats (Karege et al., 2001). Calcineurin, a phosphatase that dephosphorylates CREB, has also been found to be increased with age (Foster et al., 2001). All of these factors could result in a decrease in CREB activation, which led us to hypothesize that increasing CREB levels would ameliorate both the cognitive and biophysical

deficits observed in normal aging subjects. We tested this hypothesis by using an adeno-associated viral vector to overexpress wild-type rat CREB in CA1 of dorsal hippocampus. Spatial learning and memory were then assessed using two different Morris water maze protocols; a less challenging version, and a more difficult version. Our results revealed that CREB overexpression in dorsal CA1 ameliorated age-related long-term biophysical and behavioral deficits.

MATERIALS AND METHODS

Subjects

Young adult (3-4 mo) and aged (29-30 mo) male F1 hybrid Fischer 344XBrown Norway (F344XBN) rats were used for this study. All animals were obtained from the National Institute on Aging colony at Charles River Laboratories (Raleigh, NC). All rats were housed in a temperature-controlled facility with a 14 hr light/10 hr dark cycle and allowed free access to food and water. They were group housed, and allowed to acclimate in the Northwestern University vivarium for a minimum of one week prior to any experimentation. All procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animals were handled according to protocols approved by Northwestern IACUC (Protocol number: IS00002081, Animals Welfare Assurance: A3283-01) following NIH guidelines. All surgery was performed under isofluorane anesthesia, and every effort was made to minimize suffering.

Viral Vectors

An adeno-associated viral vector (AAV) was used to overexpress rat CREB (AAV-CREB). The construct encoded the sequence for endogenous rat CREB, downstream of a chicken β-actin promoter with a CMV enhancer. This was followed by an GFP sequence downstream from an internal ribosomal entry site. A second construct (AAV-GFP), lacking the CREB sequence served as a control (vectors were a generous gift from Dr. Corinna Burger). Both were cloned into shuttle vectors and packaged into AAV serotype 9 by Virovek (Hayward, CA). The final viral titres were 10^{13} viral particles/mL. Rats were randomly assigned to receive AAV-CREB or control AAV-GFP.

Stereotaxic injections

All surgeries were performed using sterile procedures. Animals were anesthestized with isofluorane gas, first in an induction chamber, then while secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), with lambda and bregma equal in the vertical plane. A thin layer of ophthalmic ointment was applied to keep the eyes moist. Subjects were administered Buprenex sub-cutaneously (0.03 mg/kg) to minimize discomfort. The scalp was incised and retracted, and bilateral holes were drilled through the skull above the hippocampi at the injection sites in dorsal CA1. The injection sites were 5.0mm posterior and ± 3.0mm lateral from bregma, and 2.1mm ventral from the dura. Glass micropipettes with calibrated internal diameters were pulled and cut to have 30-45 μm openings. Glass micropipettes were fitted to a blunt-tipped 10 μL Hamilton syringe, mounted to the stereotaxic frame. For each injection, 1 μL of vector was infused into the hippocampus at a rate of 0.1 μL/min using a Stoelting QSI microinjection pump. The glass micropipette was left in place for an additional 5 min before being gradually removed

from the brain. After both injections were complete the scalp was sutured, and animals were given Rimadyl sub-cutaneously (5 mg/kg) to alleviate post-surgical discomfort. Rats were allowed to recover under a heat lamp before returning to their home cage, where they remained for 2 weeks, before the start of any further experiments.

Perfusions and Coronal sectioning

Rats were deeply anesthetized, then transcardially perfused through the left ventricle, first with 0.1 M phosphate-buffer saline (PBS, pH 7.4), then with 4% paraformaldehyde. The brains were extracted, and post-fixed in 4% paraformaldehyde overnight, then stored in PBS until sectioning. After removing the cerebellum and the anterior portion of the brain (to reveal the fornix), 40 μm sections were made through hippocampus on a Leica VT100s Vibratome. Tissue was stored in PBS 4 °C.

Immunohistochemistry

Brain sections were stained using a free-floating immunofluorescence procedure. PBS-X (0.01 M phosphate-buffered saline containing 0.5 % Triton-X-100) was used to make up all other solutions, and all incubations were performed at room temperature, unless otherwise stated. Tissue was permeabilized using PBS-X for 10 min then blocked with 5 % normal goat serum (Jackson Immunoresearch, West Grove, PA) for 4 hrs. Sections were incubated overnight at 4 °C with primary antibodies. The next day, sections were washed in PBS-X then incubated with secondary antibody for 2 hrs. After further washes, tissue was mounted using ProLong gold antifade (Life Technologies, Carlsbad, CA).

Stereology utilized brain sections 600 μm apart, all within the infected area. Primary antibodies were chicken anti-GFP (1:2000, Abcam, Cambridge, MA, #13970), and mouse anti-NeuN (1:300, Millipore, Temecula, CA, #MAB377). Secondary antibodies were anti-chicken IgG conjugated to AlexaFluor633, and anti-mouse IgG conjugated to AlexaFluor594, both purchased from Life Technologies, and used at 1:500 dilution.

CREB intensity stains utilized brain sections 200 μm apart, on the edge of the infected area (to ensure sufficient number of GFP- cells were included in the analysis). Primary antibodies were chicken anti-GFP (1:2000, Abcam, #13970), and rabbit anti-CREB (1:800, Cell Signaling Technologies, Boston, MA, #9197). Secondary antibodies were anti-chicken IgG conjugated to AlexaFluor633, and anti-rabbit IgG conjugated to AlexaFluor594, both purchased from Life Technologies, and used at 1:500 dilution.

Stained tissue sections were imaged on a confocal microscope (Nikon A1R) in Northwestern University's Centre for Advanced Microscopy. Using a 40x objective, 3 z-stacks from non-overlapping fields of view were collected from CA1 of dorsal hippocampus. Laser and PMT settings were optimized for imaging each slide, but remained constant for all images taken from that slide.

Staining for NeuN and GFP was done to calculate the percent of infected neurons, in the infected area of dorsal CA1 from AAV-CREB-injected animals. For each animal (5 young and 4 aged), 3 sections from the center of the infected area (600 μm apart) were analyzed. From these sections, 3 non-overlapping z-stacks were collected from CA1 of each hippocampus, resulting in

a total of a total of 18 z-stacks for each animal. The sum of the collapsed z-stack was used for subsequent analyses. The threshold of NeuN-positive signal was first set to encompass the signal from cells, but not debris or processes. The signal was then subjected to the Watershed function, followed by Analyze Particles to count how many NeuN-positive cells that z-stack contained. A 'selection' was then created from a thresholded GFP signal. This was then applied to the NeuN signal, to count how many of the NeuN-positive cells were also GFP-positive, which was then expressed as a percentage. All image analysis was carried out using NIH's ImageJ software.

Staining for CREB and GFP was done to determine whether AAV-CREB-infected cells express more CREB protein than uninfected cells from the same animal. Three sections from each animal were analyzed (5 young and 4 aged animals). Each section was from the edge of the infected area and separated by 200 μm. From these sections 3 non-overlapping z-stacks were collected from area CA1 of each hemisphere of the hippocampus, resulting in a total of 18 z-stacks for each animal (3 sections X 3 z-stacks X 2 hemispheres). The sum of each collapsed z-stack was used for subsequent analyses. The threshold of CREB signal was first set to encompass the signal from cell bodies, but not debris or processes. After subjecting the signal to the Watershed function, the intensity of each cell was measured along with ROI size. CREB intensity was then normalized to ROI area to ensure the intensity measurement was independent of ROI size (to account for potential differences in measured area). Cells were manually separated into GFP-positive (GFP+), and GFP-negative (GFP-) groups. For each z-stack, the average intensity for all GFP+ cells was calculated, as well as the average intensity for all GFP- cells. To quantify CREB levels in GFP+ cells relative to GFP- cells, the average CREB intensity from GFP+ cells was first normalized to the average CREB intensity from GFP- cells (average

GFP+ intensity/ average GFP- intensity. This value was then multiplied by 100 to obtain a percentage. Thus, GFP- cells had an average CREB intensity of 100%, and a higher percentage for the GFP+ cells was indicative of an increase in CREB levels.

Morris Water Maze: less challenging version

We used the Morris water maze task to assess learning, short-term memory, and long-term memory in 7 young animals injected with AAV-GFP, 7 young animals injected with AAV-CREB, 17 aged animals injected with AAV-GFP, and 15 aged animals injected with AAV-CREB. All training and probe trials were conducted in a circular pool (180 cm in diameter). The water (24 ± 1 °C) was made opaque by the addition of white non-toxic paint. Ten minutes before each day of training or testing animals were individually housed in new cages. At the end of each daily session of water maze training animals were returned to their home cages. All animals were handled on 3 separate occasions, for at least 3 min each, in the week prior to the start of visible platform training.

We first trained the rats on a visible platform task to ensure they were capable of locating, navigating to, and climbing onto the escape platform. The rectangular escape platform (20.3 cm x 25.4 cm) was placed in the center of 1 of 4 quadrants of the pool, with the bottom of the platform sitting at the level of the water. No distinctive cues surrounded the pool, while the platform had several high-contrast visual cues attached to it. At the start of each trial, the animal was placed approximately 7.5 cm away from the wall of the pool, in the center of the quadrant opposite from the platform-containing quadrant. Each animal was given 60s to navigate to the

visible platform. If the animal did not reach the platform after 60s, the experimenter gently guided the animal to the platform location. The location of the platform changed after every trial, and each animal received 8 trials, with an inter-trial interval of 19 ± 1 min. This visible platform testing ensured that all animals used in subsequent behavioural experiments had no gross sensorimotor deficits, as they were able to swim, see, and climb onto the visible escape platform. All animals successfully reached the platform during the last trial of visible platform training, and no animals were excluded from further water maze training.

Three days after the conclusion of visible platform training, all animals were trained with a hidden platform task. Distinctive, high-contrast cues were placed on each of the walls surrounding the water maze pool. The circular escape platform (20 cm diameter) was placed in one quadrant, and submerged 2 cm below the water level, so that animals could not see the hidden platform. The location of the platform remained the same throughout hidden platform training. Each animal received 5 trials of hidden platform training per day, over 4 consecutive days (total 20 trials) with an inter-trial interval of 19 ± 1 min. Each trial began by placing the animal in the centre of one of the three quadrants that did not contain the hidden platform. Each animal was allowed 60 s to locate the escape platform. If the animal did not swim to the hidden platform after 60 s, the experimenter gently guided the animal to the platform. Upon reaching the platform, animals remained on the platform for a further 15 s, before being removed by the experimenter. The animals' cumulative proximity to the platform was measured for each training trial, then the average cumulative proximity was calculated for each animal for each of the 4

days of training. We chose to use the cumulative proximity measurement as it has been shown to be sensitive to age-related cognitive impairments (Gallagher et al., 1993).

To test for memory for the platform location probe trials were performed 1 hour after each day of hidden platform training, as well as 1 day and 4 days after the last session of hidden platform training. During these probe trials the escape platform was removed from the pool, and each rat was given 60 s to swim in the pool. Memory for the platform location was assessed by quantifying the percent of time each animal spent in the target quadrant of the pool during the first 20 s of the probe test. The target quadrant was the quadrant that contained the hidden platform during training. Similar to the cumulative proximity measure, percent time in target quadrant has been shown to be sensitive to age-related memory impairments (Foster, 2012). In order to prevent extinction, a reminder hidden platform trial was conducted 19 ± 1 min after each probe trial. WaterMaze software was used for computerized tracking during trials and for subsequent offline analysis (Actimetrics, Wilmette, IL).

Collection of hippocampal tissue for RT-PCR, biophysical recordings, and western blotting

Two-three weeks after the end of behavioral testing or viral infusion, rats were euthanized to collect hippocampal tissue. Animals were anesthetized with isoflurane and then were rapidly decapitated. Brains were removed and placed into ice-cold artificial cerebrospinal fluid (aCSF, in mM): 124 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 25 glucose, 2.4 $CaCl_2$, pH 7.4, oxygenated with 95%:5% O_2:CO_2. A sucrose-aCSF was used for brains from aged rats (in

mM): 206 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.1 $CaCl_2$, 3 $MgSO_4$, and 25 glucose. Hippocampi were then extracted, and 300 μm transverse sections cut on a Leica VT100s Vibratome. Sections were then used for biophysical measurements, or frozen in RNAlater (Qiagen, Valencia, CA) for RT-PCR experiments, or immediately frozen for Western blot experiments.

Biophysical recordings

The 300 μm sections from dorsal hippocampus were prepared as described above, then incubated in aCSF for 20 min at 34 °C, before being allowed to return to room temperature for at least 40 min. Slices were then transferred to a submersion chamber at 34-35 °C. Slices were visualized using an upright Hamamatsu Orca R2 camera. Patch electrodes (5-7 MΩ) were filled with (in mM) 120 KMeSO4, 10 KCl, 10 Hepes, 4 Mg2ATP, 0.4 NaGTP, 10 Na2 phosphocreatine, 0.04 AlexaFluor 594, and 0.5% neurobiotin, pH adjusted to 7.4 with KOH. No correction was made for a 10 mV liquid junction potential. All measurements were made 5 min after membrane rupture to allow for adequate solution equilibration. Neurons were held near -69mV, and recordings were obtained using an Axon Digidata 1550A amplifier, and data analyzed using pClamp 10 software (Molecular Devices, Sunnyvale, CA) and custom routines in Matlab. Matlab routines are available upon request. AHP values were observed using a train of 15 action potentials (50 Hz), evoked by direct somatic current injections. The experimenter was blind to the infection status of the neurons during collection and analysis of biophysical data.

In order to confirm infection status of patched cells sections were fixed overnight in 4% paraformaldehyde and then stored in PBS until re-sectioning. To amplify the GFP and neurobiotin signals, tissue was first re-sectioned. Each slice was embedded in 8 % agar then re-sectioned into 4-5 70 μm sections. These were permeabilized in PBS-X, and blocked in 5 % normal goat serum (Jackson Immunoresearch) for 4 hrs. Sections were incubated with chicken anti-GFP primary (1:2000, Abcam, #13970) overnight at 4 °C. The next day, the tissue was washed with PBS-X, then incubated with anti-chicken IgG conjugated to AlexaFluor488 (1:375, Jackson Immunoresearch) and Streptavidin conjugated to AlexaFluor594 (1:1000, Life Technologies). After further washes, tissue was mounted using ProLong gold antifade (Life Technologies). Patched cells were examined for the presence of neurobiotin and GFP signal to determine infection status.

RT-PCR

Four to five hippocampal slices were placed in RNAlater, and frozen. The major hippocampal sub-regions (CA1, CA3, and dentate gyrus) were manually dissected from these slices (Núñez Santana et al., 2014). Under a Stemi DV4 dissecting microscope (Zeiss), and on ice, a straight cut was first made in front of the blades of the dentate gyrus, to isolate the CA3 sub-region from each slice. The dentate gyrus was then peeled away from the CA1-subiculum, and the subiculum severed from CA1 and discarded. Tissues from the sub-regions were then frozen again before further processing. Tissue from CA1, CA3, or DG was first disrupted manually, then with a QiaShredder (Qiagen) column. RNA was subsequently extracted using RNeasy Plus Mini Kit (Qiagen), according to manufacturer's directions. All samples were eluted

in 30 uL RNase-free and DNase-free water. All samples were checked for integrity using Biorad's Bioanalzyer (Hercules, CA) and RNA 6000 Pico chips (Biorad). All samples had RNA integrity numbers (RIN) of 7 or higher and therefore none were excluded from analysis. For each sample, equal amounts of RNA were reverse transcribed into cDNA using SuperScript® VILO cDNA Synthesis Kit (Invitrogen). To ensure reagents were contaminant-free concurrent controls containing no VILO enzyme were made and carried through the rest of the real-time polymerase chain reaction (RT-PCR) experiment.

All RT-PCR reactions were carried out in 384-well plates. Each well contained sample cDNA, primer and probe against target genes, and SsoAdvanced™ Universal Probes Supermix (Biorad). Each plate included controls from the cDNA synthesis step, which lacked the reverse transcription enzyme, and wells in which water replaced the cDNA sample. These negative controls were to confirm that no genomic DNA was present, and that no reagents aside from sample cDNA contained template that could be amplified. All samples were run in triplicate, and reactions carried out on a 7900HT (Applied Biosystems, Foster City, CA).

Primers and probes against GAPDH, CREB1, and GFP were purchased from Integrated DNA Technologies. Utilizing GAPDH as the house-keeping gene, the fold change over young GFP animals were calculated for genes of interest using the ΔΔCt method.

Gene	Forward Primer	Probe	Reverse Primer
GAPDH	CCAGTAGACTCCA CGACATAC	CAGCACCAGCATCA CCCCATTTG	AACCCATCACC ATCTTCCAG

CREB	AGCACTTCCTACA CAGCCT	ATTCTCTTGCTGCTT CCCTGTTCTTCA	CACTGCCACTC TGTTCTCTA
GFP	GAACCGCATCGA GCTGAA	ATCGACTTCAAGGA GGACGGCAAC	TGCTTGTCGGC CATGATATAG

Western Blotting

Four to five hippocampal slices were immediately frozen on dry ice. While frozen and on ice, CA1 was manually isolated under a Stemi DV4 dissecting microscope (Zeiss) as described above. CA1 tissue was lysed in RIPA buffer (150 mM NaCl, 25 mM Tris, 1 mM EDTA, 0.1% SDS, 1 % Triton X-100, and 0.5 % sodium deoxycholate) containing protease and phosphatase inhibitors (Pierce, Rockford, IL). Each sample was manually dissociated with a syringe, and shaken at 4 °C for 30 min. All samples were then centrifuged at 14,000 g for 15 min. The supernatants were retained and protein concentration measured by BCA assay (Pierce). Fifteen micrograms of each sample was boiled in 2x Laemmli buffer containing 5 % β-mercaptoethanol (Bio-Rad Hercules, CA). Samples were separated using 4-20 % Mini Protean TGX gels (Bio-Rad), and transferred to Immobilin-P PVDF membranes (Millipore, Temecula, CA). Blots were probed for CREB (Millipore Cat #04-767, RRID:AB_1586959, 1:5000) and GAPDH (Thermo Fischer Scientific Cat #MA1-16757, RRID:AB_568547 1:40000, Thermo Scientific, Waltman, CA). Anti-rabbit secondary was first used to react with the anti-CREB antibody. After imaging, HRP signal was quenched using 15 % H_2O_2. An anti-mouse secondary (Jackson) was then used to react with the anti-GAPDH primary. Immunoreactive bands were visualized using a

ChemiDoc XRS+ Molecular Imager System with ImageLab Software (Bio-Rad). Offline quantification of reactive bands also used ImageLab software. All gels were loaded with the same reference sample to allow for comparison across gels. All samples were run in triplicate, and normalized for loading error using GAPDH signal. Pearson's R values were determined to test for strength of correlations between CREB protein levels and behavioral performance.

Morris Water Maze: more difficult version

We determined whether the difficulty of the water maze task affected our results by training a new group of animals on a more difficult version of the task. Similar to the first group, we assessed learning and short-term memory in 7 young animals injected with AAV-GFP, 8 young animals injected with AAV-CREB, 15 aged animals injected with AAV-GFP, and 8 aged animals injected with AAV-CREB. Unless otherwise noted, the training conditions remained the same as the previous protocol.

As before, we first trained the rats on 8 trials of a visible platform task (inter-trial interval of 19 ± 1 min) to ensure they were capable of locating, navigating to, and climbing onto the escape platform. All animals successfully reached the platform during the last trial of visible platform training, and no animals were excluded from further water maze training.

Three days after visible platform training, all animals were trained with a hidden platform task. The location of the platform remained the same throughout hidden platform training. Each animal received 3 trials of hidden platform training per day, over 4 consecutive days (total 12

trials). The inter-trial interval was 1min. The animals' cumulative proximity to the platform was measured for each training trial, then the average cumulative proximity was calculated for each animal for each of the 4 days of training. We chose to use the cumulative proximity measurement as it has been shown to be sensitive to age-related cognitive impairments (Gallagher et al., 1993).

To test for memory for the platform location probe trials were performed 1 hour after each day of hidden platform training. Memory for the platform location was assessed by quantifying the percent of time each animal spent in the target quadrant of the pool during the first 20 s of the probe test. The target quadrant was the quadrant that contained the hidden platform during training. Similar to the cumulative proximity measure, percent time in target quadrant has been shown to be sensitive to age-related memory impairments (Foster, 2012). Unlike in the less challenging version of the task, no reminder hidden platform trial was given after each probe trial. WaterMaze software was used for computerized tracking during trials and for subsequent offline analysis (Actimetrics, Wilmette, IL).

One hour after the last probe trial, animals were euthanized to collect tissue for RT-PCR. Hippocampi were isolated and frozen in RNAlater. The CA1 sub-region was dissected and RNA extracted as described above. RT-PCR reactions for CREB were carried out as described above.

Statistical analysis

Data are presented at mean ± SEM. N is number of animals, except for biophysical experiments, where n is number of cells. To minimize number of animals used, only n = 4-5 animals were used for the immunofluorescent staining. For behavior, 7 young animals were used for each treatment, while approximately double this number of aged animals were used to accommodate for their increased variance due to presence of both aged impaired and aged unimpaired animals (Curlik et al., 2014; Gallagher and Nicolle, 1993; Knuttinen et al., 2001a).

GraphPad Prism version 6 (GraphPad Software, La Jolla, CA) was used for statistical analysis. One sample t-test, two-tailed student t-tests, one-way, and two-way ANOVAs with Tukey's or Bonferroni's multiple comparisons tests were used as appropriate. Normality was tested using D'Agnostino & Pearson omnibus normality test. Three-way repeated measures ANOVAs were run using Statview version 5.0.1. Significance levels were set at $p = 0.05$. Significance for comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

RESULTS

Infusion of AAV-CREB caused CREB overexpression in infected CA1 neurons from young and aged rats

While several studies have utilized viral vectors to achieve overexpression of CREB in brains of young animals (Josselyn et al., 2001; Sekeres et al., 2010; Zhou et al., 2009), few studies have done the same in aged animals. Thus, we characterized the infection efficiency and the extent of CREB overexpression of our new adeno-associated viral vector. AAV-CREB virus with a GFP reporter (Fig. 3.1) was bilaterally injected into CA1 region of 5 young (3-4 months

old, mo), and 4 aged (29-30 mo) male, Fischer 344XBrown Norway F1 hybrid rats. Two weeks after the viral injections, animals were transcardially perfused, and their brains post-fixed to make coronal sections (40 μm) for immunofluorescent staining. Three sections from each animal were stained for NeuN and GFP, to determine the percentage of neurons that were GFP-positive (GFP+): i.e., cells that had been infected by AAV-CREB virus. By visualizing the GFP signal, we observed widespread infection through the medial-lateral axis of CA1 (Fig. 3.2a), with approximately 2 mm of spread in the anterior-posterior axis. Over 95% of cells in this infected area were positive for GFP, and there was no difference in the percentage of GFP+ cells between young (95.24 ± 0.71 %) and aged (96.30 ± 0.83 %) animals (t_7 = 1.26, n.s.; Fig. 3.2b). To confirm that viral infection lead to overexpression of CREB, another set of sections from the beginning of the infected area, with a lower infection rate to encompass sufficient number of GFP- cells, was stained for CREB and GFP (Fig. 3.2c). The average CREB intensity of GFP- neurons was set at 100%, and the CREB immunocytochemical signal from GFP+ neurons was normalized to that value. One sample t-tests against a hypothetical average of 100% revealed that GFP+ cells expressed more CREB than GFP- cells in both young (151.4 ± 5.16 %, t_4 = 9.97, p = 0.0006) and aged (141.0 ± 3.44 %, t_3 = 11.91, p = 0.001) animals, and no differences were observed between young and aged animals (t_7 = 1.59, n.s.; Fig. 3.2d). Together, these data indicate that our AAV-CREB virus infects the same percentage of neurons and achieves the same amount of CREB overexpression in both young and aged animals.

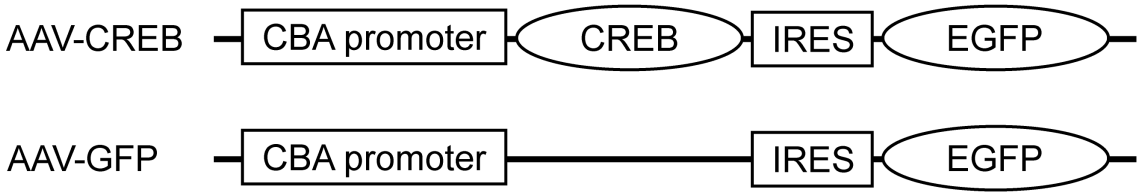


Figure 3.1. Schematic for viral constructs. AAV-CREB encoded the sequence for wild-type rat CREB, expressed from a chicken β-actin promoter with a CMV enhancer. The GFP reporter was downstream from an internal ribosomal entry site (IRES). The control AAV-GFP virus lacked the rat CREB sequence.

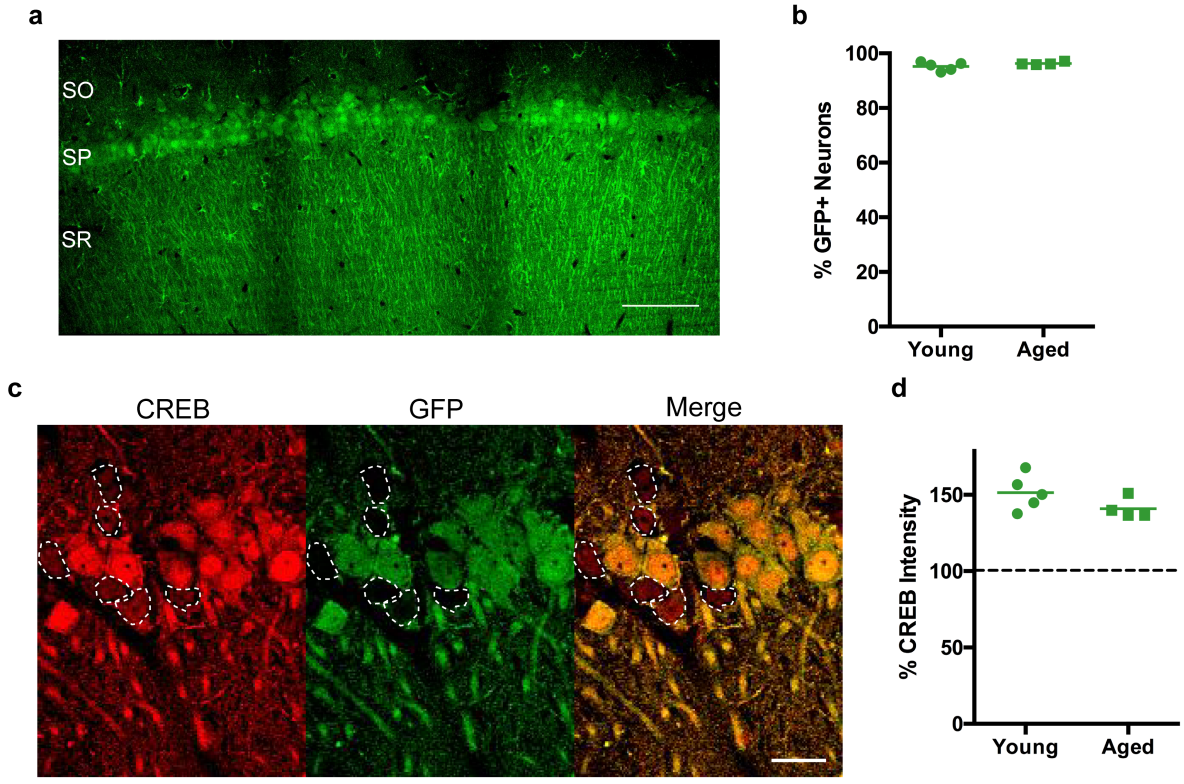


Figure 3.2. CREB expression levels are increased in CA1 neurons of both young and aged rats following stereotaxic injection of AAV-CREB vector into dorsal CA1 region. a) The AAV-CREB vector contained a GFP reporter. Example of a stitched 40x confocal image of GFP staining in CA1 area. SO – stratum oriens; SP – stratum pyramadale; SR – stratum radiatum. Scale bar = 100 μm. b) The percentage of infected CA1 neurons in young and aged animals (n = 5, 4) was quantified by dividing the number of GFP positive cells by the total number of NeuN positive cells for each animal. c) Example of CREB immunofluorescence in infected (green) and uninfected (not green, outlined) cells. Note the higher intensity in GFP positive cells. Scale bar = 20 μm. d) AAV-CREB infected cells (i.e., GFP positive cells) had higher CREB expression than uninfected cells. Relative CREB expression level of infected cells was quantified as a percentage of CREB intensity of uninfected cells (normalized to be 100%, dashed line) in young and aged animals (n = 5, 4).

AAV-CREB ameliorated age-related spatial memory deficits; less challenging water maze

To determine whether viral overexpression of CREB in dorsal CA1 facilitated learning and memory, we injected dorsal CA1 of young and aged rats with AAV-CREB (7 young and 15 aged) or a control AAV-GFP virus (7 young and 17 aged) which lacked the sequence for CREB (Fig. 3.1). We used approximately double the number of aged animals to account for their

increased variability, due to the presence of both AI and AU animals. Two weeks after AAV infusion, the rats were trained on a less challenging protocol for Morris water maze. Animals first received training with the visible platform procedure to ensure that they did not have sensorimotor deficits that would prevent them from performing the task. On the visible platform day, animals were trained with 8 trials, and animals were able to successfully acquire the task (Fig. 3.3a, inset). A repeated measures ANOVA, with age and virus as the main factors, and trial as the repeated factor, revealed significant effects of trial ($F_{7,42} = 15.76$, $p < 0.0001$) and of age ($F_{1,42} = 7.66$, $p = 0.008$), but not of virus ($F_{1,42} = 0.001$, n.s.). All animals successfully reached the visible platform on the last trial, and therefore no animals were excluded from further training.

Three days after visible platform training, animals were trained with the hidden platform task. All animals were trained with 5 trials of the hidden platform task per day, over 4 consecutive days (Fig. 3.3a). Each animal's average cumulative proximity to the hidden platform during each training session was used to assess performance. We found that young animals acquired the task more quickly than aged animals, but there were no differences amongst viral groups for either age group. A repeated measures ANOVA, with age and virus as the between groups measures, and session as the repeated measure, revealed significant main effects of session ($F_{3,42} = 63.30$, $p < 0.0001$) and of age ($F_{1,42} = 14.72$, $p = 0.0004$), but not of virus ($F_{1,42} = 0.14$, n.s.). We observed that on the last session of hidden platform training, aged GFP animals tended have higher cumulative proximities than the other groups. We examined these differences on the last session with a two-way ANOVA, with age and virus as the between groups factors.

This revealed a significant main effect of age ($F_{1, 42} = 9.70$, $p = 0.003$), but not of virus ($F_{1,42} = 1.16$, n.s.). Post-hoc comparisons (Tukey's) of cumulative proximity on the last day of training confirmed that aged GFP animals had greater cumulative proximities than both young GFP animals ($p = 0.031$), and young CREB animals ($p = 0.023$).

To assess memory for the platform location, all animals underwent probe trials 1 hour after the end of each daily hidden platform session, as well as 1 day and 4 days after the last session of training (Fig. 3.3b). Percent time in the target quadrant during the first 20s of each probe trial was used as our measure of spatial memory. A repeated measures ANOVA, with age and virus as main factors, and probe trial as the repeated factor, revealed significant main effects of trial ($F_{5, 42} = 31.66$, $p < 0.0001$) and age ($F_{1, 42} = 23.63$, $p < 0.0001$), as well as a trial by age interaction ($F_{5, 42} = 2.61$, $p = 0.026$). Together, these results indicate that aged animals did not perform as well as young animals on probe trials. Since young animals treated with GFP and CREB performed comparably across all sessions of visible platform training ($F_{1, 12} = 0.50$, n.s.), hidden platform training ($F_{1, 12} = 1.17$, n.s.), and probe tests ($F_{1, 12} = 0.38$, n.s.) we collapsed the data from these animals into one "young" group for further analyses.

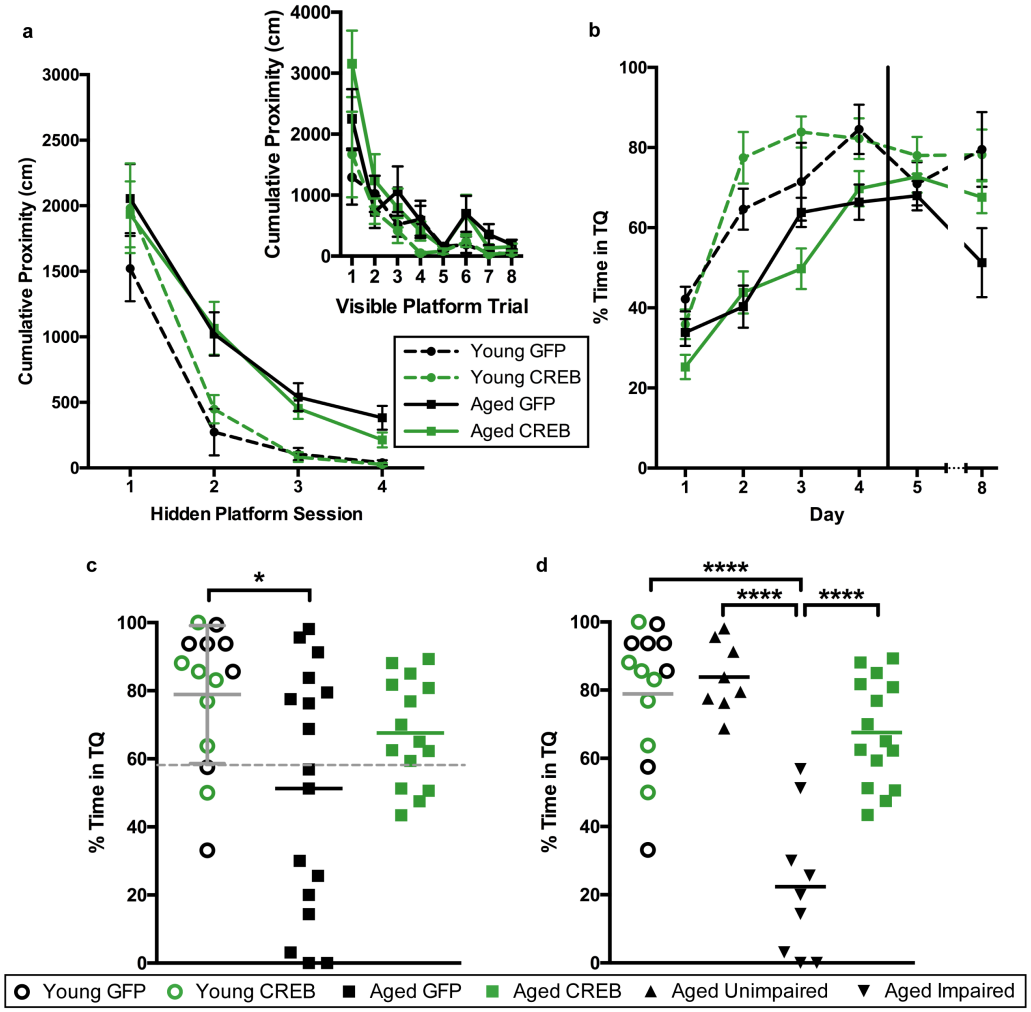


Figure 3.3. AAV-CREB ameliorates spatial memory deficits in aged animals. a) Performance on visible platform over 8 trials (inset), and hidden platform over 4 sessions of training (5 trials each session; 1 session per day) for young animals and aged animals that received control AAV-GFP, or AAV-CREB virus (n = 7, 7, 17, 15). All animals were capable of learning the visible and hidden platform task. While young animals performed better than aged on hidden platform training, no virus-based differences were observed. b) Performance of young and aged animals that received AAV-GFP or AAV-CREB on probe trials run 1 hr after each session of hidden platform training, as well as 1 day and 4 days after the end of training (n = 7, 7, 17, 15). A difference was found between young and aged animals, but no effect of virus was observed. c) Aged rats given control AAV-GFP vector were significantly impaired on memory for the platform, as measured by a probe test conducted 4 days after the end of hidden platform training. Dashed line indicates the average performance of young rats minus 1 SD. d) Aged rats classified as aged impaired (AI: n = 9) performed significantly worse on the probe test conducted 4 days after the end of the hidden platform training as compared to young adult (n = 14), aged unimpaired (AU: n = 8) and aged rats given AAV-CREB (n = 15). No significant differences were observed between young adult, AU and aged CREB rats. Results in a and b represent mean ± SEM, horizontal bars in c and d represent mean, c represents mean ± SD or mean only.

As upregulation of CREB has been shown to enhance long-term memory, we more closely examined performance during the last probe test, conducted four days after the last session of hidden platform training. A one-way ANOVA comparing performance between young, aged GFP, and aged CREB animals was significant ($F_{2, 43} = 4.45$, $p = 0.018$). Tukey's multiple comparisons test revealed that young animals spent significantly more time in the target quadrant compared to aged GFP ($p = 0.014$), but not compared to aged CREB (n.s., Fig. 3.3c). When a D'Agostino & Pearson omnibus normality test was run, we found young and aged CREB data to have a Gaussian distribution, while aged GFP did not (young n.s, aged CREB n.s., aged GFP $p = 0.045$). To determine if we had two groups within the aged GFP group, we separated the animals into two groups; AU and AI, using [young average - 1 standard deviation] as the cut off (grey dotted line, Fig. 3.3c). The two new aged GFP groups were each found to have Gaussian distributions (AU n.s., AI n.s.). Based upon these new behavioral groupings, we reanalyzed the day 4 probe data, and found a significant difference between the groups (one-way ANOVA: $F_{3, 42} = 23.78$, $p < 0.0001$, Fig. 3.3d). Tukey's multiple comparisons test revealed that the AI group was significantly different from each of the other groups: young ($p < 0.0001$), AU ($p < 0.0001$), and aged CREB ($p < 0.0001$), while young, AU, and aged CREB animals were not different from each other. These results indicate that in aged animals, AAV-CREB injection ameliorated the long-term memory deficits seen in AAV-GFP animals, to the point where their performance was no different to that of young animals.

Behavioral effects were due to CREB overexpression in CA1 only

Two to three weeks after the end of behavioral testing, animals were euthanized to collect tissue for RNA, and gather biophysical data. To verify that viral overexpression of CREB was limited to the CA1 sub-region, the three major hippocampal sub-regions were separated to examine their CREB mRNA levels. A two-way ANOVA revealed a main effect of virus on CREB mRNA levels in CA1 ($F_{1, 42}$ = 13.20, p = 0.0008, Fig. 3.4a). Bonferroni's multiple comparisons test indicated that AAV-CREB animals had significantly more CREB mRNA than those that had received control AAV-GFP virus in both young (p = 0.018), and aged animals (p = 0.037). We examined RNA from dentate gyrus and CA3 in a subset of animals. In dentate gyrus, a two-way ANOVA revealed no effect of virus ($F_{1, 16}$ = 0.065, n.s.), but did find an effect of age ($F_{1, 16}$ = 5.87, p = 0.028, Fig. 3.4b), indicating aged animals had less CREB mRNA in dentate gyrus, regardless of which virus they received. In CA3, no effect of age ($F_{1, 16}$ = 0.7330, n.s.) or virus (F (1, 16) = 0.36, n.s.) was found for CREB mRNA levels (Fig. 3.4c). These results indicate that any behavioral effects seen in these animals could be attributed to overexpression of CREB specifically in the CA1 sub-region of the hippocampus.

As hypothesized, injection of AAV-CREB rescued behavioral deficits in aged, but not young adult rats. However, it was not known if differences in basal level expression of CREB might underlie the behavioral differences. Therefore, CREB mRNA and protein levels were compared between the AU and AI animals (Fig. 3.5). Surprisingly there was no significant group difference in CREB mRNA levels between the AU and AI animals (p > 0.05). However, there was a significant positive correlation between CREB protein levels and performance on the last

day of training (probe 4: Fig. 3.6; $r = 0.78$, $p = 0.0002$). These data suggest that aged animals with higher basal levels of CREB protein may be better at learning a hippocampus dependent task than those with lower basal levels of CREB protein.

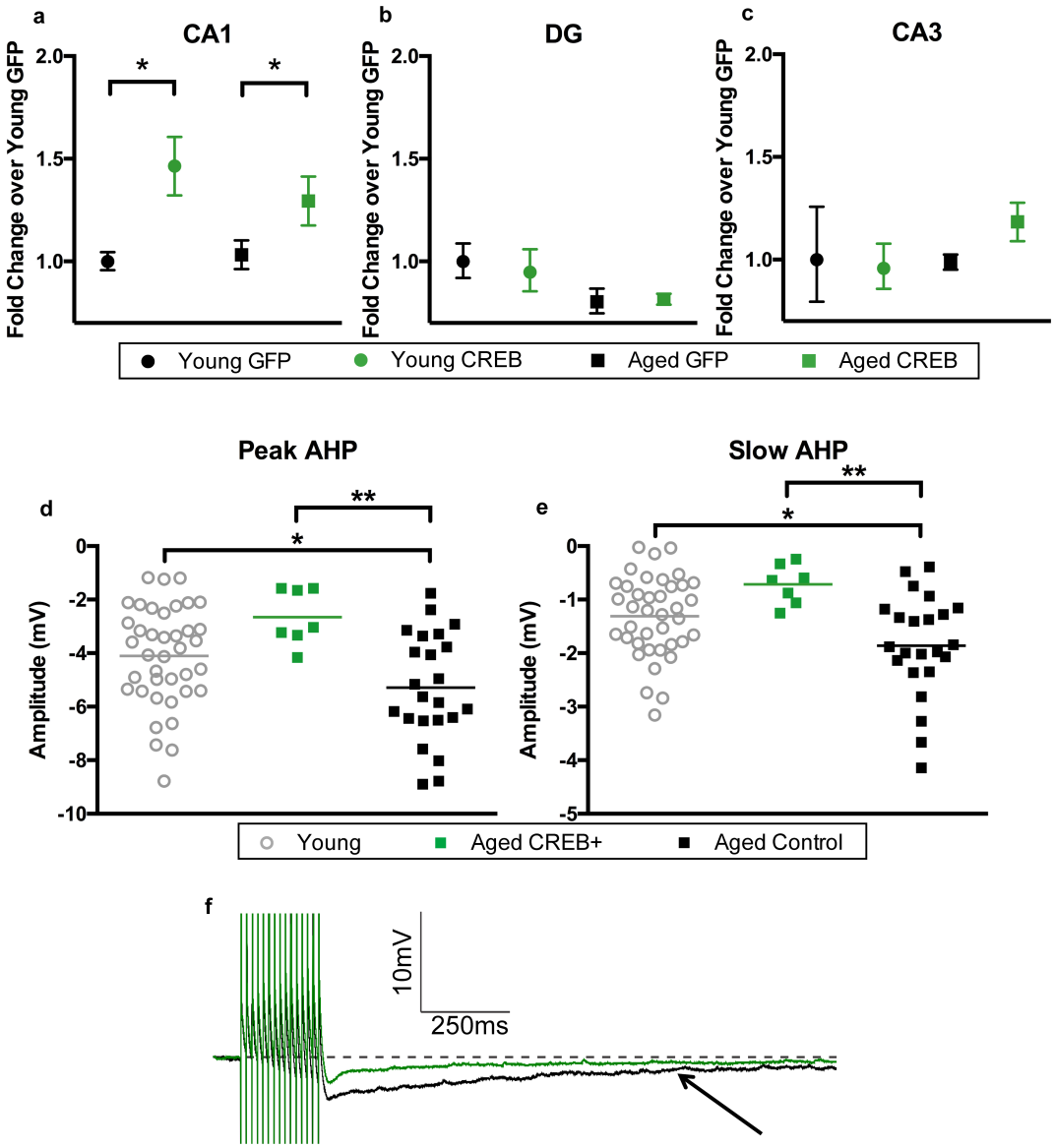


Figure 3.4. AAV-CREB results in higher CREB mRNA levels in CA1, and reduces AHP in infected aged animals. CREB mRNA levels relative to young GFP group in a) CA1, b) DG, and c) CA3. In both young and aged animals, AAV-CREB injected animals had more CREB mRNA in CA1 (n = 7, 7, 17, 15). This viral difference was not observed in DG (n = 4, 6, 4, 6) or CA3 (n = 4, 6, 4, 6). d) Peak postburst AHP is significantly reduced in CA1 pyramidal neurons (n = 7) from aged AAV-CREB animals as compared to control cells (n = 23) from aged animals. No significant differences were observed in peak postburst AHP between CA1 neurons from young adult (n = 39) and aged AAV-CREB animals. e) Similarly, the slow postburst AHP from CA1 neurons of young adult and aged AAV-CREB animals were significantly reduced as compared to control cells from aged animals. f) Example postburst AHP traces from aged CREB+ (green) and aged control (black) CA1 pyramidal neurons. Arrow indicates 1 s time point where slow postburst AHP was measured. Results in a, b, c represent mean ± SEM.

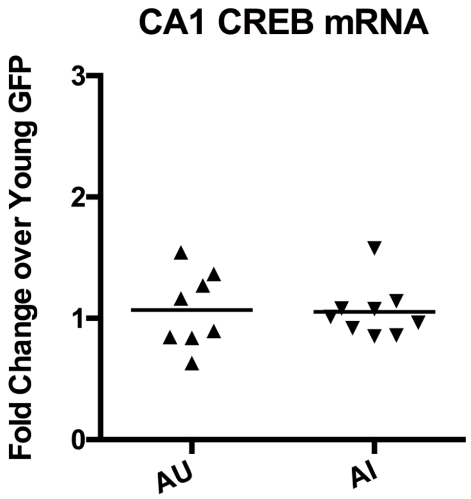


Figure 3.5 Aged unimpaired and impaired animals had the same amount of CREB mRNA. Upon separating the aged GFP group into AU and AI, CREB mRNA levels were not different between AU and AI animals. Horizontal bars represent mean.

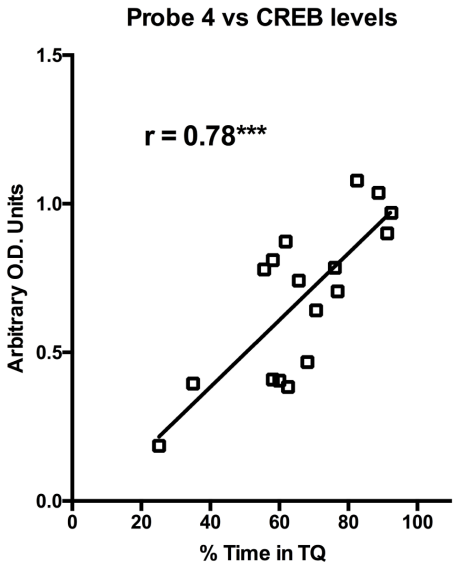


Figure 3.6. CREB protein levels are positively correlated with probe trial performance in aged GFP animals. Levels of CREB protein in CA1 of aged GFP animals are correlated with % time spent in target quadrant on a probe trial run 1 hour after the last training session. Aged rats with more basal levels of CREB protein performed better on the probe trial.

AAV-CREB infection reduced AHP amplitudes in aged neurons

In addition to the increase in CREB mRNA levels, we hypothesized that CREB overexpression would result in increased intrinsic excitability in the CA1 pyramidal neurons infected by AAV-CREB (Yiu et al., 2011; Zhou et al., 2009). Whole-cell current-clamp recordings were made from CA1 pyramidal neurons held near -69 mV. Specifically, the postburst AHP was measured following a train of 15 suprathreshold current injections. We compared three different groups of cells from young and aged animals; AAV-CREB infected cells (CREB+), their neighboring uninfected cells (CREB-), and cells from AAV-GFP injected animals. Two-way ANOVAs with main factors of age and cell type revealed similar changes in both the peak and slow AHPs: significant effect of cell type ($F_{2, 63} > 4$, p's < 0.05), but not of age ($F_{1, 63} < 1$, n.s.) (Fig. 3.7, Tables 3.1, 3.2). Tukey's multiple comparisons test revealed that aged CREB+ cells had significantly smaller peak and slow AHPs than both aged CREB- and aged GFP cells. Importantly, no differences were found between any of the young cell types (Fig. 3.7a, b), or between aged CREB- and GFP (Fig. 3.7c, d). Therefore, postburst AHP data for all young cells were combined into one group, and the postburst AHP data for aged CREB- and GFP cells were combined into one aged control group. Analyses revealed significant differences of the postburst AHP in the three groups of cells: one-way ANOVA for peak AHP, $F_{2, 66} = 6.222$, $p = 0.0033$; for slow AHP $F_{2, 66} = 6.541$, $p = 0.0026$ (Fig. 3.4d, e). Furthermore, Tukey's multiple comparisons test revealed that the postburst AHP in aged controls (peak, -5.29 ± 0.42 mV: slow, -1.86 ± 0.20 mV) were significantly larger than those from young (peak, -4.10 ± 0.30 mV: slow -1.31 ± 0.12 mV) and aged CREB+ (peak, -2.65 ± 0.39 mV: slow, -0.71 ± 0.14 mV) cells. Importantly, young and aged CREB+ were not statistically different from each other. Passive

membrane properties (such as input resistance and resting membrane potential) were not significantly different across groups of neurons (Tables 3.3, 3.4). These data indicate that in aged animals, cells infected with AAV-CREB were found to be significantly more excitable than control cells, to the extent where they were no different from young cells.

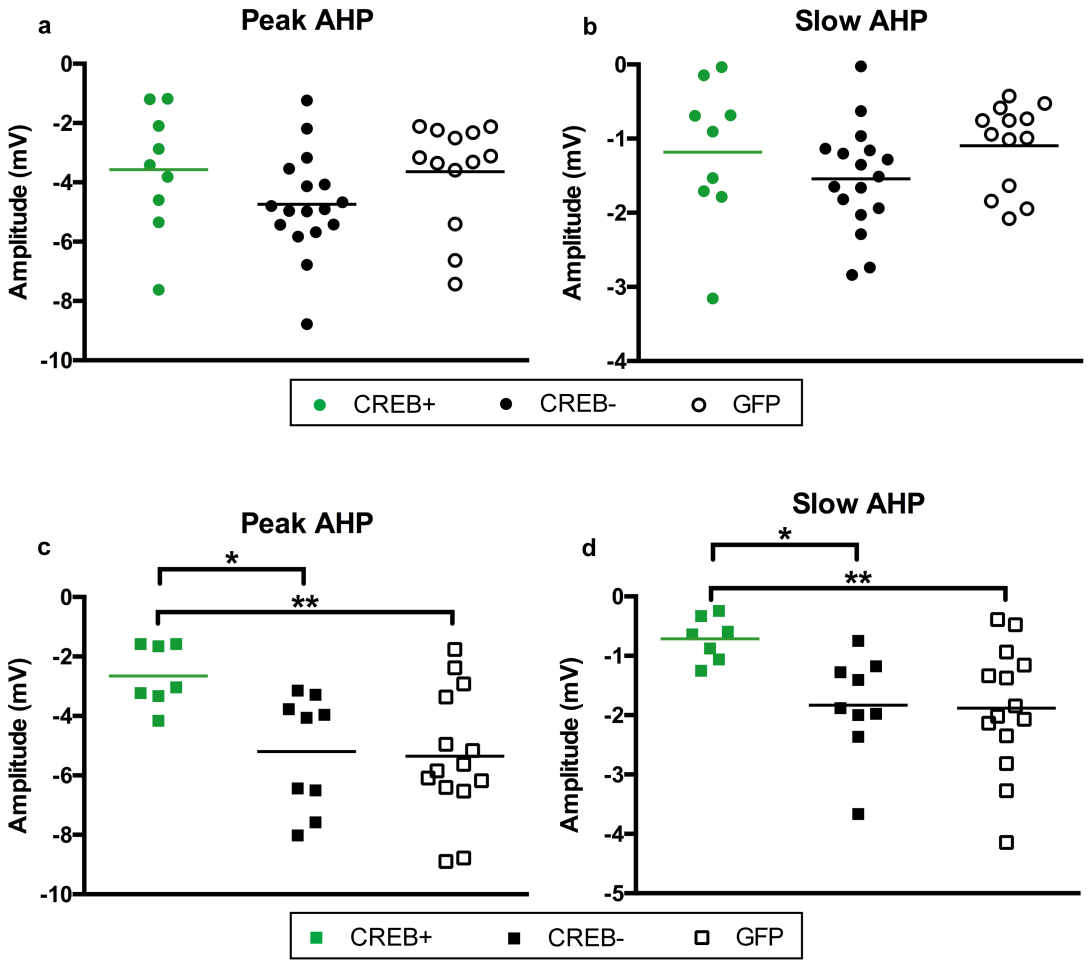


Figure 3.7. Viral infection state had no effect on postburst AHP size in young CA1 pyramidal neurons, but did affect aged neurons. a) Peak and b) slow AHP were measured in AAV-CREB infected cells (CREB+), AAV-CREB uninfected cells (CREB-), and cells from AAV-GFP injected young animals (n = 9, 17, 13). For both measurements, no differences were observed between the three groups. c) Peak and d) slow AHP were measured in AAV-CREB infected cells (CREB+), AAV-CREB uninfected cells (CREB-), and cells from AAV-GFP injected aged animals (n = 7, 9, 14). For both measurements, while CREB+ cells had significantly smaller amplitudes when comparing to both CREB- and GFP cells, the CREB- and GFP cells were not different from each other. Horizontal bars represent mean.

Table 3.1. Peak post-burst AHP amplitudes (mV)

	CREB+	CREB-	GFP
Young	-3.57 ± 0.70 (n = 9)	-4.74 ± 0.42 (n = 17)	-3.64 ± 0.49 (n = 13)
Aged	-2.66 ± 0.39 (n = 7)	-5.20 ± 0.64* (n = 9)	-5.35 ± 0.58** (n =14)

Aged CREB+ cells have smaller peak AHP's than CREB- and GFP, but young cell types do not differ from each other. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$ compared to aged CREB+.

Table 3.2. Slow post-burst AHP amplitudes (mV)

	CREB+	CREB-	GFP
Young	-1.18 ± 0.33 (n = 9)	-1.54 ± 0.17 (n = 17)	-1.09 ± 0.16 (n = 13)
Aged	-0.71 ± 0.14 (n = 7)	-1.83 ± 0.28* (n = 9)	-1.88 ± 0.28** (n = 14)

Aged CREB+ cells have smaller slow AHP's than CREB- and GFP, but young cell types do not differ from each other. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$ compared to aged CREB+.

Table 3.3. Resting Membrane Potential (mV)

	CREB+	CREB-	GFP
Young	-62.19 ± 2.28 (n = 9)	-62.04 ± 2.08 (n = 17)	-64.16 ± 2.39 (n = 13)
Aged	-58.23 ± 2.96 (n = 7)	-64.74 ± 1.50 (n = 9)	-60.20 ± 3.28 (n = 14)

Resting membrane potential does not vary across different groups of cells. A two-way ANOVA revealed no significant effect of age ($F_{1, 63} = 0.61$, n.s.) or of cell type ($F_{2, 63} = 0.61$, n.s.). Data represent mean ± SEM.

Table 3.4. Input Resistance (MΩ)

	CREB+	CREB-	GFP
Young	47.75 ± 5.18 (n = 9)	53.51 ± 4.02 (n = 17)	64.59 ± 10.95 (n = 13)
Aged	51.68 ± 4.84 (n = 7)	73.92 ± 11.40 (n = 9)	57.40 ± 5.58 (n = 13)

Input resistance does not vary across different groups of cells. A two-way ANOVA revealed no significant effect of age ($F_{1, 63} = 0.83$, n.s.) or of cell type ($F_{2, 63} = 1.61$, n.s.). Data represent mean ± SEM.

AAV-CREB ameliorated age-related spatial memory deficits; more difficult water maze

Unlike previous studies (Brightwell et al., 2007; Josselyn et al., 2001; Sekeres et al., 2010), we did not observe any difference in water maze performance between our young AAV-GFP and AAV-CREB injected animals. In past studies, animals were trained on difficult versions of the tasks, where control animals (i.e., those given control viral injections) displayed little long-term memory, but memory in animals overexpressing CREB was strongly facilitated. In the experiments described in Figure 3.2, we trained the rats over 4 sessions (1 session/day) with 5 trials per session. This training regimen may have been 'less challenging' and produced a ceiling effect in our young animals, where no further improvements could be observed with increased CREB levels. Therefore, a new group of rats were trained using a more difficult training protocol, to determine whether AAV-CREB could facilitate learning and/or memory in young animals.

Young and aged rats were infused with AAV-CREB (8 young and 8 aged) or a control AAV-GFP virus (7 young and 15 aged) in the dorsal CA1 region. Two weeks after AAV infusion, animals were trained with the visible platform procedure, which was performed exactly as we described earlier. Repeated measures ANOVA, with age and virus as the main factors and trial as the repeated factor, revealed significant effects of trial ($F_{7, 34} = 10.61$, $p < 0001$) and of age ($F_{1, 34} = 4.06$, $p = 0.052$), but not of virus ($F_{1, 34} = 0.40$, n.s.), with no significant interactions between any of the factors. All animals successfully reached the visible platform on the last trial, and therefore no animals were excluded from further training (Fig. 4a, inset).

Three days after visible platform training, animals were trained with the hidden platform task. Rather than train animals with 5 trials per each daily session, as we did previously, we trained each animal with 3 trials per day, over 4 consecutive days (Fig. 3.8a). The inter-trial interval (ITI) was also changed. Previously the ITI was 19 ± 1 min, and we reduced that to 1 min (Josselyn et al., 2001; J. C. J. Yin et al., 1995). As expected, young animals acquired the task more quickly than aged animals, however there were no differences amongst viral groups for either age group. A repeated measures ANOVA, with age and virus as the between groups measures, and session as the repeated measure, revealed significant main effects of session ($F_{3, 34}$ = 26.24, $p < 0.0001$) and of age ($F_{1, 34}$ = 11.85, $p = 0.0015$), but not of virus ($F_{1, 34}$ = 0.001, n.s.), with no significant interactions between any of the factors.

To assess memory for the platform location, all animals underwent probe trials 1 hour after the end of each daily hidden platform session (Fig. 3.8b). A repeated measures ANOVA, with age and virus as main factors, and probe trial as the repeated factor, revealed significant main effects of trial ($F_{3, 34}$ = 13.05, $p < 0.0001$), but not of age ($F_{1, 34}$ = 3.31, n.s.) or virus ($F_{1, 34}$ = 0.85, n.s.). However, a trial by age interaction was observed ($F_{3, 34}$ = 3.15, $p = 0.028$). Together, these results indicate that aged animals did not perform as well as young animals across the probe trials. Since young animals treated with GFP and CREB performed almost identically across all sessions of visible platform training ($F_{1, 7}$ = 2.039, n.s.), hidden platform training ($F_{1, 13}$ = 0.03, n.s.), and probe trials ($F_{1, 13}$ = 0.01, n.s.) we once again collapsed the data from these animals into one “young” group for further comparisons against aged animals.

As with the first set of animals, we split the aged GFP group into AU and AI. The cut off (grey dotted line, Fig. 3.8c) was determined using young animals' probe trial performance on the last day [young average – 1 standard deviation]. Two young animals (one GFP and one CREB) did not successfully learn the task, i.e. performed below chance on this probe trial, and were therefore omitted from this calculation. Upon reanalysis of the probe 4 data, we found a significant difference between the groups (one-way ANOVA: $F_{3, 34} = 6.36$, $p < 0.002$, Fig. 3.8d). Tukey's multiple comparisons test revealed that the AI group was significantly different from each of the other groups [young ($p = 0.0024$), AU ($p = 0.0034$), and aged CREB ($p = 0.028$)], while young, AU, and aged CREB animals were not different from each other. Despite being trained on a more challenging protocol for water maze the young CREB group still performed comparably to the young GFP group. However, in aged animals, AAV-CREB injection ameliorated long-term memory deficits seen in the AAV-GFP group, to the point where their performance was no different to that of young animals.

To confirm this protocol was more challenging, we compared the behavioral performance by groups on the two different versions. Given that we saw a significant effect of age, but not of virus for both tasks, the young were collapsed into one group, and the aged were collapsed into a second group for each version. A two-way ANOVA (training protocol as the between groups measure; hidden platform training session as the repeated measure) revealed significant main effects of session ($F_{3, 81} = 36.89$, $p < 0.0001$) and of training protocol ($F_{1, 27} = 4.36$, $p = 0.047$). This indicates that as expected, young animals found it more difficult to acquire the task when trained on the more difficult training protocol. The same effects were found for performance on

probe trials; a two-way ANOVA revealed a significant main effect of probe trial ($F_{3, 81}$ = 44.00, $p < 0.0001$), and of training protocol ($F_{1, 27}$ = 18.37, $p = 0.0002$). Aged animals also performed worse on hidden platform training and probe trials. A two-way ANOVA revealed a significant main effect of hidden platform training session ($F_{3, 159}$ = 55.28, $p < 0.0001$), and of training protocol ($F_{1, 53}$ = 24.40, $p < 0.0001$). A two-way ANOVA for probes revealed a significant main effect of probe trial ($F_{3, 159}$ = 26.93, $p < 0.0001$), and of training protocol ($F_{1, 53}$ = 21.35, $p < 0.0001$). These results reveal that both young and aged animals exhibited worse performance on both the learning and memory components of the more difficult water maze task, indicating that the lack of AAV-CREB facilitation of young animals' performance was not due to a ceiling effect.

One hour after the final probe trial, the animals were euthanized to collect tissue for RNA. To verify that CREB overexpression occurred, the CA1 sub-region was examined for CREB mRNA levels. A two-way ANOVA revealed a main effect of virus on CREB mRNA levels in CA1 ($F_{1, 34}$ = 84.24, $p < 0.0001$). Bonferroni's multiple comparisons test indicated that AAV-CREB animals had significantly more CREB mRNA than those that had received control AAV-GFP virus in both young (AAV-CREB 2.34 ± 0.27 fold change over AAV-GFP 1.05 ± .09; $p < 0.0001$), and aged animals (AAV-CREB 2.23 ± 0.18 fold change over AAV-GFP 1 ± .07$p < 0.0001$). These data indicate that as shown previously, the behavioral effects seen in this second group of aged animals could also be attributed to the overexpression of CREB.

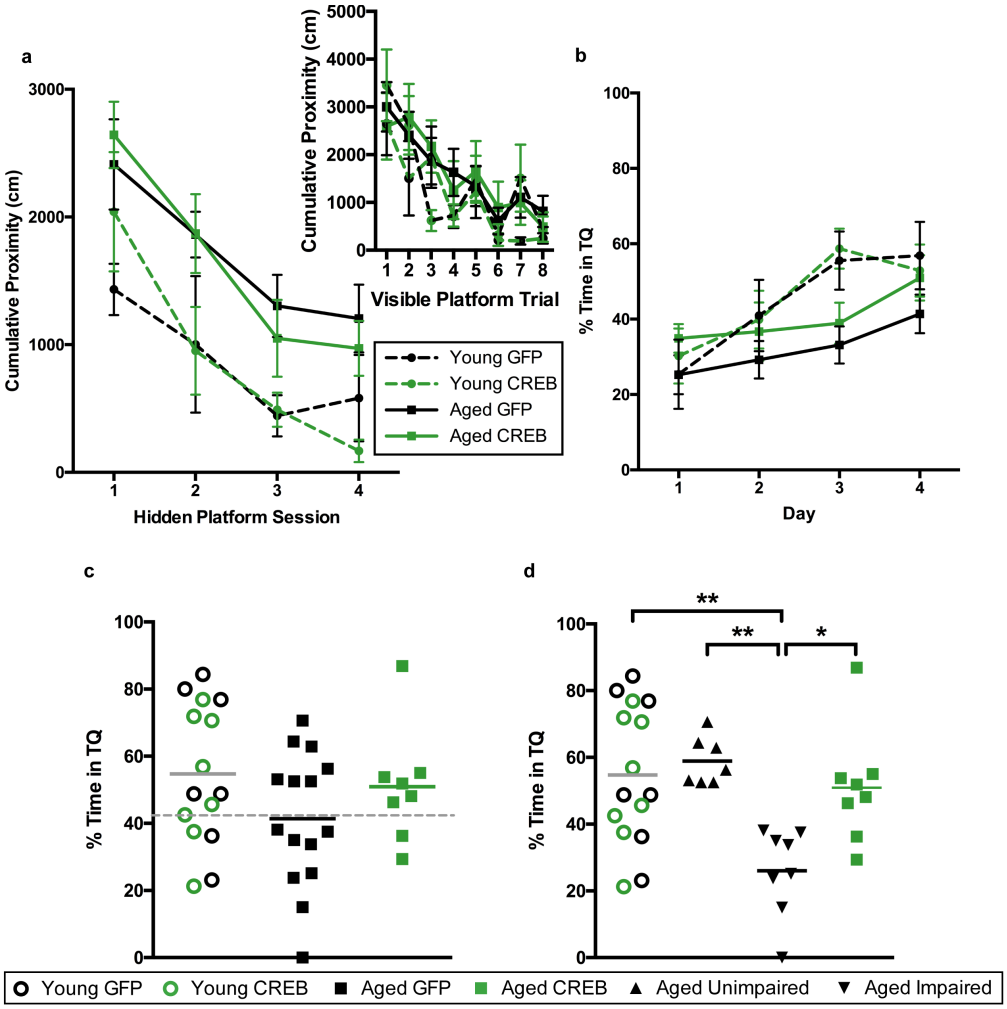


Figure 3.8. AAV-CREB ameliorates age-related spatial memory deficits on a more difficult water maze task. a) Performance on visible platform over 8 trials (inset), and hidden platform over 4 sessions of training (3 trials each session; 1 session per day) for young animals and aged animals that received control AAV-GFP, or AAV-CREB virus (n = 7, 8, 15, 8). All animals were capable of learning the visible and hidden platform task. While young animals performed better than aged on hidden platform training, no virus-based differences were observed. b) Performance of young and aged animals that received AAV-GFP or AAV-CREB on probe trials run 1 hr after each session of hidden platform training (n = 7, 8, 15, 8). A difference was found between young and aged animals, but no effect of virus was observed. c) Aged rats given control AAV-GFP vector trended towards worse performance on a probe test conducted 1 hour after the last hidden platform training session. Dashed line indicates the average performance of young rats that successfully learned minus 1 SD. d) Aged rats classified as aged impaired (AI: n = 8) performed significantly worse on the probe test conducted on day 4 of hidden platform training as compared to young adult (n = 15), aged unimpaired (AU: n = 7) and aged rats given AAV-CREB (n = 8). No significant differences were observed between young adult, AU and aged CREB rats. Results in a and b represent mean ± SEM, horizontal bars in c, d represent mean.

DISCUSSION

This is the first study to directly increase CREB levels in aged animals, and as a result, rescue their age-related cognitive deficits. Administration of AAV-CREB resulted in widespread infection of dorsal CA1 in young and aged rats, which specifically increased CREB mRNA levels in dorsal CA1. Importantly, this increase in CREB was sufficient to ameliorate age-related long-term spatial memory deficits. Furthermore, as predicted, aged pyramidal neurons infected by AAV-CREB had reduced post-burst AHPs, indicating that infected cells were more excitable. These data reaffirm that aging-related processes increase the AHP in CA1 neurons, and leads to decreased excitability that contributes to memory impairment. Increasing CREB levels is sufficient to reverse these processes and normalize memory function. Our data is clear, and is consistent with some previously reported work. However, it is at odds with other reports, and we suspect that a number of different experimental variables contribute to this inconsistency in the literature.

Aged animals, as a group, displayed robust spatial memory deficits. However, not every aged animal was impaired. Nearly all aged CREB animals, and a subset of aged GFP rats were able to learn and remember both the less challenging, and more difficult task at young-like levels. These results are consistent with those of earlier studies, indicating that approximately half of aged rats are impaired on this task (Disterhoft and Oh, 2006; Gallagher et al., 1993). With that said, our aged control animals could be separated into those with impaired long-term spatial memory, and those without impaired long-term spatial memory, whereas aged animals receiving AAV-CREB were much more homogenous, consistently performing at, or near, young-like

levels. We found water maze performance by our young and, presumably AU animals were not improved with AAV-CREB, which is supported by results of a prior study which revealed that viral administration of CREB-Y134F improved fear memory in young "poor learners", with no improvement in young "good-learners" (Cowansage et al., 2013). These findings are also supported by a study by Brightwell et al. (2004), which found that AI animals trained on water maze had lower levels of CREB protein than young animals and AU animals (2004). Together, these previous findings and our present results suggest that lower levels of CREB protein in AI animals contributes to their cognitive deficits, and increasing CREB in these animals can rescue their behavioral deficits. This rescue by CREB overexpression was most likely mediated by increased levels of pCREB. Despite reports of decreased CREB activation with aging, via increases in calcineurin (Foster 2001) and decreases in PKA (Karege 2001), we hypothesize that increasing the absolute levels of their substrate, CREB, would lead to an increase in the levels of pCREB and CREB activity. However, the detailed mechanistic relationship between the levels of CREB, pCREB, and CREB activity is unlikely to be straight forward (Briand et al., 2015).

A second parameter that might contribute to experimental variability is the nature of the CREB protein that is overexpressed. We found that CREB overexpression increased the intrinsic excitability of aged CA1 neurons. The AHPs from AAV-CREB infected neurons were reduced when compared to neighboring uninfected cells, or GFP-injected controls, as previously reported (Yiu et al., 2011; Zhou et al., 2009). These results mirror those from studies using pharmacological methods where treatment that reduced the AHP also facilitated cognitive performance in aged animals (Deyo et al., 1989; Kronforst-Collins et al., 1997; Moyer et al.,

1992; Oh et al., 1999; Weible et al., 2004). Interestingly, we found that CREB overexpression had no observable effect on the size of AHPs in young neurons. Previous studies using partially-active CREB-Y134F or VP16-CREB have demonstrated their ability to facilitate long-term potentiation and reduce the AHP in cells from young animals (Lopez de Armentia et al., 2007; Suzuki et al., 2011; Yu et al., 2016). The increase in downstream transcription is likely to be much greater when a partially activated form of CREB, as opposed to wild-type, is used. This may in part explain why we did not see any effect of virus on AHP amplitude in cells from young animals.

A third variable is the excitability state of the animal at the time of training. Studies using CREB overexpression in young animals have produced mixed results. Zhou et al. (2009) used HSV-CREB to overexpress CREB in amygdala of mice, which resulted in a smaller AHP amplitude when measured 300 ms after the final action potential, as compared to neighboring control cells. Peak AHP was not found to be different (2009). Another study transfected primary mice hippocampal neurons to overexpress CREB and found that transfected cells had increased excitability (Yiu et al., 2014). Lastly, when HSV-CREB was injected into locus coeruleus of rats, excitability was unchanged, as compared to rats injected with control virus (M.-H. Han et al., 2006). These varying results may be due to differences in slice vs. cultured neurons, mouse vs. rat tissue, or even due to differing brain regions, but we suspect that the "excitability state" of the animals is a major uncontrolled determinant. In our hands, CREB overexpression was not sufficient to cause any differences in peak or slow AHP amplitude in CA1 neurons from young animals. Although we saw similar levels of CREB overexpression in both age groups, only the

aged animals exhibited a change at the biophysical level. It is possible that while the young animals are already at an "optimal state", and have sufficient excitability or plasticity for learning and memory, the aged animals are in a "sub-optimal state", and therefore have room for improvement. This effect has been demonstrated before, where systemic application of L-type calcium channel blocker nimodipine (Deyo et al., 1989) or cholinesterase inhibitor galantamine (Weible et al., 2004) was beneficial for aged animals, but had no effect on young. Similarly, exposure to environmental enrichment, which can indirectly lead to increased CREB activation (Hu et al., 2013), reduced the AHP in neurons from aged animals, but not young (Kumar and Foster, 2007). For potential therapeutic purposes, this may be a desirable trait, so as to only affect those who have room for improvement.

Given our biophysical results, it is not surprising that AAV-CREB (vs. AAV-GFP) injections had no effect on any aspect of young animals' water maze performance. We have demonstrated that viral overexpression of CREB was achieved in these young animals, both at the mRNA and protein levels. Overexpression of wild-type rat CREB, as opposed to a mutated form of CREB, may partly explain the lack of behavioral effect. Expression of VP16-CREB in young animals has led to transient facilitation of eye-blink conditioning (Gruart et al., 2012), as well as facilitation of memory for contextual and cued fear (Viosca et al., 2009a). Additionally, expression of a CREB-Y134F in young animals has given enhanced memory for both fear conditioning and water maze (Restivo et al., 2009; Suzuki et al., 2011). These studies suggest that the increase in CREB expression we observed with our AAV-CREB was not sufficient (when compared to that achieved by mutated forms of CREB) to facilitate the behavioral

performance of young animals. However, some studies have shown facilitation of fear conditioning as a result of HSV overexpression of wild-type CREB in the amygdala (Josselyn et al., 2001; Sekeres et al., 2010) . In these studies, animals underwent weak training, where control animals failed to form a memory, but animals overexpressing CREB were able to do so. In the current study, we initially used a less challenging version of the water maze task, and delivered 4 sessions of training with 5 trials per session. We suspected this "strong" training may have produced a ceiling effect in our young animals, where increasing CREB activity could not lead to further improvements. Thus, we injected a new group of animals with AAV-GFP and AAV-CREB, and trained them on a more difficult version of the task, to determine if young CREB animals' performance would then be facilitated. The difficult version did prove more challenging, with both young and aged animals taking longer to learn the task, and spending less time in the target quadrant across probe trials as compared to the less challenging version. Interestingly, the pattern of behavioral results was very similar to that observed during the less challenging task: CREB mediated rescue in aged rats, but had no effect in young rats. This suggests that the difference between the viral overexpression of HSV and AAV may be at play. HSV is known to give strong, but transient gene expression, while AAV tends to provide more subtle, but stable expression over long periods of time (Neve et al., 2005). These data indicate that strong CREB overexpression, such as in the case of HSV, or a mutant form of CREB is required to achieve behavioral facilitation in young adult animals, given that they are already at an "optimal learning state".

Notably, CREB mRNA levels were not related to behavioral performance. Young GFP and CREB groups whose behavior did not differ showed differing CREB mRNA levels; while AU and AI groups, who by definition differ in behavior, had the same levels of CREB mRNA. However, levels of CREB protein and pCREB have repeatedly been shown to be related to behavior (Brightwell et al., 2004; Cowansage et al., 2013; Josselyn et al., 2001; Pittenger et al., 2002; Viosca et al., 2009a). Importantly, we provide evidence that basal level of CREB protein is correlated to successful learning in aged rats (Fig 3 - figure supplement 2). Together, these findings suggest that CREB mRNA and protein levels are not directly related to each other. Moreover, while CREB mRNA levels served to confirm viral overexpression, only CREB protein levels were predictive of behavioral performance.

Interestingly, CREB mRNA levels did not differ between young and aged animals in CA1, but it was reduced in DG of aged animals. While this is an important observation, given the lack of relationship between CA1 CREB mRNA levels and behavior, our focus continues to be on the CA1 sub-region. We chose to target this sub-region of the hippocampus as it has exhibited biophysical deficits with age (Bach et al., 1999; Disterhoft and Oh, 2007; Tombaugh et al., 2005). Additionally, CA1 has been targeted to successfully facilitate memory in young animals with CREB overexpression (Sekeres et al., 2010). In our present study, targeting the CA1 sub-region has successfully enhanced intrinsic excitability of aged pyramidal neurons, with a corresponding amelioration of age-related memory deficits.

The postburst AHP is influenced by numerous manipulations. One of these is

successful learning of a behavioral task, where animals that learn exhibit smaller AHPs than those who do not (Oh and Disterhoft, 2015; Moyer et al., 1996). However, the learning-related reduction in postburst AHP is transient, and AHP amplitudes return to baseline within two weeks after the end of behavioral training (Moyer et al., 1996; Thompson et al., 1996; Tombaugh et al., 2005). All biophysical recordings in this study were made between two to three weeks after the end of behavioral testing in order to measure the baseline AHP, and to avoid any learning-induced changes in the AHP. However, this still raises the question of how CREB overexpression resulted in the reduction of the AHP. CREB may modulate the AHP by indirectly influencing the levels or activity of AHP channels via one or more of CREB's downstream transcriptional targets. Alternatively, it is possible that CREB could act on AHP channels via a previously unknown non-transcriptional mechanism. Elucidating this mechanism would be of great interest. Despite not fully understanding the mechanisms involved, the consequences of CREB's enhancement of neuronal excitability have been examined. Neurons that have increased CREB activity become more excitable (Lopez de Armentia et al., 2007), and are then preferentially recruited to form new memories (J.-H. Han et al., 2007; Yiu et al., 2014; Zhou et al., 2009).

While previous studies have manipulated factors upstream of CREB, such as cAMP levels (Bach et al., 1999), this is the first study to directly test CREB's role in age-related cognitive deficits. Previous studies have shown increases in calcineurin with aging (Foster et al., 2001), decreases in PKA, (Foster et al., 2001; Karege et al., 2001), and AI animals have been shown to have reduced CREB protein (Brightwell et al., 2004). All of these could result in

decreases in CREB function, but only one study has manipulated CREB levels with aging. Mouravlev and colleagues (2006) did a longitudinal study revealing that overexpressing wild-type CREB in young rats prevents age-related cognitive deficits from arising later in life. However, it was still unclear whether CREB would be able to reverse the age-related deficits after they arose. In our present study, CREB overexpression alone was sufficient to rescue the long-term memory deficits in aged animals. We hypothesize that CREB's effects on behavioral performance may have been via the normalization of the previously reported deficits in CREB activity/function. This hypothesis is supported by our finding that CREB protein levels strongly correlate with performance on probe 4, where aged animals with the lowest levels of CREB protein performed the worst. This suggests that aged animals with low CREB protein levels are more likely to be AI. On the other hand, aged animals with high CREB protein levels, either naturally or due to AAV-CREB infusions, are more likely to perform at young-like levels. Alternatively, it is also possible that CREB overexpression may have compensated for age-related deficits via other mechanisms. Possibilities include CREB's ability to modulate synaptic plasticity (Benito and Barco, 2010), and to induce preferential recruitment of neurons to form a new memory via increasing intrinsic excitability (J.-H. Han et al., 2007; Yiu et al., 2014; Zhou et al., 2009).

In addition to these findings contributing to a better understanding of the aging process, other studies have shown that CREB and its activation are also negatively impacted in Alzheimer's disease. Amyloid –beta treatment reduces CREB levels and can cause it to be retained in the cytoplasm (Arvanitis et al., 2007; Pugazhenthi et al., 2011), while high BACE-1

levels reduce CREB activation (Chen et al., 2012), and CREB-mediated gene expression is aberrant in brains of Alzheimer's patients (Satoh et al., 2009). A recent study has also shown that Tau accumulation reduces CREB activity by increasing calcineurin levels (Y. Yin et al., 2016). Together, these results suggest that impaired, or sub-optimal CREB function in aging may make aged individuals more vulnerable to Alzheimer's disease. Furthermore, this indicates that CREB may not only be targeted for amelioration of cognitive deficits seen in normal aging, it could also be an important pharmacological target in the prevention or treatment of Alzheimer's disease.

ACKNOWLEDGEMENTS

This work was supported by the Northwestern University NUSeq Core Facility. Imaging work was performed at the Northwestern University Center for Advanced Microscopy generously supported by NCI CCSG P30 CA060553 awarded to the Robert H Lurie Comprehensive Cancer Center. Behavioral assays were performed at the Northwestern University Behavioral Phenotyping Core Facility.

We thank Dr. Shoai Hattori for writing custom Matlab routines to analyze biophysics data and Dr. Corrina Burger for the generous gift of the CREB and control constructs. This work was supported by NIH grants R37 AG008796 (JFD), RF1 AG017139 (JFD), R01 NS063245 (JCPY), T32 AG020506 (DMC and XY), P30 AG13854 (DMC) and the Glenn/AFAR Scholarship for Research in the Biology of Aging (XY).

Chapter 4 – Comparing genes, proteins, and neuronal excitability in naïve and virally-infected groups

ABSTRACT

While comparisons have typically been made between young and aged animals, or control vs. active virus, few studies compare virally-injected animals to their un-injected counterparts. This chapter characterizes differences in these groups at the protein and mRNA level. Furthermore, after not seeing changes to excitability of young neurons overexpressing wild-type CREB (Chapter 3), we tested if it was possible to increase their excitability using the partially-active VP16-CREB. We found that, similar to results from Chapter 2, behaviorally trained viral animals had lower levels of CREB and some of its downstream target genes than naïve animals. Our biophysical experiments also revealed that strong activation of CREB-mediated transcription, such as that caused by VP16-CREB is indeed sufficient to increase excitability of neurons from young rats.

INTRODUCTION

The previous two chapters compared young and aged animals, and control and CREB virally-injected animals, respectively. These are the commonly seen comparisons that are made in the literature (Brightwell et al., 2007; Josselyn et al., 2001; Restivo et al., 2009; Sekeres et al., 2010; Suzuki et al., 2011). However, it is fairly uncommon to see comparisons made between virally naïve animals, animals that received control virus, and animals that received CREB virus. This chapter examines the question of whether viral infection in CA1, or viral surgery might cause

changes compared to animals that never underwent surgery. This was examined at both the protein level, using western blotting for CREB and pCREB, and the mRNA level for CREB and a few of its downstream target genes.

Additionally, the biophysical results from Chapter 3 were not congruent with that of previous studies in young animals. It had been shown that viral overexpression of CREB was sufficient to reduce the AHP amplitude (Zhou et al., 2009), and increase firing rate (Yiu et al., 2014). However, overexpression of wild-type CREB using our AAV did not produce an increase in excitability in neurons from young animals. To ensure that the lack of increased excitability with AAV-CREB was not due to technical reasons, we virally expressed another construct, VP16-CREB, in the CA1 region of young animals.

MATERIALS AND METHODS

Hippocampal Sections

Two weeks after the end of behavioral testing or viral injection, rats were euthanized to collect hippocampal tissue. Western blotting and RT-PCR experiments used tissue from rats from Chapter 3. Biophysics experiments were carried out on a new cohort of rats. After anaesthetizing the animals with isoflurane, they were rapidly decapitated, their brains were removed, and placed into ice-cold artificial cerebrospinal fluid (aCSF, in mM): 124 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 25 glucose, 2.4 $CaCl_2$, pH 7.4, oxygenated with 95%:5% O_2:CO_2. A sucrose-aCSF was used for brains from aged rats (in mM): 206 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.1 $CaCl_2$, 3 $MgSO_4$, and 25 glucose. Hippocampi were then

extracted, and 300 μm transverse sections cut on a Leica VT1000s Vibratome. Sections were then used immediately for biophysical measurements, directly frozen for western blotting, or frozen in RNAlater (Qiagen) for RT-PCR experiments.

Hippocampal microdissection

The major hippocampal sub-regions (CA1, CA3, and dentate gyrus) were manually dissected from dorsal hippocampus slices that were either flash-frozen for western blotting, or frozen in RNAlater for RT-PCR. As previously described (Coultrap et al., 2005; Núñez Santana et al., 2014), under a Stemi DV4 dissecting microscope (Zeiss) and on ice, a straight cut was first made in front of the blades of the dentate gyrus, to isolate the CA3 sub-region from each slice. The dentate gyrus was then peeled away from the CA1-subiculum, and the subiculum severed from CA1 and discarded. Tissue from each sub-region were immediately then frozen again before further processing.

Western Blotting

Two weeks after the end of water maze probe tests, rats (from Chapter 3) were euthanized and 300 μm slices were made from dorsal hippocampus (see above). Five to six slices were flash-frozen on dry ice for western blotting. The three major hippocampal sub-regions (CA1, CA3, and DG) were manually isolated as described above. Tissue from CA1 was lysed in RIPA buffer (150 mM NaCl, 25 mM Tris, 1 mM EDTA, 0.1% SDS, 1 % Triton X-100, and 0.5 % sodium deoxycholate) containing protease and phosphatase inhibitors (Pierce). Each sample was manually dissociated with a syringe, then shaken at 4 °C for 30 min. All samples were then

centrifuged at 14,000 g for 15 min. The supernatants were retained and protein concentration measured by BCA assay (Pierce). Fifteen micrograms of each sample was boiled in 2x Laemmli buffer containing 5 % β-mercaptoethanol (Bio-Rad). Samples were separated using 4-20 % Mini Protean TGX gels (Bio-Rad), and transferred to Immobilin-P PVDF membranes (Millipore). Blots were first probed for pS133CREB (#9189, 1:1,000, Cell Signaling, Beverly, MA), and detected using an anti-rabbit secondary (Jackson). The blots were then stripped using Restore Western Blot Stripping Buffer (Pierce), and re-probed with primary antibodies against CREB (#04-767, 1:5000, Millipore) and GAPDH (#MA1-16757, 1:40000, Thermo Scientific). Anti-rabbit secondary was first used to react with the anti-CREB antibody. After imaging, HRP signal was quenched using 15 % H_2O_2. An anti-mouse secondary (Jackson) was then used to react with the anti-GAPDH primary. Immunoreactive bands were visualized using a ChemiDoc XRS+ Molecular Imager System with ImageLab Software (Bio-Rad). Offline quantification of reactive bands' optical density also used ImageLab software. All gels were loaded with a reference sample to allow for comparison across gels. All samples were normalized for loading error using GAPDH signal. All reported signals were first normalized to reference sample signal, then GAPDH signal, and lastly, normalized to GFP average, or naïve average.

RNA Extraction and cDNA synthesis

Four to five hippocampal slices were placed in RNAlater, and frozen. The three major hippocampal sub-regions (CA1, CA3, and DG) were manually isolated as described above. Tissue from CA1 was first disrupted manually, then with a QiaShredder (Qiagen) column. RNA was subsequently extracted using RNeasy Plus Mini Kit (Qiagen), according to manufacturer's

directions. All samples were eluted in 30 uL RNase-, DNase-free water. All samples were checked for integrity using an RNA 6000 Pico chip (Biorad) on the Biorad Bioanalyzer. All samples had RNA integrity numbers (RIN) of 7 or higher and therefore none were excluded from analysis. For each sample, equal amounts of RNA were reversed transcribed into cDNA using SuperScript® VILO cDNA Synthesis Kit (Invitrogen). To ensure reagents were contaminant-free, concurrent controls containing no VILO enzyme were made and carried through the rest of the RT-PCR experiment.

RT-PCR

Real-time polymerase chain reaction (RT-PCR) reactions were carried out in 384-well plates. Each well contained sample cDNA, primer and probe against target gene, and SsoAdvanced™ Universal Probes Supermix (Biorad). Each plate included controls from the cDNA synthesis step, which lacked the reverse transcription enzyme, and wells in which water replaced cDNA sample. These negative controls were to confirm that no genomic DNA was present, and that no reagents other than sample cDNA contained template that could be amplified. All samples were run in triplicate, and reactions carried out on a 7900HT (Applied Biosystems).

Primers and probes against GAPDH, CREB1, zif248, c-fos, BDNF, and EGFP were purchased from Integrated DNA Technologies. Utilizing GAPDH as the house-keeping gene, genes of interest were analyzed using the ΔΔCt method.

The following primers and probes were used:

GAPDH forward CCAGTAGACTCCACGACATAC

GAPDH probe CCAGTAGACTCCACGACATAC

GAPDH reverse AACCCATCACCATCTTCCAG

CREB forward AGCACTTCCTACACAGCCT

CREB probe ATTCTCTTGCTGCTTCCCTGTTCTTCA

CREB reverse CACTGCCACTCTGTTCTCTA

zif 248 forward GAGCGAACAACCCTACGAG

zif 248 probe ATTCTCTTGCTGCTTCCCTGTTCTTCA

zif 248 reverse GTATAGGTGATGGGAGGCAAC

c-fos forward CAGCCTTTCCTACTACCATTCC

c-fos probe CTGTCAACACACAGGACTTTTGCGC

c-fos reverse TTGGCACTAGAGACGGACA

BDNF forward TTGGCACTAGAGACGGACA

BDNF probe AGAAGTTCGGCTTTGCTCAGTGGA

BDNF reverse AAGGTGGATGAGAGTTGAAGC

EGFP forward GAACCGCATCGAGCTGAA

EGFP probe ATCGACTTCAAGGAGGACGGCAAC

EGFP reverse TGCTTGTCGGCCATGATATAG

Biophysics

After 300 μm slices were made from dorsal hippocampus, they were incubated in aCSF for 20 min at 34 °C, then allowed to cool down to room temperature for at least 40 min. One at a time, slices were transferred to a submersion chamber at 34-35 °C. Slices were visualized using a

Hamamatsu Orca R2 camera mounted on an upright Leica DMLFS microscope. Whole-cell current clamp recordings were made from visually identified infected cells (identified by GFP signal), and neighboring control, uninfected cells. Patch electrodes (5-7 MΩ) were filled with (in mM) 120 $KMeSO_4$, 10 KCl, 10 Hepes, 4 Mg_2ATP, 0.4 NaGTP, 10 Na_2 phosphocreatine, 0.04 AlexaFluor 594, and 0.5% neurobiotin, pH adjusted to 7.4 with KOH. No correction was made for a ~10 mV liquid junction potential. All measurements were made 5 min after membrane rupture to allow for adequate solution equilibration with the neuron held near -69mV. The biophysical data were collected using pClamp 10 software, an Axoclamp-2A amplifier, and an Axon Digidata 1550A. The data were analyzed using Clampfit 10 software (Molecular Devices, Sunnyvale, CA) and custom routines in Matlab. AHP values were observed using a train of 15 action potentials (50 Hz), evoked by direct somatic current injections.

RESULTS

CREB Protein Levels from Viral Tissue

Two-to-three weeks after the end of water maze testing, western blots were run on CA1 tissue to determine levels of both total and activated CREB. Levels of total CREB from AAV-CREB groups were compared to those of AAV-GFP from young and aged animals. A two-tailed students t-test showed no significant difference in total CREB levels from AAV-GFP and AAV-CREB groups in young ($t_{12} = 0.78$, n.s., Fig. 4.1A), or aged animals ($t_{30} = 0.043$, n.s., Fig. 4.1A). A similar pattern was observed for pCREB levels (young $t_{12} = 0.21$, n.s.; aged $t_{30} = 0.77$, n.s., Fig. 4.1B). Correspondingly, no difference was found in the pCREB/CREB ratio (young $t_{12} = 0.59$, n.s.; aged $t_{30} = 0.59$, n.s., Fig. 4.1C). These data indicate that despite being tested at the

same time point, CREB mRNA levels were significantly increased in AAV-CREB animals, but western blotting revealed no changes in CREB protein levels. This also contradicts the immunofluorescence data from Chapter 3.

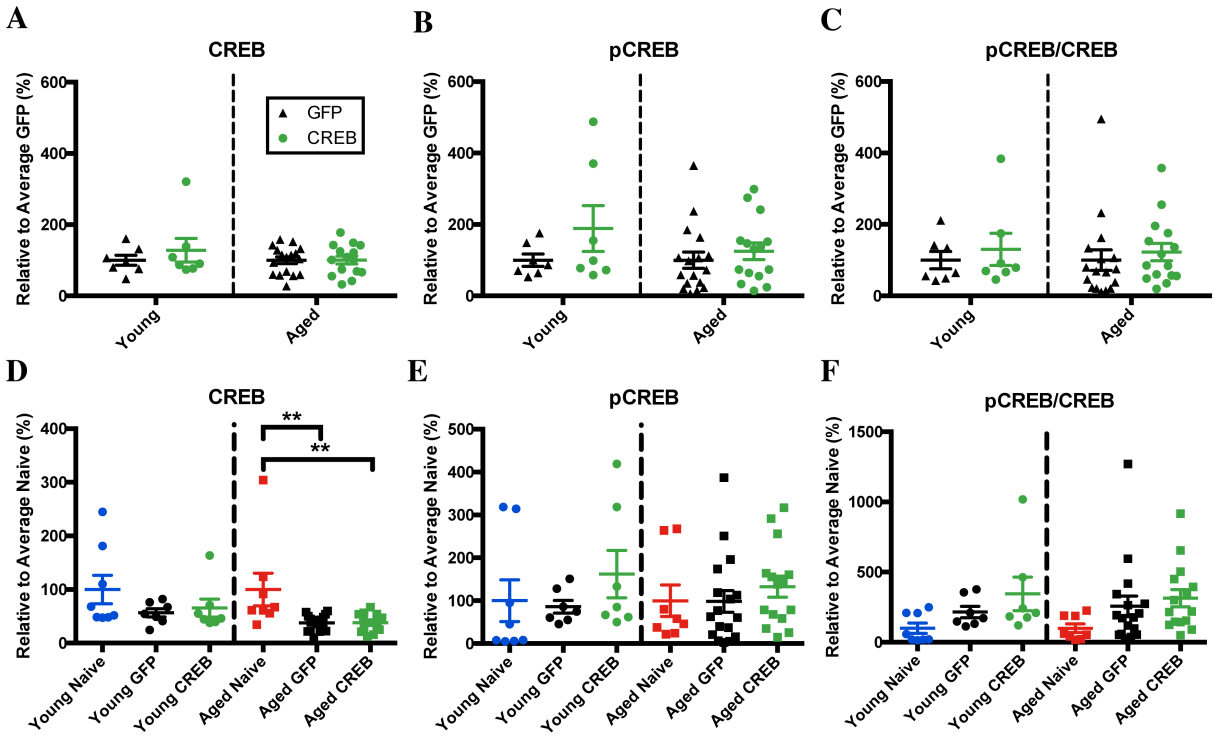


Figure 4.1. Western blotting detects few changes between naïve and viral animals. CA1 tissue from young and aged animals that received AAV-GFP (GFP, black) or AAV-CREB (CREB, green), do not differ in protein levels of A) CREB, B) pCREB, or C) pCREB/CREB ratio. D) Aged animals that received either AAV-GFP or AAV-CREB had less CREB protein than naïve aged animals. Levels of E) pCREB and F) pCREB/CREB ratio were unchanged when comparing naïve and viral animals.

Further analyses compared the AAV-GFP and AAV-CREB viral groups to naïve animals for each age group. A one-way ANOVA on total CREB levels showed no significant difference across young groups ($F_{2,19}$ = 1.43, n.s.,). However, total CREB was significantly different across aged groups ($F_{2,37}$ = 7.63, p = 0.002, Fig. 4.1D). Specifically, a Tukey's multiple comparisons test revealed that aged naïve animals had significantly more CREB than both aged AAV-GFP (p

= 0.003), and aged AAV-CREB animals (p = 0.003). These data indicate that in aged animals, viral injection and/or training results in a reduction in levels of total CREB protein. No differences were observed for pCREB levels in young ($F_{2,19}$ = 0.82, n.s.,) or aged animals ($F_{2,37}$ = 0.52, n.s., Fig. 4.1E). Similarly, no differences in pCREB/CREB ratio were found in young ($F_{2,19}$ = 2.87, n.s.,) or aged animals ($F_{2,37}$ = 1.94, n.s., Fig. 4.1F).

Downstream genes from CREB

Two-to-three weeks after the end of water maze testing, we determined levels of CREB mRNA and verified that the observed behavioral and electrophysiological effects were due to overexpression of CREB (as shown in Chapter 3). To determine if genes downstream of CREB activation were upregulated alongside CREB, we also examined mRNA levels of zif268, BDNF, and c-fos. These mRNA levels were compared across young or aged naïve, AAV-GFP, or AAV-CREB animals, with the levels of the viral groups expressed as fold change over the naïve group for each age group. As shown in Chapter 3 and also found here, a one-way ANOVA with Tukey's multiple comparison tests revealed a significant difference across the young groups ($F_{2,30}$ = 11.40, p = 0.0002; naïve vs. AAV-CREB p = 0.031; AAV-GFP vs. AAV-CREB p = 0.0001, Fig. 4.2A). These results indicate that AAV-CREB did indeed increase CREB mRNA levels in young animals. There was also a trend towards higher CREB mRNA levels in aged AAV-CREB animals ($F_{2,50}$ = 3.03, p = 0.057 ; AAV-GFP vs. AAV-CREB p = 0.066, Fig. 4.2E). No differences were seen for zif268 levels in either young ($F_{2,30}$ = 1.31, n.s., Fig. 4.2B), or aged animals ($F_{2,50}$ = 1.22, n.s., Fig. 4.2F). A somewhat different pattern emerged for BDNF. A significant difference was found in young animals ($F_{2,30}$ = 6.91, p = 0.003), where both AAV-

GFP ($p = 0.006$) and AAV-CREB ($p = 0.010$) animals were significantly different from naïve animals (Fig. 4.2C). This indicates that while there is no difference between the two viral groups, viral injection and/or water maze training caused a reduction in BDNF levels. This effect was not seen in aged animals, where no differences were observed ($F_{2,50} = 1.32$, n.s., Fig. 4.2G). We found no differences across young groups in c-fos mRNA levels ($F_{2,30} = 1.41$, n.s., Fig. 4.2D). However, the aged groups were significantly different from each other ($F_{2,50} = 4.25$, $p = 0.02$). Specifically, both viral groups had higher c-fos levels than the naïve group (AAV-GFP $p = 0.021$; AAV-CREB $p = 0.036$, Fig. 4.2H). Similar to the finding with BDNF in young animals, this suggests that either viral injection and/or water maze training resulted in an increase in c-fos levels in these aged animals.

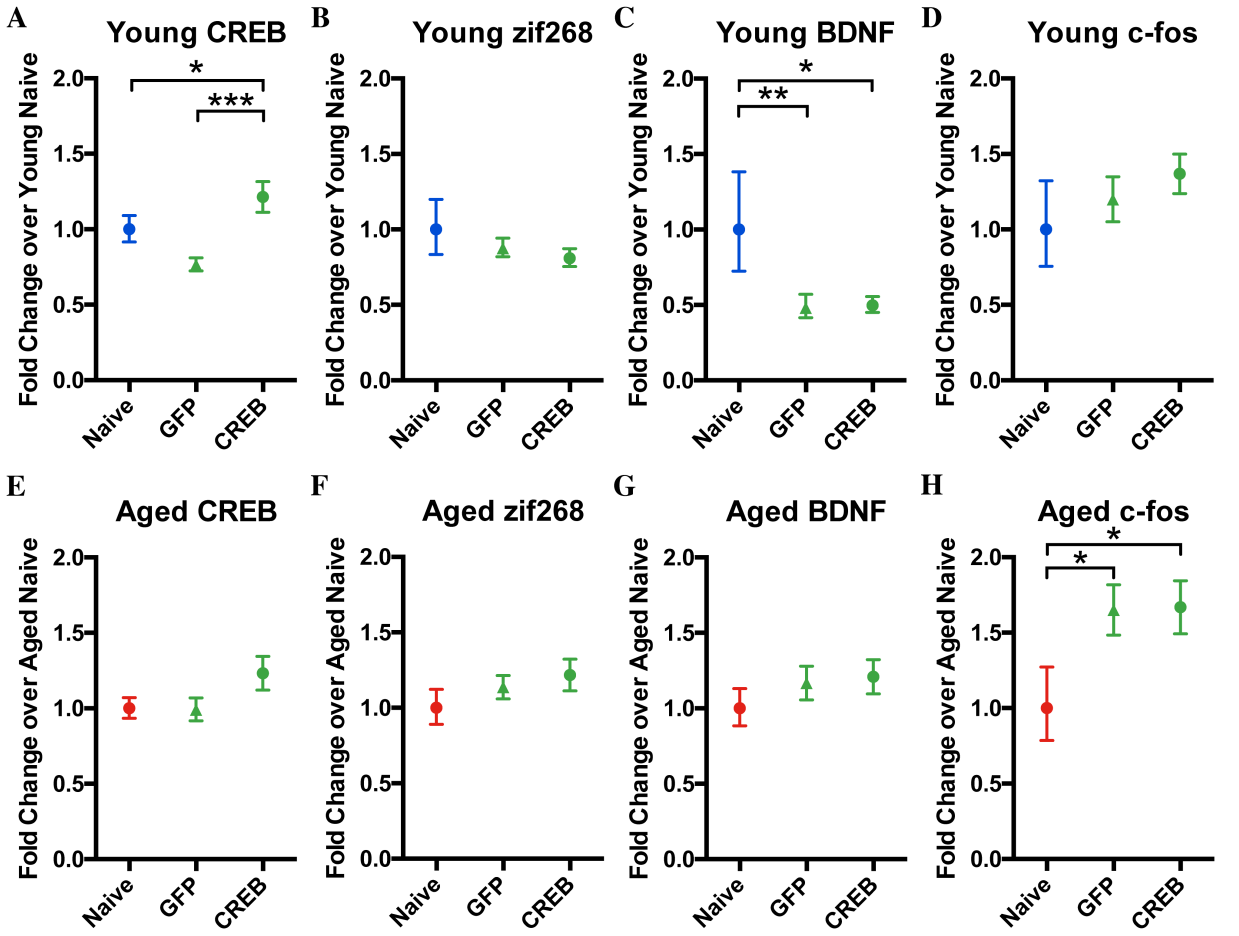


Figure 4.2. Changes in mRNA for CREB and related downstream genes. A) Young animals that received AAV-CREB (n = 11) had significantly more CREB mRNA than both naïve (n = 10) and AAV-GFP animals (n = 12). E) Similarly, aged animals received AAV-CREB trended towards having higher CREB mRNA also (n = 10, 22, 21). Levels of zif268 were unchanged for both B) young (n = 10, 12, 11), and F) aged animals (n = 10, 22, 21). C) Young animals that received AAV-GFP (n = 12) or AAV-CREB (n = 11) had less BDNF than naïve animals (n = 10), while G) aged animals showed no difference (n = 10, 22, 21). Conversely, c-fos was unchanged across D) young groups (n = 10, 12, 11), while H) aged animals that received AAV-GFP (n = 22) and AAV-CREB (n = 21) had more c-fos than naïve animals (n = 10).

Lentiviral expression of VP16-CREB

To test whether VP16-CREB expression modulates AHP size, lentivirus encoding for VP16-CREB (a generous gift from Dr. Angel Barco) was injected into CA1 of young animals, and biophysical recordings made 2-3 weeks later. Peak and slow AHPs were recorded from GFP+, infected neurons, and neighboring control, uninfected neurons. A two-tailed student t-test on peak AHP amplitudes revealed VP16-CREB-infected neurons had smaller peak AHPs than control cells (t_{19} =2.31, p = 0.032, Fig. 4.3B). A similar effect was observed with the slow AHP, where VP16-CREB neurons had smaller slow AHP amplitudes than neighboring control neurons (t_{19} = 2.99, p = 0.008, Fig. 4.3C). These results indicate that cells expressing VP16-CREB are more excitable than control, uninfected neighboring cells.

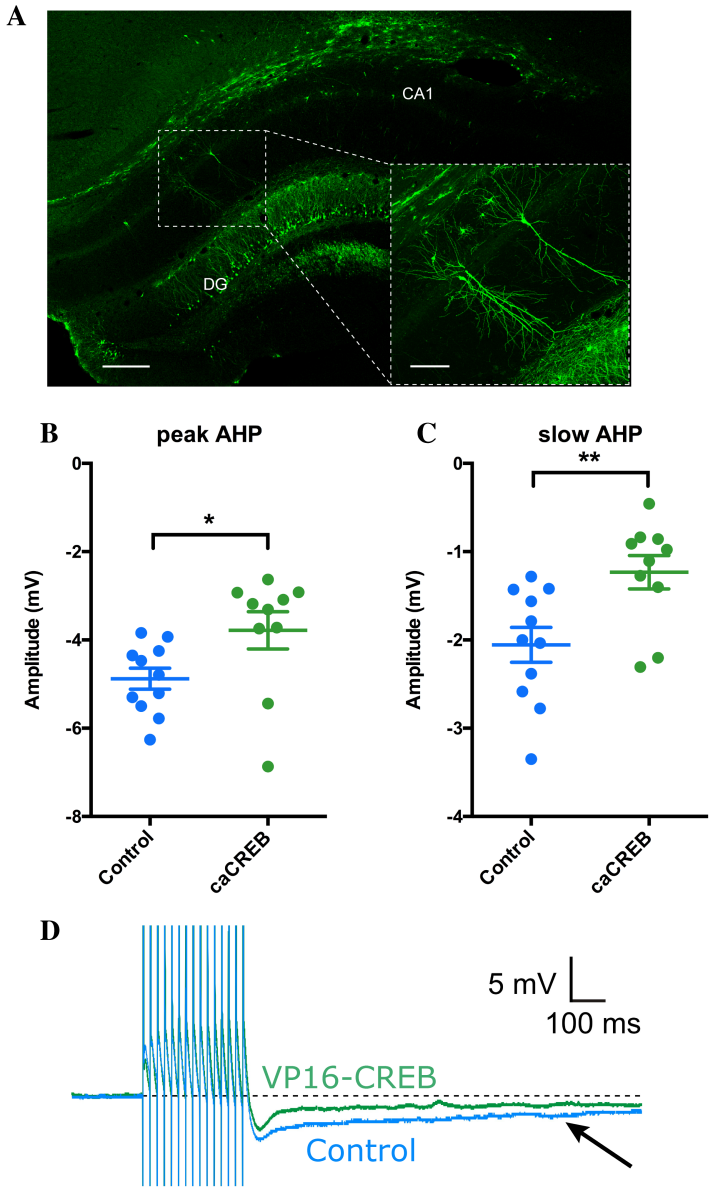


Figure 4.3. Partially-active CREB expression reduces peak and slow AHP in young cells. A) Example stitched 10x confocal image of EGFP expression 2 weeks post-injection of lentivirus into dorsal CA1 area. Summed z-stack of infected CA1 pyramidal neurons (inset). Left scale bar 250 μm, right scale bar 100 μm. B) Peak AHP amplitudes measured from VP16-CREB-infected cells (green) are smaller than that of neighboring uninfected control cells (blue), $p < 0.05$. C) Slow AHP amplitudes measured from VP16-CREB-infected cells (green) are also smaller than that of neighboring uninfected control cells (blue), $p < 0.01$. D) Representative traces from AHP recordings made from VP16-CREB-infected and control cells, arrow indicates 1 s time point where slow AHP was measured. Bars in a and b represent mean ± S.E.M.

DISCUSSION

Studies using viral vectors typically compare groups of animals that either receive a virus expressing the gene of interest, or a control virus containing only the reporter gene. Here, we investigated whether animals that received viral injections differed from naïve animals. We found that young animals injected with AAV-CREB had significantly more CREB mRNA than naïve or AAV-GFP animals. A similar trend was seen in the aged animals. At this time point, two weeks after either water maze training or after viral injection, a significant decrease in BDNF was observed in young animals that received either viral vector, relative to those who did not. Conversely an increase in c-fos was observed in aged animals that received viral injections, when compared to naïves. Surprisingly, our western blot experiments did not reveal any differences in levels of CREB or pCREB protein when comparing AAV-GFP and AAV-CREB groups. However, a significant decrease was observed in CREB levels in aged viral animals, when compared to aged naïve animals. Lastly, we tested if the AHP of young neurons could be reduced by activating CREB signaling. This was confirmed by the observation that VP16-CREB-infected neurons from young animals had smaller peak and slow AHPs, as compared to neighboring, uninfected controls.

As mentioned in the results section, the current western blot results are incongruous with both the mRNA data, and the results from immunofluorescent staining of similar tissue. In both of those cases, we found that animals injected with AAV-CREB had more CREB mRNA and protein, than that of AAV-GFP, which was not observed in the current western blot experiments. This is likely explained by differences in the methods used. First, in comparing

immunofluorescence to western blotting, the morphology of coronal sections means we can achieve high likelihood of selecting tissue from the virus infected area with good reliability, as compared to the transverse hippocampal slices used in the western blot experiments. This means that the tissue taken for western blotting may not have encompassed the infection hotspot, thereby diluting any potential increase we would have seen; contrary to the visually identified sections used for immunofluorescence experiments. Furthermore, while RT-PCR with primer-probe setups are highly sensitive and specific, it's likely that western blot experiments may be more susceptible to reduced signal to noise. Signal from processes, glia, and extracellular matrix is likely to have added to signal from our pyramidal neurons of interest, and potentially further diluted out any increases in CREB signal present in the western blot experiments. This problem was avoided in the use of immunofluorescent staining, as the analysis only used regions of interest that included cell bodies of neurons.

Interestingly, in aged animals that received either AAV-CREB or AAV-GFP, we observed a decrease in CREB protein relative to aged naïve animals. At first glance, this may point towards viral injection as the potential cause for this effect, however, another factor may be at play. All of the animals used in the western blot study first received water maze training (see Chapter 3 results). Therefore, it is possible that the decreased CREB levels may actually be a result of having undergone behavioral training, as opposed to an effect of receiving injections of viral vector. This observation parallels the trend seen in trained animals from Chapter 2, Table 2.1. There, animals that had received training tended to have lowered CREB levels as compared to naïve animals. To fully tease apart this issue, both AAV-GFP and AAV-CREB would need to

be injected into animals that do not then undergo any behavioral training. We predict that tissue from those animals would no longer display a drop in CREB levels.

We then used RT-PCR to compare levels of mRNA of CREB and some of its downstream target genes in naïve and viral animals. As expected, young animals that received AAV-CREB injections had higher levels of CREB mRNA than both naïve animals, and those that received AAV-GFP. A similar trend was observed in aged animals, evidence of the desired CREB overexpression. We also measured mRNA levels of downstream genes – zif248, BDNF, and c-fos. For several genes, there were no differences across the naïve, AAV-GFP, and AAV-CREB groups. This is likely due to the nature of their expression typically being very temporally locked to salient stimuli (Alberini, 2009). These downstream genes are not elevated in the AAV-CREB groups, despite CREB being elevated. This is most likely due to the time point at which these measurements were made – two weeks after the end of training or viral injection. As is commonly accepted, if we were to measure the levels of these genes shortly after behavioral training, for example, they are likely to be elevated, along with CREB (Barco and Marie, 2011; Benito and Barco, 2010; Davis and Squire, 1984). The observed changes in young BDNF and aged c-fos levels may be due to a compensatory effect. The current experiments do not provide enough information on why the compensation occurs in differing directions in young and aged animals, and warrants further investigation.

Lastly, in Chapter 3, we observed no change in intrinsic excitability of neurons between young animals injected with AAV-GFP and AAV-CREB. However, previous literature

suggested that CREB overexpression should have been sufficient to induce an increase in intrinsic excitability (Yiu et al., 2014; Zhou et al., 2009). Therefore, we tested if strong activation of CREB signaling via expression of VP16-CREB would result in increased neuronal excitability. We measured both peak and slow AHP for VP16-CREB-infected neurons, and neighboring uninfected control neurons. The VP16-CREB-expressing neurons had reduced AHPs at both the peak and slow time points, indicating an increased excitability. This finding is crucial, as it indicates that increasing CREB signaling can in fact increase excitability of young neurons. However, it also suggests that the level of CREB overexpression our viral vector induced may not have been sufficient to increase excitability of young neurons.

In summary, results from experiments in this chapter provide further support, in addition to data from Chapter 2, that behavioral training leads to reductions in CREB levels, even two weeks after the end of training and testing. Furthermore, we conclude that western blotting may not be sensitive enough to detect subtle changes in protein levels in a limited area of the hippocampus. Lastly, we show that with strong, constitutive activation of CREB signaling, we were able to increase intrinsic excitability of young neurons, suggesting that a higher level of CREB overexpression (as compared to that observed in Chapter 3) could also achieve the same effect.

Chapter 5 – General Discussion and Future Directions

In many different species, individuals may develop deficits in cognition, such as in spatial memory, as they age. To better prevent or treat these age-related cognitive deficits, we need to first better understand the underlying mechanisms that drive them. Alongside deficits in cognition, the Disterhoft lab and others have observed a decrease in intrinsic excitability of hippocampal CA1 pyramidal neurons in aged animals (Disterhoft and Oh, 2007). This is thought to contribute to age-related cognitive deficits, as neurons from aged-unimpaired animals have excitability that is comparable to neurons from young animals, while aged-impaired animals have decreased excitability (Disterhoft and Oh, 2006). This thesis tested the hypothesis that dysfunction or decreases in CREB levels and/or activity drives age-related deficits in both cognition, and neuronal excitability.

CREB's importance in the formation of memories, and its potential as a cognitive enhancer has been thoroughly tested in young adult animals (see reviews by Barco et al., 2006; Carlezon et al., 2005; Johannessen et al., 2004; Tully et al., 2003; J. C. J. Yin and Tully, 1996). How CREB changes in aging is not well established. Immediately after undergoing water maze training or contextual fear conditioning, aged animals have decreased levels of total CREB and pCREB levels, respectively (Brightwell et al., 2004; Koutaro Kudo et al., 2005). Additionally, binding of CREB to target gene CRE sites has also been found to be reduced with age (Asanuma et al., 1996). Luckily, these deficits have been shown to be overcome by up-regulating levels of upstream activators such as cAMP (Bach et al., 1999). In naïve, untrained animals, the literature

has not reached a consensus. Different studies have found that hippocampi from aged rats have increased pCREB with unchanged CREB levels (Foster et al., 2001), no change in total CREB (Ramos et al., 2003), and increases in pCREB with unchanged CREB levels (Monti et al., 2005b). Thus, while some studies have found that acute activation of CREB is impaired with age, basal or chronic changes in CREB levels and its activation with age is yet to be established. Specifically, CREB levels at time points distant to the experience of behavioral training, and how they might relate to cognitive performance, have not been examined. The results presented in Chapter 2 indicate that in CA1 of hippocampus, pCREB levels are decreased with aging, and that total CREB is unchanged. In both CA3 and dentate gyrus, total CREB levels were decreased, therefore, leading to decrease in pCREB levels too. This suggests that there is a specific functional deficit, likely at the level of an upstream kinase, in the CA1 sub-region of hippocampus. This functional deficit is likely to have important implications, as this is also the sub-region of hippocampus where decreases in neuronal excitability have been observed (Disterhoft and Oh, 2006). Both CaMKIV and PKA have been found to be decreased with age, so both are potential kinases that become dysfunctional with age (Fukushima et al., 2008; Karege et al., 2001). Identifying which kinase(s) are specifically dysfunctional with age would be of great interest. This could be done with an *ex vivo* kinase activity assay. One could apply agonists of different kinases to freshly-made hippocampal sections from young and aged animals. Then carry out western blots on homogenates from each hippocampal sub-region to quantitate the level of CREB activation in each case. Aged tissue with dysfunctional kinase(s) would have decreased CREB activity when compared to young tissue.

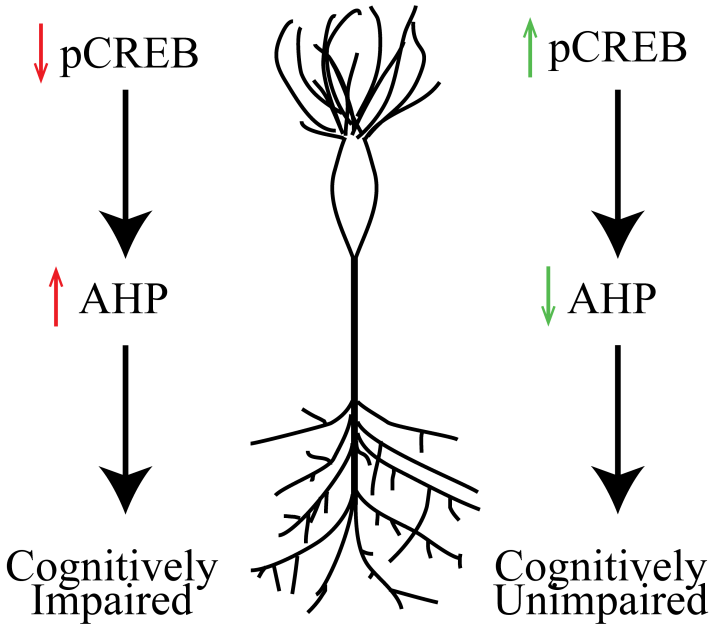
The CA1 sub-region was further identified as functionally important, as that was the only sub-region in which pCREB levels significantly correlated with short-term memory for the water maze task. This is somewhat at odds with previously published studies, which highlight CREB's relationship with long-term memory (reviewed in Lonze and Ginty, 2002). We believe two variables may help explain this discrepancy. The first is the time point at which we carried out our biochemical experiments to measure CREB and pCREB levels. We specifically chose two weeks after the final behavioral training/testing session, as at this time point, all training-induced biophysical changes had returned to baseline (Matthews and Disterhoft, 2009; Moyer et al., 1996; Thompson et al., 1996; Tombaugh et al., 2005) theoretically giving us a measure of basal levels of CREB and pCREB. Unexpectedly, rather than measuring what we expected to be baseline measurements of CREB levels/activity, we found that animals that had undergone behavioral training had reduced CREB levels as compared to behaviorally naïve animals. This result will be discussed in the next section. However, it does suggest that this time point was not sufficiently distant from the behavioral training to allow molecular changes to return to baseline. This in part, could explain the correlation observed with short-term memory, rather than long-term memory. A second contributing variable may be CREB's other post-translational modifications. Asides from the S133 phosphorylation site, CREB can also be phosphorylated at S111, S113, S117, S121, S129, and S142/143 that are likely to play a role in how strongly and long CREB-binding protein (CBP) is able to interact with CREB – this interaction is required for downstream transcription to occur (Kaleem et al., 2011). Furthermore, the redox state of sites C320 and C330 regulate DNA binding by CREB (Goren et al., 2001). Lastly, CREB has also been found to be SUMO-ylated and o-linked glycosylated which is also likely to regulate its

activity (Kaleem et al., 2011). Some or all of these may be changed in aging, and therefore change the relationship between pCREB (at S133) and an animal's cognitive performance. Therefore, more careful studies of nuclear, DNA-bound levels of activated CREB may provide clarity.

As mentioned in the previous paragraph, results from Chapters 2 and 4 indicate that trained animals have lower levels of CREB than behaviorally naïve animals, when CREB levels are measured two weeks after the end of behavioral training/testing. We suggest that, analogous to the post-burst afterhyperpolarization at the biophysical level, this is a homeostatic response of the system at a molecular level. In the case of the post-burst AHP, after incoming stimuli causes a burst of action potentials, a long-lasting hyperpolarization occurs to reduce the likelihood of further action potentials from occurring. Perhaps the CREB reduction we observed two weeks after behavioral training serves a similar purpose. After exposure to stimuli during behavioral training, it may be advantageous for the system to down-regulate its CREB levels to ensure there is sufficient signal-to-noise ratio for further incoming signals. Molecules that mediate this down-regulation could include inducible cAMP early repressor (ICER), whose transcription is turned on by CREB (Borlikova and Endo, 2009), and CREB2, which has been shown to be a transcriptional repressor (Karpinski et al., 1992)

In Chapter 3, we directly tested the hypothesis that decreased CREB signaling mediates age-related cognitive deficits by virally overexpressing wild-type rat CREB in CA1 of young and aged rats. We found that half of aged rats receiving control virus were impaired on long-term

memory for the water maze task (probe trial 4 days after end of training), but all aged rats receiving AAV-CREB performed at young-like levels. This suggests that CREB dysfunction is likely to underlie the deficits seen in aged control rats, and that overexpression of CREB rescued these deficits (Fig. 5.1). These results suggest that targeting CREB or its downstream



transcriptional targets for pharmacological therapy could ameliorate age-related cognitive deficits.

Figure 5.1. Schematic of relationship between CREB activation, AHP size, and cognition. In situations where pCREB is reduced, such as in aging, AHP amplitude is also increased, thus decreasing intrinsic neuronal excitability. Both of these factors then lead to the animals being cognitively impaired. In situations where CREB activation is normal, such as in young animals, or when CREB levels are increased in aged animals, AHP amplitude is also decreased, i.e. neuronal excitability is increased. These animals are then cognitively unimpaired. Image modified from Disterhoft & Oh (2007).

In the context of cognitive aging, the effect of disruption to intracellular calcium homeostasis or "the calcium hypothesis of aging" is often discussed (Oh et al., 2013; Thibault et

al., 1998). Studies suggest that levels of cytosolic calcium, such as from action-potential evoked calcium influx, are increased with age. In the canonical signaling/activation pathway, we would then expect there to be increased PKA activity, leading to increased CREB activation. However, we found the opposite, that rather than having higher CREB activation with age, it is actually decreased. We suggest two potential explanations for this discrepancy. The first is an uncoupling of Ca^{2+} concentration from CREB activity at the kinase level. As mentioned above, PKA levels and activity have been found to be reduced with age (Karege et al., 2001). If Ca^{2+} levels are abnormally high, it is possible that the decrease in kinase level/activity is a compensatory mechanism to prevent the aberrant Ca^{2+} signal to be passed on further in the signaling chain. A second reason could be at the post-translational level of CREB. As mentioned above, CREB has numerous sites at which post-translational modifications occur to regulate its transcriptional activity. Changes at any or all of these sites may also cause uncoupling of the upstream Ca^{2+} levels to the actual activity of CREB itself. The previously mentioned *ex vivo* kinase assay could in part shed light on where the uncoupling might occur.

In Chapter 4, we overexpressed VP16-CREB and increased excitability of neurons from young animals. This suggests that to increase the excitability of already plastic young neurons, stronger activation of CREB-mediated transcription is needed, as compared to overexpressing wild-type CREB in Chapter 3. As seen in the behavior, too, only animals that would have exhibited cognitive deficits benefited from overexpression of wild-type CREB. This trait, of having little-to-no effect on those that do not need it, may in fact be highly desirable for therapeutic purposes.

This leads us to one very important question of how CREB modulates changes in intrinsic excitability of neurons. When considering the post-burst AHP, many channels are thought to contribute to it. However, all genes coding for the proposed channels, including BK, SK2, and SK4, lack CRE sites (TGACGTCA sequence) that would allow direct transcriptional regulation via CREB. Therefore, they must be regulated either indirectly by CREB's downstream transcriptional targets, or CREB may regulate activity of these channels via a non-transcriptional mechanism. Much work remains to be done to fully elucidate the mechanism. Another possible mechanism worth considering, is that CREB may modulate neuronal excitability and cognition via separate means. Given the numerous target genes CREB affects, and the fact that CREB may work by non-transcriptional means, it is entirely possible that CREB's effects on excitability and cognition coincide, but are not dependent upon one another. One such example is seen in a study by Gruart and colleagues (2012), who showed that expressing VP16-CREB for a prolonged period of time impaired performance on trace eye blink conditioning, but still increased neuronal excitability.

The work presented in this thesis has identified CREB's crucial role in age-related cognitive deficits. This indicates that modulation of CREB activity is a viable therapeutic target. However, CREB affects the transcription of numerous down-stream target genes, and direct modulation of CREB could result in unintended side effects. Therefore, for therapeutic purposes, it will be important to identify which down-stream target(s) of CREB transcription are mediating these effects. Similar to the approach taken here, first, downstream targets could be tested for

changes in aging and relationship to cognitive performance. Then via overexpression or knock-down approaches, rescue or worsening of deficits could be observed.

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