Transcription factors NF-κB, CREB and Egr-2 and their potential role in memory formation

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Pharmacology and Therapeutics

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Winnipeg

Abstract

Statement of the problem

Human memory can be subdivided into short- and long-term forms. Current theories propose that short -term memory is regulated by substrate phosphorylation, whereas long -term memory appears to be regulated by several transcription factors. In particular, transcription factor activation leading to the initiation of target gene expression is thought to drive many of the structural and regulatory proteins thought necessary for long-term memory formation. Some well-known transcription factors that have been implicated in memory include the cAMP-response element binding protein (CREB), nuclear factor kappa B (NF-κB), and early growth response (Egr) protein. These transcription factors have been previously shown to play various biological roles in cancer, immunity, brain injury, and inflammatory processes. Now emerging data suggest that they also play roles in regulating memory. To date, a significant amount of work has been done to demonstrate the role of CREB in memory, but much less work has been accomplished on the role of NF-κB and Egr proteins in memory. Why multiple transcription factors are required for long-term memory is currently unknown. Likewise, little is known about the spatial and temporal expression profiles of these transcription factors in long term memory. To this end, our objective was to profile the expression of these transcription factors following training in the Morris water maze (MWM), a behavioral procedure for assessing hippocampal-dependent forms of long memory in rodents. We hypothesized that there would be a difference in the expression levels of CREB, NF-κB and Egr-2 following learning and memory induced by training in the MWM. This hypothesis is based on the expectation that redundant forms of transcriptional regulation would not be advantageous to the cell unless these transcription factors had distinct roles to play in memory.

Experimental Design

CD1 and C57BL/6 male mice (~30 days old) were used for two studies in this thesis. In the first study, CD1 mice were used by myself. In this case, CD1 mice were categorized into two groups. Group 1 was assigned as the non-trained control group. The second group (experimental group) underwent 9 consecutive days of MWM training, which was performed in two phases (i.e., acquisition for 6 days and retention for 3 days). The mice were then killed 24 hours after training (i.e., on day 10).

In the second study, which was conducted by members of the lab (Eric Platt, Chris Cadonic, and Dr. Wanda Snow), male C57BL/6 mice were categorized into four groups. These experiments supplemented my work, and some of the key experiments relevant to this project are included in this thesis. In this study, Group 1 was also a non-trained control group (mice were allowed to swim randomly for a comparable time in the MWM). Groups 2, 3, and 4 (experimental groups) had variations in their MWM training for the acquisition and retention phases (detailed in the methods).

The acquisition phase is conducted to train the animals to find a hidden platform, which is found with the aid of visual cues. The retention phase, which follows after the acquisition phase, is performed without the hidden platform, so that the *recollection* of the missing platform can be assessed. "Search strategies" and "escape latency" were measured in the acquisition phase, whereas, "time spent in the target quadrant" and the "number of attempts passing over the missing platform" were measured in the retention phase.

To evaluate the expression levels of transcription factors CREB, NF-κB and Egr-2, pre and post-learning, hippocampal samples from control groups and experimental groups were then

isolated and Western blots were conducted on specific subunit structures from the transcription factors listed above. In addition, immunohistochemical (IHC) procedures were applied to some hippocampal slices to evaluate regional differences in protein expression.

Results

In study one, the escape latency time decreased progressively toward the end of the acquisition phase in the trained group, indicating a mean improvement in the animals' performance as expected. The search pattern, also toward the end of the acquisition phase, showed that the mice used primarily spatial strategies, suggestive of intelligent cognitive mapping of the MWM environment. In addition, mice spent more time in the target quadrant than the other quadrants during the retention phase, indicating they recalled the general location of the missing platform. The number of passes over the missing platform also peaked on the first day of the retention phase (mean > 5 times). In addition, NF-κB and CREB were expressed significantly higher in the control group versus the MWM trained mice) ($p = 0.0031$ and $p < 0.0001$ respectively). There was no statistically significant difference between the expression of Egr-2 in experimental group (MWM) versus the control group ($p = 0.3092$).

In study two, Group 4 showed the highest levels of CREB expression as measured by Western blotting and IHC. Groups 2 and 3 were similar in expression levels but were lower than Group 2 levels. Group 1, which was a yoked control, showed the lowest levels of CREB expression. However, these results have yet to statistically verified and this study is ongoing.

Conclusions

CREB and NF-κB were decreased following MWM training in study one. These results are contrary to some data previously reported from other labs that compared expression levels of these transcription factors in different mouse strains. In study two CREB levels were highest in Group 4 samples, which were mice that were trained in the MWM for 5 days, but had a probe test on the 12th day and then killed on the 13th day. We suspect differences in our findings are due to several reasons; including a multiphasic expression pattern and/or other experimental design issues. Further studies are warranted that examines time dependent differential expression of these transcription factors in memory.

Acknowledgements

I would like to deeply appreciate Dr. Benedict Albensi, my supervisor, for his support throughout my Master's program. This study would not be possible without his guidance.
I would like to thank Dr. Francis Amara for giving me precious advice for this study.
I would like to thank Dr. Gordon Glazner for his great comments on this study. I would like to thank Dr. Paul Fernyhough for the learning opportunity he provided for me in his lab. I would like to thank Mr. Alex Ferreira for his help and contribution during the experiments. I also thank Dr. Michael Bernstein for his help with some statistical calculations. And last but certainly not least I would like to thank MHRC/MICH for providing scholarship to help me continue my MSc research project.

Dedication

I would like to dedicate this thesis to my wonderful parents for their continuous love, support and encouragement.

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MWM	Morris Water Maze
NF-κB	Nuclear factor kappa B
CREB	cAMP-response element binding protein
Egr-2	Early growth response-2
cAMP	Cyclic adenosine monophosphate
LTP	Long-term potentiation
LTD	Long-term depression
RNA	Ribonucleic acid
NMDA	N-methyl-D-aspartate
DNA	Deoxyribonucleic acid
ATF	Activating transcription factor
bZIP	Basic region-leucine zipper
CREM	cAMP-response element modulator
KID	Kinase-inducible domain
PKA	Protein kinase A
pCREB	Phosphorylation of CREB
IκB	Inhibitory κB
mRNA	Messenger ribonucleic acid
g	Gram
ml	Milliliter
M	Molar
MgCl2	Magnesium chloride

EGTA	Ethylene glycol tetra acetate
NaF	Sodium fluoride
µ	Micro
BSA	Bovine Serum Albumin
CV	Coefficient of variation
OD	Optical density
SDS	Sodium dodecyl sulphate
°C	Degree Celsius
®	Registered sign
MW	Molecular weight
KDa	Kilodalton
mAb	Monoclonal antibody
ECL	Enhanced Chemiluminescence
SEM	Standard error of the mean
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
Sec	Second(s)
No	Number
mg	Milligram
CNS	Central Nervous System
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
cm	Centimeter

IEG	Immediate early gene
HMG	3-hydroxy-3-methyl glutaryl
TAD	Transcriptional activation domains
ROS	Reactive oxygen species
IKK	IκB kinase
CRE	cAMP response elements
BDNF	Brain-derived neurotrophic factor
CBP	CREB-binding protein
NGF	Neurotrophin growth actor
APP	Amyloid precursor protein
Nab	NGF-1A binding protein
TEMED	N,N,N',N' - tetramethylethylethylenediamene
V	Volts
IHC	Immunohistochemistry

1. Introduction

Memory as defined by temporal and biochemical characteristics, can be divided into short-term and long-term types (McGaugh 1966). The hippocampus is a key brain region involved in both short- and long-term memory processes (Zhou, et al. 2013). However, as memory becomes consolidated over time, the hippocampus becomes less and less important, yielding to processes dominated in neocortical locations. In addition, short-term memory is labile and depends on posttranslational modifications of pre-existing molecules and transcriptional regulation, whereas long-term memory requires consolidation mediated through gene expression and new protein synthesis to make a permanent memory (Davis and Squire 1984; Dudai 1996). In fact, spatial learning and memory is regulated by several transcription factors, which have been previously described in several studies (Mizuno, et al. 2002). The Morris water maze (MWM) is one of the common tests to measure spatial learning and memory. It was first introduced and developed by Richard Morris at the University of St. Andrews in 1982 (Morris, et al. 1982).

In past studies, spatial learning investigations in rodents were largely performed in rats (Ammassari-Teule and De Marsanich 1996). Now, many mouse models are used, especially transgenic mouse models. The CD1 mouse is one of the most common experimental animals due to its lower costs. Studies also show that the background strain of transgenic animals has a major influence on various factors, including the expression level of transcription factors (Patil, et al. 2010). The molecular characterizations of transcription factors are crucial for understanding their potential role in hippocampal spatial memory formation.

In this study, we characterized the expression of nuclear factor kappa B (NF-κB), cAMP response element binding protein (CREB), and early growth response factor 2 (Egr-2)

transcription factors in the hippocampus, following the MWM test. Historically, CREB has been the most well-studied transcription factor in memory processing. However, more recently, several other families of transcription factors have been shown to play roles in memory formation, which include CCAAT enhancer binding protein (C/EBP), activating protein 1 (AP-1), Egr (1-4), and NF-κB (Alberini 2009). However, many questions remain unanswered about their mechanistic pathways in memory formation: For example, what genes are required to mediate memory encoding? How might these transcription factors interact to mediate memory formation? Are there differences in the expression and/or activation of these transcription factors that are important for memory formation?

1.1. Memory and synaptic plasticity

Memory is the ability to recover previously stored information about past events or knowledge. Short-term memory involves memories of recent events (i.e., on the order of seconds to minutes), while intermediate and long-term memory is concerned with recalling the more distant past. Memory consolidation is the process of transforming newly learned short-term memory into stable long-term memory (Paul, et al. 2010). Some studies suggest that the process of memory consolidation may take several months (Baddeley, et al. 2009). Different biochemical mechanisms appear to be involved in the regulation of short-term versus long-term memory and in the consolidation and reconsolidation of long term memory. For example, long-term memory formation is accompanied by the activation of several transcriptional factors. The interactions between transcription factors and their target genes are essential steps in long -term memory formation; long-term memory formation involves activation of specific signal transduction pathways. These pathways are initiated by transcriptional activity, which then regulates cellular functions, such as the cytoskeletal re-configuration associated with synaptic plasticity (Alberini

2009). This process is displayed as structural changes in dendritic spines combined with functional connectivity changes between neurons (Kalantzis and Shouval 2009).

A primary characteristic of synaptic plasticity involves the experimental paradigms of long-term potentiation (LTP) and long-term depression (LTD) (Malenka 1994). LTP is a long lasting enhancement in synaptic transmission between or among neurons when they are stimulated synchronously at high frequency. LTD, on the other hand, is a biochemical process that can reverse LTP enhancements. Furthermore, LTP can be subdivided into different phases: a) an early phase (E-LTP), which is a protein synthesis-independent phase that lasts two to three hours, and b) a late phase (L-LTP) that is protein synthesis-dependent and lasts from several hours to weeks or longer (Martin and Morris 2002). E-LTP has also been referred to as the induction phase, whereas L-LTP is synonymous with maintenance and expression phases (Malenka and Nicoll 1999).

Calcium influx into dendritic spines is necessary for the induction of synaptic plasticity (Cummings, et al. 1996). A high frequency extra-cellular stimulus, by inducing a rapid influx of calcium, initiates this step (Tabuchi 2008). The extra-cellular calcium influx occurs via *N*-methyl-D-aspartate (NMDA) receptor-ion complexes and L-type voltage-dependent calcium channels (Tabuchi, et al. 2000).

Collectively, these molecular mechanisms underlying transcription factor activity, LTP/LTD, dendritic spine changes, and calcium influx are hypothesized to be involved in key memory formation processes.

1.2. Hippocampus

The hippocampus is composed of functionally distinct subfields, and is part of the limbic system (O'Sullivan, et al. 2010). Not only does the hippocampus function in memory formation, it is also a spatial coding system that aids in navigation (Moser, et al. 2008; Nadel, et al. 1975; Scoville and Milner 2000). The hippocampus plays a crucial role in spatial memory and also in short- and long-term memory formation. Since the brain has two hippocampi, one in each cerebral hemisphere, damage to one hippocampus may result in near-normal memory functions (Di Gennaro, et al. 2006). However severe damage to both hippocampi can lead to profound difficulties in new memory formation (anterograde amnesia) and to some extent, in memories formed before the damage (retrograde amnesia).

The hippocampus is located inside the medial temporal lobe and is subdivided into several subfields known as, CA1, CA2, CA3, CA4 and dentate gyrus (Andersen 2007). The two hippocampi are connected by the hippocampal commissure that crosses the midline under the anterior corpus callosum. The entorhinal cortex (EC), which is located in the parahippocampal gyrus, is part of the cortical region adjacent to the hippocampus. The EC is connected reciprocally with many parts of the cerebral cortex and works as an interface between hippocampus and cerebral cortex (Andersen 2007; Eichenbaum, et al. 2007). The sequence of information flow is from the EC to dentate granule cells in dentate gyrus, to CA3 pyramidal cells. Then from CA3 to CA1, axons of the neural cells send information to the subiculum and finally out of the hippocampus, back to the EC. There are also complex intrinsic pathways in each of these hippocampal regions (Andersen 2007).

Cells responsible for spatial memory formation in the hippocampus consist of pyramidal cells in the CA regions and granule cells in the dentate gyrus. The rest of the hippocampal cell

population is mostly inhibitory inter-neurons that control the firing of other neurons for spatial memory formation (Moser, et al. 2008). Previous studies have shown that an intact hippocampus is required for spatial memory tasks particularly for finding a way to a hidden goal in an experimental maze (Morris, et al. 1982).

In 1973, Bliss et al. described the LTP phenomenon in the rabbit hippocampus as a change in synaptic responsiveness induced by a brief strong activation that lasts for hours, days, or longer (Bliss and Lomo 1973). These synaptic changes were proposed to depend on glutamate-activated NMDA receptors that allow Ca^{2+} to enter the postsynaptic spine only when presynaptic activation and postsynaptic depolarization occur together (Nakazawa, et al. 2004). In other words, for the NMDA channels to be opened the post synaptic membrane must be strongly depolarized and at the same time glutamate must attach to its receptor site. As a result, the NMDA receptor detects both pre- and post-synaptic co-activation (Shapiro 2001). Therefore by blocking NMDA receptors in the hippocampus, LTP induction can be blocked (Collingridge, et al. 1983). The NMDA receptor is only required for the induction of LTP. Therefore blocking these receptors doesn't prevent the subsequent expression of LTP (i.e., L-LTP) and its potentiation (Shapiro 2001).

A newly encoded memory also requires reinforcement following retrieval in order to achieve lasting storage. This so-called consolidation process for stabilization requires new gene expression (Przybyslawski and Sara 1997; Sara 2000). For instance, activation of NMDA receptors and cAMP-response element binding protein (CREB) are required for consolidation of contextual fear memory (Kida, et al. 2002).

1.3. CREB transcription factor

One of the major transcription factors involved in memory formation is CREB (Barco, et al. 2002). The CREB family of transcription factors (CREB/ATF) is encoded by its prototype genes, CREB, CREM and ATF-1. This family has a basic region-leucine zipper (bZIP) domain, which mediates binding to specific regulatory sequences, and a transcriptional activation domain (Alberini 2009). CREB binds to a regulatory DNA sequence known as cAMP response element (CRE), which is present in the regulatory regions of many cAMP-responsive genes (Mayr and Montminy 2001). Some genes whose transcription is regulated by CREB include; c-fos, the neurotrophin BDNF (brain-derived neurotrophic factor), and many neuropeptides like enkephalin and corticotropin-releasing hormone (Purves 2008).

When a molecular signal arrives at the cell surface, it activates the corresponding post-synaptic receptors such as NMDA receptors, which leads to increases in intracellular calcium ($[Ca^{2+}]_i$), which in turn through a c-AMP mediated route activates various protein kinases (Coussens and Teyler 1996). Specific protein kinases translocate to the cell nucleus to activate CREB (Lonze and Ginty 2002). In particular, the kinase-inducible domain (KID) in CREB is active only when phosphorylated in response to c-AMP at Ser-133, which in turn activates CREB (Chrivia, et al. 1993). Phosphorylation of Ser-133 is a prerequisite, but not sufficient for CREB activation and gene expression (Alberini 2009). Then the activated CREB binds to a CRE region, followed by binding to CBP (CREB-binding protein) that allows gene expression (Parker, et al. 1996). Figure 1 summarizes the sequence of steps in CREB activation and expression.

The CREB activation that is necessary for memory consolidation is mediated by protein kinase A (PKA) phosphorylation (Bernabeu, et al. 1997). Sustained phosphorylation of CREB is

through kinases that are activated during both early and late phases, but only the late phase requires protein synthesis phosphorylation, and as a result CREB is only important in late phase LTP (Leutgeb, et al. 2005). CREB-mediated transcription involves gene activation, which leads to the expression of BDNF, required for neurite outgrowth and synaptic plasticity (Ring, et al. 2006).

The role of CREB in memory formation is consistent with significant deficit in several types of memories and impaired hippocampal LTP (Bourtchuladze, et al. 1994) in animal models with knocked out CREB (CREBα/Δ) isoforms. However, the third isoform of CREB, CREB β, as well as several activator and repressor forms of cAMP response element modulator are up- regulated, which affects this phenotype (impaired hippocampal LTP) (Blendy, et al. 1996).

Additionally, some steps of memory formation are CREB independent. This independence is demonstrated by the conditional disruption of all CREB isoforms in hippocampal CA1 regions, which doesn't impair hippocampal LTD (long-term depression) (Balschun, et al. 2003). Most of the early studies, which are done in neurons, on long-term memory formation and synaptic plasticity are performed using the invertebrate *Aplysia californica*. In this model, there is a transformation from short- into long-term facilitation to stabilize synaptic plasticity and long-term memory formation (Bartsch, et al. 1995). Inhibitory avoidance training in rats has also shown that rats learned to avoid a context previously associated with a foot shock by enhancing expression of pCREB (Taubenfeld, et al. 2001). pCREB increases substantially immediately after training and is sustained for at least 20 hours (Taubenfeld, et al. 2001).

In addition to synaptic plasticity and memory formation, CREB is also involved in other functions in the nervous system such as neuronal survival, axonal growth and regeneration and neurogenesis (Barco, et al. 2003; Carlezon, et al. 2005).

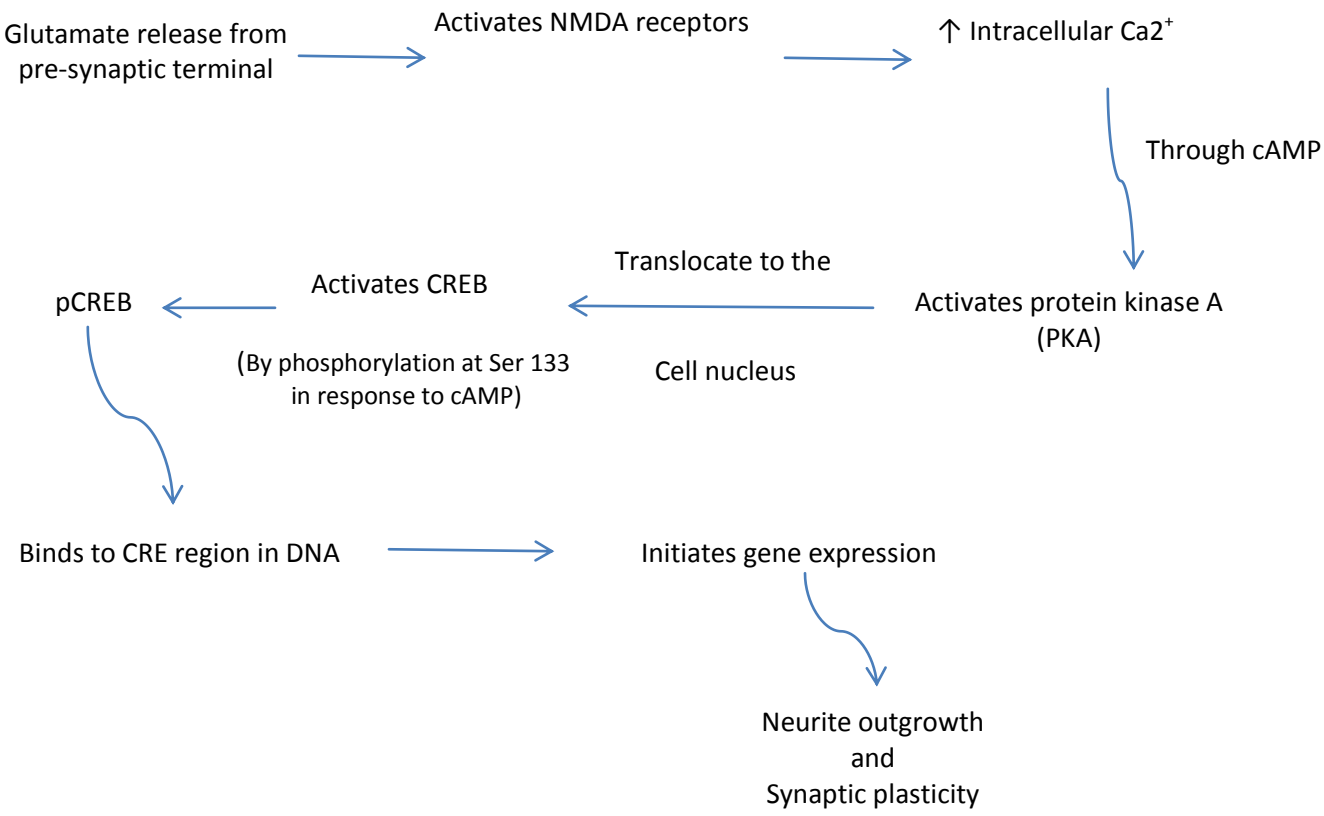


Figure 1. Summarized sequence of steps for CREB activation. CREB through specific gene expression promotes neurite outgrowth and regulates synaptic plasticity.

1.4. NF-κB transcription factor

NF-κB is found in almost all animal cell types and is involved in cellular responses to many different kind of stimuli and stressors (e.g. tumor necrosis factor (TNF)) (Brasier 2006). NF-κB belongs to the Rel family, which consists of five members: p50, p52, p65, c-Rel and Rel B (Alberini 2009). The Rel homology domain that is located in the N-terminal, structurally contains a DNA binding element, dimerization, nuclear localization, and inhibitory κB (IκB) interaction regions (Alberini 2009). Only p65, c-Rel, and RelB have transcriptional activation domains (TAD) in their C-terminal to induce transcriptional activity. The p50 and p52 subunits are generated from large precursor proteins, p105 and p100. Generally p50 and p52 are repressors of κB site transcription, but both can later form transcriptionally active heterodimers in association with the former three members (Lehmann, et al. 2010 ; Li and Verma 2002). The p50 subunit lacks a TAD, therefore p50-p50 heterodimers act mostly to slow down gene expression under resting conditions (Ghosh and Karin 2002). This "tuning" role is important in controlling immune responses (Sha, et al. 1995).

NF-κB regulates immediate early gene (IEG) expressions involved in immune responses (Li, et al. 1999). The NF-κB complex is inactive intracellularly, but following cellular stimuli, like reactive oxygen species (ROS), calcium, TNF-α or IL-1β, become rapidly activated (Chandel, et al. 2000) .

Under resting conditions, the NF-κB dimers are sequestered in the cytoplasm in an inactive form by binding to a family of inhibitory proteins, called IκBs, typically IκBα and IκBβ (Hayden and Ghosh 2008), which are proteins that have an N-terminal regulatory domain, followed by six or more ankyrin repeats. The IκB family consists of IκBα, IκBβ, IκBε and

BCL-3. Also, p105 and p100, due to the presence of ankyrin repeats in their C-terminal halves, may also act as IκB proteins (Basak, et al. 2007). Following stimulation, usually from extracellular signals, a kinase called IκB kinase (IKK) becomes activated and phosphorylates two serine residues on IκB that leads to its subsequent degradation. The degradation of IκB leads to the release of the NF-κB dimer to translocate to the nucleus where it regulates target genes that have DNA-binding sites for NF-κB (Alberini 2009). Depending on the cell, the activation of these genes leads to a given physiological response. In addition, NF-κB activates expression of its own repressor IκBα to inhibit NF-κB and forms an auto feedback loop (Nelson, et al. 2004). Figure 2 shows a summary of the NF-κB activation pathway.

In the CNS, NF-κB is expressed in neurons, glia, and Schwann cells. Common signals that activate IKK in CNS include glutamate and nerve growth factor (NGF) (Meffert and Baltimore 2005). The NF-κB subunits that are the most commonly expressed in neurons, include the p50 homodimer and p50-p65 heterodimers (Meberg, et al. 1996). The known NF-κB target genes in neurons include CaMKIIδ, μ-opioid receptors, BDNF, neural cell adhesion molecule, and amyloid precursor protein (APP), amongst many others (O'Neill and Kaltschmidt 1997). NF-κB is activated following excitatory synaptic transmission via a Ca^{2+} dependent process as well as by dopamine (Alberini 2009). LTP induction correlates with NF-κB activation and is seen as an increase in p50 and p65 heterodimers and in a decrease of IκB mRNA (Meberg, et al. 1996). *In vivo* experiments on the crab *Chasmagnathus* has shown that a fear stimulus correlates with an increase in the activated form of NF-κB in brain cell nuclei (Freudenthal, et al. 1998), and injection of IκB kinase complex inhibitor blocks its activation and disrupts memory formation (Merlo, et al. 2005). Generally, inhibition of NF-κB reduces neural growth and branching in the hippocampus (O'Sullivan, et al.2010).

In the hippocampus in particular, increased NF-κB expression and activity in different regions are associated with different roles. For example, in the dentate gyrus novelty detection, is in the dorsal (CA1) segment, context encoding, and in the ventral segment (CA3), spatial memory and consolidation (Daumas, et al. 2005; Lee and Kesner 2003). NF-κB signaling at a 3 hour post-training time-point is required for hippocampal synaptic reorganization and memory consolidation (Igaz, et al. 2002). A large proportion of learning-regulated genes at 3-hour post training contain NF-κB binding sites in their promoters (O'Sullivan, et al. 2007). Expression of these genes occur immediately prior to synaptic remodeling and plasticity, accompanying learning (O'Malley, et al. 2000).

In general, the NF-κB family has a wide range of known functions, from controlling biological responses under pathological circumstances to synaptic plasticity, memory and spatial learning (Albensi and Mattson 2000; Ghosh, et al. 1998). However, suspected roles for NF-κB in synaptic plasticity and memory are more recent, as compared to more established roles for NF-κB in inflammatory responses, etc.

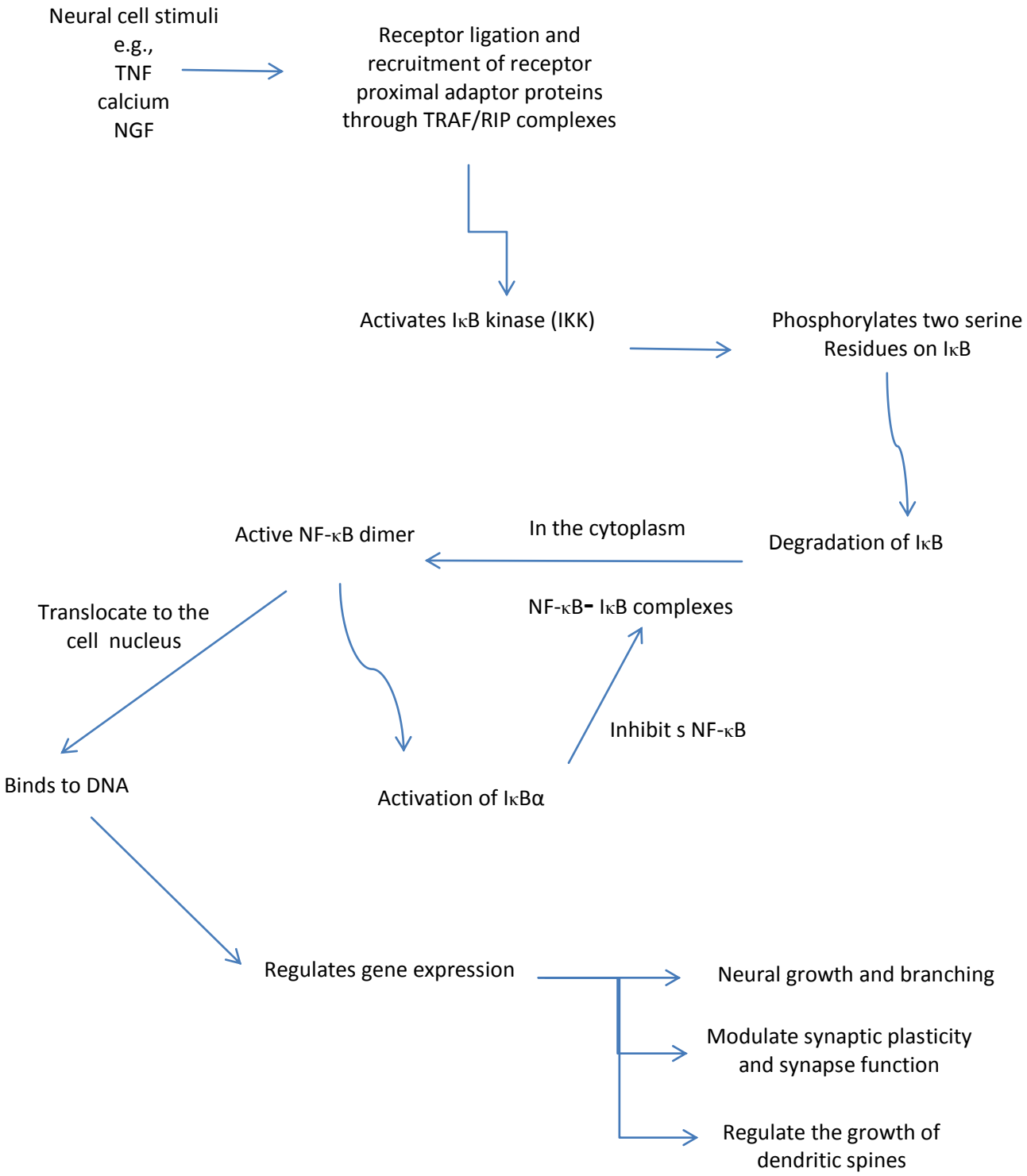


Figure 2. Summarized sequence of steps in NF-κB activation. Once activated, NF-κB leaves the cytoplasm, translocates to the nucleus, and regulates the expression of target genes that are involved in neuronal growth, branching, synaptic plasticity, and dentritic spine growth and remodeling, to name a few.

1.5. Egr-2 transcription factor

The early growth response (Egr) family of transcription factors comprises of 4 different proteins including Egr-1 (a.k.a., NGFI-A, Krox24), Egr-2 (Krox20), Egr-3 (Pilot), and Egr-4 (NGFI-C) (Poirier, et al. 2008; Williams, et al. 1995). The Egr genes that encode the expression of these transcription factors belong to the class of IEGs that, upon stimulation, share the characteristic of rapid and transient expression of proteins in response to neuronal modification (Vendrell, et al. 1998). They also encode closely related transcription factors and have common zinc-finger DNA binding domains (Swirnoff and Milbrandt 1995). However, they are different in their structural sequences, are regulated by different signaling pathways, and therefore have different functions (Beckmann and Wilce 1997). Egr-1 is specifically required for spatial navigation and consolidation of long-term memory (Jones, et al. 2001). Egr-3 is involved in short-term memory formation (Li, et al. 2007). When cells are stimulated appropriately, the cellular protein NGF-1A binding protein (Nab)-2 is expressed (Saadane, et al. 2000). Both Nab-1 and Nab-2 bind to Egr-1 (Svaren, et al. 1996). Nab-1 repressors Egr-1 mediated transcription (Saadane, et al. 2000). Egr-4 involves the encoding of inflammatory cytokines in T-cell-specific activities (Wieland, et al. 2005) and is rapidly induced following seizures in the hippocampus (Crosby, et al. 1992).

Egr-2 activates several genes that are involved in the formation and maintenance of myelin. Mutations in the Egr-2 gene can cause two forms of Charcot-Marie-Tooth disease with disruption of myelin synthesis (Nagarajan, et al. 2001). Also, some studies suggest that it has a key role in establishing persistent LTP via NMDA receptor mediation (Williams, et al. 1995). Egr-2 also has inhibitory roles on certain cognitive functions by regulating the expression of Nab1 and Nab2 proteins (Desmazieres, et al. 2008). The effect of Egr-2 can be prevented by

NMDA receptor antagonists such as MK-801 (Williams, et al. 1995). Egr-2 is also expressed in other circumstances such as following seizure activity (Bhat, et al. 1992), during developing and segmentation of hindbrain (Wilkinson, et al. 1989), and as a result of dopamine receptor activation and opiate withdrawal (Bhat, et al. 1992). Egr-2 expression levels are also different depending on the region in hippocampus; for instance, lower basal expression is seen in dentate gyrus compared to the other hippocampal regions (Richardson, et al. 1992). Furthermore, there are reports that inactivation of the adenosine 2A receptor can up-regulate Egr-2 expression in the striatum (Yu, et al. 2005). Interestingly, a review on the role of Egr-2 in forebrain reveals that its deficiency in this area does not affect memory or learning and may even facilitate memory performance in some tasks (Poirier, et al. 2008). It is thought that decreased Egr-2 levels result in decreased expression of Nab proteins that increase transcriptional activity of other Egr family where their effect is through Nab, resulting in facilitation in some types of memory (Poirier, et al. 2008).

Several Egr-2 binding sites have been recognized so far, including those on genes involved in peripheral nerve myelination such as Cx32 and Mbp (Bondurand, et al. 2001; Denarier, et al. 2005), lipid biosynthetic genes such as 3-hydroxy-3-methyl glutaryl (HMG) CoA reductase (Leblanc, et al. 2005), and ephrin A4 for controlling hindbrain gene expression (Ghislain, et al. 2003).

Collectively, these studies show that characterizing Egr regulation is important for comprehending the pathways in memory formation. Even though there are limited studies on different subtypes of Egr family transcription factors, they play significant roles in spatial memory and short-term memory formation. For example, based on the observation that there are several conserved sequences in the Egr promoter region domains (1-3), it is surprising that

Egr 1-3 show distinct functional differences as displayed by differences in short term versus long term memory regulation.

1.6. Objectives and rationale

The goal of this study is to profile the expression of CREB, NF-κB, and Egr-2 during the initial phases of memory encoding using the MWM experimental paradigm. Although these transcription factors have been implicated in learning and memory formation, little is known about their underlying mechanisms. We hypothesized that these factors will show differences in expression levels following MWM training. Differential expression profiles may give insight into the transcriptional regulation of long -term memory.

2. Materials and methods

2.1. Animals

The animal studies presented here were performed under a protocol approved by the University of Manitoba Protocol Management and Review Committee. Twenty nine to thirty two-day-old male CD1 mice were used for the study one experiment. These mice are albino in appearance, have rapid growth, and are easy to work with. Mice were acquired from the R.O. Burrell lab animal facility at the St. Boniface Research Centre, Winnipeg, Canada. Mice were maintained in cages filled with wood chips and bred and kept in the core unit of The R.O. Burrell lab. The mice were housed in their individual home cages prior to the experiments. Mice were fed standard mouse pellets *ad libitum* and typically caged in groups of two to five mice, according to R.O. Burrell lab housing protocol. All cages were supplied with a house insert to

provide shelter and enrichment. The cages were cleaned once a week. Room temperature was 22 ± 1ºC and relative humidity was 50 ± 10%. The room was illuminated with artificial light. Light was maintained on a 12-hour light-dark cycle. Ventilation was through 100% fresh air in an air change rate of 15 times per hour. The MWM experiments were done in the light phase of the cycle between 8:30 a.m. and 2:00 p.m. Housing and maintenance of mice were in the compliance with University of Manitoba regulations.

For study one, mice were categorized into two groups. Group 1 (control group), contained ten 29-32-day-old, male CD1 mice (non-trained control group). The second group (experimental group) contained ten CD1, 29-32-day-old male mice and underwent 9 consecutive days of MWM experiments. For study 2, male 30 day old C57 mice were organized into 4 groups (one control group and 3 experimental groups - described below). Following MWM training, mice were killed by decapitation using a standard decapitation device (World Precision Instruments, Inc., Sarasota, FL, USA). However, animals were first anaesthetized in a small chamber containing cotton balls saturated with isoflurane. Animals then were monitored to determine the level of consciousness by using respiratory rate, toe-pinch and the righting reflex criteria. Once the animal was unconscious (within 10 seconds), it was placed in the decapitation device and its head was quickly severed. A sagittal incision was made along the top of the skull in order to expose the brain. The brain was removed and divided into two hemispheres along the longitudinal fissure. Each hemisphere was manipulated in order to isolate the hippocampus which was then placed in ice-cold (4–6 °C) Hibrinate A solution in less than 30 s. The isolated hippocampi were stored at -80 degrees C, which occurred approximately five minutes or less following decapitation.

In the study 2 we used thirty six, six-month-old male C57BL/6 mice and used nine mice per group, with one control group and three experimental groups. Details regarding study 2 are discussed further in the other sections.

2.2. Morris water maze

Study 1. The MWM is a behavioral test that measures several factors related to memory and navigation. The MWM was first introduced by Dr. Richard Morris nearly 30 years ago (Morris, et al. 1982).The MWM test strongly correlates with hippocampal synaptic plasticity and NMDA receptor function (Lehmann, et al.). Materials for the MWM test consisted of a white fiberglass, 98 cm diameter tank (circular pool), filled with water (23-26°C) and made opaque (white in color) with powdered milk. A sufficient quantity of powdered milk (approximately 350 grams) was added in order to hide the escape platform from obvious view. The pool was conceptually divided into four quadrants. The escape platform, 10 cm in diameter, was submerged approximately 5 mm below the water surface (23 cm from the edge of the pool) in one of the quadrants and remained at that position during the acquisition phase.

On the back of the neck, the animals were marked with a black permanent marker in order to enhance visualization with the video camera (Figure 3).

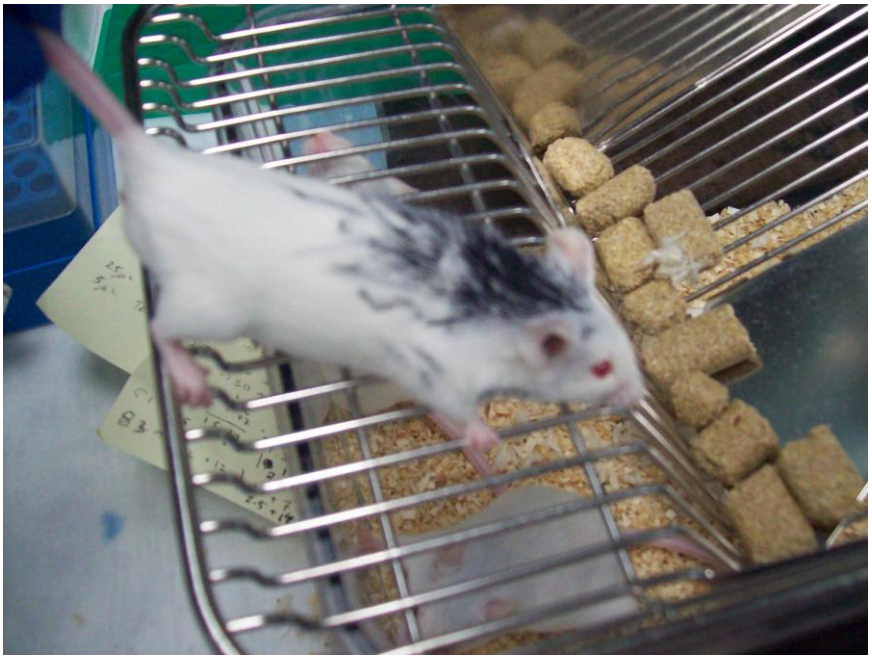


Figure 3. Mouse Marking for Visual Tracking. The photo shows a black marking on the back of an experimental animal model. The procedure helps to better visualize and track the animal during the experiments.

Four visual cues were designed and attached to the upper parts of the pool, which were placed outside high above the water. The cues consisted of different shapes including a circle, arrow, triangle, and star and were colored differently (Figure 4). The cues were placed 90 degrees apart, in order to decrease any external disturbance effects and to standardize the visual stimulation, a curtain surrounded the pool. Also, in order to standardize and decrease the stress of being placed in the water, mice were dropped gradually with a standard kitchen food strainer at the start of each experiment.

Figure 4. The Morris water maze apparatus. The photo shows the tank used for the Morris water maze experiments with the attached visual cues. The animal use these visual cues to assist with orientation as it attempts to locate the hidden the platform.

There were two phases (i.e., acquisition and retention) for the MWM tests, which occurred over 9 consecutive days (a.k.a. blocks, 1 day = 1 block). The acquisition phase consisted of 6 consecutive days in which each mouse was tested for a total of 4 trials (30-minute inter-trial interval) per day with the hidden platform. During this phase, several parameters are measured as described below. These include escape latency and search strategies. During the retention phase the platform is removed. During this phase, we measured the number of times the mouse passed over the location of the missing platform and the amount of time the mouse spent in the target quadrant. The primary purpose of the acquisition phase is to train the animals to learn the specific location of a platform using visual cues in the immediate environment.

The mice started the trial by facing the pool edge in different quadrants in each trial. The mice were given a maximum of 90 s to find the position of the platform and were required to remain seated after finding the platform for at least 10 s. If the animal was not able to find the

position of the platform after the 90-second time frame, the animal was placed directly on the platform for 10 s and then returned to its cage. The cage was then placed under a warming light (approximately 35 degrees in centigrade) during the inter-trial period. During this time the mouse was observed to be grooming itself. During the acquisition phase, escape latency was measured, which was the amount of time that it took the mouse to find the hidden platform for each trial. For those mice that did not find the hidden platform in the allotted time, a score of 90 s was given. Animals were videotaped for each trial and the video clips were analyzed for escape latency as previously described (Janus, et al. 2000). In addition, search strategies for each trial of the acquisition phase were evaluated by reviewing the videos. Search strategies based on swimming patterns were categorized into 9 types, as defined by Brody et al. (Brody and Holtzman 2006). This was first done by grouping conceptually similar search strategies into three groups for data analysis (see figure 5), which included a) spatial strategies, b) non-spatial, systemic strategies, and c) strategies based on repetitive looping (Brody and Holtzman 2006). These included spatial strategies with "spatial direct" as the mouse swims directly onto the platform, "spatial indirect" as the mouse searches the incorrect quadrant before finding the platform and "focal correct" as the mouse searches the correct quadrant before finding the platform. Non-spatial systemic strategies consists as "scanning" with the mouse searches the pool at a fixed distance from the pool wall, "random" as the mouse searches the pool randomly and "focal incorrect" as the mouse searches only a small portion of the pool that does not contain the platform. Strategies involving repetitive looping include; "chaining" as the mouse swims circularly at a fixed distance from the wall (> 15 cm), "peripheral looping" where the mouse continuously swims around the outer 15 cm of the pool and "circling" as the mouse swims in tight circles.

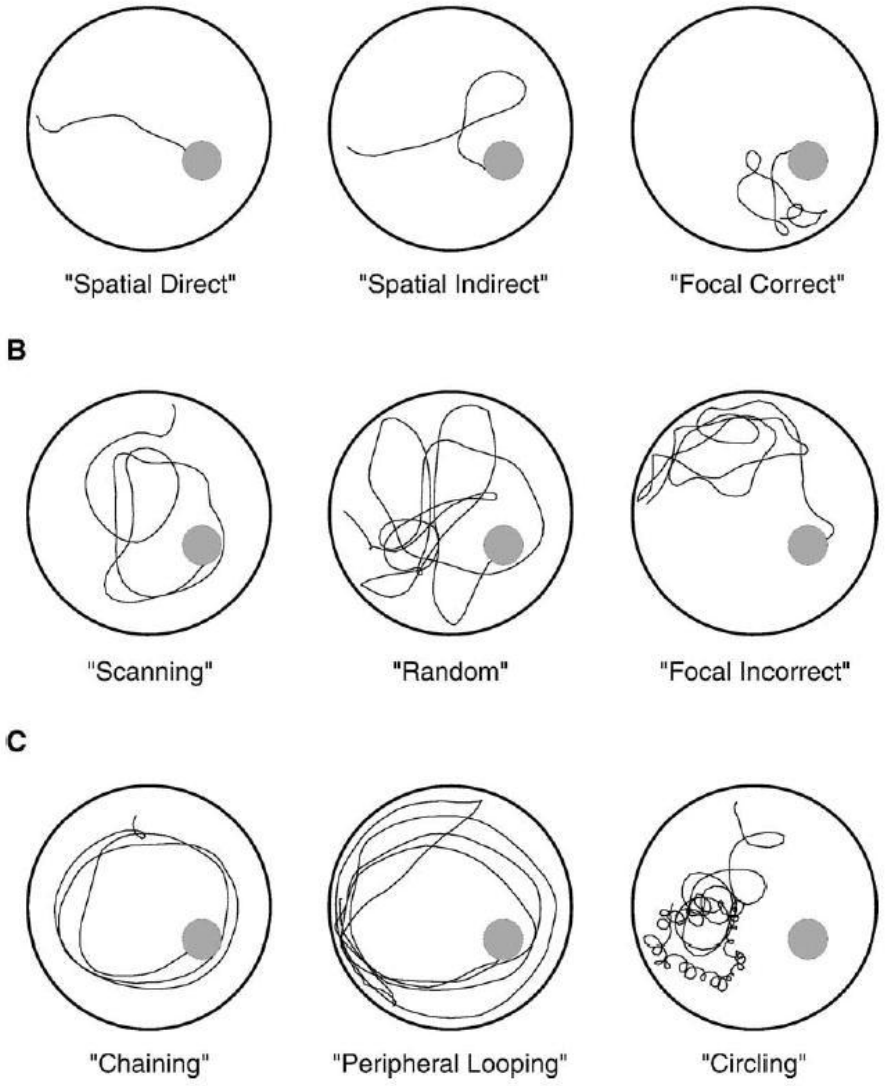


Figure 5. Search Strategies. Search strategies, as defined by Brody et al., were observed and recorded during the Morris water maze trials. Spatial strategies, such as *spatial direct*, *spatial indirect*, and *focal direct* (see above), are considered to be the most efficient for finding the platform and have been correlated with the subject forming a cognitive map in the brain. Reproduced with permission from Brody DL et al. *Exp Neurol*. 2006 February; 197(2): 330–340.

The retention phase of the MWM begins the day immediately following the last block of the acquisition phase and lasts for a total of 3 consecutive days. During the retention phase, the platform was removed and the animals were given 60 s to explore the pool after which the animal was removed from the pool and returned to its cage (as described above). Each animal

was tested for four trials per day. During this phase, we measured two parameters including the number of times the mouse passed over the location of the missing platform and the amount of time the mouse spent in the target quadrant. The primary purpose of the retention phase is to determine how well the animal can recall the position of the original platform before it was removed. The capacity to recall the specific location of the platform is a function of the animal's long term memory.

Study 2. In this case, Group 1 was also a non-trained control group (mice were allowed to swim randomly for a comparable time in the MWM). However, Groups 2 and 3 received MWM training for 6 consecutive days in the 2 phases (i.e., acquisition for 5 days and retention for 1 day). Group 1 was killed 24 hours following swimming, whereas Group 2 mice were killed 30 minutes following training (i.e., on day 6). Group 3 and Group 4 mice were killed 24 hours following training, however Group 4 mice had a delay of several days between acquisition and retention phases. In other words, Group 4 mice received acquisition training for 5 days, but were not tested in the retention phase until day 12. Group 3 mice received 5 days of acquisition training and then the retention phase was conducted on day 6.

2.3. Sample preparation and protein assay

Following the MWM experiments, all mice (10 non trained controls and 10 experimental mice exposed to the MWM) were killed by decapitation under anesthesia (as described on page 18). All efforts were made to minimize animal suffering during the process. The next step was to evaluate and compare the expression of transcription factors NF-κB, Egr-2 and CREB in control vs. experimental groups. Hippocampal tissue sampling was performed using the modified method of Moriya et al (Moriya, et al. 2011). The entire hippocampus was excised from the brains of both controls and experimental animals (as described on page 18). Isolating the hippocampus is a relatively easy process since most of the hippocampus is already free of surrounding brain structures. However, some small amounts of meningeal and other connecting tissue need to be removed as a last step before preservation. In addition, it should be noted there is no free blood associated with this dissection step, but as a matter of standard procedure, each hippocampus is rinsed by default as it is placed in the Hibrinate A solution. The hippocampi were then weighed on an analytical balance (Mettler Toledo AG104) before performing Western blotting procedures (Table 1) in order to make an appropriate weight-based homogenization solution. As shown in Table 2, by using unpaired *t* test, there was no statistical difference between hippocampal weights from control versus experimental groups.

Table 1. Hippocampal weight and the required amount of homogenization solution in a weight – based protocol.

Controls	Weight (g)	Required homogenization solution (µl)
C1	0.0254	254
C2	0.0308	308
C3	0.0800	800
C4	0.0452	452
C5	0.0454	454
C6	0.1091	1091
C7	0.0910	910
C8	0.0338	338
C9	0.0285	285
C10	0.0484	484

Preparation of homogenization solution based on the hippocampal weight in the control group.

Experiments	Weight (g)	Required homogenization solution (µl)
E1	0.0575	575
E2	0.0792	793
E3	0.0506	506
E4	0.0346	346
E5	0.0324	324
E6	0.0622	622
E7	0.0512	512
E8	0.0732	732
E9	0.0353	353
E10	0.0306	306

Preparation of homogenization solution based on the hippocampal weight in the experimental group.

Table 2. Comparison of hippocampal weight among controls and experimental groups. (p = 0.7785).

Group	Controls hippocampal weight (g)	Experiments hippocampal weight (g)	Significance
Mean	0.0537600	0.0506890	NS
SD	0.0292075	0.0174153	
SEM	0.0092362	0.0055072	
N	10	10	

Hippocampal weight were not statistically significant among the experimental and control groups. (NS= non significant)

The homogenization solution for stabilized detergent soluble and insoluble fractions from samples was prepared based on our modified protocol from Filliatreau et al. (Filliatreau, et al. 1988) (Table 3).

Table 3. The modified homogenization buffer protocol adopted from Filliatreau et al. (100 ml).

0.1 M Pipes, pH 6.9	**3.02 g**
5 mM MgCl2	**1 ml of 0.5 M**
5 mM EGTA	**1 ml of 0.5 M**
0.5% Triton X-100	**0.5 ml**
20% glycerol	**20 ml**
10 mM NaF	**0.042 g**
Water	**77.5 ml**

Following buffer preparation using the above protocol, the following solutions were added to 10 ml of the buffer.

- 10 µl of 1M PMSF
- 0.2 ml of sodium orthovanadate solution
- 0.5 ml of protease inhibitor cocktail

The frozen hippocampus was then pulverized by mortar and pestle and the adjusted weight-based homogenization buffer was added. The protease inhibitor cocktail (Roche Diagnostics, Manheim, Germany) was added to help protect the integrity of proteins during the protein extraction and purification process (Cheon, et al. 2008). The prepared samples were stored in -80 degrees C for further use. The Bradford assay was used in prepared samples and for protein content of the supernatant. Zero, 1/12, 1/10 and 1/20 dilutions of samples in homogenization buffer were also prepared. Aliquots of Bovine Serum Albumin (BSA) with different concentrations from 0-1.5 mg/ml as standards were prepared. Table 4 shows the optimal protein assay concentrations for control and experimental groups.

Table 4. Optimal and final protein assay on controls.

R-Squared: 0.9908 in 1/10 dilutions

Controls	OD (mean)	CV	Concentration
C1	0.034	22.287%	0.389
C2	0.044	8.713%	0.471
C3	0.027	22.827%	0.285
C4	0.050	17.597%	0.544
C5	0.042	19.564%	0.457
C6	0.023	73.868%	0.234
C7	0.035	12.191%	0.375
C8	0.045	24.356%	0.492
C9	0.065	8.372%	0.248
C10	0.024	23.959%	0.248
Standard 1 (S1)	0.016	8.785%	0.188
S2	0.033	1.813%	0.375
S3	0.075	15.493%	0.750
S4	0.131	4.834%	1.500

R-Squared: 0.9967 in 1/10 dilutions

Experiments	OD (mean)	CV	Concentration
E1	0.023	28.764%	0.260
E2	0.027	26.313%	0.305
E3	0.040	12.104%	0.454
E4	0.039	5.055%	0.440
E5	0.042	31.409%	0.359
E6	0.029	66.302%	0.329
E7	0.034	22.462%	0.384
E8	0.014	51.985%	0.152
E9	0.039	18.329%	0.438
E10	0.039	15.910%	0.439
Standard 1 (S1)	0.018	29.997%	0.187
S2	0.034	29.816%	0.375
S3	0.061	6.067%	0.750
S4	0.133	17.865%	1.500

Following the obtainment of the concentrations needed for Western blots from the optimal protein assay, we made samples containing 20 µg/20 µl of protein. Table 5 shows fractions of the calculated samples based on a 3 X SDS, water, and the generated protein assay derived from the Bradford protein assay.

Table 5. Planning table for using 20µg of protein in 20µl (20µg/µl).

Control group:

Samples	Protein assay mg/ml	Protein in sample 20µg	3X SDS	Water
C1	3.89	5.141	6.666	8.193
C2	4.71	4.246	6.666	9.088
C3	2.85	7.017	6.666	6.317
C4	5.44	3.676	6.666	10.342
C5	4.57	4.376	6.666	8.958
C6	2.34	8.547	6.666	4.787
C7	3.75	5.333	6.666	8.001
C8	4.92	4.065	6.666	9.269
C9	2.48	8.064	6.666	5.270
C10	2.48	8.064	6.666	5.270

Experimental group:

Samples	Protein assay mg/ml	Protein in sample 20µg	3X SDS	Water
E1	2.6	7.692	6.666	5.642
E2	3.05	6.557	6.666	6.777
E3	4.54	4.405	6.666	8.929
E4	4.4	4.545	6.666	8.789
E5	3.59	5.571	6.666	7.763
E6	3.3	6.06	6.666	7.274
E7	3.84	5.208	6.666	8.126
E8	1.52	13.157	6.666	0.177
E9	4.38	4.566	6.666	8.768
E10	4.39	4.555	6.666	8.779

2.4. Western blotting

Aliquots of 20 µg/20 µl of protein were denatured at 100ºC for 10 min to reduce and denature the proteins (Filliatreau, et al. 1988). Three Western blots were done on all controls and experimental samples for each transcription factor (NF-κB, Egr-2 and CREB) and also on actin controls to confirm the molecular weight (MW). Electrophoresis was performed with a BIORAD unit (Mini-PROTEAN® 3 Cell, USA). The proteins which were separated based on their MW on the gel, were transferred onto nitrocellulose membranes. The MWs of the transcription factors are as follows: CREB 43 kDa, NF-κB p50 50 kDa and Egr-2 (krox-20) 48 kDa. Membranes were probed with diluted primary rabbit antibodies against the transcription factors including CREB rabbit monoclonal antibody (mAb) (1:1000 dilution, Cell Signaling, Danvers, MA, USA), NF-kB p105/p50 rabbit mAb (1:500 dilution, Epitomics, Burlingame, CA, USA) and Egr-2 (krox-20) polyclonal antibody (1:500 dilution, Covance, Emeryville, CA, USA). Following incubation with primary antibodies and actin, nitrocellulose membranes were exposed to secondary goat- anti-rabbit antibodies (1:5000 dilution, Epitomics, Burlingame, CA, USA). Then enhanced chemiluminescence was detected from the blot using the ECL Plus™ (GE Healthcare, Baie d'Urfe, QC, Canada). Every transcription factor was run three times on all 20 samples (10 controls and 10 experimental). In order to normalize the results, actin controls were done on the Western blots (Figures 6-8). Then, calculation of the pixels for normalization were done accordingly.

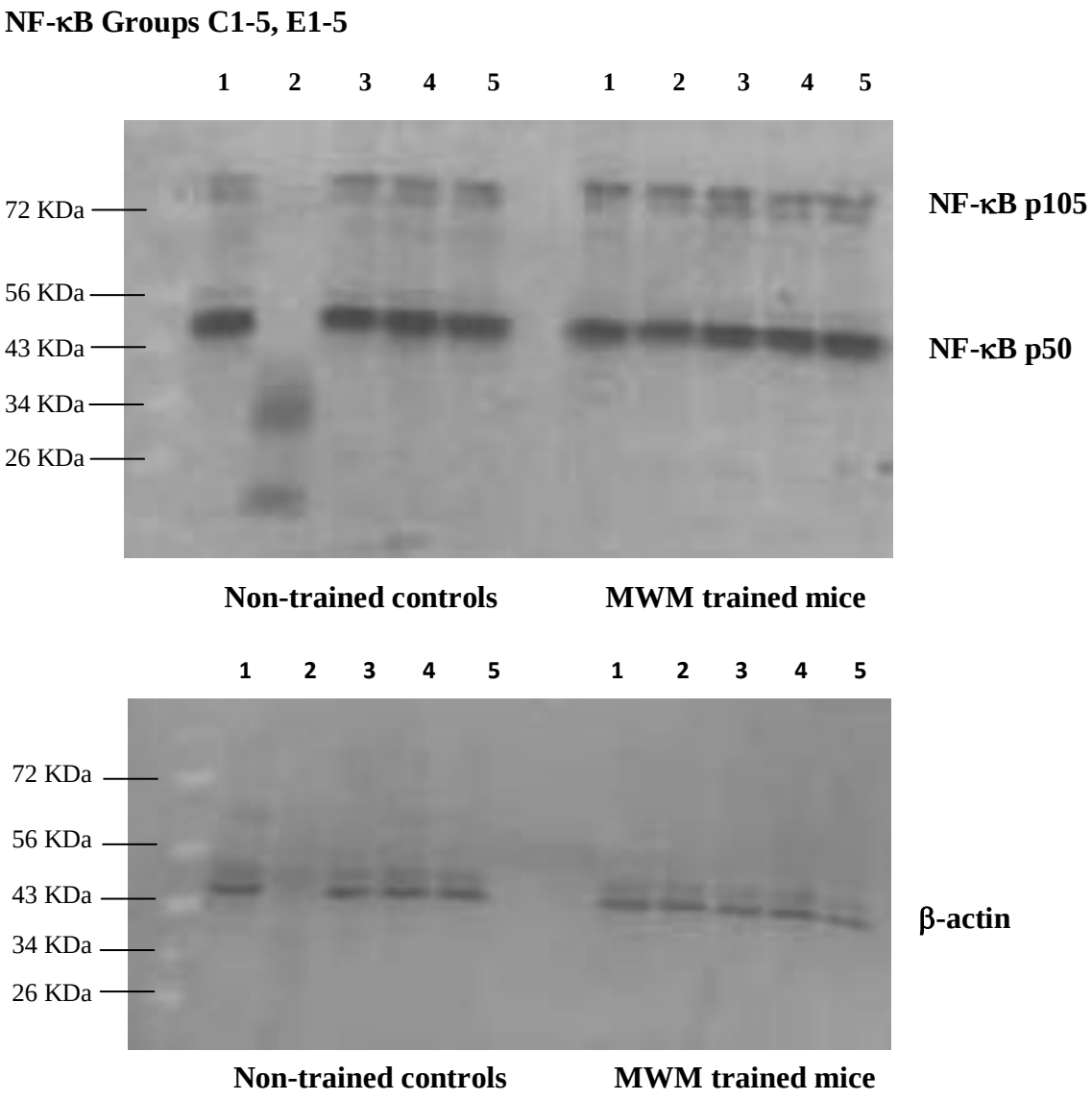


Figure 6A. NF-κB Western blots on controls and experimental samples. Lanes 1-5 represent duplicates for each group.

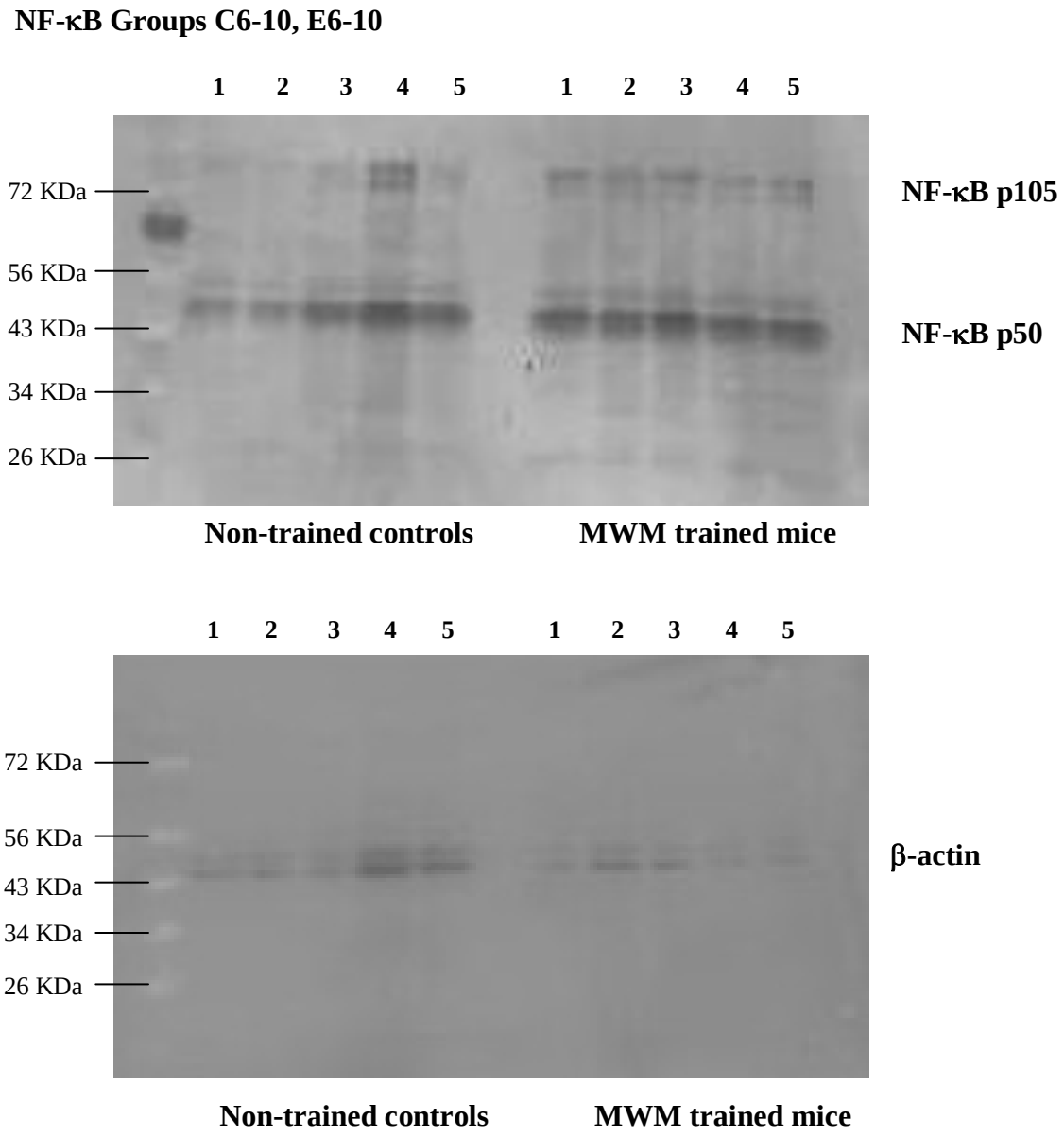


Figure 6B. NF-κB Western blots on control and experimental samples. Lanes 1-5 represent duplicates for each group.

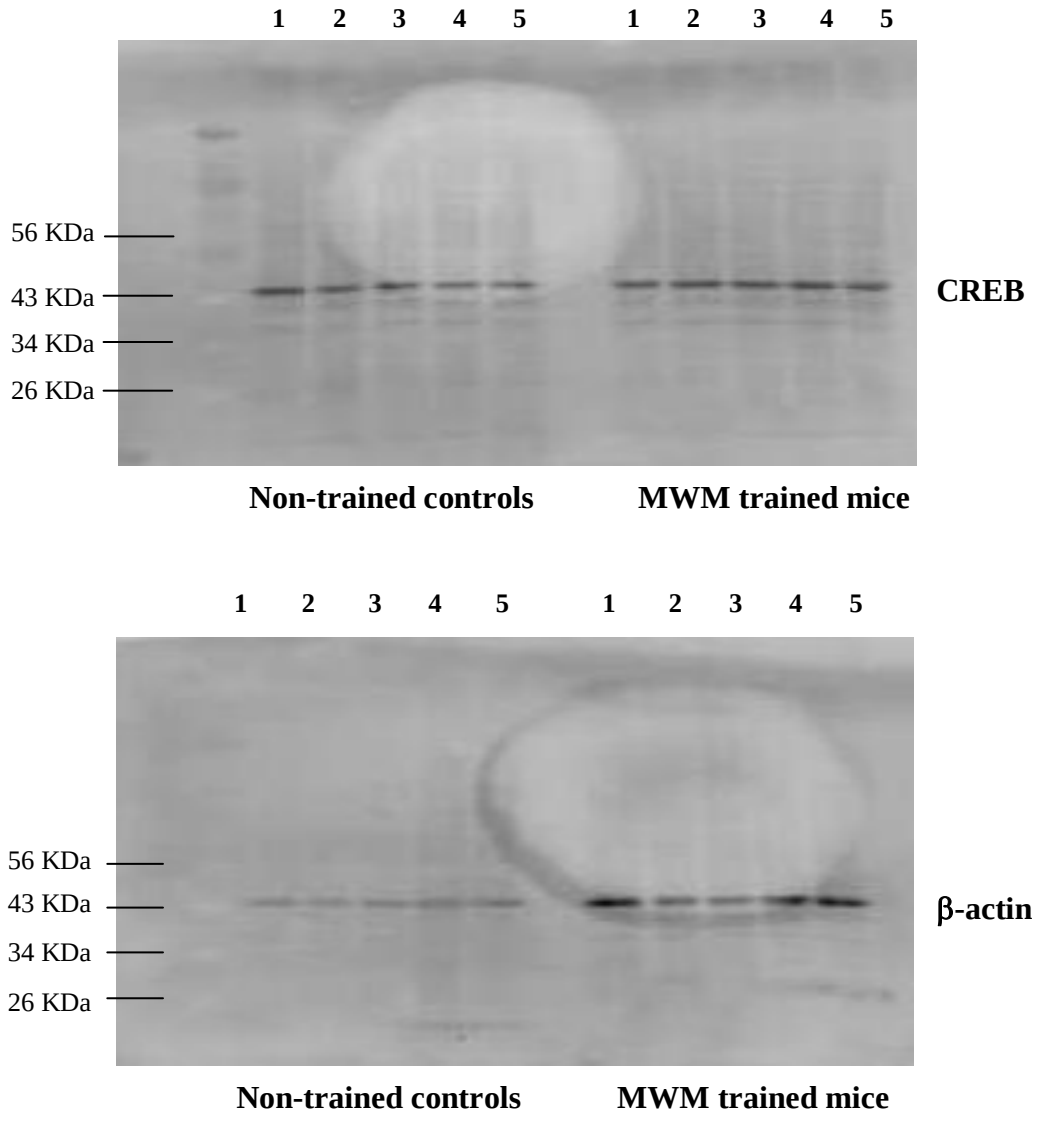


Figure 7A. CREB Western blots on control and experimental samples. Lanes 1-5 represent duplicates for each group.

CREB Groups C6-10, E6-10

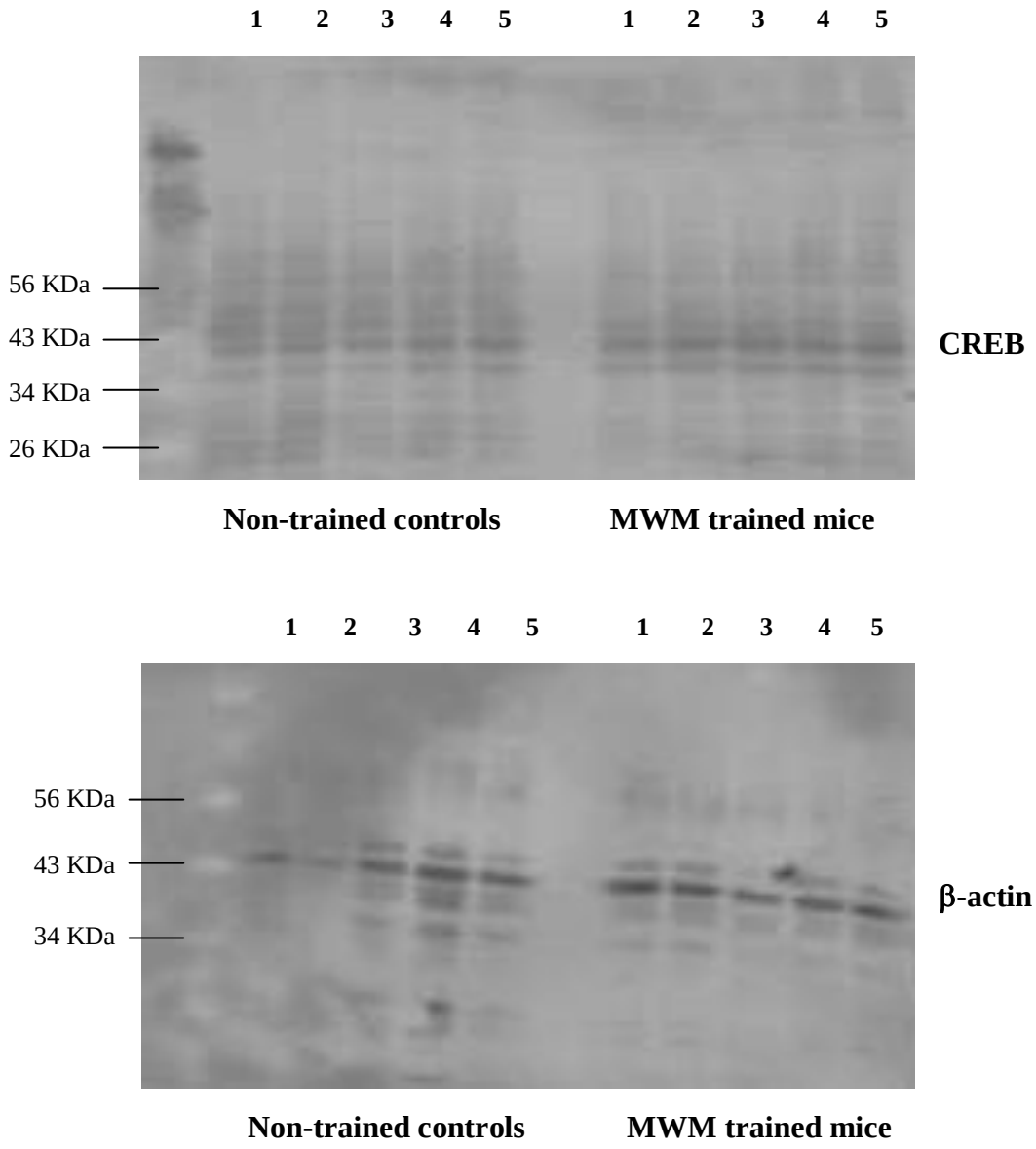


Figure 7B. CREB Western blots on control and experimental samples. Lanes 1-5 represent duplicates for each group.

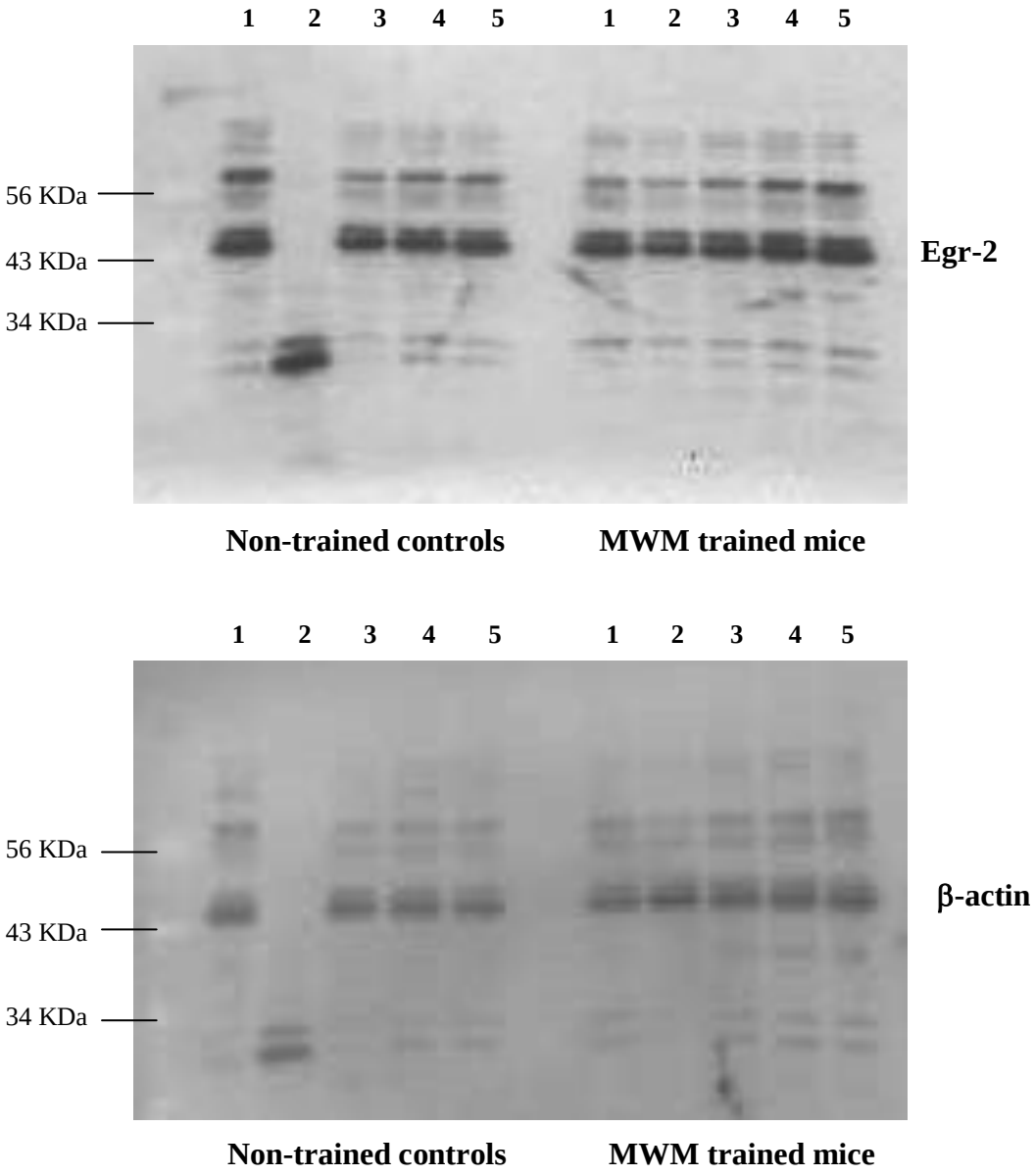


Figure 8A. Egr-2 Western blots on control and experimental samples. Lanes 1-5 are duplicates for each group.

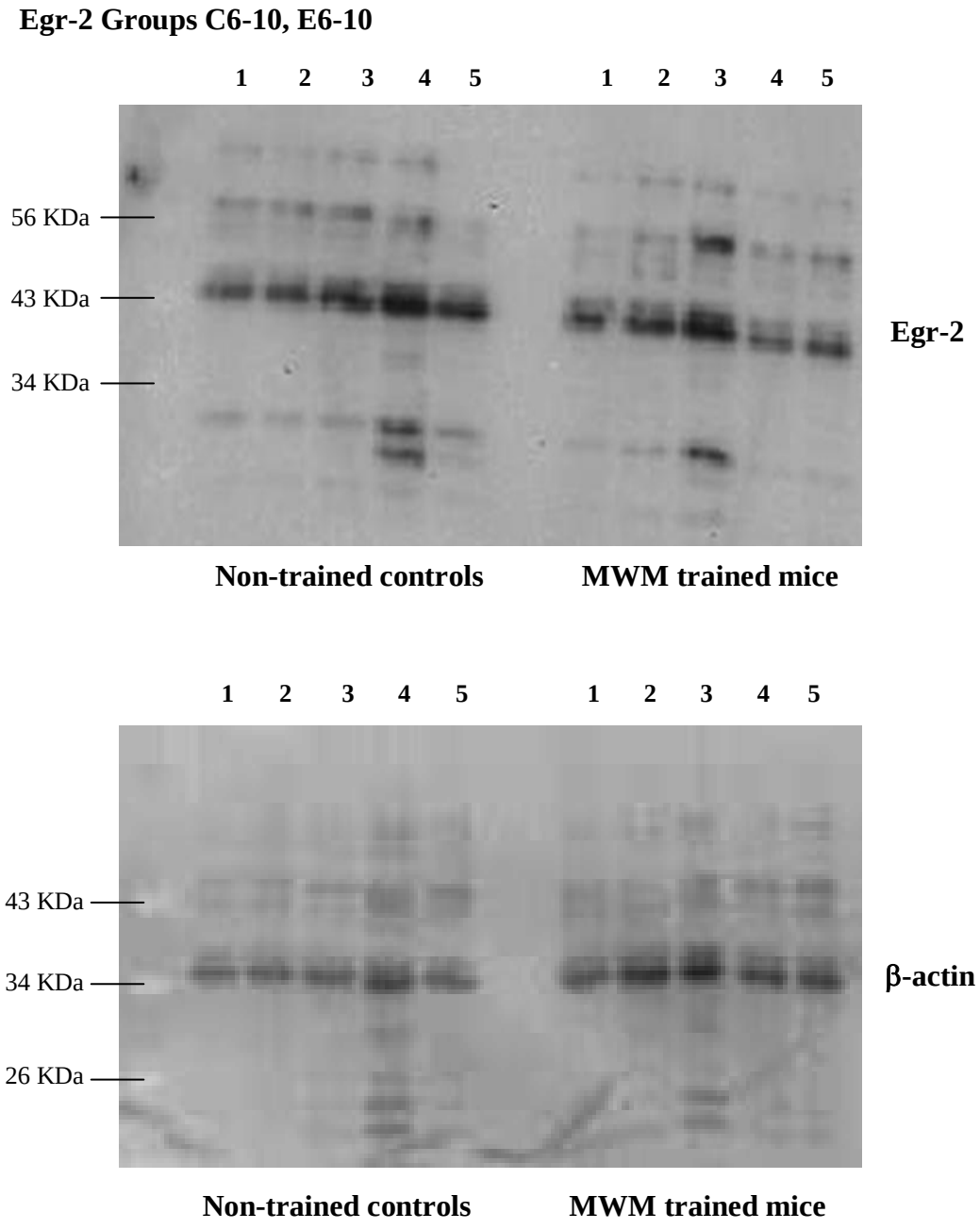


Figure 8B. Egr-2 Western blots on control and experimental samples. Lanes 1-5 are duplicates for each group.

2.5. Statistical analysis

Results are presented as mean ± SEM in all cases. Between groups differences of the Western blot experiments were performed by an unpaired student's *t*-test. The Western blot experiments data are presented as mean ± SD. Calculations were performed using SPSS (version 13.0, Chicago, IL, USA). In all cases, a *p* value < 0.05 was considered statistically significant.

3. Results

3.1. Study 1

Hippocampal weights. The range in the weight of hippocampi in the control group (0.05 ± 0.02) versus the experimental (MWM) group (0.05 ± 0.01) is shown in figure 9. There was not a statistically significant difference in these weights (*p* value = 0.7785) between the two groups.

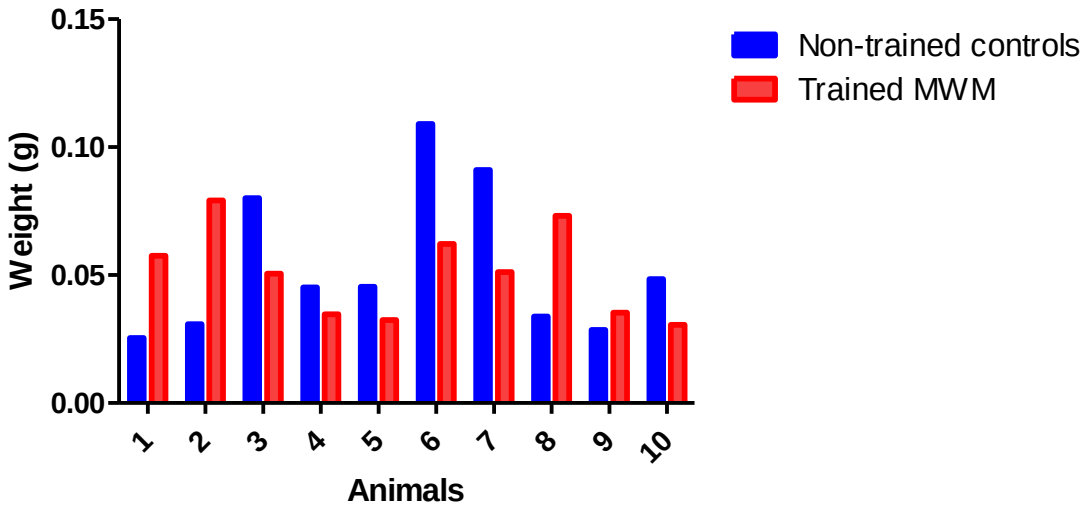


Figure 9. Hippocampal Weights. The graph shows the range of weight of hippocampi in 10 controls and 10 experimental mice.

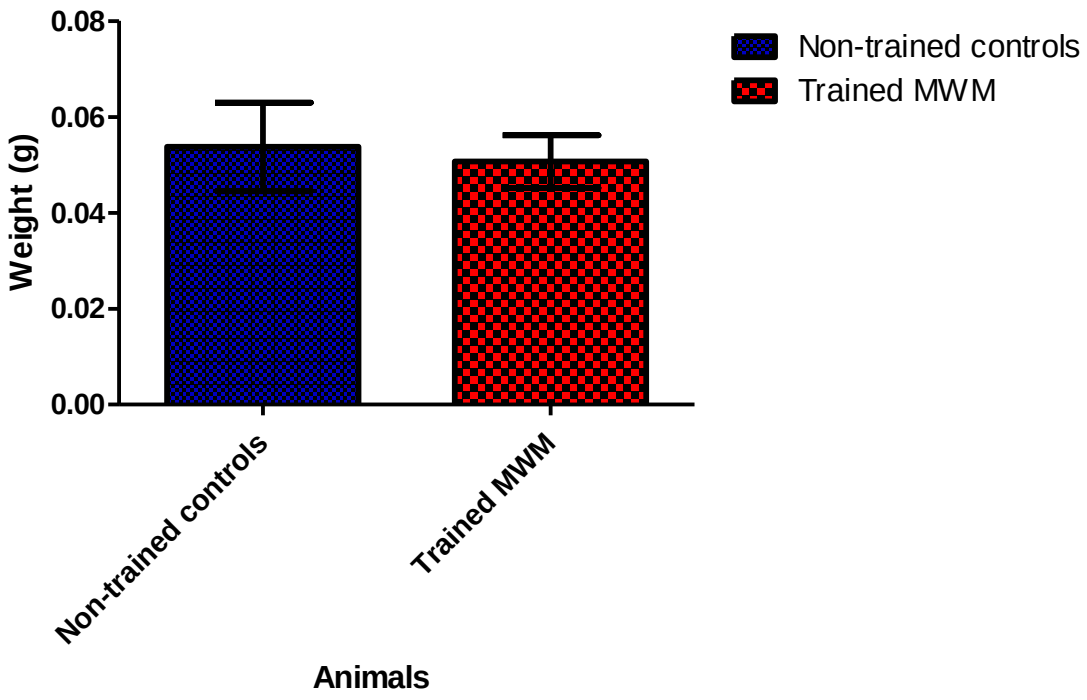


Figure 10. Hippocampal weights: controls versus experimental group. There was no statistical difference in hippocampal weights between experimental and control groups. (p = 0.7785).

MWM training. It was presumed that as the animal is trained in the water maze, each subsequent day should show an increase in the familiarity of the environment and thus demonstrate learning. We found that during acquisition, the escape latency progressively decreased toward the end of this phase from an average of 60 seconds in day 1 to an average of ~11 seconds in day 6, indicating a mean improvement in the animals' performance (Figure 11). This change in performance was statistically significant over time [$p<0.001$, $F(5, 216)=32.504$].

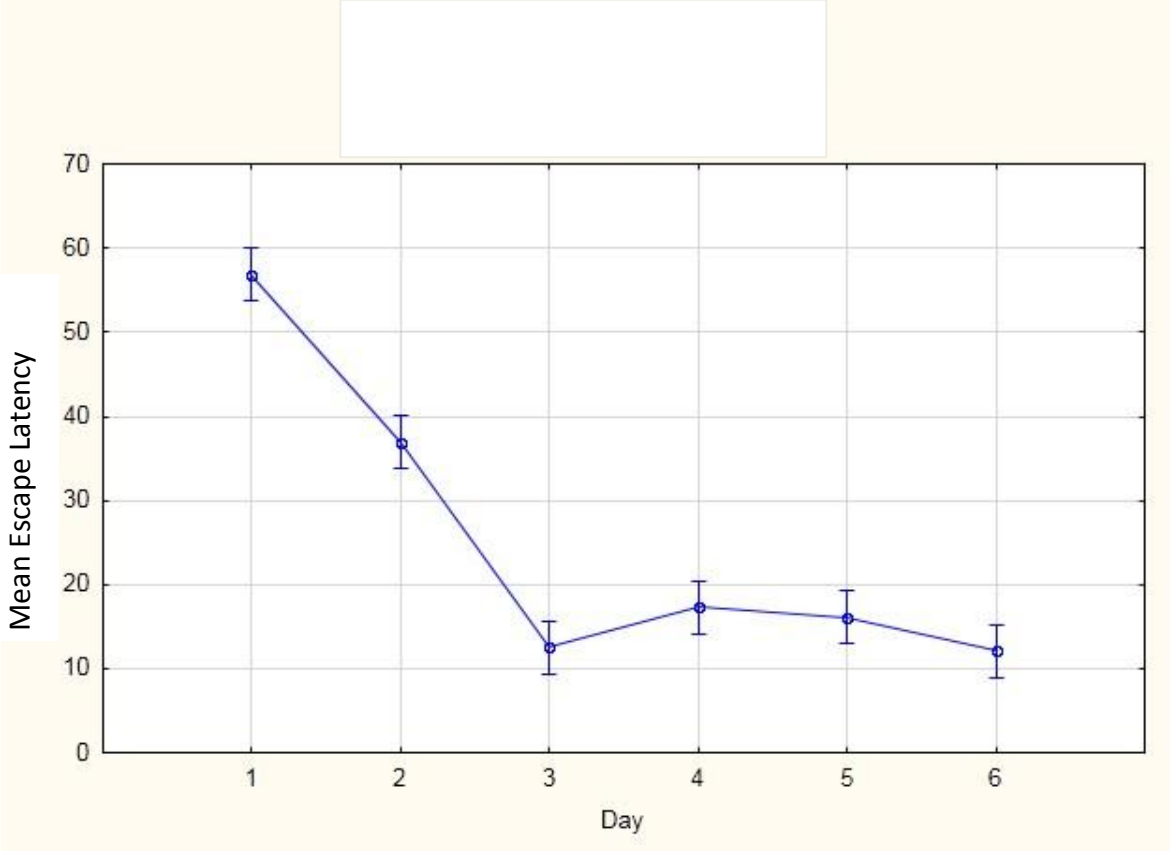


Figure 11. Escape Latency. The average escape latency is shown over time, which significantly decreased ($p < 0.001$) by the end of the acquisition phase (Error bars, ± SEM).

Over the three day retention phase, we measured the "time spent in the target quadrant" and the "number of passes over the missing platform". We found that the animals recalled the location of the missing platform, as evidenced by a substantial amount of time spent in the target quadrant and by the number of passes over the missing platform. In particular, over the 3 days of this phase, these mice spent more time in the target quadrant in the 1st and 3rd days of the retention phase (Figure 12).

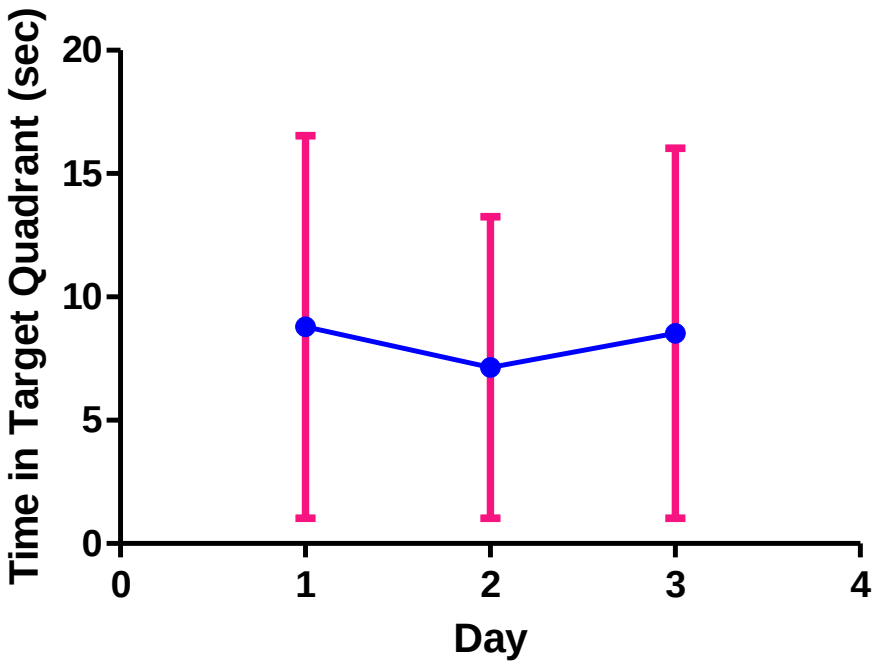


Figure 12. Time in Target Quadrant. The graph shows the average time spent by the experimental group in the target quadrant (sec) (Error bars = ± SEM).

On average mice spent approximately 17 seconds in the 1st and 3rd days in the target quadrant. Regarding the number of passes over the missing platform, the peak was the first day of the retention phase (> 5 times) then to a steady level of ~ 3 times in the remaining days of this phase (Figure 13).

Table 6. also shows that there is a per day significance passing the target quadrant over the missing platform during the retention phase.

Table 6. Summary of multivariate analysis test of significance of spending time in target quadrant. (Sigma-restricted parameterization). P < 0.05 considered significant.

Effect	Test	Value	F	Effect df	Error df	P
Intercept	Wilkis	0.115744	825.0976 1	1	108	*0.000000*
Trial	Wilkis	0.994260	0.2078	3	108	0.890782
Day	Wilkis	0.935771	3.7064	2	108	*0.027743*
Trial*Day	Wilkis	0.985396	0.2668	6	108	0.951267

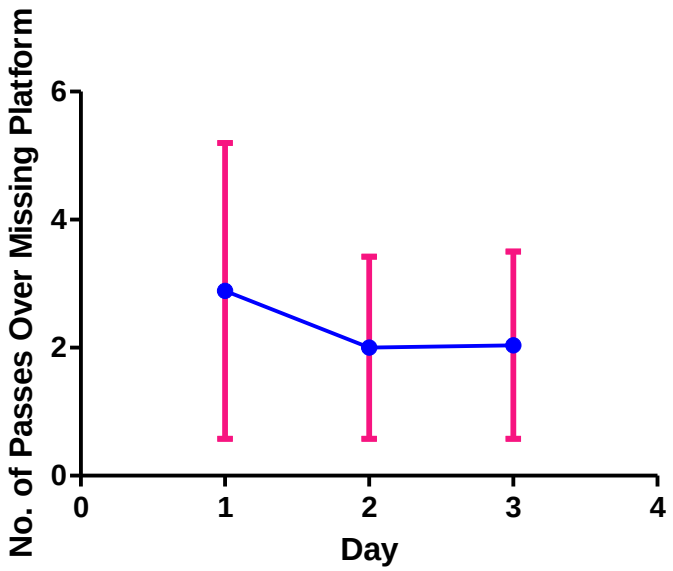


Figure 13. Number of Passes over the Missing Platform. The graph shows the average number of passes over the missing platform by experimental group (Error bars = ± SEM). Our experiment showed that the experimental animal models passed the missing platform mostly in the first day of the retention phase.

In addition to measuring the time the animal spent in the target quadrant during the retention phase (Figure 14), we also measured the time that the animals spent in another randomly selected quadrant (Figure 15).

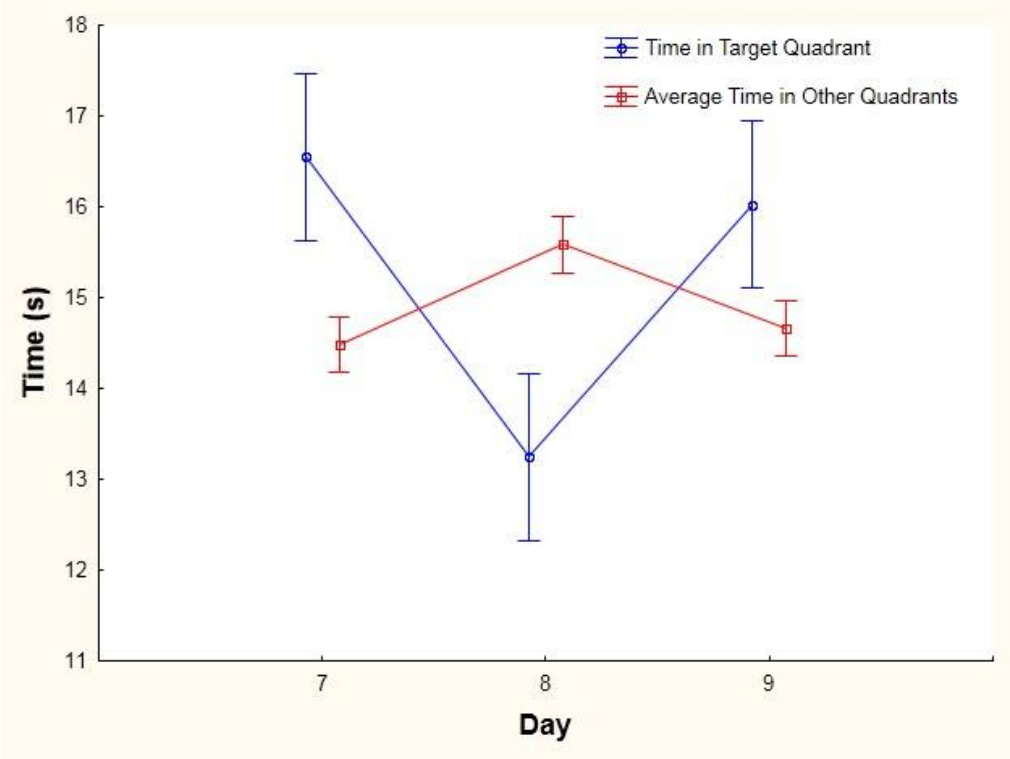


Figure 14. Time in Target Quadrant. The graph shows the time that the animals spent in the target quadrant during the retention phase (Error bars = ± SEM).

A comparison of the time that the animals spent in the target quadrant (15.27 ± 1.77) and the randomly selected quadrant (11.57 ± 1.24) showed that there was a statistically significant difference for spending more time in the target quadrant during the retention phase ($p = 0.04$) (Figure 15).

The search strategies used during the MWM were collectively assessed (Figure 16). Nine search strategies were recorded as shown in Figure 16. Given the importance of spatial

strategies, we then replotted this data by combining this data into 3 main groups, ie., repetitive looping, non-spatial, and spatial strategies (Figure 17) and found that overall spatial strategies increased in use over time.

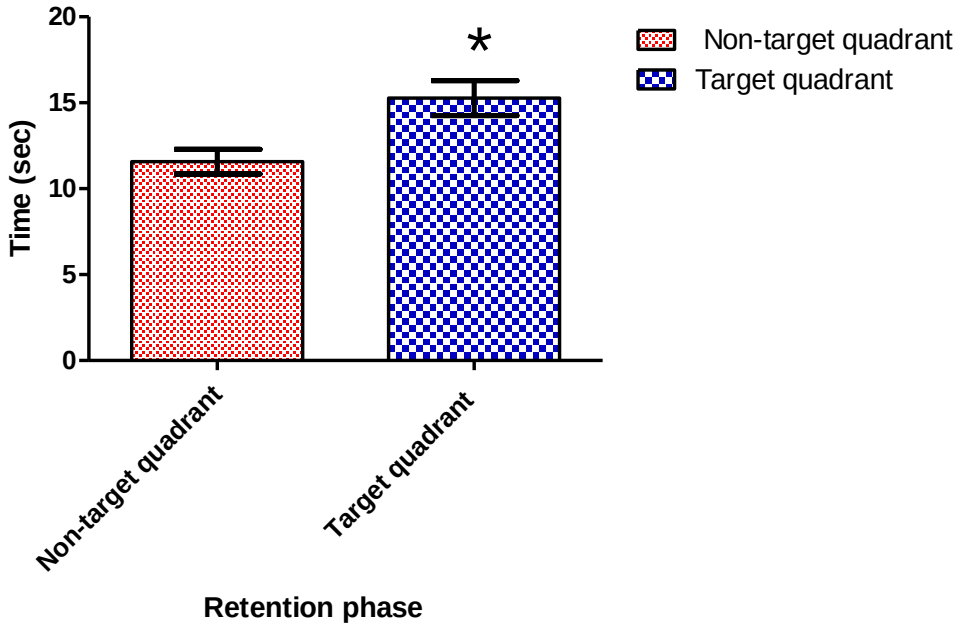


Figure 15. Comparison between Quadrants. There was a statistical difference between the time that the experimental animals spent in the target quadrant and the time they spent in a randomly selected quadrant during the retention phase (*p* value = 0.0415).

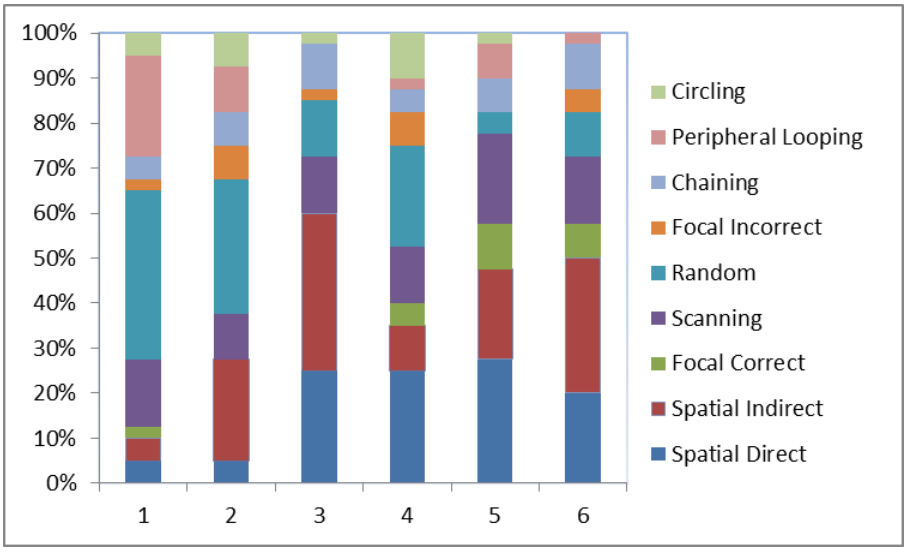


Figure 16. Assessment of Search Strategies. Nine search strategies (as defined by Brody et al.) were recorded for the experimental animals during the acquisition phase in the Morris water maze.

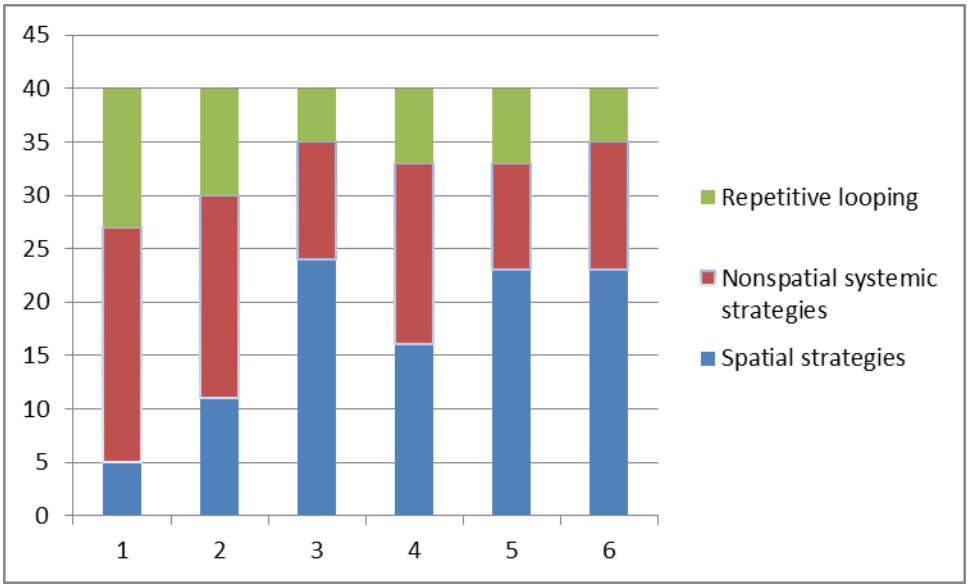


Figure 17. Spatial Search Strategy Assessment. Search strategies were combined into 3 groups based on functional similarity. Overall, spatial strategies are seen to increase in use over time. Chi square analysis was performed on a daily basis in order to quantify changes.

Table 7. Summary of *in vivo* search strategy data. Percentage of use over time during acquisition training.

Strategy	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Spatial	12.5	27.5	60	40	57.5	57.5
Non-Spatial	55	47.5	27.5	42.5	25	30
Repetitive Looping	32.5	25	12.5	17.5	17.5	12.5

Using the frequency data from Figure 17 (Table 7), we conducted a 3 (Spatial Strategy) x 6 (Day) Chi Square analysis which revealed significant overall differences in the frequencies across Day and Strategy, χ^2 (10) = 78.78, $p < .001$. Additional analyses revealed that search strategy differed at Day 1, χ^2 (2) = 27.13, $p < .001$; Day 2, χ^2 (2) = 9.13, $p = .01$; Day 3, χ^2 (2) = 35.38, $p < .001$; Day 4, χ^2 (2) = 11.38, $p = .003$; Day 5, χ^2 (2) = 27.13, $p < .001$; and Day 6, χ^2 (2) = 30.88, $p < .001$. Further, search strategies also differed across days. For Spatial Strategies, a significant difference in the frequency of use occurred across days, χ^2 (5) = 44.41, $p < .001$; differences also emerged for non-Spatial Strategies, χ^2 (5) = 19.59, $p = .001$; finally, Repetitive Looping also differed across days, χ^2 (5) = 15.59, $p = .02$. We found that mice used a mixture of strategies on the first day, including spatial (12.5%), non-spatial 55%), and repetitive looping (32.5%) and then showed a progressive increase (day 2, 27.5%; day 3, 60%; day 4, 40%; day 5, 57.5%; day 6, 57.5%; in the use of spatial strategies over the rest of the 6 day acquisition phase.

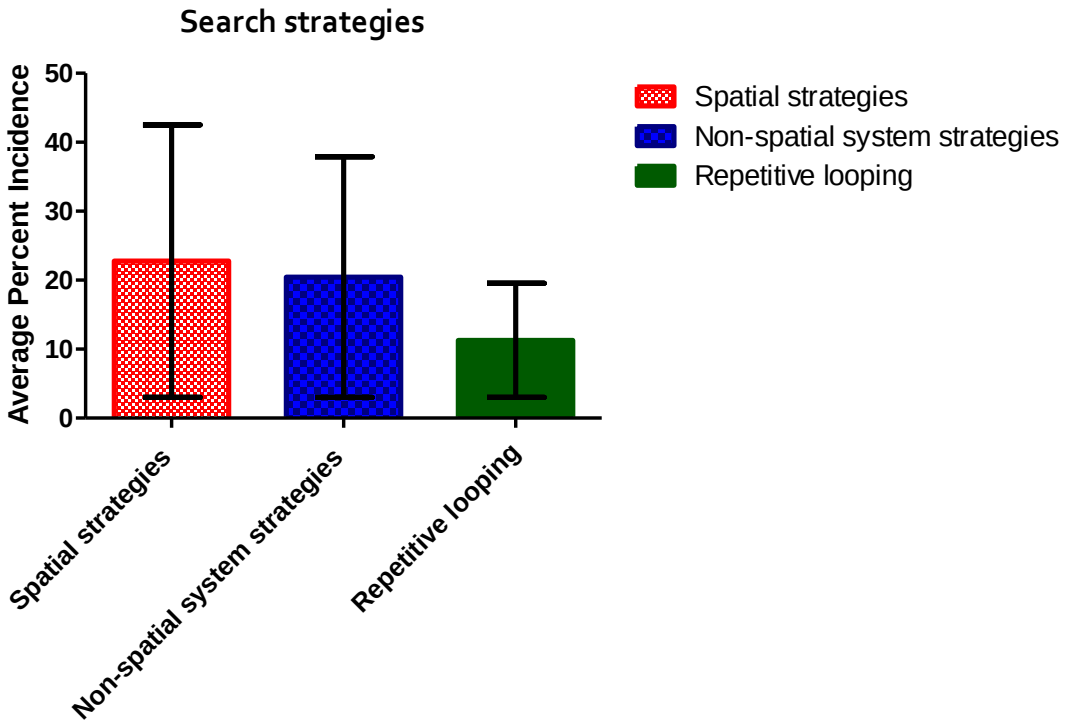


Figure 18. Quantification of search strategies during the acquisition phase. Spatial strategies consisted more than 43 percent of search strategies in our experimental animal models.

Figure 18 shows the comparison among the three different categories with regard to the average percent incidence.

Molecular data. We evaluated the expression of three different transcription factors. Tables 8-13 show the results of Western blot expression for transcription factors NF-κB, Egr-2 and CREB.

Table 8. Results of expression calculation of NF-κB, its actin control and the ratio in Western blot in the control group.

NF-κB	Actin control	NF-κB/Actin ratio
13.21	14.52	0.9097
7.24	12.7	0.57
12.26	12.69	0.9661
12.42	11.54	1.0762
6.78	8.39	0.8081
8.96	8.35	1.073
9.49	8.64	1.0983
28.43	17.36	1.6376
17.85	12.84	1.3901
15.33	9.52	1.6102
9.91	8.89	1.1147
22.04	10.11	2.18
14.01	10.13	1.383
8.45	10	0.845
10.47	9.26	1.1306
12.03	9.21	1.3061
10.7	8.78	1.2186
10.63	9.32	1.1405
12.48	11.61	1.0749
12.32	10.31	1.1949
13.92	10.09	1.3795
10.67	10.19	1.0471
4.6	5.19	0.8863
4.45	6.25	0.712
7.94	9.48	0.8375
14.22	12.54	1.1339
8.92	12.68	0.7034

Table 9. Results of expression calculation of NF-κB, its actin control and the ratio in Western blot in the experimental group.

NF-κB	Actin control	NF-κB/Actin ratio
12.55	10.41	1.2055
11.73	10.77	1.0891
11.23	9.46	1.1871
12.26	10.31	1.1891
7.11	7.6	0.9355
3.97	7.61	0.5216
7.82	11.34	0.6895
5.43	9.46	0.5739
5.54	7.47	0.7416
5.73	8.54	0.6709
9.88	12.25	0.8065
7.66	12.65	0.6055
6.26	11.56	0.5415
7.76	8.81	0.8808
7.15	7.46	0.9584
9.67	10.67	0.9062
11.12	12.05	0.9228
10.8	10.8	1
8.65	10.81	0.8001
7.49	9.1	0.823
9.3	12.01	0.7743
8.62	10.69	0.8063
10.71	11.41	0.9386
10.05	10.85	0.9262
11.93	12.85	0.9284
9.66	11.04	0.875
9.98	10.42	0.9577
13.59	10.32	1.3168
11.92	10.15	1.1743
14.71	11.94	1.2319

Table 10. Results of expression calculation of Egr-2, its actin control and the ratio in Western blot in the control group.

Erg-2	Actin control	Egr-2/Actin ratio
5.32	10.73	0.4958
14.01	6.55	2.1389
4.91	8.77	0.5598
4.34	10.01	0.4335
2.08	10.96	0.1897
3.71	8.49	0.4369
8.38	12.65	0.6624
13.27	18.1	0.7331
9.36	12.21	0.7665
11.96	8.56	1.3971
8.22	8.5	0.967
12.13	9.41	1.289
7.07	9.19	0.7693
3.08	5.29	0.5822
3.77	6.12	0.616
11.99	6.95	1.7251
22.45	11.21	2.0026
14	5.78	2.4221
5.17	15.53	0.3329
4.67	9.9	0.4717
11.61	9.65	1.2031
6.81	9.41	0.7236
2.24	8.29	0.2702
7.82	8.11	0.9642
16.83	9.85	1.7086
20.6	13.38	1.5396
10.22	9.54	1.0712

Table 11. Results of expression calculation of Egr-2, its actin control and the ratio in Western blot in the experimental group.

Erg-2	Actin control	Egr-2/Actin ratio
13.42	9.91	1.3541
21.43	8.72	2.4575
17.31	10.67	1.6223
11.36	13.88	0.8184
7.9	11.97	0.6599
11.16	8.29	1.3462
11.64	5.75	2.0243
19.15	7.2	2.6597
11.02	7.25	1.52
10.24	9.1	1.1252
12.53	10.85	1.1548
9.74	15.22	0.6399
11.47	12.38	0.9264
13.11	12.26	1.0693
13.78	13.63	1.011
10.63	8.13	1.3075
6.31	15.82	0.3988
9.21	18.6	0.4951
10.16	10.88	0.9338
8.4	11.22	0.7486
7.6	10.63	0.7149
9.39	9.76	0.962
13.64	10.9	1.2513
16.07	11.96	1.3436
19.88	12.26	1.6215
5.54	7.92	0.6994
11.94	10.88	1.0974
15.15	14.95	1.0133
4.35	8.24	0.5279
5.3	8.86	0.5981

Table 12. Results of expression calculation of CREB, its actin control and the ratio in Western blot in the control group.

CREB	Actin control	CREB/Actin ratio
18.47	8.56	2.1577
13.79	9.26	1.4892
7.28	8.14	0.894
17.96	9.54	1.8825
18.56	8.09	2.294
8.12	9.46	0.8583
9.39	7.3	1.2863
8.58	9.67	0.8872
9.97	12.55	0.7944
10.5	9.8	1.071
9.01	4.92	1.831
10.62	4.7	2.259
10.71	6.22	1.721
12.99	7.44	1.7459
12.89	7.9	1.6316
10.49	8.77	1.1961
10.91	9.7	1.1247
10.99	12.27	0.8956
10.88	17.33	0.6278
9.98	13.24	0.7537
9.49	4	2.3725
9.68	3.57	2.7114
10.18	4.59	2.2178
12.31	5.28	2.3314
13.67	5.81	2.3528
12.59	13.59	0.9264
11.98	10.24	1.1699
11.56	9.5	1.2168
10.07	5.58	1.8046
7.43	5.79	1.2832

Table 13. Results of expression calculation of CREB, its actin control and the ratio in Western blot in the experimental group.

CREB	Actin control	CREB/Actin ratio
3.62	11.41	0.3172
2.87	7.66	0.3746
5.92	11.91	0.497
5.31	13.03	0.4052
6.22	12.4	0.5016
9.17	12.39	0.7401
11.94	10.73	1.1127
9.85	7.92	1.2436
10.67	9.48	1.1252
11.8	10.7	1.1028
9.74	23.43	0.4157
8.48	4.57	1.8555
9.32	3.9	2.3897
8.96	14.54	0.6162
7.28	22.38	0.3252
11.61	8.19	1.4175
9.31	9.8	0.95
8.05	5.84	1.3784
8.46	7.01	1.2068
9.33	7.85	1.1885
11.4	25.98	0.4387
4.02	6.96	0.5775
3.84	5.28	0.7272
6.66	17.22	0.3867
18.76	21.31	0.8803
9.41	9.68	0.9721
9.1	11.63	0.7824
7.24	11.21	0.6458
9.54	12.41	0.7687
11.08	10.37	1.0684

All proteins are presented with a single band. NF-κB expression in the non trained control group was found elevated as compared to the MWM trained mice (1.12 ± 0.33 versus 0.89 ± 0.21) (Figure 19). There was a statistically significant difference in this expression for NF-κB in the control group versus the MWM group (p = 0.0031).

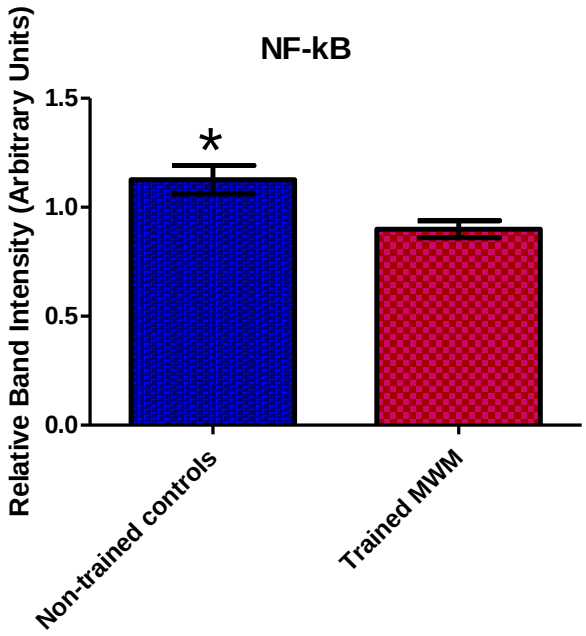


Figure 19. Comparison of NF-κB expression between control and experimental groups. This result shows that there was a statistically significant difference of more expression of NF-κB in the control group versus the experimental animal models (p = 0.0031; error bars = SEM).

However, there was no statistically significant difference in the expression of transcription factor Egr-2 between control group (0.98 ± 0.60) versus the experimental group (1.13 ± 0.54) (p value = 0.3092) (Figure 20), in spite of the slight increase in expression of Egr-2 in the trained MWM group.

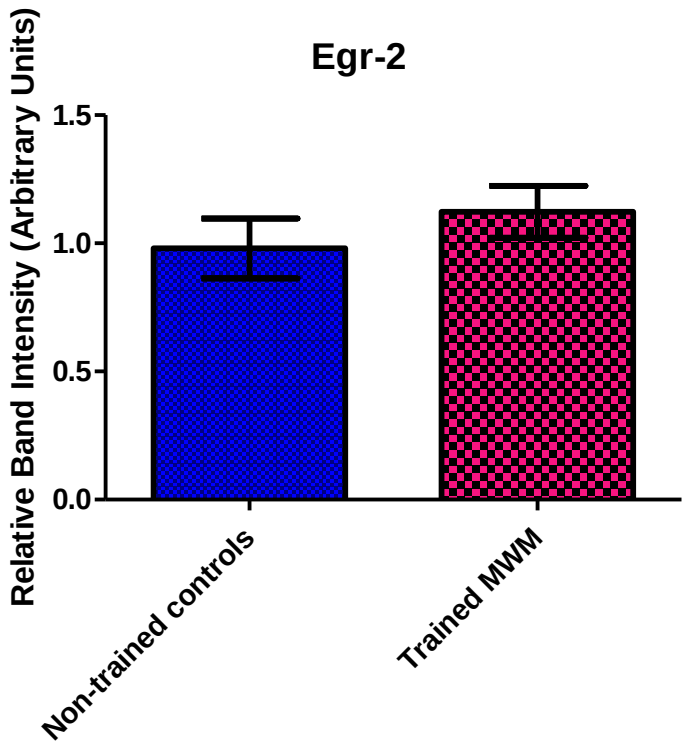
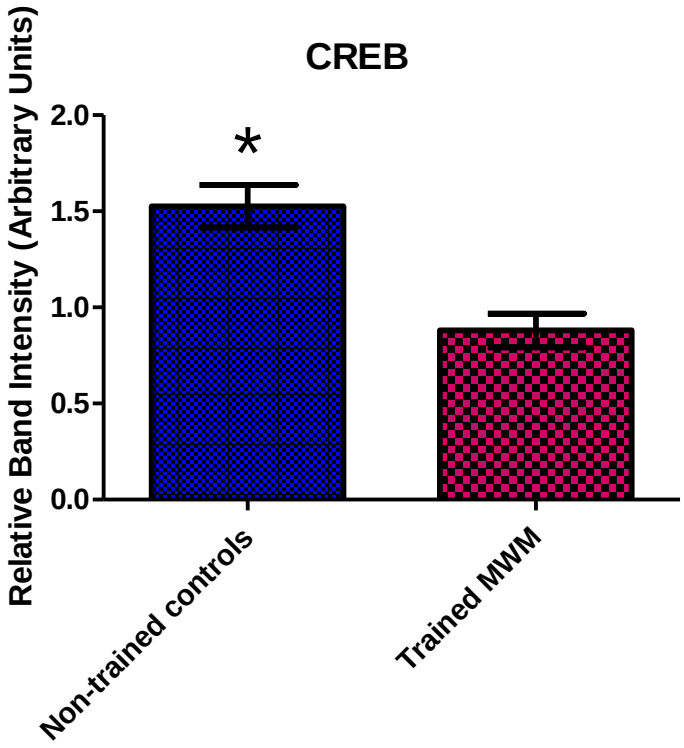


Figure 20. Comparison of Egr-2 expression between control and experimental groups with SEM bars. This result shows that there was no statistically significant difference in expression of Egr-2 among the control group and experimental animal models. ($p = 0.3092$)

In addition, we found that in the non-trained control group, CREB was expressed at higher levels as compared to the MWM trained experimental group (1.52 ± 0.60 vs. 0.88 ± 0.48). This increased level of expression for CREB was statistically significant in the control group ($p < 0.0001$) (Figure 21).



CREB quantifications in controls and experimental groups

Figure 21. Comparison of CREB expression between control and experimental groups. This result shows that there was a statistically significant difference of more expression of CREB in the control group versus the experimental animal models. (p = 0.0009).

Table 14 summarizes the results for the expression of transcription factors NF-κB, CREB, and Egr-2 in both groups.

Table 14. Summary of the expression of transcription factors in Western blot.

Transcription factor	Mean Control vs. trained (AU)	*p* value
NF-κB	1.126937 vs 0.899270	0.0031
Egr-2	0.980448 vs 1.136740	0.3092
CREB	1.526260 vs 0.880377	0.0009

AU = arbitrary units

Furthermore, to compare the expression of transcription factors between so-called poor performers vs. strong performers (defined below) as measured in the MWM test, we categorized mice by scores obtained during the retention phase. That is, the results are the cumulative numbers of passes over the missing platform for trials conducted during the retention phase (Table 15).

Table 15. Shows the cumulative number of passes over the missing platform in experimental group.

Animal													Total
E1	7	5	3	7	3	2	2	4	4	5	7	1	**50**
E2	6	4	4	2	1	4	7	4	9	6	5	5	**57**
E3	4	2	3	2	4	0	1	1	2	3	2	4	**28**
E4	3	3	3	0	2	2	2	1	3	3	3	1	**26**
E5	5	3	5	3	5	6	1	6	8	6	6	8	**62**
E6	3	2	4	3	3	2	2	3	5	3	4	6	**40**
E7	2	7	4	2	3	10	6	6	4	10	6	7	**67**
E8	2	3	0	1	4	5	4	2	4	6	8	4	**43**
E9	5	5	4	4	3	4	4	5	8	4	6	8	**60**
E10	1	8	2	4	2	6	3	2	5	7	8	4	**52**

We chose three subjects from the best group (E7, E5 and E9) and three from the worst group (E4, E3 and E6) and compared the transcription factors levels difference among them. The best group was that had the highest total number of passing the missing platform and the worst group was described on those three mice that had the lowest number of passing the missing platform during the retention phase. We found that there was no significant difference in the mean expression of transcription factors in poor versus strong performers was: NF-κB (0.90 vs 0.88) (p = 0.8651), Egr-2 (1.20 vs 1.15) (p = 0.7835) and CREB (0.85 vs 0.90) (p = 0.81). Figures 22-24 show a quantitative comparison of the transcription factors NF-κB, CREB and Egr-2 between the poor versus strong performers in the MWM.

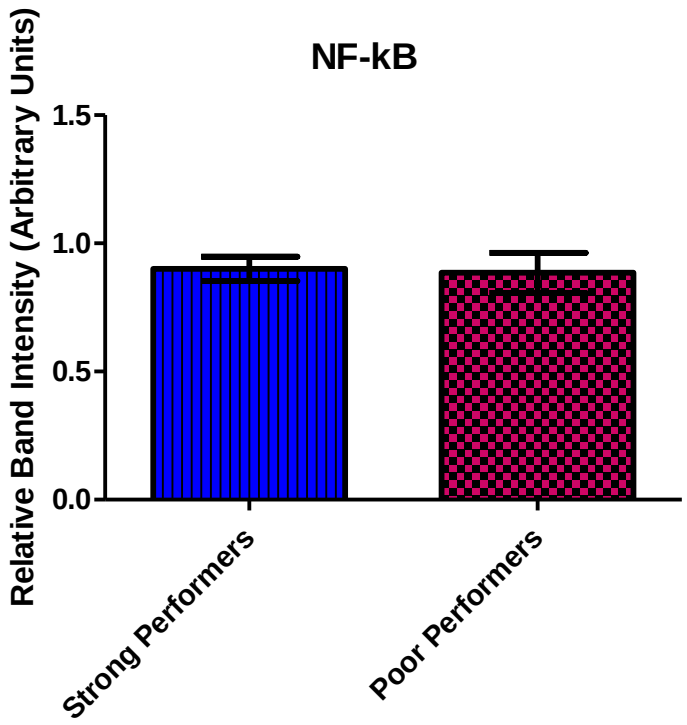


Figure 22. Comparison of NF-κB expression between poor versus strong performers in the MWM. There was no statistically significant difference between these two groups (p = 0.86).

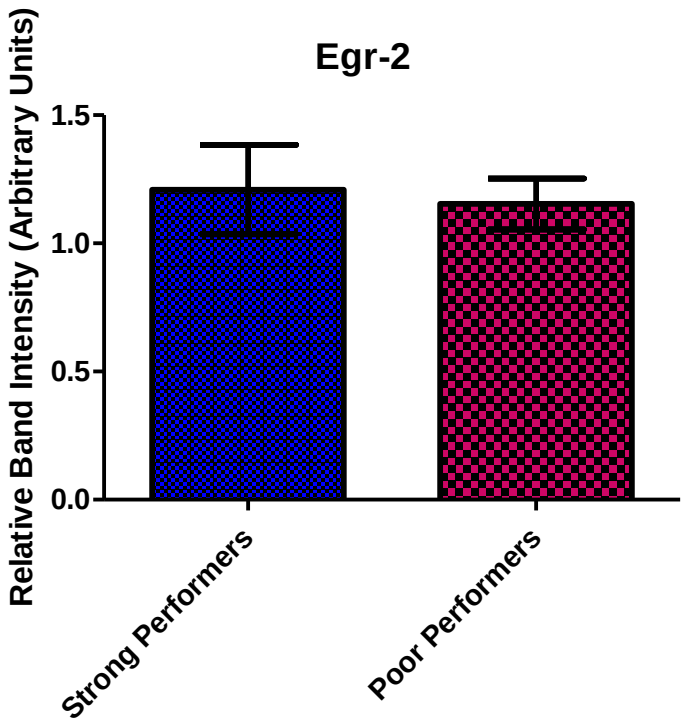


Figure 23. Comparison of Egr-2 expression between poor versus strong performers in the MWM. There was no statistically significant difference between these two groups (p = 0.78).

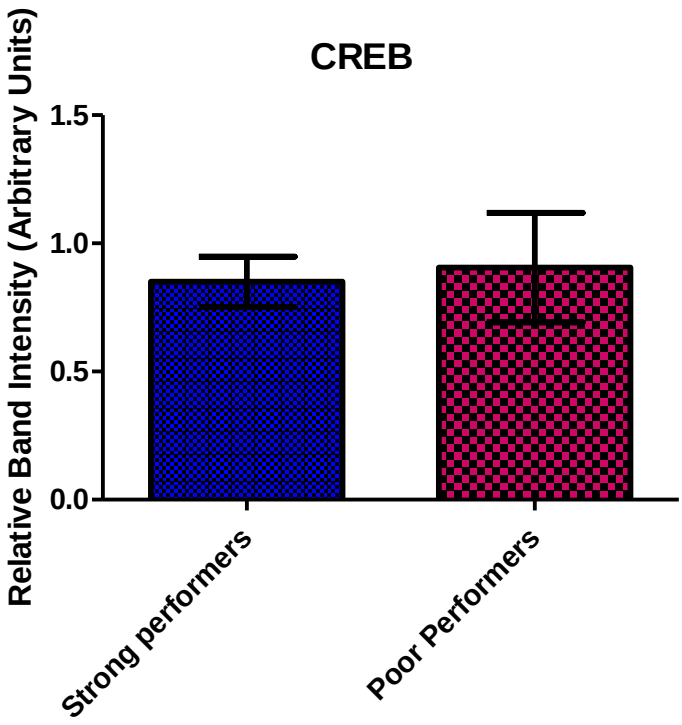


Figure 24. Comparison of CREB expression between poor versus strong performers in the MWM. There was no statistically significant difference between these two groups (p = 0.81).

3.2. Study 2

Additional experiments were conducted by members of my lab. In this case, 4 groups were assessed following training in the MWM test (described above). At the time of this writing these experiments are still ongoing. Highlights of these experiments are shown below. The Western blot data (Figure 25A and 25B) thus far (n=2) show that the Group 4, show higher levels of CREB and pCREB expression, particularly in CA1 area of the hippocampus, compared to the other groups of this study. These mice received 5 days of training in the acquisition phase with a delay of several days before they were retested in the retention phase on day 12. Animals were then killed on day 13. Samples from these mice on this day were then prepared for the Western blots shown below. Similarly, Figure 26 shows immune histochemistry (IHC) pilot data where CREB expression appears highest in Group 4 as compared to the other groups tested thus far. Next to this group (Group 4), CREB and p-CREB expressions were higher in those experimental mice which underwent MWM test and then were sacrificed 30 min and 24h after the retention phase (Group 2 and Group 3) compared to the control group.

CREB Group

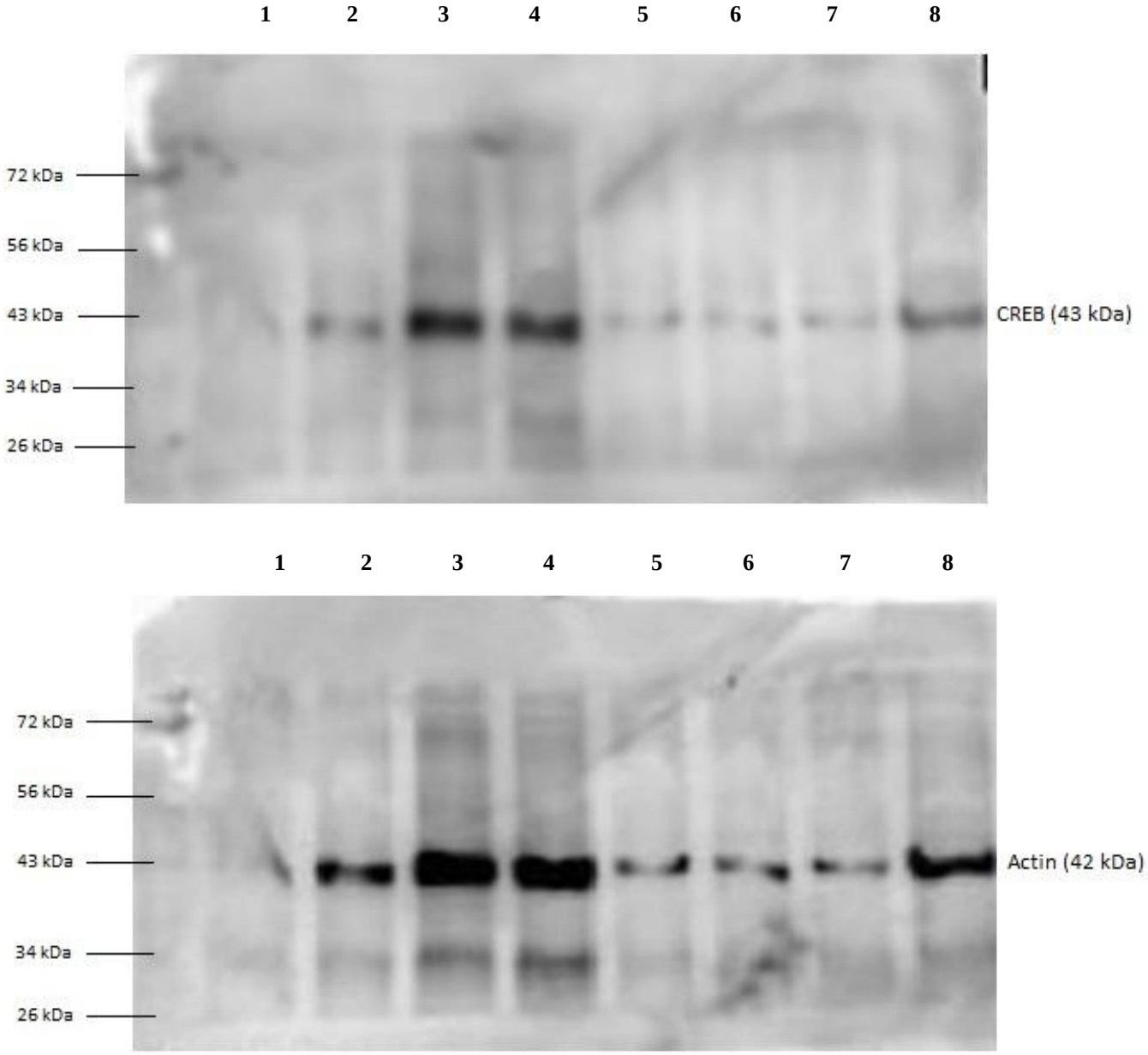


Figure 25A. CREB Western blots on control and experimental samples. Lanes 1-8 represent the 4 groups tested in the MWM. Lanes 1-2, Yoked controls; Lanes 3-4, Group 4; Lanes 5-6, Group 2; Lanes 7-8, Group 3.

pCREB Group

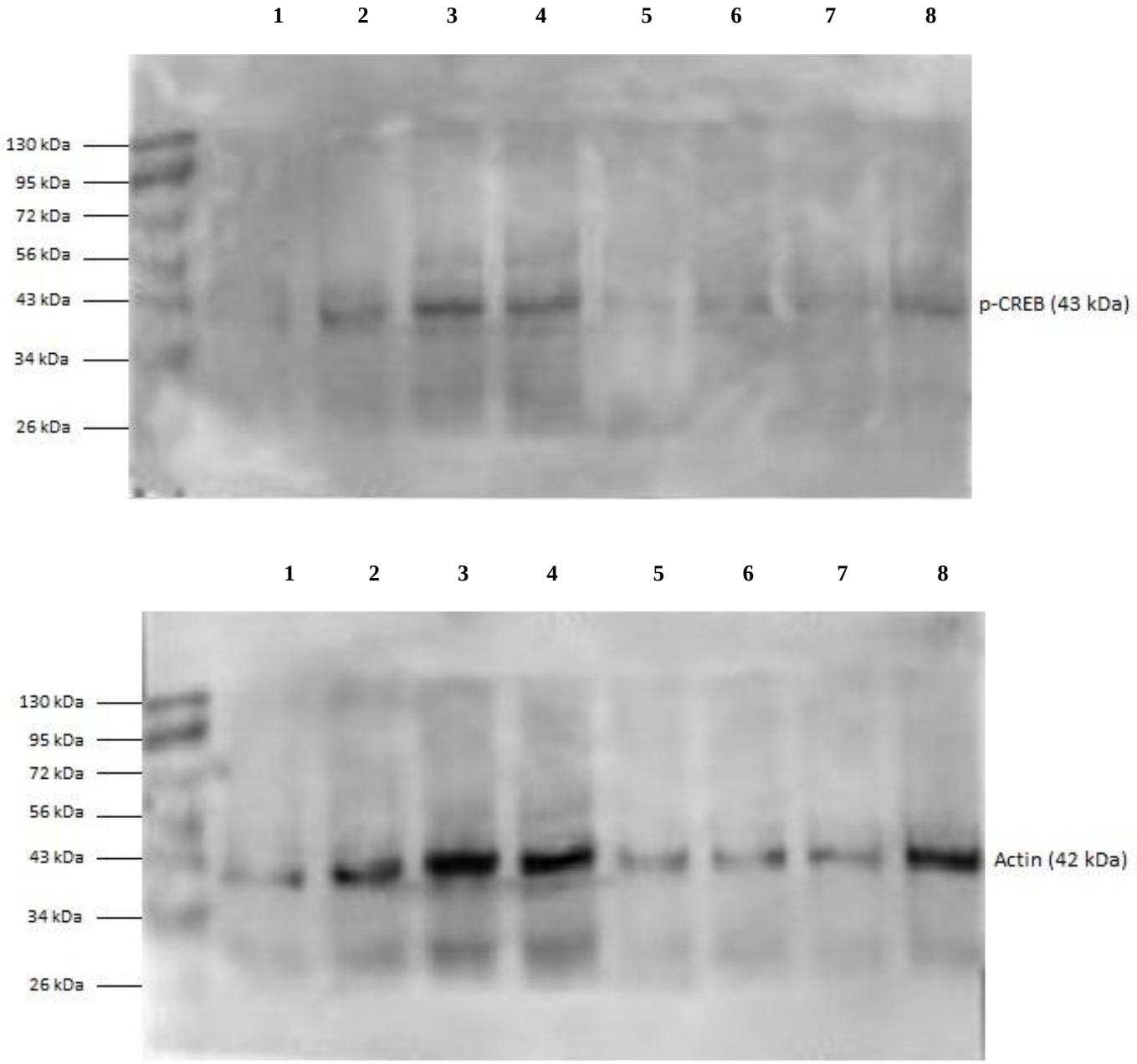


Figure 25B. pCREB Western blots on control and experimental samples. Lanes 1-8 represent the 4 groups tested in the MWM. Lanes 1-2, Yoked controls; Lanes 3-4, Group 4; Lanes 5-6, Group 2; Lanes 7-8, Group 3.

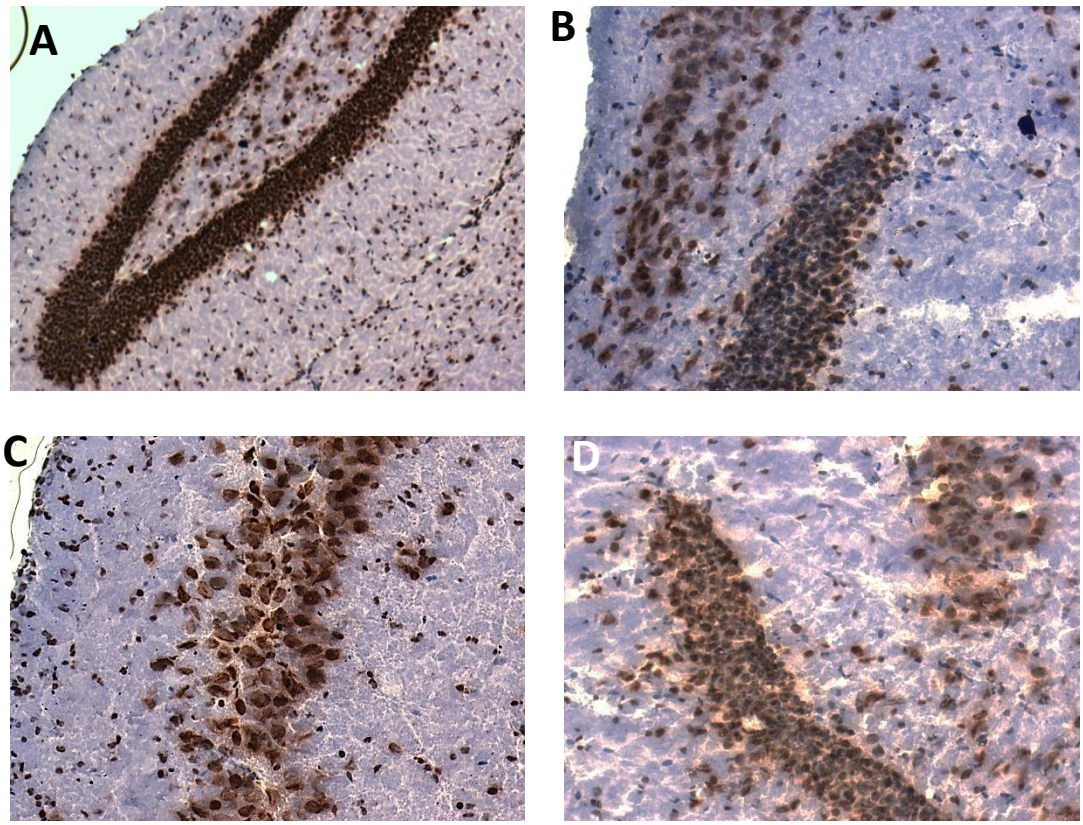


Figure 26. IHC of CREB expression in hippocampal slices. Images from immunohistochemical labeling of CREB (brown) in 20 µm-thick hippocampal cryosections counterstained with hematoxylin (purple). (A) CREB labeling was intense in mice that underwent MWM training, with assessment of memory retention after 1 week (Group 4; 10x).
(B & C) In mice tested immediately after the acquisition phase on day 6, CREB labeling was similar in tissue harvested 30 min after memory retention (Group C; 20x) to tissue harvested 24 hours post-testing (Group D; 20x). (D) In mice that did not undergo MWM testing (Yoked controls-Group 1), CREB labeling was diminished relative to all other trained groups (20x).

4. Discussion

We evaluated the expression levels of protein subunits associated with three transcription factors NF-κB (NF-κB p105 and NF-κB p50 subunits), Egr-2 and CREB, in non-trained animals versus those that were trained in the MWM in study 1. We also measured CREB and pCREB expression in control and experimental groups in study 2. In study 1, the level of CREB expressed was higher in non-trained control group compared to trained MWM group. We also observed that NF-κB was expressed at lower levels in MWM trained mice compared to the non-trained control animals. However, there was no statistically significant difference in the expression of Egr-2 between these groups. Also, there was no statistically significant difference in the expression level of these transcription factors (NF-κB, Egr-2 and CREB) between poor and strong performers in the MWM trained group.

In study 2, a higher expression of CREB and pCREB was observed in experimental Group 4 (7 day interval between the acquisition phase (5 days) and retention phase (1 day), animals killed on day 13, 24 hrs after retention test) as compared to a yoked control (swimming in the MWM only) and Groups 2 (5 days of acquisition, retention test on day 6, animals killed 30 mins later) and Group 3 (5 days of acquisition, retention test on day 6, animals killed 24 hours later).

The differences seen in our results are unclear, but memory-related signaling proteins and transcription factors in spatial learning and memory in the hippocampus are the major focus of recent studies (Ortiz, et al. 2010). For example, the phosphorylation of CREB is important for regulating hippocampus-dependent spatial memory formation (Porte, et al. 2011). Egr-1 levels are also up-regulated during spatial memory in the hippocampus (Pollak, et al. 2005), but there is still little known about Egr-2 level following learning (Cheval, et al. 2012). The role of NF-κB in

CNS functions ranges from roles in synaptic plasticity and development to learning and memory (Albensi and Mattson 2000; Meffert and Baltimore 2005). Mice lacking the p50 subunit of NF-κB (NF-κB p50 knock out) showed a significant increase in spatial learning tasks in the MWM that was not seen in other spatial learning tests like the Barnes maze (Lehmann, et al. 2010). This was, in particular, due to the level of corticosteroids that are higher in MWM test than Barnes maze test as the former is more stressful to the mice due to the water environment. However, our lab recently reported deficits in memory in the MWM and impairments in late LTP in NF-κB p50 subunit knock out mice (Oikawa et al. 2012), which is in opposition to the results of Lehmann et al.

In study 1, the expressed CREB level was significantly higher in control group versus the experimental group where we measured the total CREB level. Some studies assess p-CREB, which measures the activated form of CREB. Therefore, we conducted study 2 to measure the activity levels of both CREB and p-CREB following the MWM test. The role of CREB in hippocampal-dependent spatial learning has been widely reported in transgenic mice in learning paradigms (Guzowski and McGaugh 1997), where evidence suggests a role for CREB in long-term memory, but not short-term memory in MWM tests (Florian, et al. 2006). Higher expression levels of CREB and p-CREB in our study 2, also supports a role in long-term memory.

In addition, some previous studies report that learning-dependent activation of CREB in the hippocampus varies on demand, and is proportionate to the task that the mice are engaged in (Trifilieff, et al. 2006). We might conclude that one of the reasons that the mean CREB level in our controls was higher than the experimental group might be because a more extensive MWM training period may be needed (Porte, et al. 2008) in order to see more dramatic changes. On the

other hand, since in our study 1, we measured total CREB level and not pCREB the proportion of activated levels versus the total levels might also be changed (also see limitations section below).

There are also reports involving comparisons between MWM trained mice and controls that state there are significant differences between levels of CREB and Egr (Patil, et al. 2010). In some studies, using a cued conditioning task like MWM, resulted in a monophasic pattern of hippocampal CREB activation (Porte, et al. 2008).

Another aspect to consider for our study is that we measured total CREB in the whole hippocampus. Some studies focus specifically on the level of p-CREB in sub-fields of the hippocampus. We recommend more investigations to measure the p-CREB expression, particularly in CA1 and CA3 hippocampal subfields.

CREB expression is also diet-dependent. For example, some zinc deficiency decreases pCREB, but does not change the total CREB level (Gao, et al. 2011). Since *ad libitum* is a biotin-fortified egg white diet with variable zinc levels (Luecke, et al. 1968), we are not confident about the zinc level of this diet in our experiments which might affect the CREB expression level.

Another factor to mention is that memory in mice is strain dependent in the MWM (Crawley, et al. 1997). Evidence has shown that hippocampal proteins levels for some transcription factors are entirely strain dependent (Pollak, et al. 2005). This may explain some of the differences between transcription factor expression levels in our experiments.

In addition, some studies show that a reduction of CREB gene isoforms has only subtle effects in the MWM and on LTP parameters (Balschun, et al. 2003). For example in the study by Balschun et al., conditional deletion or reduction of CREB did not significantly alter LTP and LTD and only slightly impaired early stages of the MWM. Therefore, our study is consistent

with this limited role for CREB expression during the MWM task and spatial memory formation. This may also be explained by considering multiple signaling pathways in hippocampal-dependent spatial memory, the involvement of other transcription factors, undiscovered transcription factors, and/or regulatory roles for other CREB family proteins (eg., CREM).

Moreover, since other parts of the brain, such as the amygdala or forebrain, are significantly involved in the control of memory formation (Mayford, et al. 1996), differences in hippocampal CREB expression between our controls and experimental samples may be due to regional differences.

We also did not see a statistically significant difference in the expression of Egr-2 in our MWM trained mice vs. controls. Interestingly, previous studies have shown that Egr-2 has a paradoxical role in learning and memory and Egr-2 deficient mice show no impairment in spatial memory and even have improved performance in motor learning on rotarod tests and in tests of object recognition memory (Poirier, et al. 2007). Interestingly, some studies report that the consolidation of a spatial memory in a MWM test correlates with the expression of p-CREB and Egr-1 (Zhou, et al. 2013). Therefore, future studies might need to measure the expression levels of all known Egr family members in parallel with CREB and pCREB.

NF-κB was another transcription factor that was evaluated in our study. In the hippocampus, the most common form of NF-κB is the p65/p50 heterodimer (Crampton and O'Keeffe 2013). In our study, the expression of the NF-κB p50 subunit was lower in MWM trained mice versus the control group. Since the MWM is considered a high stress test, we might speculate that the lower expression of NF-κB p50 in the MWM trained mice group might be due to a less important role of the NF-κB p50 subunit in this context. Corticosteroids released in this high stress test bind to their receptors in the hippocampus and can, via other pathways, induce

spatial learning (Harrison, et al. 2009). Given this, we recommend another study to measure serum corticosteroid levels in CD1 mice that undergo MWM tests to check the level of anxiety, which can affect learning in the absence of NF-κB p50 subunit expression (Lehmann, et al. 2010).

It is also important to note that our study has several limitations. Even though control and experimental animals were from the same strain and age, were ordered from the same center, and were treated the same way in study 1, it is critical to mention that each group was ordered at different times (December 2010 and January 2011), which may bias our results. Therefore, it is recommended for future studies that animals be ordered at the same time and then randomized before use, so that this potential bias can be eliminated.

Another concern in our study regarding the measurement of hippocampal weights, was that even though we didn't have a statistically significant difference in the weights among controls and experimental groups, we had a high variation in the range of hippocampal weights as compared to previous studies (Lu, et al. 2001; Moriya, et al. 2011). In these other investigations the range was between 0.0232 – 0.0311 gms, and in our study the range was between 0.0254 – 0.1091 gms. Therefore, we believe that we need to modify our hippocampal removal technique in order to reduce this variation.

Our study also would be stronger if we would have measured transcription factor activation directly by using gel shift procedures (a.k.a. EMSA) or in case of NF-κB, the measurement of IκBα, to more accurately evaluate its activation.

In addition, not all mice strains perform equally in different mazes; some are better learners that others, and some perform better in specific mazes (Ammassari-Teule and De Marsanich 1996). For example, CD1 mice are considered poor performers in a radial maze

(Mizumori, et al. 1982). In addition, other factors such as motor function, motivation, and perceptual differences should be considered for each type of behavioral test (Adams, et al. 2002).

In summary, we showed differences in the expression levels of transcription factors NF-κB and CREB following the MWM test under various conditions. These results support our hypothesis that there would be a difference in the expression levels of transcription factors following learning and memory induced by training in the MWM.

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