UNIVERSITY OF CALIFORNIA,
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Mapping brain networks engaged by spaced learning

DISSERTATION

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DOCTOR OF PHILOSOPHY

in Neurobiology

by

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DEDICATION

To Grandma Dolores,

The original “Dr. Carley”,

You’ve been with me every step of the way.

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ABSTRACT OF THE DISSERTATION

Mapping brain networks engaged by spaced learning

By

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Professor Christine M. Gall, Chair

Spaced training is a powerful behavioral method for enhancing long-term memory. Despite decades of observational study of this phenomenon, a precise neurobiological mechanism remains unknown. Memory consolidation literature describes both synaptic and systems-level changes that accompany the stabilization of memory traces; the aim of the present work was to determine at which level spaced training operates. A specific spaced training schedule in the object location memory (OLM) task has been shown to dramatically lower the threshold to long-term memory in mice. Here we compare this paradigm to the standard, single-trial, "massed" schedule of training on a number of neurobiological outcomes. First we assayed, across the entire mouse brain, patterns of neuronal activation as reflected by Fos immunoreactivity. This analysis revealed a distinct network difference between spaced and massed training; specifically, the orbitofrontal cortex (OFC) showed significantly different activity. Next we chemogenetically inactivated OFC during massed and spaced training and found that it was necessary for spaced, but not massed, OLM learning. In Chapter 3 we assayed patterns of synaptic modification in hippocampus after

massed and spaced OLM training. This analysis did not significantly distinguish between the training schedules. The studies in Chapters 4 and 5 depart from the spaced vs. massed question, and instead venture into territory of intellectual disability. The Fmr1-KO mouse is a commonly-used model of Fragile-X Syndrome, the most common form of inherited intellectual disability in humans. Here we show that these mice fail to exhibit social recognition, and that this deficit is corrected with treatments which enhance TrkB signaling. In Chapter 5, we apply behavioral pattern analysis (Markov sequences) to WT vs. KO exploratory patterns, and then to behavioral tasks discussed in Chapters 1-3 (including spaced vs. massed training and OFC inactivation). The main takeaway of this ethological analysis was that exploratory patterns vary more with environmental novelty than with any other factor. Together the studies in this dissertation support a systems-level effect of spaced training upon long-term memory, which may or may not be related to the difference in exploratory movement patterns.

INTRODUCTION

Localization of memory

The “engram”, a biophysical or biochemical trace of memory, has proven an elusive target for neurobiology researchers. Despite decades of searching, an unambiguous marker for memory remains undefined (Lashley, 1950; Thompson, 1976; Eichenbaum, 2016). This has repeatedly prompted the question of whether or not memory *can* be localized. Prominent theories as to the localization of memory have polarized to two main camps: synaptic memory is thought to form rapidly via permanent modification of neurons in a discrete location while systems memory takes place in multiple locations over an extended period of time. Abundant evidence exists in support of both theories, and they are certainly not mutually exclusive (Sutherland et al., 2010). The following discussion will examine this evidence, and the chapters contained in this dissertation will attempt to reconcile these theories by investigating the mechanisms involved in consolidation of location memory following spaced training.

Evidence for synaptic consolidation

Much of the evidence for synaptic consolidation has come from studies of hippocampal long-term potentiation (LTP), the biological basis of memory (Whitlock et al., 2006). The ordered, unidirectional flow of information through hippocampus makes it an ideal system for studying synaptic changes that occur with neuronal stimulation. Hundreds of studies using this system have demonstrated involvement of various molecular signaling pathways operating in parallel over diverse time scales. The process by which LTP is

stabilized is time-dependent, occurring in at least two distinct stages. The first, involving rapid cytoskeletal modification, is complete within 30 min of stimulation, while the second, protein synthesis-dependent, stage takes place over hours (Frey and Morris, 1997; Chen et al., 2007).

Immediately after strong, patterned neuronal stimulation, NMDA receptors are activated, allowing further depolarization of the neurons involved (Morris et al., 1986; Larson and Lynch, 1988). This kicks off signaling cascades in the postsynaptic element which lead to actin cytoskeletal rearrangement and insertion of additional glutamate receptors into the membrane (Matus et al., 1982; Lu et al., 2001). Assembly of filamentous (F-) actin in dendritic spines is critical to establishing and maintaining LTP (Krucker et al., 2000; Fukazawa et al., 2003) and is modulated by multiple, parallel signaling pathways (Rex et al., 2007, 2009). Actin-relevant synaptic consolidation appears to be complete within an hour of stimulation (Babayan et al., 2012), when reversal signals (e.g. low-frequency stimulation; (Larson et al., 1993)) fail to disrupt LTP.

In the hours following stimulation and the resultant cytoskeletal remodeling, additional protein synthesis and gene expression changes will take place, resulting in higher levels of plasticity-related proteins at the recently-active synapses (Frey and Morris, 1997; Steward et al., 1998). Extended phosphorylation of regulatory proteins such as PKA mediate ongoing signaling; PKC changes extend for 1-2 h after stimulation, CaMKII for 2-3 h (Barria et al., 1997; Routtenberg et al., 2000). At 3-4 h after stimulation there is a dopaminergic D1 receptor-stimulated increase of cAMP and of cAMP-dependent protein kinase A (PKA; (Huang and Kandel, 1995)). Both PKA and PKC signaling converge on the

cAMP response element binding protein (CREB), a transcription factor critically involved in late-phase maintenance of LTP and memory (Abel et al., 1997).

Despite the decades of work describing synaptic modification in hippocampus as critical to memory, it remains possible that extra-hippocampal and extra-synaptic mechanisms also play a role. Retrograde amnesia studies since the late 1800s have suggested that memory may transfer from hippocampus to cortex over time (Ribot, 1882; SCOVILLE and MILNER, 1957), and modern computational theories of memory consolidation depend on this assumption (Fiebig and Lansner, 2014).

Evidence for systems consolidation

As described above, hippocampus is critical to memory formation and consolidation. The iconic patient H.M. had his hippocampus surgically removed in an attempt to treat his otherwise intractable epilepsy, with the result that he was never again able to form new memories. Evidence from rodent studies agrees: removal or inactivation of hippocampus abolishes memory formation and (under certain conditions) retrieval (Broadbent et al., 2004; Lehmann et al., 2009). There is an abundance of evidence, however, indicating extra-hippocampal regions in memory formation, storage and retrieval.

In a study of spatial discrimination memory, radiolabeled 2-deoxyglucose (2-DG) was injected in mice prior to either recent (5 d after training) or remote (25 d) testing in the radial arm maze. Relative metabolic activity of various brain regions could then be inferred via localization of 2-DG to compare regional involvement. Increasing the retention interval resulted in decreased activity of hippocampus and increased activity of cortex

(Bontempi et al., 1999), indicating a transient role for hippocampus and an increasing involvement of cortex over time. Interestingly, an additional cohort of animals tested remotely in a novel context showed hippocampal activity, reinforcing the role of this region in preferentially encoding new information.

A similar study of regional patterns of neuronal activity, using Fos protein rather than 2-DG as the activity marker, showed that Fos immunoreactivity was more pronounced in deep cortical layers when testing recent memory, and more pronounced in superficial layers when testing remote memory (Maviel et al., 2004). As superficial layers contain mostly cortico-cortical connections (Miller, 1996), these results support the preferential encoding of memory by cortex at later time points.

Nearly all theories of systems consolidation agree that it takes place over days to months; certainly far longer than the processes of synaptic consolidation. The complementary learning systems theory (CLS; (McClelland et al., 1995)) reconciles these time-scales best in describing the hippocampus as a "fast" and cortex as a "slow" learner. The mechanism suspected to underlie the slower learning of cortical representations is that hippocampus and cortex simply express different forms of plasticity. Hippocampal LTP, of course, is well-characterized as a mechanism for changing synaptic weights between already connected neurons. In contrast, cortical potentiation requires formation of connections between previously unconnected neurons (Trachtenberg et al., 2002). The benefits of such temporal discrimination may be to assure constant availability of hippocampus to acquire new information (Squire and Zola-Morgan, 1991) or to conserve cortical resources only for information most likely to be used in the future (Sutherland et al., 2010).

The earliest model of systems consolidation proposed a role for sleep in the transfer of memory from hippocampus to cortex (Marr, 1971). Marr proposed that memories are first encoded in both hippocampus and cortex, and that patterned reactivation of hippocampus during sleep triggers relevant activity in cortex, gradually strengthening cortico-cortical connections. More recent theories allow reactivation during both online and offline states. Mouse studies of spatial learning have shown that patterned reactivation of place cell sequences experienced during behavior are critical to memory consolidation, and that this "replay" can occur during both sleep and wake states (Carr et al., 2011). In human subjects, correlated activity between hippocampus and whatever cortical regions were engaged by a learning task is maintained during rest, and predicts recall ability (Schlichting and Preston, 2014).

Finally, there is question about whether remote (cortical) memories are even the same as recent (hippocampal) ones. Evidence suggests that, as memories are consolidated and transferred, they are also made more semantic. A case study of patient K.C., who lost function in a significant portion of his hippocampus as an adult, showed that K.C. experienced impaired remote spatial memory. However, the few representations that remained were abstract and semanticized rather than detailed and episodic (Rosenbaum et al., 2000). Interestingly, direct comparison of K.C. with patient E.L., who suffered neocortical damage, revealed opposite effects on memory composition. K.C. maintained autobiographical facts but no sense of episode; E.L. demonstrated intact episodic recall without semantic detail (Westmacott et al., 2001). It therefore appears that hippocampal and cortical representations of events are qualitatively distinct.

Spaced training enhances OLM

Since the work of German psychologist Hermann Ebbinghaus in the late 19th century there has grown an extensive literature in animals from Aplysia to humans describing the benefit of repeated study on memory (Cepeda et al., 2009; Zhang et al., 2011). It is widely accepted that more, shorter study sessions are more effective for learning than fewer, longer sessions, and hundreds of studies have confirmed that spacing provides a mnemonic advantage over massed study (Janiszewski et al., 2003; Cepeda et al., 2009).

Until very recently there has been a notable lack of evidence linking these cognitive effects to cellular mechanisms. A few years ago, Eniko Kramar and colleagues found that delivering multiple trains of theta burst stimulation (TBS) to a hippocampal slice resulted in greater and greater amplitudes of long-term potentiation, the physiological substrate of memory (Kramár et al., 2012). This was exciting not only because the strength of cellular memory could be augmented, but also because the effect was temporally limited. If the second TBS train was delivered 10-30 minutes after the first, there was no additional potentiation. If it was delivered 60-90 minutes later, however, the magnitude of LTP nearly doubled. What's more, up to 4 trains of TBS, each 1 hour apart, resulted in increasingly greater potentiation. It should be noted that each increment was not the same; LTP in Schaffer commissural afferents terminating in CA1 stratum radiatum appears to hit a ceiling around 3x baseline, which corresponded in these experiments to the third TBS. Bath application of the positive allosteric AMPA receptor modulator CX614 allowed for a greater magnitude of LTP after TBS1, but precluded any spacing effect: TBS2 did not further augment LTP. Together these data illustrate that the spacing effect does not artificially

elevate transmission in the system, but rather allows it to maximally express its full potential.

This work has since been replicated by others (Cao and Harris, 2012), and followed up with behavioral studies in mice. The latter experiments, guided by electrophysiology results, revealed that long-term memory for a hippocampus-dependent task can be enhanced by the same timing rules that lead to enhancement of hippocampal LTP (Seese et al., 2014). The object location memory (OLM) task, which typically requires at least 5 minutes of massed training to elicit long-term memory (LTM), could be accomplished with as little as 1minute of total training time if delivered in three 20-second sessions each separated by 1 hour. Notably, an inter-trial interval (ITI) of 20 min or 120 min had no effect, while an ITI of 60 min supported the expression of LTM. The number of trials was also critical. Two trials, even long ones (150 seconds each), separated by 1 hour do not result in memory. At least three trials must occur; with five trials, there is no difference in memory strength compared to that elicited by three trials.

Mechanisms thought to underlie the spaced training effect

In the absence of neural mechanisms, a multitude of cognitive theories have emerged to explain the spacing effect. Importantly, many of those theories are based on a literature that has tended toward intertrial intervals (ITIs) that are too short to account for hours-long consolidation processes (Underwood and Schultz, 1961). Theories that allow for consolidation include encoding variability (EV) and deficient processing (DP), both of

which are based on early work showing that the longer the interval between episodes, the better the retention (Melton, 1970; Wickelgren, 1974).

Encoding-variability theory assumes that aspects of the encoding process or context vary with time. Events that are further apart in time are more likely to be different than those that are close together, so each additional exposure will add new information to the episodic representation (Estes and K., 1955; Bower, 1972). Critics argue that this theory fails to account for the nonmonotonic relationship often observed between ITI and recall performance (Benjamin and Tullis, 2010). A major assumption of EV theory is that encoding of each element is an all-or-none process; to include nonmonotonicity, a modified theory might discard the all-or-none encoding assumption (Cepeda et al., 2009). This would take into account the high overlap – and thus low informational gain – between features presented close in time, and would maximize informational gain from features presented farther apart.

Deficient-processing theory, sometimes called "reminding theory", claims that memory strength is related to how long the trace is held in working memory (Rubin and Rubin, 1998). Therefore, repetition enhances long-term memory by recalling the relevant details into working memory; the more times, the better. DP theory also accounts for study-phase retrieval effects, in which repetition is thought to strengthen memory by stimulating retrieval of earlier elements at the time of encoding later elements (Hintzman, 2004). Both DP and retrieval theories account for nonmonotonicity, but retrieval theory suffers from the fact that retrieval is more likely at short lags (Benjamin and Tullis, 2010). It is therefore tempting to attribute evidence of retrieval theory instead to DP theory.

A large-scale human study tested 26 different spacing conditions to determine the relationship between intertrial interval (ITI) and retention interval (RI) on both recall (open-ended) and recognition (multiple-choice) memory. Results showed that, in general, longer ITIs support longer RIs. Optimal intervals yielded ~64% increase in recall performance and ~26% increase in recognition relative to no gap (Cepeda et al., 2008). Most important was their finding that the relationship between ITI and RI was nonlinear; rather, it was expressed as a U-shaped function of the natural log of study gap. In a verbal recall study in humans, researchers found that long-term retention benefits both from repetition and from spacing interval (Bahrick et al., 1993). Importantly, these factors appeared to affect retention independently. Taken together, one can imagine these two separate curves summing to a complex, nonlinear relationship like that described by Cepeda and colleagues. These results together refute deficient-processing and other all-or-none theories, but lend support to encoding-variability and study-phase retrieval theories.

CHAPTER ONE

Brain-wide Fos activity mapping reveals a novel learning circuit engaged by spaced training

Summary

Multiple spaced training sessions have long been known to be more efficient for learning than single-trial learning but the underlying neurobiological mechanisms for this spacing effect are not known. The present studies used the object location memory (OLM) task in mice to test the possibility that repeated spaced training sessions recruit activity of a broader network of brain regions than does a single, massed training trial. Specifically, groups of mice were exposed to two objects in fixed (initial) locations in either a single long session or in three shorter trials spaced by 1 hour with timing selected so that both training paradigms support long term OLM. The brains of these mice were subsequently evaluated for the regional distribution of neurons expressing high levels of the activity-regulated gene product c-Fos. Results show that the distribution of Fos expression differed between training paradigms with differences being prominent in frontal cortical fields. In particular, orbitofrontal cortex (OFC) activity varied robustly with spaced as compared to massed training. These results suggest that spaced training engages a fundamentally different neuronal network than does massed training and that frontal cortical fields may be critical for the greater efficacy of encoding with spacing.

Introduction

Human and animal research has indicated a critical role of the hippocampus in the formation and maintenance of declarative type memory (Eichenbaum, 2001). However, depending on the type of learning in question, other brain regions are also necessary. For instance, the amygdala is needed for fear conditioning and piriform cortex is critical for odor-based tasks (Kim et al., 1993; Zhang et al., 1998). Whatever the region, both cellular and circuit-level changes occur with learning (Frankland and Bontempi, 2005). The spaced training effect is a broadly described phenomenon showing that repeated training or study is more efficient for forming long-term memories than is a single training session. Although the powerful effect of spacing was first described over a century ago (Ebbinghaus, 1885) it is still not understood at a neurobiological level.

Theories as to the benefit of spacing, like theories of memory consolidation, have generally focused on synaptic and/or circuit mechanisms. Regarding the former, it is possible that with repeated training the strength of synaptic potentiation or possibly the number of synapses that become potentiated increases with each training session. In line with this idea, recent studies showed with repeated trains of stimulation of hippocampal field CA1 afferents, spaced by 60 minutes, the level of synaptic potentiation becomes progressively larger (Cao and Harris, 2012; Kramár et al., 2012). Further analysis provided evidence that this augmentation does indeed involve potentiation at progressively larger numbers of synapses as opposed to greater potentiation at individual synapses (Kramár et al., 2012; Lynch et al., 2013). A not mutually exclusive possibility is that with repeated training the task recruits a broader network of brain regions and that the areas engaged by spacing facilitate long term memory.

The goal of the present study was to test if a broader neuronal network is indeed engaged with spaced, as opposed to single trial, training, using mapping of the activity regulated gene *c-fos* to assess network activation in association with different object location memory (OLM) training paradigms. The OLM task is particularly well suited for this work because prior research has shown that spaced training dramatically increases the efficiency of object location encoding. Specifically, wild type mice have been shown to form long term memory of object location after exposure to two objects in an open field in a single trial lasting a minimum of 5 minutes; typically single trials of 5 or 10 minutes are used (Stefanko et al., 2009; Seese et al., 2014). In contrast, mice trained in 3 trials spaced by 60 minutes can learn with total training of 3 minutes or less (Seese et al., 2014). Moreover, OLM has been shown, for single-trial learning, to depend upon dorsal CA1 (Clark and Squire, 2010; Haettig et al., 2013).

An advantage of Fos expression analysis for assessing regional neuronal activation with training is that immunostaining for Fos protein can be used to evaluate recent neuronal activity in nearly all brain regions simultaneously, providing a broad overview of networks activated by learning. Fos mapping provides enormous power in terms of the large number of regions that can be visualized, but it is temporally limited: it is only possible to test one timepoint per animal and, for those animals, one needs to wait for a period of over an hour after training to allow levels of recently induced protein to accumulate. In contrast, a technique such as chronic recording would provide high temporal resolution at the cost of extremely limited spatial resolution and an additional challenge of extracting meaningful information from complex activity patterns. Thus, it is more efficient to begin answering the network question using a tool with high spatial

resolution. The studies described here will use Fos mapping to identify networks engaged by massed and spaced training, with the goal of detecting differences in regional activation between these paradigms if present.

Materials and Methods

Animals. Experiments used male C57Bl/6J mice at least 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME, USA). Mice were group housed with food and water ad libitum, and a 12 hr light cycle with lights coming on at 6:30 am. All procedures were conducted in accordance with NIH guidelines for the care and use of laboratory animals and using protocols approved by the University of California, Irvine's Institutional Animal Care and Use committee.

OLM Task. Training and testing was performed as described (Vogel-Ciernia and Wood, 2014). Briefly, mice were familiarized to experimenter and behavioral chambers for 5 min per day for several days. For training, mice were placed in the arena containing two identical objects (100 mL glass beakers) placed near corners and allowed to explore freely during either one single trial lasting 3, 5, or 10 minutes (termed "massed" training), or in three 60 or 90 s long trials spaced by 60 minutes (termed "spaced" training). Previous work has shown that such trials spaced by 60 min are effective for encoding whereas the same amount of training spaced by shorter (20 min) and longer (2 h) intervals were not (Seese et al., 2014). In cohorts assessed for long-term OLM mice were returned to their home cages immediately after training and then tested for retention 24 h later. For the retention testing trial, mice were returned to the training arena containing the original objects with one in the familiar location and one in a novel location. All training and testing sessions were video recorded using an overhead camera and hand-scored offline, by individuals blind to animal treatments, to assess total time spent exploring each of the

objects. The analysis determined the discrimination index (DI) which was calculated as the time spent exploring the novel location (time attending to the object) minus that attending to the familiar location divided by the total time spent attending to both objects.

Perfusion and immunohistochemistry. One hour after the final training session, mice were euthanized with an overdose of isoflurane gas and intracardially perfused, first with ice-cold 0.9% NaCl followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.2). Brains were removed, post-fixed for 1.5 h and cryoprotected in 30% sucrose in PB (30-48 h, 4°C). Brains were sectioned on the coronal plane (30 μm thickness) and separate series with sections spaced by 150 μm were mounted on Superfrost Plus slides (Fisher Scientific). For c-Fos immunostaining, sections were washed in 0.1M glycine in PB (5 min), incubated for 1 h in 10% normal swine serum plus 0.3% Triton-X in PB and then incubated for 48 h at 4°C with rabbit anti-c-Fos (1:10,000; Millipore ABE457). After washing in PB, sections were then incubated for 1 h at room temperature with donkey anti-rabbit IgG conjugated to Alexa Fluor 594 (1:1000; Invitrogen). Slides were cover-slipped using Vectashield mounting media containing DAPI nuclear stain (Vector Laboratories).

c-Fos quantification. Regional Fos immunolabeling was evaluated as described (Wei et al., 2015). Briefly, digital images were captured using a 10x objective (Plan Apo, NA 0.4) on a Leica 6000B epifluorescence microscope with PCO Scientific CMOS camera and Metamorph acquisition software. Image montages were stitched together using FIJI (Schindelin et al., 2012). Variability in background fluorescence was normalized by subtracting a gaussian-

blurred image of each image from itself. Objects of cellular size and shape were then detected using Python 2.7 and FIJI (**Fig 1.1A**). Only densely-labeled objects – above the intensity threshold of 150 on a scale of 255 - were counted as Fos+. Brain regions were traced by hand in FIJI using an atlas reference (Franklin and Paxinos, 2008), and resulting region-of-interest (ROI) coordinates were used to restrict cell counts (**Fig 1.1B**).

Cell count analysis and statistics. Counts of Fos immunopositive (+) cells were first normalized to the sample region area and to each animal's total Fos+ counts, then divided by average counts of control animals to yield a "percent change from baseline" value. Group "percent change" means and SEMs were then compared to a (Student's) *t* Distribution to yield p-values for each mean compared to 0, representing no change from baseline. Behavioral results were analyzed using a Student's *t* test to compare either total sniffing time or object preference between groups. All graphs are shown as mean $\pm$ SEM. Statistical tests were performed using Python 2.7 software, and all *t* tests were calculated from a two-tailed distribution.

Results

The first analysis used Fos expression to map the distribution of recently activated cells in brains from mice given training sufficient for encoding object location using either a single trial (10 min massed) or spaced (3 one min trials spaced by 60 min) training paradigm. The initial analysis focused on raw Fos+ cell counts across 22 different brain regions with regional boundaries defined by Paxinos and Franklin's Mouse Brain in Stereotaxic Coordinates overlaid on images of DAPI-stained sections. Fos+ cell counts were averaged across the 14 animals in each group to yield the results plotted in **Figure 1.2**. As shown, for most regions there were greater numbers of Fos+ cells in mice given 10 min massed training as compared to those given three, one minute-long, spaced training sessions. This was in line with the overall 3-fold greater exploration time in the massed training group. However, statistically significant differences in activation were only observed in a few regions including subiculum, orbitofrontal cortex, and barrel field of primary somatosensory cortex. Notably, massed and spaced training activated similar numbers of cells within the hippocampal formation (**Fig 1.2A**) suggesting that, despite synaptic changes that occur in this region with OLM encoding, the hippocampus is not preferentially engaged during effective spaced, as opposed to massed, training.

The initial analysis of Fos counts described above established the general patterns of neuronal activation with massed as compared to spaced training, but did not provide insight as to whether differences in regional activation reflect processes of encoding or, alternatively, the massive differences in handling and exploration inherent in the comparison between effective massed and spaced training. In an effort to control for differences in baseline neuronal activation between training paradigms, additional cohorts

of mice were trained in spaced or massed paradigms but using parameters of training that are not sufficient for learning: the spaced training control group were given three 1 minute long trials spaced by 20 minutes whereas the massed training control group received a single trial of 3 minutes duration (n=12 per group; (Stefanko et al., 2009; Seese et al., 2014)). Fos+ counts in each ROI from learning groups were divided by the ROI counts from their respective nonlearning controls. Comparing these normalized values from massed learning vs. spaced learning groups identified some of the same regional effects of spacing evident in the raw counts analysis (subiculum, orbitofrontal cortex; **Fig 1.3A,B**), but also revealed a large difference in activation of limbic fields that were not appreciated from the raw counts, and in particular generally greater activation in these limbic fields with spacing (**Fig 1.3D**). Even though orbital frontal cortex showed less activation in the spaced-learning group as compared to the massed-learning group, the significantly different level of activation of OFC with training schedule, and literature implicating frontal cortical fields in memory consolidation, suggest that activity of OFC may coordinate with limbic regions to influence hippocampal processing and, thus, long-term memory. This is a topic that will be addressed further in **Chapter Two**.

To identify the phase of spaced training giving rise to paradigm-specific patterns of Fos expression, we summed up Fos+ cell counts across all ROIs and all planes in the brains of separate cohorts of mice that received either one, two, or three 1 minute long trials, spaced by 60 minutes (n=5 per group). This analysis also addressed whether, given the reportedly long half-life of Fos protein (2 h according to (Morgan and Curran, 1991)), the observed group (massed vs. spaced) differences were due to an accumulation of Fos over time. As in prior analyses the animals were sacrificed for Fos analysis 60 min after the end

of the last behavioral trial. Although there was a significant increase in the number of Fos+ cells from the first to the second training trial, there was no additional increase after the third trial (**Fig 1.4A**). In fact, there was a significant decrease from the second to the third trial, suggesting that Fos protein induced by trial 2 had, in part, degraded in brains of animals assessed after trial 3. Furthermore, the elevated numbers of Fos+ cells after the second trial were evident in many of the same regions that had greater numbers of Fos+ cells in mice given spaced as compared to massed training (**Fig 1.4B**). In particular, numbers of Fos+ cells in frontal associational cortex (FrA) and OFC peaked dramatically after trial 2, but returned to trial 1 levels again by trial 3. Regions that did not change significantly across trials are not shown (e.g. PrL, PIR, LS). This analysis is of particular interest in that it detects regional engagement *within* the spacing paradigm, which may significantly contribute to encoding, but was not prominent in the post-trial 3 counts.

Discussion

The present findings support the hypothesis that spaced training engages a different neuronal network than does massed training; specifically, the data showed that spaced training had effects on neuronal activation in frontal cortical regions that were not evident after massed training. While the OFC and lateral entorhinal cortex share a sparse yet significant two-way connection (Kondo and Witter, 2014), functional connectivity between this cortical region and hippocampus is likely less direct.

One of the possible sources of input that could be used to enhance memory with spaced training is the medial prefrontal (mPFC) fields including infralimbic and prelimbic cortex; these regions share overlapping circuitry with hippocampus and play an important role in memory. Indeed, some theories attribute the earliest phases of declarative memory (so-called "working memory") to mPFC (Fiebig and Lansner, 2014) while others indicate its importance in remote memory (Bontempi et al., 1999). In addition, mPFC is known to phase-lock with CA1 theta rhythms (Jones and Wilson, 2005; Siapas et al., 2005) and to directly coordinate with CA1 to integrate trajectory information (Ito et al., 2015). All of this suggests a role of mPFC as an "integrator" in the consolidation process, serving to associate new information with existing schema (Preston and Eichenbaum, 2013) and would align nicely with the encoding-variability theory of distributed practice (Glenberg, 1979).

The case of Phineas Gage gave scientists the (perhaps mistaken) impression that OFC primarily regulates behavioral inhibition and emotional regulation (O'Driscoll and Leach, 1998). Recent evidence suggests instead a more "affirmative" role in that OFC predicts outcomes and guides behavior at choice points… both good and bad, appetitive

and aversive. Orbitofrontal cortex is a multi-modal association region involved in representing and learning about reinforcers; it is thought to be critical for stimulus-reinforcement learning and reward valuation. Evidence from rodent, nonhuman primate and human studies indicates a role for OFC in processing rewards and punishments (Kringelbach and Rolls, 2004; Rudebeck and Murray, 2014). It is often attributed a role similar to that of the amygdala due to similarities in their circuitry (Kringelbach and Rolls, 2004), and because the two regions share abundant, strong, reciprocal connections (Schoenbaum et al., 1998). In both rodents and primates, OFC receives inputs from all sensory modalities and all the "what" processing systems. It also shares reciprocal connections with other prefrontal cortical fields and hippocampus (Hoover and Vertes, 2011; Kondo and Witter, 2014). Outputs of the OFC, in addition to reciprocity with aforementioned regions, include striatum, hypothalamus, VTA and periaqueductal gray; together these connections indicate a central role for OFC in emotional processing.

OFC's diverse connectivity with reward-related regions, not to mention its apparent role in reward-related behaviors, suggests that the role of this cortical region in the spacing effect may involve recruitment of reward systems. There is abundant evidence that dopamine signaling can enhance consolidation of LTP and memory (Li et al., 2003; Moncada and Viola, 2007; Takeuchi et al., 2016). Microdialysis studies in mice demonstrated novelty-related dopamine spikes in both prefrontal cortex and hippocampus (Ihalainen et al., 1999). Novelty enhances both LTP and long-term memory (Davis et al., 2004), and this enhancement is dependent on dopamine receptor (D1/D5) activation (Li et al., 2003; Moncada and Viola, 2007; Wang et al., 2010).

Connectome data compiled by the Allen Brain Institute (Oh et al., 2014) indicate that a robust circuit exists from medial OFC to lateral entorhinal cortex. The thalamic nucleus reuniens (Re) appears to be the key intermediate in this pathway: it receives most of its input from infralimbic and prelimbic cortices, as well as hippocampus (via subiculum) and sends nearly all of its output directly to lateral entorhinal cortex. Recent work from the Moser lab has linked prefrontal cortex and nucleus reuniens to spatial navigation (Ito et al., 2015). Future studies in our lab will further probe the nature of this circuit and its possible role in memory consolidation.

Acknowledgements

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Figures

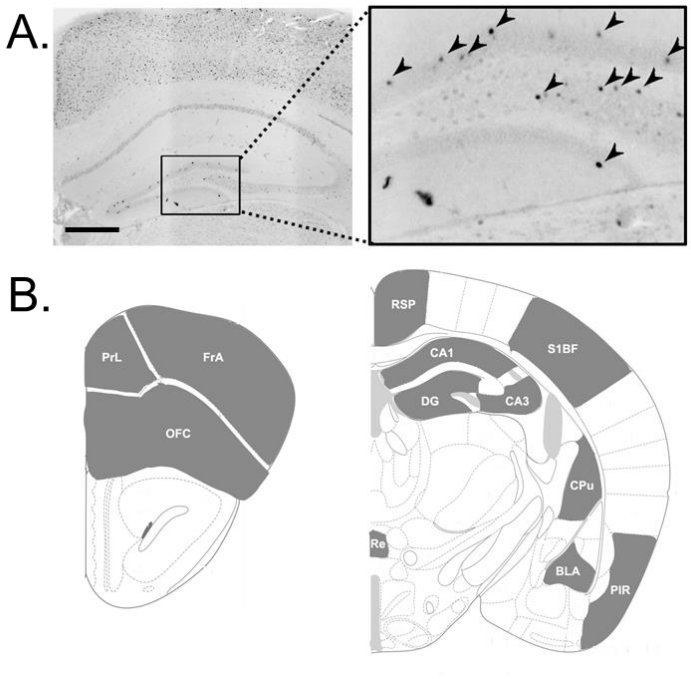

Figure 1.1 Images of c-Fos immunoreactivity and identification of regions of interest (ROIs). **A**, Image shows Fos+ nuclei in hippocampus at low magnification (left) and in a higher magnified view (right) of the boxed region; immunolabeled cells are seen as dark spots in this reverse-contrast image of immunofluorescent labeling (scale bar = 500 μm). Arrowheads indicate cells counted as Fos+ using a density threshold of 150 (used for all subsequent analyses). **B**, Representative brain atlas planes showing the ROI outlines for major regions.

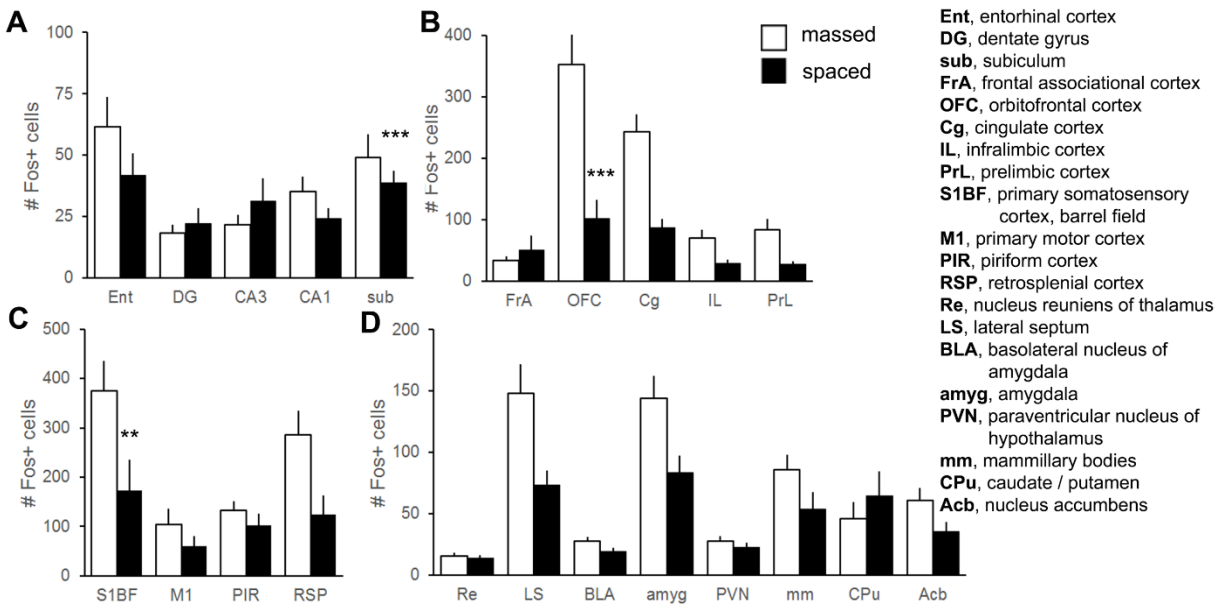

Figure 1.2. Spaced and massed training generate different regional patterns of Fos expression. These and subsequent graphs show the number of Fos+ cells in various regions of interest (ROIs), divided by the number of pixels used to define the ROI, this in order to correct for the difference in the sizes of the various fields. **A**, Major hippocampal subfields showed no significant differences between massed and spaced training, but the subiculum did with more cells in the massed group (p=0.0007). **B,** Frontal cortical fields showed significant training-related variability in Fos+ cell counts, especially in orbitofrontal cortex (p<0.0001).**C,** Among other cortical fields, somatosensory cortex showed the greatest training-related variability in Fos+ cell counts (p=0.0084).**D,** Limbic regions tended to be more active with massed training, but no differences were statistically significant. *p<0.05, **p<0.01, ***p<0.001 comparing massed to spaced training, two-way ANOVA with Sidak's multiple comparisons. n=12-14 ea.

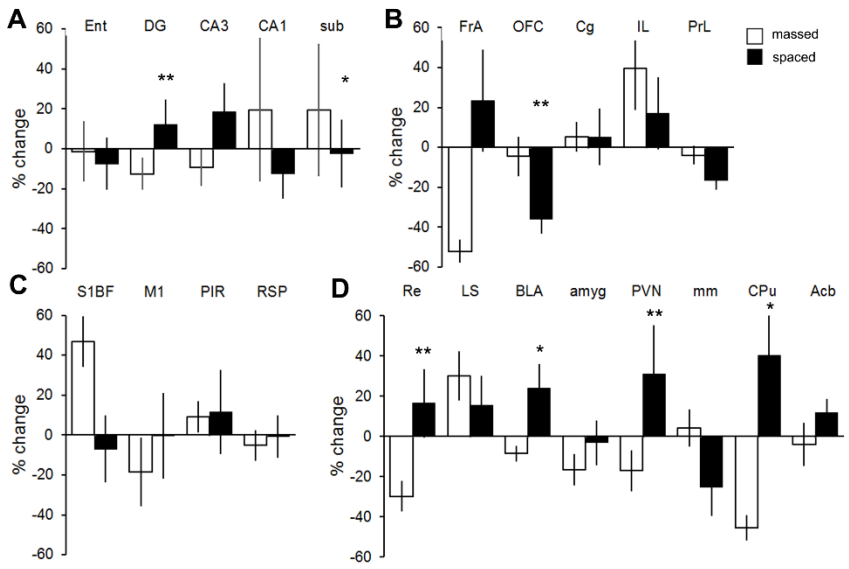

Figure 1.3. Spaced learning activates a different neuronal network than massed. Plots show the percent difference in numbers of Fos+ cells in each training group relative to its nonlearning control (learning / nonlearning x 100). **A,** Spaced training yielded higher Fos+ cell counts in dentate gyrus (p=0.0077) and lower counts in subiculum (p=0.0355). **B,** Within frontal cortical fields, orbitofrontal cortex persists as the most strikingly altered region (p=0.0086). **C,** Spaced training had no significant impact on Fos+ cell counts in other cortical fields. **D,** Normalization to nonlearning controls revealed several spacing-related changes in Fos+ cell counts in limbic regions, including thalamic nucleus reuniens (p=0.0073), basolateral nucleus of amygdala (p=0.0218), paraventricular nucleus of hypothalamus (p=0.0024), and striatum (p=0.0222). *p<0.05, **p<0.01, ***p<0.001, comparing massed to spaced training, two-way ANOVA with Sidak's multiple comparisons. n=12-14 ea.

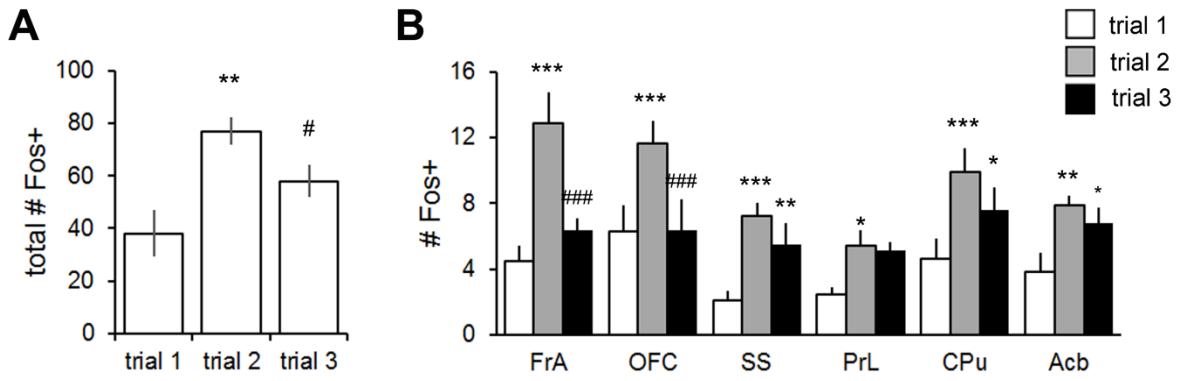

Figure 1.4. The spaced training network is most active after the second training trial. **A,** The total number of Fos+ cells (x10^2) counted per region (group mean ± SEM) increased from the

first trial to the second, but decreased again in association with trial 3. **B**, Bar graph shows the number of Fos+ cells ($x10^2$) in each region that exhibited significant trial variation. Regions that did not differ significantly are not shown. *p<0.05, **p<0.01, ***p<0.001, compared to trial 1; #p<0.05, ###p<0.001, compared to trial 2 using two-way ANOVA with Sidak's multiple comparisons. n>6 per group.

CHAPTER TWO

Orbital frontal cortex is critical for effects of spaced training on long-term object location memory

Summary

Analysis of regional neuronal activation associated with spaced as opposed to massed object location training identified paradigm-specific effects on neuronal activity in orbital frontal cortex (OFC). The present studies tested if levels of neuronal activity in OFC play a critical role in the spaced training effect on learning. A Gi-coupled Designer Receptor Exclusively Activated by a Designer Drug (DREADD) was used to inactivate OFC neurons in mice during either massed or spaced training in the object location memory (OLM) paradigm. Behavioral analyses demonstrated that chemogenetic inactivation of OFC neurons disrupted long term OLM in mice given spaced training but not in mice given a single, massed training trial. As mapping of Fos expression after OFC inactivation suggested the possible involvement of basolateral amygdala (BLA) in the spacing effect additional studies used Gi-DREADD to test inactivation of this second target. The results show that BLA is also critical for the effect of spacing on enduring OLM. Together these findings begin to define a network of structures that are recruited with spaced, as opposed to massed training, to facilitate information encoding.

Introduction

Results of using regional analysis of Fos expression in mice given spaced or massed training for object location memory (OLM) indicate that the two training paradigms differ regarding levels of neuronal activation in orbital frontal cortex (**Chapter One**). These findings, combined with evidence for OFC involvement in learning in other paradigms (Lesburguères et al., 2011; Rudebeck and Murray, 2014), suggests that the OFC may indeed be critical for the greater efficacy of spaced vs. massed training for long term memory. Orbitofrontal cortex is a multi-modal associational region involved in representing and learning about reinforcers; it is thought to be critical for stimulus-reinforcement learning and reward valuation (Kringelbach and Rolls, 2004; Rudebeck and Murray, 2014). It is often attributed a role similar to that of the amygdala due to similarities in their circuitry (Rolls, 2000), and because the two regions share abundant, strong, reciprocal connections (Schoenbaum et al., 1998; Hoover and Vertes, 2011). In particular, both regions project to medial and lateral entorhinal cortex, which provides the main cortical input to hippocampus (Burwell and Amaral, 1998; Kondo and Witter, 2014).

Coordination of activity between hippocampus and OFC has recently garnered attention as an important substrate of "cognitive maps" (Wikenheiser and Schoenbaum, 2016). The hippocampus has long been considered synonymous with mapping, both cognitive (Tolman, 1948) and spatial (O'Keefe and Nadel, 1978), whereas the OFC is thought to provide valence information to such maps (Wilson et al., 2014), monitoring internal state and guiding behavior accordingly. Functions of both regions appear to converge in reward processing, with OFC directly encoding reward contingencies (Gallagher et al., 1999; Gottfried et al., 2003) and hippocampal place cells firing together

around goal locations (Hok et al., 2007). At behavioral choice points, both hippocampus and OFC display prospective activity. Hippocampal place cells fire in anticipation of possible spatial routes, and reward-coding OFC neurons activate in anticipation of possible rewards (Johnson and Redish, 2007; Steiner and Redish, 2012).

There is evidence that simple learning tasks appear not to need OFC, whereas tasks that are more complex and require behavioral adjustment generally depend on OFC function (Schoenbaum et al., 2009). Spaced training, relative to massed, fits this description; during the second and third training trials, mice must weigh the benefits of exploring each object and act to maximize novelty. The present studies tested if the OFC is indeed required for spaced, as opposed to massed, training to support long-term memory in the OLM task. This work takes advantage of recently-developed technology for using Designer Drugs Exclusively Activated by Designer Receptors (DREADDs) coupled to an inhibitory G-protein to decrease neuronal activity of target OFC neurons (Sternson and Roth, 2014) and, thus, test for their critical involvement in the different learning paradigms.

Materials and Methods

Animals. All experiments involved male C57Bl/6J mice at least 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME, USA), and were conducted in accordance with NIH guidelines for the care and use of laboratory animals and using protocols approved by the University of California, Irvine's Institutional Animal Care and Use committee.

OLM Task. Training and testing were performed as in the previous chapter. In some experimental sets, mice were given an intraperitoneal injection of clozapine-*N*-oxide (CNO; 10 mg/kg) or saline 30 min before massed training or 30 min before the second training trial for spaced training. Mice were tested for OLM retention one day after training; the mice were returned to the training arena containing the original objects with one in the familiar location and one in a novel location for a period of 5 minutes. All training and testing sessions were video recorded using an overhead camera and hand-scored offline, by individuals blind to animal treatments, to assess total time spent exploring each of the objects. The analysis determined the balance of time spent exploring the novel location (time attending to the object) minus that attending to the familiar location divided by the total time spent attending to both objects.

AAV-DREADD Experiments. To effect regional chemogenetic inhibition of neuronal activity, the construct rAAV8/CaMKIIa-HA-hM4D(Gi)-IRES-mCitrine (Gene Therapy Core at the University of North Carolina, NC, USA) was infused bilaterally into the region of interest using a microsyringe and stereotaxic guidance. The AAV construct enables (i) expression of

the HA-tagged hM4D DREADD under control of the CaMKIIa promoter, by excitatory neurons throughout the forebrain, and (ii) inhibition of neuronal activity via systemic treatment with the otherwise inert DREADD ligand CNO. Stereotaxic surgery was performed under anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia. All mice were given carprofen (5 mg/kg) for analgesia immediately after surgery.

Stereotaxic coordinates for viral construct placement are as follows (relative to the skull surface at bregma): OFC: AP +2.82 mm, ML ±1.40 mm, DV -2.55 mm; frontal associational cortex (FrA): AP +2.82 mm, ML ±1.40 mm, DV -1.60 mm; medial prefrontal cortex (mPFC): AP +1.7 mm, ML ±0.30 mm, DV -2.3 mm; BLA: AP -1.58 mm, ML ±2.75 mm, DV -4.60 mm. Mice recovered for 10-14 d before initiating behavioral procedures. In each case a volume of 0.3 ul was deposited.

Perfusion and immunohistochemistry. One hour after the final training session, mice were euthanized with an overdose of isoflurane gas and intracardially perfused, first with ice-cold 0.9% NaCl followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.2). Brains were removed, post-fixed for 1.5 h and cryoprotected in 30% sucrose in PB (30-48 h, 4°C). Brains were sectioned on the coronal plane (30 μm thickness) and separate series with sections spaced by 150 μm were mounted on Superfrost Plus slides (Fisher Scientific). For c-Fos immunostaining, sections were washed in 0.1M glycine in PB (5 min), incubated for 1 h in 10% normal swine serum plus 0.3% Triton-X in PB and then incubated for 48 h at

4°C with rabbit anti-c-Fos (1:10,000; Millipore ABE457). After washing in PB, sections were then incubated for 1 h at room temperature with donkey anti-rabbit IgG conjugated to Alexa Fluor 594 (1:1000; Invitrogen). Slides were cover-slipped using Vectashield mounting media containing DAPI nuclear stain (Vector Laboratories).

c-Fos quantification and analysis. Regional Fos immunolabeling was evaluated as described in **Chapter 1**. Behavioral results were analyzed using a Student's *t* test to compare either total sniffing time or discrimination index between groups. All graphs show results as group mean $\pm$ SEM. Statistical tests were performed using Python 2.7 software, and all *t* tests were calculated using a two-tailed distribution.

Results

Inactivation of OFC blocks the spacing effect.

As results in Chapter One indicate that the OFC is differentially activated by spaced as compared to massed OLM training, we tested its importance to learning within the spacing regimen using chemogenetic inactivation. To this end, an HA-tagged AAV-Gi-DREADD construct was injected into OFC (**Fig 2.1A**) and then after two weeks to allow for expression and transport of the DREADD into the processes of infected cells, mice received an intraperitoneal (IP) injection of the DREADD agonist CNO or saline vehicle in association with OLM training. Separate groups of mice were trained in three trials spaced by 60 min (the spacing regimen) or in a single trial (the massed regimen); in each case the total period of training was shown to be sufficient for OLM in previous studies (Seese et al., 2014). Mice receiving spaced training were injected with CNO or vehicle 30 min prior to the second training trial because that appeared to be the time at which spacing-specific effects on Fos expression in OFC were most pronounced (**Fig 1.4**).

For the spacing regimen, we first evaluated effects of OFC inactivation in mice receiving a total of 3 minutes of training spaced across three 1-minute long trials. Mice treated with vehicle exhibited robust long-term OLM (DI: 27.6 ± 6.7, n=8), whereas those treated with CNO failed to recognize (i.e. preferentially explore) the novel location (DI: 8.2 ± 3.9, n=18; p=0.016 for vehicle vs. CNO groups; **Fig 2.1B**). The *total time* spent sniffing objects did not differ between groups during either testing (11.2 s saline vs. 10.9 s CNO, p=0.847) or training (11.1 s saline vs. 8.5 s CNO, p=0.304), although the exploration of objects during the second training trial was significantly reduced in CNO-treated mice (1.58

s saline vs. 1.02 s CNO, p=0.032; **Fig 2.1D**). Since both groups received injections prior to the second training trial, it is unlikely that this is due to stress or handling effects; rather, it suggests that OFC plays a role in guiding exploratory behavior.

The total training time used in the above experiments (3 minutes), while generally sufficient for forming long-term OLM in wild-type mice (Seese et al., 2014), is far less than the five to ten min typically used for massed training in the OLM paradigm (Stefanko et al., 2009) and may only be affected by OFC inactivation because it is near the learning threshold. Therefore we tested effects of OFC inactivation but using 5 minutes of training spaced across three trials (each trial lasting 100 s). These experiments yielded the same effect as above: OFC inactivation blocked long-term OLM (saline DI 36.8 ± 9.7, n=5; CNO DI -0.6 ± 9.0, n=8; p=0.023 vs. saline; **Fig 2.1B**).

To test if the involvement of OFC in long-term OLM is specific to spaced training, OFC-DREADD mice were injected with saline or CNO 30 min prior to either 5 or 10 min of massed training, and were tested for retention 24 hrs later. In these cases CNO (and thus OFC inactivation) had no effect on long-term memory (5 min saline DI: 37.6 ± 6.2, n=5; 5 min CNO DI: 26.4 ± 7.5, n=5; p=0.281; 10 min saline DI: 26.6 ± 7.3, n=6; 10 min CNO DI: 30.1 ± 4.9, n=9; p=0.372; **Fig 2.1C**). Treatments did not differentially influence object exploration time during training (saline 14.4 s ± 1.5, CNO 13.4 ± 1.4, p=0.628; data not shown).

Inactivation of OFC alters neuronal activity in hippocampal and limbic circuits.

The above results indicate that OFC indeed plays a role in OLM with spaced training and suggested to us that spacing may activate a broader circuit of learning related structures that include this cortical region. Therefore, we next probed the functional interactions of OFC with learning-related structures using Fos analysis in animals that had Gi-DREADD expression in the OFC. These mice were injected with the DREADD agonist CNO, returned to their home cages for 20 min to allow plasma CNO to reach peak levels, and then placed in a novel environment to explore freely for 15 min. After exploration, animals were returned to their home cages for an additional hour to allow Fos protein expression before sacrifice. Tissue sections through forebrain were processed for Fos immunofluorescence and quantification of immunolabeled neurons as above. Counts of Fos+ cells in OFC-inactivated mice were compared to counts in OFC-intact mice; for this analysis we did not evaluate OFC itself because of artifactual immunolabeling of cells in the area surrounding the DREADD injection placement. As shown in **Fig 2.2**, this analysis revealed a marked reduction in the number of Fos+ cells in BLA in mice with OFC inactivation (-41% ± 9%, p=0.002). It is noteworthy that amygdala also showed training paradigm-specific activity differences in the first round of analyses (**Fig 1.3**), suggesting that it may be part of the same functional circuit as OFC in the context of object location learning.

Basolateral amygdala is needed for effective spaced training.

To test if BLA is critical for the spacing effect on OLM, the AAV-Gi-DREADD construct used for OFC inactivation was injected into amygdala bilaterally and animals were allowed

at least two weeks recovery before behavioral testing. As in prior studies injected animals were treated with CNO or vehicle 30 min before training in the second of 3 spaced trials. The BLA-inactivated animals showed no long-term OLM compared to the normal learning in saline-injected controls (saline DI 26.59 ± 6.14, n=6; CNO DI 3.20 ± 5.48, n=9; p=0.012; **Fig 2.3C**). BLA-inactivated animals also showed a slight, yet significant, reduction in exploration of objects during testing (saline 16.40 ± 1.54, CNO 12.53 ± 1.01; p=0.048).

As medial prefrontal cortex has been implicated in complex learning (Ito et al., 1999; Takehara et al., 2003), we also examined the contribution of this frontal cortical region using the Gi-DREADD strategy. As shown in **Fig 2.3C**, bilateral DREADD inactivation of mPFC had no significant effects on either OLM retention (saline DI 24.6 ± 6.8, n=8; CNO DI 20.8 ± 7.8, n=9; p=0.723) or exploration time during OLM training (saline 15.9 s ± 1.6, CNO 13.9 s ± 2.4; p=0.505).

OFC inactivation after spaced training blocks long-term OLM.

As our experiments evaluated the effects of OFC inactivation effected between the first and second of three spaced training trials, it was unclear if the consequent impairment of long-term OLM was due to disruption of consolidating information collected during the first trial or, perhaps, initially collecting data from the second trial. Basically, the issue is whether the regional silencing is disrupting learning initiated before or after OFC inactivation. In an attempt to address this question, additional cohorts of mice received AAV-Gi-DREADD bilaterally in OFC and were injected with vehicle or CNO either 30 min before or after a single, 10 min-long training trial; it will be recalled that 10 min massed

training normally supports long term OLM. As shown in **Fig 2.4**, pre-training inactivation of OFC had no effect on OLM retention (saline DI: 22.05 ± 7.46, n=6; CNO DI: 30.15 ± 5.16, n=11; p=0.372). However, post-training inactivation of OFC resulted in dramatically lower DIs relative to those of OFC-DREADD mice given saline (saline DI: 36.57 ± 5.75, n=7; CNO DI: 7.11 ± 6.09, n=8; p=0.004). The total exploration time during OLM training was unaffected (pre-training saline 14.4 s ± 1.5, pre-training CNO 13.4 s ± 1.4; post-training saline 16.5 s ± 2.3, post-training CNO 16.0 s ± 6.1).

Discussion

Taken together, results in this Chapter suggest that OFC coordinates with BLA to mediate the spaced training effect on a traditionally hippocampus-dependent task. OFC inactivation was shown to specifically interfere with spaced training, without major disruption of exploratory behavior. Moreover, OFC inactivation altered neuronal activity in the BLA, inactivation of which *also* blocked the spacing effect.

The case of Phineas Gage gave scientists the (perhaps mistaken) impression that OFC primarily regulates behavioral inhibition and emotional regulation (O'Driscoll and Leach, 1998). Recent evidence suggests instead a more "affirmative" role in that OFC predicts outcomes and guides behavior at choice points both good and bad, appetitive and aversive. Given the nature of spaced training, it is logical to suspect that it would engage decision-making processes. Animals may retain a fuzzy working memory trace across trials which allows them to assess the relative novelty of features upon reintroduction. That is, the decision to attend to previously unexplored aspects of the arena may require an intact OFC.

Useful maps contain several overlapping layers, each contributing some new dimension of information. Likewise, cognitive maps need spatial (encoded by hippocampus), reward (OFC) and affect (amygdala) layers to encode the full depth of an experience. Perhaps repeated training trials allow a deeper map to be constructed than does a single trial; the increased informational content would then give rise to the increased robustness of long-term memory.

In the minutes to hours following a learning event, cells that were engaged by the event are reported to undergo patterned reactivation. The best described examples of this involve ordered reactivation of place cells along a previously-traveled trajectory (Foster and Wilson, 2006; Diba and Buzsáki, 2007). Such "replay" has been shown to occur both during slow-wave sleep (Wilson and McNaughton, 1994) and awake behavior (Foster and Wilson, 2006; Karlsson and Frank, 2009), and is thought to play a role in the transfer of a memory trace from short-term to long-term storage (Sirota et al., 2008). The hippocampal formation is likely involved in earlier stages of memory consolidation and neocortical fields are engaged later (Kim and Fanselow, 1992; Smith and Squire, 2009). Indeed, inactivation of rodent hippocampus abolishes fear memory only within the first couple of days following learning; after that, the memory becomes dependent on neocortex (Kim and Fanselow, 1992; Sutherland et al., 2010). Notably, this process can be accelerated by repeated training (Lehmann et al., 2009) suggesting an extra-hippocampal basis for the spaced training effect. Future studies will address the role of orbitofrontal cortex in mediating this transfer of memory.

We were surprised to observe an effect of OFC inactivation on massed learning. When OFC was inactivated after training (but not before), it blocked retention of the memory trace. Notably, previous work has shown that pre-training DREADD inactivation of dorsal hippocampus is sufficient to block long-term memory (López et al., 2016). Plasma CNO concentrations peak 20 min after systemic injection in mice, and the compound has a circulating half-life of approximately 15 min. Biological effects similarly peak around 20 min post-injection but demonstrate a slightly longer half-life; effects have been reported to linger for up to 10 hours (Guettier et al., 2009; Wess et al., 2013). Pre-training CNO was

injected 30 min prior to the beginning of training, so there would have been a significant amount of it in the bloodstream during training, but levels decrease rapidly such that effective levels would be absent by ~30 min after training. Post-training CNO was injected 30 min after the training trial, so plasma levels would be highest 40-60 min post-training. This is notable as previous studies report a delayed phase of memory consolidation occurring within this precise window of time (Babayan et al., 2012). Together these points indicate that OFC inactivation disrupts consolidation of an initial memory trace with CNO given following massed training. Future experiments will test effects of OFC inactivation at even longer post-training intervals, for both massed and spaced training. OFC effects on massed training suggest that, perhaps, the time of inactivation during spaced training may disrupt *consolidation* of the first training trial rather than *acquisition* of the second training trial. This distinction is crucial, and merits further investigation.

Acknowledgements

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Figures

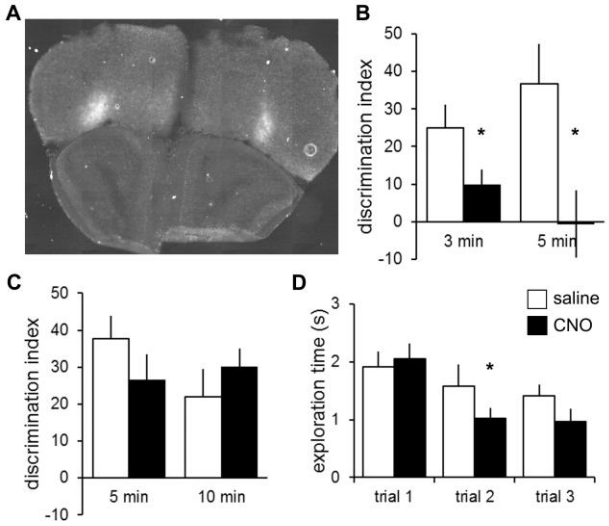

Figure 2.1 OFC is needed for long-term object location memory with spaced training. Graphs show average discrimination index (DI; exploration of novel – exploration of familiar / total exploration time) +/- sem. **A**, Representative image of a coronal tissue section through rostral forebrain showing fluorescent marker of AAV-Gi-DREADD expression bilaterally in OFC. **B-D**, Gi-DREADD injected mice were injected with saline (white bars) or CNO (black bars) before OLM training. **B**, In mice given a spaced training regimen, saline-treated mice learned with either three or five min total training spaced across three trials whereas mice given CNO did not (p=0.016 and 0.023, respectively). **C**, DREADD-mediated OFC inactivation had no effect on OLM DIs for mice given 5 or 10 min total training in a single, massed session. **D**, Although total exploration of objects did not vary between groups during training, exploration during the second trial was significantly less in OFC-inactivated animals (p=0.032). *p<0.05, CNO vs. saline, two-tailed t-test. n>10 per group.

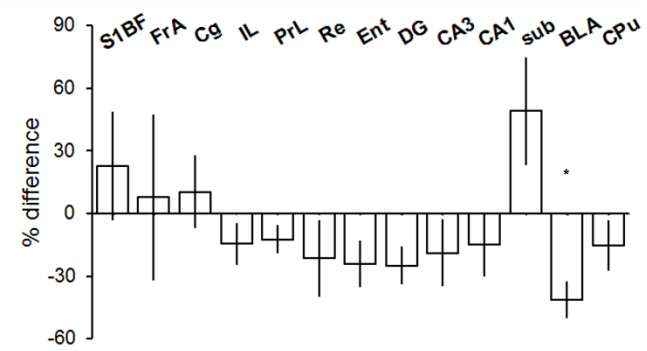

Figure 2.2 Inactivation of OFC alters neuronal activity in basolateral nucleus of the amygdala (BLA). Graph shows percent difference in Fos+ cell counts in CNO- vs. saline-treated animals (CNO / saline x 100) as described in Chapter One. Only BLA showed a significant difference

(p=0.0214). *p<0.05, comparing CNO- to saline-treated animals, two-way ANOVA with Sidak's multiple comparisons post-hoc test. n>7 per group.

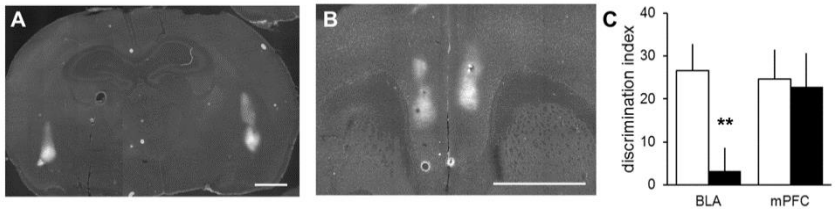

Figure 2.3 Inactivation of other regions suggests an OFC-amygdala network underlies the spaced training effect. Representative images show the placement of AAV-Gi-DREADD expression in BLA (**A**) and mPFC (**B**). **C**, Plot shows discrimination indices (DI) for long-term OLM in saline (white bar) and CNO (black bar) treated mice. DREADD-mediated inactivation of BLA significantly lowered DI (**p=0.012 vs. saline, two-tailed t-test) whereas inactivation of mPFC had no effect. n>8 per group.

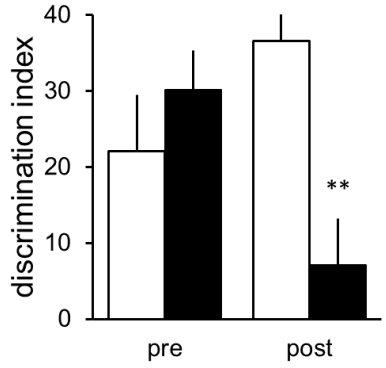

Figure 2.4 OFC inactivation after, but not before, massed training blocks long-term OLM. All mice received AAV-Gi-DREADD in OFC, and were injected with saline (open bars) or CNO (solid bars) either 30 min before or 30 min after a single 10 min long training trial. Gi-DREADD mediated inactivation of OFC prior to training did not significantly alter DIs relative to saline controls (p=0.372). In contrast, OFC inactivation after training dramatically reduced DI (p=0.004). **p<0.01, CNO vs. saline, two-tailed t-test. n>7 per group.

CHAPTER THREE

Synaptic activity mapping reveals septal-temporal "activation zones" across all hippocampal fields

Summary

One potential basis for the efficacy of spaced as compared to massed training is that temporally spaced synaptic activation leads to greater amounts of synaptic potentiation and thus a stronger memory trace. Synaptic activity-induced actin remodeling is known to be critical for hippocampal LTP and learning, and actin regulatory signaling molecules involved in this process can be used to identify recently activated synapses. Here we used immunolabeling for the phosphorylated form of the actin regulatory protein cofilin to identify synapses undergoing long term potentiation (LTP), to identify the distribution and numbers of potentiating synapses in animals recently engaged in spaced and massed training in two spatial learning paradigms: object location memory (OLM) and the Morris water maze (MWM). The goal was to test the hypothesis that there are differences in numbers of potentiating synapses with spaced as compared to massed training within a given paradigm. In agreement with past work using OLM, synaptic modifications were clustered in a discrete portion of the septal-temporal axis of hippocampus in both tasks. However, within this span there were no significant regional differences with training schedule. From this we conclude that, for hippocampus-dependent spatial learning tasks, hippocampal subfields coordinate their activity during memory encoding rather than operating as discrete, independent units.

Introduction

As discussed in previous chapters, spaced training may enhance learning through influences on synaptic or circuit mechanisms of memory encoding. My initial studies (**Chapter One**) investigated the possibility that spaced training recruits a broader, or perhaps different, neuronal network than is activated with massed training. This regional analysis showed that patterns of neuronal activation do indeed differ with spaced as compared to massed training for object location memory (OLM) with paradigm-specific differences being particularly prominent in limbic structures including basolateral amygdala and orbital frontal cortex. These findings do not, however, rule out the possibility of paradigm-specific differences in activity at the synaptic level or that, within a field, spaced and massed training promote enduring plasticity in different numbers of synapses and thereby lays down a stronger or relatively weaker memory trace. In the present studies we used a neurochemical synaptic marker of long-term potentiation (LTP) to test if spaced vs. massed training engaged different numbers and distributions of synapses in hippocampal subfields.

Previous work has shown that with induction of LTP, and specific forms of learning, there are increases in dendritic spine actin polymerization (Fedulov et al., 2007; Rex et al., 2009). Both the increase in filamentous (F-) actin and the stabilization of new actin polymers is required for maintenance of the potentiated state (Krucker et al., 2000; Fukazawa et al., 2003) and, in many instances, memory (Lamprecht, 2016). Synaptic potentiation and / or learning may therefore be inferred by the activation of actin regulatory signaling in dendritic spines during learning. Based on this logic, studies in this laboratory (Chen et al., 2007; Fedulov et al., 2007; Cox et al., 2014) and elsewhere

(Lamprecht, 2016) have been able to map the distribution and numbers of synapses undergoing structural changes associated with LTP in different learning paradigms.

Actin remodeling is regulated by a complex pattern of signaling involving mainly the small GTPases RhoA, Rac, and Ras (Spiering and Hodgson, 2011). In addition, we know that BDNF signals to actin and is critical for signaling through the RhoA pathway (Gehler et al., 2004; Rex et al., 2007). Other work has shown that CaMKII is needed for LTP and learning, although probably not via actin remodeling (Lisman et al., 2012). Studies in this lab have used pCofilin, pTrkB, pCaMKII, and pERK1/2 to look at effects of learning in a spatial memory task. The present studies used pERK1/2 and pCofilin.

To investigate patterns of synaptic activation with spaced vs. massed training, brains will be collected after each trial of spaced training and alternate spaced sections through hippocampus will be processed for immunofluorescence. Cofilin, an actin severing protein, is inactivated by phosphorylation, and activity induced signaling to Cofilin has been found to be essential for synaptic potentiation (Rex et al., 2009) and to be associated with increases in filamentous (F) actin. Although we are interested in spine synapses associated with increases in F-actin, procedures for labeling this cytoskeletal element are not readily applied to fixed tissue sections. Analysis of pCofilin is thus preferred and this marker is reliably associated with the induction of LTP.

Phosphorylated levels of the kinase ERK1/2 are also increased in dendritic spines with induction of LTP (Seese et al., 2012) and signals to the actin-organizing protein cortactin; the latter has been implicated in the stabilization and branching of activity-induced increases in F-actin and in the stabilization of LTP. ERK1/2 activity has been

shown to be critical for enduring potentiation. Moreover, studies in this lab have shown that learning increases synaptic pERK1/2 levels in field CA1 of rostral hippocampus, an area required for OLM encoding. Thus, both pCofilin and pERK1/2 levels would be expected to increase at spines undergoing potentiation with learning. The marker pERK1/2 is particularly short lived and thus suited for analysis of signaling changes with spaced trials because, for this marker, the trace from each trial should have dissipated within the 60 min inter-trial interval.

Recently, big data entered the search for the engram; in an ambitious undertaking Cox et al. counted learning-induced changes in synapses across entire cross-sections of dorsal hippocampus (Cox et al., 2014). For the first time, direct comparison was possible across all subfields of hippocampus. While previous work had focused on stratum radiatum of dorsal CA1, there was now evidence that, indeed, unsupervised learning of a novel environment preferentially activates CA1 and CA3. The question remains: does this activation pattern vary with learning paradigm? The following studies examined the hypothesis that different learning tasks would activate different regions of hippocampus. Results suggest that spatial learning tasks do generally result in synaptic modification in dorsal hippocampus, whereas the amount of handling required for the task influences the activity of more ventral regions.

Materials and Methods

Animals. All experiments involved male FVB129 mice at least 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME, USA), and were conducted in accordance with NIH guidelines for the care and use of laboratory animals and using protocols approved by the University of California, Irvine's Institutional Animal Care and Use committee.

Object Location Memory (OLM) Task. Training was performed as published (Vogel-Ciernia and Wood, 2014) and described in Chapter 1. Briefly, mice were familiarized with experimenter and behavioral chambers in daily handling sessions over 3-5 days. For OLM training, mice were placed in the arena containing two identical objects (100 mL glass beakers) placed near corners and allowed to explore freely during one massed trial lasting 5 minutes, or in three 60 s long trials spaced by 60 minutes. These periods of training are sufficient, but near threshold, for learning in the massed and spaced regimens (Stefanko et al., 2009; Seese et al., 2014).

Morris Water Maze (MWM) Spatial Learning Task. Mice were trained to find a hidden platform in a circular pool of opaque water as described previously (Brielmaier et al., 2012). All mice were habituated to the pool over the course of 4 training days in which they were placed in the pool and allowed to swim freely until they reached a visible platform, when they would be removed to a warming chamber. The start position and the location of the platform were pseudorandomized across trials. A control cohort of mice were sacrificed at the end of the 4th day of visible platform training. Another cohort

underwent an additional day of hidden platform training, during which mice were given a maximum of 60 s to locate a submerged platform. A mouse that failed to find the platform within 60 s was guided to the platform by the experimenter to allow spatial learning to occur.

Immunohistochemistry. On removal from the test apparatus, each mouse was promptly anesthetized with isoflurane and decapitated; the brains were removed, fast frozen, and then processed for immunofluorescence as described (Seese et al., 2014). Briefly, the brains were cryostat-sectioned on the coronal plane (20 μm thickness) and separate series with sections spaced by 200 μm were mounted on Superfrost Plus slides (Fisher Scientific). Sections were fixed in 4% PFA (15 min), washed in 0.1M phosphate buffer (PB; pH 7.2) and then processed for dual immunofluorescence localization using primary antisera cocktail containing goat anti-postsynaptic density-95 (PSD95; 1:1000, abcam), a protein enriched in the postsynaptic density at excitatory synapses, in combination with rabbit antisera to phosphorylated (p) ERK1/2 Thr202/Tyr204 (1:500, Cell Signaling) or p-cofilin Ser3 (1:1000, Millipore). Following primary antisera incubation for 48 h at 4°C, the tissue was rinsed in PB and then incubated in the secondary antisera mixture including Alexa Fluor 594 anti-rabbit IgG or Alexa Fluor 488 anti-goat IgG (1:1000 each).

Image collection. Digital images of immunolabeling were captured using a 63x (Plan Apo; NA 1.4) objective, a Leica DM6000B epifluorescence microscope with Hamamatsu Orca ER camera and Leica MetaMorph v1.6.0 software. To use the autofocus function of MM AF,

overlap of synaptic labeling on the green and red channels was verified, then the center of the focal plane for the red (phosphoproteins) channel was established for field CA1 stratum radiatum. Starting from this plane, the Image Autofocus function and Directional Average algorithm were used to optimize focus for images that were automatically collected at 63x from contiguous sample fields (105 x 136 μm each) covering an entire hippocampal cross section (~12 million PSDs). Individual images were then stitched together to generate a composite image of each entire hippocampal cross section.

Automated object counts. In-house software (Chen et al., 2007; Rex et al., 2009) was used to normalize background intensities and then immunolabeled puncta within the size and eccentricity constraints of synaptic elements were identified using multiple intensity thresholds, with erosion and dilation filters, to exclude nonsynaptic objects. The size and fluorescence labeling intensity of the objects thus identified as synapses were automatically measured and counted as being labeled in the green channel only, the red channel only, or in both channels (i.e., synapses double-labeled for PSD-95 and phosphoprotein). Finally, intensity frequency distributions for phosphoprotein immunolabeling associated with PSD95 were constructed for each section and only elements labeled with intensities that exceeded a fixed threshold (>100 on a scale of 255) were used to calculate the percentage double-labeled values for that section. The results are thus described as percentage of total PSD-95 immunopositive (+) synapses associated with high concentrations of the phosphoprotein of interest.

Data analysis. To evaluate the regional distribution of synapses enriched in the phosphoproteins of interest (and hence considered to be recently activated), the hippocampal image was divided into 18 zones that conformed to local cytoarchitectonic and laminar boundaries (**Fig 3.1**). Contiguous sampling blocks were then used to subdivide each lamina within a particular pyramidal cell field; the dentate gyrus (DG) was not separated into different layers. Automated methods described above were then used to calculate the percentage of double-labeled PSDs within each zone. To avoid potential contributions of differences in immunostaining intensity between tissue sections, we normalized the "percentage double-labeled" values for each zone to the mean value for that entire section.

Results

Massed OLM training activates a discrete portion of dorsal hippocampus

A single massed session of object location memory training has been shown to activate synaptic signaling, critical for LTP and learning, in a surprisingly discrete septotemporal span of field CA1 stratum radiatum. Specifically, studies evaluating levels of pERK1/2 colocalized with the postsynaptic marker PSD-95 in mice (Seese et al., 2014) detected significantly elevated pERK1/2 at excitatory synapses in dorsal fields equivalent to the more septal third of hippocampus. This finding was in agreement with the finding that spatial learning in rat stimulates synaptic signaling to actin (and specifically spine increases in pCofilin Ser3) in a narrow septal span of hippocampus (approximately the most septal quarter of the structure; (Rex et al., 2007)). As shown in **Figure 3.2A**, the present analysis replicated this finding using fluorescence deconvolution tomography (FDT), a method for quantifying in three-dimensions colocalization of signaling markers with synaptic elements (Rex et al., 2009). This validation ensured that our behavioral training was effective in producing learning-related synaptic modifications observed by multiple investigators. In comparison, full-section mapping (hereafter referred to simply as "mapping") of the same tissue and using the same signaling marker but in a 2-dimensional (single plane) analysis yielded somewhat different results. Mapping indicated that synaptic modifications were restricted to stratum radiatum (sr) of field CA3 rather than field CA1 as seen with FDT (**Figure 3.2B**). Moreover, the CA3 sr effect was consistent across septal-temporal levels of hippocampus and did not appear to experience the same topography as did the CA1 sr effect.

Spaced OLM training activates dorsal and ventral hippocampus differently

To test whether spaced training modifies hippocampal synapses in patterns different than does massed training, we counted pERK+ PSDs across hippocampal subfields after the first, second, or third of three 60 second-long training trials, spaced by 60 minutes. The numbers of pERK immunopositive (+) synapses for each training group was normalized to counts of nonlearning controls to enable comparison of changes relative to baseline. Nonlearning controls consisted of mice who had been comparably handled and then placed into an empty arena prior to tissue collection. **Figure 3.3** shows the normalized counts for each hippocampal subfield, collapsed across all septal-temporal planes. There was some regional variation in synaptic modification after the first training trial, but no significant changes from baseline. Trial 2 exhibited more variability, but again, no significant changes from baseline. Trial 3 did not differ at all from baseline.

To further probe possible changes after the second training trial, we separated regional counts by septal-temporal plane. As expected, this revealed significant changes from baseline (**Fig 3.4**). Very septal sections contained significantly more pERK+ synapses than baseline, while very temporal sections contained significantly fewer ($p<0.0001$ effect of septal-temporal plane, Two-Way ANOVA). Notably, these differences were consistent across all hippocampal subfields; all dendritic layers of dentate gyrus, CA3, and CA1 behaved similarly ($p=0.5870$, Two-Way ANOVA).

Morris water maze training preferentially activates a discrete plane of dorsal hippocampus

The training schedule-specific changes in septal-temporal activation patterns observed with the object location memory task prompted further study using other

behaviors. The Morris Water Maze (MWM) is a commonly-used rodent learning task that has been shown to depend on hippocampus (Morris et al., 1982). In the following experiments, mice trained to find a hidden platform were compared to those who had only experienced swimming to a visible platform; this allowed us to more clearly measure changes due to spatial learning while minimizing effects of handling and stress. As with OLM, we first examined synaptic modifications using FDT (**Figure 3.5A**). And as with OLM, we saw a large and significant increase in the number of pERK+ synapses in CA1 stratum radiatum of a specific plane ($p=0.00347$, $n=7$); in fact, it was the same plane as identified for OLM. Surprisingly, the mapping results showed the same effect; the number of pERK+ synapses in CA1 stratum radiatum peaked in the same discrete plane ($p=0.0178$, $n=7$ per group, **Fig 3.5B**).

Further analysis of all hippocampal subfields revealed a similar regional effect as that seen with spaced OLM training: in planes that experience learning-related synaptic modification, all subfields are equally active (**Fig 3.6**). Very septal planes experience no change relative to nonlearning controls, whereas central and temporal planes exhibit significant, coordinated activation.

Discussion

The above findings are consistent with previous reports that learning-related synaptic modifications in hippocampus occur in discrete regions (Rex et al., 2007; Cox et al., 2014; Seese et al., 2014). Moreover, these findings support the notion that different behavioral tasks require activity of distinct hippocampal fields. The most robust finding described here was the septal-temporal specificity of synaptic effects with variation in training schedule. It appears that repeated handling – as required for spaced training and MWM – influences activity in temporal regions of hippocampus independent of learning per se. Future work will focus on modulatory influences on hippocampal processing in an effort to better understand this effect. It is likely that stress hormones are involved, as ventral hippocampus is known to be more responsive to systemic modulation (Fanselow and Dong, 2010).

A major concern uncovered by these comparisons was that, for massed OLM training, FDT and mapping yielded different results (FDT localized effects to CA3 sr, mapping to CA1 sr). This may be due to either (i) three- vs. two-dimensional image collection or (ii) slight variations in the code used for analysis. Our lab has validated FDT for use with both electrophysiology and behavior, and the resulting findings have been robust and replicable (Rex et al., 2007, 2009; Seese et al., 2014). Mapping, on the other hand, is a newer technique and thus harder to evaluate its reliability. In the case of the present data, wherein the two methods contrast, conclusions are likely more reliable when drawn from the FDT set. Further study will be needed to compare these techniques in greater detail and thus determine where their respective analytical boundaries lie.

Another caveat of the present studies was that of the signaling markers used. Based on availability of antibodies over time, some studies used pCofilin while others used pERK; this makes direct comparison across experiments difficult. Moreover, these antigens mark different branches of the actin remodeling pathway, and are by no means comprehensive. A perfect study would run several experiments in parallel using markers for each major branch of the actin signaling cascade (RhoA, Rac, Ras). However, as it was, each mapping experiment required hundreds of man-hours and generated terabytes of data. The results of this tremendous undertaking were, frankly, underwhelming, and do not encourage replication. It seems that FDT is still the most expedient route for investigating regional synaptic modification with learning.

Acknowledgements

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Figures

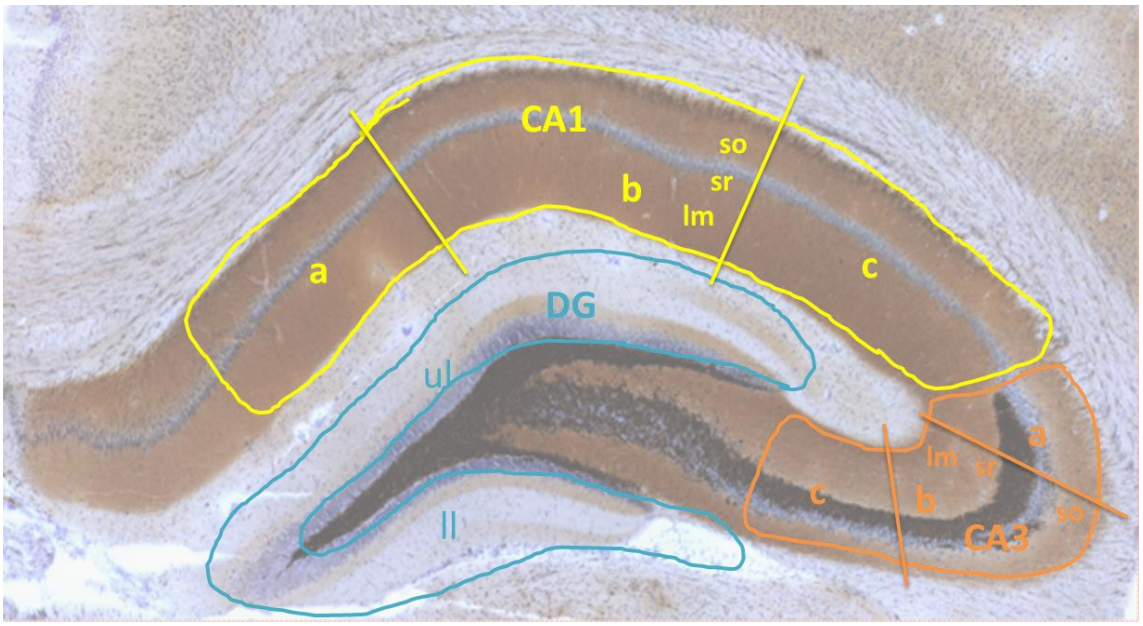

Figure 3.1 Anatomical divisions used for regional analysis. Photomicrograph of a rostral hippocampal section processed for the Timm's stain and Nissl stain to delineate major lamina and cellular layers, respectively. Sampling zones used for regional counting of synaptic elements are indicated with colored outlines. so, stratum oriens; sr, stratum radiatum; lm, lacunosum-moleculare; ul, upper leaf; ll, lower leaf.

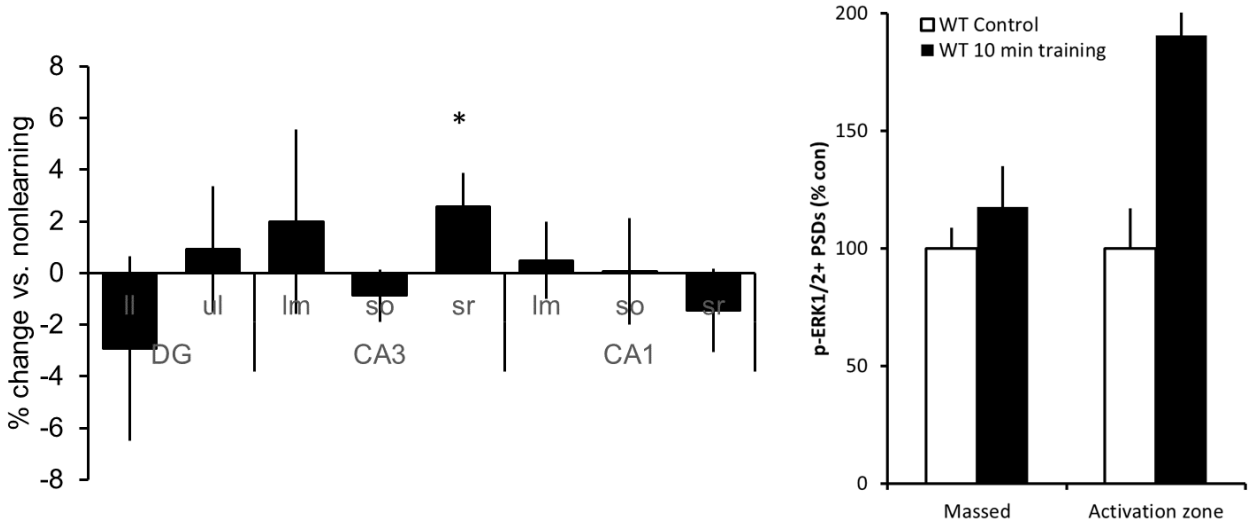

Figure 3.2 Massed OLM training increases synaptic phosphorylation of ERK1/2 primarily in CA3 stratum radiatum. **A,** Plot shows means per hippocampal subfield, calculated from all sections of the ten mice per group. Only CA3 stratum radiatum showed a significant training-related change

in synaptic ERK1/2 phosphorylation (p=0.0397). **B,** Group means within CA1 stratum radiatum were calculated from all sections (left) or only those sections which were previously shown to be activated by massed training (“activation zone”, right). *p<0.05, two-tailed t-test.

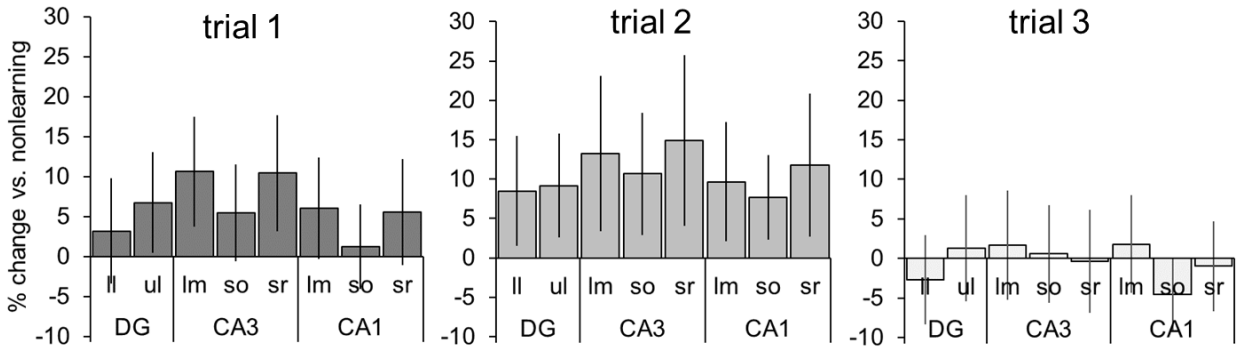

Figure 3.3 Spaced OLM training generally increases synaptic phosphorylation of ERK1/2 across all hippocampal subfields after the second training trial. Group means per hippocampal subfield were calculated from all rostral-caudal sections of the eight mice per group. No subfield in any group was significantly (p<0.05) different from arena-only controls.

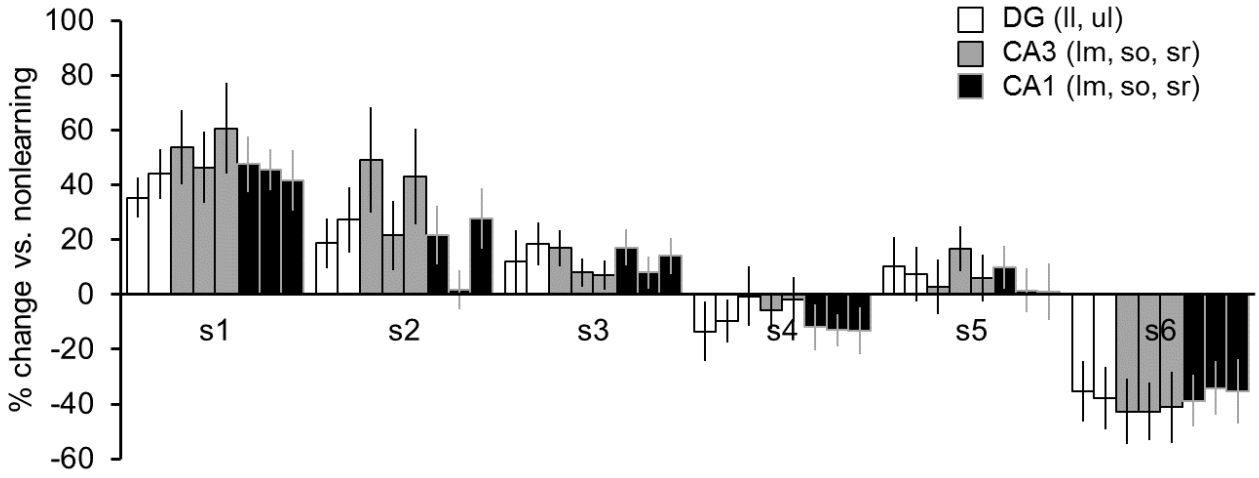

Figure 3.4 The second trial of spaced OLM training generally increases synaptic phosphorylation of ERK1/2 across all hippocampal subfields in more rostral sections, and generally decreases phosphorylation in more caudal sections. All values are expressed as % phosphorylation compared to arena-only controls. There was a significant effect of septal-temporal plane but not of subfield. p<0.0001 and p=0.5870, respectively; Two-Way ANOVA.

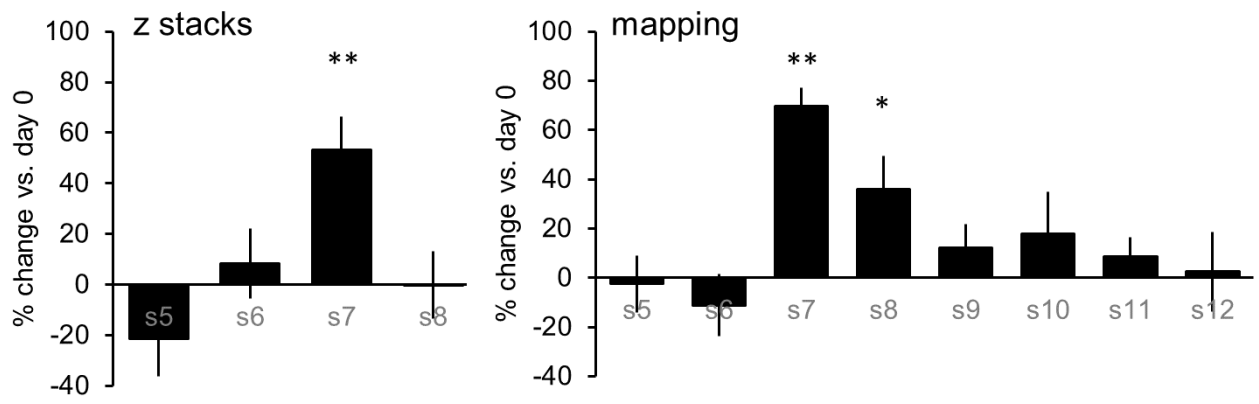

Figure 3.5 Morris Water Maze training results in increased levels of synaptic phosphorylation of the actin-severing protein cofilin in a discrete rostral-caudal plane of hippocampus. **A,** Three-dimensional fluorescence deconvolution tomography of CA1 stratum radiatum illustrates a discrete rostral-caudal plane of activation (p=0.00347, n=7). **B,** Single-plane, whole-section mapping of all hippocampal subfields yields a similarly discrete activation plane within CA1 stratum radiatum (s7 p<0.0001, s8 p=0.017, n=7 per group). All values are expressed as % phosphorylation compared to visible-platform controls.

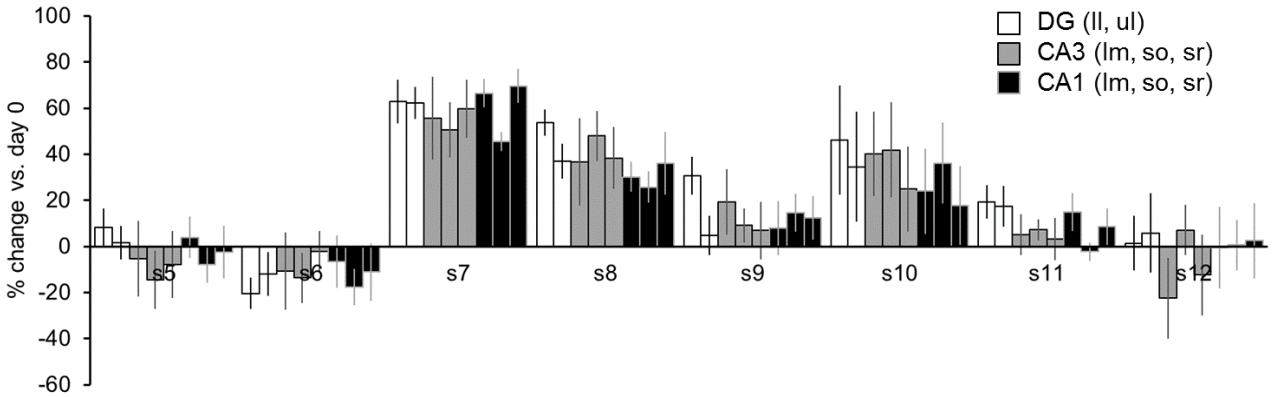

Figure 3.6 Morris Water Maze training results in increased levels of synaptic phosphorylation of the actin-severing protein cofilin in generally caudal planes of hippocampus. All values are expressed as % phosphorylation compared to visible-platform controls. Hippocampal subfields within the same plane tended to follow the same phosphorylation trends (e.g. high in section 7, low in section 12). There was a significant effect of septal-temporal plane but not of subfield. p<0.0001 and p=0.5897, respectively; Two-Way ANOVA.

CHAPTER FOUR

Increasing TrkB signaling offsets social learning deficits in Fmr1-KO mice

Summary

Fragile X syndrome (FXS) is the most prevalent form of inherited intellectual disability and the most common monogenetic cause of autism. Consequently the Fmr1-knockout (KO) mouse, which models the genetic basis of this disorder, has been the focus of intense analysis directed toward understanding the neurobiological basis and treatments for autism. Prior work has shown that the mice do not show robust disturbances in social approach but they have major deficits in social recognition memory wherein they show no recognition of previously encountered mice (i.e., no preference for novel as compared to familiar mice in behavioral testing). The present studies tested the hypothesis that increasing signaling through the synaptic TrkB receptor for brain derived neurotrophic factor (BDNF) can restore social recognition in the mutants. Indeed, both acute and chronic treatment with the TrkB agonist 7,8-DHF rescued social recognition learning in the mutants. Facilitation of learning was also obtained via ampakine treatments known to upregulate endogenous BNDF levels. These results reinforce other findings which suggest that enhancing synaptic TrkB signaling can offset at least some cognitive impairments in FXS and, perhaps, other neurodevelopmental disorders and further indicate these effects will improve some aspects of social interactions in autism.

Introduction

Fragile-X Syndrome (FXS) is the most common form of inherited intellectual disability, and is often comorbid with autism (Kazdoba et al., 2014). Behavioral disturbances with FXS include repetitive behaviors, hypersensitivity to sensory stimuli, cognitive and learning deficits, and social and communication deficits. In the brains of persons with FXS there are disturbances in spine morphology most particularly for pyramidal cells of cerebral cortex (Comery et al., 1997). At present there are no effective treatments for the cognitive or social impairments exhibited in FXS or other autism disorders.

The Fmr1-knockout (KO) mouse is a widely-used model for FXS, and has been well-characterized using a number of neurobiological and behavioral assays (Kazdoba et al., 2014). Previously our lab has shown that these mice have a robust impairment in both novel object recognition (NOR) and hippocampus-dependent object location memory (OLM) tasks (Seese et al., 2014). These memory impairments are associated with disturbances in synaptic levels of the phosphorylated (p-) form of extracellular signal-regulated kinase 1/2 (ERK1/2), a signaling molecule intimately associated with long-term memory consolidation (Selcher et al., n.d.; Atkins et al., 1998; Blum et al., 1999; Hebert and Dash, 2002). Specifically, dendritic spine p-ERK1/2 levels were found to be constitutively elevated in the KOs and, in contrast to effects in wild-types (WTs), synaptic p-ERK1/2 levels were not increased by LTP-inducing synaptic activity (Seese et al., 2012) or hippocampus-dependent learning (Seese et al., 2014). ERK1/2 contributes to dynamic modification of both actin- and microtubule-based cytoskeletal systems (Harrison and Turley, n.d.; Sánchez et al., 2000; Cosen-Binker and Kapus, 2006), both of which are

abnormal in Fmr1-KO hippocampus (Chen et al., 2010a; Seese et al., 2012). Thus, normalization of ERK1/2 activity in the mutants may influence, and perhaps rescue, many of the synaptic events thought to be critical for memory encoding.

Brain-derived neurotrophic factor (BDNF) has been shown to be involved in both the formation and retention of spatial memory (Mizuno et al., 2000). The expression of BDNF is positively regulated by neuronal activity, and upregulating BDNF expression with peripheral ampakine treatment has been shown to rescue forms of synaptic plasticity and learning in many animal models of cognitive impairment (Rex et al., 2006; Simmons et al., 2009; Baudry et al., 2012). Moreover, applying BDNF to Fmr1-KO hippocampal slices completely restores the otherwise deficient long-term potentiation (LTP) in hippocampal field CA1 (Lauterborn et al., 2007). The TrkB agonist 7,8-dihydroxyflavone (7,8-DHF) selectively activates BNDF's receptor TrkB and enhances learning. A recent study has found that chronic consumption of 7,8-DHF via drinking water rescued learning in the Morris Water Maze (MWM) and fear conditioning in Fmr1-KOs, but had no effect on learning in WT mice (Tian et al., 2015). Similarly, in our own lab, recent work showed that both acute and chronic treatment with 7,8-DHF normalized synaptic signaling through ERK1/2 and restored object location memory in Fmr1-KO mice (Seese et al., unpublished). These findings raise the possibility that enhancing TrkB signaling might rescue other facets of cognitive function and, in particular, what might be considered social learning, in the FXS model mouse.

The three-chambered sociability task is a commonly used paradigm for testing basic social behavior in rodents without introducing stress of aggressive behavior (Nadler et al.,

2004; Yang et al., 2011). It is highly odor-based, likely through the main olfactory circuitry (i.e. olfactory bulb > piriform / entorhinal cortex > hippocampus), which processes complex, volatile odor cues and is modifiable through experience (Lledo et al., 2005; Moncho-Bogani et al., 2005). Studies have also indicated a role of the basolateral nucleus of amygdala (BLA) in mediating social approach behavior (Ferri et al., 2016). The BLA receives second order olfactory input, expresses relatively high levels of TrkB, and is known to undergo synaptic changes with 7,8-DHF treatment (Tian et al., 2015).

The present studies used the three-chambered sociability task to investigate if the same treatments that enhance TrkB activation, and rescue OLM in Fmr1-KO mice (Seese et al., unpublished), can rescue aspects of social behavior in the mutants. Specifically we tested if acute and chronic treatment with an ampakine or 7,8-DHF can influence social recognition memory. The results show that acute and chronic 7,8-DHF, and chronic ampakine, increases synaptic levels of activated (phosphorylated) TrkB and rescues social memory functions in the mutants.

Materials and Methods

Animals. Adult (3-5 mo old) male Fmr1-KO mice on the FVB 129 background and age, sex and background-matched WTs, were used for all studies. Animals were standard group housed with littermates (2-5 mice per cage) in rooms maintained at 68° C and 55% humidity, on 12 hr on/12 hr off light cycle and with food and water ad libitum. Experiments were performed during the animal's light cycle (began at 6:30 am) and in accordance with NIH guidelines for the care and use of laboratory animals and protocols approved by the Institutional Animal Care and Use Committee at University of California, Irvine.

Object location memory task (OLM). Animals were handled, trained, and tested for the OLM task as described in detail in Chapters One and Two.

Novel object recognition task (NOR). Animals were handled and trained identically as for the OLM task. For NOR retention testing, one beaker was replaced with a novel object and both objects remained in the same positions as in the training episode. The novel and familiar objects were counterbalanced across animals to ensure that the animals did not prefer one object over another.

Social approach and recognition tasks. Social approach and social recognition tests were carried out as described previously (Nadler et al., 2004; Yang et al., 2011). The test

apparatus was a three-chambered polycarbonate box which included a central empty chamber attached, with doors, to side chambers that could be 'baited' with an object or a mouse to provide the test mouse the decision as to which bait to explore. Mice were habituated to the apparatus, first for 10 min in the center chamber, then for 10 min in the entire apparatus (doors to side chambers open). After this habituation, animals were tested for social approach (SA). An unfamiliar adult C57BL/6J male mouse (stranger 1) was placed inside a small wire cup in one of the end chambers and an empty wire cup was placed in the opposite chamber; the test mouse was allowed to explore freely for 10 min and movements were monitored to determine times spent in the side/baited chambers. Immediately after this exploration, the mouse was tested in the social recognition task (SRT) wherein stranger 1 was moved to the opposite chamber (previously containing the empty cup) and its place taken by a new unfamiliar mouse (stranger 2). The target mouse was again allowed to explore freely for 10 min with movements monitored.

To assess behavior, animals were video recorded and scored offline by raters blind to genotype and experimental group. Mice were scored as interacting with an object, or mouse, when sniffing or nose touching within 0.5 cm of the overlying wire cage. Interaction was not scored when (a) the animal was within this radius but grooming or digging, or (b) did not show intent to interact (e.g., they fell within this zone when turning). Total exploration time was quantified as the time interacting with both objects. A discrimination index was also calculated as $(t_{novel}-t_{familiar})/(t_{novel}+t_{familiar})$, where "familiar" denotes the object that was unchanged from the training trial and "novel" refers to the object that was

changed (either moved to a new location in OLM or swapped for a new mouse in SRT). Thus, a positive discrimination index represents a preference for the novel object.

Drug administration. The positive AMPA receptor modulator (ampakine) CX929 was prepared as a stock solution at 7.5 mg/ml in sterile 30% cyclodextrin (CDX) before being diluted with sterile 0.9% NaCl to a working concentration of 2.5 mg/ml in 10% CDX. For chronic injection studies, animals were given two intraperitoneal (IP) injections daily (9am and 4pm) containing equivalent volumes of sterile saline (first 2 days), 10% CDX (next 4 days), and then either 10% CDX or CX929 (5 mg/kg; next 4 days) (Simmons et al., 2009). The beginning of these injections corresponded with the first day of handling such that the final injection occurred on the afternoon of the 5th day of habituation, e.g. approximately 17 hr prior to training. For acute injection studies, mice were injected IP once daily with sterile saline for 2 days, 10% CDX for 4 days, and then either 10% CDX or CX929 (5 mg/kg; 1 day). The beginning of these injections corresponded with the fifth day of handling such that CX929 was injected 15 min prior to the training episode.

The TrkB agonist 7,8-DHF (Jang et al., 2010), was dissolved in 100% dimethyl sulfoxide (DMSO) before 0.1M sterile phosphate-buffered saline (PBS) was slowly added to reach a diluted solution of 17.5% DMSO and a final concentration of 0.67 mg/ml 7,8-DHF. Animals were injected IP twice daily (as above) with sterile saline (2 days), 17.5% DMSO (4 days), and then either 17.5% DMSO or 7,8-DHF (5 mg/kg: 4 days). The first of these injections corresponded with the first day of animal handling, and an episode of handling or

habituation followed each injection. A final 7,8-DHF injection was given 1 hr before training. For acute injection studies, mice were injected once daily with sterile saline (2 days), 17.5% PBS (4 days), and then either 17.5% PBS for controls or 7,8-DHF (5 mg/kg; 1 day) for experimentals. The first of these injections corresponded with the fifth day of handling such that 7,8-DHF was injected 60 min prior to the training episode. We chose this time point and dose based on prior studies (Jang et al., 2010; Andero et al., 2011; Devi and Ohno, 2012). In some experiments 7,8-DHF was given chronically in the drinking water as described (Johnson et al., 2012). In these cases the 7,8-DHF was dissolved in DMSO to generate a stock solution of 50 mg/ml; 160 µl of this stock was added to every 100 ml of tap water containing 1% sucrose. Cages of 3-5 mice each were provided this 7,8-DHF solution or vehicle (1% sucrose in tap water). Drinking bottles containing these solutions were replaced every 3-4 days.

Tissue collection and immunohistochemistry. Mice with euthanized 2 min after a single 5 min massed training episode with deep isoflurane anesthesia followed by decapitation. As described (Seese et al., 2012), brains were rapidly harvested, fast frozen in -50°C 2-methylbutane, cryostat sectioned (20 µm, coronal) and then fixed in -20°C methanol (15 min). After being air dried, the tissue was incubated (48 hr, room temperature) in a cocktail of primary antisera including mouse anti-PSD95 (1:1000; Thermo Scientific) and either rabbit anti-phosphorylated (p-) TrkB Y515 (dilution, source) or p-ERK1/2 Thr202/Tyr204 (1:500; Cell Signaling). Slides were rinsed 3x in 0.1 M phosphate buffer (PB) and then incubated with donkey anti-mouse IgG and donkey anti-rabbit IgG tagged with AlexaFluor

488 and 594, respectively (each 1:1000; Invitrogen) for 1 hr. The diluent for both primary and secondary cocktails included 4% BSA and 0.1% Triton X in PB. Following the second incubation, slides were washed in PB, air dried, and cover slipped with VectaShield containing DAPI (Vector Labs).

Fluorescence deconvolution tomography. Image collection and automated synapse quantification was performed as described in **Chapter Three**.

Statistical analysis. Two-way repeated-measures ANOVAs and two-tailed Student's t tests were used to test for statistical significance (considered as $p \leq 0.05$). A single n was considered to be an animal for both behavioral tests and immunohistochemical analyses. Values in text and figures show group means ± SEM.

Results

KO mice perform normally on social approach, but not social recognition, tasks

We first tested if Fmr1-KOs display impairments in social behaviors. Wildtype and Fmr1-KO mice were tested for social approach (preference for novel mouse vs. an inanimate object) followed by social recognition (preference for a novel vs. a familiar mouse). As shown in **Figure 4.1A (left)**, both genotypes exhibited equally robust preference for the novel mouse in the initial, social approach task (WT average DI = 62.5 ± 6.37; KO average DI = 55.87 ± 9.75, p=0.560); there was no effect of genotype on this measure. However, in the social recognition task (SRT) only WT mice showed preference for the novel mouse; the Fmr1-KOs failed to distinguish between novel and familiar mice (WT average DI = 37.7 ± 7.65; KO average DI = -2.40 ± 8.57, p=0.002, n=15). There were no significant interactions between object exploration time and genotype for either approach or recognition trials (**Fig 4.1A, right**).

To ensure that we were not simply observing a general deficit in short-term memory, the same mice were also tested on short-term object recognition memory (ORM). To recapitulate the timeline of the social recognition task, well-handled mice were trained in the ORM paradigm in a single 10 min massed trial and tested for retention immediately afterwards. Both WT and KO mice expressed robust discrimination indices indicative of strong preference for the novel object (**Fig 4.1C;** p=0.555, n=10 per group).

The Fmr1 KO social recognition phenotype is recapitulated using social odors alone.

To better define the nature of the Fmr1-KO deficit in social recognition, a variant of the social approach / recognition task was performed using social odors only. Rather than being provided an empty cup and a cup containing a novel mouse for the social approach test, the test mouse was allowed to explore either a cup containing fresh bedding or a cup containing soiled bedding from the cage of novel mice. Importantly, the test mouse was not permitted to make physical contact with the bedding; this ensured that only volatile (socially relevant; (Lledo et al., 2005)) odorants were taken into account. Similarly, in the social recognition phase of the task, the test mouse explored either the same soiled bedding as in the approach phase or soiled bedding from an entirely novel cage. Just as in the original version of the behavior task, both genotypes robustly prefer soiled bedding over fresh (**Fig 4.2A;** WT mean DI = 49.7 ± 5.23; KO mean DI = 49.6 ± 5.56), and only WTs preferred novel soiled bedding over familiar (**Fig 4.2B,** p=0.0025; WT mean DI =41.4 ± 7.36; KO mean DI = 4.03 ± 7.40; n=9).

As an additional control for baseline preference of cage odor, WT mice were exposed to soiled bedding from each of the novel cages simultaneously. In a ten-minute test trial, they did not exhibit a preference for either odor (DI average = -0.55 +/- 4.52, n=11; data not shown).

7,8 DHF treatment normalized social recognition behavior in Fmr1-KO mice.

Previous studies have shown that systemic treatment with the TrkB agonist 7,8-DHF increases in synaptic phosphorylated (p)-TrkB levels and lowers the threshold to long-term memory in Fmr1 KOs (Seese et al., unpublished). Here we tested if a 7,8-DHF

treatment schedule expected (from the prior work) to increase pTrkB levels during the period of training also lowers the threshold, and could perhaps normalize, social recognition in these mice. Fmr1-KO mice were given an intraperitoneal (IP) injection of 7,8-DHF 60 min prior to beginning training for the social recognition task. As shown in **Figure 4.3B**, vehicle-treated Fmr1-KOs exhibited the impairment in social recognition observed in prior groups. In striking contrast, acute 7,8-DHF-treatment fully restored social recognition in the mutants to levels found in WT mice (vehicle average DI = 5.11 ± 6.42, DHF average DI = 42.59 ± 9.81, p= 0.028, n=6 per group); thus, a treatment known to activate synaptic TrkB signaling restored this form of memory. There were no significant interactions between object exploration time and treatment for either approach or recognition phases (data not shown).

Notably, 7,8-DHF is reported to be potent for effects on different behaviors following oral administration (Johnson et al., 2012; Tian et al., 2015), making it an appealing therapeutic approach. Therefore we asked if social recognition memory could be restored in Fmr1-KO mice with the more clinically-relevant oral treatment strategy. KO mice were given 7,8-DHF or vehicle in their drinking water for 14 days prior to social recognition assessment. Vehicle-treated KOs exhibited the typical impairment in social recognition, while discrimination indices of agonist-treated mice were significantly higher and comparable to WT DIs (**Fig 4.3C**, vehicle DI: 8.04 ± 6.85, 7,8-DHF DI: 25.79 ± 7.09; p=0.038, n=19).

To critically test the involvement of the TrkB receptor in social recognition behavior, additional experiments were performed in WT mice receiving acute injections of

the TrkB antagonist ANA-12 prior to training. Social approach was unaffected by TrkB antagonist treatment, whereas discrimination indices for the social recognition phase of the task were significantly lower in drug- vs. vehicle-treated mice (**Fig 4.3D**, vehicle DI: 51.6 ± 6.08, ANA-12 DI: 9.57 ± 12.3; p=0.019, n=6) thereby indicating that TrkB signaling is important for this form of learning.

Since earlier experiments indicated that the Fmr1-KO deficit on social recognition could be replicated in a paradigm using social odors alone, we next treated KO mice with acute 7,8-DHF or vehicle and then tested them on SAT. The result was ambiguous; while agonist-treated mice (n=11) expressed robust discrimination indices, suggesting rescue, vehicle-treated mice (n=6) also trended toward recognition (**Fig 4.4**). Additional studies will be needed to clarify this finding.

Extensive handling rescues Fmr1 KO social recognition memory.

Previous studies in the lab have investigated learning-related effects of positive AMPA receptor modulators ("ampakines"); semi-chronic, but not acute, injection of the ampakine CX929 rescued long-term spatial memory in Fmr1-KO mice (Seese et al., unpublished). Moreover, chronic ampakine treatment has been shown to modulate TrkB signaling and BDNF levels (Lauterborn et al., 2009). Therefore, we tested whether a similar treatment would restore social recognition learning in mutants.

We first tested acute administration of the ampakine CX929. Briefly, mice were sham-injected with vehicle once per day for 4 days, then injected once with either CX929 or vehicle 30 min prior to social approach. As shown in **Fig 4.5A**, this treatment had no

discernable effect on social recognition (vehicle DI: 7.71 ± 7.90, CX929 DI: 14.8 ± 11.0; p=0.615, n>12 per group). This result was not surprising, as it paralleled that of Seese et al. (unpublished) using the same treatment in conjunction with object location memory.

The compound CX929 does not readily dissolve in aqueous solution, making it difficult to administer via drinking water as was possible with 7,8-DHF. We therefore tested semichronic administration via daily IP injection. Mice were sham-injected with vehicle once per day for 2 days, then injected twice per day with either CX929 or vehicle. While this treatment appeared to rescue social recognition in the mutants (CX929 DI: 24.8 ± 6.43, n=22), the vehicle treatment also dramatically increased social recognition (vehicle DI: 17.3 ± 4.39, n=23) with the result that there was no significant difference between the groups (**Fig 4.5B**; p=0.354). However, when we reduced the pre-task injection schedule to include only four days of single injection, a significant treatment effect emerged (**Fig 4.5C**; vehicle DI: 3.23 ± 6.77, CX929 DI: 30.9 ± 3.70; p=0.0022, n>8). Interestingly, when we repeated the handling-intensive treatment schedule (2 days of sham followed by 4 days of twice-daily injections) with 7,8-DHF, we also observed a robust increase in social recognition DIs for both vehicle and drug-treated animals (**Fig 4.5D**; vehicle DI: 34.0 ± 6.03, 7,8-DHF DI: 35.6 ± 9.20; p=0.889, n>7).

Discussion

Together these studies demonstrate a pervasive effect of the FXS mutation on discrimination of socially relevant cues. In addition, the results point to critical contributions of TrkB signaling to social learning. Multiple and varied treatments which modulate TrkB activity were shown to affect social recognition memory in both wildtype and Fmr1-KO mice.

Fmr1-KO mice are known to express deficits in Morris Water Maze (Baker et al., 2010), novel object recognition (Ventura et al., 2004), and object location memory (Seese et al., 2014) tasks. They also exhibit altered patterns of ultrasonic vocalization (Roy et al., 2012) and other features of social behaviors (McNaughton et al., 2008; Dahlhaus and El-Husseini, 2010). The present study demonstrated a pronounced deficit in Fmr1-KO social recognition, integrating both the learning and social impairments noted in other studies. Moreover, we demonstrate here that a treatment (7,8-DHF administration) previously shown to restore spatial learning in these mice (Seese et al., unpublished) also restores social learning. The therapeutic implications for this are staggering, especially because the compound is orally available and could thus be implemented easily in clinical populations.

The present studies also showed that in wild type mice, treatment with a TrkB antagonist blocked social recognition. The necessity of TrkB signaling for normal social recognition (and thus normal social learning) suggest that this form of encoding depends on mechanisms of synaptic plasticity that depend on the BDNF/TrkB system. Studies of hippocampus and amygdala have shown that TrkB function is needed for synaptic long term potentiation (Korte et al., 1995; Patterson et al., 1996) whereas other work has shown

that TrkB signaling is activated (Chen et al., 2010b) and necessary (Ou and Gean, 2007) for learning. Our studies showing that this is also the case for social recognition suggest that the initial exposure phase (novel mouse vs. cup) induces synaptic changes that have mechanistic overlap with LTP and perhaps induce LTP itself, and that as with studies of hippocampal slices, this potentiation and encoding depends on BDNF and signaling through its receptor TrkB (Rex et al., 2007). This earlier work also showed that the functions of BDNF and TrkB needed for learning included effects of the trophic factor on dendritic spine actin remodeling. These findings further suggest that social recognition memory, found here to be enhanced by 7,8-DHF and blocked by the TrkB antagonist ANA-12, also depends on actin remodeling.

Both acute and chronic treatment with 7,8-DHF restored social recognition, while only chronic (and not acute) treatment with CX929 was effective. For both drugs, chronic treatment may have affected BDNF-dependent pathways via sustained activation of TrkB, which itself induces BDNF expression (Lewin and Barde, 1996; Lauterborn et al., 2000). Indeed, this is likely the case, as animals treated chronically were not injected on the day of testing: no drug was in their systems at the time. Further work will investigate the mechanistic differences (if any) between acute and chronic treatment with 7,8-DHF. While the effects of acute 7,8-DHF indicate a role for TrkB signaling in social learning, the fact that acute CX929 had no effect suggests that AMPA receptor modulation, or at least that attained with doses used here, is less influential. Indeed previous electrophysiological studies indicate that basic glutamatergic transmission in hippocampal field CA1 is not seriously affected by the FXS mutation: input/output curve and baseline synaptic transmission is not abnormal in the mutants (Lauterborn et al., 2007). This is consistent

with previous reports showing memory rescue with chronic ampakine treatment (Simmons et al., 2009; Baudry et al., 2012; Lauterborn et al., 2016).

Acknowledgements

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Figures

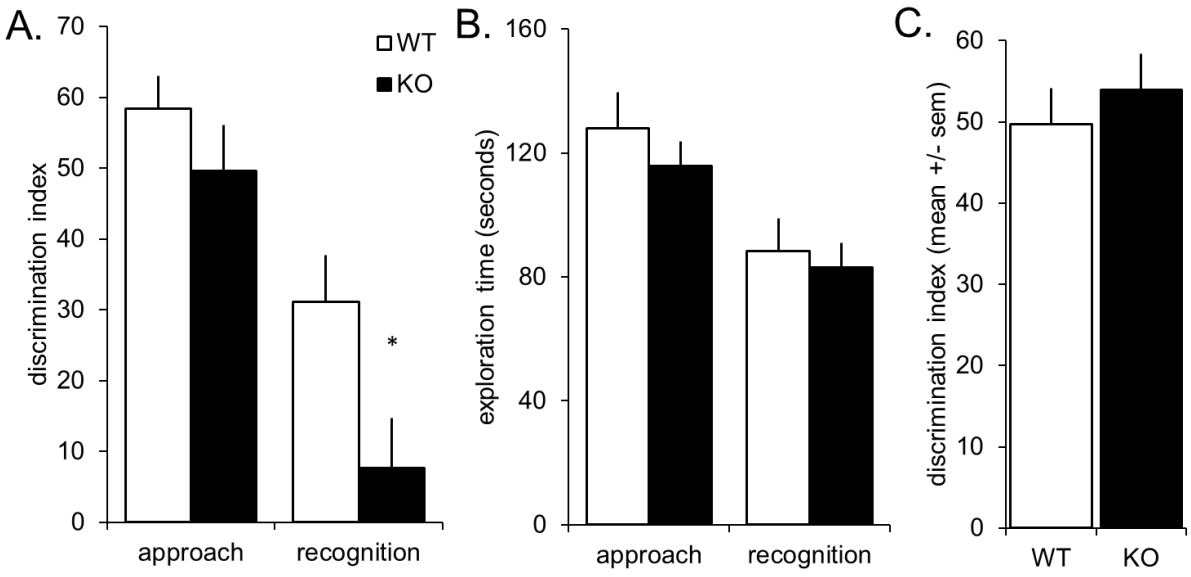

Figure 4.1 Fmr1-KO mice fail to discriminate a novel mouse from a familiar one in the three-chambered social recognition task (SRT). Plots show the proportion of time the animal spent exploring the novel mouse as compared to a blank cup in the social 'approach' task, and the time spent exploring a novel mouse as compared to a familiar mouse in the social 'recognition' task; absence of a preference would be evident as a discrimination index near zero. **A,** Discrimination indices show that Fmr1 KO and wild type (WT) mice both show preference for exploring the mouse in the social approach task whereas the WT mouse showed preference for the novel mouse in the recognition task whereas the KO mouse did not. **B,** In the social recognition test, WT and Fmr1explored the objects the same amount in both phases of the task. **C,** Given the same training schedule, but using inanimate objects rather than mice, KOs perform as well as WTs. *p<0.05 as compared to WT, two-tailed t-test.

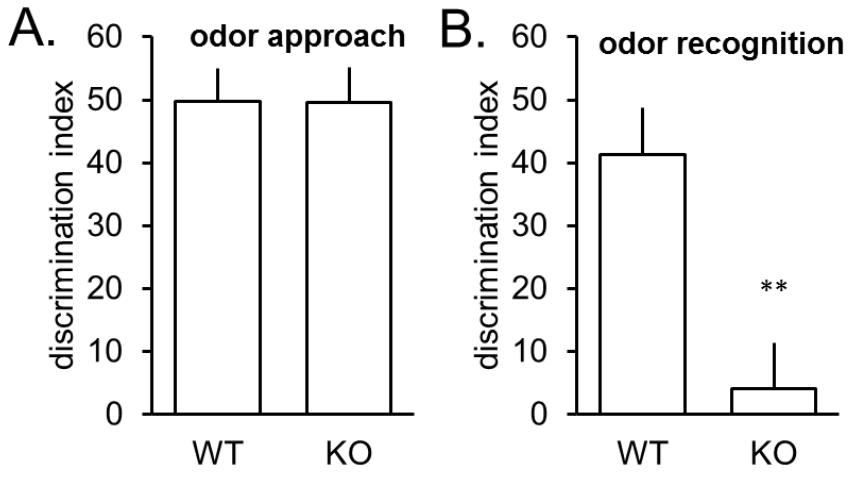

Figure 4.2 The Fmr1 KO social recognition deficit is recapitulated in a modified version of the task that uses social odors alone. **A,B**) Plots show discrimination indices denoting the relative time spent in the chamber containing soiled bedding vs clean bedding (**A**) and bedding from a cage of novel mice vs the soiled begging previously explored (**B**). Results show that both WT and KO mice preferentially explored the soiled vs clean bedding (**A**) whereas given the option to explore novel vs familiar bedding the WTs preferentially explored the novel bedding whereas the KOs showed no preference. **p<0.01, Two-Tailed t-test, n=9 per group.

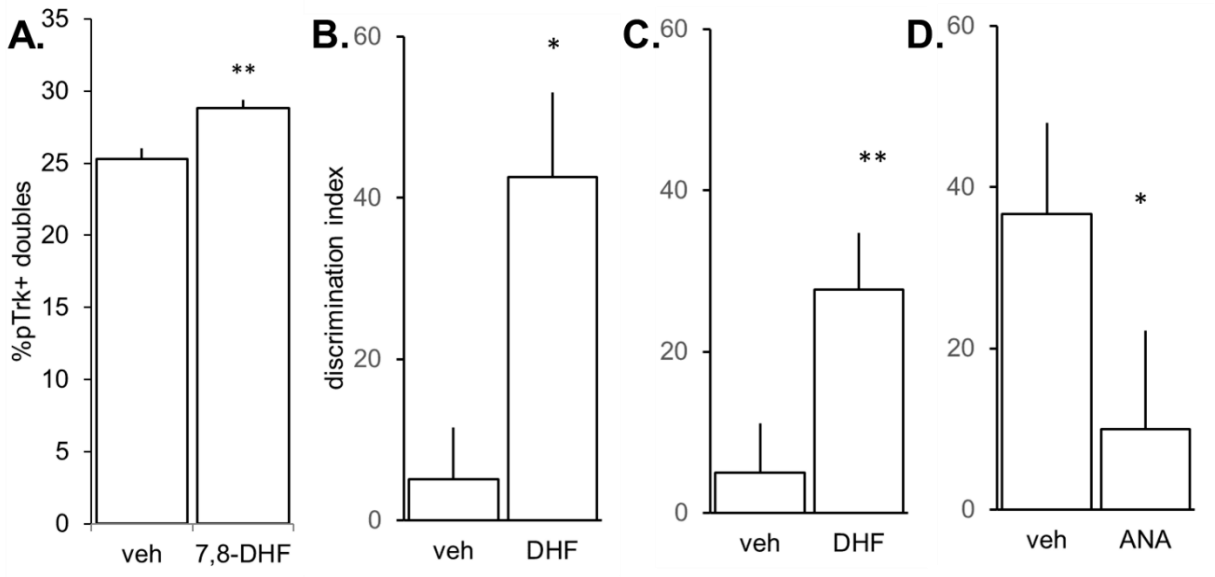

Figure 4.3 Enhanced TrkB signaling restores social recognition behavior in Fmr1-KO mice. Bars show the amount time the test mice spent with the novel as compared to the familiar mouse in social recognition behavior task. **A**, Chronic 7,8-DHF delivered via drinking water significantly increases synaptic levels of phosphorylated TrkB as indicated by the proportion of PSD-95 immunolabeled synapses that were also enriched in pTrkB Y515 (p=0.0023, n>9 per group). **B**, A single injection of 7,8-DHF restored social recognition in Fmr1-KOs. **C**, Providing mice 2 weeks of chronic 7,8-DHF treatment by inclusion in drinking water also restored social recognition in Fmr1-KO mice. **D**, Acute injection of the TrkB antagonist ANA-12 disrupts social recognition behavior and thus induces the Fmr1- KO behavioral phenotype in WT mice. *p<0.05, **p<0.01.

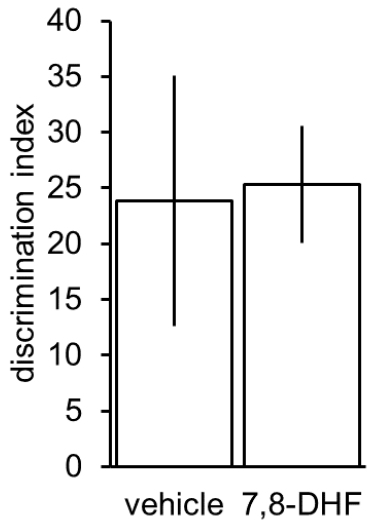

Figure 4.4 TrkB signaling modulates odor recognition behavior. Fmr1-KO mice were injected with either vehicle or the TrkB agonist 7,8-DHF one hour before exposure to two cups of soiled bedding. Plot shows discrimination index of each group when faced with bedding from familiar and novel cages; treatment had no effect (p=0.89, n = 6 vehicle, 11 DHF).

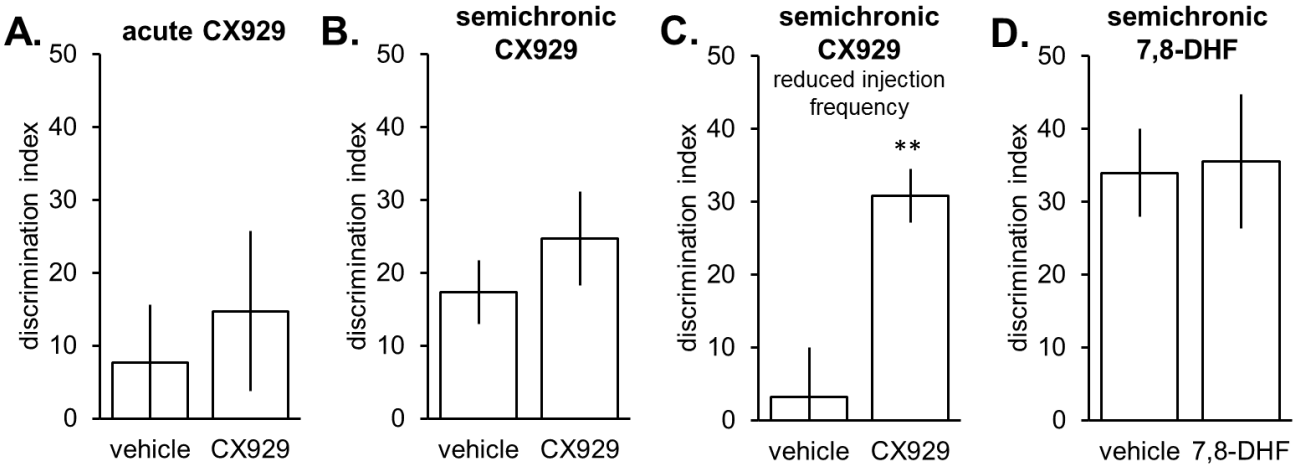

Figure 4.5 Handling frequency modulates social recognition behavior. Fmr1-KO mice were handled and injected with vehicle or drug once a day (**A,C**) or twice a day (**B,D**) for four days prior to social recognition testing. **A**, Acute injection with the positive AMPA receptor modulator CX929 had no significant effect on discrimination index (p=0.615, n=12 vehicle, 13 drug). **B**, Semichronic administration of CX929 resulted in significantly positive discrimination indices for both vehicle- and drug-treated groups (n=22 vehicle, 23 drug). **C**, Semichronic administration of CX929 that used a lower frequency of pre-testing injections produced a significant increase in discrimination index of drug-treated as compared to vehicle-treated mice (**p=0.00216, n=8 vehicle, 9 drug). **D**, Semichronic administration of the TrkB agonist 7,8-DHF resulted in high, positive discrimination indices for both treated and untreated mice (n=7 vehicle, 8 drug).

CHAPTER FIVE

Leveraging behavioral structure to predict learning

Summary

Introduction

The preceding chapters have concerned themselves with the search for an engram as may be represented at the level of circuit involvement (Chapters 1,2) and at the level of synaptic signaling (Chapter 3). Here we will pivot to consideration of an ethological engram. Specifically, we will attempt to describe, in mice, a pattern of behavioral sequences that distinguishes nonlearning from learning events.

Markovian analysis of sequences is useful for predicting stochastic processes such as weather and, in the present case, complex animal behaviors. Briefly, using this approach, all possible states are defined, and the probability of transitioning from one state to any other is arranged into a *transition matrix*. This matrix is then multiplied by itself until some future time is met. This entire set of possible states and transitions is finally compared to observed values (Tejada et al., 2010). Markov models assume stochasticity; that is, the current state has a set probability of transitioning to any other state regardless of past states. Of course, we know that complex behaviors depend largely upon past experience. Thus, Markov modeling is most useful as an ethological tool when we can quantify the deviation from the random, Markovian model itself. The following experiments will measure the *prediction error* of mouse behavioral patterns compared to a stochastic Markov model, to ask questions about whether the predictability of an animal's behavior is reflective of their cognitive processing.

Such ethological analysis is hardly a novel concept. In 1997, researchers applied Markovian analysis to mouse performance on the elevated plus maze, a common assay of anxiety (Espejo, 1997). Their analysis characterized specific sequences of behaviors that

were predictive of anxiety, and recommended that such in-depth analysis be used in future studies of anxiolytic drugs and manipulations. Likewise, here we will attempt to employ detailed sequential analysis of mouse behavioral patterns to distinguish between learning states. Specifically, we will compare learning to nonlearning states, normal learning to enhanced learning, and normal to congenitally impaired learning.

Materials and Methods

Animals. Adult (3-5 mo old) male Fmr1 KO mice on the FVB 129 background and age, sex and background-matched wildtypes (WTs), were used for all studies. The animals evaluated here included groups studied for other outcome measures in Chapters 2 and 4. Animals were standard group housed with littermates (2-5 mice per cage) in rooms maintained at 68 C and 55% humidity, on 12 hr on/12 hr off light cycle and with food and water ad libitum. Experiments were performed during the animal's light cycle (began at 6:30 am) and in accordance with NIH guidelines for the care and use of laboratory animals and protocols approved by the Institutional Animal Care and Use Committee at University of California, Irvine.

Object location memory task (OLM). Animals were handled for 2 min daily for 5 days and habituated to a white behavioral chamber (24 x 30 x 30 cm) containing sawdust bedding for 5 min daily for 5 days and then on the following day were given OLM training as described in the previous chapters. Long-term retention of object location was assessed 24 hr after training. During the 5 min retention trial, one beaker remained in the original training location and one was moved toward the center of the apparatus.

Novel object recognition task (NOR). For NOR retention testing, one beaker was replaced with a novel object and both objects remained in the same positions as in the training episode. The novel and familiar objects were counterbalanced across animals to ensure that the animals did not prefer one object over another.

Social recognition task (SRT). Social approach and social novelty tests were carried out as described previously (Nadler et al., 2004; Yang et al., 2011) and in **Chapter Four**.

In all behavioral tasks, animals were video recorded and scored offline by raters blind to genotype and experimental group. Mice were scored as interacting with an object when sniffing or nose touching within 0.5 cm of the object. Interaction was not scored when (a) the animal was within this radius but grooming or digging, or (b) did not show intent to interact (e.g., they fell within this zone when turning). Total exploration time was quantified as the time interacting with both objects. A discrimination index was also calculated as $(t_{novel}-t_{familiar})/(t_{novel}+t_{familiar})$, where "familiar" denotes the object that was unchanged from the training trial and "novel" refers to the object that was changed (either moved to a new location in OLM or swapped for a new mouse in SRT). Thus, a positive discrimination index represents a preference for the novel object.

Drug administration. The TrkB agonist, 7,8-dihydroxyflavone (7,8-DHF) was delivered as described in **Chapter Four**.

Markov sequence analyses for behavior. To analyze the sequences of movement decisions made by mice when exploring an environment, a Markov chain analysis was performed (Espejo, 1997). The goal was to determine (1) how well the short-term decisions mice

make during exploration predict longer movement sequences and (2) at what scale information was being added. Using Ethovision software, video recordings were processed to obtain trajectories of each animal's position at ~0.1 s intervals. For this analysis, the arena was divided into 9 locations as defined by a 3x3 grid; custom scripts in Python version 2.7 were used to chunk each animal's trajectory while in the arena into 2.5 s intervals, and the animal's position at the start and end of each time segment was determined. This information was used to create a matrix of transition probabilities between the 9 possible arena locations, and then a set of "ideal prediction matrices" was constructed for successive n and the original 2.5 s matrix was raised to the nth power; here n represents the number of positions predicted forward (e.g., n = 2 indicates the prediction of the animal's location using 5 s bins; n = 3 indicates the prediction on 7.5 s, etc.; (Walsh and Cummins, 1976)). Data were then split into the different bin sizes, and the corresponding empirical transition matrices were calculated per animal. The predicted position was subtracted from the actual position and squared to yield the error; this error was multiplied by the time at each location to correct for the influence of rare events. On any session day, if an animal's prediction error was >4 SDs from the group mean for >50% of the prediction steps, then the animal was dropped from analysis; this exclusion factor resulted in one to two animals per group being excluded per session.

The equation $\boldsymbol{Prediction\ Error = \Sigma(T1^n - T_n)^2 \times Proportion}$ shows the prediction error calculation where n is the number of steps forward predicted, T1 is the original prediction matrix, which is raised to the nth power (yielding the nth ideal prediction matrix), T_n is the

empirical prediction matrix for that n, and Proportion is the relative time spent at a given location.

Results

Spaced and massed training yield different exploration patterns during retention testing.

The first question we sought to answer was whether we could detect the effect of learning in a commonly-used test of spatial memory, the object location memory task (OLM). Mice were exposed to a pair of identical, novel objects for 3 or 5 minutes, then returned to their home cages overnight. The following day they were re-exposed to the same objects, but with one in a different location than before. "Learning", as established in numerous previous studies (Seese et al., 2014; Vogel-Ciernia and Wood, 2014), can be observed when a mouse spends more time examining the moved object upon re-exposure (expressed as discrimination index, DI). Moreover, 5 minutes of training is generally sufficient to elicit long-term memory, while 3 minutes is not (Stefanko et al., 2009). Accordingly, the mice first tested here showed predictable DIs – those that received 3 min of training expressed low DIs, while those receiving 5 min of training expressed high DIs (3 min DI: 2.8 ± 4.0, 5 min DI: 32.5 ± 9.5; p=0.0061, data not shown). Video recordings of these mice exploring the objects upon re-exposure ("testing") were subjected to Markov sequence analysis, yielding the plots of prediction error over time shown in **Figure 5.1**. Surprisingly, both groups of mice showed identical patterns of predictability, regardless of time bin (effect of training time: p=0.724, Two-Way ANOVA, n=6 per group); thus, the movement patterns did not reflect learning.

We next tested whether a different training paradigm would affect exploration patterns at retrieval. Spaced training is known to dramatically enhance long-term OLM (Seese et al., 2014), although the precise mechanism remains unclear; Markov analysis of

behavioral sequences may help shed light on the behavioral effects of spacing. Mice given a total of 3 min of training, distributed across three training trials each separated by one hour (Seese et al., 2014), demonstrated robust DIs at testing 24 hr later (DI: 20.3 ± 3.0, data not shown). Markov analysis revealed a significant shift in behavioral patterns in the 3 min spaced group as compared to the massed training groups described above (**Fig 5.1,** training x time bin interaction: p=0.0311, Two-Way ANOVA, n=6 per group).

Inactivation of orbital frontal cortex (OFC) alters patterns of exploratory behavior.

As indicated in previous chapters of this document, the OFC has been strongly implicated in mediating, or at least being critical for, the spaced training effect. Accordingly, we investigated whether Gi-DREADD-mediated inactivation of this cortical region impacts Markov sequences of mouse exploratory behavior during a variety of tasks. For mice given a single training trial (10 min long), both OFC-intact and OFC-inactivated groups explored the novel objects for equivalent amounts of time (saline: 9.97 s ± 0.90, CNO: 9.77 s ± 1.19; p=0.909, n=6 saline, 10 CNO). However, the *pattern* of this novel object exploration differed significantly, particularly in the 12-18 s time range (**Fig 5.2A**, training x time bin interaction: p<0.0001, F(29,348)=2.448, n=6 saline, 10 CNO). In contrast, during OLM retention testing for the same mice, the activity state of the OFC had no impact on exploratory patterns (**Fig 5.2B**, training x time bin interaction: p=0.3503, F(28,348)=1.087, n=6 saline, 10 CNO).

To further investigate the role of OFC in determining patterns of exploration of novelty, mice with Gi-DREADD injections into the OFC were allowed to explore a complex

novel arena for ten minutes a day, 5 days in a row. These mice were randomly assigned to receive the DREADD agonist CNO on either the first or last day to examine how OFC inactivation affects exploratory patterns of novel (first day) vs. familiar (last day) environments. Given the results described above (OFC inactivation altered exploratory patterns during OLM training but not testing) we expected altered movement patterns on the first but not last day of exploration; this was somewhat the case. On the first day of exploration, OFC-inactivated mice were slightly less random in their patterns of movement, particularly in the time bins between 6 and 18 s (**Fig 5.3A**, drug x time bin interaction: $p=0.1558$, $F(28,448)=1.281$). In contrast, on the last day of exploration, OFC inactivation had no effect on Markov sequences (**Fig 5.3B**, drug x time bin interaction: $p=0.6701$, $F(28,448)=0.8626$, n=8 per group).

Finally, we compared Markov sequences of OFC-intact vs. OFC-inactivated mice during each trial of spaced training. Animals were injected with either saline vehicle or the DREADD agonist CNO 30 min before the second training trial; we saw no impact of CNO on exploratory patterns in trial 1 (drug x time bin interaction: $p=0.4593$, $F(28,435)=1.006$) or trial 2 (**Fig 5.4**; drug x time bin interaction: $p=0.4685$, $F(28,435)=0.9994$). However, for trial 3 CNO-mediated inactivation of the OFC influenced behavior yielding a significant interaction effect between drug and time bin ($p<0.0001$, $F(28,435)=2.789$, n=6 saline, 10 CNO).

KO and WT mice express different exploratory patterns.

After determining that there was no learning-related shift in exploration in WT mice, we considered if there were distinct exploratory patterns in the Fmr1-KO mouse model of intellectual disability. As noted in Chapter 4, the Fmr1-KO mouse is used to model Fragile-X Syndrome, the most common form of inherited intellectual disability in humans. These mice express robust deficits in both object location and object recognition memory (Seese et al., 2014; Seese et al., *in preparation*). We evaluated movements during OLM training for WT and Fmr1-KO mice handled in parallel. The WT mice showed a large, significant shift in behavioral predictability from training to testing (**Fig 5.5A**, effect of task phase: $p<0.0001$, $F(1,406)=78.28$) across all time bins. The Fmr1-KOs, however, showed a much smaller (although still significant) shift in predictability (**Fig 5.5B**, effect of task phase: $p<0.0001$, $F(1,464)=73.60$). Notably, the WT mice expressed robust long-term memory and KOs did not ($p<0.001$, data shown in **Chapter 4**). To probe whether the shift in predictability of movement from training to testing was due to learning specifically, or alternatively to genotype, both genotypes were treated with chronic 7,8-DHF as described in Chapter 4. This treatment is known to improve LTP and learning in Fmr1-KOs (Lauterborn et al., 2007; Seese et al., unpublished). Indeed, in our hands the KO mice treated with 7,8-DHF expressed robust OLM discrimination indices comparable to those of WT mice ($p=0.358$, data not shown). However, 7,8-DHF treatment did not affect the shift in exploration patterns observed between training and test trials (**Fig 5.5D**).

To further characterize potential effects of genotype in the Fmr1-KO mice, we next analyzed behavioral sequences of WTs and KOs performing the three-chambered social approach task (SAT). In this task, mice are first exposed to a novel mouse and an inanimate object ("social approach"), then to that same mouse and a second novel mouse ("social

recognition"). As described extensively in Chapter 4 of this document, Fmr1-KO mice perform normally on the approach phase of the task but fail to discriminate a novel mouse in the recognition phase. When video recordings of these mice were subjected to Markov analysis, familiar patterns emerged: WT mice showed a significant shift in predictability from approach to recognition, while KO mice did not (**Fig 5.6A**, WT $p<0.0001$, $F(1,360)=26.27$; KO $p=0.1261$, $F(1,390)=2.350$). Recall that this is a similar result as that observed with object location memory training and testing. Additional groups of Fmr1-KO mice were treated with 7,8-DHF, which restored social recognition (see **Chapter Four**). This treatment induced a significant shift in predictability (**Fig 5.6B**, vehicle $p=0.9299$, $F(1,780)=0.007736$; 7,8-DHF $p<0.0001$, $F(1,780)=47.39$) which greatly resembled that evident in WT mice. We next asked if we could induce the KO social recognition phenotype in WT mice via treatment with the TrkB antagonist ANA-12. As described in Chapter 4, ANA-12 blocked social recognition in WT mice. Markov analysis for these groups yielded the data shown in **Figure 5.6C**. Despite the robust suppression of learning evident with ANA-12 treatment, there was no such effect on exploratory patterns during the period of analysis (vehicle $p<0.0001$, $F(1,348)=143.7$; ANA-12 $p<0.0001$, $F(1,348)=30.29$). The WT mice continued to express a robust predictability shift across task phases.

Discussion

The above results describe a novel analysis of rodent learning; behavioral sequencing, itself an established field, was applied to determine how various paradigms and genotypes affect behavior. A major question when beginning this study was whether we could use Markov analysis to distinguish between learning and nonlearning states. The results above indicate that, with the current analysis, this is not feasible. Or, said another way, learning did not influence the pattern of exploratory movements during the initial phases of the behavioral session. However, this analysis did distinguish between training schedules, and to a degree between exploration of novel vs. familiar arenas.

In general, across genotypes (WTs and Fmr1-KOs), conditions involving more novelty (e.g. OLM training) resulted in lower levels of prediction error than their corresponding low-novelty conditions (e.g. OLM testing). Markov chains in these experiments were constructed using the position, over time, of each animal in its behavioral arena; chains were then compared to a stochastic matrix. Conditions with more novelty likely drive stereotyped exploratory behaviors in mice, resulting in Markov plots with lower prediction error. In contrast, if animals were exposed to a relatively familiar arena, their exploration patterns would be more reflective of internal state, and thus more individualized, resulting in higher error. The one exception to this trend was Fmr1-KO mice that were given chronic 7,8-DHF. These animals, like their WT counterparts, significantly shifted their exploratory patterns between approach and recognition phases of the social task, but in the opposite direction. Further studies are needed to determine whether this is an effect specific to the drug or the mutant.

Inactivation of OFC lowered prediction error relative to that exhibited by vehicle-treated controls during both massed and spaced OLM training. As discussed extensively in **Chapter Two**, OFC is involved in behavioral planning and prediction. The present results support the notion that without OFC involvement, long, complex strings of behavior are difficult, as OFC-inactivated mice demonstrated reduced prediction error especially at later time points. Whether this effect is related to OFC's role in the spaced training effect remains to be seen.

The Fmr1-KO mouse is a commonly used model for Fragile X Syndrome and autism (see **Chapter Four**). Here we show that, in addition to robust learning impairments, KOs also express altered behavioral patterns as compared to WTs. In general this shift was expressed as a severely reduced gap between training and testing phases of each task. This could reflect a failure to adapt to changing task demands, or an altered sense of novelty. Or, it may be that training did not leave some effect of experience on behavior (independent of learning) in these mice as compared to the WTs. Notably, chronic treatment with 7,8-DHF restored both spatial and social learning to WT levels, but only altered exploratory patterns in the social task (and then in the opposite direction of the WT shift). The clear genotypic difference, and the sensitivity of KOs to 7,8-DHF treatment, suggest Markov analysis as a potential diagnostic tool in human populations: sequences of patient movements or verbal expressions could be used to detect autism and possibly other, more subtle, neurodevelopmental disorders. Indeed, some researchers have begun to model autism as, fundamentally, a disorder of prediction (Sinha et al., 2014). One utility of this approach could be to provide an index against which effects of therapeutics could be tested.

Acknowledgements

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Figures

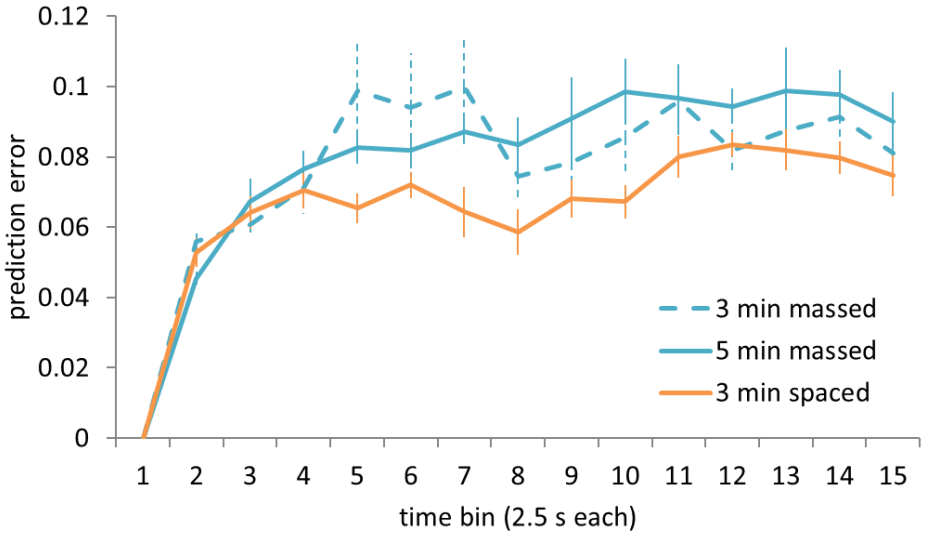

Figure 5.1 Behavioral patterns observed during object location memory (OLM) retention testing vary more with training schedule than with learning. As noted in the text, mice receiving 3 min spaced training learned in the OLM task. Markov plots of movement patterns for mice receiving spaced and massed OLM training show overlap in the prediction error for animals receiving massed training ($p=0.724$), whereas those receiving spaced training were significantly less predictable (i.e. there was lower prediction error; $p=0.0311$). Two-Way ANOVA, n=6 per group.

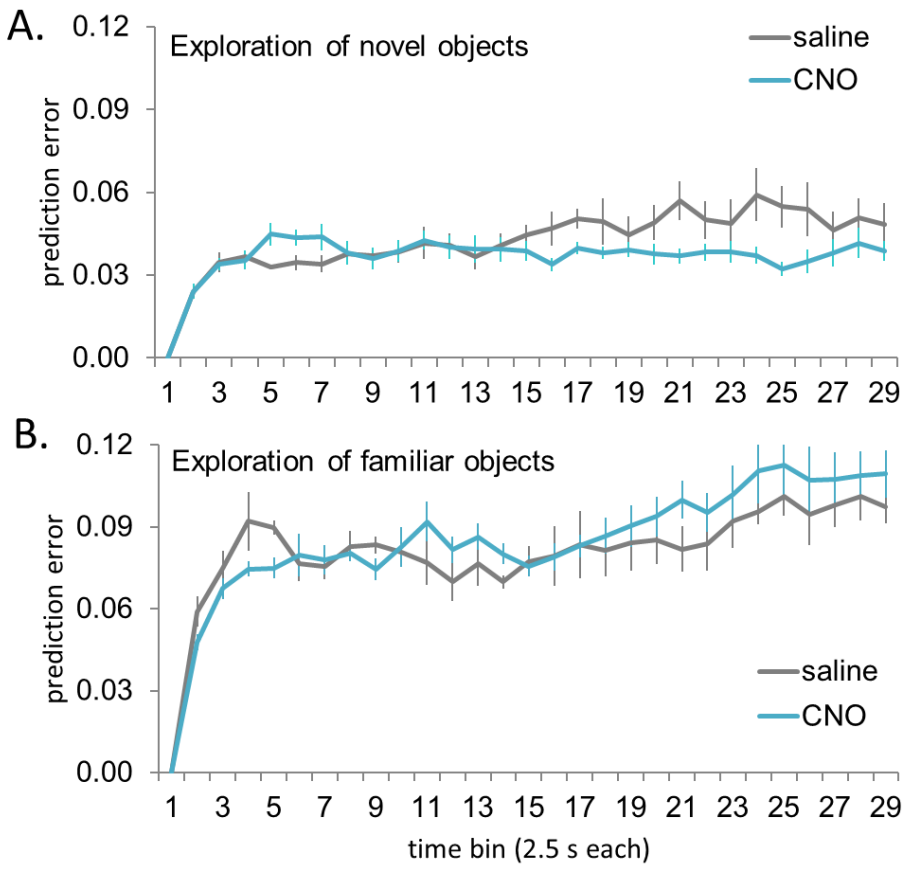

Figure 5.2 Orbital frontal cortex (OFC) inactivation alters Markov sequences during OLM training but not testing. Mice were injected bilaterally in OFC with an AAV-Gi-DREADD construct, then given either saline vehicle or DREADD agonist CNO 30 min before behavior. **A**, OFC inactivation during OLM training significantly altered behavioral predictability over time ($p<0.0001$). **B**, OFC inactivation during OLM retention testing had no effect on behavioral predictability ($p=0.3503$). Two-Way ANOVA, n=6 saline, 10 CNO.

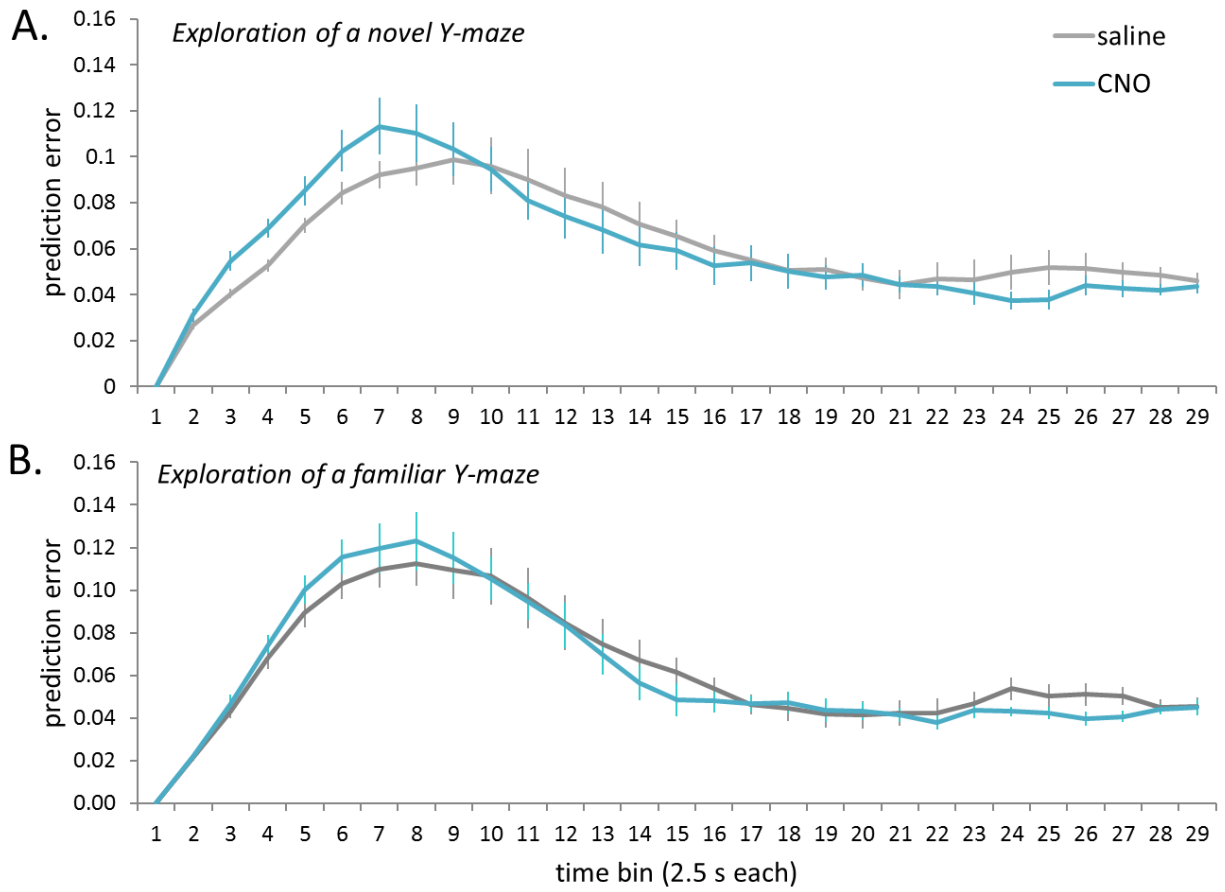

Figure 5.3 Orbital frontal cortex (OFC) function is more influential to exploration patterns in a novel than a familiar environment. Mice were infected with AAV-Gi-DREADD in the OFC bilaterally and later given either saline or DREADD agonist CNO 30 min before behavioral testing. **A**, During initial exposure to a novel arena, CNO-treated mice exhibited slightly altered behavioral patterns but the difference from the saline treatment group was not significant (p=0.1558). **B**, During exploration of the same arena after 4 days of habituation, CNO-treated mice were indistinguishable from saline-treated mice (p=0.6701). Two-Way ANOVA, n=8 per group.

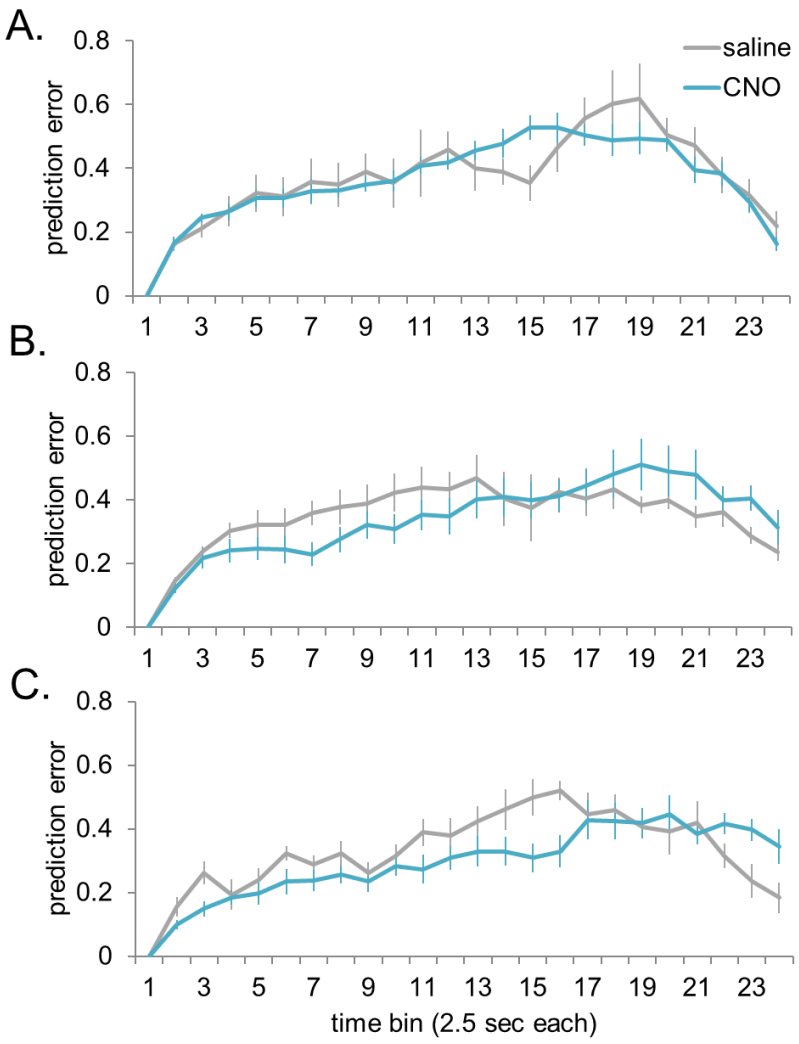

Figure 5.4 Orbital frontal cortex (OFC) inactivation affects exploration patterns in the third of 3 spaced training trials. Mice were infected bilaterally in OFC with an AAV-Gi-DREADD, then later given either saline or DREADD agonist CNO 30 min after the first training trial. Exploration patterns were monitored for the initial period of the three spaced training trials. The movement patterns did not vary between saline and CNO-treated mice in the first (**A**) or second (**B**) training trial (p=0.4593 and 0.4685, respectively). **C**, In contrast, CNO treatment significantly altered behavioral patterns in the third training trial (p<0.0001). Two-Way ANOVA, n=6 saline, 10 CNO.

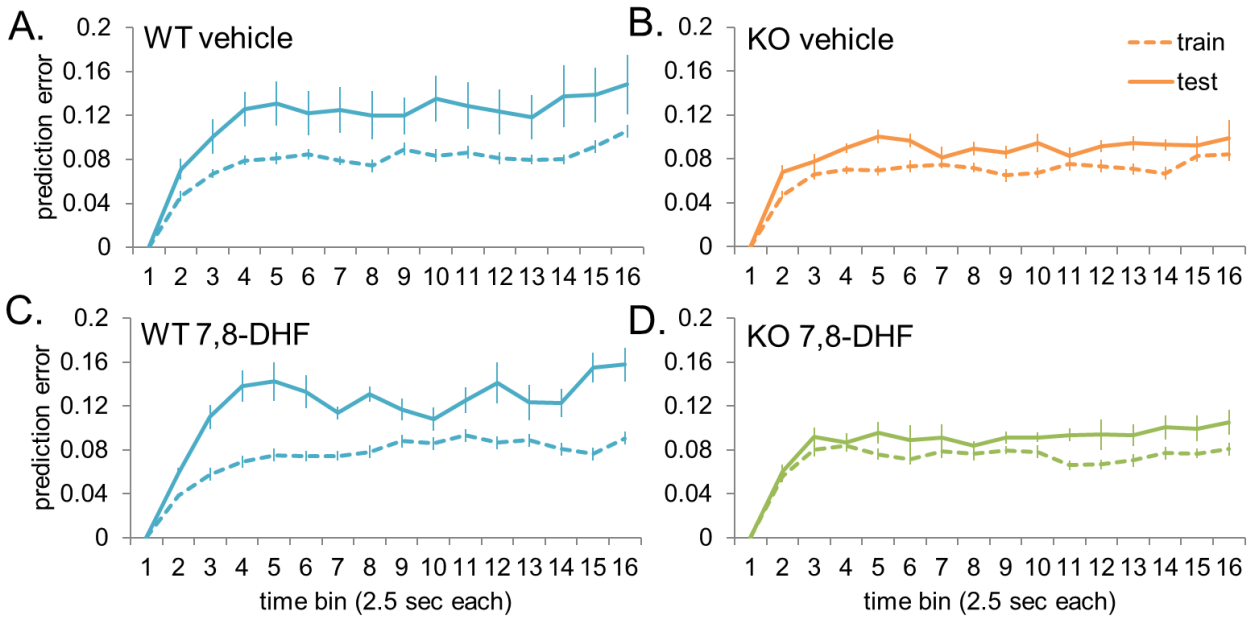

Figure 5.5 Behavioral patterns in the object location memory (OLM) task differ between wild type (WT) and Fmr1-KO mice. Mice from both genotypes were treated with saline or 7,8-DHF (groups from Chapter 4), trained for 5 min in the OLM task, and then tested for retention 24 hrs later. Movement patterns analyzed and plotted here are for both training (dashed lines) and testing (solid lines) phases. **A**, WT mice exhibited significantly different behavioral predictability between the training and testing phases of the task ($p<0.0001$). **B**, Fmr1-KO mice also exhibit significant predictability shift between task phases ($p<0.0001$), albeit of a smaller magnitude than for WTs. Chronic administration of 7,8-DHF did not significantly change WT (**C**) or KO (**D**) behavioral patterns thereby reinforcing the conclusion that the patterns do not reflect the presence of learning. Two-Way ANOVA, n=8 per group.

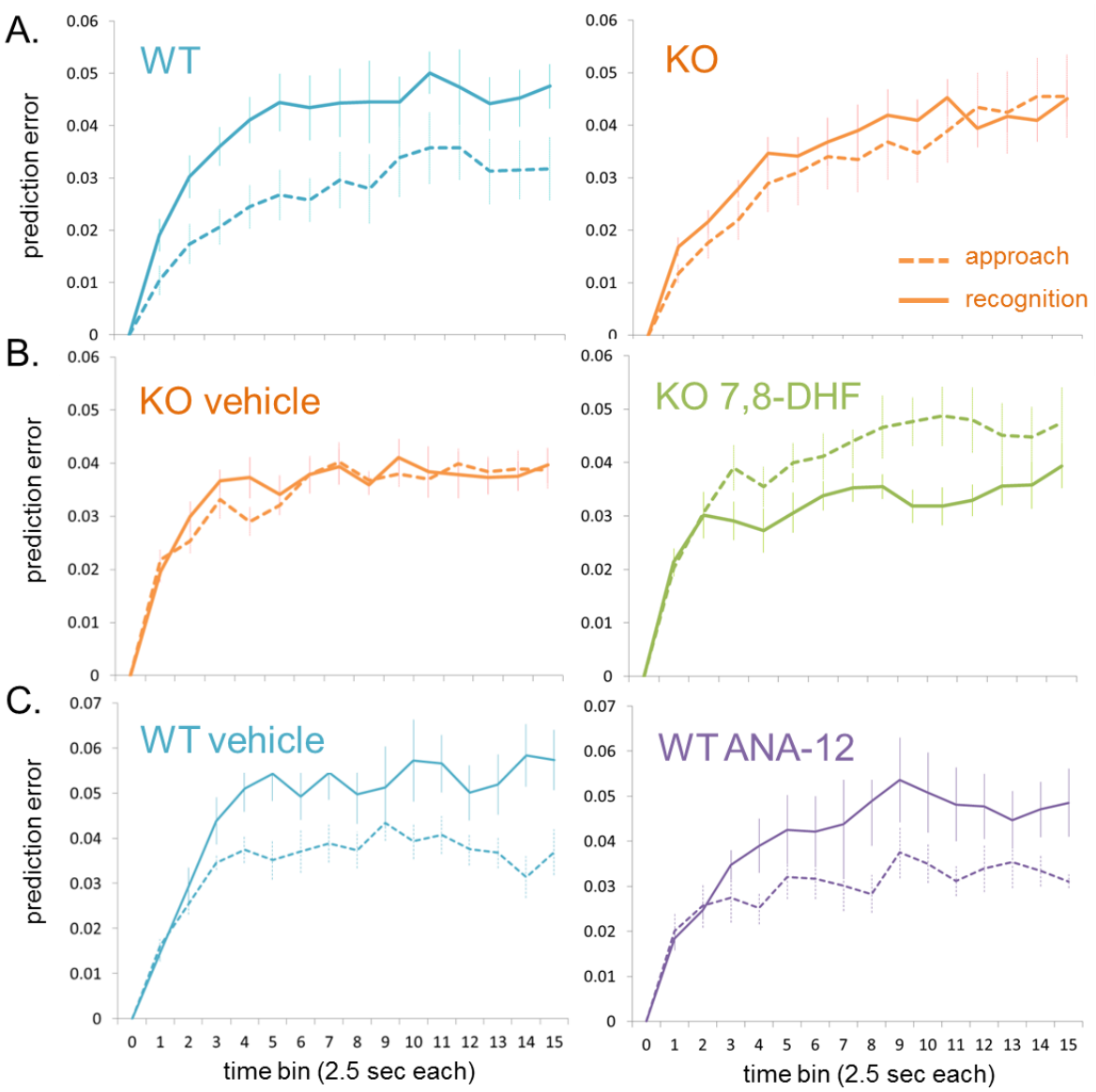

Figure 5.6 Behavioral patterns in the social approach task vary with genotype. Dashed lines represent social approach and solid lines represent social recognition phases of the task. **A,** WT mice shift behavioral patterns between phases of the task ($p<0.0001$, n=7), while KO mice do not ($p=0.1261$, n=14). **B,** Chronic 7,8-DHF induces a shift in behavioral patterns between phases of the task for KO mice (vehicle $p=0.9299$, DHF $p<0.0001$, n=13 per group). **C,** ANA-12 does not alter WT behavioral patterns relative to vehicle-treated controls. Two-Way ANOVA.

DISCUSSION

The aim of this dissertation was to investigate the mechanistic underpinnings of the memory enhancement gained through distributed, as compared to single trial, study. As discussed in the **Introduction**, psychologists have explained the spaced training effect in terms of encoding variability (Estes, 1955; Bower, 1972) and deficient processing (Rubin and Braun, 1998); the present results are more supportive of the latter. Neurobiologically, encoding variability effects would manifest, across training trials, as consistent patterns of activity in the same circuit. Within a single node of that circuit, activity patterns may expand with each repeated trial as marginally more information is added to the existing representation (see **Fig D.1A**). In contrast, and in line with data presented in **Chapters 1-3**, deficient processing would recruit auxiliary circuits, particularly noticeable when comparing the first trial to any later trial (Fig **D.1B**).

In accord with this prediction, Fos mapping after massed and spaced OLM training revealed a robust difference in network activity with each training paradigm (**Fig 1.3**) that was most pronounced after the second training trial (**Fig 1.4**). Notably, the region that exhibited the greatest training schedule-dependent variation was the orbitofrontal cortex (OFC), a frontal cortical field heretofore unassociated with object location learning. Experiments in **Chapters 2 and 5** further probe the role of OFC in the spacing effect via chemogenetic inactivation before OLM training. OFC activity was necessary for expression of long-term OLM with spaced, but not massed, training (**Fig 2.1**). In accord with deficient processing theory, the "auxiliary" OFC circuit preferentially modulated behavior on later training trials as compared to the first trial (**Fig 2.1D, Fig 5.4**), suggesting that it is critical to the "reminding" aspect of the theory. Indeed, previous work on OFC has defined its

importance in goal-directed behaviors (Gremel and Costa, 2013; Rudebeck and Murray, 2014), which necessarily involve memory (e.g. of the goal itself, paths to the goal, and rewards associated with the goal). We might therefore characterize the role of OFC in the spaced training effect as one of a "goal-orienter"; that is, it integrates past memories to inform future decisions. In the context of spaced OLM training, the OFC would not be necessary during the first training trial, when every feature is equally novel and worthy of exploration, but *would* be engaged during later trials when it benefits the animal to attend more to previously-unexplored aspects of the arena (see **Fig D.2**).

Certainly the OFC is not alone in mediating goal-directed behaviors. Indeed, it is known to interact closely with striatum (Gremel and Costa, 2013) and VTA (Takahashi et al., 2011) to calculate predicted outcomes and thus influence behavior. While our Fos analysis failed (for methodological reasons) to look at patterns of VTA activity, we did see a significant increase in striatal activity with spaced, as compared to massed, training (**Fig 1.3**). Another recent study identified a medial prefrontal – thalamic nucleus reuniens – hippocampal field CA1 circuit critical to goal-directed spatial navigation (Ito et al., 2015). As noted in **Chapters 1 and 2**, we saw no significant interaction between medial prefrontal cortex and spaced OLM learning. However, we did observe a spacing-related difference in nucleus reuniens (Re) activity (**Fig 1.3**) which may or may not be related to the spacing-related difference in OFC activity. Future studies will probe the involvement of striatum and nucleus reuniens to OFC's role in the spaced training effect.

Another major finding of this dissertation was that the spacing effect likely occurs via enhancement of systems consolidation of memory, rather than at the synaptic level. Synaptic studies presented in **Chapter Three** showed that, within the hippocampus,

learning engaged a global pattern of cytoskeletal remodeling. Similar distributions of phosphorylated (activated) signaling molecules were observed for both massed and spaced training. In contrast, the regional analysis of **Chapter One** revealed robust spacing-specific differences at the network level, especially among limbic and frontal cortical regions (**Fig 1.3**). These findings concur with other studies that demonstrate accelerated systems consolidation (i.e. transfer to cortex) with repetition (Tse et al., 2007; Lehmann et al., 2009).

Evidence in favor of systems-level consolidation of spaced learning does not rule out the possibility of synaptic-level changes. Previous work has clearly demonstrated synaptic effects of repetition (Kramar et al., 2012; Cao and Harris, 2012; Seese et al., 2014), and future work should examine whether these synaptic changes are driven by, or otherwise interact with, the network changes observed in the present studies. However, as the synaptic mapping results described in **Chapter Three** were largely inconclusive, a new approach will be necessary. As discussed above, and depicted in **Fig D.1A**, one model to explain the efficacy of spaced training predicts local expansion of representations. Kramar and colleagues reported just such an effect with spaced stimulation of *ex vivo* hippocampal slices: dendritic spines recruited by a second stimulation train were more often than not within a mere 5 μm of those recruited by the first stimulation train (Kramar et al., 2012). Such local "clustering" of activity would not have been discernable using the analysis of **Chapter Three**, but may be worthy of future study. A particularly informative study might use a chemogenetic approach to selectively modulate OFC-hippocampal projections, then assess clustering of synaptic activity in hippocampus after massed vs. spaced training. In

this way it would be possible to examine both systems / synaptic interactions and to further probe the role of OFC in the spacing effect.

In a study of human memory, spaced training for word pairs preferentially affected relational rather than item memory (Lisman and Davachi, 2008). This latter point strongly suggests that spacing operates on the hippocampus rather than on cortex (Hanslmayr et al., 2016; Shimamura et al., 2009) and aligns with other studies linking repetition to preferential encoding of gist over detail (Kornell and Bjork, 2008; Reagh and Yassa, 2014).

Several questions remain to be resolved. In particular, in contrast to Melton's early work (1970) demonstrating in human subjects that longer ITI gave rise to a longer-lasting memory trace, Seese et al., (2014) showed in mice that there was a very discrete ITI window that enhanced long-term memory. This may be because of fundamental differences in cognition and / or consolidation between mice and humans, it may be due to the nature of the tasks tested in each case, or it may be that the mouse studies didn't test retention at the necessary time points. Whatever the reason, it is critical to address this discrepancy so as to ensure generalizability of these and future findings on the mechanisms of spaced training.

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