Molecular Mechanisms of Memory Formation: Using Activity Regulated Genes to Identify Active Neural Circuits

By

Kartik Ramamoorthi

Submitted to the Department of Brain and Cognitive Sciences
in partial fulfillment of the requirements for the degree of

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Signature of Author: _____

Department of Brain and Cognitive Sciences

October 28 2013

Certified by: _____

Yingxi Lin

Assistant Professor, Department of Brain and Cognitive Sciences

Thesis Supervisor

Accepted by: _____

Matthew Wilson
Sherman Fairchild Professor of Neuroscience
Director of Graduate Education for Brain and Cognitive Sciences Program

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A fundamental goal in neuroscience is to understand mechanisms underlying the ability to create memories from sensory experience. While large structures such as the hippocampus are known to be critical for certain types of learning, memories are ultimately thought to be represented in sparsely distributed neuronal ensembles within these larger structures. Currently, there are few tools that allow for the identification and manipulation of these ensembles, which has limited our understanding of the molecular and cellular processes underlying learning and memory. We have previously reported that the activity-regulated transcription factor Npas4 is selectively induced in a sparse population of CA3 following contextual fear conditioning. Global knockout or selective deletion of Npas4 in CA3 both resulted in impaired contextual memory, and restoration of Npas4 in CA3 was sufficient to reverse the deficit in global knockout mice. Taking advantage of the critical role of Npas4 in contextual memory formation, we developed a set of novel molecular tools to gain access to cell populations activated by experience. Using this system, we identified and manipulated the properties of neurons activated by behavioral experience in a variety of neural circuits in mice, rats, and *Drosophila*. We believe that the tools developed in this thesis can provide a major advancement in the field, and will allow researchers to target any neural circuit activated by experience in a variety of species.

Thesis Supervisor: Yingxi Lin
Title: Assistant Professor, Department of Brain and Cognitive Sciences

Dedication

This thesis is dedicated to my scientific parents, Yingxi Lin and Tim Otto. Tim, you introduced me to the world of science and how to tackle interesting scientific problems. Yingxi, you taught me what it takes to be a rigorous scientist and made me the researcher I am today.

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List of Abbreviations

4AP	4-aminopridine
AAV	Adeno associated virus
AP	Action potential
AP5	2-amino-5-phosphonopropionic acid
Arc	Activity-regulated cytoskeleton-associated protein
BDNF	Brain Derived Neurotrophic Factor
bHLH	Basic helix-loop-helix
Bic	Bicuculline
C	Context Exposure
c-Fos	Proto-oncogene
CA1	Cornu Ammonis Area 1
CA2	Cornu Ammonis Area 2
CA3	Cornu Ammonis Area 3
CaMKII	Ca2+/calmodulin-dependent protein kinase II
catFISH	Cellular compartment analysis of temporal activity by fluorescence in situ hybridization
CFC	Contextual Fear Conditioning
ChIP	Chromatin Immunoprecipitation
ChR2	Channelrhodopsin-2
CMV	Cytomegalovirus promoter
CNO	Clozapine-N-oxide
Cre	Cre-recombinase
CREB	cAMP response element-binding protein
d2mCh	Destabilized mCherry
DAPI	4',6-diamidino-2-phenylindole
DD	Destabilized domain
DG	Dentate Gyrus
DH	Dorsal Hippocampus
DHFR	Dihydrofolate reductase
DIV	Days in vitro
Dox	Doxycycline
DPI	Days post injection
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
EC	Entorhinal Cortex
EGFP	Enhanced Green Fluorescent Protein
EPSC	Excitatory postsynaptic current
GCAMP	Genetically Encoded Calcium Indicator
GFAP	Glial fibrillary acidic protein
GFP	Green Fluorescent Protein
hM4Di	Inactivation DREADD

IEG	Immediate Early Gene
IHC	Immunohistochemistry
IPSC	Inhibitory postsynaptic current
KA	Kainic Acid
KO	Knockout
LSL	Lox stop lox
LTP	Long-term potentiation
Luc	Luciferase
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
mCherry	Monomeric red fluorescent protein
MCS	Multiple Cloning Site
MEF2	Myocyte enhancer factor-2
MEK	MAPK kinase
MODC	Mouse ornithine decarboxylase
MTL	Medial Temporal Lobe
MWM	Morris Water Maze
NMDA	N-methyl-D-aspartate
Npas4	Neuronal PAS domain protein 4
NR1	N-methyl-D-aspartate Receptor Subunit 1
pA	Poly A
PKC	Protein kinase C
POI	Protein of interest
Ptz	Metrazole
RAM	Robust Activity Monitor
S	Shock-Only
Seq	Sequencing
tdTom	tdTomato Red Fluorescent Protein
TetxLC	Tetanus Toxin Light Chain
TMP	Trimethoprim
TRE	Tetracycline response element
tTA	Tetracycline-controlled Trans-Activator protein
VH	Ventral Hippocampus
WT	Wildtype

Chapter 1

Introduction

The work in this thesis describes our characterization of a novel transcriptional program required for contextual memory formation in the CA3 region of hippocampus. Using the findings from this study as a foundation, we developed a novel molecular reporter system to gain access to neurons expressing Npas4 and other activity regulated genes following behavioral experiences. We characterize the development of this tool and its application *in vitro* and *in vivo* in mouse, rat, and *Drosophila*.

1.1 Organization

Chapter 1: Introduces the thesis

Chapter 2: Provides a description of the learning and memory field from a historical perspective including a discussion of model systems from humans to invertebrates. We integrate these findings with the molecular mechanisms of neural plasticity and memory formation. We end with a discussion of activity-regulated genes and how their properties can be harnessed to identify active neural circuits.

Chapter 3: Describes the identification of a novel transcriptional program in the CA3 region of hippocampus required for contextual memory formation. Through a combination of genetic knockouts and region specific overexpression we define Npas4 as a critical mediator of memory consolidation.

Chapter 4: Describes a framework of how one can take advantage of genetic programs activated by experience. We employ this approach to create an AAV-based tool to

identify and manipulate active neural circuits. We characterize this system in the context of our findings described in Chapter 3 and employ its use in a variety of mouse, rat, and *Drosophila* neural circuits.

Chapter 5: Summarize the work presented in the thesis and discusses future experiments that can be readily employed based on the findings and developed technologies.

Chapter 6: References

Appendix A: Describes the role of the GABAergic system on neurodevelopmental disorders, a review article written with my advisor Yingxi Lin.

Chapter 2

Background

2.1 Memories and Experience

People often think of memory as our ability to recall information, both factual and episodic. However, the importance of our memories goes far beyond simple information recall. Learned knowledge is used to extrapolate into the future, not simply a recapitulation of past experience. In this way, our experience and memories shape our actions, motivations, and behaviors in adaptive ways. While memories are fundamental to how we perceive and behave in the world, the neural substrate of memory and learning, remains poorly understood.

2.1.1 Defining Learning & Memory

To experimentally dissect these processes it is imperative to operationally define the parameters at hand. While often considered a semantic issue, learning, memory, and recall are fundamentally different processes with unique biological underpinnings. Colloquially, these terms are used interchangeably, however it is critical to disambiguate them in order to study them rigorously. For the purposes of this thesis, we define *learning* as the acquisition of a novel behavioral response to a stimulus, *memory* as the storage of learned information, and *recall* as the retrieval and subsequent behavioral expression of a learned behavior (Kandel, 2001).

2.1.2 Categories of learning and memory

To further define learning and memory in the context of human behavior, Larry Squire, Howard Eichenbaum, and Eric Kandel have developed a framework to categorize different types of memory (Eichenbaum, 2003; Kandel and Squire, 2000).

Human memory can be subdivided into two main categories: declarative (explicit) and non-declarative (implicit). Declarative memory refers to information that can be consciously recalled (Clark and Squire, 2013), such as facts or events. The medial temporal lobe (MTL) is believed to be the storage site for declarative memories. Non-declarative memories include processes such as procedural learning (skills/habits), priming, simple classical conditioning, and non-associative learning. A variety of brain regions are involved with these processes, including the striatum, cortex, and the cerebellum (Sweatt, 2003).

David Sweatt further breaks down learning and memory into unconscious and conscious categories. Behavioral paradigms such as contextual fear conditioning, trace fear conditioning, and conditioned place aversion are unconsciously learned, but consciously recalled. Classic Pavlovian conditioning and cued fear conditioning are thought to be unconsciously learned and recalled, while declarative learning and spatial learning are thought to be consciously learned and recalled (Sweatt, 2003).

It is important to note that all forms of memory are subject to different phases, short-term and long-term memory. Each phase is mediated by distinct biological processes, but the phases are ultimately dependent on each other for permanent storage of information (Kandel, 2001).

2.2 Model Systems to Study Learning and Memory

The learning and memory field takes advantage of a variety of model systems. The major systems include humans, primates, rodents (rats and mice), and invertebrates (*Aplysia* and *Drosophila*). Each system has distinct advantages, but

integration of theories and findings across these systems has greatly advanced our understanding of the mechanisms of memory. Additionally progress in genetic and functional imaging techniques now allow for real-time imaging of circuits (both invasive and non invasive) during behavioral tasks.

2.2.1 Humans

The study of learning and memory in humans has largely focused on patients with severe medical disorders that required the removal of brain regions. Initial pioneering work revealed that, depending on the location of the lesion, unique behavioral deficits could be observed. Through systematic analysis of memory performance in hippocampal and medial temporal lobe (MTL) lesion patients, Brenda Milner and William Scoville were able the establish the importance of this circuit to learning and memory (Scoville and Milner, 1957). Examination of these patients revealed that lesions of the MTL, both unilateral and bilateral, resulted in memory deficits with severity consistent with the size of the lesion. Of the patients in the initial study, the most popular and well-characterized was patient H.M.. H.M. lacked the ability to form new declarative memories and lacked memories for events that occurred within sixteen years of the surgical operation (Sagar et al., 1985). However, H.M. had intact memories for experiences and events from his distant past, including his childhood. These observations were some of the first data suggesting that newly formed episodic memories are transiently stored in the hippocampus and surrounding cortical areas and subsequently transferred to other brain regions for long-term storage. Further analysis of H.M. revealed several cognitive impairments that were consistent with failures in the

MTL recognition system. For example, H.M was able to detect odors and their intensity, but he was unable to identify specific odors or discriminate between them (Eichenbaum, 2013; Eichenbaum et al., 1983). Extensive work in rodent models has shown that hippocampal lesions recapitulate H.M.'s inability to discriminate between odors (Eichenbaum et al., 1987; Otto and Eichenbaum, 1992; Young et al., 1997). Interestingly, H.M. was able to acquire trace conditioning despite the generalized acceptance that this form of conditioning is highly dependent on the hippocampus (Bangasser et al., 2006; Woodruff-Pak, 1993). Further examination of the study revealed that H.M. was initially trained in a delay conditioning protocol. Employing a similar training paradigm to rats removes the necessity of the hippocampus (Bangasser et al., 2006) . These observations highlight the importance of multiple model systems to understanding the mechanisms of memory formation.

2.2.2 Commonly-Used Behavioral Paradigms to Study Learning & Memory in Rodents

Given the huge variety in types of learning and memory, it is critical to choose a behavioral paradigm that is specific and appropriate for the biological process of interest. Currently, there are several commonly-used behavioral paradigms to study the biological basis for memory formation. The selection of a specific behavioral paradigm is largely a practical decision that is dependent upon the complexity of the behavior, the circuits activated by the behavior, and the memory process required for the behavior.

Of the various paradigms used to explore the neural substrates of learning and memory, Pavlovian fear conditioning has been critical to our understanding of behaviorally-relevant brain circuits. In this paradigm, a neutral conditioned stimulus (CS)

is paired with an aversive unconditioned stimulus (US). After repeated pairings, the CS comes to elicit responses originally evoked only by the US, including changes in heart rate, respiration, and a robust "freezing" response characterized by the lack of movement other than those required for respiration (Fanselow, 1980). The emergence of these responses, to the previously neutral CS, is thought to reflect a learned association between the CS and US. Various manipulations in the timing and characteristics of the CS and US have led to the formation of a number of Pavlovian fear conditioning paradigms, the most common of which are contextual conditioning, delay conditioning, and trace conditioning. In both delay conditioning and trace conditioning, an explicit CS (e.g. a tone) precedes the US (e.g. a shock). The important difference between these two paradigms is that in delay conditioning the onset of the US occurs during the CS, followed by CS and US co-terminating. In trace conditioning, the offset of the CS and the onset of the US are separated by the trace interval, during which no significant event occurs.

Contextual fear conditioning (CFC) differs from both delay and trace conditioning in that the US is delivered in the absence of an explicit cued CS. In this paradigm, an aversive footshock is delivered in the presence of a novel context. This form of aversive learning is very popular, as an association between the footshock and the context can last for the lifetime of the animal and can be formed within one training session. The hippocampus is thought to acquire and consolidate a representation of the context, based on its known role in spatial learning (Anagnostaras et al., 2001).

With respect to the brain areas underlying these forms of associative learning, there is now widespread agreement that acquisition, retention, and expression of

Pavlovian fear conditioning, as well as generalized fear, are critically dependent on the amygdala (Kim and Fanselow, 1992; LeDoux, 2003, 2007; Maren et al., 1996a; Maren and Fanselow, 1996; Phillips and LeDoux, 1992). Manipulations of the hippocampus have revealed that it too plays a vital role in several subtypes of Pavlovian fear conditioning. Specifically, damage to or temporary inactivation of the hippocampus leads to dramatic impairments in trace fear conditioning (Chowdhury et al., 2005; McEchron et al., 1998; Quinn et al., 2005; Yoon and Otto, 2007), trace eye blink conditioning (Weiss et al., 1996), and contextual fear conditioning (Kim et al., 1993; Phillips and LeDoux, 1992). However, hippocampal manipulations generally seem to have a minimal effect on delay conditioning (Phillips and LeDoux, 1992; Quinn et al., 2008), suggesting that in contextual and trace conditioning, the hippocampus may participate in the maintenance of a memory for context (Rudy and O'Reilly, 2001; Solomon et al., 1986).

Outside of associative learning, the Morris water maze (MWM) has been used extensively to study spatial learning (Morris et al., 1982). In this task, rodents are trained to locate a submerged platform in a large pool of water. In order to escape the water, rodents learn the location of the platform over the course of several sessions using a variety of spatial cues in the room. Learning is measured by quantifying the path length (distance traveled to reach the platform) and time to platform. Spatial memory performance is measured by removing the platform and quantifying percentage of time spent in the location of the platform. Acquisition and recall of MWM are both highly sensitive to manipulations of the hippocampus (lesions, pharmacological, and genetic) and other components of the medial temporal lobe (Neves et al., 2008).

Novel object recognition (NOR) relies on neural circuitry that is distinct from but overlaps with the circuit involved in Pavlovian conditioning. During the training phase rodents are presented with two identical objects. Due to the natural exploratory behavior of rodents, both objects are explored. In a subsequent testing phase, generally twenty-four hours after training, one of the original objects is replaced with a novel object. Time spent exploring each object is quantified and preference for the novel object reflects an intact memory for the objects presented during the training phase. Several forebrain-specific gene manipulations and lesion studies suggest that this task is sensitive to perturbations of the hippocampus or surrounding cortical areas such as the perirhinal and postrhinal cortices (Bevins and Besheer, 2006; Piterkin et al., 2008; Stefanko et al., 2009; Wood et al., 2006), though the absolute contribution of the hippocampus to this task is still unknown. Both CFC and NOR are attractive paradigms because they require only a single trial rather than extensive training.

2.3 Memory Circuits

While the hippocampus is only one of the numerous structures in the mammalian brain, its study has been essential to the advancement of various neural sciences. Grounded in the observations that humans with lesions in the MTL suffer from severe memory deficits (*2.2.1*), detailed analysis of memory circuits in rodents has identified functional dissociations across multiple memory systems. Analysis of the hippocampus has revealed an overwhelming amount of valuable information ranging from electrophysiological phenomena, such as long-term potentiation (LTP) (Bliss and Lomo, 1970), to the recent implantation of false memories in the adult brain (Liu et al., 2012;

Ramirez et al., 2013). While the specific role of the hippocampus in learning has yet to be determined, converging electrophysiological, neuropsychological, and genetic evidence suggests that the hippocampus plays a particularly prominent role in memory. In addition, accumulating evidence suggests that several psychological, emotional, and neurological disorders reflect a hippocampal- and amygdala-dependent learned emotional response to the environment. Therefore, examining the role of the hippocampus in emotional learning paradigms is fundamental to uncovering the neural substrates of memory, as well its associated disorders.

The neuronal organization and structure of the hippocampus reflects many of the functional qualities associated with the region. Located in the temporal lobe, the hippocampus is generally divided along its two major axes. Along its transverse axis, the major subdivisions of the hippocampus are the CA1 field, CA3 field, and dentate gyrus (DG) (Anderson et al., 1971). The transverse cut also reveals the trisynapitc circuit, which is composed of the perforant pathway, mossy fiber system, and Schaffer Collateral system (Fig 2-1) (Amaral and Witter, 1989; Witter, 2007). Along its sepotemporal axis the hippocampus is divided in dorsal and ventral subregions (Andersen, 2007).

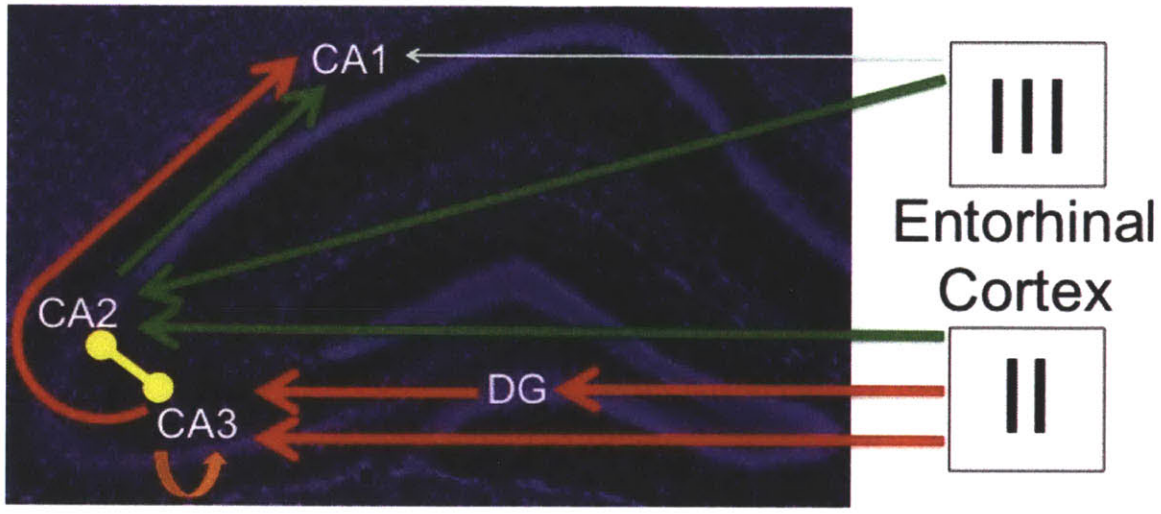

Figure 2-1. Schematic of entorhinal-hippocampal connections. Red lines: Trisynaptic pathway: ECII → DG/CA3 → CA1. Orange line: Recurrent CA3 connections. Green lines: CA2 pathway: ECII/III → CA2 → CA1. Light blue line: TA pathway: ECIII → CA1. Thick lines: dense projections. Thin line: light projections. Yellow line: inhibitory connections.

2.3.1 The CA1 Region

The CA1 region of the hippocampus is one of the most heavily-studied regions of the mammalian central nervous system largely due to its robust synaptic responses and ability to preserve major inputs to this region. CA1 neurons receive two major inputs: 1) CA3 neurons through the Shaffer Collateral pathway (Huerta et al., 2000; McHugh et al., 1996; Tsien et al., 1996) and 2) layer three neurons in the entorhinal cortex through the temporoammonic pathway (Fig 2-2) (Suh et al., 2011). Each pathway exhibits different synaptic properties, which are thought to reflect unique types of information being held by each input stream. Several theorists have proposed that CA1 may function as a "mismatch comparator", integrating input from the current environment from CA3 and stored environmental information from entorhinal cortex (Kesner et al., 2004; Suh et al., 2011). Processed information in CA1 is then passed to the neocortex through the subiculum (Andersen, 2007).

Genetic techniques restricting manipulations in a cell-type and region-specific manner have helped further determine the function of CA1 in learning and memory. Using a cross between a CA1-specific Cre mouse line (CamKII-Cre) and a loxp flanked NMDA receptor (NR1) mouse line, the Tonegawa laboratory was able to create a CA1 specific knockout of NMDA receptor function (McHugh et al., 1996; Tsien et al., 1996). Extensive work on this mouse has revealed that ablation of the NR1 subunit effectively abolishes plasticity in the CA1 region of hippocampus. Behaviorally, this mouse line exhibits impairments in the acquisition and recall of MWM-based memory (McHugh et al., 1996; Tsien et al., 1996), *2.2.2*), as well as odor discrimination (Rondi-Reig et al., 2001), consistent with a deficit in episodic memory. Using a behavioral paradigm to

separate the temporal and spatial aspects of episodic memory, Place and colleagues were able to determine that CA1 selectively contributes to the spatial component of episodic memories (Place et al., 2012). Consistent with this observation, place cell formation was degraded in the CA1 knockouts, indicating that the spatial component of the environmental representation requires CA1 plasticity (McHugh et al., 1996).

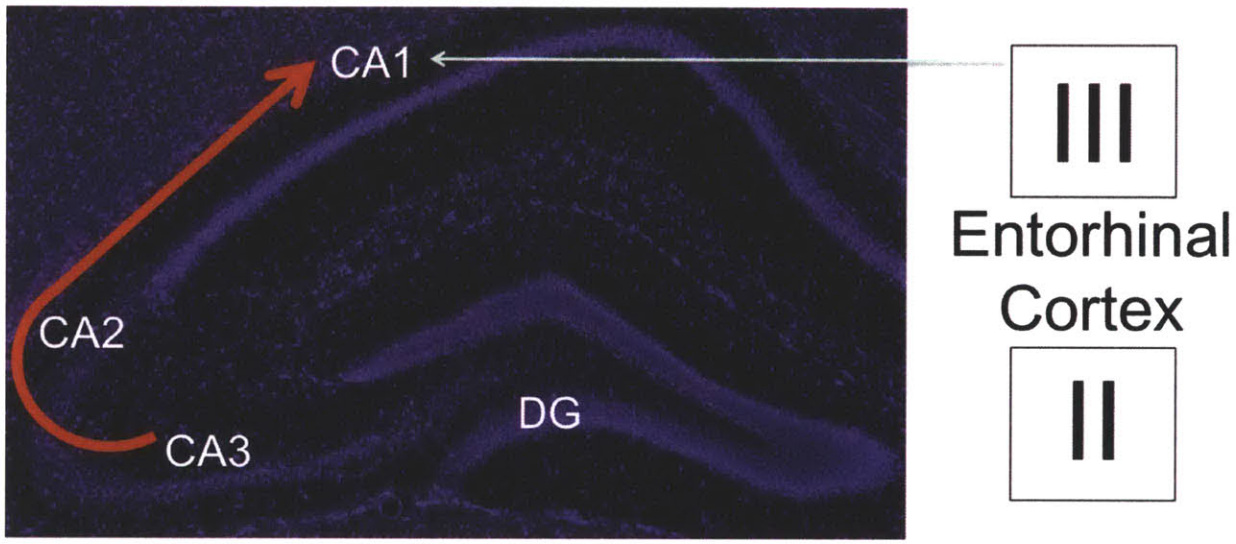

Figure 2-2. Schematic of CA1 inputs. Red line: Trisynaptic pathway: CA3 → CA1. Light blue line: TA pathway: ECIII → CA1. Thick lines: dense projections. Thin line: light projections.

2.3.2 The CA3 Region

Within the MTL, the CA3 region of hippocampus receives input from dentate gyrus (DG), entorhinal cortex, and CA3 itself. Much attention has been paid to the recurrent connections within CA3, as it is the only circuit within hippocampus to exhibit recurrent properties (Fig 2-3) (Andersen, 2007). Each input to CA3 exhibits unique forms of plasticity, which are thought to reflect the types of information being processed by the region. Several region- and pathway-specific manipulations of CA3 have begun identifying specific behavioral functions of this loci within the hippocampal circuit.

To selectively disrupt recurrent collateral (RC) activity, Nakazawa and colleagues knocked out the NR1 subunit in CA3. This effectively disrupted RC plasticity, while keeping other forms of plasticity within CA3 intact. In comparison to control mice, the CA3-NR1 knockouts were unable to use partial spatial cues to navigate the Morris water maze. However, when presented with the entire set of spatial cues, these mice performed at control levels. Consistent with this behavioral deficit, place cell recording of CA1 neurons exhibited disrupted specificity during partial cue presentation, but not during full cue presentation (Nakazawa et al., 2002; Nakazawa et al., 2003). Based on these observations and computational models of recurrent collaterals (Marr, 1971; Rolls, 1996), it is thought that CA3 plays a critical role in pattern completion.

Rapid one-trial learning is thought to rely heavily on pattern-completion strategies (Rolls, 2007). NMDA receptor antagonism of CA3, but not CA1 or DG, results in impairments in a delayed non-matching-to-place task (DNMP), where environmental contexts were rapidly changed (Lee and Kesner, 2002, 2003). Physiologically, *in vivo* recordings show that CA3 place fields can rapidly shift (Lee et al., 2004), an NMDA-

dependent plastic process in which the size of the place fields change when presented with a new context (Ekstrom et al., 2001). While this effect happens within one day in CA3 it takes multiple days in CA1, consistent with the requirement of CA3 for rapid learning (Lee et al., 2004).

Similarly, CA3 function is required for contextual fear conditioning *(2.2.2)*. Rats with CA3-specific lesions exhibit impairments in CFC memory recall (Lee and Kesner, 2004), while suppression of synaptic transmission from CA3 to CA1 impairs the encoding and recall of contextual fear conditioning (McHugh and Tonegawa, 2009; Nakashiba et al., 2009; Nakashiba et al., 2008). Spatial tuning of CA1 place cells, as well as ripple-associated reactivation of CA1 ripples, were impaired in the absence of CA3 synaptic output. These physiological alterations may provide a mechanism by which CA3 contributes to learning and memory.

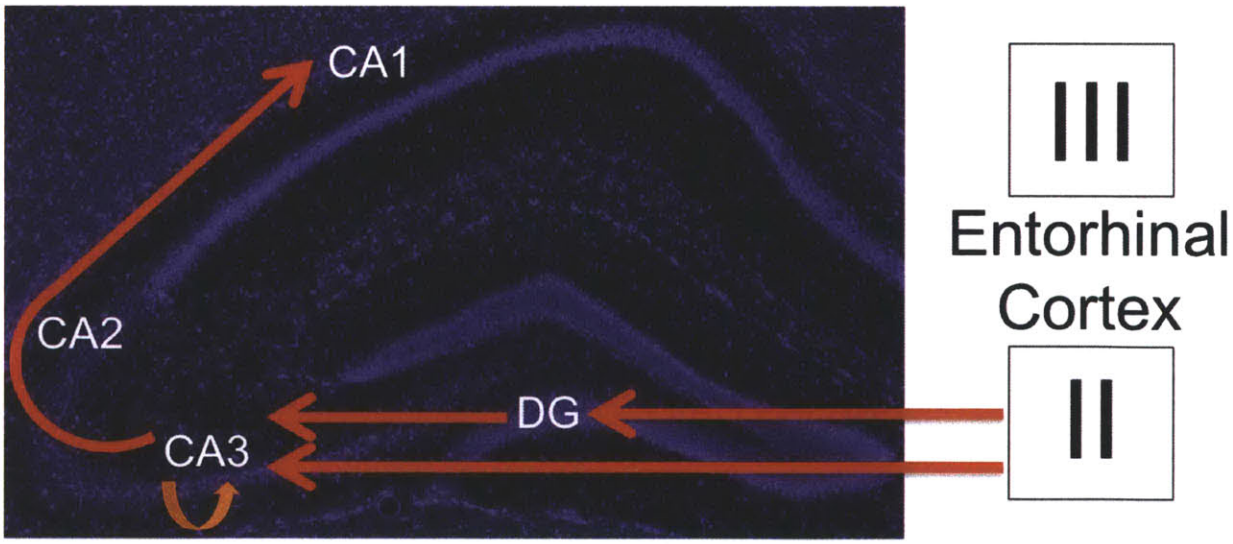

Figure 2-3. Schematic of CA3 connections. Red lines: Trisynaptic pathway: ECII → DG/CA3 → CA1. Orange line: Recurrent CA3 connections. Thick lines: dense projections. Thin line: light projections. Yellow line: inhibitory connections.

2.3.3 Dentate Gyrus

DG granule cells project to CA3 through the mossy fiber system. The synapses from this pathway terminate on the soma of CA3 neurons where they form large synapses that can reliably trigger CA3 activity (Fig 2-4) (Andersen, 2007). Activity within DG is thought to be relatively sparse, therefore creating a situation where sparse amounts of processed information are reliably transferred to CA3 (Andersen, 2007). As the number of granule cells is far greater than the number of neurons in CA3, several computational models suggest that DG may play a particularly important role in pattern separation, the ability to disambiguate overlapping cues (Rolls, 1996; Wallenstein et al., 1998).

To directly test the role of DG granule cells in pattern separation, the Tonegawa laboratory generated a mouse line in which the NR1 subunit of the NMDA receptor was selectively knocked out in DG granule cells. Using a sophisticated CFC paradigm in which one context predicted safety and another predicted shock this study was able to test the role of DG in pattern seperation. The two environments were highly similar; therefore appropriate freezing would require intact pattern separation. DG-NR1 mutants froze at equivalent levels in both the safe and unsafe context, indicating a requirement for DG plasticity in pattern separation. In contrast, control animals were able to discriminate between environments (McHugh et al., 2007). Place cell recordings in CA3 has revealed that DG-NR1 mutants exhibit impaired spatial remapping to novel environments, providing a physiological mechanism for DG to support pattern separation. Consistent with the genetic manipulations of DG, lesions of the region in rats have produced a variety of deficits in spatial pattern separation including location-

based maze navigation (Gilbert et al., 1998; Gilbert et al., 2001), radial arm maze (McLamb et al., 1988; Walsh et al., 1986), and Morris water maze (Costa et al., 2005; Hernandez-Rabaza et al., 2007; Xavier et al., 1999)

Surprisingly, blockade of synaptic transmission from mature DG granule cells, while leaving transmission of young adult-born granule cells intact, leads to an enhancement in pattern separation. The authors hypothesize that this is a consequence of a competitive process between pattern separation and pattern completion (Nakashiba et al., 2012). In support of this theory, genetically enhancing the survival of adult-born neurons in dentate gyrus results in enhanced pattern separation (Sahay et al., 2011a; Sahay et al., 2011b). These studies highlight the importance of adult-born neurons in dentate gyrus to cognitive function, though determining the function of this cell population will require further investigation.

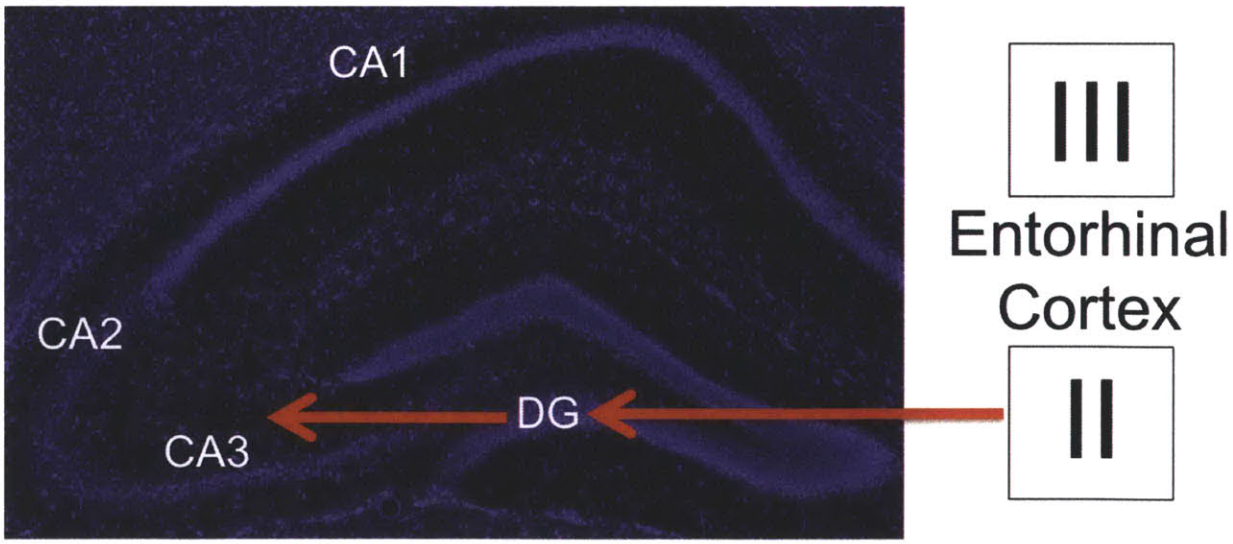

Figure 2-4. Schematic of DG connections. Red lines: Trisynaptic pathway: ECII → DG/CA3. Thick lines: dense projections. Thin line: light projections. Yellow line: inhibitory connections.

2.3.4 CA2

The majority of work studying the functional role of hippocampal subregions has focused on dentate gyrus, areas CA3, and CA1. Few studies have examined the contribution of CA2, though recent anatomical and electrophysiogical examinations of the CA2 subfield have identified several unique properties of the region (Fig 2-5) (Caruana et al., 2012; Chevaleyre and Siegelbaum, 2010; Jones and McHugh, 2011).

CA2 was originally defined as a transition region between CA3 and CA1 that lacked recurrent connectivity (Jones and McHugh, 2011). Current work has revealed that CA2 neurons are anatomically and genetically distinct from neurons in CA1, CA3, and DG. CA2 is the only region receiving strong and convergent excitatory input from both layer III and layer II of the medial and lateral EC, which are thought to participate in spatial and non-spatial information processing, respectively (Chevaleyre and Siegelbaum, 2010; Hargreaves et al., 2005; Yoganarasimha et al., 2011). CA2 output to CA1 is strongly excitatory, while CA3 input to CA2 is largely inhibitory (Chevaleyre and Siegelbaum, 2010). This pattern of connectivity positions CA2 as a critical integrator of spatial and non-spatial information from EC, independent of the trisynaptic and temporoammonic (TA) pathways (Andersen, 2007). Recent observations have shown that transgenic mice with either suppressed synaptic transmission from ECIII or deletion of genes restricted to CA2 are able to acquire simple contextual associations, but not able to form associations between spatial and non-spatial or temporally discontiguous stimuli (DeVito et al., 2009; Lee et al., 2010; Suh et al., 2011).

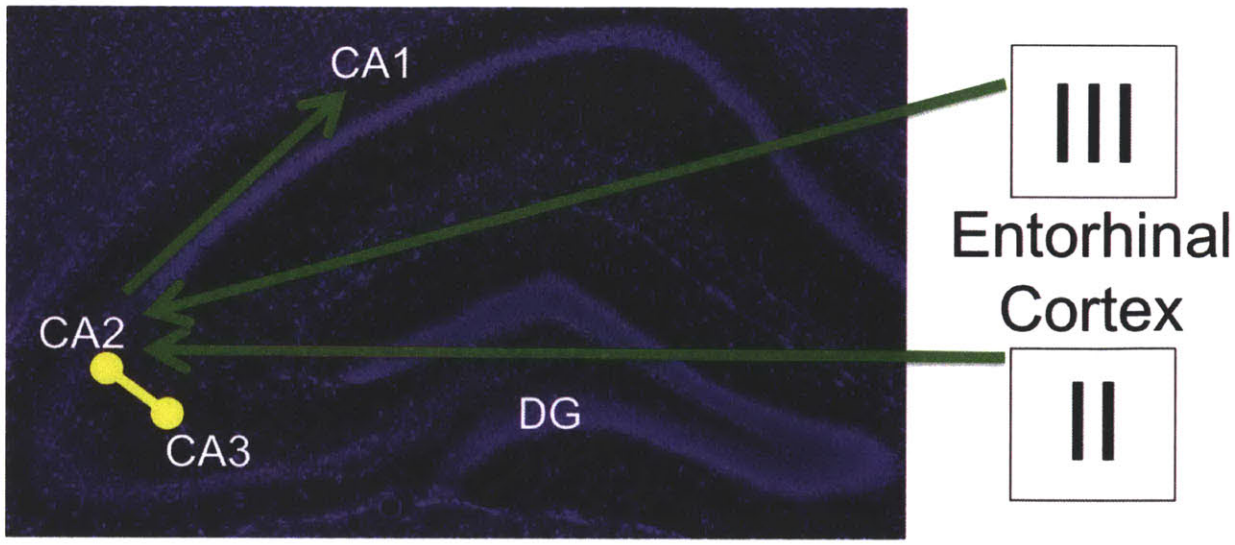

Figure 2-5. Schematic of CA2 connections. Red lines: Green lines: CA2 pathway: ECII/III → CA2 → CA1. Thick lines: dense projections. Thin line: light projections. Yellow line: inhibitory connections.

2.3.5 Dorsal and Ventral Hippocampus

Along its septotemporal axis the hippocampus can be divided into dorsal hippocampus (DH), composed of the septal two-thirds, and ventral hippocampus (VH), consisting of the remaining one-third (Moser and Moser, 1998; Richmond et al., 1999). Anatomically, DH and VH differ in their connections to other brain regions. Both dorsal and ventral hippocampus receive inputs from entorhinal cortex (EC), however the DH receives projections from the lateral and caudomedial portion of EC, while the VH receives projections from the rostromedial portion of EC. This difference in connectivity results in DH being the primary hippocampal subregion receiving visual, auditory and somatosensory information (Dolorfo et al., 1998a,b; Witter et al., 1989; Sahay and Hen, 2007). VH connects to the basal, accessory basal, and amygdalohippocampal transition area of the amygdala through CA1, while DH connects to amygdala only though VH (Pitkanen et al., 2000). Specifically, the anatomical differences between these regions suggest that DH may be more involved with roles traditionally assigned to the hippocampus, such as spatial learning, while VH may be more involved in fear and anxiety related learning, such as fear conditioning (Felix-Ortiz et al., 2013).

In addition to the anatomical studies, several groups have dissected the hippocampus at a molecular level, reporting distinct patterns of mRNA and protein expression in DH and VH of naïve animals. Genome wide microarray analyses have shown different genes and differing patterns of gene expression in these subregions (Leonardo et al., 2006). Additionally, *in situ* hybridization and western blotting have revealed differential protein expression of NMDA and AMPA receptor subunits in DH and VH (Pandis et al., 2006). With respect to experience-dependent gene expression,

to date, few studies have looked at differences between DH and VH during fear conditioning. However, numerous studies have found differential expression profiles in these subregions after spatial learning (Gusev et al., 2005), as well as in the whole hippocampus after a variety of learning paradigms (Donahue et al., 2002; Hess et al., 1995a, b; Huff et al., 2006; Runyan et al., 2004).

2.4 Molecular Mechanisms of Memory Formation

The integration of experimental work from a variety of model systems with basic learning theory from psychology has lead to a generalized model for the molecular mechanisms of memory formation.

2.4.1 Acquisition

Theories on the acquisition of learned associations are largely influenced by Hebbian plasticity, in which co-incident activity in connected neurons results in a strengthening of synaptic activity. (Sejnowski, 1999). With respect to associative fear learning, Hebbian theory suggests that weak inputs carrying contextual information (sounds, odors, visual cues etc.) can be associated with strong inputs carrying aversive information (i.e. shock) if the inputs are temporally overlapping or contiguous (Kandel, 2001; Sah et al., 2008). On a molecular level, learning and initial acquisition are thought to engage protein trafficking and post-translational modification of existing proteins, the trigger for these processes being calcium influx through the NMDA receptor and voltage-gated calcium channels (Andersen, 2007; Kandel, 2001).

2.4.1.1 NMDAR-dependent plasticity

The NMDA receptor plays a central role in almost all forms of synaptic plasticity characterized (Andersen, 2007). These receptors are thought to act as coincidence detectors on the postsynaptic neuron and trigger calcium influx upon opening. Calcium influx through the NMDA receptor is thought to result in post-translational modifications of regulators such as CREB, CAMKII, PKA, and MAPK (Abel et al., 1997; Dash et al., 1990; Dash et al., 1991; Kandel, 2001; Silva et al., 1992a; Silva et al., 1992b; Sweatt, 2003).

Pharmacological antagonists to perturb the function of the NMDA receptor (AP5 or MK-801) can effectively block memory acquisition in a variety of behavioral paradigms, as well as suppress synaptic plasticity (Kim et al., 1991; Morris, 1989). Pre-training infusion of APV into the hippocampus impairs acquisition of the Morris water maze and prevents *in vivo* long-term potentiation, consistent with the finding that lesions of the hippocampus impair performance in the water maze. The concentration of AP5 required to suppress long-term potentiation was sufficient to impair learning suggesting the recruitment of NMDAR-dependent plasticity during memory formation (Morris, 1989; Morris et al., 1982). Consistent with these observations, pre-training AP5 administration leads to impairments in contextual fear conditioning (Kim et al., 1991), trace fear conditioning (Czerniawski et al., 2012), and delay fear conditioning (Gewirtz and Davis, 1997; Maren, 1996; Maren et al., 1996b). Genetic manipulations of NMDA receptor function support these observations, but lack the temporal specificity to disambiguate effects on acquisition from effects on consolidation (Nakazawa et al., 2002; Nakazawa et al., 2003; Tsien et al., 1996). Importantly, pre-training delivery of AP5 blocks the

learning-induced expression of genes such as *Arc*, providing a molecular mechanism by which NMDA receptor antagonism impairs memory acquisition (Czerniawski et al., 2011). Activity regulated gene programs triggered by learning-related neural plasticity will be discussed in more detail below.

2.4.1.2 CaMKII

Following NMDA receptor activation, calcium influx results in the phosphorylation of Calcium-/Calmodulin-dependent protein kinase II (CaMKII) (Fox et al., 1996; Silva et al., 1992a; Silva et al., 1992b). CaMKII is a critical regulator of several signaling pathways and calcium homeostasis. The ability for CaMKII to auto-phosphorylate is thought to play a particularly important role in the persistent storage of memories (Irvine et al., 2006).

CaMKII has been show to be phosphorylated by fear conditioning at the autophosphorylation site Thr268. Pharmacological antagonism of CaMKII, using KN-62, impairs the acquisition of Pavlovian fear conditioning, as well as NMDA receptor-dependent plasticity (Rodrigues et al., 2004). Genetic manipulations of CaMKII, ranging from global knockouts to point mutations in Thr268, consistently result in robust memory impairments and spatial representations (Buard et al., 2010; Cho et al., 1998; Fox et al., 1996; Giese et al., 1998; Glazewski et al., 1996; Hinds et al., 1998; Irvine et al., 2006; Mayford et al., 1996; Rotenberg et al., 1996; Silva et al., 1992b; Taha et al., 2002).

2.4.2 Consolidation

Consolidation is the process by which initially acquired experiences are stabilized and stored for long-term memory (Kandel, 2001). While studies of the molecular mechanisms of memory acquisition are generally focused on post-translational modifications of existing proteins, memory consolidation requires the *de novo* synthesis of mRNA and protein from sets of key transcriptional regulators (Alberini, 2009). Initial work from Flexner and colleagues established that inhibiting protein synthesis prevented long-term memory formation (Flexner et al., 1963; Flexner et al., 1962; Flexner et al., 1964). Detailed analysis of the neural structures involved in learning and memory has established that all forms of long-term memory, long-term plasticity, and long-term spatial representations require protein synthesis (Andersen, 2007; Kandel, 2001; Sweatt, 2003). Using advanced pharmacological and genetic techniques, specific kinase pathways and transcription factors have been manipulated to further dissect the molecular mechanisms of memory consolidation.

2.4.2.1 Protein Kinase A (PKA)

PKA is the major target of cyclic adenosine 3',5'monophosphate (cAMP) and functions as a critical regulator of intracellular signaling pathways. PKA is thought to phosphorylate proteins such as CREB and CaMKII and can play a direct role in RNA synthesis (Johansen et al., 2011). Initial work examining the contribution of PKA to memory revealed that genetic deletion of the PKA gene or expression of an inhibitor form of PKA dramatically impaired Schafer collateral to CA1 long term potentiation, as well as various forms of hippocampal-dependent behavioral tasks including Morris water maze and contextual fear conditioning (Abel et al., 1997; Wu et al., 1995). Interestingly,

expression of inhibitory PKA had no effect on amygdala-dependent Pavlovian conditioning (Abel et al., 1997). However, pharmacological approaches in the amygdala have shown a critical role for PKA and its molecular substrates for the consolidation of fear memories (Goosens et al., 2000; Schafe et al., 1999; Selcher et al., 2002).

2.4.2.2 Protein Kinase C (PKC)

Similar to PKA, PKC is considered a major component of a signaling pathway mediating neuronal function. PKC is activated by increased levels of intracellular calcium originating from NMDA receptors and voltage-gated calcium channels. Following phosphorylation, PKC is able to contribute to gene expression through signal cascade regulation (Alberini, 2009; Johansen et al., 2011; Ventura and Maioli, 2001). PKC activity and subcellular localization is highly correlated with learning-related neuronal activity (Olds et al., 1989). Similar to PKA, pharmacological or genetic manipulation of PKC results in severe consolidation deficits across several species (Goosens et al., 2000; Roberson et al., 1999; Sossin et al., 1994).

2.4.2.3 Activity-Regulated Genes

The major downstream consequence of kinase pathways such as PKA and PKC is the activation of gene expression through transcriptional regulators such as CREB, SRF, and MEF2 (Flavell and Greenberg, 2008; Kandel, 2001; Knoll and Nordheim, 2009; Sweatt, 2003; Yin and Tully, 1996). In turn, these transcriptional regulators lead to the expression of a gene program that is ultimately thought support long-term changes in synaptic and circuit function (Flavell and Greenberg, 2008; Leslie and Nedivi, 2011;

Loebrich and Nedivi, 2009). Therefore, activity-regulated gene expression potentially represents a molecular readout of circuits undergoing plastic modifications.

CREB

The ability for cAMP to initiate signaling cascades triggered by extracellular cues was initially characterized using hormonal stimulation. Upon activation, cAMP is able to phosphorylate factors such as cAMP responsive element binding protein (CREB), and subsequently trigger gene transcription, providing a mechanism for extracellular cues to initiate intracellular molecular processes (Alberini, 2009; Montminy, 1997; Montminy and Bilezikjian, 1987; Montminy et al., 1990).

CREB is part of a family of transcription factors that contains a basic lucine zipper domain (bZIP), which can interact with other transcriptional regulators to form dimers and ultimately bind DNA. The transactivation domain of CREB is located in the N-terminus and contains a kinase inducible domain (KID) which regulates CREB's interactions with partners such as p300 and CBP. The activity-dependent nature of CREB function is regulated by a KID domain which is only activated when phosphorylated at Ser-133, a cAMP site (Alberini, 2009; Chrivia et al., 1993). Therefore, upon neuronal activity, cAMP is able to phosphorylate Ser-133 on CREB, allowing for CREB to interact with p300 and CBP at promoter regions of CREB gene targets.

CREB plays numerous roles in neuronal function such as development, survival, axonal outgrowth, neurogenesis, and memory (Carlezon et al., 2005; Dragunow, 2004). Due to the plethora of activities that require CREB, it has been difficult to separate its role in memory formation from other cellular functions. However, based on observation

from numerous species, neural circuits, and targeting approaches it is clear that CREB is a central mediator of synaptic plasticity and long-term memory formation (Alberini, 2009; Kandel, 2001).

Initial work identifying the importance of CREB in long-term plasticity and memory came from *Aplysia* and *Drosophila.* Delivery of CREB binding element (CRE) oligonucleotides into *Aplysia* sensory neurons prevented long-term facilitation in response to serotonin pulses, but left short-term facilitation intact. This was a result of the CRE oligonucleotides outcompeting endogenous gene target for CREB binding (Dash et al., 1990). Similarly, a *Drosophila* line expressing a dominate-negative form of CREB was unable to form long-term memories (Yin et al., 1994).

CREB knockout mice exhibit a variety of deficits in both long-term plasticity and memory formation (Bourtchuladze et al., 1994). Several next-generation approaches to altering CREB function, including inducible and reversible perturbation of CREB activity, also illustrate the importance of CREB to long-term memory formation, though the extent of the deficits are less robust than global knockouts (Kida et al., 2002; Pittenger et al., 2002). These observations, in combination with RNAi and antisense-oligodeoxynucleotide approaches in rats have firmly established CREB as a central regulator of long-term memory formation (Guzowski and McGaugh, 1997; Taubenfeld et al., 1999).

Arc

Arc is an attractive marker for neuronal activity because of the dynamics of its expression and regulation. Immediate early genes (IEGs), such as *Arc*, are a class of

genes that are rapidly expressed after various cellular and neural stimuli. IEGs can be divided into two major functional classes, effector IEGs (*Arc*, BDNF, Homer 1a, and others) and transcription factor IEGs (ICER, c-fos, zif268, and others), but see (Korb et al., 2013). Effector IEGs directly alter cellular function, while transcription factor IEGs regulate the expression of genes that eventually alter cellular function. While much of the early work using IEGs to map brain region activation was performed using c-fos and zif286 (Herrera and Robertson, 1996; Hess et al., 1995a, b; Okuno et al., 1995), *Arc* has recently gained a great deal of interest due to its cellular and molecular properties.

After synaptic stimulation, *Arc* expression is highly upregulated and product mRNA is specifically targeted to active dendrites (Plath et al., 2006). Translation of localized *Arc* is regulated by a number of factors highly correlated with long-term memory formation, including NMDA receptor activation, BDNF, and the mTOR pathway (Steward and Worley, 2002; Yin et al., 2002). Additionally, *Arc* is a component of both the postsynaptic density (Lyford et al., 1995) as well as NMDA receptors (Steward and Worley, 2002). Studies using primary neurons have identified a role for *Arc* in cytoskeletal destabilization, through microtubule associated protein 2, suggesting a possible function in dendritic remodeling (Fujimoto et al., 2004). Furthermore, through interactions with dynamin and endophilin, *Arc* can mediated endocytosis of AMPA receptors (Shepherd et al., 2006), providing evidence that *Arc* expression may modulate synaptic strength (Shepherd et al., 2006). Finally, in cultured neuroblastoma cells, *Arc* can interact with calcium cadmoldulin kinase II (CaMKII) to induce neurite outgrowth (Donai et al., 2003). Collectively, these *in vitro* studies indicate that *Arc* is either

dependent on or can modulate various mechanisms and pathways at least speculatively involved with learning and memory.

In addition to synaptic stimulation, *Arc* mRNA and protein expression is highly upregulated in response to novel environmental experiences (Guzowski et al., 1999) and training (Guzowski et al., 2000; Guzowski et al., 2001a; Huff et al., 2006; Plath et al., 2006). Furthermore, behavioral and electrophysiological experiments have identified *Arc* as a critical component of the maintenance of long-term memory and long-term potentiation. Behaviorally, forebrain-specific knockout mice lacking *Arc* display no deficits in the memory acquisition in a variety of learning paradigms such as Morris water maze, contextual fear conditioning, cued fear conditioning, conditioned taste aversion, and novel object exploration. However, the consolidation and maintenance of these tasks are severely impaired, suggesting that *Arc* is a necessary component of long-term memory formation (Plath et al., 2006). The same study reported that LTP was significantly altered in *Arc* knockout mice. Specifically, early phase LTP was enhanced, but rapidly terminated, while late phase LTP was completely abolished (Plath et al., 2006). The use of antisense oligodeoxynucleotides, directed towards hippocampal *Arc*, has revealed that *Arc* is vital for the long-term maintenance of a spatial version of the Morris water maze as well as the maintenance of hippocampal long-term potentiation (Guzowski et al., 2000). Importantly, using fornix lesions to impair hippocampal spatial learning, but keeping neural activity intact, it was shown that *Arc* expression is not merely induced by transient neuronal activity, but is representative of learning-associated synaptic plasticity (Fletcher et al., 2007).

c-Fos

c-Fos is a proto-oncogene found in a variety of cell types that forms hetrodimers with c-Jun family transcriptional regulators. Together, this heterodimer forms the AP-1 complex to promote gene expression (Alberini, 2009). The expression of *c-Fos* is upregulated in the presence of a variety of extracellular stimuli and was originally identified by serum stimulation of fibroblasts (Greenberg and Ziff, 1984; Sheng et al., 1990). The neuronal expression of *c-Fos* is dependent on CREB phosphorylation (Greenberg and Ziff, 1984; Sheng and Greenberg, 1990; Sheng et al., 1990).

c-Fos has been extensively used as a molecular marker of neural activity as it is reliably induced by stimuli such as LTP, seizures, sensory, and injury (Curran et al., 1996; Curran et al., 1985; Curran and Morgan, 1985, 1987, 1995; Morgan et al., 1987). Taking advantage of this pattern of expression, several groups have used *c-Fos* to identify neural circuits activated by behavioral experience, and levels of *c-Fos* have been correlated to memory performance (Garner et al., 2012; Guzowski et al., 2001b; Lin et al., 2011). Advanced *in situ* hybridization techniques, such as cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH), have taken advantage of the temporal characteristics of *c-Fos* mRNA synthesis to map circuits activated by multiple behavioral experiences (Guzowski et al., 2001b; Guzowski and Worley, 2001; Lin et al., 2011).

Mice lacking *c-Fos* expression in the CNS exhibit impairments in hippocampal-dependent spatial and associative learning tasks, but display intact amygdala-dependent learning tasks (Fleischmann et al., 2003). Acute knockdown of *c-Fos* expression using antisense oligodeoxynucleotides impairs the long-term consolidation

of recognition memory, conditioned taste aversion, water maze training, and discrimination learning (Grimm et al., 1997; Guzowski, 2002; Lamprecht and Dudai, 1996; Seoane et al., 2012). Taking advantage the expression pattern of *c-Fos* and its involvement in a variety of behavioral paradigms, several transgenic mouse lines allow for visualization and/or perturbation of neurons expressing *c-Fos* following behavioral experience (Barth et al., 2004; Guenthner et al., 2013; Reijmers et al., 2007). Using these systems to manipulate neurons expressing *c-Fos* after fear conditioning, several recent studies have been able to augment memory representations, providing novel insights into the encoding of long-term memories (Garner et al., 2012; Liu et al., 2012; Ramirez et al., 2013). The implication and further advancements of these technologies will be further discussed in this thesis.

Npas4

Neuronal PAS domain protein 4 (*Npas4*, originally referred to as Nxf) was discovered in a search for homologs of the basic-helix-loop-helix PAS (bHLH-PAS) protein Sim2 (Ooe et al., 2004). *Npas4* was found to contain a transactivation domain and bHLH-PAS domain indicating a role as a transcriptional regulator. The expression of *Npas4* was localized to neurons and regulated by cerebral ischemia, suggesting that *Npas4* may act as a stimulus-induced neuronal-specific transcriptional regulator (Ooe et al., 2004; Shamloo et al., 2006). Npas4 shares a high degree of homology to similar proteins in *C. elegans* and *Drosophila* and was found to form heterodimers with Arnt2 in order to regulate gene transcription (Ooe et al., 2007).

In a screen to identify genes regulated by calcium influx and membrane depolarization, *Npas4* was identified as one of three hundred targets whose expression was dependent on these parameters and expressed at a development stage coincident with inhibitory synapse development (Lin et al., 2008). Similarly, *Npas4* was induced in a kinase-specific manner following chemical-LTP and chemical-LTD stimulation (Coba et al., 2008). The expression of *Npas4* is selective to membrane depolarization and calcium influx; distinguishing it from several other activity-regulated genes. Knockdown of *Npas4* resulted in a decrease in inhibitory synapse formation as measured by immunocytochemistry and electrophysiology. In contrast, no change in excitatory synapse number was observed. Overexpression of *Npas4* resulted in an increase in inhibitory synaptic function. *Npas4* was found to regulate the expression of a variety of activity-regulated and synaptic gene programs, and is a particularly prominent regulator of *BDNF* expression through binding the promoter region of exon I of *BDNF* (Lin et al., 2008).

Following this foundational study from Lin and colleagues, several groups have identified a critical role for *Npas4* in transcriptional regulation (Kim et al., 2010; Rudenko et al., 2013) and several plastic processes (Coutellier et al., 2012; Maya-Vetencourt et al., 2012; Ploski et al., 2011; Ramamoorthi et al., 2011). The role for *Npas4* in memory formation was initially identified in the lateral amydgala, where acute RNAi-mediated knockdown resulted in impairments in fear memory formation and reconsolidation, but had no effect on innate fear and memory expression (Ploski et al., 2011). Using a combination of genetic knockouts and overexpression approaches, we were able to identify a critical role for CA3-specifc expression of *Npas4* in the consolidation of

contextual fear memories (Ramamoorthi et al., 2011). The details of this study will be further discussed in this thesis. Supporting a role for *Npas4* in neural circuit plasticity, *Npas4* overexpression was sufficient to re-open the critical period in adult visual cortex, while knockdown of *Npas4* prevented fluoxetine-induced critical period plasticity (Maya-Vetencourt et al., 2012). These observations suggest that *Npas4* maybe a critical regulator of neural circuit plasticity underlying processes such as visual cortex function and memory formation. Finally, using *Npas4* knockouts as a model for neurodevelopmental disorders, this mouse line was found to exhibit several phenotypes consistent with disorders such as autism and schizophrenia (Coutellier et al., 2012).

2.5 Goal of thesis

The goal of the work presented in this thesis was to bridge the gap between our deep understanding of neural structures and individual genes required for contextual memory formation. Initially, we set out to establish a role of *Npas4* and other activity-regulated genes in the formation of hippocampal-dependent contextual memories. Using the observations from this study, we developed a novel molecular tool that takes advantage of the expression pattern of Npas4 and other activity-regulated genes and allows us to monitor and perturb experience-activated neural circuits.

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Chapter 3

Npas4 Regulates a Transcriptional Program in CA3 Required for Contextual Memory Formation

Conception: Kartik Ramamoorthi and Yingxi Lin

Creation of constructs: Yingxi Lin, Gabi Belfort, Kartik Ramamoorthi, and Ross McKinney

Stimulation assays in vitro: Kartik Ramamoorthi (qPCR), Gabi Belfort (western blots)

Molecular assays after behavior: Kartik Ramamoorthi and Tim Otto

Luciferase assays: Yingxi Lin and Kartik Ramamoorthi

ChIP Assays: Kartik Ramamoorthi and Robin Fropf

Initial in vivo validation: Kartik Ramamoorthi

Surgeries: Robin Fropf (90%) and Kartik (10%)

Behavioral Analysis: Kartik Ramamoorthi, Robin Fropf, and Tim Otto

Virus Production Rachael Neve

Histology: Robin Fropf and Kartik Ramamoorthi

Mouse Husbandry: Kartik Ramamoorthi and Helen Fitzmaurice

Paper Written by: Kartik Ramamoorthi and Yingxi Lin

3.1 Summary

The rapid encoding of contextual memory requires the CA3 region of hippocampus, but the necessary genetic pathways remain unclear. We found that the activity-dependent transcription factor Npas4 regulates a transcriptional program in CA3 that is required for contextual memory formation. Npas4 was specifically expressed in CA3 after contextual learning. Global knockout or selective deletion of Npas4 in CA3 both resulted in impaired contextual memory, and restoration of Npas4 in CA3 was sufficient to reverse the deficit in global knockout mice. By recruiting RNA Polymerase II to promoters and enhancers of target genes, Npas4 regulates a learning-specific transcriptional program in CA3 that includes many well-known activity-regulated genes, suggesting that Npas4 is a master regulator of activity-regulated gene programs and is central to memory formation.

3.2 Background

The ability to form a long-term memory after a single experience is essential for the survival of higher organisms. In rodents and humans, memory of places or contexts can be formed after a single brief exposure to a novel environment, and this process requires the hippocampus (Neves et al., 2008; Scoville and Milner, 2000). It has been suggested that hippocampal area CA3 is required for rapid encoding of contextual memory (Kesner, 2007; Lee and Kesner, 2004; Nakashiba et al., 2008; Nakazawa et al., 2003). However, CA3-specific molecular pathways underlying contextual memory formation remain uncharacterized.

The formation and maintenance of long-term memories requires new gene and protein synthesis (Alberini, 2009; Davis and Squire, 1984). Learning-induced expression of activity-regulated genes, especially immediate early genes (IEGs), provides a link between behavioral experience and the molecular events required to encode memory (Kubik et al., 2007; Tischmeyer and Grimm, 1999). Genetic perturbations of IEGs or transcription factors that control activity-regulated gene expression thus often lead to deficits in neuronal plasticity and memory (Fleischmann et al., 2003; Guzowski, 2002; Jones et al., 2001; Kida et al., 2002; Plath et al., 2006). However, most IEGs can be induced by a wide range of stimuli and are involved in processes essential to normal cellular function and survival (Lonze et al., 2002; Ramanan et al., 2005), suggesting that their function may not be specific to learning-related neuronal activity. Therefore, identifying IEGs whose function is selectively correlated with both synaptic activity and learning may help to reveal the genetic programs required for memory encoding.

The expression of the activity-dependent transcription factor Npas4 (neuronal

PAS domain protein 4) was previously shown to selectively coupled to neuronal activity (Lin et al., 2008). We therefore investigated whether it regulates a learning-specific transcriptional program underlying the formation of contextual memories.

3.3 Results

3.3.1 Npas4 expression is selectively induced by neuronal activity and contextual learning

We first characterized the induction of Npas4, together with several other IEGs, in cultured mouse hippocampal neurons. Membrane depolarization resulted in robust expression of Npas4 mRNA, which was independent of new protein synthesis, suggesting that it is an IEG (Fig. 3-1A). Npas4 was selectively induced by depolarization and Ca^{2+} influx, but not by activators of several other signaling pathways that induce IEGs such as c-Fos, Arc (activity-regulated cytoskeleton-associated protein) and Zif268 (Fig. 3-1A and 3-1B), similar to what has been observed in dissociated rat neurons (Lin et al., 2008).

To examine experience-induced expression of Npas4, we trained mice in a hippocampus-dependent contextual fear conditioning (CFC) paradigm, which is thought to be dependent on de novo mRNA and protein synthesis (Alberini, 2009), and examined Npas4 mRNA expression in dorsal hippocampus (DH). We focused on DH on the basis of extensive work showing that DH is required for CFC (Fanselow and Dong, 2010).

Mice were sacrificed at various time points following CFC to measure mRNA expression for Npas4, c-Fos, and Arc using quantitative PCR (qPCR) (Fig. 3-1C).

Npas4 mRNA reached peak levels 5 minutes after training and returned to baseline levels 4.5 hours later. c-Fos and Arc reached their peak level by 30 minutes and returned to baseline levels 4.5 hours after training (Fig. 3-1C).

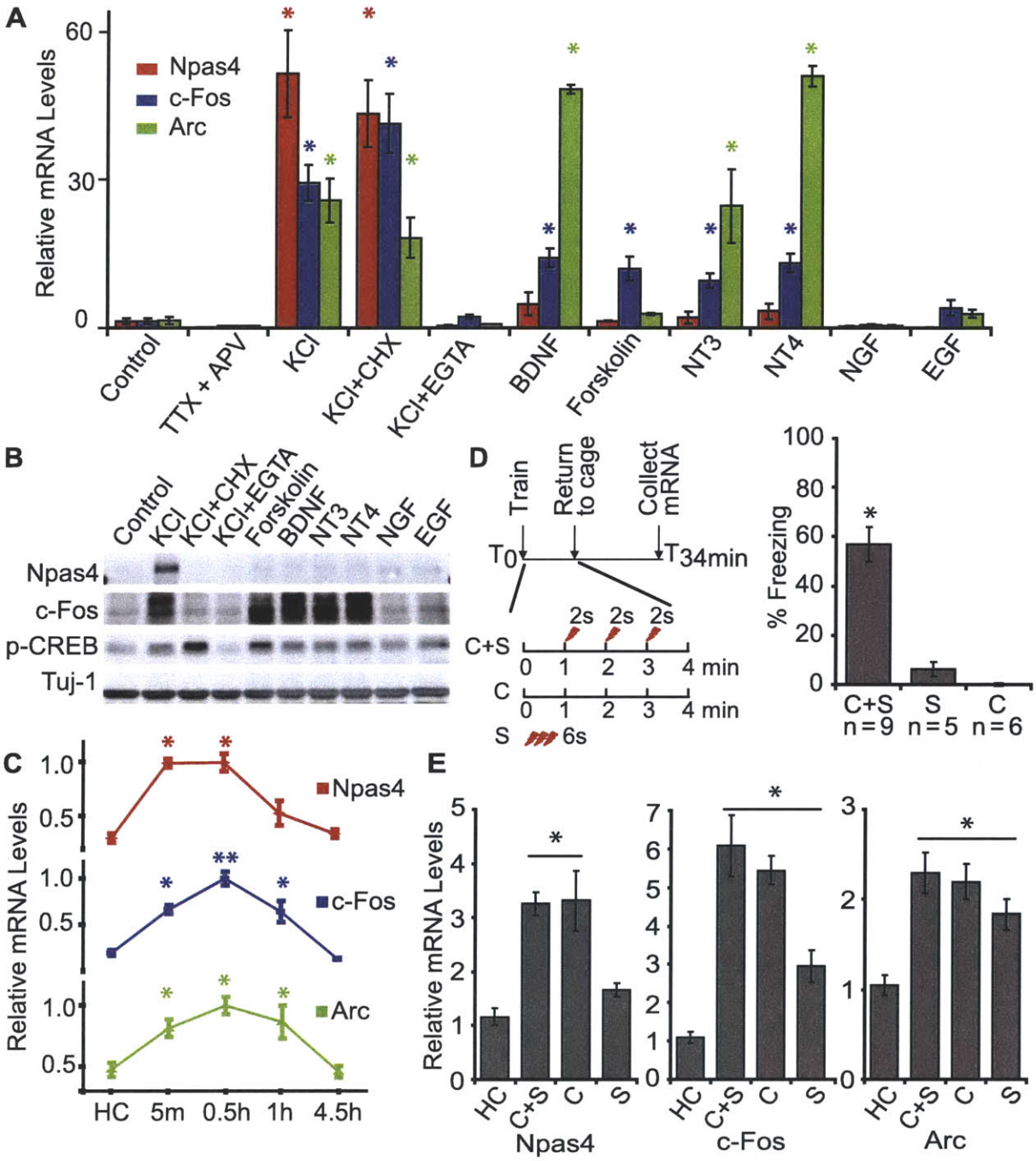
A
Relative mRNA Levels
60
30
0
Npas4
c-Fos
Arc
Control
TTX + APV
KCl
KCl+CHX
KCl+EGTA
BDNF
Forskolin
NT3
NT4
NGF
EGF
B
Control
KCl
KCl+CHX
KCl+EGTA
Forskolin
BDNF
NT3
NT4
NGF
EGF
Npas4
c-Fos
p-CREB
Tuj-1
C
Relative mRNA Levels
1.0
0.5
Npas4
c-Fos
Arc
HC
5m
0.5h
1h
4.5h
D
Train
Return to cage
Collect mRNA
T0
T34min
2s 2s 2s
C+S
0 1 2 3 4 min
C
0 1 2 3 4 min
S
6s
% Freezing
100
80
60
40
20
0
C+S n=9
S n=5
C n=6
E
Relative mRNA Levels
HC
C+S
C
S
Npas4
c-Fos
Arc

Fig. 3-1. Npas4 expression is selectively induced by neuronal activity in vitro and by learning in vivo.

(A) PCR showing that Npas4 mRNA expression in cultured hippocampal neurons (DIV 7) is selectively induced by depolarization (55mM KCl, 1hr), but not by forskolin (10μM, 1hr), BDNF (50ng/ml, 1hr), NT3 (50ng/ml, 1hr), NT4 (50ng/ml, 1hr), NGF (100ng/ml, 1hr), or EGF (100ng/ml, 1hr). Induction of Npas4 is prevented by pretreatment with the Ca^{2+} chelator EGTA (5mM, 10min), but not by treatment with cycloheximide (10ug/ml, 1 hr). Neurons were stimulated in the presence of TTX (1μM) and APV (100μM). n = 4 independent cultures. *p < 0.001 compared to control, one-way ANOVA followed by Dunnett's test

(B) Western blot showing that Npas4 protein expression in cultured hippocampal neurons (DIV 7) is selectively induced by depolarization, but not by forskolin, BDNF, NT3, NT4, NGF, and EGF. Npas4 protein expression is prevented by pretreatment with the Ca^{2+} chelator EGTA (5mM, 10min), and by treatment with cycloheximide. All treatments were the same as in (A) except applied for 2h.

(C) qPCR analysis of IEG expression in DH after CFC. Separate groups of mice were sacrificed 5min (n = 8), 30min (n = 9-11), 1hr (n = 6), or 4.5hr (n = 5) after CFC and compared to naive home cage mice (HC, n = 10). Values are plotted relative to peak timepoint. Npas4 mRNA reaches peak expression 5 minutes after CFC, c-Fos reaches peak expression 30 min after CFC. *p < 0.001 compared to HC, one-way ANOVA followed by Holm-Sidak posthoc test.

(D) Schematic of experimental design. C+S: context + shock; C: context exposure; S: immediate shock; HC: home cage. Mice trained in CFC (C+S) showed significantly

higher freezing behavior in comparison to all groups 24 hours later. Immediate shock (S), and context exposure (C) failed to produce significant freezing behavior. *p < 0.001, one-way ANOVA followed by Holm-Sidak posthoc test.

(E) Npas4 mRNA expression is induced under C+S (n = 8-10) and C (n = 8) conditions in comparison to S (n = 8) and HC (n = 10) conditions. c-Fos and Arc mRNA are induced by all conditions in comparison to HC. All groups were sacrificed 30min after training and compared to naive home caged mice. Please note differences in scale. * p < 0.001 one-way ANOVA followed by Holm-Sidak posthoc test.

Next we trained mice under CFC conditions that provided both context learning and shock association (C+S), or under conditions that involved just context learning (C) or shock (S) alone (Fig. 3-1D). Both C+S and C represent learning conditions, because the hippocampus forms contextual representations independent of shock delivery (Fanselow, 2000; Rudy and O'Reilly, 1999), but only C+S provides a behavioral readout of learning (Fig. 3-1D). Immediate shock (S) fails to induce long-term contextual memories, as the context exposure is not long enough for the hippocampus to form a representation (Figure 3-1D) (Landeira-Fernandez et al., 2006; Rudy and O'Reilly, 1999). Therefore, this served as a control condition, allowing us to distinguish IEG induction specific to context learning from induction due to the shock.

Gene expression analysis in mice sacrificed 30 minutes after training indicated that, compared to naive subjects, Npas4 was induced in the C+S and C groups, but not in the S group. In contrast, c-Fos and Arc were significantly induced in all behavioral conditions (Fig. 3-1E).

3.3.2 Learning and memory deficits in Npas4 global knockout mice

We next determined whether CFC is impaired in Npas4 knockout ($Npas4^{-/-}$) mice (Lin et al., 2008). During the training session and the memory test 5 minutes later, we observed robust freezing behavior in both $Npas4^{-/-}$ and wildtype ($Npas4^{+/+}$) littermates, suggesting that the ability to acquire CFC is normal in $Npas4^{-/-}$ mice (Fig. 3-2A and 3-2B). Furthermore, locomotor activity, anxiety levels, footshock sensitivity, and hippocampal morphology were similar across genotypes (Figs. 3-3 and 3-4). Despite having intact memories five minutes after training, freezing elicited by the context 1 hour and 24 hours after training was significantly reduced in $Npas4^{-/-}$ mice (Fig. 3-2C and

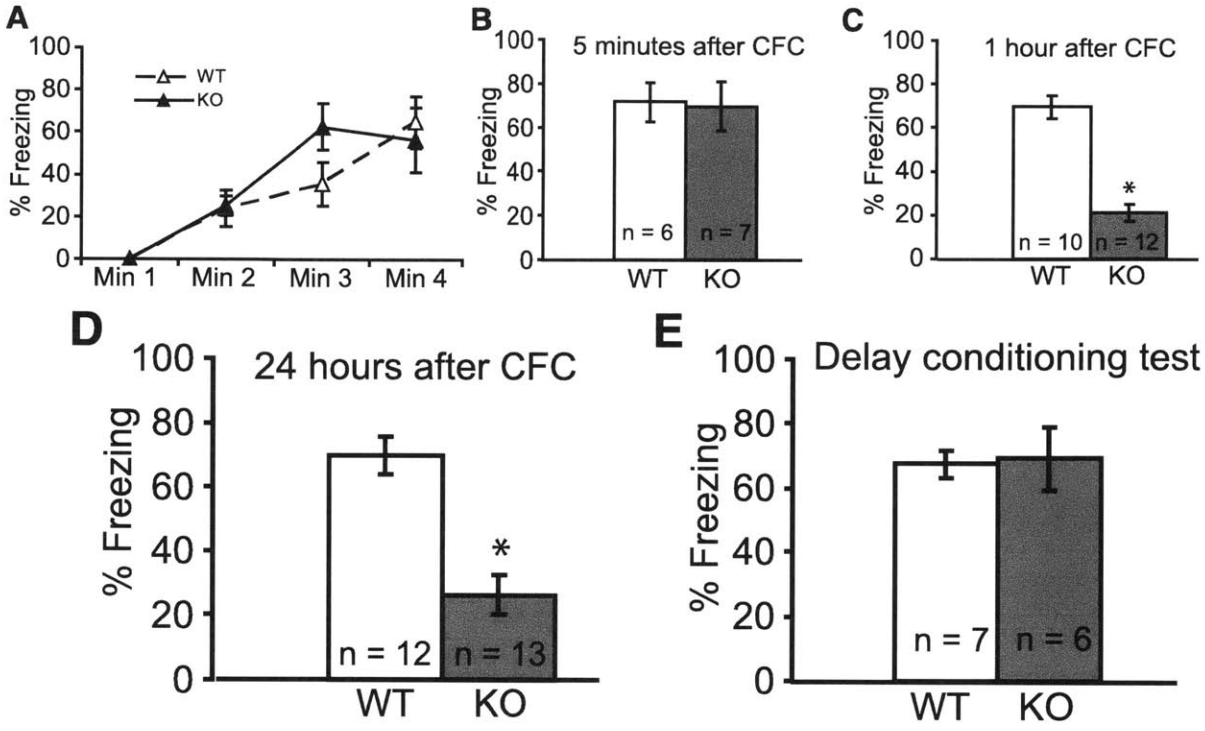

Fig. 3-2. Npas4 global knockout mice exhibit impaired hippocampal-dependent STM and LTM.

(A,B) Npas4$^{-/-}$ and Npas4$^{+/+}$ littermates exhibit similar freezing during the training session (A) and 5min after training (B). $p = 0.879$, Student's t-test.

(C,D) 1h (C) and 24h (D) after CFC Npas4$^{-/-}$ mice freeze at a significantly lower level than Npas4$^{+/+}$ littermates. *$p \leq 0.001$, Student's t-test.

(E) 24h after auditory delay conditioning, Npas4$^{-/-}$ mice exhibit similar freezing to Npas4$^{+/+}$ mice during a tone memory test. $p = 0.859$, Student's t-test.

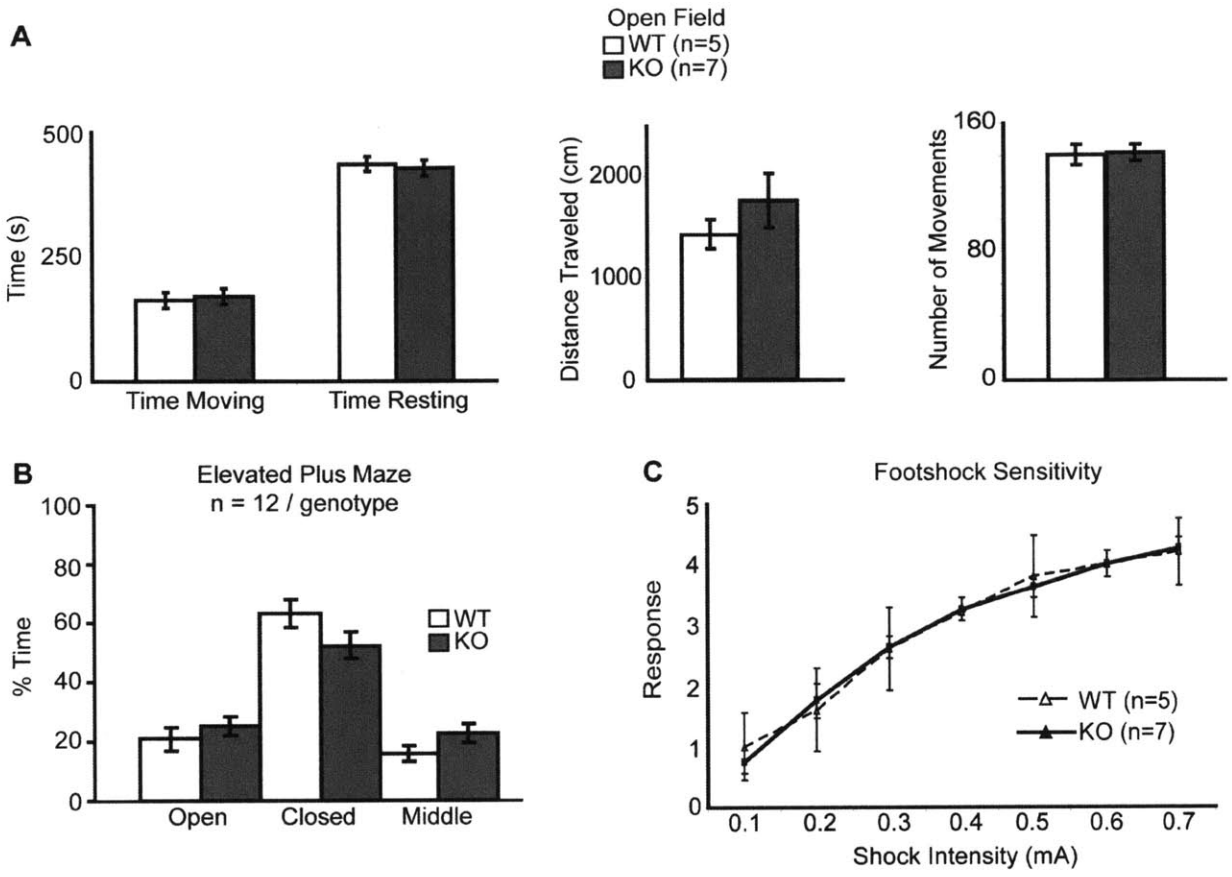

Fig. 3-3. Control behaviors in Npas4$^{-/-}$ and Npas4$^{+/+}$ littermates. No differences were observed between Npas4$^{-/-}$ and Npas4$^{+/+}$ littermates in open field, elevated plus maze, and footshock sensitivity assays. Open field: time moving ($p = 0.713$), time resting ($p = 0.718$), distance traveled ($p = 0.354$), and number of movements ($p = 0.841$), Student's t-test. Elevated plus maze: similar amounts of time were spent in the open ($p = 0.427$), closed ($p = 0.097$), and middle (0.100) arms, Student's t-test. Footshock reactivity: response to increase shock intensity was not different across genotypes ($p = 0.395$), two way repeated measures ANOVA.

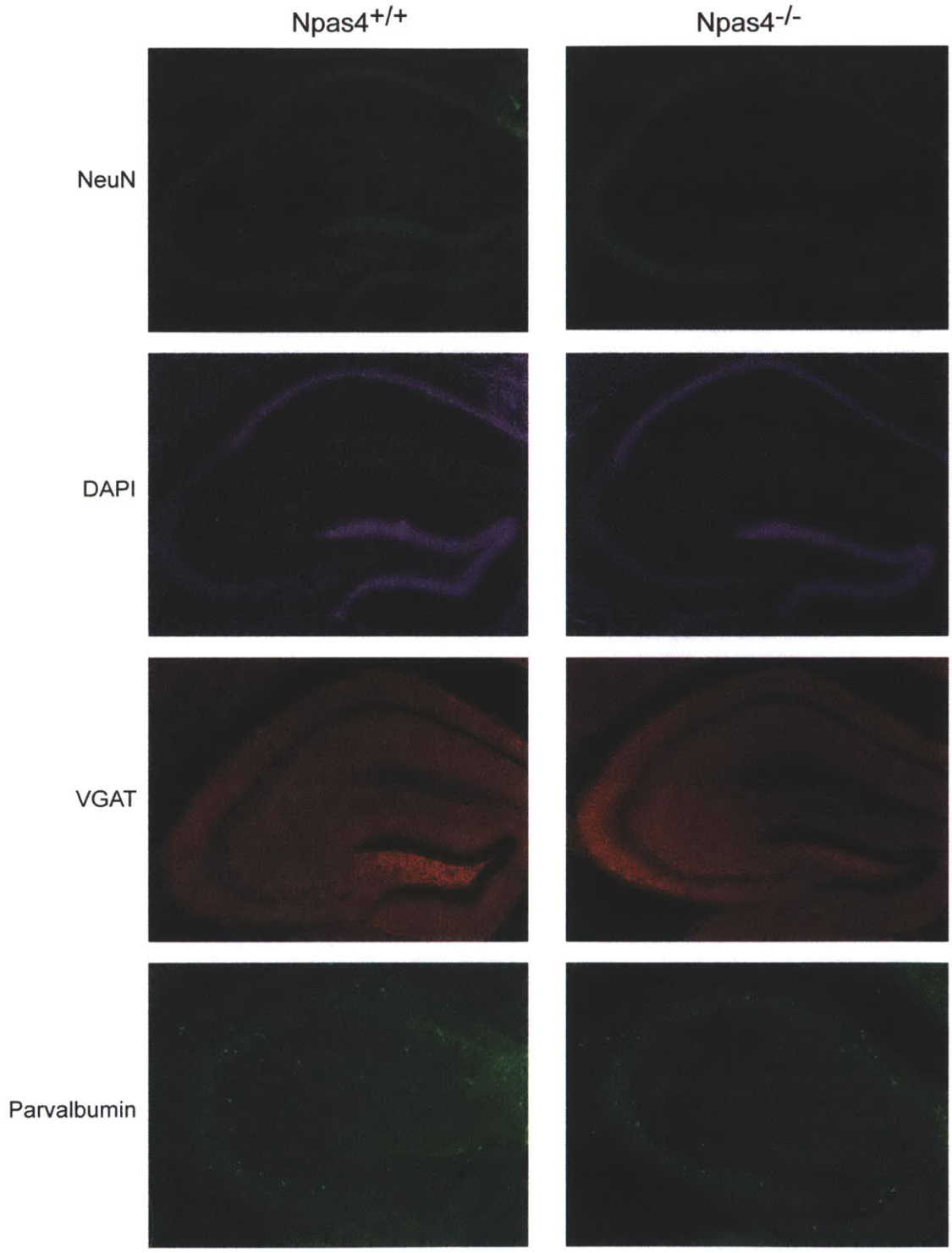

Fig. 3-4. No gross alterations in hippocampal morphology in Npas4$^{-/-}$ mice as observed by NeuN, DAPI, VGAT, or parvalbumin staining.

Figure 3-2D), suggesting that both short-term memory (STM) and long-term memory (LTM) are impaired in Npas4$^{-/-}$ mice.

There is now a general consensus that the amygdala is required for all forms of fear conditioning, while only a subset of fear conditioning paradigms (including CFC) rely on hippocampal integrity (Anagnostaras et al., 2001; Maren, 2008). We therefore tested whether Npas4$^{-/-}$ mice were deficient in auditory delay conditioning, a form of fear conditioning known to depend on the amygdala but not the hippocampus (Anagnostaras et al., 2001). We saw no difference between Npas4$^{-/-}$ and wildtype mice when tone-induced freezing was measured 24 hours after training, confirming that sensory detection and fear memory acquisition are normal in Npas4$^{-/-}$ mice and suggesting that the impairment we observed in CFC was likely due to a deficit in the hippocampus, and not the amygdala (Fig. 3-2E).

3.3.3. Selective deletion of Npas4 from CA3, but not CA1, impairs long-term contextual memory

We hypothesized that the memory impairment observed in the global knockout was due to a loss of learning-induced Npas4 expression in DH, based on its selective expression after context learning (Fig. 3-1E). Because the different subregions within DH may play dissociable roles in contextual memory formation (Kesner, 2007), we examined whether CFC resulted in a regionally-selective expression of Npas4. While Npas4 was expressed broadly in several brain regions after CFC, including amygdala and entorhinal cortex (fig. 3-5), within the hippocampus Npas4 expression after CFC was largely restricted to the CA3 subregion (Fig. 3-6A), with higher expression in dorsal CA3 than in ventral CA3 (fig. 3-7). In contrast, c-Fos was robustly expressed in both

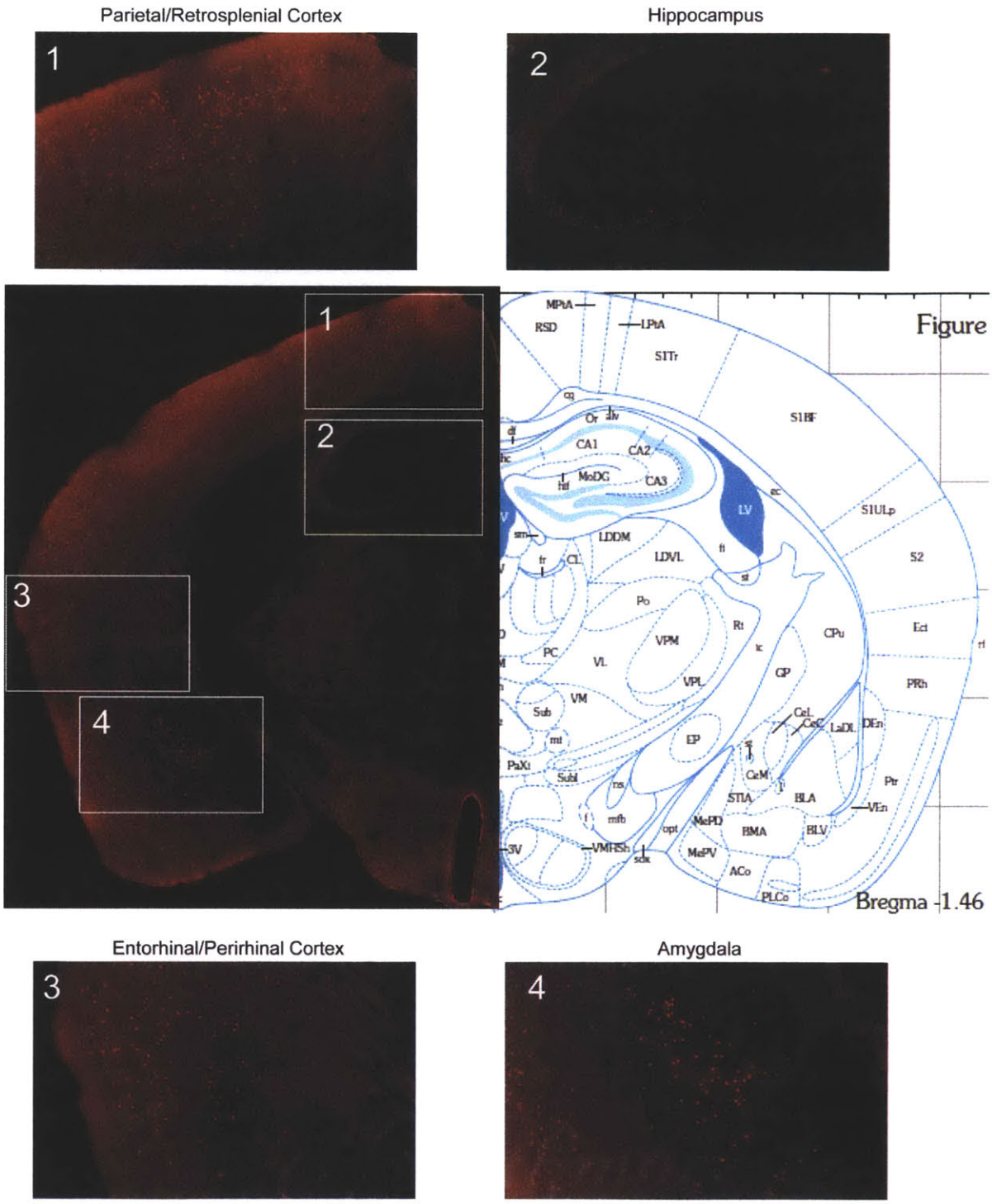

Fig. 3-5. Immunostaining showing that Npas4 was expressed in several brain regions 1h after CFC. Expression was observed in hippocampus (CA3), amygdala, and various cortical regions including entorhinal cortex.

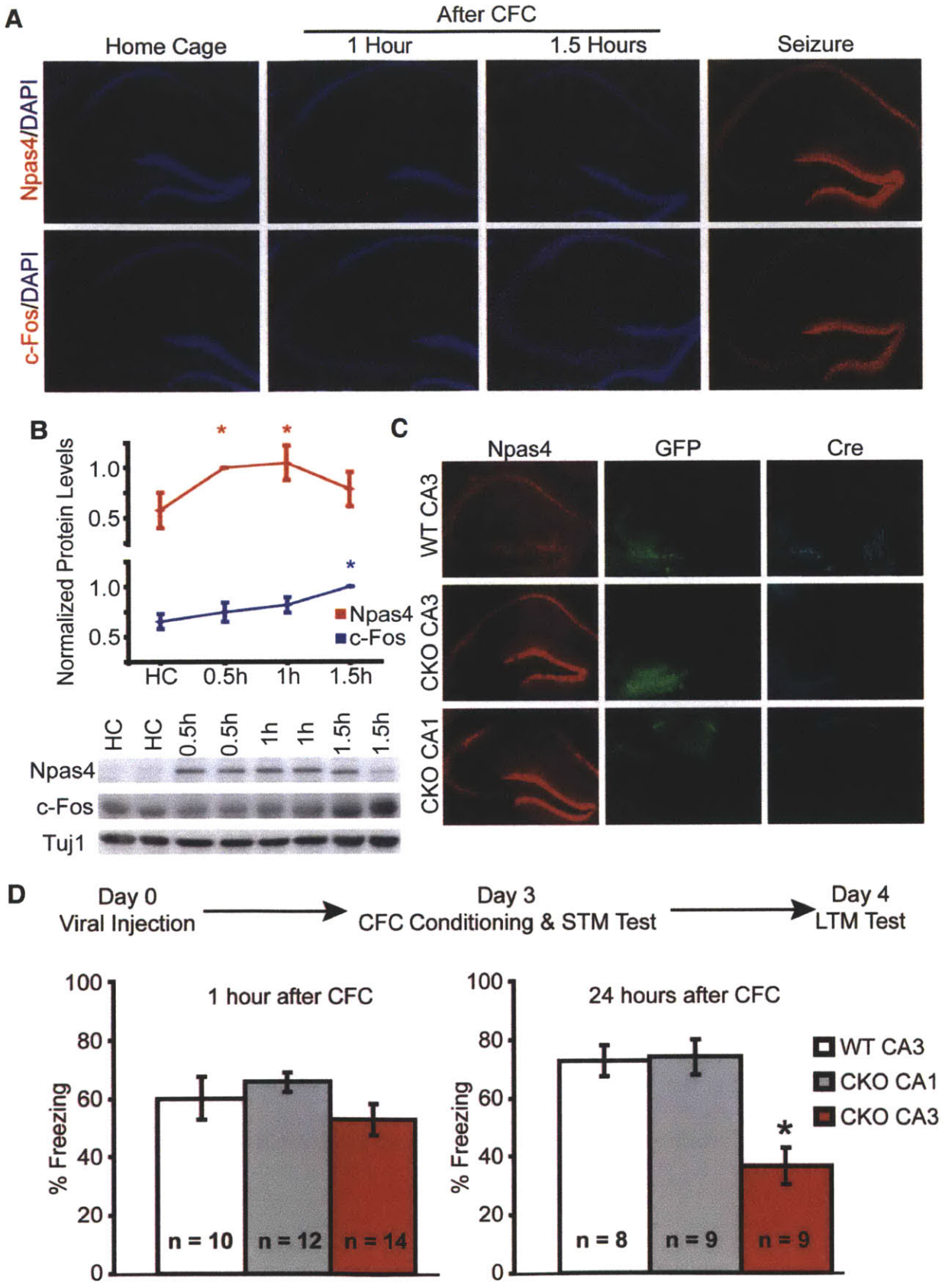
A
After CFC
Home Cage
1 Hour
1.5 Hours
Seizure
Npas4/DAPI
c-Fos/DAPI
B
Normalized Protein Levels
1.0
0.5
Npas4
c-Fos
HC
0.5h
1h
1.5h
Tuj1
C
Npas4
GFP
Cre
WT CA3
CKO CA3
CKO CA1
D
Day 0
Viral Injection
Day 3
CFC Conditioning & STM Test
Day 4
LTM Test
1 hour after CFC
24 hours after CFC
% Freezing
WT CA3
CKO CA1
CKO CA3
n = 10
n = 12
n = 14
n = 8
n = 9
n = 9

Fig. 3-6. Npas4 expression in CA3 is required for contextual fear conditioning.

(A) Upper panel: Npas4 expression is increased in CA3 and to a lesser extent in the dentate gyrus after CFC. Peak expression is observed at the 30 minute timepoint, which sustained expression 1h after CFC. Lower panel: c-Fos expression is induced in all subregions of DH. Peak expression is observed 1.5h after CFC. Seizure induces Npas4 and c-Fos in all subregions of hippocampus (kainic acid, 12mg/kg, 2h). Npas4 and c-Fos are shown in red, overlaid with blue DAPI stain. DAPI stain is not shown for seizure condition for easier viewing.

(B) Upper panel: Western blot quantification of Npas4 and c-Fos expression in DH at various time after CFC. Npas4 is significantly expressed 30 minutes and 1h after CFC, while c-Fos is significantly induced 1.5h after CFC. n = 5 mice/condition. Values are plotted relative to peak timepoint. *$p < 0.04$. Lower panel: Western blot with two animals per condition.

(C) Immunostaining showing specific targeting of CA3 or CA1 three days after viral injection. Injection of Cre into wild type animals does not impair the expression of Npas4, while injection into $Npas4^{flx/flx}$ animals abolishes Npas4 expression.

(C) Mice were injected on day 0 and trained in CFC 3 days post-injection. Memory tests were given 1h and 24h (day 4) after training. 1h after CFC all groups freeze at similar levels. $p = 0.212$, one-way ANOVA followed by Holm-Sidak posthoc test. 24h after CFC $Npas4^{flx/flx}$ animals injected with Cre in CA3 exhibit impaired freezing in comparison to CA1-injected or WT controls. *$p < 0.001$, one-way ANOVA followed by Holm-Sidak posthoc test.

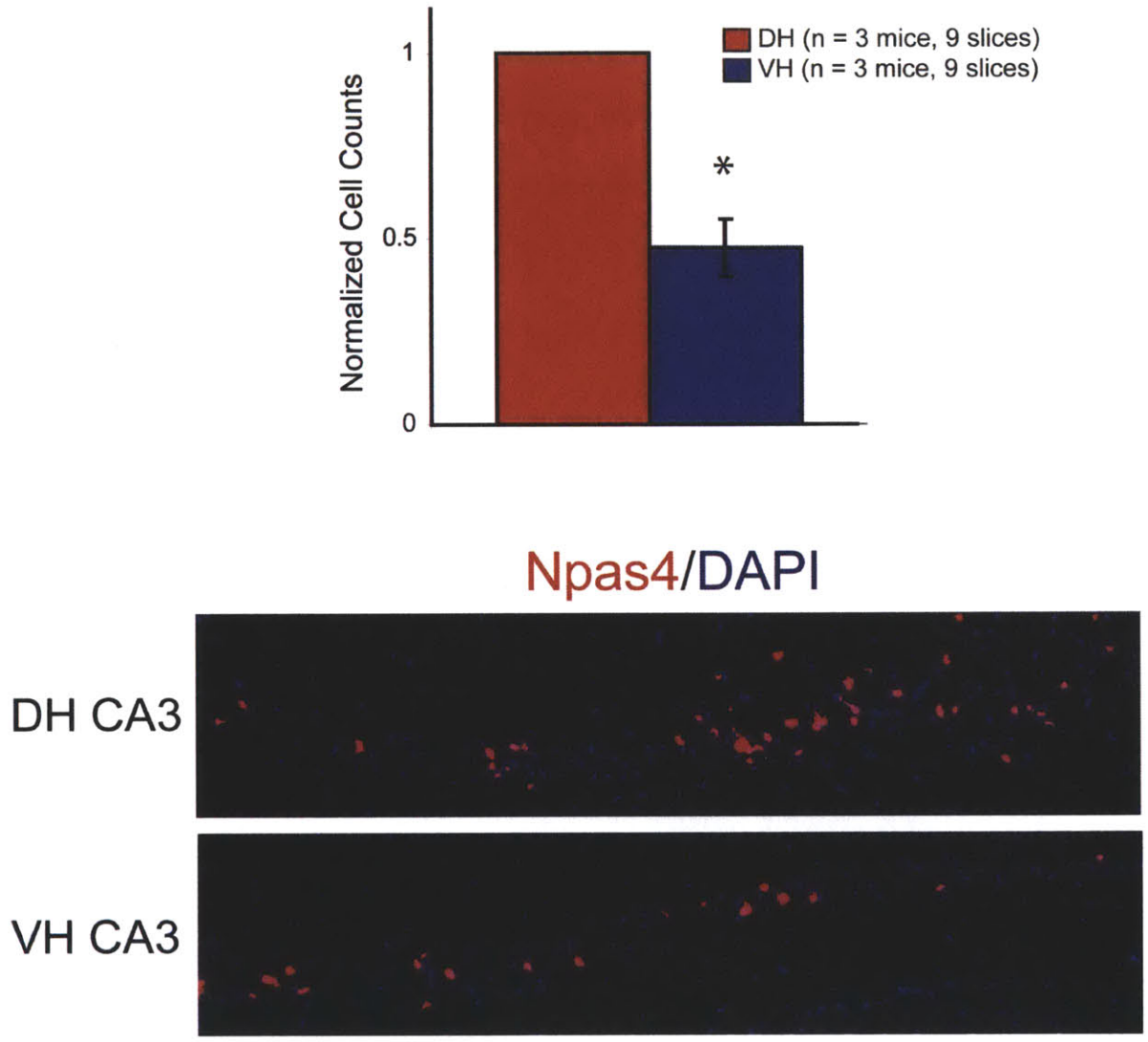

Fig 3-7. Immunostaining showing increased expression of Npas4 in dorsal CA3 in comparison ventral CA3 1h after CFC. *p = 0.003, Student's t-test.

CA1 and CA3 (Fig. 3-6A) and similar patterns of induction have been reported for Arc and Zif268 (Guzowski et al., 1999; Lonergan et al., 2010). We also noted that the highest level of Npas4 was observed 30 minutes after CFC, 1 hour before the peak expression of c-Fos (Fig. 3-6B). This observation suggests that pathways activating Npas4 may be distinct from those for other IEGs. Importantly, induction of Npas4 in CA3 appears to be specific to contextual learning, because Npas4 was induced in all regions of hippocampus following kainic acid-stimulated seizures (Fig. 3-6A). While the CA3 region is known to be required for rapid contextual learning, these data are the first to indicate the selective, learning-induced expression of a specific IEG within CA3 by this form of learning.

If induction of Npas4 in dorsal CA3 is required for contextual memory, then deleting Npas4 in CA3 should replicate the memory impairments seen in the global knockout (Fig. 3-2). We acutely deleted Npas4 by stereotaxically injecting a herpes simplex virus (HSV) expressing Cre recombinase (HSV-Cre) into the CA3 region of Npas4 conditional knockout ($Npas4^{flx/flx}$) mice (Fig. 3-6C). HSV is naturally neurotropic and reaches peak expression within three days of delivery (Barrot et al., 2002; Han et al., 2007). In another group of mice, we used an equivalent amount of virus to delete Npas4 from a similar volume of cells in dorsal CA1, where we see no activation of Npas4 after CFC (Fig. 3-6C). To control for any effects of expressing Cre recombinase we also injected HSV-Cre into CA3 of wild type mice. Mice were injected with HSV-Cre 3 days before CFC and tested 1 hour and 24 hours after training (Fig. 3-6D). All animals showed similar freezing during the 1 hour context test. However, 24 hours after training animals with Npas4 deletions in CA3 had attenuated freezing responses compared to

animals with Npas4 deletions in CA1 or wild type animals injected with HSV-Cre in CA3 (Fig. 3-6D).

3.3.4 Npas4 regulates an activity-dependent genetic program that includes several IEGs

As an activity-dependent transcription factor, Npas4 likely contributes to CA3-dependent encoding of contextual memory through the regulation of a genetic program. Npas4 expression peaks prior to that of several other IEGs (Fig 3-1C and 3-6B), and its acute deletion abolished expression of c-Fos (Fig 3-8A); together these data suggest that Npas4 may regulate the activity-dependent expression of other IEGs. To explore this possibility, we acutely deleted Npas4 in a high percentage of cultured $Npas4^{flx/flx}$ hippocampal neurons by infecting them with HSV-Cre and assayed the mRNA expression of several IEGs following membrane depolarization. Compared to uninfected and HSV-GFP infected controls, deletion of Npas4 abolished depolarization-induced expression of Arc, c-Fos and Zif268 mRNA (Fig. 3-8B). Expression of the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was not altered.

Deletion of Npas4 could affect expression of activity-regulated IEGs indirectly, for example by generally disrupting the cellular response to neuronal activity. To examine this possibility, we designed a series of luciferase reporter assays to determine whether other activity-dependent transcriptional pathways function normally in the absence of Npas4. We first characterized transcription from the promoter of Npas4 (P_{Npas4}-Luc), to measure the state of pathways upstream of Npas4 protein, and found that the reporter was induced in response to KCl depolarization but not to activators of other signaling pathways, similar to endogenous Npas4 (fig. 3-9; compare Fig. 3-1A and 3-1B). When

Npas4 was acutely deleted by the expression of Cre recombinase in cultured hippocampal neurons generated from Npas4$^{flx/flx}$ mice, activity of P_{Npas4}-Luc in response to KCl depolarization was unchanged (Fig. 3-8C). We also examined the activity of the transcription factors CREB (cAMP responsive element binding protein) and MEF2 (myocyte enhancer factor-2). Unlike Npas4, these proteins are constitutively expressed, and are activated by post-translational modifications in response to depolarization (Kornhauser et al., 2002; Mao et al., 1999). Reporters expressing luciferase under the control of CREB and MEF2 response elements (CRE and MRE) were unaffected by acute deletion of Npas4 (Fig. 3-8C).

We then directly determined whether Npas4 binds to the genomic DNA of two activity-regulated genes: BDNF and c-Fos. These genes are dependent on Npas4 for their expression in response to neuronal activity (Fig. 3-8A-C and (Lin et al., 2008)), have well characterized genomic structures (Coulon et al., 2010; Sheng et al., 1988; Treisman, 1985, 1986; Wagner et al., 1990), and have been implicated in learning and memory (Fleischmann et al., 2003; Lu et al., 2008; Minichiello, 2009). We examined one of the activity-regulated promoters of BDNF, promoter I (PI_{BDNF}), the proximal promoter region of c-Fos and one of its upstream enhancer regions, E2 (Kim et al., 2010), using chromatin immunoprecipitation (ChIP). After depolarization, Npas4 bound to PI_{BDNF} and c-Fos E2, but not to the c-Fos proximal promoter (Fig. 3-8D).

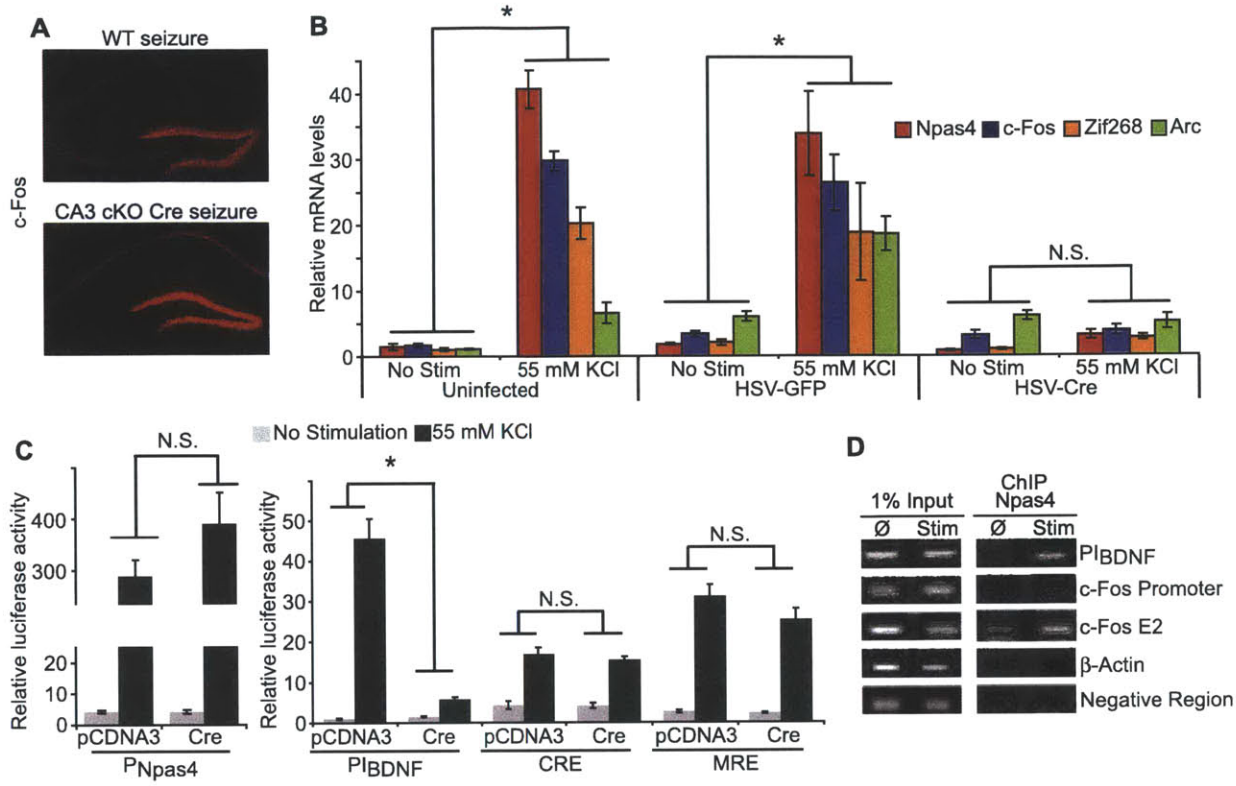

Fig. 3-8. Npas4 regulates the expression of several IEGs.

(A) Conditional deletion of Npas4 in CA3 results in loss of c-Fos expression in CA3 *in vivo* (kainic acid, 12mg/kg, 2h)

(B) All IEGs are significantly induced by KCl treatment (55mM, 1h) in both uninfected and HSV-GFP-infected Npas4$^{flx/flx}$ hippocampal neurons (7DIV), however no induction is observed when Npas4 is deleted by HSV-Cre. Data were compiled from 3 independent cultures each conducted in triplicate. The basal level of Arc was elevated by viral treatment. The fold induction of Arc following KCl depolarization is very similar in the uninfected and GFP infected conditions, suggesting that viral application alone does not alter the activity-regulated expression of Arc. *$p < 0.001$, two-way ANOVA followed by Holm-Sidak posthoc test.

(C) Activity of BDNF promoter I (PI_{BDNF}), Npas4 promoter (P_{Npas4}), CREB (CRE) and MEF2 (MRE) reporter constructs. $Npas4^{flx/flx}$ hippocampal cells were transfected (DIV 5) with one of the reporter constructs, with or without Cre. Neurons were treated with TTX (1μM) and APV (100μM) 1hr prior to KCl treatment for 6hr. PI_{BDNF} reporter is significantly induced by depolarization, but the activity of the reporter is abolished in the absence of Npas4. P_{Npas4}, CRE, and MRE reporters show similar induction in the presence or absence of Npas4. Data were compiled from 4 independent cultures each conducted in triplicate. *$p \leq 0.001$, two-way ANOVA followed by Holm-Sidak posthoc test.

(D) ChIP experiments showing that under depolarized conditions (cultured cortical neurons, DIV 7, 55mM KCl, 2hr) Npas4 binds to PI_{BDNF} and enhancer II of c-Fos (c-Fos E2). No binding is observed at the c-Fos promoter, the β-actin promoter, or a negative control region.

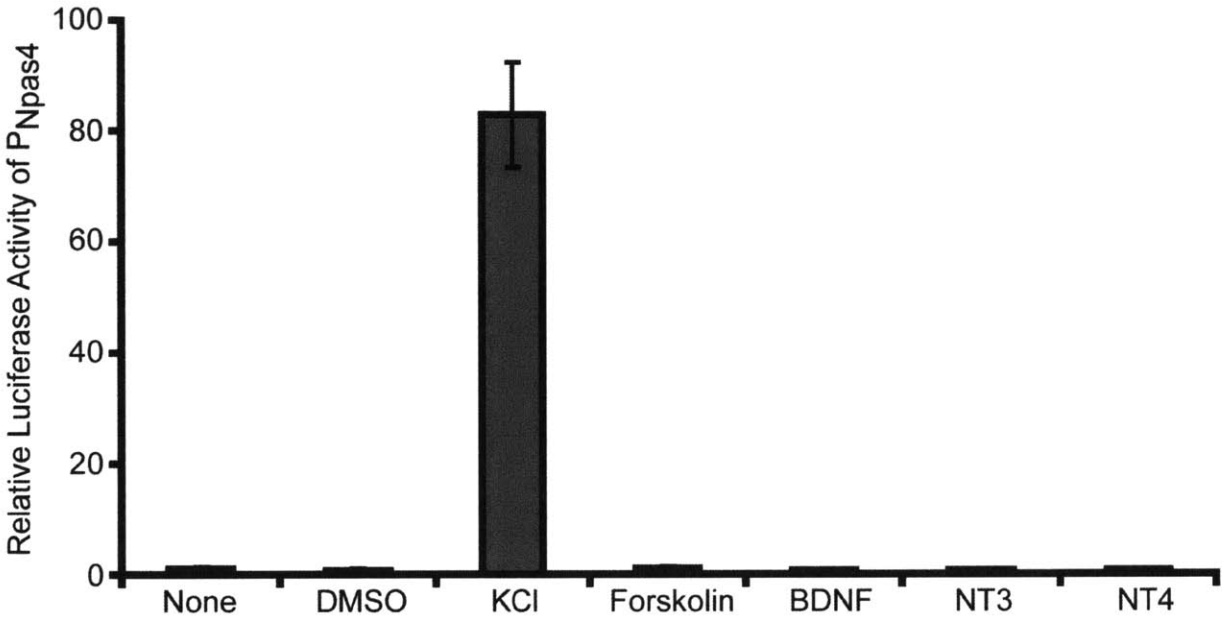

Fig. 3-9. Validation of the P_{Npas4} construct. P_{Npas4} was selectively activated by membrane depolarization. Cultured hippocampal neurons were transfected with P_{Npas4} at DIV 5 and at DIV 7 treated with DMSO (1:1000), KCl (55mM), forskolin (10μM), BDNF (50ng/ml), NT3 (50ng/ml), or NT4 (50ng/ml) for 6h in the presence of TTX (1 mM) and APV (100 mM). Only KCl treatment significantly activated P_{Npas4}. Data were compiled from 2 independent experiments each conducted in triplicate.

3.3.5 Npas4 is required for recruitment of RNA polymerase II to target genes

Genome-wide ChIP-sequencing has revealed that Npas4 co-localizes with RNA polymerase II (Pol II) at enhancer and promoter sites of many activity-regulated genes, including BDNF and c-Fos (Kim et al., 2010). However, it is not known whether this co-localization plays an important role in regulating transcription of these genes. We hypothesized that Npas4 is required for activity-dependent recruitment of Pol II to promoter and enhancer regions of its targets, in order to activate their transcription.

We acutely deleted Npas4 using HSV-Cre in a high percentage of cultured $Npas4^{flx/flx}$ cortical neurons and then performed ChIP for Pol II after 2 hours of membrane depolarization. In control neurons infected with HSV-GFP, Pol II localized to PI_{BDNF}, the c-Fos enhancer E2, the c-Fos promoter region, and the b-actin promoter after depolarization (Fig. 3-10A). When Npas4 was deleted by HSV-Cre, localization of Pol II to PI_{BDNF} and c-Fos E2 was impaired (Fig. 3-10A). As we described above, Npas4 binds to both of these regions. Pol II binding to the promoter regions of c-Fos and b-actin, where we did not observe Npas4 binding (Fig. 3-8D), was not affected by deletion of Npas4. To confirm that the Npas4-dependent binding of Pol II is important for gene expression, we compared luciferase reporters driven by PI_{BDNF} and the c-Fos promoter and found that expression from PI_{BDNF} was abolished by deletion of Npas4, while expression from the c-Fos promoter was not attenuated (Fig. 3-10B).

To confirm our findings *in vivo,* we performed ChIP for Pol II from hippocampal tissue extracted from adult $Npas4^{+/+}$ and $Npas4^{-/-}$ littermates. Npas4 is expressed only in a sparse population of neurons following CFC (Fig. 3-6A) making it difficult to detect Pol

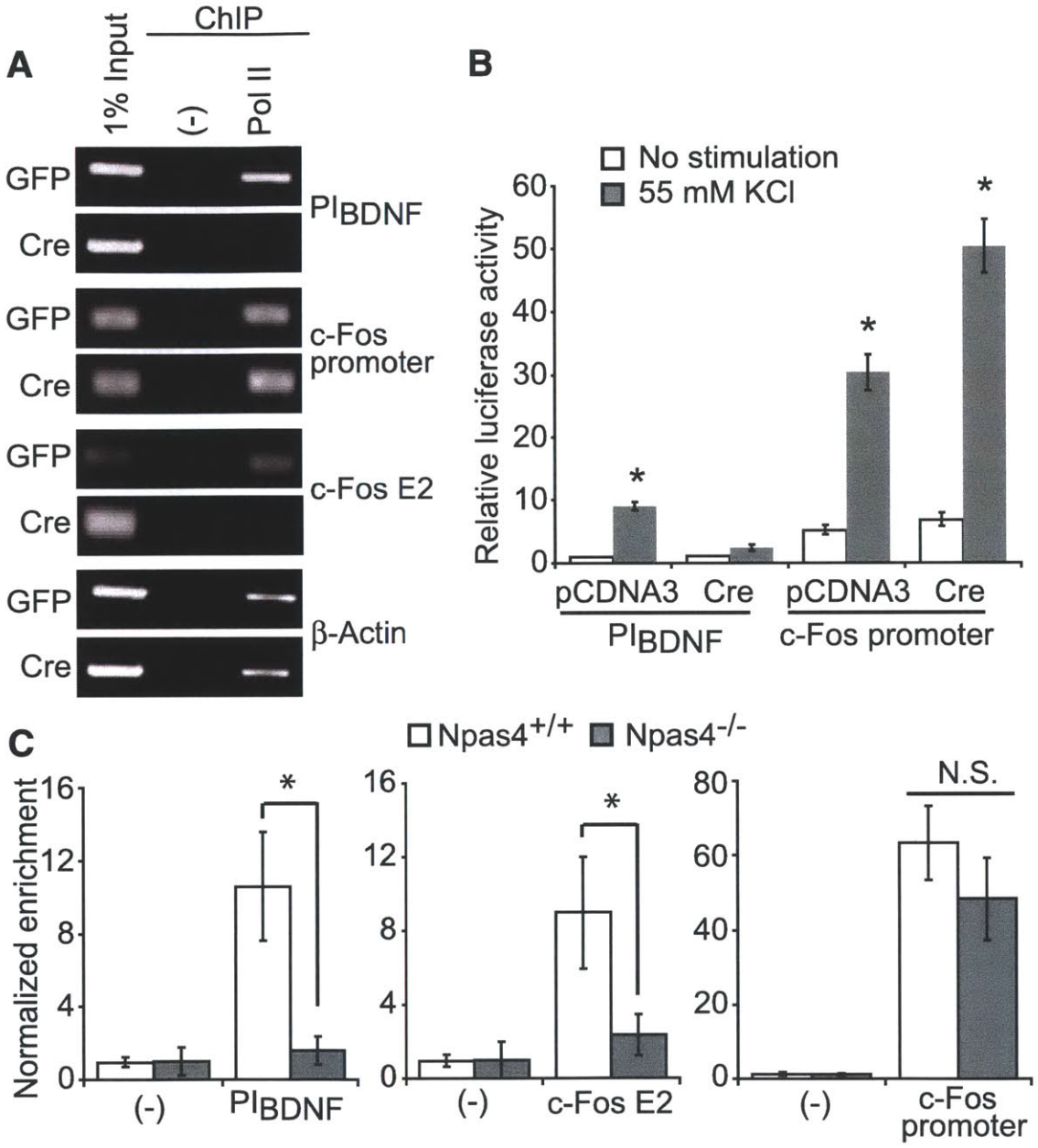

Fig. 3-10. Npas4 is required for the recruitment of RNA Polymerase II to enhancer and promoter regions of activity-regulated genes.

(A) Localization of Pol II to PI_{BDNF} and c-Fos enhancer region E2 is dependent on Npas4. Acute deletion of Npas4 results in reduced binding of Pol II at these regions. No change is observed in Pol II binding at the c-Fos or β-actin promoter.

(B) $Npas4^{flx/flx}$ cortical cells were transfected (DIV 5) with luciferase reporters driven by either PI_{BDNF} or c-Fos promoter, with or without Cre. Neurons were treated with TTX (1μM) and APV (100μM) overnight prior to KCl treatment for 6hr The activity of PI_{BDNF} reporter is significantly induced by depolarization (55mM, 6h), but its activity is abolished when Npas4 is removed by Cre. The activity of c-Fos promoter is not attenuated, but rather heightened, in the absence of Npas4. Data were compiled from 4 independent experiments each conducted in triplicate. *$p < 0.001$, two-way ANOVA followed by Holm-Sidak posthoc test.

(C) qPCR analysis of ChIP samples from seized $Npas4^{-/-}$ and $Npas4^{+/+}$ littermates (kainic acid, 12mg/kg, 2h, hippocampus). $Npas4^{-/-}$ samples showed diminished Pol II binding to PI_{BDNF} (*$p \leq 0.008$, Student's t-test, n=7/genotype) and c-Fos E2 (*$p \leq 0.048$, Student's t-test, n=6/genotype) relative to $Npas4^{+/+}$ littermates. No change is observed in Pol II binding at the c-Fos promoter ($p = 0.333$, Student's t-test, n=6/genotype). Data are normalized to a negative control region and are presented as mean +/- s.e.m. from separate experiments run in triplicate.

II binding in these cells. We therefore used kainic acid-induced seizures to activate all neurons in order to determine the genomic localization of Pol II *in vivo*. Seizure has been shown to robustly induce activity-regulated genes, many of which have been implicated in memory formation, and under certain conditions can induce potentiation similar to LTP (Ben-Ari and Represa, 1990). In line with our *in vitro* observations, localization of Pol II to PI_{BDNF} and c-Fos E2 was impaired in $Npas4^{-/-}$ mice compared to $Npas4^{+/+}$ littermates, while Pol II binding to the promoter regions of c-Fos and b-actin was similar across genotypes (Fig. 3-10C and fig. 3-11).

3.3.6 Expression of Npas4 in CA3 rescues transcription and memory formation in global knockouts

We next investigated whether re-expressing Npas4 in CA3 of $Npas4^{-/-}$ mice would induce expression of its genetic program and subsequently rescue memory formation in the mutant mice.

The CA3 region of $Npas4^{-/-}$ mice was infected with HSV expressing Npas4 (HSV-Npas4) (Fig. 3-12A) and activation of Npas4 gene targets was examined using immunostaining. HSV-Npas4 induced the expression of c-Fos (Fig. 3-12B), but a transcriptionally inactive version of Npas4 (ΔNpas4) did not, confirming that the transcription activation ability of Npas4 is required. We also tested whether expression of Npas4 is sufficient to induce BDNF by measuring the activity of a PI_{BDNF} reporter construct *in vitro*. We transfected Cre into $Npas4^{flx/flx}$ neurons and found that activity of the PI_{BDNF} reporter was abolished. Co-transfecting Npas4, but not ΔNpas4, rescued the activity of the PI_{BDNF} reporter (Fig. 3-12C).

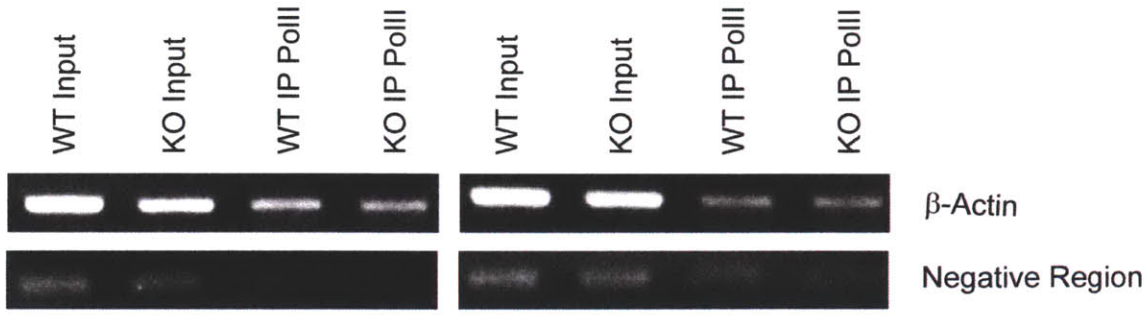

Fig. 3-11. ChIP from seized Npas4$^{-/-}$ and Npas4$^{+/+}$ littermates (kainic acid, 12mg/kg, 2h, hippocampus) showing that localization of Pol II to the β-actin promoter is not altered in Npas4$^{-/-}$ mice. Results from 2 representative pairs of animals are shown here.

We then determined whether expressing Npas4, and activating its downstream genetic program (Fig 3-12B and 3-12C), would restore long-term memory formation in Npas4$^{-/-}$ mice. We targeted CA3 because Npas4 is required in CA3, but not CA1, for CFC (Fig 3D) and Npas4$^{-/-}$ mice have normal amygdala function (Fig. 3-2E). The use of HSV allowed us to acutely express Npas4, with a peak expression 3 days after injection (Barrot et al., 2002; Han et al., 2007). Mice were injected with virus, trained three days after injection, and tested 1 hour and 24 hours after training (Fig. 3-12D). Expressing Npas4 in CA3 completely reversed both the short-term and long-term contextual memory deficits observed in the global knockouts, since Npas4 knockout mice with HSV-Npas4 injected into CA3 showed similar freezing behavior to wild type control animals injected with GFP. Global knockouts with HSV-Npas4 delivered to CA1 showed no such recovery (Fig. 3-12D). Expressing ΔNpas4 in CA3 failed to overcome the memory deficits in Npas4$^{-/-}$ mice, confirming that activation of the genetic program regulated by Npas4 is required for rescue of memory formation.

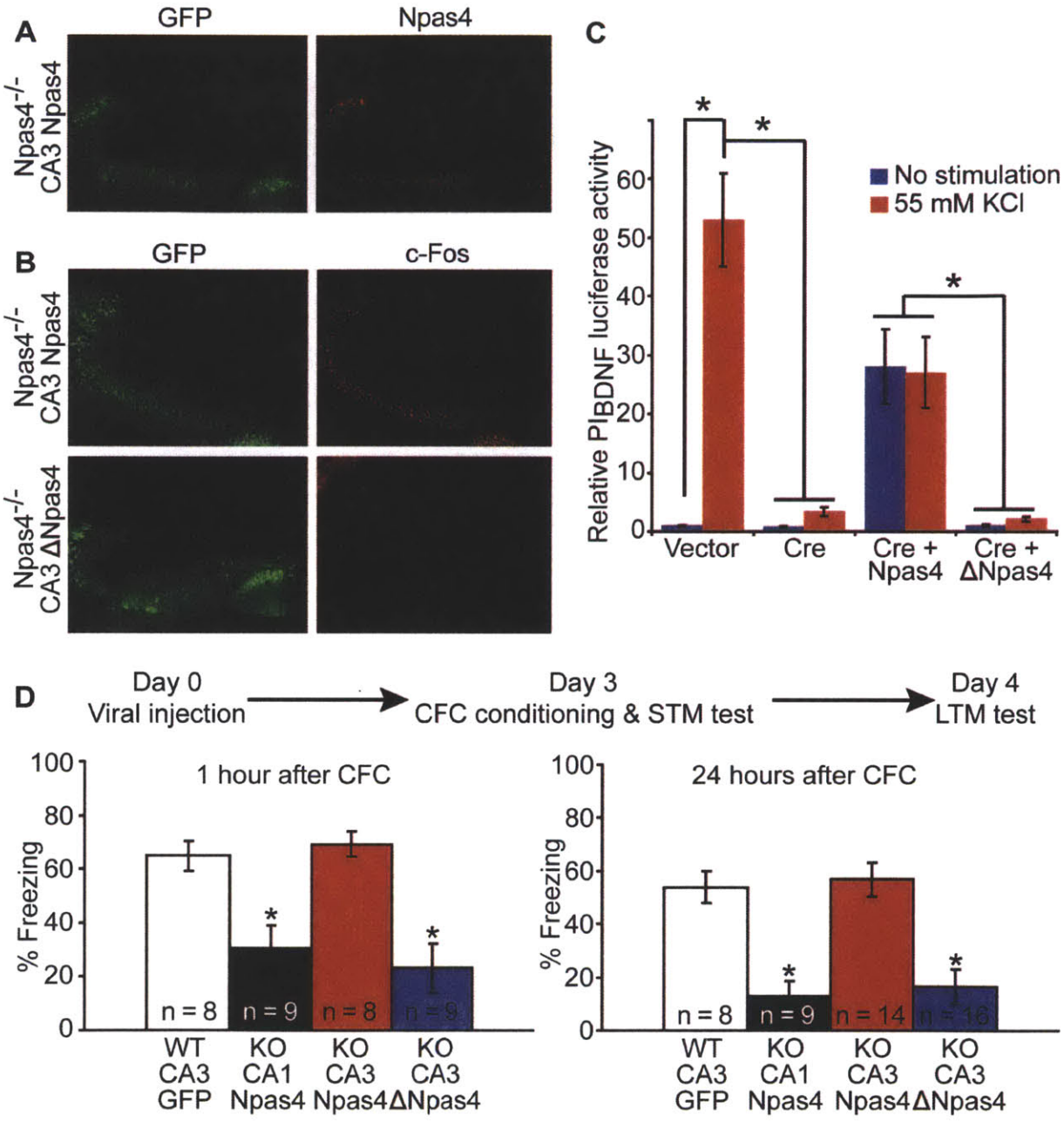

Fig. 3-12. Acute expression of Npas4 in CA3 reverses STM and LTM deficits observed in Npas4$^{-/-}$ mice.

(A) Immunostaining showing specific targeting and expression of Npas4 in CA3 of Npas4$^{-/-}$ mice using HSV-Npas4.

(B) Expression of HSV-Npas4 in Npas4$^{-/-}$ mice resulted in activation of c-Fos *in vivo*. Expression of ΔNpas4 in CA3 did not result in c-Fos activation.

(C) Expression of Npas4 resulted in activation of a PI_{BDNF} reporter. PI_{BDNF} reporter was co-transfected into Npas4$^{flx/flx}$ hippocampal cells (DIV 5) with Cre and either Npas4 or ΔNpas4. Neurons were treated with TTX (1μM) and APV (100μM) 1hr prior to KCl treatment for 6hr. Depolarization significantly induced PI_{BDNF} when Npas4 was present. Expression of Npas4, but not ΔNpas4, drives activity of PI_{BDNF} independent of KCl depolarization. *$p \leq 0.001$, two-way ANOVA followed by Holm-Sidak posthoc test. Data were compiled from 4 independent experiments each conducted in triplicate.

(D) Mice were injected on day 0 and trained in CFC 3 days post-injection. Memory tests were given 1h and 24h (day 4) after training. Npas4$^{-/-}$ mice with Npas4 injected into CA3 freeze at similar levels to Npas4$^{+/+}$ mice injected with GFP 1hr and 24hrs after training. CA1 injection of Npas4 or CA3 injection of ΔNpas4 did not rescue the memory deficit. *$p < 0.001$, one-way ANOVA followed by Holm-Sidak posthoc test.

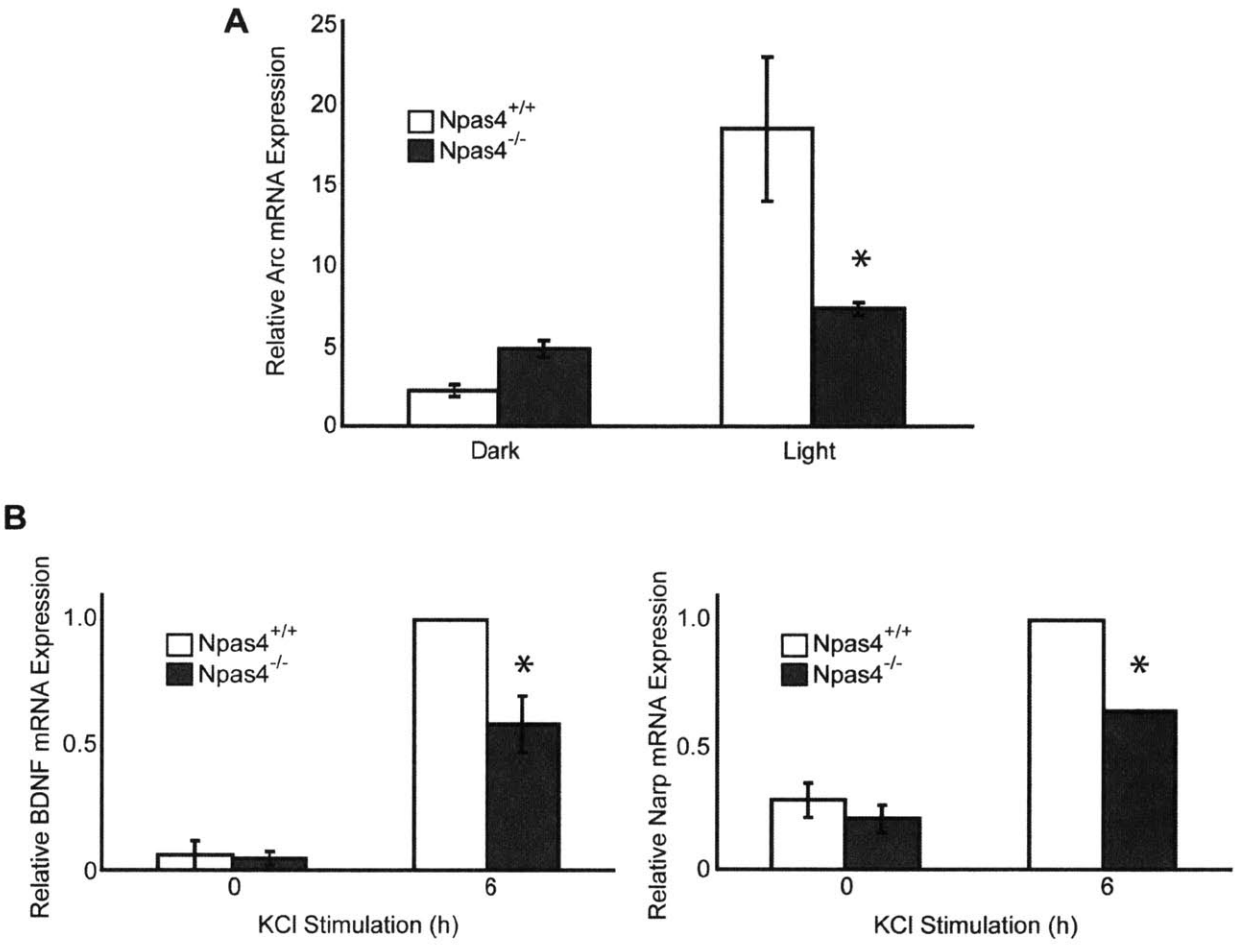

Fig 3-13. Expression of the activity regulated genes Arc, BDNF, and Narp is attenuated in Npas4 global knockouts. Mice were dark-reared for one week and then light stimulated for 1h. Induction of Arc mRNA in response to light stimulation was impaired in Npas4$^{-/-}$ mice. The expression of BDNF and Narp mRNA in response to KCL (55mM) is impaired in cortical neurons cultured from Npas4$^{-/-}$ mice.

3.4 Discussion

We have identified a genetic pathway in CA3 required for rapid encoding of hippocampal-dependent contextual memory. While several studies have identified CA3 function and output as essential to the encoding of contextual information (Kesner, 2007; Lee and Kesner, 2004; Nakashiba et al., 2008; Nakazawa et al., 2003), very little is known about the molecular mechanisms underlying this process. We found that acute deletion of Npas4 from CA3 resulted in a dramatic reduction in IEG expression and impaired contextual memory formation, and that expression of transcriptionally active Npas4 in CA3 was sufficient to restore both IEG expression and memory formation in the global knockout. Additionally, we found that expression of Npas4 in CA1 is neither necessary nor sufficient for contextual memory formation. While our viral strategy cannot target all of CA1, these findings are in line with other studies using transgenic mouse lines targeting CREB in CA1 (Balschun et al., 2003; Pittenger et al., 2002), but see (Athos et al., 2002)). Our data indicate that regulation of a transcriptional program by Npas4 is a mechanism through which CA3 supports the rapid acquisition and consolidation of contextual information.

Activity-dependent gene expression is thought to be required for LTM, but not for STM (Alberini, 2009). We observed a STM deficit in the Npas4 global knockout mice, but not in the conditional CA3 knockout (Fig. 3-6D). Although the STM impairment could be due to a developmental deficit caused by germline deletion of Npas4 in the global knockout, the rescue by acute expression of Npas4 argues against this explanation. It is possible that basal levels of neuronal activity maintain a low level of Npas4, which in turn provides a moderate level of the downstream molecules required for STM. Then,

while acute deletion of Npas4 does not reduce the level of those genes below that required for STM, chronic deletion in the global knockout results in insufficient levels to support STM.

It is intriguing that Npas4 global knockout mice function normally in auditory delay conditioning, which is hippocampus-independent but amygdala-dependent, because long-term memory formation in the amygdala is thought to be dependent on activity-regulated gene expression. We observed that the expression of Npas4 gene targets is attenuated in the Npas4 global knockout, but not to the degree that was observed in the conditional deletion (fig. 3-13), suggesting that compensatory pathways may result in some expression of target IEGs. Conceivably these pathways are sufficient to support memory formation in the amygdala, but IEG expression fails to reach a level sufficient to support the hippocampal learning required for CFC. Alternatively, or additionally, the activity-regulated genetic program induced through compensating pathways independent of Npas4, although including certain IEGs such as c-Fos and BDNF, may not contain all the components necessary for CFC. These hypotheses suggest that acute deletion of Npas4 in the amygdala will result in impairment of auditory delay conditioning.

Our findings suggest a hierarchical genetic program in which Npas4 is upstream of several activity-regulated genes. However, Npas4 itself is regulated by activity at the mRNA level and though it reaches peak expression slightly earlier than other rapidly responding IEGs (Fig. 3-6B), it is unclear whether Npas4 protein is synthesized quickly enough to initiate the first wave of IEG expression. It seems more likely that Npas4, through the recruitment of Pol II, only enhances and sustains IEG expression at later

time points, as suggested recently for Npas4-dependent regulation of BDNF transcripts (Pruunsild et al., 2011).

The mechanism by which Npas4 affects Pol II recruitment to its target genes is not immediately obvious. It could directly recruit Pol II to genomic regions in a manner similar to CBP, or it could be indirectly involved through interactions with other proteins, such as CREB (Kwok et al., 1994; Nakajima et al., 1997).

Our previous work identified a role for Npas4 in the activity-dependent regulation of inhibitory synapse development (Lin et al., 2008). Thus the genetic program controlled by Npas4 may be involved in contextual memory formation, at least in part, through the modulation of inhibitory synapses in the hippocampal circuit. Consistent with this idea, learning-induced increases in inhibitory synaptic transmission have recently been reported in the hippocampus (Cui et al., 2008; Ruediger et al., 2011).

We have focused here on the role of Npas4 in hippocampus-dependent contextual learning, but the genetic program regulated by this transcription factor likely contributes to several other experience-dependent processes. We hope to leverage the function of Npas4 in order to dissect specific neural circuits actively engaged in information processing in order to ultimately understand the molecular and cellular mechanisms underlying learning and memory.

3.5 Methods

Mice

Npas4$^{-/-}$ and Npas4$^{flx/flx}$ mutants were previously generated (Lin et al., 2008). All mouse lines were backcrossed at least 9 times into the C57Bl6 background (Charles River Laboratory). Heterozygous mice were bred to produce Npas4$^{-/-}$ and Npas4$^{+/+}$ littermates and Npas4$^{flx/flx}$ mice were bred as homozygotes. Mice were weaned at postnatal day 20, housed by sex in groups of 3-5, and used for experiments at 8-12 weeks of age. For gene expression and IHC experiments mice were housed individually for one week prior to conditioning. All mice were housed with a 12 hour light-dark schedule and received food and water *ad libitum*. Animal protocols were performed in accordance with NIH guidelines and approved by the Massachusetts Institute of Technology Committee on Animal Care.

Fear conditioning. On day 1, mice (8-12 weeks old) were trained in one of the following conditions:

Contextual Fear Conditioning. Mice were placed in the chamber, allowed to explore for 58s and then given three 2s 0.55mA footshocks at 58s intervals. Following the last shock mice were left in the chamber for 1 minute and then returned to their home cage.

Auditory Delay Conditioning. Mice were placed in the chamber, allowed to explore for 1 minute and then given three tone stimuli (85dB, 20s, 2.8kHz) at 58s intervals that co-terminated with a 2s 0.55mA footshock. Following the last shock mice were left in the chamber for 1 minute and then returned to their home cage.

Context Only. Mice were placed in the training chamber for 4min and then returned to their home cage.

Immediate Shock. Mice were placed in the training chamber, immediately given a 6s shock (0.55mA) and then returned to their home cage.

5min, 1hr or 24hr after training, mice were returned to the conditioning chamber for 4min to test memory recall. Separate sets of subjects were used at each timepoint to prevent extinction effects. Memory for the context was measured by recording freezing behavior, defined as the total absence of movement aside from that required for respiration. Memory for the tone was measured by recording freezing in a novel context during presentation of the tone. Training and testing sessions were video recorded and behavioral scoring was conducted by scorers blind to the experimental genotypes. In every case, the chamber was cleaned with 70% ethanol between subjects. Subjects used for gene expression or immunohistochemical analysis were sacrificed at various time points after the conditioning. Genotypes were compared using a one-way ANOVA followed by Holm-Sidak posthoc test or Student's t-test.

Elevated Plus Maze

The elevated plus maze consisted of two open arms, two closed arms, and a center. Animals were always placed in the same orientation and observed for 5min. Genotypes were compared using Student's t-test.

Open Field Activity

Open field activity in a novel context was measured using a Versamax Activity Monitor. One hour prior to testing mice were placed in the testing room to habituate to the room. Mice were placed in the open field and were monitored for 10 minutes.

Foot Shock Sensitivity Assay

Assay was performed similar to Alexander et al 2009. Responses to a range to footshocks (0.1mA to 0.7mA, 0.1mA steps) were measured by assigning a numerical value by a blinded scorer. 0 – no response, 1 - move, 2 – flinch, 3 – run, 4 – jump, 5- maximum response.

Immunohistochemistry

Mice were overdosed with avertin (1000mg/kg, 1.25% 2,2,2-tribromoethanol and 2.5% 2-methyl-2-butanol) and perfused with 4% paraformaldeyde in PBS. Brains were removed and post-fixed for 12hr then cryo-protected in 30% sucrose overnight. Subsequently, brains were immersed in OCT-Tissue Tek, flash frozen on dry ice, and sectioned on a cryostat at 50μm thickness. All sections were blocked for 1hr at room temperature in a solution containing 0.3% Triton X-100, 0.2% Tween-20, 3.0% BSA, and 3.0% goat serum then incubated with primary antibody overnight at 4°C. The next day, sections were rinsed in PBS and incubated in secondary antibody for 1hr at room temperature. Sections were mounted on Superfrost, slides.

The following antibodies were used: c-Fos (rabbit, 1:500, Santa Cruz sc-52), Cre (mouse, 1:100, Millipore MAB3120), GFP (chicken, 1:1000, Aves GFP-1020), NeuN (mouse, 1:1000, Millipore MAB377), Parvalbumin (mouse, 1:1000, Sigma P3088), and VGAT (rabbit, 1:1000, Synaptic Systems 131002). The Npas4 antibody (rabbit, 1:10,000) was produced and validated as previously described (Lin et al., 2008).

Viral Vectors

HSV vectors were prepared as previously described (Barrot et al., 2002; Han et al., 2007; Han et al., 2009). Npas4, ΔNpas4, or Cre cDNA were cloned into the bicistronic

p1005+ vector and driven by the constitutive promoter immediate-early gene IE 4/5. EGFP was expressed from a CMV promoter.

Viral Injection Surgery

Mice were anesthetized with avertin (250mg/kg, 1.25% 2,2,2-tribromoethanol and 2.5% 2-methyl-2-butanol) and monitored for depth of anesthesia throughout the procedure. Animals were secured in a stereotax (Kopf Instruments) and the skull was exposed. After resection, holes were drilled bilaterally overlying dorsal CA3 (AP -2.0, ML +/- 2.3; DV -2.3) or CA1 (AP -2.0; ML +/- 1.6; DV -1.4) using bregma as a reference point. Injections consisted of 1µL of virus ($1.5x10^8$ infectious units/mL) delivered at a rate of 50nL/minute and the needle was left in place for 20min post-injection. Incision sites were closed using Vet-Bond glue (3M) and treated with topical antibiotic and anesthetic. Mice were given i.p. injections of Buprenex (1mg/kg) and allowed to recover for 3d before behavior or expression experiments were conducted. Following testing, GFP-immunoflourescence was used to verify proper targeting of the virus. Only mice with bilateral expression of virus into the target structure (CA3 or CA1) were included for analysis. Multiple sections were taken from each animal and only animals with at least 50% of the target structure hit in the section with the highest GFP expression were included. Additionally, any mice exhibiting GFP expression within hippocampus, but outside of the target region were excluded. In total, five mice from the Cre condition and six mice from the rescue condition were removed from analysis following histological verification.

Dissociated Neuron Culture

Dissociated cortical and hippocampal neurons were prepared from P1 mouse pups and maintained in a humidified incubator with 5% CO_2 at 37˚C, as previously described (Lin et al., 2008). Cultures were maintained in Neurobasal A medium supplemented with B27 (Invitrogen) and glutamine. Neurons were plated at 100,000 per well in a 24 well plate, 1,000,000 per well in a 6 well plate, or 8,000,000 per 10cm plate. Plates were coated with poly-D-lysine. Neurons were transfected using calcium phosphate precipitation on DIV5/6. For viral transfection, neurons were infected overnight on DIV 6 with 1µL of $1.5x10^8$ infectious units/mL per 1,000,000 cells and collected in the morning on DIV 7. For stimulation experiments neurons were depolarized for 1h for qPCR, 2h for western blots and ChIP, and 6h for luciferase with 55mM KCl. Neurons were treated for 1h (qPCR), 2h (western blots), or 6h (luciferase) with the following drugs: forskolin (10µM), BDNF (50ng/ml), NT3 (50ng/ml), NT4 (50ng/ml), NGF (100ng/ml), or EGF (100ng/ml). Prior to stimulation for luciferase neuronal activity was block for 1h or overnight with TTX (1µM) and APV (100µM).

RNA Extraction and Quantitative PCR (qPCR)

RNA was purified from dissociated neurons (1,000,000 neurons/prep) or dorsal hippocampal tissue (bilateral dorsal hippocampus/prep). 1000ng of RNA was reverse transcribed to cDNA using iScript Reverse Transcriptase and qPCR was performed using SybrGreen Supermix on an iQ5 thermal cycler. Primers were verified with standard curves to ensure reliability. Optimal primer pairs were then used to evaluate levels of cDNA samples. Genes of interest were normalized to Gapdh and presented as fold changes over baseline using the delta-delta CT method (Livak and Schmittgen, 2001). “n” represents the number of mice used. Data were complied from independent

experiments each conducted in triplicate. For fear conditioning experiments a one-way ANOVA followed by a Holm Sidak posthoc test was used to compare expression at different time points after training or different training procedures. For culture experiments a two-way ANOVA followed by a Holm Sidak posthoc test was used to compare the condition (No Stim or KCl) and virus (Uninfected, GFP, or Cre).

Primers:

Npas4: forward 5'- CTGCATCTACACTCGCAAGG-3', reverse 5'-GCCACAATGTCTTCAAGCTCT-3'

c-Fos: forward 5'- ATGGGCTCTCCTGTCAACACAC-3', reverse 5'-ATGGCTGTCACCGTGGGGATAAAG-3'

Arc: forward 5'- TACCGTTAGCCCCTATGCCATC-3', reverse 5'-TGATATTGCTGAGCCTCAACTG-3'

Zif268: forward 5'- TATGAGCACCTGACCACAGAGTCC-3', reverse 5'-CGAGTCGTTTGGCTGGGATAAC-3'

GAPDH: forward 5'- CATGGCCTTCCGTGTTCCT-3', reverse 5'-TGATGTCATCATACTTGGCAGGTT-3'

Chromatin Immunoprecipitation (ChIP)

For *in vitro* experiments eight million cortical neurons were used per ChIP. For *in vivo* experiments bilateral hippocampi were used per ChIP. Samples were fixed in 1% formaldehyde, quenched with 2M glycine, and washed with cold PBS + cocktail protease inhibitor tablets. Samples were lysed in 10% SDS lysis buffer then sonicated (10% output, 5s on/30s off repeated 24 times). Samples were precleared with agarose beads for 1h at 4°C, and then supernatant was incubated with primary antibody

overnight at 4˚C. The next day, samples were incubated with agarose beads for 1hr at 4˚C. Beads were washed 2 times each with low salt, high salt, LiCl, and TE solutions. Samples were eluted in Elution Buffer then reverse crosslinked at 65˚C for at least 6hr. Samples were purified using a PCR Purification Kit (Qiagen) and processed by qPCR. "n" represents the number of mice used. Each qPCR was conducted in triplicate and normalized to a negative control region. Genotypes were compared using Student's t-test.

The following antibodies were used: RNA Polymerase II (mouse, 1:500, Convance 8WG16), and the Npas4 antibody (rabbit, 1:500).

Primers:

BDNF promoter I: forward: 5′-GTGCCTCTCGCCTAGTCATC-3′, reverse: 5′-AGGGAACAACTGCGTGAATC-3′

c-Fos promoter: forward: 5'-GCCCAGTGACGTAGGAAGTC-3', reverse: 5'-GTCGCGGTTGGAGTAGTAGG-3'

c-Fos E2: forward: 5'- CACAGATGACATCGCTCCAT-3', reverse: 5'-GCCGACGTCCTGACACTAA-3'

ß-actin promoter: forward: 5'- CCCATCGCCAAAACTCTTCA-3', reverse: 5'-GGCCACTCGAGCCATAAAAG-3'

Negative control region: forward 5'- GGACAATTCAACCGAGGAAA-3', reverse 5'-TGAACTGGTTTGGTGTGCTC-3'

Luciferase Assay

Plasmids were transfected on DIV 5/6. TK-Renilla, which expresses renilla luciferase, was co-transfected in every experiment to control for transfection efficiency. On DIV 7

cells were depolarized (55mM KCl, 6hr) and lysed in passive lysis buffer. Firefly luciferase levels were measured and normalized to renilla luciferase levels. Data were compiled from separate experiments each conducted in triplicate. Two-way ANOVA followed by a Holm-Sidak posthoc test was used to compare conditions (no stimulation vs KCl depolarization) and plasmids (pcDNA3 vs Cre).

Gel Electrophoresis and Immunoblotting

Samples were lysed in Laemmli buffer, separated by SDS-PAGE, and transferred to 0.45μm nitrocellulose membrane. Following transfer, the membrane was blocked with 10% nonfat milk in TBST for 1hr at 25°C and probed with specific antibodies overnight. The following day membranes were washed 3 times with TBST and incubated with horseradish peroxidase-labeled secondary antibody for 1h at room temperature. After 3 more washes, the membranes were incubated in ECL Plus reagent for 5min and then developed. The following antibodies were used: c-Fos (rabbit, 1:1000, Santa Cruz sc-52 for cultured neurons), c-Fos (rabbit, 1:1000, Synaptic Systems 226033 for tissue samples), phosphor-serine 133 CREB (mouse, 1:2000, Upstate) and the Npas4 antibody (rabbit, 1:10,000).

Statistical Analysis

All data are shown as mean ± s.e.m. Data were analyzed with one-way, two-way, or repeated measure ANOVA followed by Holm-Sidak posthoc or Dunnett's post hoc tests. Student's *t*-test was used when two groups were compared.

3.6 References

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Chapter 4

Molecular Interrogation of Active Neural Circuits

Conception: Kartik Ramamoorthi and Yingxi Lin
Creation of construct: Kartik Ramamoorthi
Chip-Seq Code: Kartik Ramamoorthi
Luciferase validation: Kartik Ramamoorthi
Initial in vivo validation: Kartik Ramamoorthi
Cloning of DD/TTA plasmids: Kartik Ramamoorthi
Luciferase testing of DD: Kartik Ramamoorthi and Colleen Schneider
Luciferase test of d2TTA: Kartik Ramamoorthi
Creation of Tag backbone: Kartik Ramamoorthi
Virus Production: Virovek (high titer)/Kartik Ramamoorthi (low titer)
In vitro validation of virus: Kartik Ramamoorthi (infection, collection, cell counts) and Riana Bo Lu (cell counts), Lauren Blachorsky (cell counts)
Live imaging Timer analysis: Kartik Ramamoorthi
Surgeries: Colleen Schneider (60%), Casper Gotsche (30%), and Kartik Ramamoorthi (10%)
Electrophysiology: Andrew Young
Behavioral Activation of Reporter: Kartik Ramamoorthi, Colleen Schneider, Andrew Young
SHELAC: Andrew Young and Kartik Ramamoorthi
Cortex Analysis: Andrew Young and Kartik Ramamoorthi
Behavior Analysis: Kartik Ramamoorthi
Histology: Kartik Ramamoorthi (collect, staining, and cell counts), Colleen Schneider (collect, staining, and cell counts) and Riana Bo Lu (staining and cell counts)
Drosophila: Robin Fropf (creation of fly and luciferase assay) and Kartik Ramamoorthi (creation of construct)
Rat: Mike Baratta (injections and behavior)
Mouse Husbandry: Kartik Ramamoorthi

4.1 Summary

A fundamental goal in neuroscience is to understand mechanisms underlying the ability to create memories from sensory experience. While large structures such as the hippocampus are known to be critical for certain types of learning, memories are ultimately thought to be represented in sparsely distributed neuronal ensembles within these larger structures. Currently, there are few tools that allow for the identification and manipulation of these ensembles, which has limited our understanding of the molecular and cellular processes underlying learning and memory. We have previously reported that the activity-regulated transcription factor Npas4 is selectively induced in a sparse population of CA3 and required for contextual learning. The goal of this research proposal is to determine if neurons expressing activity regulated genes following contextual learning comprise a memory circuit in CA3. Using a genetic reporter that is activated by Npas4, we examined and manipulated the properties of neurons activated by behavioral experience. We believe that the tools developed for this project can provide a major advancement in the field, and will allow researchers to target any neural circuit activated by experience in a variety of species.

4.2 Background

Long-term memory formation is dependent on *de novo* mRNA and protein synthesis within neuronal structures such as the hippocampus and amygdala (Alberini, 2009a). Electrophysiological and cellular imaging studies suggest that only subpopulations of neurons within these structures actively participate in a given memory and these neurons may represent a "memory trace" (Guzowski, 2002; Han et al., 2007; Wilson and McNaughton, 1993). This suggests that memories are held in sparsely distributed neuronal ensembles in which the cellular and molecular changes required for memory formation occur. However, gaining access to these cell populations remains a challenge.

The expression of immediate early genes (IEGs) has been used extensively as molecular correlates for neuronal activity and plasticity *in vivo*. Several studies have exploited the specific induction of these genes to identify neuronal ensembles activated by behavioral experience (Guzowski et al., 1999, 2001a; Guzowski et al., 2001b; Guzowski and Worley, 2001a). Furthermore, IEG knockout mice generally exhibit profound impairments in long-term memory formation (Jones et al., 2001; Plath et al., 2006). Together, these findings have led to the hypothesis that IEG expression may serve as a marker for neurons actively engaged in information processing and memory formation.

We recently found that the transcription factor Npas4 is rapidly and selectively induced by neuronal activity *in vitro*. Npas4 mRNA was selectively induced by depolarization and Ca^{2+} influx, but not by activators of several signaling pathways that induce other IEGs such as c-Fos, Arc, and Zif268. This specificity suggests that Npas4

may regulate a genetic program important for experience-dependent processes such as learning and memory. In order to examine this, we trained mice in a hippocampus-dependent contextual fear conditioning (CFC) paradigm and examined Npas4 mRNA expression in dorsal hippocampus (DH). We found that Npas4 mRNA was induced by CFC and context exposure (C), but not by the shock-only condition (S). Learning-induced Npas4 protein was localized to a subpopulation of neurons in the CA3 subregion of the hippocampus, a region known to be required for rapid encoding of one-trial experiences (Kesner, 2007; Kubik et al., 2007; Nakashiba et al., 2009; Nakashiba et al., 2008; Nakazawa et al., 2002; Nakazawa et al., 2003)

We next examined the requirement of Npas4 for contextual memory formation. Npas4 knockout mice ($Npas4^{-/-}$) exhibited profound impairments in long-term contextual memory recall, suggesting that the learning-induced expression of Npas4 in CA3 is required for contextual memory formation. Consistent with this hypothesis, acute deletion of Npas4 in CA3, but not CA1, resulted in long-term contextual memory deficits. Restoring Npas4 in CA3, but not CA1, in Npas4 global knockout was sufficient to reverse the contextual memory deficit observed in these mice. Furthermore, expression of a transcriptionally-inactive version of Npas4 (ΔNpas4) in CA3 failed to rescue the contextual memory deficit observed in the global knockouts, suggesting that the genetic program regulated by Npas4 is required for long-term memory formation (Ramamoorthi, 2011). Npas4 has also been shown to be re-expressed during memory recall and important for reconsolidation (Ploski et al., 2011). Outside of learning-related neuroplasticity, Npas4 has been show to play a critical role in visual cortical plasticity

(Maya-Vetencourt et al., 2012). Together these findings suggest that Npas4 is required for a variety of experience-dependent neuroplastic events.

Our data suggest that Npas4 is induced by context learning and is necessary in the CA3 region of dorsal hippocampus for context memory formation. Due to the unique synaptic connectivity of CA3 pyramidal neurons, several computational and behavioral studies suggest that CA3 is required for contextual learning (Kesner, 2007). Consistent with this notion, manipulation of CA3 neurons using lesion or pharmacological techniques has consistently been reported to impair learning and memory, suggesting that plasticity within CA3 may be required for contextual learning and memory (Daumas et al., 2004; Gilbert and Kesner, 2006; Gold and Kesner, 2005; Kesner, 2007; Kesner et al., 2008; Kesner and Warthen, 2010; Lee and Kesner, 2004; Rolls and Kesner, 2006). Gaining access to cell populations activated by experience will lead to a far greater understanding of the neural substrates of learning and memory.

Probing for immediate early gene expression using techniques such as immunohistochemistry (IHC) or in situ hybridization has allowed for the identification of neurons activated soon after learning, but the transient expression of IEGs prevents labeling neurons activated by two separate behavioral experiences. Advanced *in situ* techniques, such as catFISH (Guzowski and Worley, 2001b), allow for imaging neuronal activity induced by two distinct behavioral experiences, but the experiences must be separated by a very short time window (less than 20 minutes) (Guzowski and Worley, 2001b). Genetically encoded calcium indicators allow for the visualization of active neural circuits, but the timescale (milliseconds to seconds) for this reporter is far too fast for long-term analysis of neural circuit dynamics (Chen et al., 2012; Tian et al., 2009).

Several recently generated transgenic mouse lines allow for long-term labeling of activated neuronal populations but are focused on single genes (c-Fos or Arc) and/or impair endogenous expression of activity-regulated genes (Guenthner et al., 2013; Reijmers et al., 2007). See Table 5-1 for details. Therefore, in order to gain access to neuronal populations activated by experience, we developed a novel reporter system that integrates the actions of several activity-regulated transcriptional regulators, permitting us to identify active neurons in multiple neural circuits and species.

4.2.1 Motivation to Create a Novel Reporter System

The motivation to develop a novel reporter system stemmed from the realization that current technology was too limited for the experimental parameters we wanted to test. Therefore we set out to create a genetically-encodable reporter system that exhibited the following properties: 1) induced by neural activity, 2) low expression in control conditions, 3) robust expression in induced conditions to allow for strong endogenous labeling, 4) small DNA elements compatible with AAVs and 5) a platform design so that promoters and transgenes could easily be swapped to address specific experimental questions. An additional major focus in our development plans was to create a technology that could be applied to species other than the mouse. We

Technique	Pro	Con
Immunohistochemistry	Straightforward brain-wide labeling of proteins	Single snapshot of activity. No potential for perturbation experiments, in/ex vivo electrophysiology, or long-term analysis
CatFISH	Allows for visualizing multiple behavioral experiences	Very short time course (5-20 minutes) and dependent on in situ hybridization. No potential for perturbation experiments, in/ex vivo electrophysiology, or long-term analysis
In vivo electrophysiology	Detailed analysis of synaptic and circuit activity	Low throughput and difficult to target specific cell populations
Genetically encoded calcium or glutamate indicators	Allows for visualization of active neurons on a rapid time scale. Currently the best tool to identify active neural circuits	Does not allow for perturbation experiments and very rapid timescale
TetTag and TRAP mice	Allows for visualization and perturbation	Limited to single genes (Fos or Arc) and mice. TRAP mice are heterozygous for Arc and Fos

Table 4-1. Current Technology. Current molecular tools used to study active neural circuits.

reasoned that a technology that could fulfill these parameters would be a powerful tool for the neuroscience community as a whole.

4.3 Results

We started our development process by cloning out the promoter regions of several activity-regulated genes. We selected these candidates (*Arc, Npas4, c-Fos, and BDNF)* based on their known ability to express during conditions of high synaptic activity (Alberini, 2009b). The size of each promoter region was determined by including highly conserved regions of DNA, which are generally thought to indicate critical regulatory elements (Santini et al., 2003). Additionally, we included two reporters that consisted of small conserved DNA binding motifs for the transcription factors CREB and MEF2 upstream of a minimal promoter.

We created an *in vitro* assay to monitor genetic reporters of neuronal activity in cultured hippocampal neurons by using promoter and enhancer elements of *Arc, Npas4, c-Fos, BDNF, CREB, and, MEF2,* to drive the expression of luciferase. Following transfection of these plasmids cultured neurons were treated with 35mM potassium chloride to induce membrane depolarization and high levels of synaptic activity (Fig. 4-1). Each of the reporters responded to membrane depolarization. However, the levels of induction were different across the plasmids. The reporters with the best signal-to-noise ratio (SNR) were also the largest in size (*Arc, Npas4,* and *c-Fos*), restricting their use in viral vectors. While the smaller reporters (*CREB and MEF2)* were induced by neural activity, their level of expression was too low for robust *in vivo* use. To overcome this limitation, we set out to develop a synthetic reporter construct containing regulatory

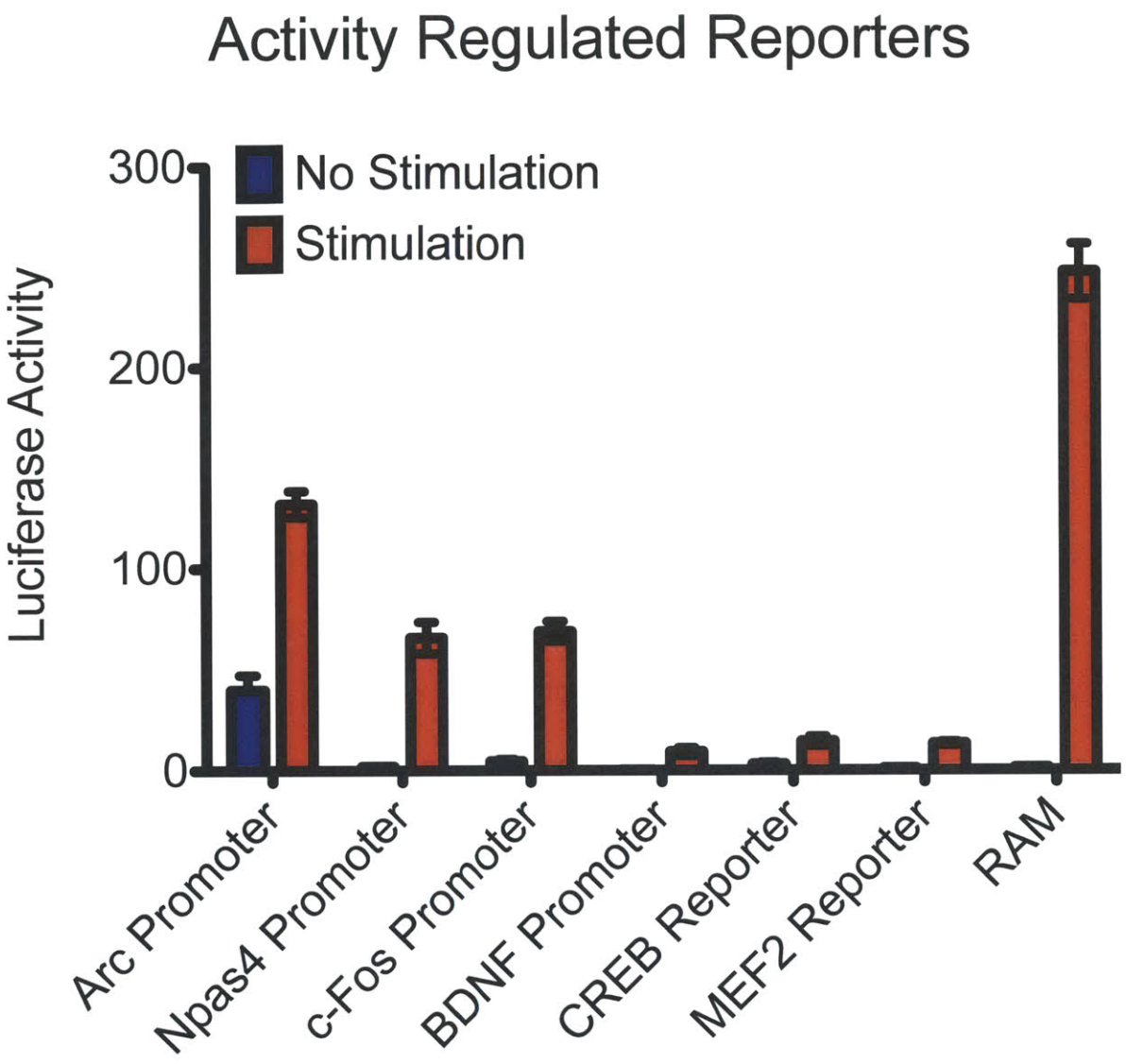


Fig 4-1. Characterization of RAM *in vitro*. Cultured hippocampal neurons were transfected on DIV 5 and stimulated on DIV 7 for 6h with 35mM potassium chloride to induce membrane depolarization. All reporters responded to treatment (Arc ~9kb, Npas4 ~10kb, c-Fos ~2kb, BDNF ~0.5kb, CREB ~0.2kb, MEF2 ~0.2kb, and RAM ~0.2kb, $p < 0.0001$), with the larger reporters showing the most robust response to stimulation. RAM exhibits a dramatically higher response to stimulation in comparison to all other reporters ($p<0.001$). N = 3 independent cultures run in triplicate. Two-way ANOVA with Bonferroni post test.

elements from activity-regulated genes, with a focus on keeping the size of the construct minimal.

4.3.1 Creation of RAM

To rationally design our reporter system, we used a genome-wide chromatin immunoprecipitation sequencing (ChIP-Seq) study to understand the dynamics of activity-regulated transcription factors in neurons (Kim et al., 2010). We generated a custom Python script to parse this dataset and found that under conditions of high neuronal activity Npas4 binds to several activity-regulated genes and co-localizes with major transcriptional regulators such as CBP, SRF, and RNA polymerase II at these sites. Furthermore, the co-localization of Npas4 and CBP is dramatically higher on genes such as *Npas4*, *c-Fos*, and *NR4A1* in comparison to genes that are not regulated by neuronal activity (Fig 4-2). The pattern of Npas4 binding indicates that a reporter based on an Npas4 binding sequence may act as a robust marker of neuronal activity, integrating activity from several regulatory factors. These characteristics would allow us to gain access to neuronal populations of interest without restricting focus to a single gene. Importantly, the bHLH-PAS domain of Npas4, which plays a major role in the protein's ability to regulate transcription, is highly conserved in rats, Drosophila, and C. elegans (Ooe et al., 2004; Ooe et al., 2007) suggesting that a reporter based on the binding of Npas4 could be applicable in a variety of model systems.

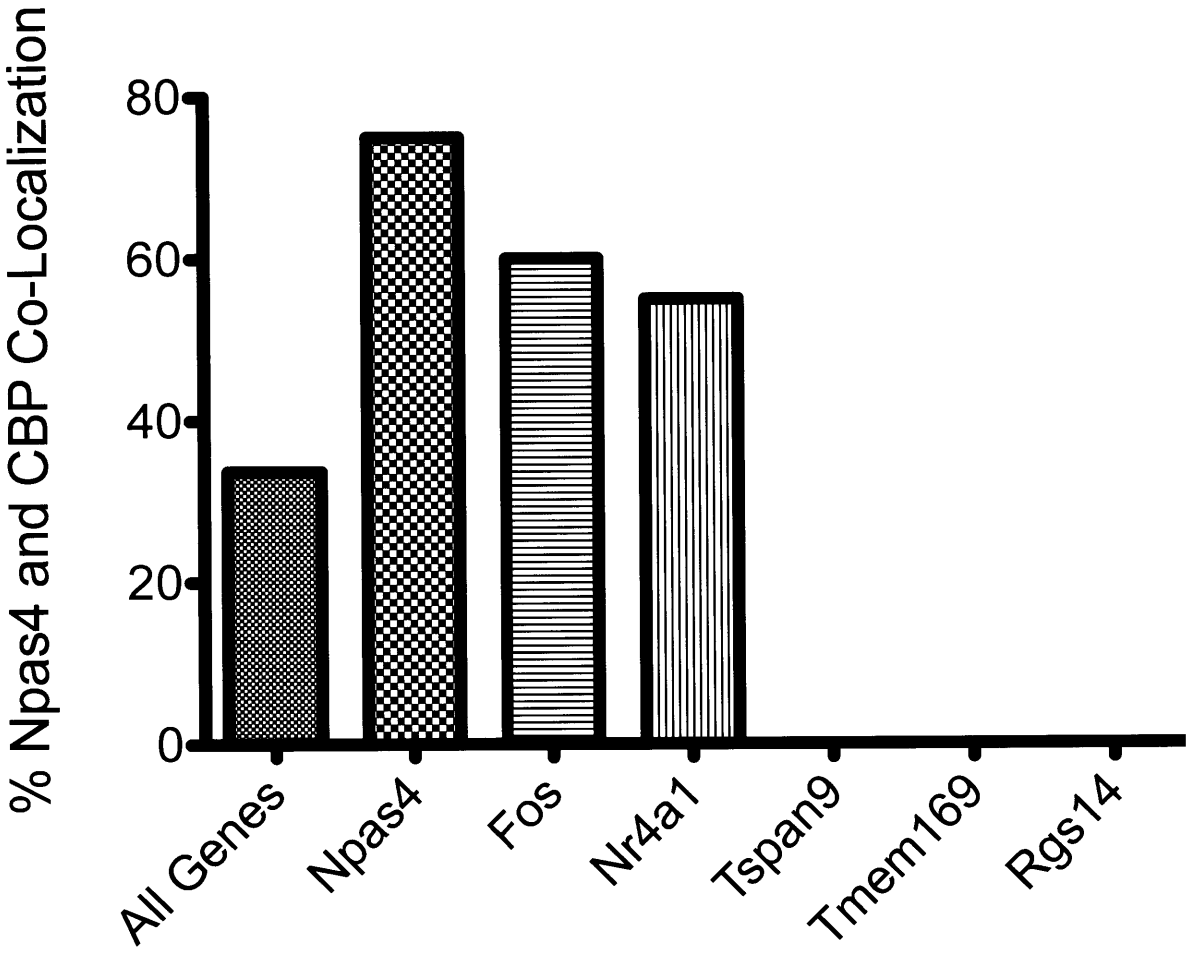


Fig 4-2. Characterization of Npas4 and CBP co-localization following conditions of high neuronal activity. Npas4 co-localizes with CBP at approximately 30% of Npas4 binding sites across the genome. Co-localization dramatically increases at activity-regulated genes (Npas4, Fos, Nr4a1) in comparison to example genes that do not respond to depolarization (Tspan9, Tmem169, and Rgs14). Custom Python script was used to mine data from (Kim et al., 2010).

We developed a synthetic activity reporter referred to as RAM (**R**obust **A**ctivity **M**onitor) by coupling four repeats of a putative binding sequence of Npas4 (Ooe et al., 2004) with the c-Fos minimal promoter. RAM-Luciferase exhibited a dramatically improved SNR in comparison to other activity-regulated reporters, at a fraction of the size (0.2kb vs. 2kb – 10kb, Fig 4-1). Expression of RAM-EGFP *in vitro* was consistent with this observation, showing robust expression following depolarization without requiring an antibody for visualization (Fig 4-3).

Encouraged by these results, we generated an AAV vector that contained RAM-EGFP and could easily be delivered to brain regions of interest. Because the expression of activity-regulated genes is dramatically increased in the hippocampus by novel context exposure (Ramamoorthi, 2011), we initially tested AAV-RAM-EGFP in this setting. However, we observed high *in vivo* reporter expression even under control conditions. Within the hippocampus, percentages of AAV-RAM expressing cells were indistinguishable between control and novel context treatments (Fig 4-4). Pharmacologically-induced seizures were able to induce the reporter above control conditions. Therefore, while AAV-RAM-EGFP exhibited some activity-dependent properties in this form, it lacked the sensitivity to detect experience-induced changes in neural circuit function.

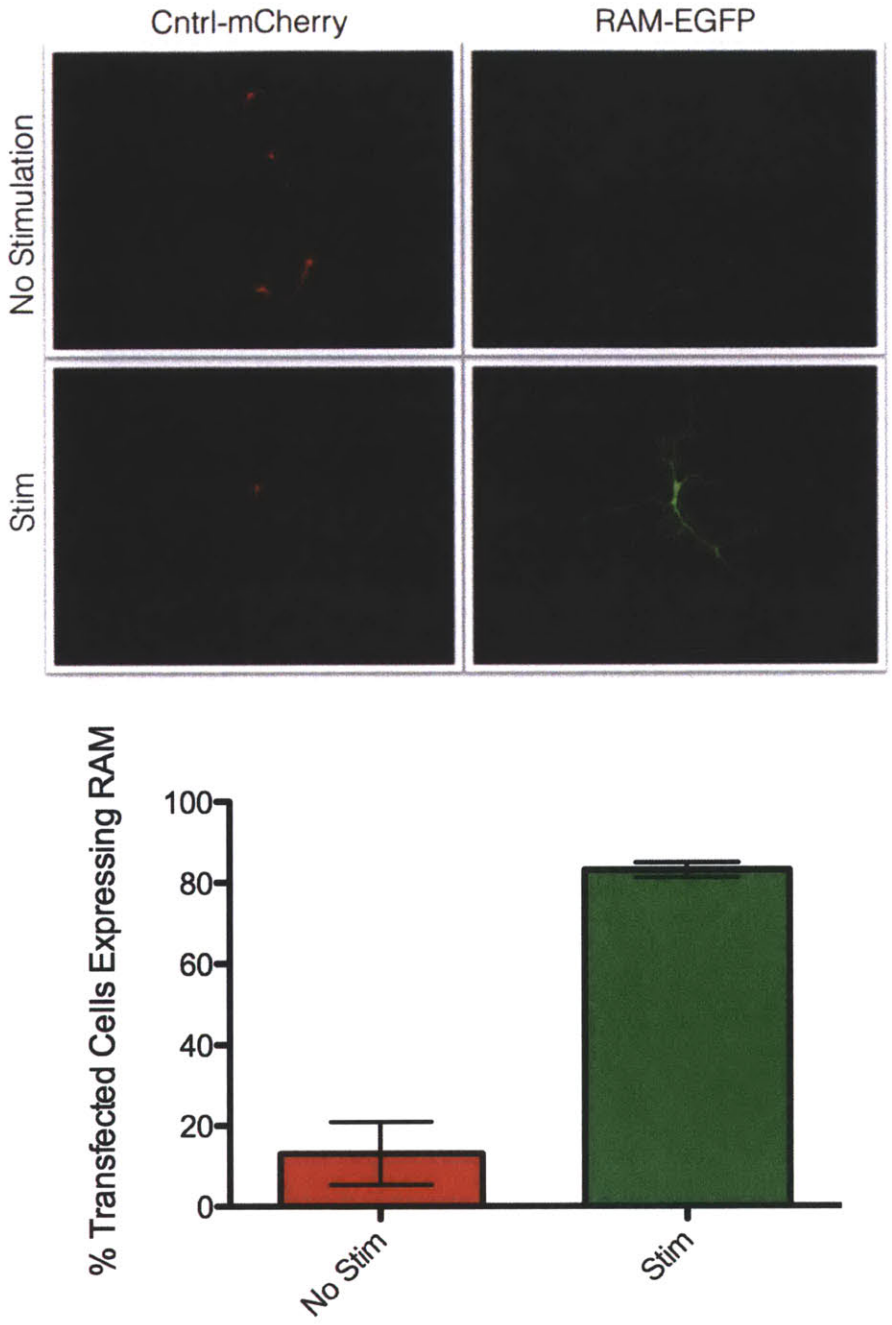


Fig 4-3. Characterization of RAM-EGFP in vitro. Top: RAM-EGFP expression is highly upregulated by 35mM KCl treatment. **Bottom**. Prior to stimulation approximately 10% of transfected neurons express RAM-EGFP. Following stimulation 80-90% of transfected neurons are EGFP positive (N = 2 independent cultures/ ~45 neurons per condition). Unpaired t-test, ($p < 0.05$).

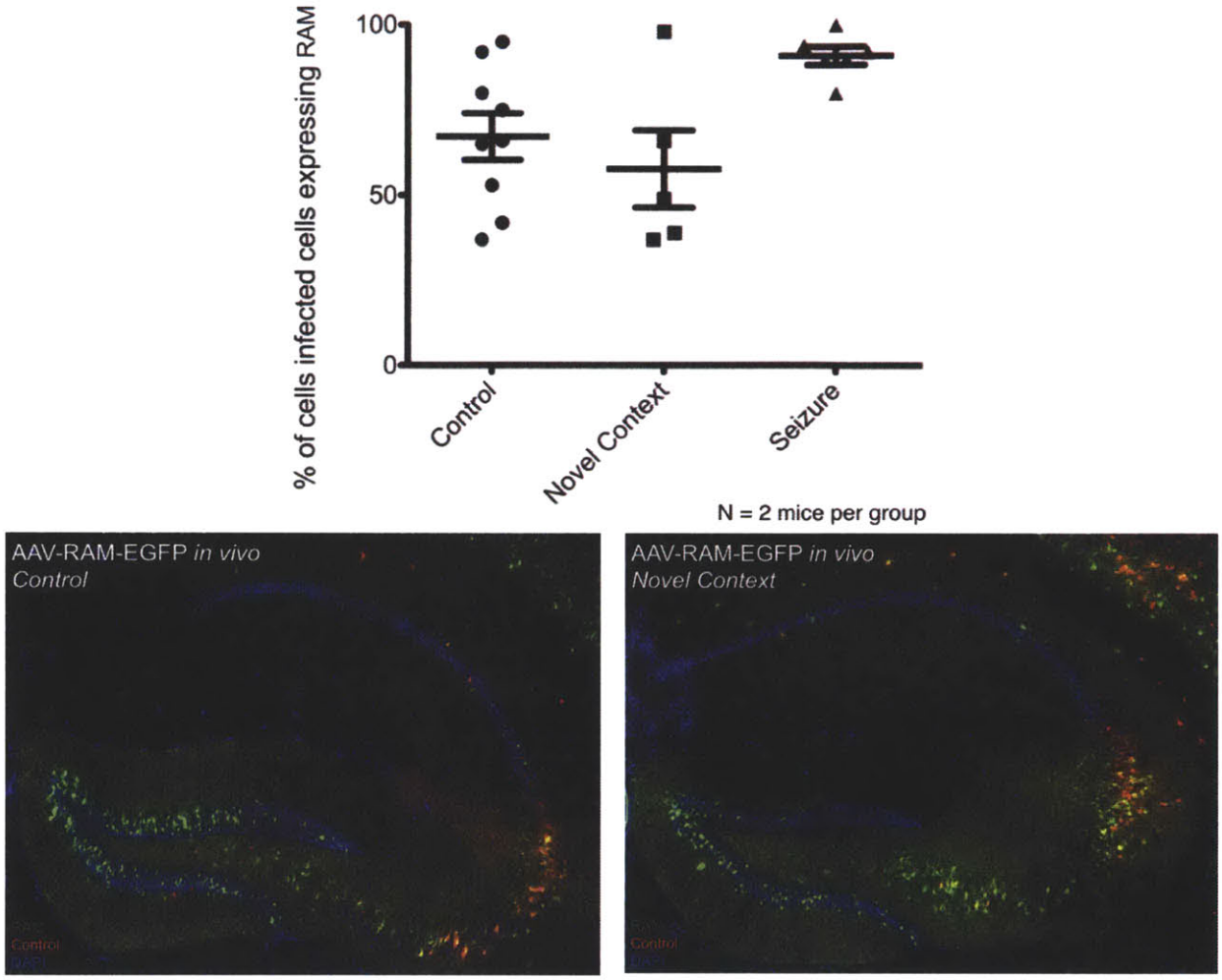


Fig 4-4. Characterization of RAM-EGFP *in vivo*. Top: Expression of RAM-EGFP following novel context exposure was not higher than control conditions ($p > .05$). Seizure activates the reporter above novel context conditions ($p < .05$). **Bottom:** CA3 and DG labeling of RAM-EGFP following control or novel context exposure. RAM-EGFP: Green, CMV-mCherry: Red, DAPI: Blue. N = 2 mice per condition. One-way ANOVA with Bonferroni post test.

4.3.2 Modifications of Gene Regulatory Systems

To reduce the baseline activity of RAM *in vivo*, we reasoned that it would be necessary to regulate its expression such that behavioral experiences prior to our experimental manipulation would not result in labeling. Several gene regulatory systems currently exist that allow for control of the expression or stabilization of transgenes of interest. The first system tested was the destabilized domain (DD) system developed in the laboratory of Tom Wandless (Iwamoto et al., 2010). The DD allows for inducible stabilization by fusing the E. coli dihydrofolate reductase (DHFR) enzyme to a protein of interest (POI). The DHFR domain results in proteasomal degradation of the POI. In the presence of trimethoprim (TMP), a potent inhibitor of DHFR, proteasomal degradation is prevented and the expression of the POI is maintained. In addition to DD we also tested the Tet-Off system, which allows for control of transgene expression through transcriptional regulatory mechanisms. In this system, a transgene of interest is expressed under control of a TTA-responsive promoter. Transcription from this promoter is dependent on the binding of the tetracycline transactivator (TTA) and can be blocked by the antibiotic doxycycline.

We simultaneously tested the sensitivity of both systems using a stringent *in vitro* assay. A highly sensitive way to test leakiness from either system involves controlling the expression of Cre-recombinase-dependent activation of luciferase. Minute amounts of Cre recombinase are sufficient to induce recombination and subsequent high expression of luciferase, therefore making this assay highly responsive to any amount of Cre expression. We transfected cultured neurons with a constitutive CMV promoter driving the expression of TTA, a TTA-responsive promoter driving the expression of

Cre-DD, and a lox-stop-lox luciferase reporter as a readout of Cre activity (Fig 4-5, top). Cultures were treated with either 1) Dox alone, OFF condition, 2) Dox and TMP, Leak Dox condition, 3) Nothing, Leak TMP, or 4) TMP alone, ON condition. These four conditions allowed us to test the leakiness of the Dox system (condition 2) and the leakiness of the DD system (condition 3). We found that in comparison to background levels of luciferase expression (LSL condition), both the Dox system and DD system exhibited significant luciferase expression. However, we found that be combining both systems (condition 1) we were successfully able to block all leakiness down to the level of background luciferase expression (Figure 4-5, bottom).

While the combined system showed a vastly improved level of control over transgene expression, the requirement of multiple antibiotics rendered it difficult to use *in vivo*. Based on the high leakiness in our *in vitro* assay, the use of the DD or Dox systems individually was also not an option. Comparing the two systems we found that the Dox-based approach resulted in the lowest level of leakiness. Based on these results, we reasoned that modifying the stability of TTA may sufficiently decrease background levels of TTA expression and confer a high degree of specificity to our reporter system.

We fused mouse ornithine decarboxylase (MODC) to the N terminal of TTA (d2TTA) to decrease its stability. MODC contains a PEST sequence that results in targeting TTA for degradation. This strategy has been used extensively to degrade the expression of EGFP for various transcriptional reporter assays (Li et al., 1998). We reasoned that destabilizing TTA, would decrease background transgene expression by requiring an increased amount of TTA to drive the TRE promoter.

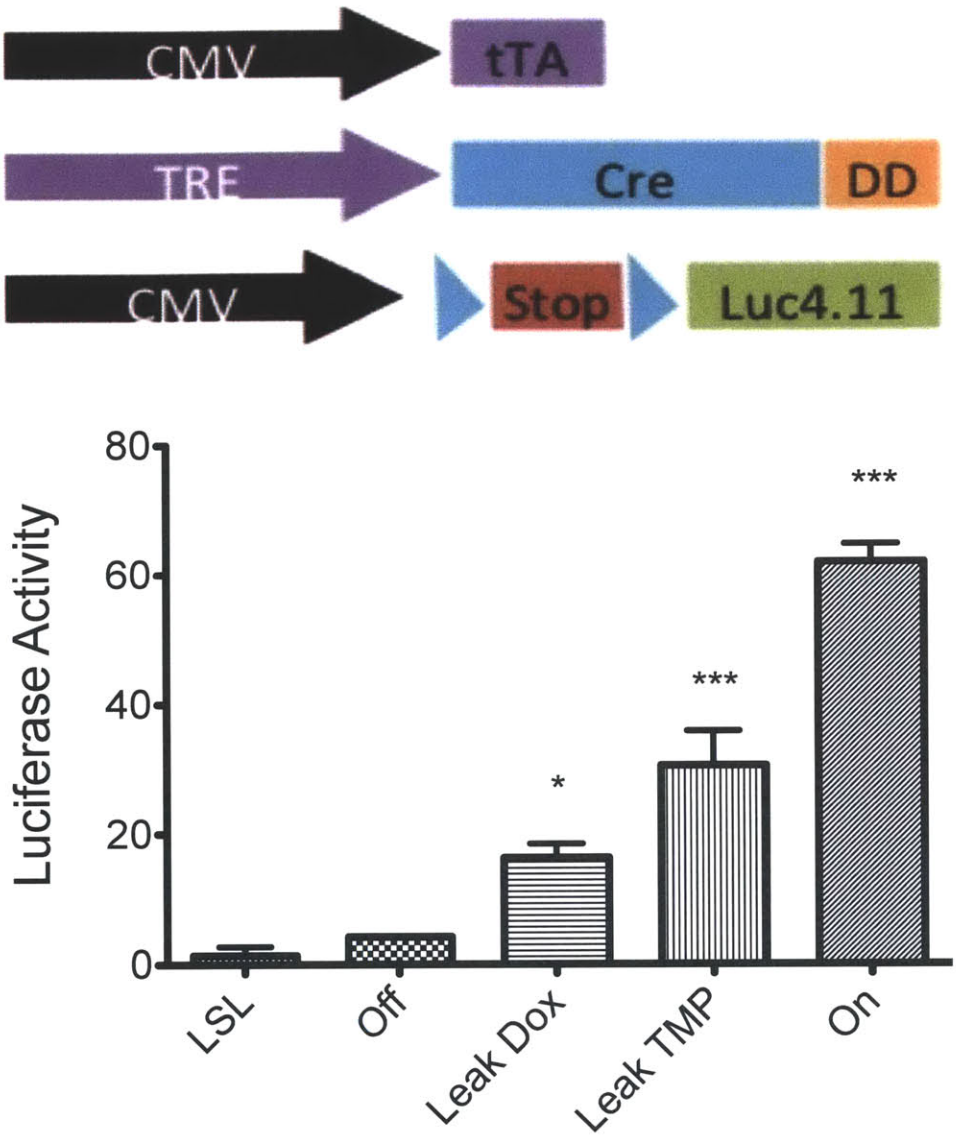


Fig 4-5. Testing gene regulatory systems. Top. Construct and experimental design. **Bottom** Comparison of DD (Leak TMP) and Tet-Off (Leak Dox) gene regulation systems revealed a significant degree of leakiness in both conditions. Combining both systems (Off) resulted in expression levels similar to background expression of luciferase (LSL). N=3 independent cultures run in triplicate. One-way ANOVA with Dunnett's post test to compare to single control group (LSL). *** $p < .001$, * $p < .05$.

Therefore, only under conditions of high activity will there be enough TTA to drive downstream transcriptional events.

To test this hypothesis, we cloned the RAM promoter upstream of TTA or d2TTA and directly compared their ability to drive the expression of TRE-luciferase. We found that RAM-d2TTA exhibited a significantly improved SNR in comparison to RAM-TTA (Fig 4-6A) and this was due to a large reduction in TTA expression at baseline conditions (Fig 4-6B). One negative consequence of destabilizing TTA was an overall reduction in RAM-d2TTA expression under stimulated conditions (Fig 4-6C), but this was coupled with a complete silencing of RAM-d2TTA under Dox conditions (Fig 4-6D). Encouraged by these results we developed the rest of the RAM system around our modified d2TTA protein.

4.3.3 Platform Design

Following the development of our activity-regulated promoter and gene regulatory switch, we next generated our RAM-Tag AAV vector. A key element in this design was to treat each element of the vector as an interchangeable module so that promoters and transgenes could easily be swapped for the specific experimental conditions. Furthermore, because of the small size of the RAM promoter, we were able to create a single construct that contained RAM-d2TTA and TRE-MCS all within the size of AAV packaging limitations. Each element in the vector is flanked by cloning sites allowing us to change promoters, transgenes, and stop signals with ease (Fig 4-7). The initial vectors focused on labeling active neuron populations with fluorescent proteins while the more updated version of the vectors allow us to probe the function of cells in more detail. Refer to Table 5-2 for a catalog of vectors created using the RAM system.

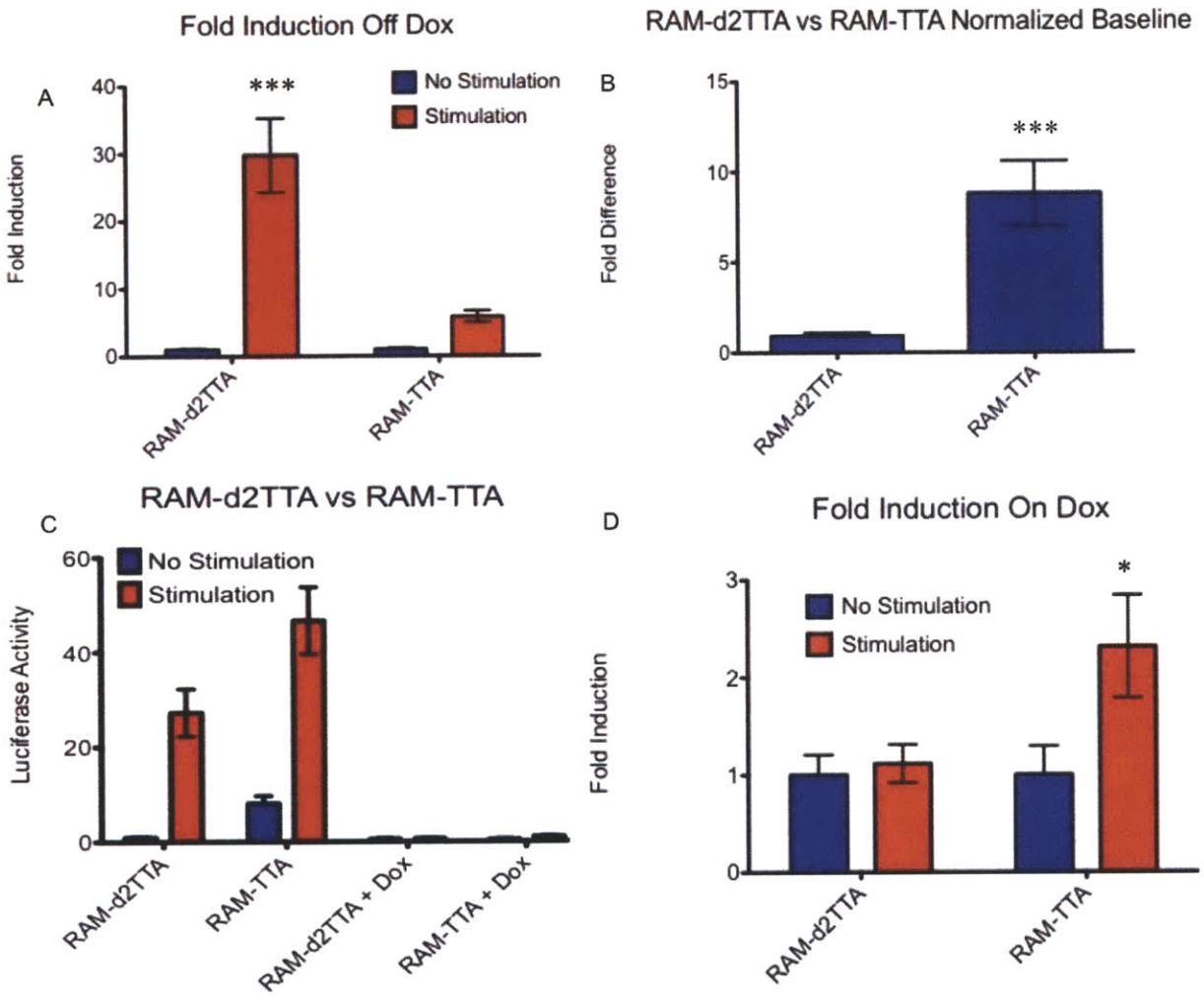
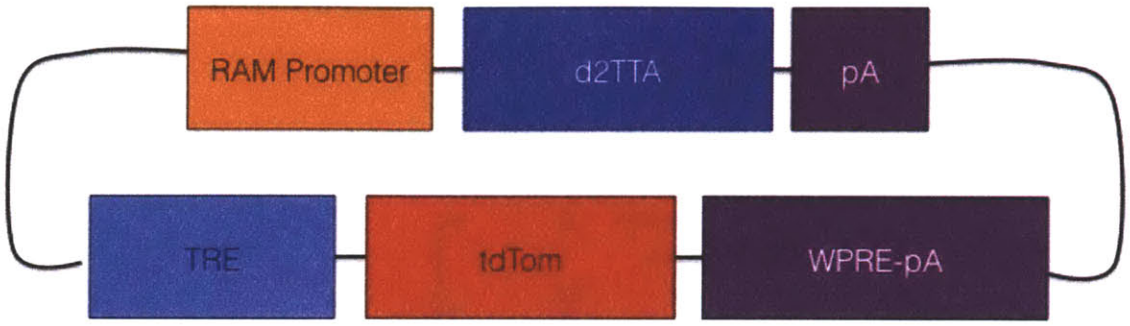


Fig 4-6. d2TTA characterization. A. RAM-d2TTA exhibits a dramatically improved SNR in comparison to RAM-TTA ($p < .001$). **B.** Baseline expression (no stimulation) of RAM-d2TTA is approximately ten times lower than RAM-TTA ($p < .001$). **C.** Raw luciferase values show that the overall expression of RAM-d2TTA is lower than RAM-TTA. **D.** In the presence of doxycycline RAM-d2TTA is completely silenced ($p < .05$). N=3 independent cultures run in triplicate. Two way ANOVA with Bonferroni post test.



Virus	Serotype
RAM-EGFP	AAV 5.2
RAM-d2mCh-tTA —TRE-EGFP	AAV 5.2
RAM-d2mCh-tTA —TRE-CreDD	AAV 5.2
RAM-d2tTA— TRE-EGFP-NLS	AAV 5.2
RAM-d2tTA— TRE-tdTom	AAV 8.2
RAM-d2tTA— TRE-Cre	AAV 5.2
RAM-d2tTA— TRE-DREADD	AAV 8.2
RAM-d2tTA— TRE-TimerGO	AAV 8.2
RAM-d2tTA— TRE-EGFP-TeTxLC	AAV 5.2

Table 4-2. Catalog of RAM vectors.

Fig 4-7. RAM-Tag Vector. AAV RAM-Tag consists of the RAM promoter driving the expression of d2TTA. Under conditions of high activity d2TTA will drive the expression of the second cassette, which in this example contains tdTomato. Convenient cloning sites allow for each element of the vector, represented as blocks, to be swapped as needed.

Construct	Description
RAM-d2TTA-TRE-MCS	RAM tag backbone without WPRE
RAM-d2Tag-TimerNLS	RAM tag backbone expressing TimerNLS
RAM-d2Tag-Gi	RAM tag backbone expressing DREADD
RAM-d2Tag-Ribo	RAM tag backbone expressing RiboTag
RAM-d2Tag-TdTom	RAM tag backbone expressing tdTom
RAM-d2Tag-FastNLS	RAM tag backbone expressing fluorescent timer protein Fast
RAM-d2Tag-mKate2	RAM tag backbone expressing mKate
RAM-d2Tag-Fast	RAM tag backbone expressing fluorescent timer protein Fast
RAM-d2Tag-TimerGO	RAM tag backbone expressing mOrange-NLS IRES EGFP
RAM-d2Tag-eTox	RAM tag backbone expressing EGFP-Tetanus Toxin
RAM-d2Tag-EGFP-NLS	RAM tag backbone expressing EGFP NLS
RAM-d2Tag-Cre	RAM tag backbone expressing Cre
RAM-d2EGFPTTA-TRE-MCS	RAM tag backbone expressing d2EGFP-TTA without WPRE
RAM-Tag-CreDD	RAM tag backbone expressing d2mchTTA and CreDD
RAM-d2mchTTA-TRE-MCS	RAM tag backbone expressing d2mchTTA
RAM-d2EGFPTTA-TRE-MCS	RAM tag backbone expressing d2EGFPTTA
RAM-Tag V032	V032 AAV vector that contains RAM driving d2mChTTA
RAM-TagEGFP-hm4d	RAM d2EGFPTTA expressing DREADD
RAM-TagmCh-hm4d	RAM d2mCHTTA expressing DREADD
RAM-d2EGFPTTA-TRE-RiboTag	RAM d2EGFPTTA expressing RiboTag
RAM-d2mCHTTA	RAM driving d2mChTTA
RAM-EGFP-IRES-TTA	RAM driving EGFP IRES TTA
RAM-d2TTA-TRE-MCS-WPRE-pA	RAM tag backbone with WPRE

Table 4-3. Catalog of RAM constructs.

4.3.4 AAV-RAM-Tag In Vitro

We initially cloned and generated an AAV8 virus that contained RAM-tdTomato (RAM-d2TTA: TRE-tdTom) and validated its functionality in vitro. Cultured hippocampal neurons were infected on DIV 7 and stimulated on DIV 14. As before, we found RAM expression was highly upregulated by synaptic activity (4AP/ Bicuculline treatment) in comparison to baseline conditions (Fig 4-8 A and B). The robust activity-induced expression of RAM was completely suppressed in the presence of doxycycline (Fig 4-8 A and B). Characterizing the cell-types that express RAM has revealed that the reporter is neuron-specific and can be expressed in all neuron subtypes (present in cultured neurons) under appropriate stimulation conditions (Fig 4-8 C and D).

Electrophysiological characterization of RAM-positive neurons has revealed that this cell population exhibits unique synaptic properties (recordings collected by Andrew Young). RAM-positive neurons in DIV14-21 cultures show an increased frequency of sEPSCs and mEPSCs in comparison to neighboring RAM-negative neurons (Fig 4-9 A and B). In contrast, no change was observed in mIPSCs or sIPSCs (Fig 4-9 C and D). In addition to increased frequency of EPSCs, RAM positive neurons exhibited a higher frequency of action potentials (Fig 4-9E). Taken together, these *in vitro* findings suggest that the RAM reporter can tag and robustly label a unique cell population that preferentially responds to high levels of neuronal activity and exhibits synaptic properties consistent with an active neural circuit.

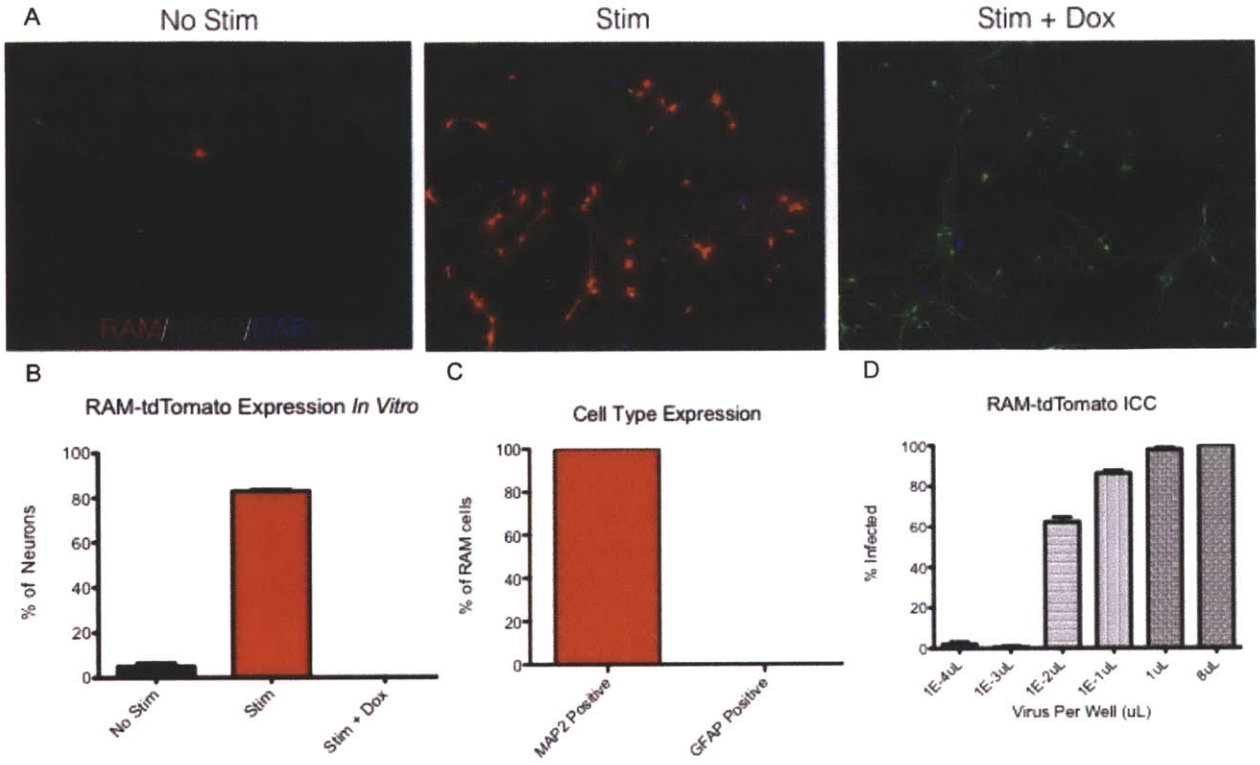


Fig 4-8. RAM-tdTomato Characterization. A. ICC visualizing the expression of AAV-RAM-tdTom (red-RAM, green-MAP2). **B.** Quantification of RAM-positive neurons reveals that 4AP/Bic stimulation results in ~90% of neurons expressing RAM in comparison to ~10% of neurons at baseline. Stimulation induced RAM expression can be suppressed by treating the cultures with doxycycline. **C.** RAM expression is selective to neurons and does not show any expression in GFAP-positive glia. **D.** Exposing cultures to high-titer RAM virus results in all neurons expressing RAM following stimulation. N = 3 independent cultures/condition. One way ANOVA or t-test, ($p < .001$).

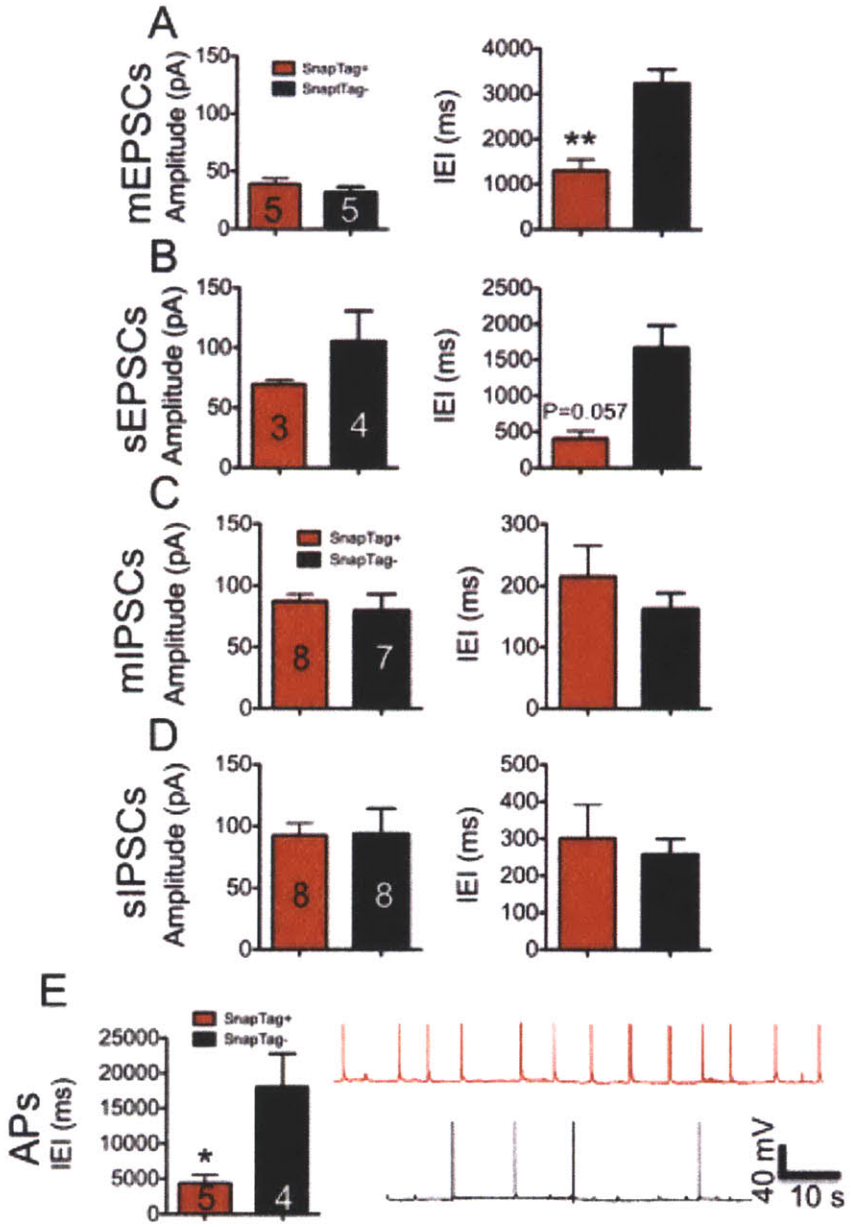


Fig 4-9. Electrophysiological characteristics of RAM-positive neurons. A. RAM-positive neurons exhibit increased frequency of mEPSCs **B.** and sEPSCs. No change in amplitude was observed. **C and D.** Frequency and amplitude of mIPSCs and sIPSCs are similar across conditions. **E.** RAM-positive neurons exhibit increased frequency of action potentials, holding potential = -70mV. N refers to cell number, DIV 14-17. Unpaired t-test, ($p < .05$). Cells infected by Kartik Ramamoorthi, recordings and analysis by Andrew N. Young.

To define the temporal dynamics of the reporter system, we created a dual-florescent RAM construct, RAM-Timer. In this system, RAM initially drives a transiently expressed mCherry, followed by the expression of a stable EGFP. Over time the mCherry signal degrades, while the EGFP will persists and acts as a long-term label for neurons activated by the initial experience. When subsequent experiences activate the transient mCherry, cellular localization of mCherry and EGFP differentiates ensembles of neurons activated by distinct stimuli. Neuronal depolarization will lead to the activation of RAM and the expression of a short half-life mCherry-TTA fusion protein (d2mCh). Through the Tet-Off system, TTA will drive the expression of EGFP. Application of doxycycline will prevent the expression of new EGFP, only allowing one wave of EGFP expression. In cultured neurons, RAM-Tag is robustly induced by neuronal activity. d2mCh is expressed within hours of neuronal depolarization, followed by EGFP. The expression of d2mCh is transient and returns to baseline levels at a much faster timescale than EGFP. Labeled EGFP neurons are capable of being reactivated, as depolarization results in the activation of d2mCh in this cell population (Fig 4-10). The application of this system *in vivo* would permit the identification of neuronal populations of neurons that encode distinct stimuli that guide behavior.

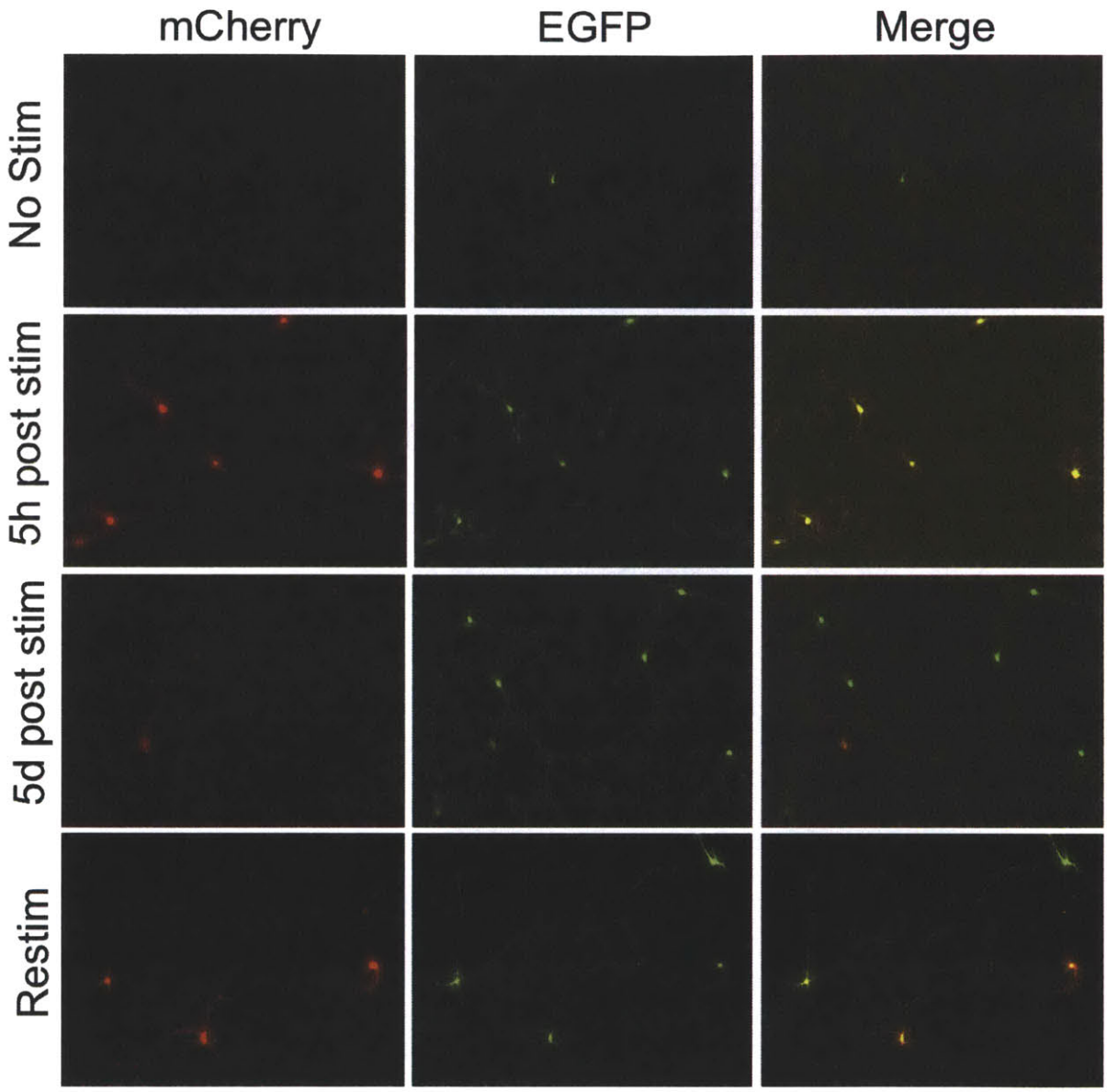


Fig 4-10. Characterization of RAM-Timer. Cultured neurons (DIV 7) were depolarized (55mM KCl, 2h) and collected at different time points (No Stim, 5h, 5d, Restim). d2mCherry-TTA drives the expression of EGFP. d2mCherry returns baseline levels by 5 days, while EGFP expression is sustained. A second depolarization (Restim) results in the reactivation of d2-mCherry.

4.3.5 AAV-RAM-Tag In Vivo – Visualizing Mouse and Rat Circuits

To test the functionality of this reporter system *in vivo*, an AAV5-RAM-TagEGFP (RAM-d2mCH-TTA: TRE-EGFP) vector was delivered to the CA3 region of adult C57Bl6 mice (AAV5, 1uL, ~1E13 vg/mL). We selected AAV5 based on screening various AAV serotypes for optimal expression in CA3 and DG. Initial characterization tested whether pharmacologically-induced seizures (kainic acid 12mg/kg or metrazole 50mg/kg), which drive high levels of neuronal activity and activity-regulated gene expression in the hippocampus, could induce EGFP expression. To determine whether learning-related neuronal activity can activate RAM-TagEGFP, injected mice were trained in contextual fear conditioning (3x2s 0.55mA shock, 58s ITI, 4min). In comparison to homecage controls, RAM-TagEGFP was highly induced by seizure and CFC, with the highest expression observed in the seizure condition (Fig 4-11).

While RAM-Tag is induced by behavioral experience, we have observed some degree of variability in background labeling from different behavioral cohorts. We believe this is a reflection of the unique behavioral experiences of each mouse (cage changes, housing, etc). To control for variable background expression levels the dox system can be used to define a very specific behavioral window.

To test the versatility of RAM, we have begun applying it to other neural circuits in the mouse brain. We initially focused on somatosensory cortex due to the strong relationship between whisker stimulation and neural activity within the somatosensory cortex. We used an AAV8-RAM-tdTomato virus and exposed mice to an enriched environment or metrazole-induced seizures.

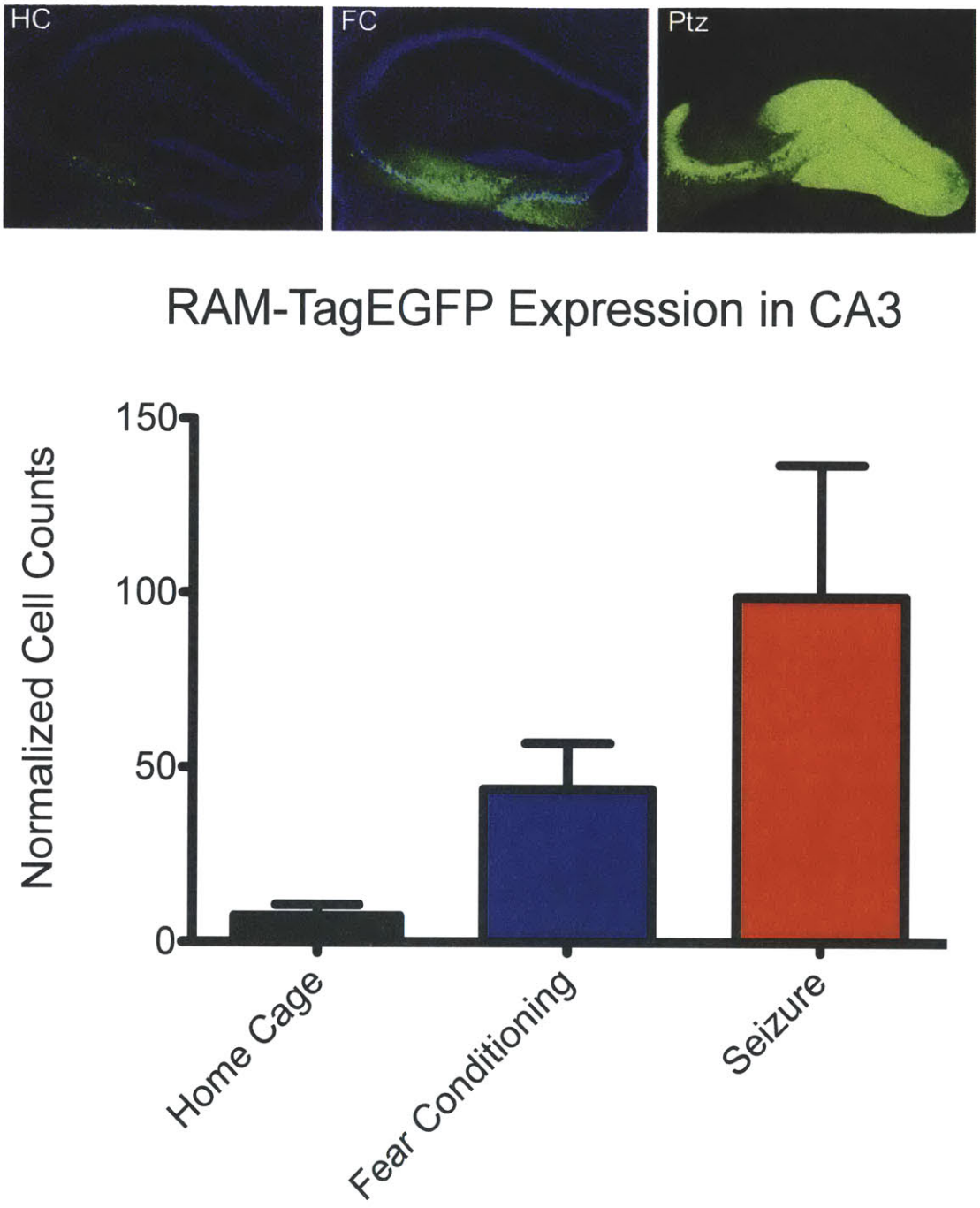


Fig 4-11. RAM-TagEGFP *in vivo* hippocampus. Top. RAM-TagEGFP was targeted to the CA3 and DG region of hippocampus. Injected mice were exposed to contextual fear conditioning (FC) or metrazole induced seizures (Ptz) and compared to home cage (HC) controls. DAPI is removed from the Ptz condition for easier viewing. **Bottom**. Quantification reveals that RAM-EGFP is induced by FC and Ptz in comparison to home cage controls (HC). N=3-6 mice per condition, 3-6 sections counted per mouse and averaged.

Similar to the hippocampus, we observed robust expression of RAM following both seizure and enriched environment exposure (Fig 4-12). Furthermore, the expression of RAM-tdTomato is strong enough to visualize without antibody enhancement.

To further validate the use of RAM in a variety of circuits we have developed the SHELAC paradigm (**S**timulating **H**ousing **E**nriched **L**iving **a**nd **C**onflict) to activate multiple circuits with several behavioral paradigms. Specifically, mice were injected with RAM-tdTomato in hippocampus, hypothalamus, and amygdala. Following surgery, mice were exposed to a sequence of behavioral paradigms that are known to robustly activate these circuits, including enriched environment, mating, fighting, food/water deprivation, and exercise. Twenty-four hours after the last exposure mice were collected and compared to HC controls that were removed from doxycycline at two different time points (24h and 96h). Similar to fear conditioning or novel environmental exposure, we found that the SHELAC paradigm robustly induced RAM-tdTomato expression in the amygdala, hippocampus, and hypothalamus suggesting that this tool can be used in identify active neurons using a behaviors and neural circuits.

To validate the RAM reporter system in different animal models, we are collaborating with Robert Froemke (NYU, experiments run by Ioana Carcea) and Steven Maier (University of Colorado, experiments run by Michael Baratta) to test the utility of RAM in rat auditory cortex and prefrontal cortex respectively. In auditory cortex, the ability for RAM to identify the tonotopic maps in A1 will be tested by exposing rats to tones of a specific frequency and visualizing the expression pattern of RAM. We have found that in rat prelimbic cortex, uncontrolled stress is able to drive AAV5-RAM-

TagEGFP expression with peaks levels being observed ten days after viral injections (Fig 4-14).

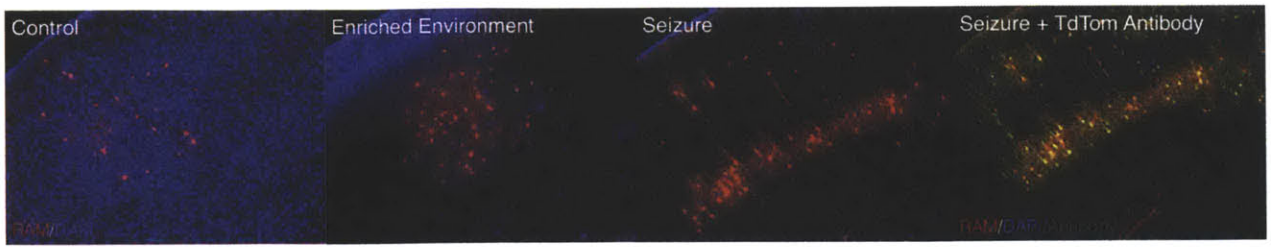


Fig 4-12. RAM-tdTom *in vivo* cortex. AAV8-RAM-tdTom (1uL @ 2E13vg/mL) was targeted to somatosensory cortex. Injected mice were exposed to an enriched environment (3d) or metrazole-induced seizures. RAM-tdTom expression (red) was highly upregulated by both conditions in comparison to home cage controls. A high degree of overlap between autofluorescent TdTom (red) labeling and antibody based TdTom labeling (green) indicates the robust nature for RAM labeling.

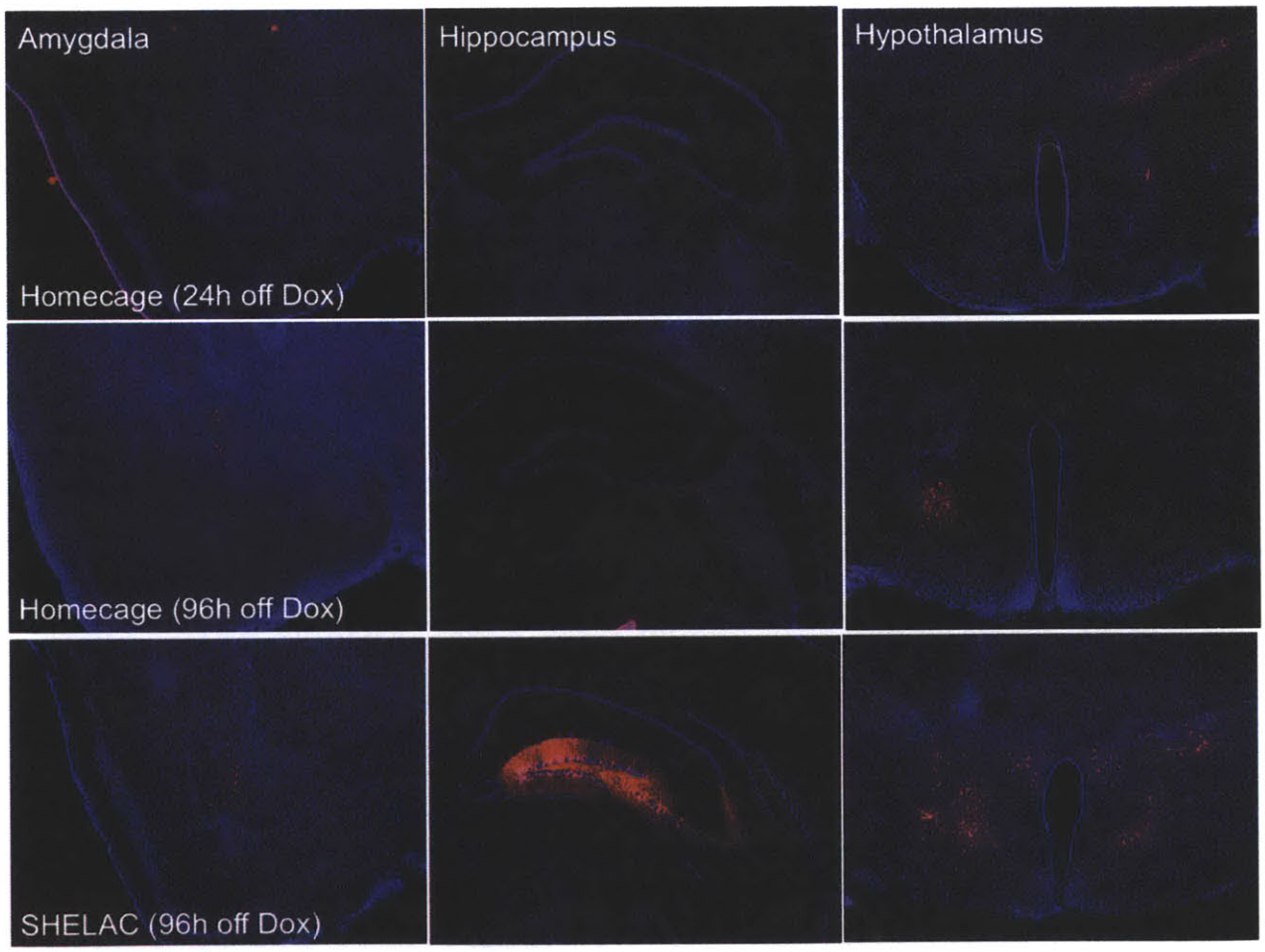


Fig 4-13. RAM-tdTom *in vivo* SHELAC. AAV8-RAM-tdTom (1uL @ 2E13vg/mL) was targeted to amygdala, hippocampus, and hypothalamus. Mice were taken off of doxycycline 24h or 96h before being exposed to the SHELAC paradigm. RAM-tdTom expression (red) was upregulated in the amygdala, hippocampus, and hypothalamus by the SHELAC paradigm in comparison to homecage controls off dox. Experiments run by Kartik Ramamoorthi and Andrew N. Young.

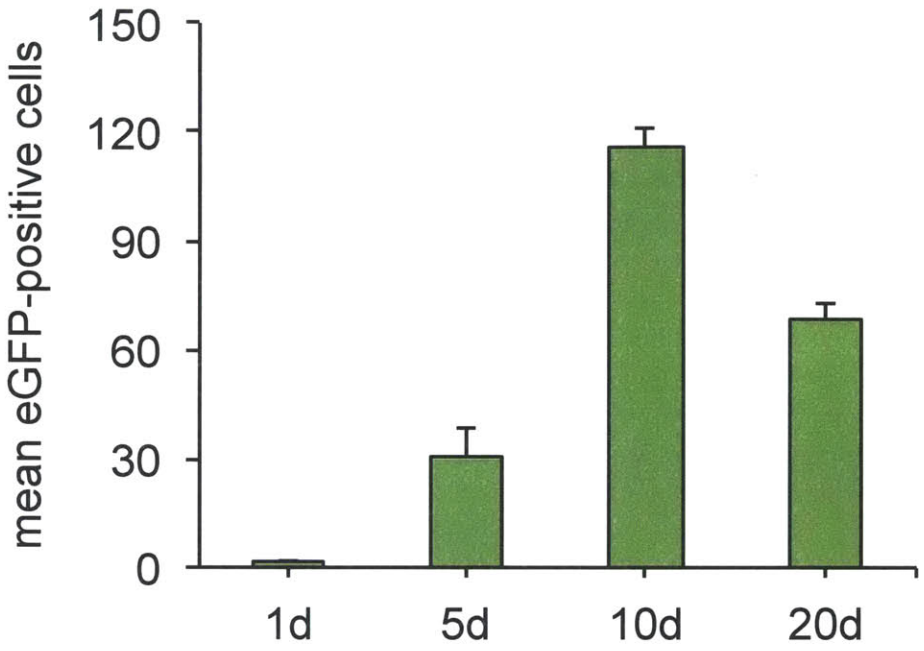


Fig 4-14. RAM-TagEGFP *in vivo* Rat Prelimbic Cortex. AAV5-RAM-TagEGFP was unilaterally targeted to rat prelimbic cortex (1uL @ 2E13vg/mL). Following injections rats were allowed to recover of 1, 5, 10, or 20 days and exposed to 100 trials of inescapable tail shock twenty-hour hours prior to collection. Peak expression of RAM-TagEGFP in prelimbic cortex was observed 10 days after viral delivery. Experiments coordinated and planned by Kartik Ramamoorthi, planned and executed by Michael Baratta. N = 2 rats per condition

Using RAM-Tag we are now in a position to selectively deliver effector genes to neural circuits activated by behavioral experience, in order to systematically analyze their contribution to learning, memory, and animal behavior. Please refer to Table 4-2 and 4-3 to see the current list of RAM AAV vectors that have been created.

4.3.6 AAV-RAM-Tag In Vivo – Manipulating Mouse Circuits

To explicitly test whether the neurons that express RAM following behavioral experience are required for subsequent memory recall, we employed the use of inducible and reversible channels selectively expressed in RAM-positive neurons. We hypothesized that silencing RAM-positive neurons during a contextual memory test would suppress the memory representation formed during contextual fear conditioning and subsequently impair recall. To selectively silence RAM neurons, we introduced a ligand-activated G-protein coupled receptor (hM4Di) driven by RAM (AAV5-RAM-d2TTA:TRE-hM4Di, referred to as RAM-hM4Di). Activation of hM4Di, with the pharmacologically inert ligand clozapine-N-oxide (CNO), results in the opening of Kir3 potassium channels, which in turn causes membrane hyperpolarization and subsequent silencing of the neuron (Armbruster et al., 2007). hM4Di provides a simple method to selectively silence neurons, as CNO can be delivered through an IP injection.

Mice were injected with RAM-hM4Di or human Synapsin (hSyn)-hM4Di into dorsal CA3 and trained in CFC two weeks later (Fig 4-15A). hSyn is a constitutively active neuronal promoter that served as a positive control to ensure that hM4Di-based inactivation of hippocampus was able to impair contextual memory recall. Mice were trained in Context A. 24 hours after training mice were given an IP injection of saline (RAM or hSyn ON) or CNO (RAM or hSyn OFF 1mg/kg) followed by a memory test.

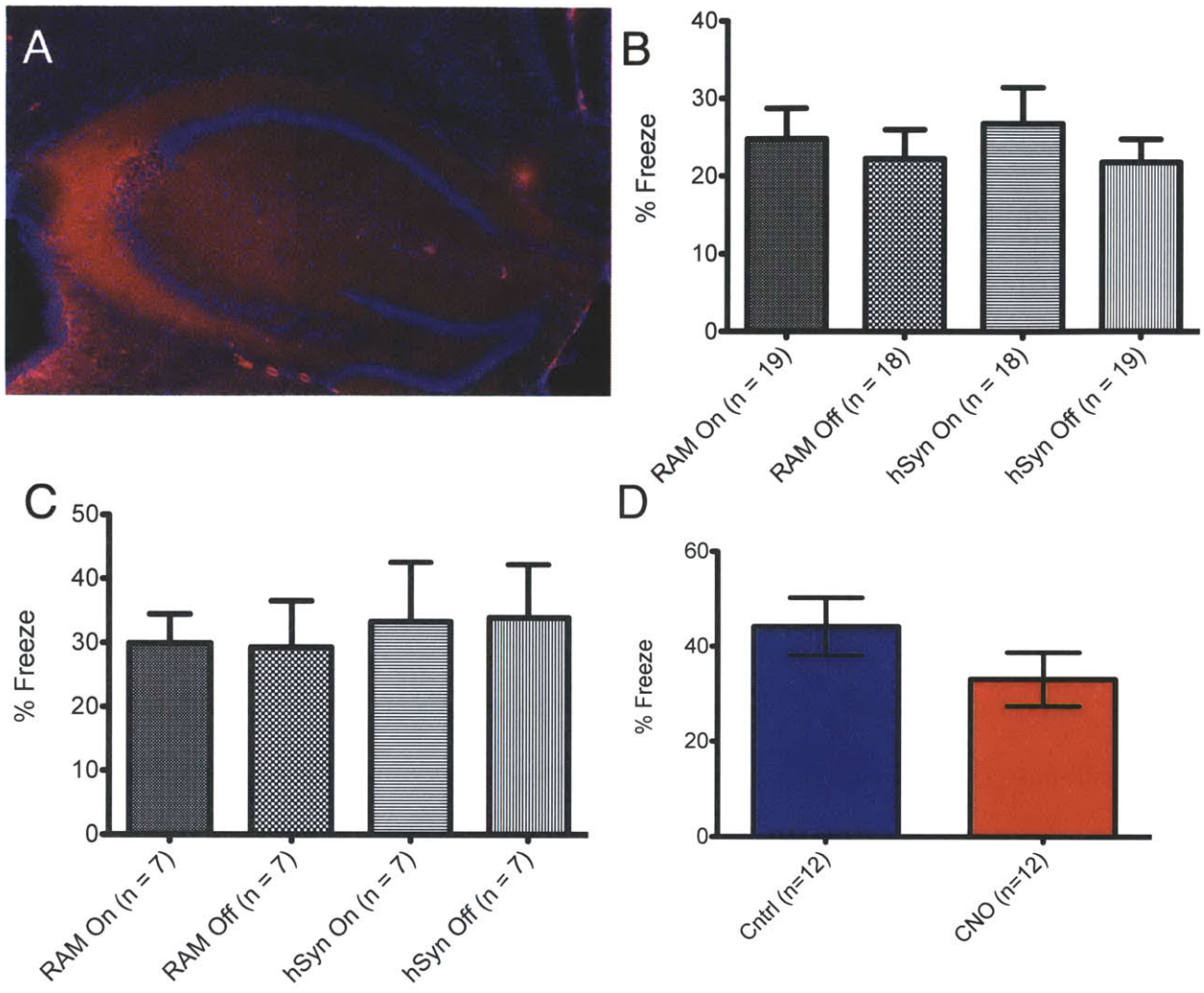


Fig 4-15 Perturbing neural circuits using RAM-hM4Di. A. AAV5-RAM-hM4Di or AAV5-hSyn-hM4Di were targeted to the CA3 region of hippocampus. Visualization of HA tagged hM4Di (red) in the CA3 region of hippocampus from a fear conditioned RAM mouse. **B.** Twenty-four hours after contextual fear condition mice were given IP injections of saline (On) or CNO (Off, 1mg/kg) followed by a contextual memory test. Inactivation of RAM positive or hSyn positive neurons had no contextual effect on freezing. N = 18-19 mice per group. **C.** Twenty-four hours after auditory delay fear condition mice were given IP injections of saline (On) or CNO (Off, 1mg/kg) followed by a tone memory test. Inactivation of RAM positive or hSyn positive neurons had no effect on tone cue induced freezing. N = 7 mice per group. **D.** AAV5-RAM-hM4Di was targeted to retrosplenial cortex. Twenty-four hours after contextual fear condition mice were given IP injections of saline (Cntrl) or CNO (1mg/kg) followed by a contextual memory test. No difference was seen in freezing across all groups. N = 12 mice per group. One way ANOVA or unpaired t-test, ($p > .05$)

Surprisingly, in both RAM and hSyn conditions we were unable to detect an impairment in contextual memory recall with CNO delivery (Fig 4-15B). It is well-established that inactivation of hippocampus impairs contextual memory recall (Eichenbaum et al., 1992), therefore we reasoned that the failure to see an effect was an indication of a failure to express the hM4di receptor, a failure to activate the hM4Di receptor, or an inability for the receptor to hyperpolarize CA3 neurons. We verified expression of the receptor through IHC (Fig 4-15A) and confirmed that our concentration of CNO had been successfully used in vivo to activate this class of receptors in the hippocampus (Alexander et al., 2009; Garner et al., 2012). However, we failed to detect a consistent CNO-induced inactivation of neurons by patching onto neurons in hippocampal slices. To ensure that our effects were circuit-specific, we tested a separate cohort of mice in auditory delay conditioning, which does not rely on the hippocampus. As expected, we saw no effect on delay fear conditioning, as freezing responses to a tone cue were similar across groups (Fig 4-15C). We attempted a similar series of experiments in retrosplenial cortex, where we see robust expression of activity-regulated genes following fear conditioning and where the receptor has been successfully used (Garner et al., 2012), and again failed to observe an effect of using the receptor (Fig 4-15D).

In a second attempt to inactivate RAM positive neurons in hippocampus, we used an orthogonal strategy by expressing tetanus toxin in this cell population. Tetanus toxin cleaves synaptobrevin and subsequently prevents synaptic transmission in expressing cell populations (Nakashiba et al., 2008). The experimental conditions were

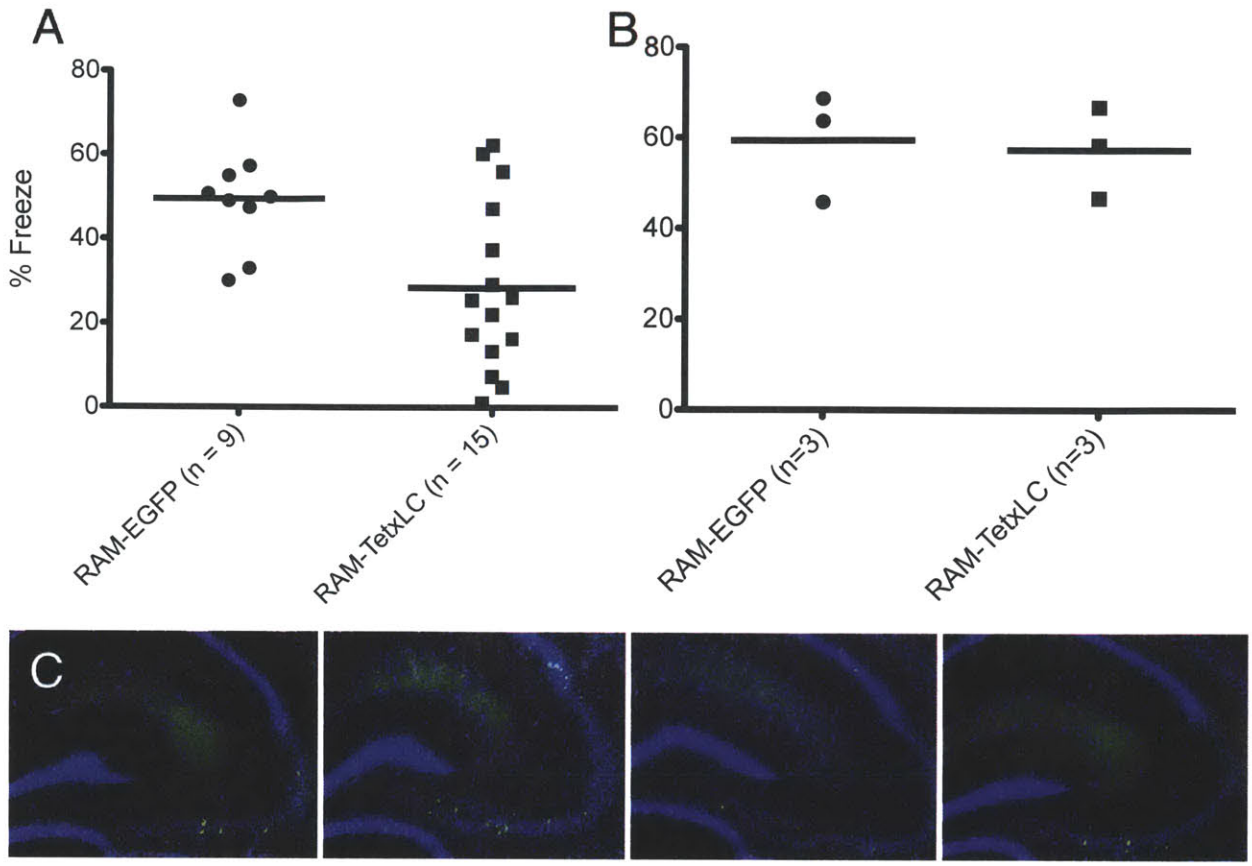


Fig 4-16. Perturbing neural circuits using RAM-TetxLC. A. AAV5-RAM-EGFP (n = 9) or AAV5-RAM-TetxLC-EGFP (n=15) were targeted to the CA3 region of hippocampus. Twenty-four hours after contextual fear condition mice were give a contextual memory test. RAM-TetxLC mice exhibit impaired freezing in comparison to RAM-EGFP mice ($p < .01$). **B.** Expressing TetxLC in neurons tagged from an alternate context had no effect on freezing to the training context (n = 3 mice per group). **C.** Visualization of EGFP tagged TetxLC in four separate mice revealed sparse and degraded expression of TetxLC. Unpaired t-test.

identical to the hM4Di experiments. In this setting, we observed an impairment in contextual memory recall when comparing mice expressing RAM-TetxLC-EGFP to RAM-EGFP controls (Fig 4-16A). In control mice where neurons were tagged in an alternate context we did not observe an impairment in freezing behavior, suggesting that the behavioral impairment was context specific (Fig 4-16B). However, the expression of EGFP tagged TetxLC was so weak and variable we were unable to verify the location and size of our injections (Fig 4-16C). Therefore, without histological verification, it is difficult to interpret the consequence of this perturbation.

4.3.7 AAV-RAM-Tag In Vivo – Invertebrates

A second major goal in the development of the RAM system was to create a reporter that could be used ubiquitously by the neuroscience community. The use of an Npas4 binding motif was guided by our lab's focus on the transcription factor, but also due to the fact that the bHLH-PAS domain of Npas4 is high conserved in *drosophila* and *C. elegans* (Ooe et al., 2004; Ooe et al., 2007). Furthermore, *drosophila* and *C. elegans* homologs of Npas4 have been shown to interact with known binding partners of mammalian Npas4 and were both able to activate a mammalian Npas4 reporter plasmid (Ooe et al., 2007). Together, these observations support the hypothesis that the RAM reporter may be functional in *drosophila* and *C. elegans*.

To generate a RAM fly line we collaborated with former Lin lab member, Robin Fropf, and her advisor Jerry Yin. We developed a *drosophila* version of the RAM plasmid and the Yin lab generated a pan-neuronal RAM-luciferase fly line. Using an *in vivo* luciferase assay we were able to identify robust circadian oscillations of the RAM

reporter with daytime peaks and nighttime troughs (Fig 4-17A). Furthermore, olfactory conditioning leads to enhanced expression of RAM in forward-conditioning groups in comparison to groups presented the stimuli in a non-contingent fashion (backwards-conditioning) (Fig 4-17B). These data support the hypothesis that the RAM reporter is functional in non-mammalian neural circuits and, similar to the mammalian systems, may act as a robust activity monitor in invertebrates.

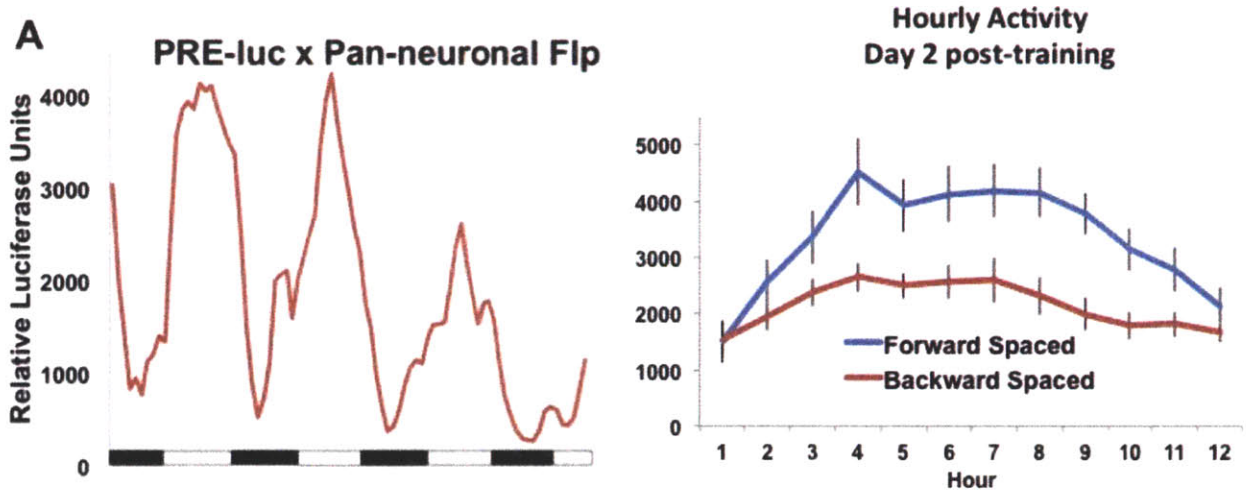


Fig 4-17. RAM reporter fly. A. Pan-neuronal RAM-Luc fly (referred to as PRE-luc) exhibits strong nighttime (black bar) and daytime (white bar) circadian rhythms. **B.** Associative conditioning results in an increase RAM-Luc activity that is not observed in backwards conditioning controls. Plasmid created by Kartik Ramamoorthi, experiments carried out by Robin Fropf.

4.4 Discussion

We have developed a novel genetic system to identify and manipulate active neural circuits using Npas4 as a molecular handle. The system takes advantage of our previous observation that Npas4 is activated by and required for contextual memory formation (Ramamoorthi et al., 2011). Using a putative Npas4 binding sequence we designed a series of AAV viral vectors that allowed for the experience dependent expression transgenes in a doxycycline dependent manner (Ooe et al., 2004; Ooe et al., 2007). We initially validated the system in cultured neurons and observed a robust activity dependent response that was completely doxycycline dependent. We found that neurons labeled with the reporter exhibited a variety of electrophysiological properties that were consistent with an active neural circuit.

We next validated the system *in vivo*, where we observed experience dependent expression of RAM in a variety of murine neural circuits. Furthermore, we were able to observe a similar patterns of effects in rat cortex, suggesting that the RAM reporter can be applied to a variety of neural circuits in both rat and mouse. The creation of the RAM reporter fly further broadens the applicability of the reporter and suggests that our approach takes advantage of an evolutionarily conserved genetic pathway that responds to neural activity.

To begin perturbing active neural circuits, we used the reporter system to express effector genes to functionally silence experience-activated neurons. While our initial observations support the hypothesis that neurons activated by learning are required for subsequent memory recall, technical limitations and inconsistent observations across effector genes have complicated the interpretation of these data.

However, these studies further reinforce the robust nature of the RAM reporter system and highlight potential future applications of the system. Chapter 5 will highlight several future studies that can take advantage of the specific and robust nature of the RAM reporter. The combination of our "all-in-one" AAV approach with invertebrate reporter lines will make RAM a ubiquitous tool for the neuroscience community.

4.5 Methods

Mice

Wildtype C57BL6/N mice were purchased from Charles River Laboratories. Mice were used for experiments at 8-12 weeks of age. For gene expression and IHC experiments mice were housed individually for one week prior to conditioning. All mice were housed with a 12 hour light-dark schedule and received food and water *ad libitum*. Animal protocols were performed in accordance with NIH guidelines and approved by the Massachusetts Institute of Technology Committee on Animal Care.

Fear conditioning. Injected mice (8-12 weeks old) were trained in one of the following tasks 5 days after injections:

Contextual Fear Conditioning. Mice were placed in the chamber, allowed to explore for 58s and then given three 2s 0.55mA footshocks at 58s intervals. Following the last shock mice were left in the chamber for 1 minute and then returned to their home cage.

Auditory Delay Conditioning. Mice were placed in the chamber, allowed to explore for 1 minute and then given three tone stimuli (85dB, 20s, 2.8kHz) at 58s intervals that co-terminated with a 2s 0.55mA footshock. Following the last shock mice were left in the chamber for 1 minute and then returned to their home cage.

Context Only. Mice were placed in the training chamber for 4min and then returned to their home cage.

Immediate Shock. Mice were placed in the training chamber, immediately given a 6s shock (0.55mA) and then returned to their home cage.

Memory Testing. 24hr after training mice were returned to the conditioning chamber for 4min to test memory recall. Memory for the context was measured by recording freezing behavior, defined as the total absence of movement aside from that required for respiration. Memory for the tone was measured by recording freezing in a novel context during presentation of the tone. Training and testing sessions were video recorded and behavioral scoring was conducted by scorers blind to the experimental conditions. In every case, the chamber was cleaned with 70% ethanol between subjects. Subjects used for gene expression or immunohistochemical analysis were sacrificed 24h after conditioning.

SHELAC

Mice were exposed to several experiences known to activate the amygdala, hippocampus, and hypothalamus. 96h before exposure mice were removed from doxycycline diet (40mg/kg). Mice were place in a novel enriched environment that contained various tubes, toys, and a running wheel. Following an initial acclimation period to the novel environment, mice were exposed to two breeder females for two hours. Following the mating experience, mice were exposed to a single CD1 retired breeder aggressor male. After 10 minutes the male was removed and mice were left in the enriched environment for 24h and collected for histological analysis.

Immunohistochemistry

Mice were overdosed with avertin (1000mg/kg, 1.25% 2,2,2-tribromoethanol and 2.5% 2-methyl-2-butanol) and brains were drop-fixed in 4% paraformaldeyde in PBS. Brains

were removed and post-fixed for 12hr then cryo-protected in 30% sucrose overnight. Subsequently, brains were immersed in OCT-Tissue Tek, flash frozen on dry ice, and sectioned on a cryostat at 50μm thickness. All sections were blocked for 1hr at room temperature in a solution containing 0.3% Triton X-100, 0.2% Tween-20, 3.0% BSA, and 3.0% goat serum then incubated with primary antibody overnight at 4°C. The next day, sections were rinsed in PBS and incubated in secondary antibody for 1hr at room temperature. Sections were mounted on Superfrost, slides. 3-6 sections were taken from dorsal hippocampus of each mouse and GFP-positive neurons were counted. Cell counts were averaged to represent normalized cell counts per mouse.

The following antibodies were used: GFP (chicken, 1:1000, Aves GFP-1020), GFP (rabbit, 1:1000 Millipore AB3080), dsRed (rabbit, 1:1000 ClonTech 632496), MAP2 (mouse 1:1000, Sigma M9942), and GFAP (mouse, 1:1000 Sigma G3893).

Viral Vectors

AAV vectors were produced by Virovek or within the laboratory using transient transfection of VG and pAAV helper into a 293T cell line expressing adenovirus helper protein. Cells were harvested by freeze thaw and centrifugation. Lysates were purified using heparin binding columns from the CellBioSystems purification kit. Purchased vectors were produced at 2E13vg/mL range. In all vectors, the RAM promoter drove the expression of a modified TTA protein (d2TTA), which in turn drove the expression of an upstream cassette in a doxycycline dependent manner. Upstream cassettes used in this study were: EGFP, tdTomato, hM4di-HA, and TetxLC.

Viral Injection Surgery

Mice were anesthetized with isoflourane (1-3%) and monitored for depth of anesthesia throughout the procedure. Animals were secured in a stereotax (Kopf Instruments) and the skull was exposed. After resection, holes were drilled overlying dorsal CA3 (AP -2.0, ML +/- 2.3; DV -1.8) using bregma as a reference point. Injections consisted of 1µL of virus ($2x10^{12}$ vg/mL) delivered at a rate of 150nL/minute and the needle was left in place for 3min post-injection. For SHELAC experiments mice were injected in three sites: amygdala (AP -1.34, ML -3.0, DV -3.8), hippocampus (AP -2.0, ML -2.3, DV -1.8), and hypothalamus (AP -0.7, ML -0.5, DV -5.0). Incision sites were closed using Vet-Bond glue (3M) and treated with topical antibiotic and anesthetic. Mice were given i.p. injections of Buprenex (1mg/kg) and allowed to recover for at least 5d before behavior or expression experiments were conducted.

Dissociated Neuron Culture

Dissociated cortical and hippocampal neurons were prepared from P1 mouse pups and maintained in a humidified incubator with 5% CO_2 at 37°C, co-cultured with rat astrocytes. Cultures were maintained in Neurobasal A medium supplemented with B27 (Invitrogen) and glutamine. Neurons were plated at 40,000 per well in a 24 well plate. Plates were coated with poly-D-lysine. Neurons were transfected using lipofectamine on DIV5/6. For viral transfection, neurons were infected on DIV 7 with 0.2µL/well of AAV8 at 2E12vg/mL, stimulated on DIV 14 with 4AP (5uM)/Bic (50uM) and collected on DIV 15. Cultures were treated with doxycycline where indicated at 40ng/well on DIV 7.

Luciferase Assay

Plasmids were transfected on DIV 5/6. TK-Renilla, which expresses renilla luciferase, was co-transfected in every experiment to control for transfection efficiency. On DIV 7 cells were depolarized (35mM KCl, 6hr) and lysed in passive lysis buffer. Firefly luciferase levels were measured and normalized to renilla luciferase levels. Data were compiled from separate experiments each conducted in triplicate.

ChipSeq Data Analysis

We generated a command line Python script to parse the genome-wide ChipSeq dataset from (Kim et al., 2010). The script aligns ChIP-Seq reads with additional metadata on gene location and transcription start site (TSS) information. The user inputs the upstream and downstream ranges and the program calculates the number of binding sites for indicated transcription factors on genes of interest within the specified range. The script then generates gene-specific box plots of transcription factor binding profiles and can output co-localization data to various file formats.

We used this program to assess the percent co-localization of Npas4 and CBP on 2248 activity-regulated and non-activity-regulated genes within a range 75 kB upstream and 15 kB downstream of the TSS. For each gene, we calculated the percentage of CBP sites also bound by Npas4, as well as the percentage of Npas4 sites bound by CBP.

Drosphila Reporter Line

RAM binding sites were placed upstream of a minimal promoter and CaSpeR TATAA sequence, followed by FRT-flanked stop codons and the luciferase open reading

frame. A short sequence coding for a poly-glycine run is downstream of the second FRT site, and placed so that it is in the same reading frame as the ATG start codon, regardless of which FRT site remains after site-specific recombination. The luciferase-coding region (minus its normal ATG start codon) is placed downstream, and in frame with, the poly-glycine run. In the absence of FLP, the transgene will produce no luciferase protein. After FLP-mediated recombination, a fusion protein would be encoded that contains amino acids from the FRT sequence and a poly-glycine run, all fused to luciferase. Reporter constructs were inserted at the NotI/XhoI sites of pCaSper5. Standard methods were used to generate transgenic flies (BestGene).

Statistical Analysis

All data are shown as mean ± s.e.m. Data were analyzed with unpaired t-tests or one-way or two-way ANOVA.

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Chapter 5

Summary and Future Directions

5.1: Summary

The studies described in this thesis summarize my work in the Lin Lab. The goal of this work was to gain a deeper understanding of the molecular mechanisms of memory formation and using that foundation to develop a novel reporter system to gain access to neurons activated by experience. Future experiments can take advantage of the RAM system to address fundamental questions surrounding experience-dependent changes in neural circuits. I will highlight three experiments that can be immediately pursued based on our developed technology.

5.2 Memory Perturbation

Our initial studies focused on identifying active neural circuits revealed that a subpopulation of neurons in the hippocampus and cortex are activated by exposure to a novel environment. To test whether the cell population activated by experience is required for subsequent memory recall, the RAM system can be used to selectively express effector genes to perturb cellular function. Our initial studies proved to be inconclusive, as we struggled with technical limitations. With the DREADD system (Alexander et al., 2009; Armbruster et al., 2007) we were able to confirm expression and transport of the channel, but we were unable to detect an electrophysiological or behavioral effect of channel activation. Using tetanus toxin (Yu et al., 2004) to prevent synaptic transmission we were able to detect a behavioral impairment, but failed to confirm robust expression of tetanus toxin.

To overcome these limitations, it is necessary to use a well-validated channel whose expression and physiological effect can be reliably induced and detected. Our

laboratory has recently had success both visualizing expression and validating a physiological effect using the Pharmacologically Selective Actuator Module (PSAM) channels developed by Scott Sternson's laboratory (Atasoy et al., 2012; Magnus et al., 2011).

To explicitly test whether the neurons that activate RAM following CFC are required for contextual memory recall, inducible and reversible channels selectively expressed in RAM-positive neurons will be used. It is hypothesized that silencing learning-activated neurons will impair contextual memory recall, while activating these neurons will induce contextual memory recall. To selectively silence RAM-expressing neurons, the ligand-activated ionotropic receptor PSAM driven by RAM (RAM-d2TTA;TRE-PSAM, referred to as RAM-PSAM) will be delivered. Activation of PSAM, with the pharmacological inert ligand Pharmacologically Selective Effector Molecule (PSEM), results in chloride influx, which in turn causes membrane hyperpolarization and subsequent silencing of the neuron (Magnus et al., 2011). PSAM provides a simple method to selectively silence neurons, as PSEM can be delivered through an IP injection (Atasoy et al., 2012; Magnus et al., 2011).

Mice will be injected with AAV-RAM-PSAM or AAV-CMV-PSAM into dorsal CA3 and trained in CFC 2 weeks later. The amount of each virus will be adjusted so that a similar number of cells will be infected in each condition. The CMV condition serves as a control, to ensure that any effects seen in the RAM condition can be attributed to manipulating the function of RAM-expressing cells, as opposed to manipulating the function of CA3. Using RAM-PSAM, we will target neurons activated following CFC. It is hypothesized that inactivation of this population prior to testing will impair contextual

memory recall, while inactivation of a random population of CA3 cells (CMV condition) should have no effect. Mice will be trained in Context A. Training should lead to the activation of RAM and the expression of PSAM. The next day, mice will be given an IP injection of PSEM to induce silencing, and given a memory test in Context A. If activity within RAM-expressing neurons is required, then freezing in the RAM group should be impaired in comparison to the CMV group. As silencing is transient, each animal serves as its own control. 48hrs after training subjects will be injected with saline and given a memory test in Context A. Because neuronal activity in RAM-expressing neurons will be restored, we hypothesize that memory recall will be unaltered.

To test whether the activation of RAM-expressing neurons is sufficient to induce contextual memory recall, mice will be injected with AAV-RAM-ChR2 or AAV-CMV-ChR2 and trained in CFC 2 weeks later. The use of ChR2 requires surgical implantation of optical fibers. Our initial experiments suggest that we are able to accurately implant the fiber optic cables and selectively activate CA3, as measured by c-Fos expression (experiments conducted in collaboration with M. Baratta from E. Boyden's lab). The pattern of ChR2 stimulation was determined based on several recent studies using ChR2 to modulate behavioral output (Johansen et al., 2010; Lobo et al., 2010). Mice will be trained in Context A. Training should lead to the activation of RAM and the expression of ChR2. Following training, mice will be placed in a neutral chamber, Context B. Under standard conditions Context B will not induce a robust fear response, as mice can distinguish between two different contexts. Using ChR2 light-stimulation, we will activate RAM-expressing neurons in Context B. If this manipulation induces freezing behavior under the RAM, but not the CMV conditions, it would suggest that the

neuronal population expressing Npas4 represents a memory trace that is necessary and sufficient for recall of a contextual representation. To ensure that the context memory is intact, mice can be tested 24h later in the absence of light stimulation in Context A.

5.3 Memory Transcriptome

To determine the components of the experience-activated gene program, RNAseq provides a powerful, unbiased method to identify novel transcripts, splice variants, and rare species from our samples (Wang et al., 2009). However, these strengths are negated if the starting material consists of a heterogeneous population of cells. We originally identified Npas4 using a homogenous culture system (Lin et al., 2008). Under these robust conditions, screening transcripts using microarray technology was sufficient. However, identifying learning-activated RNA transcripts requires a more sophisticated approach as the cell population of interest is sparse and the transcripts of interest are unknown and in low abundance.

To address these issues, we have generated a genetic system in which the RAM reporter construct expresses Cre recombinase. This construct will be virally delivered to a newly generated mouse line, RiboTag, which will allow for the purification of mRNA from a specific cell population (Sanz et al., 2009). In RiboTag mice, the ribosomal protein RPL22 has been modified such that the last exon of the gene is flanked by loxp sites and is immediately followed by a hemagglutinin tagged (HA) version of the exon. Prior to Cre-mediated recombination, wildtype RPL22 will be expressed. Following the expression of Cre, endogenous exon 4 is removed and RPL22-HA is expressed.

RPL22-HA is then incorporated into polysomes, which contain actively translated mRNA transcripts, and can be immunoprecipitated using an HA antibody. mRNA will be purified from the immunoprecipitated polysome complexes and then analyzed. As ribosomal depletion strategies have been previously used for RNAseq (Cloonan et al., 2008), it is likely that the RiboTag strategy will allow for the purification of pure RNA from a select population of cells. An additional advantage of this technique will be that it does not require extensive harvesting steps, compared to methods such as FACS sorting and laser dissection. This is especially important for activity-regulated genes, whose expression is transient and very sensitive.

To examine the genetic program activated by learning, RiboTag mice will be injected with RAM-Cre into dorsal CA3 and trained in contextual fear conditioning. Following fear conditioning, the expression of RAM-Cre should result in the incorporation of RPL22-HA into polysomes. To ensure that transcripts identified in this condition are specific to RAM-positive neurons, separate sets of mice will be injected with Synapsin-Cre and fear conditioned. Unlike the targeted expression of RAM, the Synapsin promoter is highly active in *all* neurons and will therefore randomly label CA3 neurons. Hippocampus will be dissected 1, 3, 6, 12, 24, 48, 72hrs, 7d, 15d, and 30d after fear conditioning and mRNA transcripts will be purified by RPL22-HA immunoprecipation. This system allows for the unique opportunity to examine learning-induced gene transcripts at timepoints far beyond what has been traditionally examined, because the activation of the ribosomal tag is permanent. These time points were selected based on the proposed duration hippocampal involvement in contextual memories (Neves et al., 2008). As RNA will be sampled from a sparse population of neurons it will be necessary

to pool samples to generate sufficient starting material for RNAseq (0.1ug-0.4ug, Illumina TruSeq Guide).

Analysis should focus on targets uniquely regulated in the RAM condition, with at least a two-fold difference between the RAM and Synapsin conditions. While large differences in known downstream targets of Npas4, such as BDNF, are expected (Lin et al., 2008; Ramamoorthi et al., 2011), it is difficult to predict what types of targets will be induced by learning. Changes in transcripts encoding synaptic proteins are likely, as alterations in synaptic plasticity are thought to be the cellular substrate of long-term memories (Neves et al., 2008). All results should be cross-referenced with our previous microarray data and qPCR results examining the effects of Npas4 knockdown (Lin et al., 2008) or knockout (Ramamoorthi et al., 2011) on expression of downstream gene targets.

5.4 Electrophysiological signature of experience activated neurons

A longstanding question in the learning and memory field is how is information stored in neural circuits. Examination of this question has focused on to major areas: 1) evaluation of the molecular and cellular events that are induced by and required for plastic processes such as long-term potentiation and 2) extracellular recordings of hippocampus in awake behaving rodents. Both areas have made tremendous strides in identifying unique learning-related electrophysiological signatures, but technical limitations have made it difficult to integrate the two areas.

Several theories suggest that, at the synaptic level, information storage can be represented as a shift of synaptic weights within select populations of neurons (Kandel,

2001; Neves et al., 2008). Plastic processes, such long-term potentiation, have been studied extensively and provide a sense of the molecular and cellular changes that could subserve memory formation. A plethora of molecules have been identified as necessary for the maintenance long-term potentiation and the majority of these molecules are also required for long-term memory formation. Since the first use of genetically modified mice in the study of synaptic plasticity and memory formation (Grant et al., 1992; Silva et al., 1992a; Silva et al., 1992b), tremendous progress has been made in identifying molecules that contribute to these processes. Genetic manipulations such as altering receptor function (McHugh et al., 1996; Tsien et al., 1996), modulating kinase activity (Abel et al., 1997; Mayford, 2007), and inhibiting transcriptional activity (Alberini, 2009; Bourtchuladze et al., 1994; Jones et al., 2001; Lee and Silva, 2009; Mayford et al., 1995; Silva et al., 1998) have all shown a variety of deficits. From these studies, it is generally thought that learning induces enhanced excitatory synaptic transmission within sparse populations of neurons. Within this population, NMDA receptor activation and subsequent opening of voltage-gated calcium channels results in the post-translational modification of transcription factors (e.g. CREB and SRF) and expression of activity-regulated programs of gene expression. Expression of genes such as Arc and BDNF are ultimately thought to enhance synaptic transmission, the presumed substrate of long-term information storage. Blockade of any of these steps (e.g. NMDA receptor antagonists, voltage gated calcium channel antagonists, transcriptional or translational inhibitors, or single gene knockdown/knockout) is sufficient to impair long-term plasticity and memory formation.

Moving past individual synapses and cells, multicellular recordings from awake and behaving rodents has revealed that populations of neurons within the hippocampus exhibit location-specific patterns of activity. These neurons, referred to as place cells (O'Keefe & Dostrovsky 1971), fire at specific locations within a given environment such that an entire environment can be represented across the hippocampus. Firing fields for these cells are specific to an environment such that in different environments the firing fields change (O'Keefe and Conway 1978), suggesting a unique neural representation for each location. Furthermore, recent evidence suggests that the hippocampus can encode multiple types of information, far beyond spatial location (Eichenbaum, 2003; Sweatt, 2003). These findings suggest that the pattern of activity within the hippocampus functions as a neural representation of experience.

Despite our increasing understanding of the cellular and circuit mechanisms of memory formation, connecting the two areas has been difficult. The most obvious obstacle is that there are no clear ways to identify place other than multicellular recordings during environmental exploration, preventing in-depth synaptic and molecular analysis of these cells. Furthermore, for any given environment only a percentage of hippocampus (5-20%) (Guzowski et al., 1999) is thought be part of the representation, preventing non-targeted patching experiments.

The RAM system provides an opportunity to gain access to experience-activated cell populations and determine their electrophysiological properties, potentially revealing an electrophysiological signature of experience-activated neurons. Mice will be implanted with tetrodes in dorsal CA3 and simultaneously injected with a ChR2 expressing RAM virus (RAM-ChR2-EYFP). After recovering, mice will be exposed to a

novel environment "A" which will drive the expression of ChR2-EYFP in experience-activated neurons. 24 hours after exposure to environment "A", mice will be exposed to blue-light ChR2 stimulation in their homecage, allowing for identification of which single units on the tetrode are recording from RAM positive neurons. Mice will then be returned to environment A where place cells will be detected using standard methodology and place fields will be correlated to individual units known to be RAM positive. Using this approach one can directly determine the environmental and spatial elements being encoded for by neurons activated by experience (RAM positive). Following *in vivo* recordings, sections can be prepared and *ex vivo* slice recordings can be made allowing for direct analysis of synaptic properties of experience-activated neurons.

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Appendix

The GABAergic system and neurodevelopmental disorders

Abstract:

As the major inhibitory component in the brain, the GABAergic system maintains the balance between excitation and inhibition (E/I balance) that is required for normal neural circuit function. Perturbations in the development of this system, from the generation of inhibitory neurons to the formation of their synaptic connections, have been shown to cause several neurodevelopmental disorders as a result of E/I imbalance. In this review we discuss how impairments at different stages in GABAergic development can lead to disease states. We also highlight recent studies showing that modulation of the GABAergic system can successfully reverse cognitive deficits in disease models and suggest that therapeutic strategies targeted at the GABAergic system may be effective at ameliorating impairments in neurodevelopmental disorders.

Introduction:

The basic wiring of our nervous system is established during the early developmental period. Key events during this period include birth and migration of neurons, synapse formation and maturation, and experience-dependent refinement of circuit connections. Any perturbation of these early steps can lead to life-long cognitive and emotional disabilities, such as epilepsy, autism and schizophrenia, for most of which we have no effective treatments. Basic research into early development has promoted advancements in clinical diagnosis. As a result, disorders such as schizophrenia that were traditionally thought to have late onsets are now being linked to deficits during early development (Lewis and Levitt, 2002).

A common feature of neurodevelopmental disorders is a disrupted excitatory/inhibitory (E/I) balance, often due to dysfunctional GABAergic system (Table 1). GABA is the major inhibitory neurotransmitter in the brain. The components of the GABAergic system are GABAergic neurons, the GABAergic synapses they form onto their targets and the synaptic inputs they receive. The development of the GABAergic system coincides with the onset of many neurodevelopmental disorders, suggesting a critical role for inhibition in neural circuit development and function. A nice example of this is the Zoghbi laboratory's recent work on Rett syndrome (Chao et al.). Rett syndrome, one of the autism spectrum disorders, is caused by mutations in the transcriptional regulator MeCP2 (methyl-CpG-binding protein 2), resulting in predominantly neurological symptoms despite its ubiquitous expression. Dr. Zoghbi and her colleagues show that selectively disrupting MeCP2 in GABAergic neurons can

recapitulate a majority of Rett phenotypes, signifying the important role of the GABAergic system in the etiology of Rett syndrome and likely autism in general.

Table 1 lists major classes of neurodevelopmental disorders in which deficits in the GABAergic system have been implicated. It is evident that disrupted E/I balance is a common feature of these disorders. Although both excitatory and inhibitory systems are important for maintaining E/I balance, the GABAergic system will be the focus of this review. Limited by space, we will focus on three major aspects of GABAergic development: generation and migration of GABAergic neurons, development of GABAergic synapses, and the impact of the GABAergic system on neural circuit function. Our knowledge of the development of the GABAergic system has grown rapidly in recent years thanks to newly developed genetic tools, giving us an unprecedented opportunity to examine the process of GABAergic development in the context of neurodevelopmental disorders and hopefully to reevaluate and even redesign therapeutic strategies.

1. Generation and migration of GABAergic neurons

The first stage of neural GABAergic development, generation and migration of GABAergic neurons, is responsible for producing the right numbers and kinds of GABAergic neurons and placing them in the appropriate locations. This stage seems likely to have the most significant and longest-lasting impact on the final neural circuits. Alteration in specific subtypes of GABAergic neurons has been observed in several neurodevelopmental disorders (Casanova et al., 2002; Kalanithi et al., 2005; Kataoka et al., 2010; Levitt et al., 2004; Lewis et al., 2005). For instance, in Tourette's syndrome

the number of PV-positive neurons is increased in striatum, but decreased in globus pallidus (Kalanithi et al., 2005).

The majority of GABAergic neurons are derived from lateral, medial and caudal ganglionic eminences (LGE, MGE and CGE, respectively) in the ventral part of the telecephalon (subpallium) (Batista-Brito and Fishell, 2009). GABAergic neuronal fate is determined by transcription factors, *Mash1* and *Dlx* family members, whose expression is restricted to the subpallium (Anderson et al., 1997b; Fode et al., 2000). It has been shown that *Dlx5* may be directly regulated by *MeCP2*, suggesting a direct link between GABAergic neurogenesis and autism (Horike et al., 2005). Furthermore, the *Dlx1/2* and *Dlx5/6* gene clusters are near autism susceptibility loci mapped on chromosomes 2q and 7q. However, the link between *Dlx* genes and autism remains to be firmly established (Nabi et al., 2003; Schule et al., 2007).

A striking feature of GABAergic neurons is their diversity. Though no single parameter can unequivocally differentiate all subtypes of GABAergic neurons, they can be classified by morphology (basket, chandelier, bipolar, double bouquet cells, etc.), physiology (fast-spiking, regular firing, bursting, stuttering etc.), and by the molecular markers they express (Markram et al., 2004). Molecular markers have proven useful for examining postmortem tissues from patients. Commonly used ones include calcium-binding proteins such as parvalbumin (PV), calretinin (CR) and calbindin (CB) and neuropeptides such as somatostatin (SST), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and cholecystokinin (CCK).

The molecular mechanism by which the diverse subtypes of GABAergic neurons are specified is not fully understood. A gene profiling study to identify genes enriched in

cortical interneuron precursors, carried out in the Fishell lab, suggests that GABAergic subtypes are already determined at the progenitor stage, shortly after they become postmitotic and long before they are functionally integrated into neural circuits (Batista-Brito et al., 2008). Genetic fate-mapping studies suggest that GABAergic subtype specification is regulated by both spatial and temporal factors. For example, neurons from MGE preferentially express PV and SST/CB, while CGE-derived neurons tend to be CR/VIP or NPY positive (Batista-Brito and Fishell, 2009). Temporally, SST neurons are born before PV neurons, which are followed by VIP neurons (Miyoshi et al., 2007). This is consistent with the fact that neurogenesis in MGE (producing SST and PV neurons) happens earlier than in CGE (producing VIP neurons) (Butt et al., 2005). The next challenge is to identify the genetic programs that determine the fate of the diverse repertoire of GABAergic neurons.

Most newly born GABAergic neurons first migrate tangentially away from the ganglionic eminence, following two migratory streams into regions above and below the developing cortical plate (Danglot et al., 2006). Once there, they adopt various radial migration modes to settle into specific cortical layers in an inside-out fashion according to their birth order, with cells born earlier taking the deeper layers and cells born later occupying the superficial layers (Batista-Brito and Fishell, 2009).

Many factors have been identified as regulating the tangential migration of GABAergic neurons. These include a set of attractant and repellent guidance molecules similar to those used by glutamatergic neurons, growth factors and their receptors, and neurotransmitters (Danglot et al., 2006; Hernandez-Miranda et al., 2010; Powell et al., 2001). Dysregulation of a number of those factors has been implicated in neurological

disorders (Campbell et al., 2006; Martinowich et al., 2007). The schizophrenia susceptibility gene Nrg1 and its receptor ErbB4 are worth special mention (Stefansson et al., 2003; Stefansson et al., 2002). Tangentially migrating GABAergic neurons express ErbB4. Ngr1 secreted in cortical regions can serve as a potent chemoattactant to guide ErbB4-expressing interneurons to migrate tangentially into the cortex (Corfas et al., 2004). Impaired GABAergic neuron migration resulting from defects in Nrg1-ErbB4 signaling may contribute to the pathology of schizophrenia in at least a subset of schizophrenia patients (Corfas et al., 2004). As we discuss in the following sections, Ngr1-ErB4 signaling is also involved in other aspects of GABAergic system development.

An interesting aspect of GABAergic neuron migration is that there is interplay between guidance cues and GABAergic neuron subtype-specific transcription factors (Anderson et al., 1997a; Nobrega-Pereira et al., 2008). For example, a recent study showed that Nkx2.1 negatively regulates the semaphoring receptor Nrp2 (Nobrega-Pereira et al., 2008). Because striatum expresses the repellent ligands of Nrp2, Sema3A and Sema3F, neurons expressing low levels of Nkx2.1 (thus high levels of Nrp2) avoid striatum and migrate to cortex, while high Nkx2.1-expressing neurons migrate into striatum. Further exploration of migration mechanisms important for different subtypes of GABAergic neurons is necessary, as they might be relevant to altered distributions of certain subtypes in neurodevelopmental disorders such as Tourette's syndrome (Kalanithi et al., 2005).

In contrast to tangential migration, very little is known about regulation of radial migration. The only signaling pathway implicated in this process involves the chemokine

Cxcl12 and its receptor Cxcr4, which affect the timing of switching from tangential to radial migration (Li et al., 2008; Lopez-Bendito et al., 2008). While tangential migration distributes GABAergic neurons longitudinally around the cortical plate, radial migration is responsible for sorting GABAergic neurons into the correct cortical layers, where they are integrated into the local cortical circuits within each layer. Therefore radial migration of GABAergic neurons may have a profound impact on the computational power of cortical circuits and can directly affect behavioral output. It is important to investigate what governs this aspect of GABAergic neuron development and determine whether different mechanisms are at work in different subtypes of GABAergic neurons.

II. Development of GABAergic synapses

While generation of GABAergic neurons occurs during the mid-embryonic stage, the formation and maturation of inhibitory synapses occurs mostly postnatally. Proper development of GABAergic synapses is critical to achieve an optimal E/I balance within a neural circuit, impairments of which are associated with a wide spectrum of neurodevelopmental disorders (Rubenstein and Merzenich, 2003). Although we focus on the inhibitory role of GABAergic synapses here, there is an important developmental stage during which these synapses are excitatory, and interested readers can find several reviews covering this topic (Ben-Ari and Holmes, 2005).

The development of GABAergic synapses is regulated by both genetically hard-wired and neuronal activity-dependent processes, but these two processes are interconnected and should not be considered independent of each other (Lu et al., 2009). As for glutamatergic synapses, the initial formation of GABAergic synapses is thought to be genetically determined, through elaborate cell-cell recognition processes

that are mediated by transmembrane cell adhesion molecules. Neurexin (NRXN) and neuroligin (NLGN) family members are the best characterized ligand/receptor partners important for the development of GABAergic synapses (Sudhof, 2008). NRXNs are thought to be predominantly presynaptic proteins mediating the formation of specializations that contribute to neurotransmitter release. NLGNs are postsynaptic NRXN ligands. Together they form complexes, with specific isoforms localizing to excitatory or inhibitory synapses (Sudhof, 2008). Complexes containing Nlgn2 are mainly found at GABAergic synapses (Varoqueaux et al., 2004) and mutations result in impaired inhibitory synapse development and aberrant inhibitory transmission (Chubykin et al., 2007; Sudhof, 2008).

Several mutations have been observed in Nrxn1, Nlgn3, and Nlgn4 that are linked to autism (Jamain et al., 2003; Kim et al., 2008; Yan et al., 2008). Importantly, mouse models mimicking the human mutations in Nlgn3 and Nlgn4 produce autistic phenotypes: impaired social interaction and communication (Jamain et al., 2008; Tabuchi et al., 2007). It is particularly interesting that knock-in mice with an Arg451 to Cys mutation in the Nlgn3 gene not only have autistic behavioral phenotypes, similar to human patients with the same mutation, but they also exhibit an increase in inhibitory synaptic transmission with no obvious changes in excitatory transmission (Tabuchi et al., 2007), indicating that a GABAergic system deficit might underlie the disease associated with this mutation in humans.

In addition to the NRXN-NLGN pair, Neuregulin 1 (Nrg1) and its receptor ErbB4 have recently been shown to be important regulators of GABAergic synapses and, as we mentioned earlier, have been repeatedly identified as risk genes for schizophrenia

(Mei and Xiong, 2008). A recent study by the Rico and Marin laboratories identified a role for this complex in GABAergic neurons. They found that ErbB4 expression in PV-positive basket and chandelier cells contributes to the formation of inhibitory synapses on pyramidal neurons, as well as excitatory synapses on the PV inhibitory neurons (Fazzari et al., 2010). This suggests that the Nrg1-ErbB4 complex has an important role in regulating inhibitory drive, alteration of which is one of the defining hallmarks of schizophrenia (Karam et al., 2010). As we discuss later, this might indicate a critical role for the GABAergic system in schizophrenia etiology.

Recently added to the limited number of receptor-ligand complexes functioning selectively at GABAergic synapses is FGF7, a member of the fibroblast growth factor family, and its receptor FGFR2 (Terauchi et al., 2010). Expressed in CA3 pyramidal neurons, FGF7 regulates GABAergic synapse development in this region of the hippocampus by selectively promoting the organization of GABAergic presynaptic terminals. FGF7-deficient mice are prone to epileptic seizure, presumably as a result of reduced inhibition (Terauchi et al., 2010). Whether the FGF signaling pathway is affected in epileptic patients has not been examined.

While the receptor-ligand complexes we have discussed play an important role in creating GABAergic synapses, neuronal activity is critical for their further maturation (Lu et al., 2009). For example, postnatal development of inhibitory synapses in brain regions such as primary sensory cortex is modified by neuronal activity and sensory experience (Chattopadhyaya et al., 2004; Knott et al., 2002). This positions inhibition to mediate neural circuit development based on experience, but the molecular and cellular mechanisms underlying this process are largely unknown. Recently the transcription

factor Npas4 has been shown play a critical role in the activity-dependent regulation of inhibitory synapse formation (Lin et al., 2008). Npas4 expression is rapidly activated by excitatory synaptic activity and regulates a genetic program that triggers the formation and/or maintenance of inhibitory synapses on excitatory neurons. Although the Npas4 gene has not been found to be associated with any disorders, one of its potential transcriptional targets, the $(Na^+, K^+)/H^+$ exchanger Nhe9, is deleted in some autistic patients (Morrow et al., 2008). It is currently not clear whether mutations in Nhe9 result in reduced levels of inhibition, nevertheless Nhe9 deficiency often leads to co-morbid epilepsy, suggesting that the genetic program downstream of Npas4 may contribute to E/I balance.

A genome-wide screen for Npas4 targets identified several genes with known roles in inhibitory synapse formation, including BDNF (Lin et al., 2008). While the role of BDNF in CNS function seems to be quite broad (Lu, 2003), its function as a mediator of inhibitory synapse formation and maintenance is well documented (Chattopadhyaya et al., 2004; Huang et al., 1999; Lu et al., 2009). Since BDNF is highly regulated by activity, it is considered a major player in activity-dependent development of GABAergic synapses. This hypothesis is supported by recent work from the Greenberg and Lu laboratories (Hong et al., 2008; Sakata et al., 2009). By genetically abolishing the function of one of BDNF's activity-dependent promoters, promoter IV, both labs observed impairments in GABAergic synapses with no change in excitatory synapses in cortical areas. These studies establish an important role for activity-driven BDNF expression in the development of inhibition. Impaired BDNF function has been

implicated in several neurodevelopment disorders (Hong et al., 2005), but its ubiquitous role in CNS function makes it difficult to identify its precise role in these disease states.

III. Impact of the GABAergic system on neural circuit function

Given that the GABAergic system is indispensible for establishing and maintaining E/I balance, it is not surprising that impairments in the development of this system have a profound impact on neural circuit and cognitive functions. The ability of neural circuits to modify their connections based on changing external inputs is thought to underlie core processes such as learning and memory (Neves et al., 2008). Usually this modification is measured by changes in excitatory synaptic transmission, which can exhibit plasticity such as long-term potentiation (LTP) and depression (LTD) (Neves et al., 2008). Until recently, the contributions of inhibition to these processes were relatively unknown, but it is becoming evident that altered inhibitory function can profoundly impair excitatory synaptic transmission and subsequent circuit plasticity (Cui et al., 2008; Fernandez and Garner, 2007; Fernandez et al., 2007; Kleschevnikov et al., 2004). For example, the onset of visual cortex plasticity can be accelerated or delayed by increasing or decreasing inhibitory transmission respectively (Hensch, 2005). In this section we will highlight several diseases that have recently been shown to involve aberrant inhibitory transmission to show how alterations to the GABAergic system can profoundly alter neural circuit function (Fig 1).

Down syndrome, a triplication of chromosome 21 that results in an extra copy of approximately 300 genes, is the most common genetic cause of mental retardation. While there are several mouse models of Down syndrome, the best characterized is the Ts65Dn line which is trisomic for a large region of chromosome 16 (homologous to

human chromosome 21) (Reeves et al., 1995). These mice exhibit a variety of behavioral deficits that are consistent with learning and memory impairments (Fernandez et al., 2007; Reeves et al., 1995; Rueda et al., 2008) (Table 1). In addition to various structural abnormalities observed at synapses (Belichenko et al., 2004), electrophysiological recordings from dentate gyrus (DG) of Ts65Dn mice show increased inhibitory transmission and enhanced feedback inhibition, with no change in excitation. Furthermore, these mice exhibit impaired DG LTP (Fernandez et al., 2007; Kleschevnikov et al., 2004). Taken together, these findings suggest that the plasticity and behavioral impairments seen in the Ts65Dn mouse line may be due to altered E/I balance due to enhanced inhibition.

Neurofibromatosis, caused by genetic mutations in the NF1 gene, is characterized by similar increases in inhibition that probably contribute to plasticity and behavioral impairments (Cui et al., 2008). Within the CNS, mutations in NF1 lead to deficits in learning and memory, attention, and visuospatial tasks (Costa and Silva, 2003). These behavioral phenotypes have been successfully recapitulated in a mouse model of neurofibromatosis in which one copy of the NF1 gene is deleted (Table 1) (Costa et al., 2002; Cui et al., 2008). At the synaptic level, NF1 mutant mice exhibit an overall increase in inhibitory synaptic transmission and no change in excitatory synaptic transmission. Possibly as a consequence of heightened inhibition, these mice exhibit attenuated excitatory synaptic plasticity as measured by hippocampal LTP (Costa et al., 2002; Cui et al., 2008). Remarkably, deleting one copy of NF1 from inhibitory neurons, but not excitatory neurons or glial cells, was sufficient to reproduce the inhibitory transmission, LTP, and behavioral phenotypes seen in the global NF1 mutant,

suggesting that dysfunction of the GABAergic system is responsible for the cognitive phenotypes seen in neurofibromatosis (Cui et al., 2008). It is believed that the loss of NF1 leads to hyperactive Ras-Erk signaling, which subsequently causes an increase in GABA release and a suppression of the plasticity required for cognitive function (Fig 1) (Cui et al., 2008). Consistent with this model, reducing Ras activity either genetically or pharmacologically leads to a reversal of the behavioral and plasticity impairments in NF1 mutant mice (Costa et al., 2002).

Down syndrome and neurofibromatosis provide clear examples of the impairments caused by excessive inhibition. However, decreases in inhibition can also disrupt circuit function. One of the most consistent observations in schizophrenia is the decreased inhibitory drive on to glutamatergic neurons (Karam et al., 2010). Postmortem studies show decreases in NMDA receptor expression on PV neurons (Bitanihirwe et al., 2009; Woo et al., 2004), which would result in a decrease in inhibitory drive. This is in line with other postmortem brain studies of schizophrenic patients that have identified decreases in overall GABA levels, as well decreases in GAD65, GAD67, and other presynaptic components of the GABAergic system (Torrey et al., 2005).

The Nakazawa laboratory recently showed that the specific deletion of NR1 in cortical and hippocampal interneurons resulted in several phenotypes seen in schizophrenic patients, both behavioral (Table 1) and molecular (decreased GAD67 and PV expression) (Belforte et al., 2010). Similarly, the loss of ErbB4 from hippocampal PV-positive GABAergic neurons (PV-ErbB4), which results in impaired inhibitory synapse formation (Fazzari et al., 2010), produced schizophrenia-like phenotypes and impaired GABAergic modulation of hippocampal LTP (Wen et al., 2010, Chen, 2010

#440). Subsequent treatment of the PV-ErbB4 mutant mice with diazepam, a GABA agonist, resulted in a recovery of some of the behavioral deficits, suggesting that impairment in inhibition may contribute to the behavioral phenotypes (Wen et al., 2010).

Despite the range of cognitive and behavioral phenotypes seen in Down syndrome, neurofibromatosis, and schizophrenia, the studies described here strongly support the idea that deficits in the GABAergic system might be a common underlying factor contributing to the etiology of these diseases. However, these observations are still limited to mouse models and further investigation is required to establish these links in human patients.

IV. Implications & conclusion

From the studies we have reviewed here, it is clear that altering the level of inhibition either upwards or downwards can have profound effects on circuit function. While the various disease states we have considered are different in their causes and phenotypes, one common theme that has emerged is the improper regulation of inhibitory function. Any perturbation in the development of the GABAergic system, from the generation and migration of these neurons to the formation and refinement of their synaptic connections, can lead to severe neurological impairments. Furthermore, it is now becoming clear that selectively modulating the function of susceptibility genes such as MeCP2, NF1, and ErbB4 in GABAergic neurons may be sufficient to cause deficits once only seen in global knockouts. As a consequence of having a basic understanding of the general mechanisms mediating GABAergic development, there is potential for generating targeted and specific therapeutic interventions.

Recent work from the Stryker lab has shown that transplantation of inhibitory neurons (MGE precursors) into primary visual cortex is sufficient to induce ocular dominance plasticity after the closing of the critical period (Southwell et al., 2010). This strategy also showed promise to alleviate epilepsy in animal models (Sebe and Baraban, 2010). These findings suggest that the developmental program of inhibitory neurons may regulate and induce plasticity. Transplanting these neurons may provide a new therapeutic intervention to treat neural circuits impaired by disease.

Pharmacological modulation of GABAergic synapses has also been shown to be able to restore circuit plasticity (Cui et al., 2008; Fernandez et al., 2007; Rueda et al., 2008). Several groups have shown a potential causal relationship between the regulation of inhibition and impaired neural circuit function. For example, work from the Silva lab has shown that treatment of the NF1 mutant mouse with a subthreshold dose of $GABA_A$ antagonist rescues both the behavioral and plasticity deficits (Cui et al., 2008). This finding is particularly significant because NF1 is a developmental disorder, yet the cognitive impairments can be reversed in adult mice with acute modulation of the GABAergic system. A similar observation was seen in the mouse model of Down syndrome in which chronic treatment with $GABA_A$ antagonists could improve both plasticity and memory (Fernandez et al., 2007; Kleschevnikov et al., 2004; Rueda et al., 2008). The implication of these studies is that pharmacological modulation of the GABAeric system may provide a potential treatment for a number of neurodevelopmental disorders. Studying how specific GABAergic regulators, such as those shown in Figure 1, modulate inhibition can identify targets for specific therapeutic interventions.

Our understanding of the GABAergic system is still in its infancy. The advent of new molecular tools will provide the ability to examine and modulate the GABAergic system with exquisite specificity. This will surely lead to a greater understanding of the GABAergic system, providing key insight into disease states and their potential treatments.

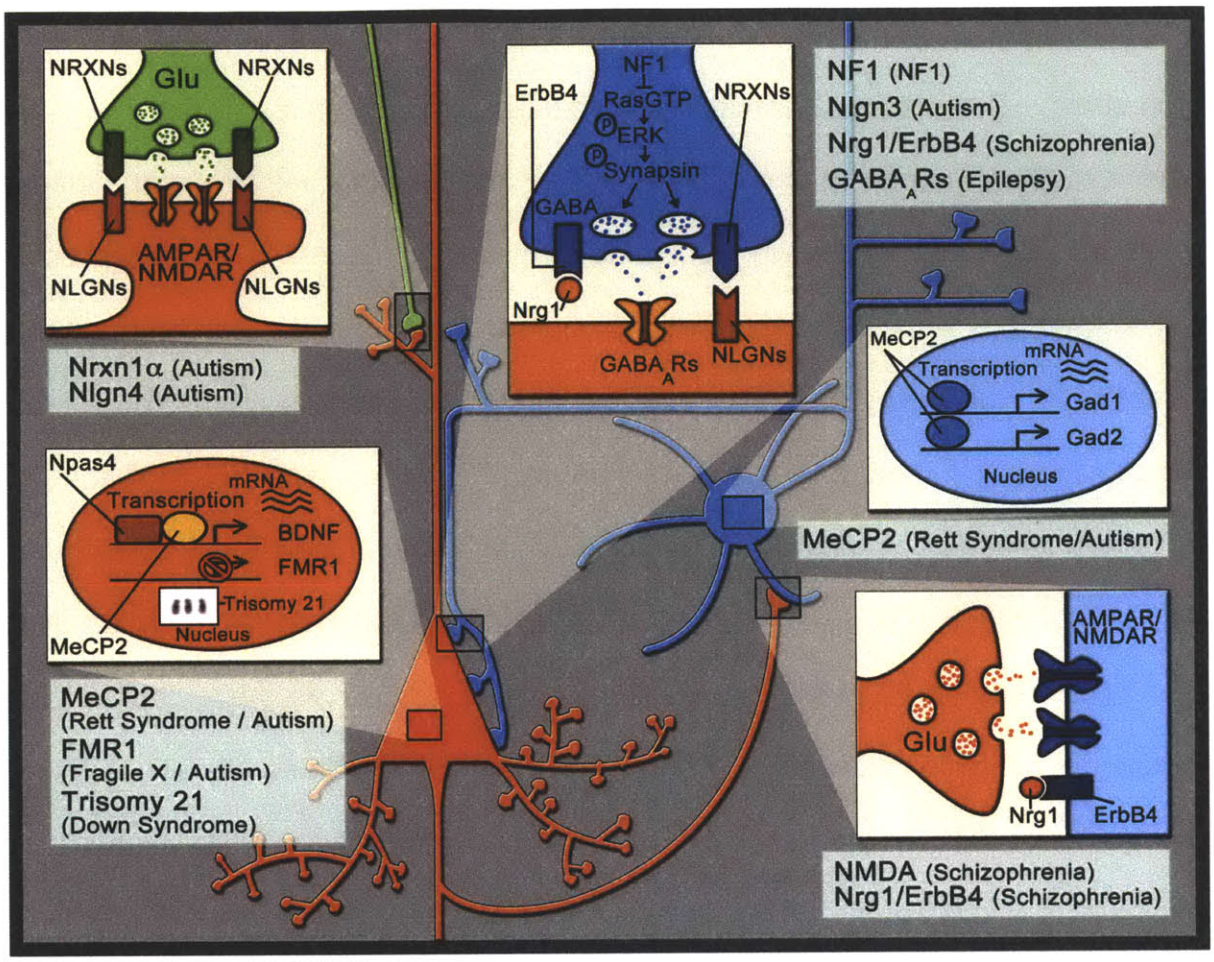
NRXNs
Glu
NRXNs
AMPAR/
NMDAR
NLGNs
NLGNs
Nrxn1α (Autism)
Nlgn4 (Autism)
ErbB4
NF1
RasGTP
ERK
Synapsin
NRXNs
GABA
Nrg1
GABA A Rs
NLGNs
NF1 (NF1)
Nlgn3 (Autism)
Nrg1/ErbB4 (Schizophrenia)
GABA A Rs (Epilepsy)
MeCP2
mRNA
Transcription
Gad1
Gad2
Nucleus
MeCP2 (Rett Syndrome/Autism)
Npas4
mRNA
Transcription
BDNF
FMR1
Trisomy 21
Nucleus
MeCP2
MeCP2
(Rett Syndrome / Autism)
FMR1
(Fragile X / Autism)
Trisomy 21
(Down Syndrome)
AMPAR/
NMDAR
Glu
Nrg1
ErbB4
NMDA (Schizophrenia)
Nrg1/ErbB4 (Schizophrenia)

Figure 1. Sites of action of molecules implicated in neurodevelopment disorders. The figure shows a pair of interconnected pyramidal neuron (orange) and GABAergic neuron (blue). The orange pyramidal neuron also receives an excitatory input from another excitatory neuron (green). Details of the nuclei of the neurons and synapses formed between them are shown, according to their color codes, in expanded boxes. Depending on their subcellular localizations, disease-relevant molecules and pathways are depicted in the boxes and related disorders indicated next to them. Top left box: Excitatory synapse formed on a pyramidal neuron. Mutations in Nrxn1α and Nlgn4 have been reported in autistic patients, mutant mice exhibit similar phenotypes(Etherton et al., 2009; Sudhof, 2008; Zhang et al., 2009). Bottom left box: Nucleus of a pyramidal neuron. Suppression of FMR1 expression leads to Fragile X, while triplication of chromosome 21 leads to Down Syndrome. MeCP2 mutations cause alterations in gene expression (e.g. BDNF) contributing to Rett Syndrome and Autism(1994; Chahrour and Zoghbi, 2007; Reeves et al., 1995). Bottom right box: Excitatory synapse formed on an inhibitory neuron. Alterations in Nrg1/ErbB4 complexes as well as NMDA receptor function have been reported in schizophrenic patients and produce schizophrenia-like phenotypes in mutant mice(Belforte et al.; Fazzari et al.; Hall et al., 2006). Middle right box: Nucleus of an inhibitory neuron. Mouse models with selective impairment MeCP2 function in inhibitory neurons produces Rett syndrome phenotypes and a decrease in GABA transmission(Chao et al.). Center box: Inhibitory synapse on a pyramidal neuron. Selective deletion of NF1 from GABAergic neurons results in hyperactive GABA release. Mutations in Nlgn3 have been reported in autistic patients, mutant mice exhibit enhanced inhibitory transmission and autistic-like phenotypes. Alterations in ErbB4 in

inhibitory neurons results in a decrease in the number of GABAergic synapse on pyramidal neurons and produces schizophrenia like phenotypes in mouse models. Modulations in GABAergic system seem to be a major factor in seizure disorders(Cui et al., 2008; Fazzari et al.; Noebels, 2003; Tabuchi et al., 2007). Abbreviations: Nrxn1α: neurexin 1 alpha, Nlgn4: neuroligin 4, FMR1: fragile X mental retardation 1, MeCP2: methyl CpG binding protein 2, BDNF: brain derived neurotrophic factor, Nrg1: Neuregulin 1, ErbB4: Receptor tyrosine-protein kinase 4, NMDA: *N*-Methyl-D-aspartic acid, NF1: Neurofibromatosis type I.

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